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der Christian-Albrechts-Universität zu Kiel

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# Improved Rapeseed as Fish Feed in Aquaculture

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## General Introduction

The human population is estimated to reach 9.7 billion by the year 2050 (United Nations, 2019) and an increase by 25-70 % in the supply of food would be necessary to support the global inhabitants (Hunter et al., 2017). Additionally, ascending prosperity causes a greater demand of diets increasingly rich in animal protein (Hua et al., 2019). Both the livestock and aquaculture sector have been growing over the past decades, however, aquaculture has been the fastest growing food production sector in the world, reaching 17 % of animal protein consumed globally in 2014 (Gasco et al., 2018). Aquaculture production constituted 46 % (82 million tons) of global fish production in 2018 (FAO, 2020) and is expected to increasingly contribute to the global production of proteins with animal origin. Between 2001 and 2018, global production of aquatic animals grew on average by 5.3 % annually and was only decelerated to some extent by reduced production in China in recent years (FAO, 2020). The aquaculture sector can generally be divided into “fed” and “unfed” aquaculture. Fed aquaculture relies on providing farmed fish with nutrient-rich feed sources such as formulated diets and whole or processed fish. Although both sectors of aquaculture have been growing over recent years, unfed aquaculture only accounted for 30.5 % of total aquaculture production in 2018 with decreasing perspectives (FAO, 2020). Fishmeal was utilized as the main source of protein in fish diets of fed aquaculture in the past and is still included in diets for the aquaculture sector until today (FAO, 2020; Hardy, 2010; Luo et al., 2008). Although about 25-35 % of fishmeal (and fish oil) now originates from by-products of fish processing, a major portion still depends on catches of small pelagic fish (FAO, 2020; Hua et al., 2019). Aforementioned dependency creates additional pressure on wild fish stocks, which have been dealing with decreases in sustainable exploitation over the past decades (FAO, 2020). While production of fishmeal from wild caught fish and its inclusion in commercial diets has been decreased, production of feed for aquaculture is expected to increase by 75 % by 2025 compared to production in 2015 (Tacon & Metian, 2015). With feed formulations currently used, amounts of sustainably caught small pelagic fish cannot meet this demand as their availability has not increased in decades (Hua et al., 2019). Additionally, prices of fishmeal increased significantly over the past decades and are expected to stay at a high level due to the rising demand. As feed accounts for about 60 % of operating costs in aquaculture (Daniel,

2018), inclusion levels of fishmeal in diets for aquaculture have to decrease not only from an ecological standpoint but also due to economic concerns.

### **Rapeseed as an alternative**

Plant protein products have been extensively investigated as alternatives for fishmeal in fish feed in the past and continue to be of major interest for fish nutrition (Bonaldo et al., 2011; Bu et al., 2018; Burel et al., 2000b; Burel et al., 2000c; Higgs et al., 1982; Luo et al., 2012b; Men et al., 2014; Nagel et al., 2012; Thiessen et al., 2003, 2004). Global availability and a promising protein content and amino acid composition led to rapeseed protein products being regarded as potential replacements for fishmeal (Higgs et al., 1982; Luo et al., 2012a; Mwachireya et al., 1999; Suárez et al., 2009). Global production of rapeseed meal reached 40.5 million metric tons in 2017 and ranked second behind soybean meal in most widely traded protein ingredients (Dossou et al., 2018). In addition to these advantages, rapeseed protein products have a sustainable origin, being a by-product of the rapeseed oil industry. However, rapeseed protein products like meals and oilcakes as well as more purified products like concentrates and isolates had impaired nutritional quality when being included in diets for multiple species of fish in comparison with fishmeal (Burel et al., 2000a; Burel et al., 2000b; Burel et al., 2000c; Nagel et al., 2012; Slawski, 2012). These limitations led to reduced diet acceptance, decreased digestibility of nutrients as well as health issues and were considered to be mainly caused by various anti-nutritive factors (ANFs) present in rapeseed (Bu et al., 2018; Burel et al., 2000a; Burel et al., 2000c; Francis et al., 2001; Mwachireya et al., 1999). Additionally, rapeseed is limited in some amino acids compared with fishmeal, which could potentially affect protein utilization and subsequently growth performance of fish (Cheng et al., 2010; Luo et al., 2012a; Men et al., 2014).

### **Anti-nutritive factors**

Glucosinolates, phenolic compounds like sinapic acid and its esters, phytic acid, protease inhibitors and tannins are commonly present in rapeseed and their presence can lead to a negatively affected fish growth performance (Francis et al., 2001; von Danwitz & Schulz, 2020). Additionally, indigestible carbohydrates are considered to be of major importance for digestibility of nutrients (Greiling et al., 2018; Mwachireya et al., 1999).

Glucosinolates are secondary plant metabolites present in plants of the *Brassicaceae* family and other species. Myrosinase (thioglucosylase), an endogenous enzyme, is responsible for the degradation of glucosinolates into their breakdown products. These degradation products include nitriles, isothiocyanates, thiocyanates, epithionitriles and vinyl oxazolidinethiones (Possenti et al., 2017). Glucosinolates and their breakdown products were made responsible for impairment of diet palatability, tissue damage in liver and kidney as well as altering thyroid functions in different species of fish (Burel et al., 2000a; Burel et al., 2000c; Duncan, 1991; Hermann et al., 2016; Slawski, 2012). Rapeseed also contains various phenolic compounds, majorly consisting of sinapic acid and its esters (Shahidi & Naczek, 1992; Vuorela et al., 2005). Both substances were shown to exude bitter taste consequently reducing diet palatability (Naczek et al., 1998). Additionally, sinapine might reduce the digestibility of proteins (von Danwitz & Schulz, 2020). Phytic acid is the main storage form for phosphorus in rapeseed (50-85 % of total phosphorus in plants; Reddy et al., 1982), mostly existing as salts of Ca, Mg and K (Mills & Chong, 1977; Thompson, 1990). The presence of these phytates can lead to impaired digestibility of proteins and minerals in various species of fish as they can chelate with bi- and trivalent mineral ions and can form protein-phytate complexes, which are not digestible for non-ruminants (Francis et al., 2001; Richardson et al., 1985; Thompson, 1990). Various dietary ingredients with plant origin contain protease inhibitors and the potency of latter mentioned ANFs is contingent on its source and target enzyme (Norton, 1991). Rapeseed meal was shown to include trypsin inhibitors, which can potentially reduce protein digestibility (Francis et al., 2001; Kroghdal et al., 1994). Tannins occur in numerous plant species and are secondary compounds of different chemical structures (Francis et al., 2001). Similar to effects from phytic acid, tannins can reduce the digestibility of proteins and minerals by binding with aforementioned substances and they can affect the activity of digestive enzymes (Bell, 1993; Liener, 1989).

Processing methods for production of rapeseed protein products included techniques to reduce content of most ANFs (Adem, 2014; Slawski, 2012). Small amounts of trypsin inhibitors can be compensated by most fish and effects from other ANFs could be of greater importance (Francis et al., 2001). Rainbow trout for example were shown to elevate their enzyme secretion to counteract effects from trypsin inhibitors to some extent (Kroghdal et al., 1994). Similarly, glucosinolates seem to play a more prominent role in impairment of diet palatability in comparison with sinapine (Josefsson & Uppström, 1976). Additionally, von Danwitz and

Schulz (2020) recently demonstrated that dietary levels of sinapic acid comparable to those found in processed rapeseed protein products do not affect growth performance of turbot significantly. Tannins can effectively be removed by de-hulling of rapeseed seeds (Griffiths, 1991) and their dietary content should not have a significant impact on growth performance of fish when processed rapeseed protein products are being included in diets for fish (Adem, 2014). Content of glucosinolates and phytic acid in rapeseed protein products has been reduced as well, however, their inclusion is still considered to cause negative effects on growth performance and health of fish (Adem, 2014; Bu et al., 2018; Slawski, 2012; von Danwitz & Schulz, 2020).

### **Processing of rapeseed protein products**

Depending on origin and strain, rapeseed seeds contain 20-25 % protein (Yiu et al., 1982) and hulls comprise about 17-18 % of seed weight (Carré et al., 2016). About 90 % of the nitrogen content in rapeseed seeds is present in the form of two storage proteins (Eklund et al., 1971) and around 70 % of hulls can be total fiber on a defatted dry matter basis (Carré et al., 2016). The ratio between both storage proteins, namely napin (albumin) and cruciferin (globulin) can vary, also depending on strain (Adem, 2014). Levels of glucosinolates range between 10-15  $\mu\text{mol/g}$  in rapeseed varieties in Germany (Schumann, 2005) and rapeseed seeds can contain 2-4 % of phytic acid (Reddy et al., 1982).

The product of oil removal via cold or hot pressing is a rapeseed press cake or a rapeseed meal respectively. Press cakes contain 28-31 % protein and rapeseed meals have a protein content of 38-45 % (Östbring et al., 2019). These products are the basis for further processing to obtain protein concentrates or isolates. Four steps can be used to produce rapeseed meal, including conditioning, mechanical pressing, n-hexane solvent extraction and desolventization (Adem, 2014). These resemble the processing steps conventionally used for rapeseed oil removal and an additional step involving ethanol extraction subsequent to the n-hexane extraction can be implemented after conventional oil removal procedure to reduce content of ANFs. The resulting rapeseed meal typically contains reduced levels of glucosinolates due to the ethanol treatment, however, content of phytic acid can be elevated in comparison with seeds. Adem (2014) achieved a crude protein content of 41.6 % in rapeseed meal, containing 3.0  $\mu\text{mol/g}$  glucosinolates and 3.3 % phytic acid. These values were comparable to rapeseed meals used in other literature (Burel et al., 2000a; Cheng et al., 2010). The content of total fiber from a



commercial ethanol-treated rapeseed meal can be around 33 % (Ivanova et al., 2017). Conditioning of rapeseed seeds at high temperatures is the first key step to reduce effects from ANFs, as myrosinase can be deactivated with heat (Björkman & Lönnerdal, 1973). Inclusion of glucosinolates and phytic acid in rapeseed meal, however, is still high in comparison with the original seeds at this processing point.

Further processing of rapeseed meals can include extraction, separation, filtration and drying, resulting in the production of a rapeseed protein concentrate. These processing steps elevate protein content and reduce levels of ANFs in resulting product. This processing protocol was used in Adem (2014) and resulted in elevation of crude protein content from 41.6 % in rapeseed meal to 71.2 % in rapeseed protein concentrate and reduction of nitrogen-free extracts (NfE) plus crude fiber from 46.7 % to 12.1 %. Content of tannins decreased from about 4 % in rapeseed meal to 0.43 % in rapeseed protein concentrate while levels of sinapic acid and sinapine were reduced to 0.01 %, demonstrating the effective removal of phenolic compounds and tannins with applied methods. Glucosinolate content was reduced (from 3.03  $\mu\text{mol/g}$  in rapeseed meal to 1.32  $\mu\text{mol/g}$  in protein concentrate) as well as content of phytic acid (from 3.34 % to 1.77 %; Adem, 2014). The achieved composition of rapeseed protein concentrate is comparable to rapeseed protein concentrates used in different studies (Hermann et al., 2016; Higgs et al., 1994; Nagel et al., 2017; Slawski, 2012).

### **Breeding of rapeseed**

Breeding rapeseed for reduced glucosinolate content has always been a major breeding goal. Glucosinolate levels have been reduced from 60-100  $\mu\text{mol/g}$  from early rapeseed cultivars to the modern day low-glucosinolate varieties containing <25  $\mu\text{mol/g}$  in seeds (Snowdon et al., 2007; Wittkop et al., 2009). The introduction of the "00" cultivars which were low in erucic acid and glucosinolates was one noteworthy product of breeding efforts in the past and the Polish variety "Bronowski" has been the genetic source for reduced glucosinolate content in seeds of commercially bred rapeseed cultivars. Although some environmental factors can influence glucosinolate content in plants, genetic variation determines content of glucosinolates most dominantly (Verkerk et al., 2009). Further reduction of glucosinolate content in new rapeseed varieties could aid in the development of a more cost-effective rapeseed protein product, since processing of rapeseed seeds to obtain a high-quality product with low content of ANFs is currently connected to major economic expenses.

*Arabidopsis thaliana* has been utilized as a model species to improve the understanding of the biosynthesis pathway of glucosinolates. This led to the identification of various genes involved in biosynthesis of these ANFs (Verkerk et al., 2009). The profile of glucosinolates could be altered through genetic variation at different glucosinolate loci, while their amount can be influenced via allelic variation at the quantitative trait loci (QTLs; Verkerk et al., 2009). Previous literature has demonstrated for instance that three major QTLs on chromosome A9, C2 and C9 control the content of glucosinolates (Harper et al., 2012; Howell et al., 2003; Li et al., 2014; Lu et al., 2014; Qu et al., 2017). Gene products of the CYP79 family play a major role in the first steps of biosynthesis where a primary or chain elongated amino acid is converted to an aldoxime (Verkerk et al., 2009). Alternatively, content of methionine-derived glucosinolates could be influenced by MAM genes, as the entry of methionine into the pathway could be dependent on the activity of these genes (de Quiros et al., 2000; Sarikamis et al., 2006). Additionally, the transcription factor MYB28 is responsible for the biosynthesis of aliphatic glucosinolates in *Arabidopsis* and the removal of its orthologs in rapeseed led to a reduction in glucosinolate content (Wang et al., 2018). Although multiple candidate genes potentially responsible for glucosinolate biosynthesis have been identified in *Brassica* species, the coherence between alteration in glucosinolate content and sequence variation of most candidate genes has not been shown yet (Wang et al., 2018). During the course of this project, new candidate genes have been tested to demonstrate their association with glucosinolate variation and the results will be included in a different thesis.

### **Aim of the thesis**

The aim of this study was to determine if, in comparison to previous processed rapeseed protein products, further reduction of glucosinolates and phytic acid content together with reduced content of other substances like fiber and NfE via improved processing techniques is an effective method to enhance the nutritional value of these products as fishmeal alternatives in diets for carnivorous fish. Determining which factors still limit the use of rapeseed protein products could improve their effectiveness in replacing fishmeal in diets for carnivorous fish in the future.

### Chapter 1

Supplementation of two major ANFs, namely glucosinolates and phytic acid was applied to determine threshold concentrations of both ANFs in diets for rainbow trout (*Oncorhynchus mykiss*). Effects on growth performance, health status and thyroid hormone levels were taken into consideration. Results should give insight on what concentrations of both ANFs can be tolerated by rainbow trout.

### Chapter 2

Suitability of a highly purified rapeseed protein isolate with limited amounts of fiber, glucosinolates, NfE and phytic acid was tested as an alternative for fishmeal in diets for rainbow trout (*Oncorhynchus mykiss*). Nutrient digestibility was determined as a key factor for the potential of rapeseed protein products. In the subsequent growth trial, effects on overall growth performance as well as on health status were investigated.

### Chapter 3

A highly purified rapeseed protein isolate with a similar phytic acid content but a reduced content of glucosinolates and a differing amino acid profile in comparison to the isolate in chapter 2 was tested as an alternative for fishmeal in diets for rainbow trout (*Oncorhynchus mykiss*). Processing techniques applied in production of this rapeseed protein isolate decreased costs of production and reduced content of ash, fiber and NfE to a minimal amount. Consequently, nutrient digestibility was determined in chapter 3 as well. Furthermore, an encapsulation technique of experimental diets as well as supplementation of a diet with free amino acids was utilized to improve growth performance of rainbow trout in the subsequent growth trial.

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## Chapter 1

### Effects of supplemented anti-nutritive substances from rapeseed on growth and health performance of rainbow trout (*Oncorhynchus mykiss*)

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**Abstract**

Inclusion of certain plant protein products in fish feed is limited by multiple anti-nutritive factors (ANF) present in plants. Since these factors negatively affect growth performance and health of fish, the aim of this study was to investigate threshold concentrations for negative effects and to determine if a specific factor could be responsible for reduced growth and health. Eight identical basal diets including rapeseed protein concentrate were supplemented with five tiered levels of phytic acid (PA) or three tiered levels of glucosinolates (GLS) respectively. One basal rapeseed diet served as control. Diets were fed twice per day to apparent satiation to triplicate groups of juvenile rainbow trout (*Oncorhynchus mykiss*). After 38 and 56 days of feeding, growth performance as well as health parameters were analyzed for diets containing glucosinolates (38 feeding days) and phytic acid (56 feeding days), respectively. Performance and health parameters were determined for control group at both sampling times. Dietary phytic acid showed significant effects on growth performance and whole body composition. The lowest concentration of supplemented phytic acid (1.0 %) significantly improved overall growth performance while higher inclusion percentages significantly decreased voluntary feed intake and subsequently growth performance. Supplementation with glucosinolates had no significant effect on any of the investigated parameters.

## 1. Introduction

Fishmeal has been the main protein source in fish feed for several decades and continues to be of major importance today (FAO, 2018; Hardy, 2010; Luo et al., 2008). Due to an increasing demand and stagnating production, fishmeal prices are expected to increase from now to 2030 by 20 % (FAO, 2018). Proteins originating from different plants have been extensively studied as alternatives for fishmeal in aquaculture feeds for many years, including corn (Men et al., 2014), lupine (Burel et al., 2000b; Burel et al., 2000c), pea (Burel et al., 2000c; Thiessen et al., 2003), rapeseed (Bu et al., 2018; Higgs et al., 1982; Luo et al., 2012b; Nagel et al., 2012), soybean (Bonaldo et al., 2011) and wheat gluten (Bonaldo et al., 2011). Due to high availability (Suárez et al., 2009) and a favorable amino acid composition in comparison to other plant proteins (Higgs et al., 1982; Luo et al., 2012a; Mwachireya et al., 1999), rapeseed protein products are regarded to be a promising alternative to fishmeal in fish feed. However, presence of anti-nutritive factors (ANFs) limits the use of rapeseed protein in fish diets. These factors, including complex carbohydrates, glucosinolates, fiber, phytic acid, sinapine and others, potentially caused negative effects like reduced feed intake (Bu et al., 2018; Burel et al., 2000b; Nagel et al., 2012; Slawski, 2012), lowered digestibility of nutrients (Burel et al., 2000c; Francis et al., 2001; Mwachireya et al., 1999) and health issues (Francis et al., 2001) in different species of fish.

Glucosinolates (GLS) are secondary plant metabolites and the main ANFs in rapeseed (Duncan, 1991). After cell rupture, in presence of the enzyme myrosinase, GLS are being broken down into substances including nitriles, isothiocyanates, thiocyanates and others (Duncan, 1991; Possenti et al., 2017). GLS and their degradation products are reported to be responsible for causing a bitter taste in diets (Nagel et al., 2012; Slawski, 2012; Tan et al., 2011; Tripathi & Mishra, 2007), tissue damage to liver and kidney in animals (Duncan, 1991; Griffiths et al., 1998) and alteration of thyroid activity in fish (Burel et al., 2000a; De Pedro et al., 2003; Hermann et al., 2016).

Phytic acid (PA; myo-inositol 1,2,3,4,5,6-hexakis [dihydrogen phosphate]) is the main storage form for phosphorus in rapeseed (Reddy et al., 1982), mostly existing as salts of Ca, Mg and K (Mills & Chong, 1977; Thompson, 1990). Consequently, rapeseed meal can contain relatively high amounts of latter mentioned phytate salts (Bedford, 2000; Francis et al., 2001; Ravindran et al., 1999) compared to other plant based meals, causing multiple negative effects when

rapeseed is included in diets for aquatic animals. These effects include reduction of availability of phosphorus from rapeseed protein (Liener, 1989), impairment of protein digestibility (Richardson et al., 1985) and lowered availability of bi- and trivalent mineral ions (Sugiura et al., 2001; Thompson, 1990).

Content of both GLS and phytic acid was reduced via different processing and treatment methods over the last decades (Adem, 2014; Kalaydzhiev et al., 2019; Shahidi & Naczki, 1990). Nevertheless, low inclusion levels of both ANFs putatively caused negative effects on growth performance of fish (Nagel et al., 2012; Slawski, 2012). Research about effects of single ANF was done in the past (Denstadli et al., 2006; Hossain & Jauncey, 1988; Richardson et al., 1985; von Danwitz & Schulz, 2020), however, results are inconsistent and could not clarify precisely which inclusion levels are mainly responsible for reduced growth performance in fish. Consequently, this study aimed towards clarifying if a single ANF of those commonly present in rapeseed protein products, namely GLS and phytic acid, could be responsible for impaired growth performance and health status in rainbow trout and at which dietary concentration negative effects could occur. Both ANFs caused significant effects on growth performance in rainbow trout in previous studies (Francis et al., 2001) and both can be further reduced via processing (Kalaydzhiev et al., 2019; Shahidi & Naczki, 1990) and/or possibly breeding. This leads to a great potential for improving rapeseed protein products for future use in aquaculture. Information about effects of these specific ANFs and their resulting threshold concentrations can be crucial for future breeding and processing of rapeseed plants. Both breeding and processing are critical steps in the development of a nutritious and cost efficient alternative for fishmeal.

## 2. Materials and methods

### 2.1 Experimental setup

Juvenile rainbow trout (*Oncorhynchus mykiss*, mean = 14.6 g  $\pm$  standard deviation = 4.2 g) were obtained from Forellenzucht Troststadt GmbH & Co. KG, Troststadt, Germany. Fish were fed a commercial trout diet (Aller Gold, 2 mm) for 14 days while being adapted to the aquaculture facilities of the Gesellschaft für Marine Aquakultur mbH, Büsum, Germany. Prior to the experiment, fish were starved for two days and distributed in triplicate groups of 20 fish per tank (50 l). Distribution of groups was semi-random to avoid potential tank effects.

Water parameters were measured daily to ensure an optimal rearing environment for fish. Temperature during the experiment was held at  $12.8 \pm 0.8$  °C. The oxygen saturation was  $107.0 \pm 3.5$  % (Handy Polaris, Oxy Guard International A/S, Birkerød, Denmark), pH  $7.5 \pm 0.3$  (pH-Messgerät GMH 5550, GHM Messgerät GmbH, Regenstauf, Germany), salinity  $3.0 \pm 0.7$  ppt (HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA), total ammonia nitrogen (TAN)  $0.2 \pm 0.2$  mg l<sup>-1</sup> and total nitrite nitrogen  $0.5 \pm 0.1$  mg l<sup>-1</sup> (Microquant test kits for NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup>, Merck KGaA, Darmstadt, Germany).

During the feeding trial, fish were fed manually twice per day until apparent satiation for 38 or 56 days depending on treatment. Since acquisition of high quality glucosinolates from rapeseed proved to be challenging and was coupled with extreme financial expenses, feeding of glucosinolate-groups had to be terminated early (38 days of feeding). Feeding of remaining control and phytic acid treatments continued for 56 days. After 38 days of feeding, samples were taken from all treatments and feeding continued with 14 fish per tank for control group and phytic acid treatments. After each feeding event, excess pellets were collected and counted. An average pellet weight was measured prior to the feeding trial to calculate the exact feed intake.

### 2.2 Diet formulation and preparation

Nine diets were formulated and processed for the fish feeding trial. All diets were based on a diet with high rapeseed protein concentrate levels and only differed in type and amount of supplemented anti-nutritive substances. All diets were formulated to be isonitrogenous and isoenergetic as well as to meet the nutritional requirements of rainbow trout (*Oncorhynchus mykiss*, table 1.1, 1.2 and 1.3). The rapeseed protein concentrate was produced by Pilot

Pflanzenöltechnologie Magdeburg e.V., Magdeburg, Germany according to methods applied in Adem (2014), with a differing filtration method. Diafiltration was used to reduce the salt content in extraction solution until a conductivity between 4 and 5 mS/cm was reached. Resulting solution was spray dried.

Eight diets were supplemented with either phytic acid (Phytic Acid solution, 50 % in H<sub>2</sub>O, Sigma-Aldrich, St. Louis, USA) or a mixture of glucosinolates isolated from *Brassica napus* (Rapeseed, glucosinolate mixture, Cfm Oskar Tropitzsch GmbH, Marktrechwitz, Germany). Added amount of either ANF replaced the same amount of cellulose in the basal diet. The basal rapeseed protein concentrate diet without supplementation was used as the control diet (CD RP). Due to inconsistent results in literature with different concentrations of both ANFs, a wide range of concentrations was used for either ANF, however, this range still remained within relevant dimensions for rapeseed protein used in fish feed. Five different concentrations of phytic acid ranging from 1.0 to 2.6 % (PA1, PA2, PA3, PA4, PA5, table 1.4) were added to the diets. The remaining three diets were supplemented with glucosinolates in concentrations from 0.8 to 3.2 µmol/g (GLS1, GLS2, GLS3, table 1.4).

The anti-nutritive substance for each diet was dissolved in 100 ml distilled water and sprayed evenly over homogeneously premixed mealy feed ingredients while mixing was continued. After lipids were added, each mixture was pressed to pellets of 2 mm in diameter (L 14-175, AMANDUS KAHL, Reinbeck, Germany). To prevent any thermic damage to the ingredients, a temperature of 50 °C was not exceeded during processing of diets. Pellets were air-dried and subsequently stored at 4 °C.



Table 1.1 Feed composition of basal diet.

<b>Ingredient (% DM)</b>	<b>Basal diet rapeseed</b>
<b>Fishmeal<sup>1</sup></b>	13.0
<b>Rapeseed protein concentrate<sup>2</sup></b>	19.0
<b>Blood meal<sup>3</sup></b>	5.0
<b>Gelatine<sup>4</sup></b>	3.0
<b>Pea protein isolate<sup>5</sup></b>	14.3
<b>Wheat gluten<sup>6</sup></b>	6.7
<b>Wheat starch<sup>6</sup></b>	23.0
<b>Rapeseed oil<sup>7</sup></b>	5.0
<b>Fish oil<sup>1</sup></b>	6.0
<b>Vitamin premix<sup>5</sup></b>	0.5
<b>Mineral premix<sup>5</sup></b>	0.4
<b>Ca<sub>2</sub>PO<sub>4</sub><sup>8</sup></b>	0.5
<b>Cellulose<sup>9</sup></b>	3.6

<sup>1</sup> Bioceval GmbH & Co. KG, Cuxhaven; Germany; <sup>2</sup> Pilot Pflanzenöltechnologie Magdeburg e. V., Magdeburg, Germany; <sup>3</sup> Saria SE & Co. KG, Selm, Germany; <sup>4</sup> Gustav Ehlert GmbH & Co. KG, Verl, Germany; <sup>5</sup> Emsland-Aller Aqua GmbH, Golßen, Germany; <sup>6</sup> Kröner-Stärke GmbH, Ibbenbüren, Germany; <sup>7</sup> Cargill GmbH, Riesa, Germany; <sup>8</sup> Lehmann & Voss & Co. KG, Hamburg, Germany; <sup>9</sup> Mikro-Technik GmbH & Co. KG, Bürgstadt, Germany.

Table 1.2 amino acid composition in basal diet rapeseed.

<b>Amino acid composition (% DM)<sup>1</sup></b>	<b>CD RP</b>	<b>Requirements<sup>2</sup></b>
<b>Arginine</b>	3.4	1.4 - 4
<b>Histidine</b>	1.3	0.5 - 0.6
<b>Isoleucine</b>	2.0	0.7 - 1.4
<b>Leucine</b>	3.7	1.1 - 1.4
<b>Lysine</b>	2.8	1.4 - 2.5
<b>Methionine</b>	0.7	0.4 - 0.9
<b>Phenylalanine</b>	2.2	0.7
<b>Threonine</b>	1.8	1.0
<b>Valine</b>	2.3	0.8 - 1.6

<sup>1</sup> based on analysis of feed ingredients; <sup>2</sup> according to National Research Council (2011).

Table 1.3 Analyzed composition of experimental diets.

Feed composition (% DM)	CD RP	PA1	PA2	PA3	PA4	PA5	GLS1	GLS2	GLS3
Moisture	7.3	8.3	8.3	8.1	8.1	9.1	7.9	7.7	7.9
Crude protein	54.6	54.4	54.0	53.5	53.6	52.5	54.9	55.0	54.0
Crude lipid	14.5	14.3	14.1	14.4	14.3	14.2	14.5	14.5	14.4
Crude ash	7.8	8.0	8.2	8.4	8.5	8.8	7.8	7.8	7.8
NfE + crude fiber <sup>1</sup>	23.2	23.2	23.7	23.8	23.5	24.5	22.8	22.7	23.8
Gross energy (MJ kg <sup>-1</sup> )	22.5	22.2	22.1	22.1	21.9	21.9	22.3	22.4	22.3

<sup>1</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash).

Table 1.4 Amount of anti-nutritive substances in experimental diets.

Anti-nutritive factor	CD RP	PA1	PA2	PA3	PA4	PA5	GLS1	GLS2	GLS3
Phytic acid added (% of feed) <sup>1</sup>	0	1.0	1.4	1.8	2.2	2.6	0	0	0
Phytic acid (% analyzed) <sup>2</sup>	0.7	1.7	2	2.6	2.8	3.0	0.6	0.7	0.6
Glucosinolates added ( $\mu\text{mol/g}$ of feed) <sup>1</sup>	0	0	0	0	0	0	0.8	1.6	3.2
Glucosinolates ( $\mu\text{mol/g}$ analyzed) <sup>3</sup>	0.05	0.05	0.06	0.04	0.05	0.05	0.5	1.0	1.7

<sup>1</sup> discrepancy between added and analyzed content of ANFs was most likely due to losses during supplementation process; <sup>2</sup> analyzed using phytic acid enzyme test (Megazyme Kit) and high performance liquid chromatography (HPLC) according to Sashidhar et al. (2020); <sup>3</sup> determined via enzyme test (myrosinase/glucose test kit according to Heaney et al., 1988) and HPLC (desulfatase C18 RP column according to Fiebig & Arens, 1992, UV detection of 15 compounds).

### 2.3 Samplings

After the initial adaptation and prior to the experiment, 15 fish were randomly selected for analysis of initial whole body composition and calculation of hepatosomatic index (HSI), viscerosomatic index (VSI), spleen somatic index (SSI) and fulton condition factor (FCF). These factors were also determined for glucosinolate treatments plus control and later for phytic acid treatments and control, in each case for three fish per tank. Fish were anaesthetized with a blunt hit on the central nervous system and subsequently euthanized by piercing the heart. In addition, blood was sampled with syringes at the caudal vein from three fish per tank for analysis of thyroidal hormone concentrations for glucosinolate treatments and control. Blood samples were centrifuged and the plasma was stored on ice until deep freezing at -80 °C. Three fish per tank were used for histological analysis of liver, spleen and gut health of all treatments after 38 days of feeding. All fish were starved for 48 hours before sampling. All samples for

whole body composition were freeze dried (Alpha 1-2 LD plus, Christ, Osterode, Germany) and stored at -20 °C until analyses.

#### 2.4 Analysis of nutrients, anti-nutritive factors and thyroidal hormones

Macro nutrients of diets and whole body were analyzed according to the European Commission Regulation (EC) No 152/2009 (European Commission, 2009). Crude protein was determined by standard Kjeldahl methods while methods according to the Soxhlet-protocol were used to determine crude lipid content of samples (Soxtherm, C. Gerhardt GmbH & Co. KG, Königswinter, Germany). A combustion oven was used for the determination of crude ash content at 550 °C for 12 hours (P300, Nabertherm, Lilienthal, Germany). Dry matter content was determined by drying samples for 4 hours at 103 °C (ED 53, Binder GmbH, Tuttlingen, Germany). Remaining portion was defined as nitrogen-free extracts (NfE) plus crude fiber.

Amino acid concentration in feed ingredients was analyzed by LUFA-ITL GmbH, Kiel, Germany according to the regulation of the European Commission Regulation (EC) No 152/2009, III, F: 2009-02 (European Commission, 2009).

Phytic acid content in rapeseed protein and diets was analyzed using phytic acid enzyme test and high performance liquid chromatography (HPLC) according to methods used by Sashidhar et al. (2020). Glucosinolate content in rapeseed protein and diets was determined via enzyme test (myrosinase/glucose test kit according to Heaney et al., 1988) and HPLC (desulfatase C18 RP column according to Fiebig & Arens, 1992, UV detection of 15 compounds).

Solid phase enzyme-linked immunosorbent assays (ELISA) based on the principle of a competitive binding were used to measure total triiodothyronine (T3) levels in plasma samples (IBL International GmbH, Hamburg, Germany). Afterwards, hormone levels were measured photometrically at 450 nm.

#### 2.5 Histological analysis

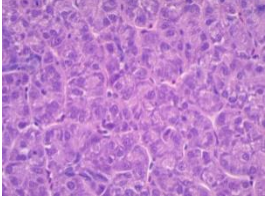
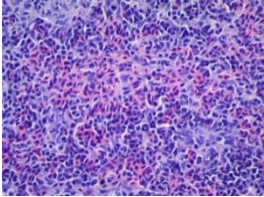
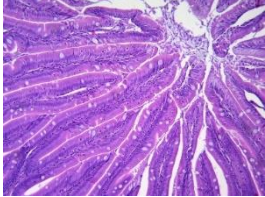
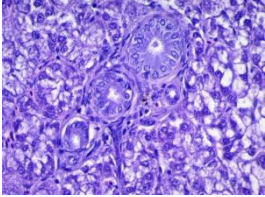
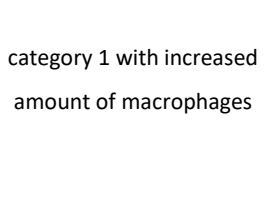
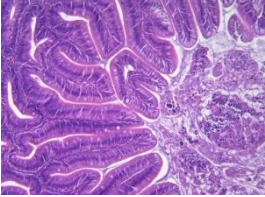
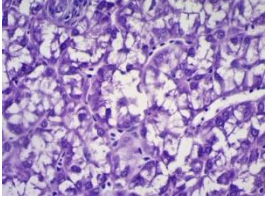
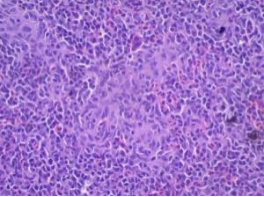
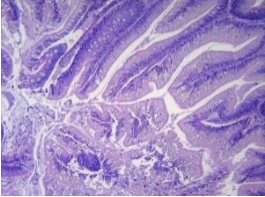
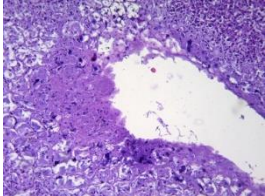
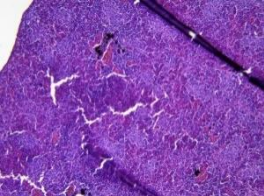
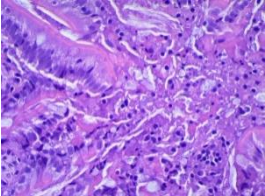
Organ samples were taken from liver, spleen and intestine and were subsequently fixed with 4 % buffered formalin solution. Every sample was dehydrated and embedded in paraffin, cut in 4 µm thick slices and stained with haematoxylin and eosin according to routine methods of the histopathological surgery, Bleyer and Gruber-Dujardin, Göttingen, Germany. Examination of samples was done randomly with a light microscope (Primo Star, Carl Zeiss Microscopy GmbH, Göttingen, Germany). An ordinal ranking was assigned to each slide, based on

categories shown in table 1.5. The median was subsequently used for statistical comparison among treatments. Analysis was done according to modified methods from Bruce et al. (2018).

## 2.6 Statistical analysis

All data analysis was done using SPSS (Version 20). The Kolmogorov-Smirnov test and the Levene test were used to determine normal distribution and homogeneity of variances of data respectively. Depending on results from Kolmogorov-Smirnov and Levene test, data was either analyzed using an analysis of variances (ANOVA) or the Kruskal-Wallis test. Multiple comparisons among means were made using Tukey`s HSD test. Kruskal-Wallis test was also applied for comparison of medians. The aggregate type I error was defined at 5 % ( $p < .05$ ) for each set of comparisons to determine statistical significance.

Table 1.5 Histological scoring system with sample picture for each examined organ. Images were taken in either 100x or 400x magnification.

Category	Liver	Spleen	Intestine
1	Mild contents of glycogen, no pathohistological changes 	No pathohistological changes, decreased amounts of melanomacrophages 	No pathohistological changes; mild amounts of cell detritus and mucus in enteral lumen; small multifocal absorptive vacuoles in enterocytes 
2	Moderate contents of glycogen, minor fat deposition; no histopathological changes 	No histopathological changes, normal amount of melanomacrophages  category 1 with increased amount of macrophages 	Moderate amounts of cell detritus and mucus in enteral lumen, moderate diffuse absorptive vacuoles in enterocytes 
3	Severe contents of glycogen, major fat deposition, no other histopathological changes 	Mild, diffuse lymphocytic depletion, medium amount of melanomacrophages 	Severe amounts of cell detritus and mucus in enteral lumen, severe diffuse absorptive vacuoles in enterocytes 
4	Severe amounts of glycogen and fat deposits with focal to multifocal corresponding necrosis or inflammation, perivascular inflammatory cell infiltration, oedema and/or necrosis 	Mild and diffuse follicular hyperplasia with increased amounts of melanomacrophages 	Severe amounts of cell detritus and mucus in enteral lumen, severe diffuse absorptive vacuoles in enterocytes, villi are focally ruptured with inflammatory cell infiltration 

### 3. Results

#### 3.1 Growth performance

No significant differences were observed when comparing GLS treatments to the CD RP in any of the growth parameter factors (table 1.6). Despite the shortened experimental period, all treatments at least tripled their weight after 38 days of feeding.

PA1 had a significantly increased FBW in comparison with CD RP. With increasing inclusion levels of phytic acid, FBW decreased (table 1.7). FBW of PA5 was significantly lower than of all other dietary groups. Significant differences in feed conversion ratio were observed between PA4 and CD RP, where PA4 had a lower FCR than the rapeseed protein control group. Specific growth rate of PA1 and PA2 was significantly increased while SGR of PA5 was significantly decreased when compared to CD RP. Similar to FBW and SGR, daily feed intake decreased with increasing amount of dietary phytic acid, with significant differences between RP CD and the two highest phytic acid groups (PA4 and PA5). Dietary groups PA2, PA3, PA4 and PA5 had significantly increased levels of PER compared to control group. Similar to results in PER, levels of PRE were elevated in PA groups; PRE was significantly higher in PA3 and PA4 compared with control (table 1.7).

Table 1.6 Growth performance parameters of fish fed glucosinolates and control diets (mean  $\pm$  standard deviation (SD), n=3).

	CD RP	GLS1	GLS2	GLS3
<b>IBW</b>	12.2 $\pm$ 0.1	12.3 $\pm$ 0.1	12.2 $\pm$ 0.1	12.2 $\pm$ 0.2
<b>FBW</b>	37.0 $\pm$ 1.3	38.3 $\pm$ 0.4	36.8 $\pm$ 0.2	37.9 $\pm$ 0.5
<b>FCR</b>	0.87 $\pm$ 0.01	0.90 $\pm$ 0.01	0.93 $\pm$ 0.04	0.86 $\pm$ 0.02
<b>SGR</b>	2.91 $\pm$ 0.08	3.00 $\pm$ 0.04	2.92 $\pm$ 0.01	2.99 $\pm$ 0.02
<b>DFI</b>	2.55 $\pm$ 0.07	2.70 $\pm$ 0.04	2.70 $\pm$ 0.11	2.58 $\pm$ 0.07
<b>PER</b>	2.26 $\pm$ 0.02	2.19 $\pm$ 0.02	2.14 $\pm$ 0.09	2.33 $\pm$ 0.06
<b>PRE</b>	33.8 $\pm$ 0.6	32.9 $\pm$ 0.4	31.4 $\pm$ 2.1	33.6 $\pm$ 1.3

IBW (initial body weight); FBW (final body weight); FCR (feed conversion ratio) = g feed intake / g weight gain; SGR (specific growth rate) = (ln (FBW) – ln (IBW))/ feeding days \* 100; DFI (daily feed intake) = daily feed intake in % body weight; PER (protein efficiency ratio) = g body weight gain / g crude protein intake; PRE (protein retention efficiency) = g crude protein gained / g crude protein intake \*100. Values in the same row without superscript letters are not significantly different ( $P > .05$ ).

Table 1.7 Growth performance parameters of fish fed phytic acid and control diets (mean  $\pm$  SD, n=3).

	CD RP	PA1	PA2	PA3	PA4	PA5
<b>IBW</b>	12.2 $\pm$ 0.2	12.2 $\pm$ 0.1	12.2 $\pm$ 0.1	12.3 $\pm$ 0.5	12.3 $\pm$ 0.2	12.2 $\pm$ 0.1
<b>FBW</b>	54.5 $\pm$ 2.7 <sup>bc</sup>	63.0 $\pm$ 1.3 <sup>a</sup>	61.4 $\pm$ 1.5 <sup>ab</sup>	55.8 $\pm$ 5.3 <sup>bc</sup>	52.4 $\pm$ 3.0 <sup>c</sup>	42.6 $\pm$ 3.0 <sup>d</sup>
<b>FCR</b>	0.87 $\pm$ 0.03 <sup>b</sup>	0.84 $\pm$ 0.03 <sup>ab</sup>	0.82 $\pm$ 0.02 <sup>ab</sup>	0.81 $\pm$ 0.03 <sup>ab</sup>	0.78 $\pm$ 0.01 <sup>a</sup>	0.81 $\pm$ 0.01 <sup>ab</sup>
<b>SGR</b>	2.67 $\pm$ 0.08 <sup>c</sup>	2.93 $\pm$ 0.05 <sup>a</sup>	2.88 $\pm$ 0.04 <sup>ab</sup>	2.70 $\pm$ 0.09 <sup>bc</sup>	2.58 $\pm$ 0.07 <sup>c</sup>	2.22 $\pm$ 0.10 <sup>d</sup>
<b>DFI</b>	2.32 $\pm$ 0.09 <sup>bc</sup>	2.46 $\pm$ 0.10 <sup>ab</sup>	2.35 $\pm$ 0.07 <sup>bc</sup>	2.20 $\pm$ 0.09 <sup>cd</sup>	2.02 $\pm$ 0.03 <sup>d</sup>	1.81 $\pm$ 0.08 <sup>e</sup>
<b>PER</b>	2.27 $\pm$ 0.08 <sup>a</sup>	2.40 $\pm$ 0.10 <sup>ab</sup>	2.47 $\pm$ 0.08 <sup>bc</sup>	2.50 $\pm$ 0.10 <sup>bc</sup>	2.60 $\pm$ 0.03 <sup>c</sup>	2.57 $\pm$ 0.04 <sup>bc</sup>
<b>PRE</b>	36.9 $\pm$ 0.8 <sup>a</sup>	38.9 $\pm$ 1.0 <sup>ab</sup>	40.1 $\pm$ 1.1 <sup>ab</sup>	40.4 $\pm$ 0.5 <sup>b</sup>	40.4 $\pm$ 1.0 <sup>b</sup>	40.1 $\pm$ 0.2 <sup>ab</sup>

IBW (initial body weight); FBW (final body weight); FCR (feed conversion ratio) = g feed intake / g weight gain; SGR (specific growth rate) = (ln (FBW) – ln (IBW)) / feeding days \* 100; DFI (daily feed intake) = daily feed intake in % body weight; PER (protein efficiency ratio) = g body weight gain / g crude protein intake; PRE (protein retention efficiency) = g crude protein gained / g crude protein intake \* 100. Values in the same row with different superscript letters are significantly different ( $P < .05$ ).

### 3.2 Body composition

No significant differences in whole body composition were observed among glucosinolate treatments and control (table 1.8).

Table 1.8 Proximate whole body composition of fish fed glucosinolate treatments and control (mean  $\pm$  SD, n=3).

(% OS)	CD RP	GLS1	GLS2	GLS3
<b>Moisture</b>	71.9 $\pm$ 0.9	71.6 $\pm$ 0.2	71.9 $\pm$ 0.5	71.6 $\pm$ 0.1
<b>Crude ash</b>	1.5 $\pm$ 0.1	1.4 $\pm$ 0.1	1.6 $\pm$ 0.1	1.6 $\pm$ 0.2
<b>Crude protein</b>	15.2 $\pm$ 0.2	15.2 $\pm$ 0.2	15.1 $\pm$ 0.3	14.8 $\pm$ 0.2
<b>Crude fat</b>	11.0 $\pm$ 0.7	11.2 $\pm$ 0.3	10.7 $\pm$ 0.6	11.5 $\pm$ 0.3
<b>NfE + crude fiber<sup>1</sup></b>	0.4 $\pm$ 0.2	0.5 $\pm$ 0.3	0.8 $\pm$ 0.1	0.6 $\pm$ 0.3
<b>Energy (MJ/kg)</b>	7.9 $\pm$ 0.4	8.1 $\pm$ 0.1	7.9 $\pm$ 0.2	8.1 $\pm$ 0.1

<sup>1</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash). Values in the same row without superscript letters are not significantly different ( $P > .05$ ).

Significant differences were observed in moisture, crude ash, crude fat and energy content between phytic acid treatments and control. Moisture content was elevated in PA5 differing significantly from all other treatments. CD RP had a decreased crude ash content compared with all other dietary phytic acid groups; these differences were significant in comparison with PA1, PA3, PA4 and PA5. PA5 differed significantly in comparison to all other groups in crude fat and energy content. Energy content of PA4 was significantly increased compared to group

PA5 and significantly lower than of all other dietary groups. Overall, crude fat and energy content followed trends in FBW, SGR and DFI. With increasing inclusion of phytic acid, crude fat and energy content decreased (table 1.9).

Table 1.9 Proximate whole body composition of fish fed phytic acid and control diets (mean  $\pm$  SD, n=3).

(% OS)	CD RP	PA1	PA2	PA3	PA4	PA5
<b>Moisture</b>	71.0 $\pm$ 0.2 <sup>bc</sup>	69.9 $\pm$ 0.5 <sup>a</sup>	70.4 $\pm$ 0.3 <sup>ab</sup>	71.0 $\pm$ 0.2 <sup>b</sup>	71.8 $\pm$ 0.2 <sup>c</sup>	74.3 $\pm$ 0.3 <sup>d</sup>
<b>Crude ash</b>	1.3 $\pm$ 0.0 <sup>a</sup>	1.5 $\pm$ 0.0 <sup>bc</sup>	1.5 $\pm$ 0.1 <sup>ab</sup>	1.5 $\pm$ 0.1 <sup>b</sup>	1.6 $\pm$ 0.1 <sup>bc</sup>	1.6 $\pm$ 0.0 <sup>c</sup>
<b>Crude protein</b>	15.6 $\pm$ 0.1	15.5 $\pm$ 0.2	15.4 $\pm$ 0.4	15.5 $\pm$ 0.1	15.2 $\pm$ 0.2	15.2 $\pm$ 0.2
<b>Crude fat</b>	11.8 $\pm$ 0.5 <sup>bc</sup>	12.8 $\pm$ 0.4 <sup>a</sup>	12.2 $\pm$ 0.3 <sup>ab</sup>	11.9 $\pm$ 0.2 <sup>ab</sup>	10.9 $\pm$ 0.3 <sup>c</sup>	8.7 $\pm$ 0.3 <sup>d</sup>
<b>NfE + crude fiber<sup>1</sup></b>	0.3 $\pm$ 0.3	0.2 $\pm$ 0.1	0.6 $\pm$ 0.1	0.2 $\pm$ 0.2	0.5 $\pm$ 0.3	0.2 $\pm$ 0.1
<b>Energy (MJ/kg)</b>	8.3 $\pm$ 0.1 <sup>b</sup>	8.7 $\pm$ 0.2 <sup>a</sup>	8.5 $\pm$ 0.0 <sup>ab</sup>	8.3 $\pm$ 0.1 <sup>b</sup>	7.9 $\pm$ 0.1 <sup>c</sup>	7.0 $\pm$ 0.2 <sup>d</sup>

<sup>1</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash). Values in the same row with different superscript letters are significantly different ( $P < .05$ ).

### 3.3 Health parameters

No significant differences were observed between glucosinolates and control treatments in any of the health parameters (table 1.10). FCF was significantly elevated in PA1 compared with PA5 (table 1.11).

Table 1.10 Health parameters of fish fed glucosinolates and control diets (mean  $\pm$  SD; VSI, HSI, SSI: n=9; FCF: n=18).

	CD RP	GLS1	GLS2	GLS3
<b>VSI</b>	17.4 $\pm$ 2.2	18.5 $\pm$ 2.3	18.2 $\pm$ 1.4	17.2 $\pm$ 1.9
<b>HSI</b>	2.4 $\pm$ 0.3	2.6 $\pm$ 0.4	2.8 $\pm$ 1.0	2.4 $\pm$ 0.3
<b>SSI</b>	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
<b>FCF</b>	1.4 $\pm$ 0.0	1.5 $\pm$ 0.0	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1

VSI (viscerosomatic index) = (g fish weight - g gutted fish weight) / g fish weight \* 100; HSI (hepatosomatic index) = g liver weight / g fish weight \* 100; SSI (spleen somatic index) = g spleen weight / g fish weight \* 100; FCF (fulton condition factor) = g fish weight \* 100 \* cm fish length<sup>-3</sup>. Values in the same row without superscript letters are not significantly different ( $P > .05$ ).



Table 1.11 Health parameters of fish fed phytic acid and control diets (mean  $\pm$  SD; VSI, HSI, SSI: n=9; FCF: n=18).

	CD RP	PA1	PA2	PA3	PA4	PA5
<b>VSI</b>	17.4 $\pm$ 2.5	17.5 $\pm$ 1.5	18.0 $\pm$ 1.7	18.0 $\pm$ 0.8	19.3 $\pm$ 1.8	19.2 $\pm$ 0.8
<b>HSI</b>	2.0 $\pm$ 0.2	1.9 $\pm$ 0.3	2.1 $\pm$ 0.2	2.1 $\pm$ 0.3	2.2 $\pm$ 0.4	2.2 $\pm$ 0.3
<b>SSI</b>	0.1 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
<b>FCF</b>	1.5 $\pm$ 0.1 <sup>ab</sup>	1.6 $\pm$ 0.1 <sup>a</sup>	1.5 $\pm$ 0.1 <sup>ab</sup>	1.5 $\pm$ 0.1 <sup>ab</sup>	1.5 $\pm$ 0.1 <sup>ab</sup>	1.4 $\pm$ 0.1 <sup>b</sup>

VSI (viscerosomatic index) = (g gutted fish weight / g fish weight) \* 100; HSI (hepatosomatic index) = (g liver weight / g fish weight) \* 100; SSI (spleen somatic index) = (g spleen weight / g fish weight) \* 100; FCF (fulton condition factor) = (g fish weight / cm fish length) \* 10. Values in the same row with different superscript letters are significantly different ( $P < .05$ ).

### 3.4 Thyroidal hormones

Statistical analysis of thyroidal hormones concentration showed no significant differences between glucosinolates and control groups (table 1.12).

Table 1.12 Thyroidal hormone concentrations in plasma of fish fed glucosinolates and control diets (mean  $\pm$  SD, n=9).

	CD RP	GLS1	GLS2	GLS3
<b>T3 (ng/ml)</b>	1.5 $\pm$ 1.5	0.8 $\pm$ 0.4	0.8 $\pm$ 0.4	1.9 $\pm$ 1.9

Values in the same row without superscript letters are not significantly different ( $P > .05$ ).

### 3.5 Histological analysis

There were no significant differences between any of the dietary treatments in analysis of histological samples (table 1.13). Overall, almost no severely damaged organs were observed in the samples examined during this study.

Table 1.13 Overall histological health status of liver, spleen and gut (median  $\pm$  median absolute deviation (MAD), n = 9) according to table 1.5.

Organ	CD RP	PA1	PA2	PA3	PA4	PA5	GLS1	GLS2	GLS3
<b>Liver</b>	1 $\pm$ 0	2 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 0	2 $\pm$ 1	3 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 1	3 $\pm$ 1
<b>Spleen</b>	2 $\pm$ 0	2 $\pm$ 1	2 $\pm$ 0	2 $\pm$ 0	2 $\pm$ 0	2 $\pm$ 1	2 $\pm$ 0	2 $\pm$ 0	2 $\pm$ 0
<b>Gut</b>	3 $\pm$ 1	2 $\pm$ 0	4 $\pm$ 0	3 $\pm$ 1	3 $\pm$ 1	3 $\pm$ 1	3 $\pm$ 1	3 $\pm$ 1	3 $\pm$ 1

Values in the same row without superscript letters are not significantly different ( $P > .05$ ).

## 4. Discussion

### 4.1 Growth performance

Previous studies have used a wide variety of different rapeseed protein products, consequently having many different concentrations and compositions of ANFs in produced diets (Burel et al., 2001; Burel et al., 2000b; Burr et al., 2013; Greiling et al., 2018; Nagel et al., 2012; Slawski, 2012). In addition to the impact of single compounds, there might also be synergistic effects between different ANFs. Therefore, it is challenging to determine exact thresholds of particular ANFs or to be definite if a certain ANF created a distinct negative impact on growth performance parameters or if a combination of multiple ANFs was responsible. Nevertheless, due to their chemical attributes, specific effects are expected from single ANFs when included in fish feeds.

In accordance with von Danwitz and Schulz (2020), supplementation with glucosinolates did not adversely affect growth performance of fish in this study. While the aforementioned authors used turbot (*Psetta maxima*), adverse effects have been reported for different concentration levels of glucosinolates originating from various rapeseed protein products in numerous species of fish (Bu et al., 2018; Burel et al., 2001; Burel, et al., 2000a; Burel et al., 2000b; Francis et al., 2001; Nagel et al., 2012). Results of this study showed no negative effects on growth performance although comparable levels of dietary glucosinolates were used (0.5; 1.0; 1.7  $\mu\text{mol/g}$ ) and supplemented glucosinolates were isolated from rapeseed, ensuring a similar composition of different GLS. Adverse effects on growth performance of fish are mainly associated with breakdown products of glucosinolates such as isothiocyanates and nitriles (Francis et al., 2001). This was demonstrated with supplementation of allyl isothiocyanate in diets for common carp (*Cyprinus carpio*, Hossain & Jauncey, 1988). The aforementioned breakdown product caused a reduction in overall growth, FCR and PER (Hossain & Jauncey, 1988). Isothiocyanate is suspected to cause changes in thyroid function (Duncan, 1991), which was observed for various species of fish in studies with dietary rapeseed protein product inclusion (Burel et al., 2001; Burel, et al., 2000a; Davies et al., 1990; Higgs et al., 1982; Teskeredžić et al., 1995). Breakdown of GLS is dependent on the activity of the specific enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), which is located mainly in myrosinase cells. These cells are in close proximity to GLS containing cells, separating enzyme and GLS. Cellular damage leads to rapid breakdown of GLS in multiple products (Mawson et al., 1993).

Composition of breakdown products is dependent on various factors like the structure of the R group or pH. In acidic conditions, for example, the portion of nitriles in breakdown products will rise, while the content of isothiocyanates will decrease (Mawson et al., 1993). Contingent on GLS composition and various conditions, the nature of breakdown products will vary, presumably creating different effects on growth performance. Until conditions under which breakdown of GLS in diets and/or the gastrointestinal tract of fish occurs are understood, exact effects from GLS on growth performance cannot be predicted accurately. The enzyme myrosinase will likely denature at temperatures above 60 °C (Björkman & Lönnerdal, 1973). Processing of different rapeseed protein products can exceed this temperature, likely preventing breakdown of GLS. Production of the rapeseed protein concentrate used in this study exceeded 60 °C to inactivate myrosinase, however, negative effects on growth performance were observed previously with myrosinase being almost absent (Josefsson & Uppström, 1976). Since latter study was conducted with mice, it is possible that negative effects from GLS in absence of myrosinase are less severe in fish. Insignificant effects on growth performance by supplemented GLS in this study could be attributed to the circumstance that the amounts of certain breakdown products were not sufficient to cause any significant harmful effects because of distinct circumstances in which added GLS were broken down. Secondly, a possible lack of active myrosinase caused by high temperatures during processing of the rapeseed protein concentrate could have led to an absence of breakdown products from supplemented GLS. Finally, if GLS cause negative effects on growth performance of fish without being broken down, the causing amount of GLS was not reached in this experiment. Additionally, the short duration of the experiment could have led to reduced effects on growth performance.

Negative effects on growth performance were mainly associated with the ability of phytic acid to chelate with di- and trivalent mineral ions or to form phytate-protein complexes (Francis et al., 2001) limiting availability of both minerals (Liener, 1989) and proteins (Carnovale et al., 1988; Richardson et al., 1985; Spinelli et al., 1983) from plants containing phytates. Additionally, phytates are the main storage form for phosphorus in multiple plant species, also limiting the availability of phosphorus (Reddy et al., 1982). Usage of plant protein products containing various levels of phytic acid in fish feed therefore resulted in reduced growth performance, elevated FCR and reduced protein efficiency (Hermann et al., 2016; Nagel et al., 2012; Slawski, 2012; Takii et al., 1999; Teskeredžić et al., 1995). Due to the presence of

multiple ANFs in plant protein products, however, negative effects could not exclusively be associated with phytic acid. Nevertheless, most studies using supplemented levels of phytic acid showed similar results supporting a direct negative effect from phytic acid on growth performance (Denstadli et al., 2006; Richardson et al., 1985; Spinelli et al., 1983; Zhong et al., 2019).

In accordance with previous studies in which diets of various species of fish were supplemented with phytic acid (Denstadli et al., 2006; Richardson et al., 1985; Riche & Garling, 2004; Zhong et al., 2019), significant negative effects on growth from phytic acid were observed at inclusion levels above 2% in diets. Other studies investigating effects from supplemented phytic acid found significant effects on growth performance at lower inclusion levels (Liu et al., 2018; Spinelli et al., 1983; von Danwitz & Schulz, 2020).

Previous studies have demonstrated that organic acids can increase availability of minerals (P, Ca, Fe, Mg) in fish diets (Sarker et al., 2007, 2012; Sugiura et al., 1998), probably due to solubilisation of bone minerals from fishmeal (Sarker et al., 2007). The acidifying effect of phytic acid could have increased availability of minerals by a higher amount than the chelation with mineral ions decreased its availability. Although available phosphorus in RP CD was possibly not at a deficient level for optimal growth for rainbow trout at this size (Ketola & Richmond, 1994; Skonberg et al., 1997; Sugiura et al., 2000), higher percentage of dietary phosphorus could increase growth performance (Ketola & Richmond, 1994). Whole body crude ash content in this study was significantly lowered in RP CD compared to almost all other dietary groups including added phytic acid (table 1.9), indicating increased amounts of available dietary phosphorus in phytic acid supplemented groups (Ketola & Richmond, 1994; Sugiura et al., 2004). Therefore, supplementation with phytic acid could improve feed conversion, PER, PRE and subsequently growth performance via increase of available minerals from fishmeal. Consequently, higher availability of phosphorus via solubilisation of bone material could compensate for negative effects from phytic acid on FCR, PER and PRE to some extent and delay a negative impact on growth performance. However, aforementioned effect will likely only be effective in diets, which contain a source of phosphorus susceptible to solubilisation and are low in available phosphorus relative to requirements of experimental species. Therefore, observed positive effect from phytic acid supplementation was likely highly circumstantial and probably caused by specific diet formulation used in this study. Additionally, supplemented phytic acid might have been used as a minor source for

phosphorus, further increasing availability of phosphorus. Phytase-producing bacteria were found in multiple freshwater teleosts and Atlantic salmon (Ray et al., 2012), indicating a possible presence of similar bacteria in the digestive tract of rainbow trout. Furthermore, high inclusion levels of phytic acid might also elevate phosphatase activity (Denstadli et al., 2006). In accordance with studies investigating effects from supplemented phytic acid, feed intake in this study decreased with high amounts of added phytic acid (Denstadli et al., 2006; Liu et al., 2018; Rasid et al., 2017; Zhong et al., 2019). This seems to be the main cause for impaired growth performance in this study, since feed conversion was not negatively affected by phytic acid supplementation (see above). Whether this effect on feed intake is due to palatability or due to physiological mechanisms could not have been clarified in aforementioned literature. Since reduction in feed intake was already observed after the first week of feeding in high-dose phytic acid groups and no negative effect on feed efficiency was observed, an impairment of diet palatability seems likely in this study. Formation of protein-phytate complexes can change protein availability (Carnovale et al., 1988; Richardson et al., 1985; Spinelli et al., 1983) through a structure change of those proteins (Cheryan & Rackis, 1980). This change in protein structure could have an impact on palatability of diets, since fish have a pronounced sense for protein sources (Morais, 2017). Formation of protein-phytate complexes is dependent on protein source, since both protein and amino acids vary in their capacity to bind to phytic acid (Dendougui & Schwedt, 2004). Depending on the total number of cationic groups of the protein, certain proteins are preferred substrates for binding with phytic acid (Adeola & Sands, 2003), which enables the formation of protein-phytate complexes with certain proteins over others. If a structure change of proteins caused by formation of protein-phytate complexes could possibly alter diet palatability positively by altering less palatable proteins remains to be elucidated. This could explain the minor improvement in DFI in PA1 compared to CD RP. Another factor determining diet palatability could be dietary pH. Although supplementation with certain acids improved palatability of diets for rainbow trout (Jones, 1990; Kasumyan & Döving, 2003), only specific members of each class of substances, including acids, seem to be palatable (Jones, 1990). Consequently, while certain organic acids or acidic dietary pH could improve palatability of diets for rainbow trout, phytic acid might still act as a deterrent. Additionally, taste preferences are dependent on species (Kasumyan & Döving, 2003), explaining differences in studies investigating effects from supplemented phytic acid. Conclusively, high levels of phytic acid could either influence

diet palatability through formation of phytate-protein complexes, a direct influence from phytic acid or a combination of both.

Effects from supplemented phytic acid on growth performance could have been caused by a combination of an acidifying effect on availability of minerals, chelating of minerals, formation of protein-phytate complexes and changing of diet palatability. Depending on species of fish and feed formulation, negative effects from phytic acid could occur at different inclusion levels.

#### 4.2 Whole body composition and health parameters in fish

Differences in moisture, crude fat and energy content as well as in FCF between dietary groups are most likely due to aforementioned differences in DFI. Reduced crude ash content in CD RP can be a sign of phosphorus deficiency, not necessarily for growth but for non-optimal bone development (Ketola & Richmond, 1994; Skonberg et al., 1997). Fish can compensate for phosphorus deficiency for optimal growth by resorption of scale-phosphorus (Carragher & Sumpter, 1991; Persson et al. 1994, 1995). This serves as a reserve for phosphorus and calcium and leads to a reduced ash content (Skonberg et al., 1997). As mentioned in 4.1, supplementation with organic acids can lead to higher availability of phosphorus from fishmeal, thus increasing ash content in diets supplemented with phytic acid.

#### 4.3 Thyroidal hormones

No significant effect from glucosinolates on thyroidal hormone concentration could be observed in this study. This is in contrast to results of previous studies showing decreased T3 levels in blood of rainbow trout at concentrations of 1-2  $\mu\text{mol/g}$  GLS or GLS breakdown products in experimental diets (Burel et al., 2001; Burel, et al., 2000a; Hardy & Sullivan, 1983; Leatherland et al., 1987). Effects of GLS on thyroidal functions were attributed to their derivatives impairing the activity of thyroid follicles (Burel et al., 2001; Mawson et al., 1994) and competing with iodine for thyroidal transport (Burel et al., 2001; Fenwick & Heaney, 1983). Since aforementioned effects depend on certain breakdown products like thiocyanate anions in case of impairment of iodine uptake, restrictive effects from GLS on thyroid activity are likely linked to presence and concentration of these derivatives. Similar to effects on growth, no impairment of thyroid activity from GLS supplementation could have been observed due to absence or low concentrations of certain breakdown products. The short

duration of the experiment might also have led to reduced effects on thyroid activity due to ANF supplementation. Additionally, high standard deviation in some treatments might have prevented significant effects. Due to small fish size and consequent limited amounts of blood, analysis of thyroidal hormones could not have been repeated to improve standard deviation.

#### 4.4 Histological analysis

Toxicity of GLS and resulting detoxification in liver and kidney can lead to hypertrophy, hyperplasia and necrosis of cells in both organs (Woyengo et al., 2017). This hypertrophy of liver was inter alia shown in pigs (Tripathi & Mishra, 2007) and rats (Nishie & Daxenbichler, 1980). However, liver damage caused by GLS may depend on species, susceptibility, formation of GLS hydrolysis products in gastro-intestinal tract and detoxification pathways (Mawson et al., 1994). Accordingly, Hossain and Jauncey (1988) observed no histopathological changes in gills, liver, muscle, intestine and kidney due to supplementation of diets with isothiocyanate. Similar to effects on growth performance, effects on health status of different organs could be circumstantial and dependent on a multitude of factors. Necessary amounts of GLS or breakdown products were likely not reached in this experiment to adversely affect organ morphology. Consequently, up to 1.7  $\mu\text{mol/g}$  of GLS in diets seem to have no negative effect on overall health status of liver, spleen and gut if similar circumstances (e.g. inactivation of myrosinase, see 4.1) apply. Similar to results in thyroidal activity, the short time period in which the experiment was conducted could have led to reduced effects on histopathology. A similar conclusion could be drawn for results of phytic acid supplementation. While Zhong et al. (2019) demonstrated that phytic acid supplementation caused morphological changes in the intestine of grass carp (*Ctenopharyngodon idella*) at inclusion levels of 2.4 and 4 % respectively, no changes were observed in Atlantic salmon at inclusion of 2.1 %. Latter results would be in line with our findings. Consequently, levels necessary for impaired organ health were likely not reached during this experiment. A dietary inclusion of up to 3 % of phytic acid could be a safe threshold in terms of organ health of rainbow trout.

## 5. Conclusion

Supplementation with glucosinolates showed no significant negative effect on growth performance or health status in concentration levels expected from inclusion of plant protein products in fish feed (up to 1.7  $\mu\text{mol/g}$  GLS in diets). Denaturation of myrosinase during processing of the rapeseed protein concentrate might have been the decisive factor in preventing effects from GLS in previously mentioned concentration levels. However, the shortened experimental duration should be considered as well when drawing conclusions for further experiments. High inclusion levels of phytic acid can lead to impaired feed intake and consequently decreased growth performance. However, the level at which supplemented phytic acid significantly reduces growth could vary, depending on species and feed formulation.

It will be crucial to improve our understanding of the mechanisms involved in formation of breakdown products of certain ANFs, their interactions with other substances during feed production and in the digestive tract of fish and their effect on diet palatability.

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## Chapter 2

### **Effects of highly purified rapeseed protein isolate as fishmeal alternative on nutrient digestibility and growth performance in diets fed to rainbow trout (*Oncorhynchus mykiss*)**

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**Abstract**

Effective inclusion of rapeseed protein products as fishmeal alternatives in diets for carnivorous fish is still limited. Previous studies observed restrictions in both nutrient utilization and feed intake. Contents of fiber, nitrogen-free extracts (NfE) and anti-nutritive factors (ANFs) were made responsible for latter mentioned limitations. Consequently, a highly purified rapeseed protein isolate with high protein content and low levels of fiber, NfE and ANFs was investigated in this study. In the first experiment, digestibility of the rapeseed protein isolate was determined. In the second experiment, the fishmeal portion (19.0 %) of a control diet was gradually replaced by rapeseed protein isolate to 33, 66 and 100 % of digestible protein. Diets were fed twice per day to apparent satiation to triplicate groups of rainbow trout (*Oncorhynchus mykiss*). After 56 days of feeding, growth performance as well as health parameters were evaluated. Protein digestibility of the rapeseed protein isolate was 95.2 % (mean = 95.2 %  $\pm$  standard deviation (SD) = 1.7 %) and up to 66 % of dietary fishmeal could be replaced without significantly affecting growth or health parameter. Total replacement of fishmeal led to significantly reduced feed intake and consequently reduced growth performance.

## 1. Introduction

The importance of viable fishmeal alternatives continues to increase as fishmeal production is stagnating while its demand is rising (FAO, 2020). Various plant proteins have been studied over the past decades as more sustainable alternatives for fishmeal including corn (Men et al., 2014), lupine (Burel et al., 2000b; Burel et al., 2000c), pea (Burel et al., 2000c; Thiessen et al., 2003), rapeseed (Bu et al., 2018; Higgs et al., 1982; Luo et al., 2012b; Nagel et al., 2012), soybean (Bonaldo et al., 2011) and wheat gluten (Bonaldo et al., 2011). Rapeseed protein products emerged as a potentially suitable alternative in fish feeds due to high availability and a favorable amino acid composition (Higgs et al., 1982; Luo et al., 2012a; Mwachireya et al., 1999; Suárez et al., 2009). However, different substances commonly present in rapeseed seeds, including complex carbohydrates, glucosinolates, fiber, phytic acid and others potentially decreased feed intake (Bu et al., 2018; Burel et al., 2000b; Nagel et al., 2012; Slawski, 2012), reduced digestibility of nutrients (Burel et al., 2000c; Francis et al., 2001; Mwachireya et al., 1999) and caused health issues (Francis et al., 2001) in various species of fish. The content of these anti-nutritive substances was reduced via multiple processing methods in the past (Adem, 2014; Bérot et al., 2005; Kalaydzhiev et al., 2019; Shahidi & Naczki, 1990; Slawski, 2012), however, even rapeseed protein products with small amounts of ANFs caused negative effects on growth performance in diets for different species of fish (Nagel et al., 2012; Slawski, 2012). Fiber, nitrogen-free extracts (NfE) and ANFs were made responsible for reductions in nutrient digestibility and feed intake in fish (Mwachireya et al., 1999; Nagel et al., 2012). Slawski (2012) managed to replace 100 % of fishmeal with rapeseed protein products in diets for rainbow trout without reducing growth. However, latter mentioned author included high amounts of blood and crustacean meal respectively in diets acting as potent feed attractants, possibly masking potential negative effects from rapeseed protein products on feed intake. Additionally, despite using a purified rapeseed protein isolate, Slawski (2012) observed a reduced crude protein digestibility of the isolate (84.6 % in rapeseed diet) in comparison with fishmeal (89.2 % in control diet).

Rapeseed processing methods applied in this study aimed towards reduction of fiber and NfE as well as ANFs, while simultaneously increasing protein content. Processing techniques resembled processing of rapeseed seeds in Adem (2014) however, the extraction process with 0.5 M salt solution was carried out in a one-step process and filtration ended at a lower

conductivity of 2 mS/cm<sup>2</sup> compared to literature (4-5 mS/cm<sup>2</sup>, Kaiser et al., 2021; 5-6 mS/cm<sup>2</sup>, Slawski, 2012). This filtration step increased protein content while simultaneously reducing levels of fiber, NfE and ANFs like glucosinolates and phytic acid. The use of ethanol in early protein processing steps was omitted, reducing production costs without impairing product quality. Latter mentioned novelties in already established methods resulted in exceptionally high protein contents and highly reduced levels of fiber and NfE as well as reduced ANF content. These improvements should lead to elevated nutrient digestibility and increased palatability of the resulting rapeseed protein isolate.

Conclusively, this study used a highly purified rapeseed protein isolate as a replacement for fishmeal in diets for rainbow trout to test its viability as an alternative protein source.

## 2. Materials and methods

### 2.1 Digestibility trial

For the digestibility trial, 120 rainbow trout (*Oncorhynchus mykiss*, initial weight: mean = 79.9 g  $\pm$  SD = 9.4 g) from Forellenzucht Trostade GmbH & Co. KG, Trostade, Germany were randomly assigned to six tanks (20 fish per tank, 150 l). Prior to the experiment, fish were fed a commercial trout diet (Aller Gold, 4 mm) for 14 days during adaptation to the recirculation system of the Gesellschaft für Marine Aquakultur mbH, Büsum, Germany. After adaptation, fish were starved for two days.

During the experiment, temperature was held at  $13.1 \pm 1.4$  °C. Oxygen saturation was at  $95.1 \pm 2.6$  % (Handy Polaris, Oxy Guard International A/S, Birkerød, Denmark), pH at  $7.9 \pm 0.2$  (pH-Messgerät GMH 5550, GHM Messgerät GmbH, Regenstauf, Germany), total ammonia nitrogen at  $0.7 \pm 0.4$  mg l<sup>-1</sup>, total nitrite nitrogen at  $3.9 \pm 2.6$  mg l<sup>-1</sup> (Microquant test kits for NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup>, Merck KGaA, Darmstadt, Germany) and salinity at  $5.7 \pm 1.2$  ppt (HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA).

Two diets were formulated and produced for the digestibility trial, a basal diet with fishmeal as the main source of protein (BD FM, table 2.1 and 2.2) and a test diet, consisting of 70.0 % basal diet and 30.0 % rapeseed protein isolate (RI T, table 2.1 and 2.2). Both diets contained titanium dioxide as inert marker for determination of apparent digestibility coefficients. Production of rapeseed protein isolate was conducted by Pilot Pflanzenöltechnologie Magdeburg e.V. (PPM), Magdeburg, Germany. Rapeseed seeds ("Raffiness", Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Holtsee, Germany) were conditioned in a vacuum dryer for 20 min at 70 – 80 °C to inactivate the enzyme myrosinase. Subsequently, rapeseed oil was extracted via oil pressing (60 – 65 °C) and hexane extraction (50 – 60 °C). After desolventising of hexane, rapeseed seeds were crushed to a particle size of 0.2 – 0.5 mm. Protein content of resulting rapeseed meal was elevated through liquid salt (0.5 M) water extraction at 50 °C. Suspension was decanted and then filtrated (membrane size: 10 kDa) without including residue from decantation. Filtration ended when conductivity reached 2 mS/cm<sup>2</sup> (Multi 3320, WTW GmbH, Weilheim, Germany). Conclusively gained solution was spray dried.

Mealy feed ingredients were mixed evenly before addition of oily substances. Resulting mixture was pressed to pellets of 4 mm diameter (L 14-175, AMANDUS KAHL, Reinbeck,

Germany). During feed processing, temperature was kept below 60 °C to prevent thermic damage to feed ingredients. Pellets were air-dried for 24 hours before storing at 4 °C.

Table 2.1 Nutrient composition, amino acid composition and content of anti-nutritive factors in fishmeal and rapeseed protein isolate.

	Fishmeal	Rapeseed protein isolate
<b>Nutrient composition (% DM)</b>		
Moisture	6.80	5.35
Crude protein	71.55 <sup>5</sup>	93.87 <sup>5</sup>
Crude lipid	13.93	1.46
Crude ash	14.05	4.58
NfE + crude fiber <sup>1</sup>	0.00	0.08
Gross energy (MJ kg <sup>-1</sup> )	22.34	21.48
<b>Amino acid composition (% DM)<sup>2</sup></b>		
Arginine	4.4	6.3
Histidine	1.6	2.7
Isoleucine	2.7	3.9
Leucine	4.7	6.8
Lysine	4.7	5.1
Methionine	1.7	1.6
Phenylalanine	2.6	3.9
Threonine	2.7	3.6
Valine	3.3	4.9
<b>ANF content</b>		
Phytic acid (%) <sup>3</sup>		1.28
Glucosinolates (µmol/g) <sup>4</sup>		0.11

<sup>1</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash); <sup>2</sup> analyzed by AGROLAB LUFA GmbH, Kiel, Germany according to European Commission Regulation (EC) No 152/2009, III, F: 2009-02 (European Commission, 2009); <sup>3</sup> analyzed using phytic acid enzyme test (Megazyme Kit) and high performance liquid chromatography (HPLC) according to Sashidhar et al. (2020); <sup>4</sup> determined via enzyme test (myrosinase/glucose test kit according to Heaney et al., 1988) and HPLC (desulfatase C18 RP column according to Fiebig & Arens, 1992, UV detection of 15 compounds); <sup>5</sup> conversion factor for calculation of crude protein was 6.25\*N.

Table 2.2 Feed ingredients and feed composition of diets for digestibility trial.

	Base diet fishmeal (BD FM)	Rapeseed protein isolate test diet (RI T)
<b>Ingredient (% DM)</b>		
Fishmeal <sup>1</sup>	55.5	38.9
Rapeseed protein isolate	0	30
Gelatine <sup>2</sup>	5.0	3.5
Wheat starch <sup>3</sup>	25.0	17.5
Fish oil <sup>4</sup>	10.0	7.0
Vitamin premix <sup>4</sup>	0.5	0.4
Mineral premix <sup>4</sup>	0.4	0.3
Ca <sub>2</sub> PO <sub>4</sub> <sup>5</sup>	0.5	0.4
Cellulose <sup>6</sup>	2.1	1.5
Marker (TiO <sub>2</sub> ) <sup>7</sup>	1.0	0.7
<b>Feed composition (% DM)</b>		
Moisture	15.3	7.7
Crude protein	47.0	61.3
Crude lipid	17.3	13.5
Crude ash	9.9	8.3
NfE + crude fiber <sup>8</sup>	25.9	16.9
Gross energy (MJ kg <sup>-1</sup> )	22.3	22.3

<sup>1</sup> Bioceval GmbH & Co. KG, Cuxhaven; Germany; <sup>2</sup> Gustav Ehlert GmbH & Co. KG, Verl, Germany; <sup>3</sup> Kröner-Stärke GmbH, Ibbenbüren, Germany; <sup>4</sup> Emsland-Aller Aqua GmbH, Golßen, Germany; <sup>5</sup> Lehmann & Voss & Co. KG, Hamburg, Germany; <sup>6</sup> Mikro-Technik GmbH & Co. KG, Bürgstadt, Germany; <sup>7</sup> Kronos Titan GmbH, Nordenham, Germany; <sup>8</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash).

Fish were fed manually once per day at 1.5 % of their body weight for 14 days. 5 hours after each feeding event, faeces were collected by manual stripping of all fish. To avoid any injuries, fish were sedated using clove oil (1 ml per 40 l of water) during stripping procedure.

Apparent digestibility coefficients (ADC) of different nutrients in diets were calculated according to Maynard and Loosli (1979) subsequent to faeces and diet nutrient and marker analysis:

$$\text{ADC}_{\text{Nutrient, Diet}} (\%) = 100 * (1 - (\text{Marker}_{\text{Diet}} / \text{Marker}_{\text{Faeces}}) * (\text{Nutrient concentration}_{\text{Faeces}} / \text{Nutrient concentration}_{\text{Diet}}))$$

The formula for calculation of ADC of test substance was derived from Bureau and Hua (2006):

$$\text{ADC}_{\text{Test substance}} = (\text{ADC}_{\text{Test diet}} - \text{ADC}_{\text{Base diet}}) * ((30 * \text{Nutrient concentration}_{\text{Test diet}} + 70 * \text{Nutrient concentration}_{\text{Base diet}}) / 30 * \text{Nutrient concentration}_{\text{Test diet}})$$

## 2.2 Growth trial

Prior to the growth trial, juvenile rainbow trout (*Oncorhynchus mykiss*, 79.9 ± 9.4 g) from Forellenzucht Troststadt GmbH & Co. KG, Troststadt, Germany were adapted to the recirculating aquaculture facilities of the Gesellschaft für Marine Aquakultur mBH, Büsum, Germany for 14 days. During adaptation, fish were fed a commercial trout diet (Aller Gold, 4 mm). Before experimental feeding commenced, fish were starved for two days. For the main growth performance experiment, 240 juvenile rainbow trout were distributed in triplicate groups of 20 fish. Each group was assigned to a tank (150 l) at semi-random to avoid potential tank effects. In a semi-random distribution, each tank at the corners of the system is stocked with a different dietary group. Additionally, distribution of triplicates is even across floor-level tanks and tanks which are located above aforementioned tanks.

During rearing of fish, water parameters were measured daily, to ensure optimal conditions. Oxygen saturation was 100.7 ± 2.4 % (Handy Polaris, Oxy Guard International A/S, Birkerød, Denmark) and temperature was held at 14.1 ± 0.8 °C. Salinity was 4.5 ± 0.8 ppt (HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA), pH at 7.2 ± 0.2 (pH-Messgerät GMH 5550, GHM Messgerät GmbH, Regenstauf, Germany), total ammonia nitrogen 0.7 ± 0.4 mg l<sup>-1</sup> and total nitrite nitrogen 2.6 ± 2.4 mg l<sup>-1</sup> (Microquant test kits for NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup>, Merck KGaA, Darmstadt, Germany).

Fish were fed manually twice per day for 56 days to apparent satiation. To calculate exact feed intake, an average pellet weight was measured prior to the feeding experiment and excess pellets were collected via siphoning and counted after feeding.

For the main feeding trial, four diets were formulated and produced. A low fishmeal diet based on commercial trout diets served as positive control (CD FM). In remaining diets, fishmeal was gradually replaced by rapeseed protein isolate on digestible protein basis, based on results from digestibility trial. Consequently, fishmeal was replaced to 33, 66 and 100 % in diets on digestible protein basis (RI33, RI66, RI100). All diets were formulated to meet nutritional demands of rainbow trout (National Research Council, 2011) and experimental diets of main



feeding trial were formulated to be isonitrogenous and isoenergetic on digestible level (table 2.3 and 2.4).

Processing of experimental diets was carried out according to methods described in 2.1. To activate gelatin and stabilize resulting pellets, water was added at 8.0 (RI100); 15.0 (RI66); 15.0 (RI33) and 20.0 (CD FM) % of feed. Feed mixture was pressed to pellets of 4 mm diameter (L 14-175, AMANDUS KAHL, Reinbeck, Germany).

Table 2.3 Feed ingredients, feed composition and anti-nutritive factors content of diets for main feeding trial.

	CD FM	RI33	RI66	RI100
<b>Ingredient (% OS)</b>				
Fishmeal <sup>1</sup>	19.0	12.7	6.3	0
Rapeseed protein isolate <sup>2</sup>	0	4.4	8.7	13.0
Blood meal <sup>3</sup>	5.0	5.0	5.0	5.0
Gelatine <sup>4</sup>	5.0	5.0	5.0	5.0
Pea protein isolate <sup>5</sup>	24.0	24.0	24.0	24.0
Wheat gluten <sup>6</sup>	10.0	10.0	10.0	10.0
Wheat starch <sup>6</sup>	13.5	13.5	13.5	13.5
Corn starch <sup>6</sup>	12.0	12.0	12.0	12.0
Rapeseed oil <sup>7</sup>	3.3	3.3	3.3	3.3
Fish oil <sup>1</sup>	6.0	6.8	7.5	8.3
Vitamin premix <sup>5</sup>	0.5	0.5	0.5	0.5
Mineral premix <sup>5</sup>	0.4	0.4	0.4	0.4
Ca <sub>2</sub> PO <sub>4</sub> <sup>8</sup>	0.5	0.5	0.5	0.5
Cellulose <sup>9</sup>	0.8	2.0	3.2	4.4
<b>Feed composition (% DM)</b>				
Moisture	20.7	15.4	16.2	9.2
Crude protein	54.0	53.6	53.2	52.6
Crude lipid	17.8	16.5	18.5	19.1
Crude ash	5.1	4.4	3.4	2.9
NfE + crude fiber <sup>10</sup>	23.1	25.6	24.9	25.4
Gross energy (MJ kg <sup>-1</sup> )	23.4	23.5	23.5	23.7
<b>Anti-nutritive factors<sup>11</sup></b>				
Phytic acid (% of feed)	0	0.06	0.11	0.17
Glucosinolates (µmol/g of feed)	0	0.005	0.010	0.014

<sup>1</sup> Bioceval GmbH & Co. KG, Cuxhaven; Germany; <sup>2</sup> Pilot Pflanzenöltechnologie Magdeburg e. V., Magdeburg, Germany; <sup>3</sup> Saria SE & Co. KG, Selm, Germany; <sup>4</sup> Gustav Ehlert GmbH & Co. KG, Verl, Germany; <sup>5</sup> Emsland-Aller Aqua GmbH, Golßen, Germany; <sup>6</sup> Kröner-Stärke GmbH, Ibbenbüren, Germany; <sup>7</sup> Cargill GmbH, Riesa, Germany; <sup>8</sup> Lehmann & Voss & Co. KG, Hamburg, Germany; <sup>9</sup> Mikro-Technik GmbH & Co. KG, Bürgstadt, Germany; <sup>10</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash); <sup>11</sup> Based on analysis of feed ingredients.

Table 2.4 Amino acid composition in main trial diets.

Amino acid composition (% DM diet) <sup>1</sup>	CD FM	RI33	RI66	RI100	Requirements <sup>2</sup>
Arginine	3.7	3.7	3.7	3.6	1.4 – 1.8
Histidine	1.3	1.3	1.3	1.3	0.5 – 0.6
Isoleucine	2.2	2.2	2.2	2.2	0.7 – 1.4
Leucine	4.0	4.0	4.0	4.0	1.1 – 1.4
Lysine	2.9	2.8	2.7	2.7	1.4 – 2.5
Methionine	0.7	0.7	0.6	0.6	0.4 – 0.9
Phenylalanine	2.3	2.3	2.3	2.3	0.7
Threonine	1.8	1.8	1.8	1.7	1.0
Valine	2.47	2.5	2.5	2.5	0.8 – 1.6

<sup>1</sup> based on analysis of feed ingredients; <sup>2</sup> according to National Research Council (2011).

### 2.3 Samplings

Prior to the main experiment and subsequent to the adaptation phase, 15 fish were randomly selected for initial analysis of whole body composition and calculation of hepatosomatic index (HSI), viscerosomatic index (VSI), spleen somatic index (SSI) and fulton condition factor (FCF). After 56 days of feeding, same factors were determined for each treatment, with samplings of three fish per tank. Fish were euthanized by manual percussive stunning followed by heart piercing. Before sampling, all fish were starved for 48 hours. All samples for whole body composition were freeze-dried (Alpha 1-2 LD plus, Christ, Osterode, Germany) and stored at -20 °C prior to analyses.

### 2.4 Analysis of nutrients and anti-nutritive factors

Macronutrients of proteins, diets and whole body were analyzed according to the European Commission Regulation (EC) No 152/2009 (European Commission, 2009). Analysis of diets and homogenized whole bodies was done in duplicates. Crude lipid content of samples was determined using methods according to the Soxhlet-protocol (Soxtherm, C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Standard Kjeldahl methods were utilized to determine crude protein content. Samples were dried for 4 hours at 103 °C to determine dry matter content (ED 53, Binder GmbH, Tuttlingen, Germany) and subsequently combusted in a combustion oven for 12 hours at 550 °C for determination of crude ash content (P300, Nabertherm, Lilienthal, Germany). Remaining portion was defined as nitrogen-free extracts (NfE) plus crude fiber. Amino acid concentration of feed ingredients was analyzed by AGROLAB

LUFA GmbH, Kiel, Germany according to the regulation of the European Commission (EC) No 152/2009, III, F (European Commission, 2009).

High performance liquid chromatography (HPLC) and phytic acid enzyme test (Megazyme Kit) according to methods used by Sashidhar et al. (2020) were used to measure phytic acid content in rapeseed protein isolate and diets at Christian-Albrechts-University, Kiel, Germany. Determination of Glucosinolate content was done at Christian-Albrechts-University, Kiel, Germany as well, using an enzyme test (myrosinase/glucose test kit after Heaney et al., 1988) and HPLC analysis (desulfatase C18 RP column after Fiebig & Arens, 1992, UV detection of 15 compounds).

Titanium content in collected faeces was analyzed using methods according to DIN EN ISO 11885:2009 (DIN EN ISO 11885:2009-09, 2009; AGROLAB LUFA GmbH, Kiel, Germany).

## 2.5 Statistical analysis

All data analysis was done using SPSS (Version 20). The Kolmogorov-Smirnov test and the Levene test were used to determine normal distribution and homogeneity of variances of data respectively. Depending on results from Kolmogorov-Smirnov and Levene test, data was either analyzed using an analysis of variances (ANOVA) or the Kruskal-Wallis test. Multiple comparisons among means were made using Tukey's HSD test. The aggregate type I error was defined at 5 % ( $p < .05$ ) for each set of comparisons to determine statistical significance.

### 3. Results

#### 3.1 Digestibility

Apparent digestibility coefficient (ADC) of dry matter and protein was significantly lower in basal diet fishmeal (BD FM) compared to rapeseed protein isolate test diet (RI T, table 2.5). ADC of protein of rapeseed protein isolate (RPI) was  $95.2 \pm 1.7$  % and ADC of energy  $94.6 \pm 3.0$  %.

Table 2.5 Apparent digestibility coefficients of test diets and test ingredients (mean  $\pm$  SD, n=3).

Apparent digestibility coefficient (%)	BD FM	RI T
Dry matter diet	$80.4 \pm 0.3^a$	$83.9 \pm 0.2^b$
Crude protein diet	$88.3 \pm 0.9^a$	$91.6 \pm 0.8^b$
Crude protein of test ingredient		$95.2 \pm 1.7$
Energy of test ingredient		$94.6 \pm 3.0$

Values in the same row with different superscript letters are significantly different ( $P < .05$ ).

#### 3.2 Growth performance

After 56 feeding days, overall growth performance was not significantly affected by an exchange rate of up to 66% of dietary fishmeal (table 2.6). However, final body weight (FBW), specific growth rate (SGR), daily feed intake (DFI), protein efficiency ratio (PER) and protein retention efficiency (PRE) were significantly decreased in comparison to control when 100% of fishmeal was exchanged for rapeseed protein isolate (RI100, table 2.6). Over the first 14 days of feeding, DFI decreased significantly with each subsequent inclusion level of rapeseed protein isolate with the exception of RI33 and RI66 which did not differ significantly. No significant effect on feed conversion ratio (FCR) was observed. However, when FCR is calculated on dry matter basis, FCR between CD FM and RI100 were significantly different (table 2.6).

Table 2.6 Growth performance parameter of rainbow trout after 56 days of feeding (mean  $\pm$  SD, n=3).

	CD FM	RI33	RI66	RI100
<b>IBW</b>	77.58 $\pm$ 0.52	77.88 $\pm$ 0.31	77.83 $\pm$ 0.94	77.36 $\pm$ 0.24
<b>FBW</b>	269.83 $\pm$ 12.21 <sup>a</sup>	268.10 $\pm$ 8.91 <sup>a</sup>	248.93 $\pm$ 4.37 <sup>a</sup>	211.73 $\pm$ 9.14 <sup>b</sup>
<b>FCR</b>	1.21 $\pm$ 0.02	1.20 $\pm$ 0.01	1.21 $\pm$ 0.01	1.18 $\pm$ 0.06
<b>FCR DM</b>	2.75 $\pm$ 0.09 <sup>a</sup>	2.98 $\pm$ 0.19 <sup>ab</sup>	2.95 $\pm$ 0.13 <sup>ab</sup>	3.08 $\pm$ 0.23 <sup>b</sup>
<b>SGR</b>	2.20 $\pm$ 0.07 <sup>a</sup>	2.19 $\pm$ 0.05 <sup>a</sup>	2.06 $\pm$ 0.04 <sup>a</sup>	1.78 $\pm$ 0.08 <sup>b</sup>
<b>DFI</b>	2.67 $\pm$ 0.11 <sup>a</sup>	2.62 $\pm$ 0.04 <sup>a</sup>	2.50 $\pm$ 0.06 <sup>a</sup>	2.10 $\pm$ 0.04 <sup>b</sup>
<b>DFI 1-14</b>	2.62 $\pm$ 0.08 <sup>a</sup>	2.45 $\pm$ 0.02 <sup>b</sup>	2.32 $\pm$ 0.07 <sup>b</sup>	1.87 $\pm$ 0.04 <sup>c</sup>
<b>PER</b>	1.93 $\pm$ 0.02 <sup>a</sup>	1.84 $\pm$ 0.02 <sup>ab</sup>	1.85 $\pm$ 0.01 <sup>ab</sup>	1.77 $\pm$ 0.09 <sup>b</sup>
<b>PRE</b>	32.61 $\pm$ 0.69 <sup>a</sup>	31.25 $\pm$ 0.48 <sup>ab</sup>	30.66 $\pm$ 0.34 <sup>ab</sup>	29.86 $\pm$ 1.43 <sup>b</sup>

IBW (initial body weight); FBW (final body weight); FCR (feed conversion ratio) = g feed intake / g weight gain; FCR DM (feed conversion ratio on dry matter basis) = g dry feed intake / g dry weight gain; SGR (specific growth rate) = (ln (FBW) – ln (IBW)) / feeding days \* 100; DFI (daily feed intake) = daily feed intake in % body weight; DFI 1-14 = DFI over the first 14 days of feeding; PER (protein efficiency ratio) = g body weight gain / g crude protein intake; PRE (protein retention efficiency) = g crude protein gained / g crude protein intake \*100. Values in the same row with different superscript letters are significantly different ( $P < .05$ ).

### 3.3 Body composition

Proximate whole body composition differed significantly in crude ash content. Both dietary treatments RI66 and RI100 had significantly decreased crude ash content in comparison with RI33 and control (table 2.7). No significant differences occurred in remaining body composition parameters.

Table 2.7 Proximate whole body composition of fish after 56 days of feeding (mean  $\pm$  SD, n=3).

(% OS)	CD FM	RI33	RI66	RI100
<b>Moisture</b>	67.68 $\pm$ 1.17	67.55 $\pm$ 1.18	67.12 $\pm$ 0.41	68.11 $\pm$ 0.96
<b>Crude ash</b>	2.12 $\pm$ 0.09 <sup>a</sup>	1.96 $\pm$ 0.04 <sup>a</sup>	1.71 $\pm$ 0.12 <sup>b</sup>	1.53 $\pm$ 0.08 <sup>b</sup>
<b>Crude protein</b>	16.53 $\pm$ 0.11	16.59 $\pm$ 0.29	16.30 $\pm$ 0.05	16.41 $\pm$ 0.09
<b>Crude fat</b>	13.66 $\pm$ 1.27	13.90 $\pm$ 1.47	14.72 $\pm$ 0.44	13.80 $\pm$ 1.23
<b>NfE + crude fiber<sup>1</sup></b>	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.14 $\pm$ 0.03	0.14 $\pm$ 0.15
<b>Energy (MJ/kg)</b>	9.23 $\pm$ 0.49	9.42 $\pm$ 0.57	9.60 $\pm$ 0.19	9.21 $\pm$ 0.49

<sup>1</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash). Values in the same row with different superscript letters are significantly different ( $P < .05$ ).

### 3.4 Health parameters

No significant differences were observed in any of the health parameters investigated in this study (table 2.8).

Table 2.8 Health parameters of fish after 56 days of feeding (mean  $\pm$  SD; VSI, HSI, SSI: n=9; FCF: n=18).

	<b>CD FM</b>	<b>RI33</b>	<b>RI66</b>	<b>RI100</b>
<b>HSI</b>	1.51 $\pm$ 0.13	1.50 $\pm$ 0.24	1.37 $\pm$ 0.18	1.45 $\pm$ 0.15
<b>SSI</b>	0.15 $\pm$ 0.05	0.20 $\pm$ 0.10	0.16 $\pm$ 0.05	0.20 $\pm$ 0.05
<b>VSI</b>	13.34 $\pm$ 1.03	14.36 $\pm$ 1.03	14.46 $\pm$ 1.55	14.98 $\pm$ 1.15
<b>FCF</b>	1.18 $\pm$ 0.11	1.19 $\pm$ 0.09	1.28 $\pm$ 0.12	1.18 $\pm$ 0.07

VSI (viscerosomatic index) = (g fish weight - g gutted fish weight) / g fish weight \* 100; HSI (hepatosomatic index) = g liver weight / g fish weight \* 100; SSI (spleen somatic index) = g spleen weight / g fish weight \* 100; FCF (fulton condition factor) = g fish weight \* 100 \* cm fish length<sup>-3</sup>. Values in the same row without superscript letters are not significantly different ( $P > .05$ ).

## 4. Discussion

### 4.1 Apparent digestibility

In comparison with various studies investigating digestibility of multiple rapeseed protein products, both ADC of dry matter ( $83.9 \pm 0.2$  % of diet) and crude protein ( $95.2 \pm 1.7$  % of test ingredient) recorded in this study were among the highest observed in rainbow trout using the stripping method. Mwachireya et al. (1999) stated that indigestible carbohydrates either alone or with phytic acid have the strongest effect on digestibility of rapeseed protein products. Correspondingly, various other studies showed negative impacts from rapeseed protein products containing comparatively high amounts of carbohydrates and/or ANFs on nutrient digestibility in different species of fish (Bu et al., 2018; Burel et al., 2000a; Cheng et al., 2010; Greiling et al., 2018; Luo et al., 2012a; Nagel et al., 2012; Sławski, 2012). Since rainbow trout are unable to digest cellulose, other non-starch polysaccharides (NSP) or lignin (Greiling et al., 2018), fiber was made responsible for most effects on impaired digestibility. However, the extent to which impairment of crude protein digestibility occurs due to NSP, might depend on which NSP are present and at which concentration (Glencross, 2009). This was demonstrated by studies, showing little or no effect on crude protein digestibility in spite of high fiber content (Burel et al., 2000a; Glencross, 2009). Therefore, contents of fiber were mainly made responsible for impairment of digestibility of dry matter and energy (Burel et al., 2000a; Glencross, 2009). This would also be in line with our results, since one of the highest apparent digestibilities of dry matter was recorded for rapeseed protein products in rainbow trout, while fiber and NfE content was one of the lowest ever reached in a rapeseed protein product. Mwachireya et al. (1999) observed an even higher crude protein digestibility with a rapeseed protein isolate (97.6% crude protein digestibility). This could have been due to applied methods for fecal collection and/or due to exceptionally low contents of ANFs, mainly phytic acid. Phytic acid can form protein-phytate complexes, limiting availability of proteins for fish by reducing digestibility of proteins (Francis et al., 2001). ANFs like tannins were also made responsible for reducing activity of multiple digestive enzymes, which was demonstrated for protease, lipase, amylase (Cheng et al., 2010) and pepsase (Bu et al., 2018), decreasing digestibility of nutrients. Remaining contents of phytic acid and other ANFs could therefore have limited digestibility of crude protein in this study. Conclusively, fiber, NfE and



ANF content should be reduced to an absolute minimum to reach best possible nutrient digestibility of rapeseed protein products in rainbow trout.

#### 4.2 Growth performance

It appears likely that reduced DFI caused impaired growth performance when fishmeal was replaced completely by rapeseed protein isolate in this study. Latter assumption is in line with results of various studies observing reduced feed intake due to increasing dietary content of rapeseed protein concentrate or isolate in different species of fish (Hermann et al., 2016; Kissil et al., 2000; Nagel et al., 2012, 2017; Slawski, 2012). ANFs such as glucosinolates and phytic acid were made responsible for reduced feed palatability when rapeseed protein products were used as fish feed ingredients (Francis et al., 2001). Consequently, different purification methods were introduced to reduce contents of ANFs (Bérot et al., 2005; Slawski, 2012). However, as mentioned above, even highly purified protein products like concentrates and isolates can still lead to reduced feed palatability in different species of fish (Nagel et al., 2012, 2017; Slawski, 2012). In contrast to results of latter mentioned studies and results of this experiment, Slawski (2012) replaced 100% of fishmeal with a rapeseed protein concentrate or isolate respectively without significantly reducing feed intake of rainbow trout. Both protein products contained higher or comparable levels of glucosinolates (1.32 and 0.13  $\mu\text{mol/g}$ ) and higher or lower levels of phytic acid (1.77 and 0.69 %) compared with the rapeseed protein isolate used in this study (0.11  $\mu\text{mol/g}$  glucosinolates; 1.28 % phytic acid). However, Slawski (2012) used potent feed attractants in both studies, possibly masking effects on palatability by rapeseed protein products. Blood meal and crustacean meal were shown to act as strong attractants in fish diets (Espe et al., 2006, 2007; Torstensen et al., 2008; Tusche, 2012) and might have compensated for negative effects from rapeseed protein products on feed intake. A similar effect was demonstrated with a mixture of krill meal and krill concentrate (Zhang et al., 2012). Increasing dietary levels of phytic acid were shown to negatively affect feed intake (Kaiser et al., 2021), however, inclusion levels in this study are comparatively low. A significant influence from phytic acid on feed intake is therefore unlikely but should not be excluded. Similarly, effects from other ANFs like sinapic acid (Francis et al., 2001), not being analyzed in this study, are unlikely due to high purification of the rapeseed protein isolate but cannot be precluded.

Additionally, other anti-nutritive substances present in rapeseed protein isolates, whose effects on feed intake were not intensively investigated in literature could possibly be responsible for impaired palatability as well. Hald et al. (2018) identified Kaempferol 3-O-(2''-O-Sinapoyl- $\beta$ -sophoroside) to contribute to the bitter taste of canola/rapeseed protein isolates. Effects of several substances which are, like the Kaempferol derivative of bitter taste in humans, were observed in different species of fish, including rainbow trout (Hara, 2006; Kasumyan & Döving, 2003; Oike et al., 2007; Yamashita et al., 2006). One of these substances (Q-HCl) depressed feeding behavior in rainbow trout to some extent in concentrations of  $10^{-7}$  M (Hara, 2006). If the Kaempferol derivative can have a significant effect on diet palatability for rainbow trout remains to be elucidated.

Since DFI was already significantly reduced after the first 14 days of feeding, palatability seems to be the determining factor influencing DFI in comparison with metabolic issues caused by ANFs. If negative effects from ANFs are not the main cause of reduced feed intake due to low content of the latter, protein and/or amino acid composition could be the decisive factor determining diet palatability. While olfactory senses are mainly responsible for detection of food, gustatory senses determine whether food is being swallowed or ejected (Hara, 2006; Kasumyan & Sidorov, 2012; Kasumyan, 2019). Fish possess a highly pronounced sense of taste (Kasumyan, 2019; Kasumyan & Mouromtsev, 2020; Morais, 2017), however, mechanisms involved in how olfactory and gustatory reception contribute to regulation of feed intake in fish is not fully understood yet. Observations by Comesaña et al. (2018a); Comesaña et al. (2018b) and Comesaña et al. (2020) could offer some first insight. Latter mentioned authors observed significant effects on different parts of the brain following intracerebroventricular (ICV) or intraperitoneal (IP) treatment respectively with different amino acids in rainbow trout (Comesaña et al., 2018a; Comesaña et al., 2018b). Both treatments induced expression of neuropeptides involved in the metabolic control of feed intake and/or parameters related to metabolism of different substances including amino acids in the hypothalamus and the telencephalon. Additionally, results from Comesaña et al. (2020) demonstrated for the first time in rainbow trout, that the pre-absorptive detection of certain amino acids also triggers a satiety signal in the hypothalamus, resulting in decreased feed intake. Consequently, consumption of amino acids could regulate feed intake via pre- and/or post-absorptive signaling pathways. This feed intake regulation in fish not only depends on specific amino acids, but also their concentration and their interaction with other amino acids (Comesaña et

al., 2020). Additionally, differences in effects on feed intake regulation of amino acids were observed, depending on type of absorption (pre-/post-absorptive). Results of this study cannot assess adequately if pre- or post-absorptive effects from amino acids were the determining factor for impairment of palatability.

Conclusively, DFI of treatment RI100 was most likely reduced due to palatability issues. These issues could have been caused by ANFs, however, effects from amino acids or protein composition seem more likely given low content of ANFs. Further studies will be necessary investigating what determines the palatability of diets devoid of fishmeal including high amounts of rapeseed protein products. Discovering whether pre- or post-absorptive effects are responsible for impairment of feed intake could help improve application of rapeseed protein products in diets for carnivorous fish.

In addition to reduced DFI, PER and PRE were significantly decreased and FCR DM was significantly increased in RI100 compared to control. These differences in feed and protein utilization could be caused by differences in amino acid ratios between diets (Kaushik & Seiliez, 2010; National Research Council, 2011) or non-optimal content of single amino acids like lysine for protein utilization (Rodehutscord et al., 1997). Dietary amino acid composition can determine postprandial amino acid composition to a high degree (Rolland et al., 2015). Consequently, excessive amino acids will be oxidized if the absorbed amino acid profile is not similar to the required amino acid profile (Carter et al., 2001). Hence, non-optimal ratios between dietary amino acids or content of single amino acids could have led to impaired protein utilization given the exceptionally high protein digestibility of rapeseed protein isolate. Due to the strong association between live weight gain and protein deposition, FCR DM was affected as well (Dumas et al., 2007; Shearer, 1994). High moisture content in diets CD FM, RI33 and RI66 most likely prevented observations of significant differences in FCR, however, addition of water was necessary to achieve a pellet water stability sufficient enough for the growth trial.

Changes in dietary amino acid profile could improve protein utilization of diets including rapeseed protein products. This would be in line with Kaushik and Seiliez (2010) who stated that it might be necessary to mimic the amino acid profile of fishmeal to successfully replace it with alternate protein sources.

### 4.3 Body composition

Whole body crude ash content decreased significantly with increasing levels of dietary rapeseed protein isolate. This effect could have been caused by differences in available phosphorus in experimental diets. Inclusion of available phosphorus in diets was probably not at a deficient level for optimal growth of rainbow trout at this size (Ketola & Richmond, 1994; Skonberg et al., 1997; Sugiura et al., 2000) due to dietary inclusion of dicalcium phosphate. However, reduced crude ash content can be a sign of phosphorus deficiency for non-optimal bone development (Ketola & Richmond, 1994; Skonberg et al., 1997).

## 5. Conclusion

The rapeseed protein isolate used in this study could replace 66% of dietary fishmeal without impairing growth performance of rainbow trout significantly. Novel processing methods of rapeseed protein isolate were able to improve nutrient digestibility, however, diet palatability and protein utilization were negatively influenced by the rapeseed protein isolate at 100% exchange rate. It could not have been definitely clarified if remaining ANFs or fiber and NfE content are mainly responsible for reduced protein digestibility. This could be determined in a future study by dietary exclusion of either group of substances. Further reduction of ANFs could improve palatability, however, protein and amino acid composition could be determining factors for diet palatability and protein utilization in already highly purified rapeseed protein products.

To use rapeseed protein products as fishmeal alternatives effectively, palatability of rapeseed protein should be improved or potent feed attractants could be used to maintain feed attractiveness. Protein palatability and utilization of rapeseed protein products could be improved by altering protein composition via breeding or processing.

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## Chapter 3

### **Fat encapsulation and supplementation with free amino acids cannot compensate for negative effects from dietary rapeseed protein isolate on growth performance of rainbow trout (*Oncorhynchus mykiss*)**

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**Abstract**

There are still restrictions when using rapeseed protein products as fishmeal alternatives in diets for rainbow trout. Digestibility of a particularly purified rapeseed protein isolate (protein content: 98.7 %) was determined in rainbow trout (initial body weight: mean = 178.2 ± standard deviation (SD) = 20.9 g) and it was used to exchange fishmeal gradually (0 %, 66 % and 100 %) in diets based on commercial trout diets. To overcome potential limiting factors on growth performance from rapeseed protein isolates, duplicates of these experimental diets were encapsulated with stearic acid and one diet devoid of fishmeal included rapeseed protein isolate as well as a mixture of free amino acids. The highest recorded crude protein digestibility (99.8 ± 1.6 %) of a rapeseed protein was reached using the stripping method. Daily feed intake (DFI) of the dietary group devoid of fishmeal (DFI: 2.1 ± 0.06) was significantly reduced in comparison with control (DFI: 2.6 ± 0.07) at the end of the experiment. Similarly, feed conversion ratio (FCR) was significantly impaired when comparing control (FCR: 0.9 ± 0.01) to the dietary group with complete exchange of fishmeal (FCR: 1.1 ± 0.02). Neither encapsulation of diets nor supplementation with free amino acids could overcome negative effects on growth performance from dietary rapeseed protein isolate inclusion. Results suggest that even highly purified rapeseed protein products can impair growth performance of rainbow trout and that factors other than ANFs could determine the effectiveness of rapeseed protein products as fishmeal alternatives.

## 1. Introduction

Although the proportion of fishmeal obtained from fish processings will likely have increased by 2030 (from 22 % in 2018 to 28 % in 2030), fishmeal prices are expected to rise due to elevated global demand (FAO, 2020). Hence, importance of sustainable alternatives is still increasing. Effects from multiple plant protein products on fish growth performance and health have been investigated in the past (Bonaldo et al., 2011; Bu et al., 2018; Burel et al., 2000b; Burel et al., 2000c; Greiling et al., 2018; Luo et al., 2012b; Men et al., 2014; Nagel et al., 2012, 2017) and rapeseed was determined to be a suitable candidate for replacing fishmeal due to its high global availability and desirable amino acid profile in comparison with other plant proteins (Higgs et al., 1982; Luo et al., 2012a; Mwachireya et al., 1999; Suárez et al., 2009). However, different issues negatively affecting fish growth performance occurred when using rapeseed protein products in the past (Francis et al., 2001; Nagel et al., 2012; Slawski, 2012). Anti-nutritive factors (ANFs), like glucosinolates and phytic acid present in rapeseed negatively affected feed intake, protein digestibility and caused various health issues in fish (Burel et al., 2000c; Francis et al., 2001; Mwachireya et al., 1999; Nagel et al., 2012; Slawski, 2012). Different processing techniques (Adem, 2014; Bérot et al., 2005; Kalaydzhiev et al., 2019; Shahidi & Naczki, 1990; Slawski, 2012) reduced content of ANFs in rapeseed protein products to a great extent, however, growth performance of fish was still impaired even at comparatively low levels of ANF inclusion (Kaiser et al., in press; Nagel et al., 2012; Slawski, 2012). Exceptionally high digestibility of crude protein was reached in rainbow trout using rapeseed protein isolates, following reduction in fiber, nitrogen-free extracts (NfE) and phytic acid (Mwachireya et al., 1999; Kaiser et al., in press). Reduction of these substances is presumed to be the most effective way to increase digestibility of rapeseed protein products (Mwachireya et al., 1999) and could lead to even higher nutrient digestibility.

When highly purified rapeseed protein products are used, effects from ANFs could be insignificant and a total replacement of fishmeal is possible without significantly affecting fish growth performance, as demonstrated by results of Slawski (2012). At high levels of purification, dietary amino acid composition could become a major factor for growth performance of rainbow trout (Kaiser et al., in press), since amino acid profiles are commonly known to affect protein utilization (Kaushik & Seiliez, 2010) and were demonstrated to influence feed intake via a hypothalamic regulation system triggered by pre- and post-

absorptive signaling pathways (Comesaña et al., 2018a; Comesaña et al., 2018b; Comesaña et al., 2020).

Consequently, bypassing of pre-absorptive evaluation of feed palatability by rainbow trout or changes in dietary amino acid composition could improve voluntary feed intake and growth performance when substantial amounts of fishmeal are being replaced by highly purified rapeseed protein products in experimental diets. Application of capsules leads to the effect, that fish ingest diets based on post-prandial signals and not due to taste (Brännäs & Strand, 2015). Consequently, encapsulation of diets with stearic acid and fish oil was used as an available and cost-effective tool to investigate if this masking of diet taste can compensate for negative effects from high dietary inclusion of rapeseed protein isolates on voluntary feed intake of rainbow trout. In fish nutrition, supplementation of plant based diets with amino acids improved growth performance in earlier studies (Ahmed et al., 2019; Gaylord & Barrows, 2009; Ren et al., 2017) and imitation of the amino acid profile of fishmeal could be necessary to exchange fishmeal with alternate proteins (Kaushik & Seiliez, 2010). One experimental diet in which fishmeal was completely replaced by rapeseed protein isolate was supplemented with crystalline amino acids to achieve the amino acid profile of the control group. Latter mentioned step was implemented to compensate for negative effects on growth performance of rainbow trout fed diets including high levels of rapeseed protein isolate. Additionally, a new processing method for a rapeseed protein isolate including precipitation as the main step was applied to increase protein yield, limit content of ANFs like glucosinolates and phytic acid as well as fiber and nitrogen-free extracts (NfE) and reduce costs of processing. The resulting isolate consisted mainly of cruciferin, one of the two major storage proteins in rapeseed, changing protein as well as amino acid composition in comparison to previous studies (Kaiser et al., in press; Slawski, 2012).

The aim of the present study was to investigate the effectiveness of a rapeseed protein isolate, which was purified using new techniques, as an alternate protein source in diets for rainbow trout and to test if encapsulation of experimental diets as well as supplementation with free amino acids can overcome negative effects from rapeseed protein products on growth performance of rainbow trout.

## 2. Materials and methods

### 2.1 Digestibility trial

225 rainbow trout (*Oncorhynchus mykiss*,  $178.2 \pm 20.9$  g) were obtained from Forellenzucht Trostadt GmbH & Co. KG, Trostadt, Germany for the initial digestibility trial. All fish were assigned evenly and at random to nine tanks (150 l) of a recirculating aquaculture system in the Gesellschaft für Marine Aquakultur, Büsum, Germany. During an adaptation phase of 14 days to the recirculating system, fish were fed a commercial trout diet (Aller Gold, 4 mm) at 1.5 % of their body weight and were subsequently starved for two days prior to the start of the trial.

Oxygen saturation was at  $98.0 \pm 4.0$  % (Handy Polaris, Oxy Guard International A/S, Birkerød, Denmark), temperature at  $14.5 \pm 0.1$  °C, pH at  $7.5 \pm 0.2$  (pH-Messgerät GMH 5550, GHM Messtechnik GmbH, Regenstauf, Germany), total ammonia nitrogen at  $0.3 \pm 0.2$  mg l<sup>-1</sup>, total nitrite nitrogen at  $1.7 \pm 0.6$  mg l<sup>-1</sup> (Microquant test kits for NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup>, Merck KGaA, Darmstadt, Germany) and salinity at  $2.5 \pm 1.0$  ppt (HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA) for the entirety of the digestibility experiment. Photoperiod was set at 14 hours of light and 10 hours of darkness. Each tank was provided with filtered water at 10 l/min.

For the digestibility trial, three diets were prepared. The basal diet (basal diet fishmeal: BD FM) was formulated with fishmeal (table 3.1) as the main source of protein and the two test diets consisted of 70.0 % basal diet and 30.0 % test substances (rapeseed protein test diet: RI T; fishmeal test diet: FM T; table 3.2). Fishmeal and rapeseed protein isolate were used as test substances (table 3.1). Titanium dioxide served as an inert marker to determine apparent digestibility coefficients. Processing of rapeseed seeds ("Raffiness", obtained from Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Holtsee, Germany) was done by Pilot Pflanzenöltechnologie Magdeburg e.V., Magdeburg, Germany following the processing protocol described in Adem (2014) with some alterations to reduce content of ash, fiber, NfE, glucosinolates and phytic acid while achieving an increased protein content. Rapeseed seeds were conditioned in a vacuum dryer for 20 min at 70 – 80 °C to inactivate the enzyme myrosinase. Subsequently, rapeseed oil was extracted via oil pressing (60 – 65 °C) and hexane extraction (50 – 60 °C). After desolventising of hexane, rapeseed seeds were crushed to an average particle size of 0.2 mm. The resulting rapeseed meal was subsequently processed by



extraction and separation, however, the filtration process described in Adem (2014) was omitted and replaced by precipitation. Latter mentioned alteration led to elevated protein content while being more cost-effective. The resulting rapeseed protein isolate mostly consists of cruciferin. After precipitation, gained material was freeze-dried.

Before addition of oily substances, mealy feed ingredients were evenly mixed. Resulting mixture was pressed to pellets of 4 mm diameter (L 14-175, AMANDUS KAHL, Reinbeck, Germany). Temperature was kept under 60 °C during processing of feed to prevent thermic damage to ingredients. Pellets were air-dried and subsequently stored at 4 °C.

Table 3.1 Nutrient composition, amino acid composition and anti-nutritive factors content in fishmeal and rapeseed protein isolate.

	Fishmeal	Rapeseed protein isolate
<b>Nutrient composition (% DM)</b>		
Moisture	5.01	2.19
Crude protein	72.38 <sup>5</sup>	98.70 <sup>5</sup>
Crude lipid	11.87	0.87
Crude ash	15.74	0.43
NfE + crude fiber <sup>1</sup>	0.01	0.00
Gross energy (MJ kg <sup>-1</sup> )	21.50	22.66
<b>Amino acid composition (% DM)<sup>2</sup></b>		
Alanine	4.3	4.5
Arginine	4.6	7.6
Aspartic acid	5.8	11.1
Cysteine	0.6	1.2
Glutamic acid	8.1	20.8
Glycine	4.9	5.8
Histidine	1.5	2.4
Isoleucine	2.9	4.9
Leucine	4.9	8.8
Lysine	4.9	3.3
Methionine	1.8	2.0
Phenylalanine	2.5	5.2
Proline	3.2	5.2
Threonine	2.9	4.1
Tryptophan	0.6	1.5
Tyrosine	2.0	3.1
Valine	3.5	5.8
<b>ANF content</b>		
Phytic acid (%) <sup>3</sup>		1.30
Glucosinolates (μmol/g) <sup>4</sup>		0.04

<sup>1</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash); <sup>2</sup> analyzed by AGROLAB LUFA GmbH, Kiel, Germany according to European Commission Regulation (EC) No 152/2009, III, F: 2009-02 (European Commission, 2009) or European Commission Regulation (EC) No 152/2009, III, G: 2009-02 (European Commission, 2009); <sup>3</sup> analyzed using phytic acid enzyme test (Megazyme Kit) and high performance liquid chromatography (HPLC) according to Sashidhar et al. (2020); <sup>4</sup> determined via enzyme test (myrosinase/glucose test kit according to Heaney et al., 1988) and HPLC (desulfatase C18 RP column according to Fiebig & Arens, 1992, UV detection of 15 compounds); <sup>5</sup> conversion factor for calculation of crude protein was 6.25\*N.

Table 3.2 Feed ingredients and feed composition of diets for digestibility trial.

	<b>Basal diet fishmeal (BD FM)</b>	<b>Rapeseed protein isolate test diet (RI T)</b>	<b>Fishmeal test diet (FM T)</b>
<b>Ingredient (% DM)</b>			
<b>Fishmeal<sup>1</sup></b>	55.5	38.9	68.9
<b>Rapeseed protein isolate</b>	0.0	30	0.0
<b>Gelatine<sup>2</sup></b>	5.0	3.5	3.5
<b>Wheat starch<sup>3</sup></b>	25.0	17.5	17.5
<b>Fish oil<sup>4</sup></b>	10.0	7.0	7.0
<b>Vitamin premix<sup>4</sup></b>	0.5	0.4	0.4
<b>Mineral premix<sup>4</sup></b>	0.5	0.4	0.4
<b>Ca<sub>2</sub>PO<sub>4</sub><sup>5</sup></b>	0.5	0.4	0.4
<b>Cellulose<sup>6</sup></b>	2.0	1.4	1.4
<b>Marker (TiO<sub>2</sub>)<sup>7</sup></b>	1.0	0.7	0.7
<b>Feed composition (% DM)</b>			
<b>Moisture</b>	11.8	10.2	10.7
<b>Crude protein</b>	46.5	64.0	54.1
<b>Crude lipid</b>	17.3	12.0	15.1
<b>Crude ash</b>	10.9	7.8	12.2
<b>NfE + crude fiber<sup>8</sup></b>	25.3	16.2	18.7
<b>Gross energy (MJ kg<sup>-1</sup>)</b>	21.9	22.1	21.8

<sup>1</sup> Bioceval GmbH & Co. KG, Cuxhaven; Germany; <sup>2</sup> Gustav Ehlert GmbH & Co. KG, Verl, Germany; <sup>3</sup> Kröner-Stärke GmbH, Ibbenbüren, Germany; <sup>4</sup> Emsland-Aller Aqua GmbH, Golßen, Germany; <sup>5</sup> Lehmann & Voss & Co. KG, Hamburg, Germany; <sup>6</sup> Mikro-Technik GmbH & Co. KG, Bürgstadt, Germany; <sup>7</sup> Kronos Titan GmbH, Nordenham, Germany; <sup>8</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash).

Collection of faeces was done manually by stripping subsequently to sedation of rainbow trout in clove oil (1 ml per 40 l of water). Stripping was carried out with all fish 5 hours after each manual feeding event. Fish were fed daily at 1.5 % of their body weight for 14 days.

Subsequent to faeces and diet nutrient and marker analysis, apparent digestibility coefficients (ADC) of nutrients in diets were calculated according to Maynard and Loosly (1979):

$$\text{ADC}_{\text{Nutrient, Diet}} (\%) = 100 * (1 - (\text{Marker}_{\text{Diet}} / \text{Marker}_{\text{Faeces}}) * (\text{Nutrient concentration}_{\text{Faeces}} / \text{Nutrient concentration}_{\text{Diet}}))$$

Calculation of ADC of the test substances was derived from the formula used in Bureau and Hua (2006):

$$\text{ADC}_{\text{Test substance}} = (\text{ADC}_{\text{Test diet}} - \text{ADC}_{\text{Base diet}}) * ((30 * \text{Nutrient concentration}_{\text{Test diet}} + 70 * \text{Nutrient concentration}_{\text{Base diet}}) / 30 * \text{Nutrient concentration}_{\text{Test diet}})$$

## 2.2 Growth trial

An adaptation period of 14 days to the recirculating aquaculture system of the Gesellschaft für Marine Aquakultur mBH, Büsum, Germany was carried out prior to the growth trial. Fish (*Oncorhynchus mykiss*,  $23.6 \pm 0.2$  g, Forellenzucht Troststadt GmbH & Co. KG, Troststadt, Germany) were fed a commercial trout diet (Aller Gold, 2 mm) at 1.5 % of their body weight during adaptation and were starved for two days before the start of the experiment. Rainbow trout were assigned to 21 tanks (50 l) in triplicate groups of 17 fish each. Assignment of groups was semi-random to avoid potential tank effects.

Optimal rearing conditions were ensured during the growth trial by daily observation of water parameters. Oxygen saturation was at  $101.3 \pm 4.2$  % (Handy Polaris, Oxy Guard International A/S, Birkerød, Denmark) and temperature was held at  $13.9 \pm 0.3$  °C. Salinity was  $5.4 \pm 0.8$  ppt (HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA), pH was  $7.1 \pm 0.3$  (pH-Messgerät GMH 5550, GHM Messgerät GmbH, Regenstauf, Germany), total ammonia nitrogen was  $0.3 \pm 0.2$  mg l<sup>-1</sup> and total nitrite nitrogen was  $1.1 \pm 0.4$  mg l<sup>-1</sup> (Microquant test kits for NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup>, Merck KGaA, Darmstadt, Germany). Photoperiod was set at 14 hours of light and 10 hours of darkness. Each tank was provided with filtered water at 6 l/min.

Fish were fed manually twice per day (10:00 am and 3:00 pm) to apparent satiation for 56 days. Prior to the feeding experiment, an average pellet weight was measured to calculate exact feed intake subsequent to collection of excess pellets via siphoning.

Seven diets were prepared for the main feeding trial (table 3.3). A diet containing 19 % fishmeal served as positive control (CD FM). In two diets, fishmeal of control diet was replaced by rapeseed protein isolate to 66 and 100 % of digestible protein content of fishmeal respectively (RI66, RI100), based on results from digestibility trial. To reach the same amino acid profile as in the positive control group (CD FM, table 3.4), a mixture of free amino acids replaced a portion of the rapeseed protein isolate in RI100 for the fourth diet (RI100 AA). The

remaining three diets were duplicates of CD FM, RI66 and RI100 with reduced rapeseed oil content (CD FM C; RI66 C; RI100 C); however, all three diets were encapsulated with stearic acid (95 % of capsule) and fish oil (5 % of capsule). This encapsulation was carried out by Katz Biotech AG, Berlin, Germany with a hotmelt-process using Romaco Innojet Ventilus V-2.5 (Romaco Holding GmbH, Karlsruhe, Germany). Subsequent to the encapsulation procedure, capsulated pellets were checked on a random basis for a continuous solid capsule by Katz Biotech AG, Berlin, Germany using a Leica S9i microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 55x magnification.

Formulation of all diets was carried out to meet nutritional demands of rainbow trout (National Research Council, 2011). Additionally, diets of the main feeding trial were isonitrogenous and isoenergetic on digestible level (table 3.3). Processing of pellets followed the same protocol as for diets of digestibility trial, however, diameter of pellets was 2 mm (2.1). At the end of the growth trial, growth performance parameters were calculated including feed conversion ratio ( $FCR = \text{g feed intake} / \text{g weight gain}$ ), specific growth rate ( $SGR = (\ln (FBW) - \ln (IBW)) / \text{feeding days} * 100$ ), daily feed intake ( $DFI = FCR * SGR$ ), protein efficiency ratio ( $PER = \text{g body weight gain} / \text{g crude protein intake}$ ) and protein retention efficiency ( $PRE = \text{g crude protein gained} / \text{g crude protein intake} * 100$ ).

Experimental procedure has been ethically approved by the “Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung” in Schleswig-Holstein, Germany.

Table 3.3 Feed ingredients, feed composition and anti-nutritive factors content of diets for main feeding trial.

	CD FM	RI66	RI100	RI100 AA	CD FM C	RI66 C	RI100 C
<b>Ingredient (% OS)</b>							
Fishmeal <sup>1</sup>	19.00	6.27	0	0	19.00	6.27	0
Rapeseed protein isolate <sup>2</sup>	0	7.59	11.08	8.80	0	7.59	11.08
Blood meal <sup>3</sup>	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Gelatine <sup>4</sup>	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Pea protein isolate <sup>5</sup>	24.00	24.00	24.00	24.00	24.00	24.00	24.00
Wheat gluten <sup>6</sup>	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Biolys <sup>7</sup>	0	0.20	0.50	1.15	0	0.20	0.50
MetMet <sup>7</sup>	0	0	0.10	0.15	0	0	0.10
Amino acid mix <sup>8</sup>	0	0	0	1.75	0	0	0
Wheat starch <sup>6</sup>	13.49	13.49	13.49	13.49	13.49	13.49	13.49
Corn starch <sup>6</sup>	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Rapeseed oil <sup>9</sup>	3.30	3.30	3.30	3.30	0.30	0.30	0.30
Fish oil <sup>1</sup>	6.59	7.93	8.90	8.90	6.59	7.93	8.90
Vitamin premix <sup>5</sup>	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral premix <sup>5</sup>	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Ca <sub>2</sub> PO <sub>4</sub> <sup>10</sup>	0.70	1.00	1.20	1.20	0.70	1.00	1.20
Cellulose <sup>11</sup>	0.27	3.57	4.78	4.61	0.27	3.57	4.78
Stearic acid + fish oil <sup>12</sup>	0	0	0	0	3.00	3.00	3.00
<b>Feed composition (% DM)</b>							
Moisture	6.1	6.3	6.8	6.32	4.25	3.98	4.14
Crude protein	53.4	52.1	52.0	51.5	50.5	49.3	49.3
Crude lipid	16.3	15.8	16.4	17.4	15.4	14.6	14.7
Crude ash	5.2	3.8	3.0	2.9	4.9	3.6	2.9
NfE + crude fiber <sup>13</sup>	25.1	29.0	28.6	28.3	29.3	32.5	33.1
Gross energy (MJ kg <sup>-1</sup> )	23.0	23.1	23.2	23.1	23.9	23.8	24.1
<b>Anti-nutritive factors<sup>14</sup></b>							
Phytic acid (% of feed)	0	0.01	0.14	0.11	0	0.01	0.14
Glucosinolates (µmol/g of feed)	0	0.003	0.004	0.003	0	0.003	0.004

<sup>1</sup> Bioceval GmbH & Co. KG, Cuxhaven; Germany; <sup>2</sup> Pilot Pflanzenöltechnologie Magdeburg e. V., Magdeburg, Germany; <sup>3</sup> Saria SE & Co. KG, Selm, Germany; <sup>4</sup> Gustav Ehlert GmbH & Co. KG, Verl, Germany; <sup>5</sup> Emsland-Aller Aqua GmbH, Golßen, Germany; <sup>6</sup> Kröner-Stärke GmbH, Ibbenbüren, Germany; <sup>7</sup> Evonik Industries AG, Herne, Germany, Biolys, MetMet; <sup>8</sup> Amino acid mix, Evonik Industries AG, Herne, Germany (% OS): L-Alanine (0.40), ThreAMINO (0.16), L-Tyrosine (0.10) Carl Roth GmbH + Co. KG, Schoemperlenstraße 3-5, 76185, Karlsruhe, Germany (% OS): L-Arginine (0.15), L-Aspartic acid (0.10), Glycine (0.40), L-Leucine (0.10), L-Proline (0.10), L-Tryptophan (0.12), L-Valine (0.12); <sup>9</sup> Cargill GmbH, Riesa, Germany; <sup>10</sup> Lehmann & Voss & Co. KG, Hamburg, Germany; <sup>11</sup> Mikro-Technik GmbH & Co. KG, Bürgstadt, Germany; <sup>12</sup> Katz Biotech AG, Berlin, Germany; <sup>13</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash); <sup>14</sup> Based on analysis of feed ingredients.

Table 3.4 Amino acid composition in growth trial diets.

Amino acid composition (% DM) <sup>1</sup>	CD FM	RI66	RI100	RI100 AA	CD FM C	RI66 C	RI100 C	Requirements <sup>2</sup>
Alanine	2.6	2.3	2.2	2.5	2.6	2.3	2.2	-
Arginine	3.7	3.7	3.7	3.7	3.7	3.7	3.7	1.4 – 1.8
Aspartic acid	4.2	4.3	4.4	4.2	4.2	4.3	4.4	-
Cysteine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-
Glutamic acid	7.9	8.4	8.7	8.2	7.9	8.4	8.7	-
Glycine	3.1	2.9	2.8	3.1	3.1	2.9	2.8	-
Histidine	1.2	1.2	1.2	1.2	1.2	1.2	1.2	0.5 – 0.6
Isoleucine	2.2	2.2	2.2	2.1	2.2	2.2	2.2	0.7 – 1.4
Leucine	4.0	4.1	4.1	4.0	4.0	4.1	4.1	1.1 – 1.4
Lysine	2.9	2.7	2.6	2.9	2.9	2.7	2.6	1.4 – 2.5
Methionine	0.7	0.6	0.7	0.7	0.7	0.6	0.7	0.4 – 0.9
Phenylalanine	2.3	2.4	2.4	2.3	2.3	2.4	2.4	0.7
Proline	3.1	3.1	3.0	3.0	3.1	3.1	3.0	-
Threonine	1.8	1.8	1.7	1.8	1.8	1.8	1.7	1.0
Tryptophan	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1 - 0.2
Tyrosine	0.8	0.7	0.7	0.8	0.8	0.7	0.7	-
Valine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.8 – 1.6

<sup>1</sup> based on analysis of feed ingredients; <sup>2</sup> according to National Research Council (2011).

### 2.3 Sampling

All fish were starved for 48 hours before collection of samples. After the initial adaptation phase as well as after 56 days of feeding, fish were sampled for determination of whole body composition and calculation of hepatosomatic index (HSI), viscerosomatic index (VSI), spleen somatic index (SSI) and Fulton condition factor (FCF). 15 fish were randomly selected for the initial sampling and all factors were determined after 56 days of feeding for each treatment using three randomly selected fish per treatment. Fish were anaesthetized with a blunt hit on the central nervous system and subsequently euthanized by piercing the heart. Whole body samples were freeze-dried before analysis (Alpha 1-2 LD plus, Christ, Osterode, Germany). Prior to freeze-drying and analysis, samples were stored at -20 °C.

#### 2.4 Analysis of nutrients and anti-nutritive factors

Analysis of amino acid concentration (AGROLAB LUFA GmbH, Kiel, Germany) and macronutrients of raw materials as well as macronutrients analysis of diets and whole body was carried out according to the European Commission Regulation (EC) No 152/2009 (European Commission, 2009). For the analysis of amino acid composition, methods according to European Commission Regulation (EC) No 152/2009, III, F: 2009-02 (European Commission, 2009) were used. Amino acids were extracted with hydrochloric acid and the resulting solution is set at a pH value of 2.2. Amino acids are being separated by ion exchange chromatography and subsequent to a reaction with ninhydrin, amino acids are being identified by photometric analysis at 570 nm. For analysis of tryptophan, methods according to European Commission Regulation (EC) No 152/2009, III, G: 2009-02 (European Commission, 2009) were used. Analysis of diets and homogenized whole bodies was carried out in duplicates. For determination of crude lipid content in whole body, methods according to the Soxhlet-protocol (Soxtherm, C. Gerhardt GmbH & Co. KG, Königswinter, Germany) were applied. Crude protein content was determined using standard Kjeldahl methods. Before determination of crude ash content via a combustion oven (P300, Nabertherm, Lilienthal, Germany) at 550 °C for 12 hours, dry matter content of samples was ascertained by drying (ED 53, Binder GmbH, Tuttlingen, Germany) for 4 hours at 103 °C. Nitrogen-free extracts plus crude fiber content was defined as remaining portion of macronutrients in diets and whole body. Methods applied in Sashidhar et al. (2020) were used to measure phytic acid content (Christian-Albrechts-University, Kiel, Germany). The enzyme test as described by Heaney et al. (1988) and HPLC analysis after Fiebig and Arens (1992) were used for determination of glucosinolate content (Christian-Albrechts-University, Kiel, Germany).

Content of titanium in faeces was determined according to DIN EN ISO 11885:2009 (DIN EN ISO 11885:2009-09, 2009; AGROLAB LUFA GmbH, Kiel, Germany).



### 2.5 Statistical analysis

The statistical program SPSS (version 20) was used for data analysis of the digestibility trial. Prior to application of T-test or one-way analysis of variances (ANOVA), Kolmogorov-Smirnov and Levene test were applied to determine normal distribution and homogeneity of variances. Tukey's HSD test was used for multiple comparisons when differences among groups were identified. The aggregate type I error was defined at 5 % ( $p < .05$ ) for each set of comparisons to determine statistical significance.

The statistical software R (2020) was used to evaluate the data of the growth trial. The data evaluation started with the definition of an appropriate linear pseudo one-way model. This model included a pseudo factor, which represents a mixture of the actual factors exchange rate of fishmeal (0 %, 66 %, 100 %), encapsulation (encapsulated and non-encapsulated) and amino acid supplementation (not supplemented and supplemented). This pseudo factor was necessary as the actual factors are not orthogonal (Schaarschmidt & Vaas, 2009). The residuals were assumed to be normally distributed and to be homo- or heteroscedastic. These assumptions are based on a graphical residual analysis. Multiple contrast tests (Hasler & Hothorn, 2008; Hothorn et al., 2008) were conducted in order to compare the several levels of exchange rate of fishmeal, encapsulation and amino acid supplementation. Additionally, appropriate contrast was used for the consideration of the interaction effects between exchange rate of fishmeal and encapsulation (Kitsche & Schaarschmidt, 2015).

### 3. Results

#### 3.1 Digestibility

Rapeseed protein isolate inclusion resulted in significantly elevated dry matter and crude protein apparent digestibility coefficient (ADC) compared with control diet (BD FM) and fishmeal diet (FM T, table 3.5). Additionally, rapeseed protein isolate had a significantly higher ADC of crude protein ( $99.8 \pm 1.6 \%$ ) and energy ( $96.2 \pm 2.9 \%$ ) in comparison with fishmeal ( $84.7 \pm 3.3 \%$ ;  $85.2 \pm 2.8 \%$ ; table 3.5).

Table 3.5 Apparent digestibility coefficients of test diets and test ingredients (mean  $\pm$  SD, n=3).

Apparent digestibility coefficient (%)	BD FM	RI T	FM T
Dry matter diet	$77.6 \pm 0.5^a$	$83.2 \pm 1.0^b$	$76.2 \pm 1.1^a$
Crude protein diet	$84.0 \pm 1.0^a$	$91.7 \pm 0.8^b$	$84.3 \pm 1.4^a$
Crude protein of test ingredient		$99.8 \pm 1.6^a$	$84.7 \pm 3.3^b$
Energy of test ingredient		$96.2 \pm 2.9^a$	$85.2 \pm 2.8^b$

Values in the same row with different superscript letters are significantly different ( $P < .05$ ).

#### 3.2 Growth performance

With increasing dietary levels of rapeseed protein isolate, final body weight (FBW) was decreased, both in capsulated and non-capsulated diets (table 3.6). Each level of fishmeal exchange rate was significantly different to the former in FBW (table 3.7). Additionally, both control groups (CD FM and CD FM C) differed significantly in FBW and RI100 AA had a significantly reduced FBW in comparison with CD FM and RI66 (table 3.7). FCR was rising with increasing levels of fishmeal exchange rate (table 3.6). Every treatment was significantly different in FCR to the previous with a smaller portion of fishmeal (table 3.7). RI66 had a significantly higher FCR in comparison with RI66 C. FCR of RI100 AA ( $1.04 \pm 0.01$ ) was significantly elevated in comparison with CD FM ( $0.91 \pm 0.02$ ) and RI66 ( $1.00 \pm 0.03$ ). Differences in SGR resemble differences in FBW (table 3.7). DFI also decreased with increasing exchange rate of fishmeal (table 3.6), however, 66 % exchange did not lead to a significantly reduced DFI in comparison with control (table 3.7). Capsulation of diets led to a significant decrease in DFI (table 3.6 and 3.7). This difference was not significant between RI100 and RI100 C (table 3.7). RI100 AA had a significantly decreased DFI in comparison with CD FM and RI66 (table 3.6 and 3.7). With each increase in fishmeal exchange rate, PER is significantly

reduced (table 3.6 and 3.7). Additionally, capsulation of diets significantly elevated PER (table 3.6 and 3.7). PER of RI100 AA ( $2.01 \pm 0.02$ ) was elevated in comparison with RI100 ( $1.94 \pm 0.01$ ), however, this difference was not significant. CD FM had a significantly higher PER ( $2.20 \pm 0.05$ ) compared with RI100 AA, while PER of RI66 ( $2.08 \pm 0.06$ ) and RI100 AA were similar (table 3.7). PRE was significantly elevated when CD FM was compared to RI100 and CD FM C had a significantly higher PRE than RI66 C and RI100 C (table 3.7). Capsulated and non-capsulated diets had a similar PRE (table 3.6 and 3.7) while PRE of RI100 AA ( $30.08 \pm 0.97$ ) was not significantly different to any of the non-capsulated diets (table 3.7).

There was no significant interaction detected between exchange rate of fishmeal and capsulation for any of the growth performance parameters.

Table 3.6 Growth performance parameter of rainbow trout after 56 days of feeding (mean  $\pm$  SD, n=3).

	CD FM	RI66	RI100	RI100 AA	CD FM C	RI66 C	RI100 C
<b>IBW</b>	23.5 $\pm$ 0.26	23.7 $\pm$ 0.10	23.5 $\pm$ 0.29	23.7 $\pm$ 0.19	23.8 $\pm$ 0.11	23.6 $\pm$ 0.17	23.5 $\pm$ 0.39
<b>FBW</b>	118.2 $\pm$ 1.37	93.8 $\pm$ 6.02	71.1 $\pm$ 2.60	78.7 $\pm$ 5.24	100.8 $\pm$ 4.22	87.7 $\pm$ 5.15	67.2 $\pm$ 3.14
<b>FCR</b>	0.91 $\pm$ 0.02	1.00 $\pm$ 0.03	1.07 $\pm$ 0.01	1.04 $\pm$ 0.01	0.89 $\pm$ 0.02	0.95 $\pm$ 0.02	1.03 $\pm$ 0.01
<b>SGR</b>	2.88 $\pm$ 0.01	2.45 $\pm$ 0.11	1.98 $\pm$ 0.04	2.14 $\pm$ 0.12	2.58 $\pm$ 0.08	2.34 $\pm$ 0.12	1.88 $\pm$ 0.11
<b>DFI</b>	2.62 $\pm$ 0.06	2.45 $\pm$ 0.06	2.11 $\pm$ 0.06	2.24 $\pm$ 0.12	2.30 $\pm$ 0.09	2.22 $\pm$ 0.10	1.94 $\pm$ 0.10
<b>PER</b>	2.20 $\pm$ 0.05	2.08 $\pm$ 0.06	1.94 $\pm$ 0.01	2.01 $\pm$ 0.02	2.33 $\pm$ 0.05	2.22 $\pm$ 0.04	2.05 $\pm$ 0.03
<b>PRE</b>	32.93 $\pm$ 2.21	30.18 $\pm$ 1.08	28.06 $\pm$ 2.21	30.08 $\pm$ 0.97	35.45 $\pm$ 1.19	30.12 $\pm$ 0.53	28.00 $\pm$ 1.62

IBW (initial body weight); FBW (final body weight); FCR (feed conversion ratio) = g feed intake / g weight gain; SGR (specific growth rate) = (ln (FBW) – ln (IBW)) / feeding days \* 100; DFI (daily feed intake) = daily feed intake in % body weight; PER (protein efficiency ratio) = g body weight gain / g crude protein intake; PRE (protein retention efficiency) = g crude protein gained / g crude protein intake \* 100.

Table 3.7 Adjusted P values of multiple contrast tests comparing the several levels of exchange rate of fishmeal, encapsulation and amino acid supplementation for growth performance parameters of rainbow trout after 56 days of feeding.

Compared groups	FBW	FCR	SGR	DFI	PER	PRE
<b>Exchange rate of fishmeal</b>						
CD FM – RI66	<.001	<.001	<.001	.12	.02	.19
CD FM – RI100	<.001	<.001	<.001	<.001	<.001	.008
RI66 – RI100	<.001	.003	<.001	.002	.004	.41
CD FM C – RI66 C	.01	.007	.04	.80	.04	.004
CD FM C – RI100 C	<.001	<.001	<.001	<.001	<.001	<.001
RI66 C – RI100 C	<.001	<.001	<.001	.006	<.001	.40
<b>Encapsulation</b>						
CD FM – CD FM C	<.001	.53	.004	.001	.005	.17
RI66 – RI66 C	.27	.02	.42	.02	.002	.99
RI100 – RI100 C	.61	.11	.49	.08	.01	.99
<b>Supplementation with amino acids</b>						
RI100 AA – CD FM	<.001	<.001	<.001	<.001	<.001	.09
RI100 AA – RI66	.002	.02	.003	.03	.11	.99
RI100 AA – RI100	.11	.42	.12	.20	.14	.28

FBW (final body weight); FCR (feed conversion ratio) = g feed intake / g weight gain; SGR (specific growth rate) =  $(\ln(\text{FBW}) - \ln(\text{IBW})) / \text{feeding days} * 100$ ; DFI (daily feed intake) = daily feed intake in % body weight; PER (protein efficiency ratio) = g body weight gain / g crude protein intake; PRE (protein retention efficiency) = g crude protein gained / g crude protein intake \*100.

### 3.3 Body composition

No significant differences occurred in moisture, crude fat, NfE and crude fiber or energy content in any of the analyzed fish bodies (table 3.8). Crude ash content of CD FM C ( $2.23 \pm 0.16$ ) differed significantly ( $P = .007$ ) from RI100 C ( $1.75 \pm 0.20$ ). Additionally, RI100 AA ( $1.54 \pm 0.08$ ) had a significantly ( $P = .007$ ;  $P = .02$ ) reduced crude ash content in comparison with CD FM ( $1.98 \pm 0.14$ ) and RI100 ( $1.93 \pm 0.16$ ). Crude protein content of RI66 C ( $14.26 \pm 0.28$ ) was significantly ( $P = .02$ ) reduced in comparison with CD FM C ( $15.46 \pm 0.22$ ).

There was no significant interaction detected between exchange rate of fishmeal and capsulation for any of the whole body composition measurands.

Table 3.8 Proximate whole body composition of fish after 56 days of feeding (mean  $\pm$  SD, n=3).

(% OS)	CD FM	RI66	RI100	RI100 AA	CD FM C	RI66 C	RI100 C
<b>Moisture</b>	69.71 $\pm$ 0.81	70.26 $\pm$ 1.05	71.53 $\pm$ 1.37	70.36 $\pm$ 0.80	69.92 $\pm$ 0.53	70.44 $\pm$ 1.40	71.44 $\pm$ 1.09
<b>Crude ash</b>	1.98 $\pm$ 0.14	1.78 $\pm$ 0.11	1.93 $\pm$ 0.16	1.54 $\pm$ 0.08	2.23 $\pm$ 0.06	1.99 $\pm$ 0.22	1.75 $\pm$ 0.20
<b>Crude protein</b>	15.22 $\pm$ 0.54	14.94 $\pm$ 0.06	15.04 $\pm$ 0.72	15.34 $\pm$ 0.23	15.46 $\pm$ 0.22	14.26 $\pm$ 0.28	14.52 $\pm$ 0.54
<b>Crude fat</b>	12.76 $\pm$ 0.64	12.85 $\pm$ 1.08	11.43 $\pm$ 0.90	12.62 $\pm$ 1.23	12.37 $\pm$ 0.31	12.97 $\pm$ 1.54	12.27 $\pm$ 0.59
<b>NfE + crude fiber<sup>1</sup></b>	0.33 $\pm$ 0.30	0.18 $\pm$ 0.19	0.08 $\pm$ 0.13	0.13 $\pm$ 0.12	0.01 $\pm$ 0.02	0.34 $\pm$ 0.09	0.01 $\pm$ 0.02
<b>Energy (MJ/kg)</b>	8.52 $\pm$ 0.36	8.46 $\pm$ 0.46	7.82 $\pm$ 0.61	8.43 $\pm$ 0.41	8.35 $\pm$ 0.13	8.32 $\pm$ 0.60	8.05 $\pm$ 0.33

<sup>1</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash).

### 3.4 Health parameters

There were no significant differences between any of the dietary treatments in SSI, VSI and FCF (table 3.9). HSI of RI100 AA (1.47  $\pm$  0.16) was significantly decreased in comparison to RI66 ( $P = .01$ ; 1.92  $\pm$  0.17) and RI100 ( $P = .001$ ; 2.08  $\pm$  0.59). Additionally, RI100 AA had a reduced HSI in comparison with control (1.81  $\pm$  0.23, table 3.9), however, statistical comparison was not significant ( $P = .05$ ).

There was no significant interaction detected between exchange rate of fishmeal and capsulation for any of the health parameters.

Table 3.9 Health parameters of fish after 56 days of feeding (mean  $\pm$  SD; n=3).

	CD FM	RI66	RI100	RI100 AA	CD FM C	RI66 C	RI100 C
<b>HSI</b>	1.81 $\pm$ 0.23	1.92 $\pm$ 0.17	2.08 $\pm$ 0.59	1.47 $\pm$ 0.16	1.72 $\pm$ 0.30	1.86 $\pm$ 0.22	1.90 $\pm$ 0.32
<b>SSI</b>	0.12 $\pm$ 0.03	0.10 $\pm$ 0.03	0.13 $\pm$ 0.05	0.10 $\pm$ 0.03	0.14 $\pm$ 0.06	0.11 $\pm$ 0.02	0.14 $\pm$ 0.05
<b>VSI</b>	16.51 $\pm$ 1.54	17.84 $\pm$ 1.50	18.52 $\pm$ 2.69	16.85 $\pm$ 1.15	16.88 $\pm$ 1.06	18.14 $\pm$ 1.25	18.56 $\pm$ 1.45
<b>FCF</b>	1.17 $\pm$ 0.08	1.24 $\pm$ 0.11	1.12 $\pm$ 0.12	1.18 $\pm$ 0.07	1.12 $\pm$ 0.06	1.19 $\pm$ 0.08	1.21 $\pm$ 0.15

VSI (viscerosomatic index) = (g fish weight - g gutted fish weight) / g fish weight \* 100; HSI (hepatosomatic index) = g liver weight / g fish weight \* 100; SSI (spleen somatic index) = g spleen weight / g fish weight \* 100; FCF (fulton condition factor) = g fish weight \* 100 \* cm fish length<sup>-3</sup>.

## 4. Discussion

### 4.1 Digestibility

In comparison with previous studies investigating digestibility of rapeseed protein products (Mwachireya et al., 1999; Kaiser et al., in press; Slawski, 2012), content of ash, fiber and NfE in rapeseed protein isolate was reduced to a minimal amount, while content of phytic acid was similar. The highest apparent crude protein digestibility of a rapeseed protein product in rainbow trout using stripping as the faeces collection method was subsequently measured. In previous literature, indigestible carbohydrates with or without phytic acid were made responsible for being decisive factors for nutrient digestibility of rapeseed protein products (Bu et al., 2018; Burel et al., 2000a; Cheng et al., 2010; Greiling et al., 2018; Luo et al., 2012a; Mwachireya et al., 1999; Nagel et al., 2012; Slawski, 2012). This study showed that phytic acid might be negligible at low dietary levels, in terms of its influence on protein digestibility. In comparison with results from Kaiser et al. (in press) higher crude protein digestibility was reached in this study at almost identical phytic acid contents (1.28 and 1.3 %), faeces collection method and diet formulation. Additionally, studies using different experimental designs also did not reach a protein digestibility as high as in this study despite lower levels of phytic acid in rapeseed protein isolates (Mwachireya et al., 1999; Slawski, 2012). Additionally, crude ash content of rapeseed protein isolate used in this experiment was exceptionally low, which could also have improved digestibility of nutrients (Gomes et al., 1995). This effect, together with reduced fiber content could also have led to the increase in energy digestibility (Burel et al., 2000a; Glencross, 2009) in comparison with previous results (Mwachireya et al., 1999; Kaiser et al., in press). Mean and standard deviation of crude protein digestibility of the rapeseed protein isolate indicate that crude protein digestibility was above 100% in one of the triplicates. This overestimation of digestibility was likely due to minor inaccuracies in nutrient analysis.

Conclusively, exceptionally high purification of the rapeseed protein product in terms of ash, fiber and NfE content was most likely responsible for reaching the highest apparent digestibility of crude protein from rapeseed products in rainbow trout. These results demonstrate that plant proteins have the potential to reach an apparent protein digestibility of virtually 100% in carnivorous species of fish if they are being highly purified. Minor inclusions of phytic acid might be negligible for crude protein digestibility in this species of fish.

#### 4.2 Growth performance

Feed intake and feed conversion were significantly affected by inclusion of rapeseed protein isolate. In contrast to results from Kaiser et al. (in press) and Slawski (2012), 66% of fishmeal could not have been exchanged with rapeseed protein isolate without significantly affecting growth performance of rainbow trout. Reduced growth performance parameters were also observed in other studies investigating effects from different rapeseed protein concentrates and isolates in multiple species of fish (Hermann et al., 2016; Nagel et al., 2012, 2017; Stickney et al., 1996). The majority of adverse effects from rapeseed proteins were traced back to ANFs present in rapeseed (Francis et al., 2001). Glucosinolates and phytic acid were made responsible for reductions in DFI in different species of fish at certain levels of inclusion (Kaiser et al., 2021; Slawski, 2012). Additionally, aforementioned ANFs seemingly caused impairments in feed conversion via negative effects on thyroid activity (glucosinolates; Burel et al., 2000a) or reduction of protein and mineral digestibility (phytic acid; Francis et al., 2001). Similar to effects on DFI from glucosinolates and phytic acid, other potential and lesser-known ANFs like the Kaempferol derivate Kaempferol 3-O- (2''-O-Sinapoyl- $\beta$ -sophoroside) could influence feeding behavior via impaired diet taste (Kaiser et al., in press). However, levels of glucosinolates and phytic acid in diets are probably below amounts with significant impact on growth performance (Burel et al., 2000a; Kaiser et al., 2021). Phytic acid can form protein – phytate complexes, which would lower protein digestion for fish (Francis et al., 2001). As described in 4.1, exceptionally high crude protein digestibility also indicates that the dietary amounts of phytic acid did not affect protein digestibility significantly and therefore did not affect protein utilization in this study. This would also be in line with an assumption from Teskeredžić et al. (1995), stating that the native form of phytic acid in rapeseed protein concentrate has a negligible effect on protein availability in salmonids. Effects from other ANFs present in rapeseed on feed conversion cannot be precluded. However, a significant impact appears unlikely due to the exceptionally high purification of the rapeseed protein isolate used in this study.

Slawski (2012) was able to replace 100% of fishmeal with canola protein isolate in diets fed to rainbow trout (initial body weight:  $31.5 \pm 0.5$  g) without affecting feed intake or feed conversion. Comparison between the 100% exchange diet of previously mentioned literature and RI100 shows that they are almost identical in terms of nutrient composition, similar in glucosinolate and phytic acid content, but different in amino acid composition (table 3.10).

Table 3.10 Feed composition, anti-nutritive factors content and amino acid composition of CD FM, RI100 and control group as well as the dietary group with 100% exchange of fishmeal from Slawski (2012).

	CD FM	RI100	Control (Slawski, 2012)	100% exchange (Slawski, 2012)
<b>Feed composition (% DM)</b>				
Crude protein	53.4	52.0	48.9	50.6
Crude lipid	16.3	16.4	15.9	15.9
Crude ash	5.2	3.0	8.0	3.7
NfE + crude fiber <sup>1</sup>	25.1	28.6	27.2	29.8
Gross energy (MJ kg <sup>-1</sup> )	23.0	23.2	22.8	23.7
Glucosinolates (μmol/g) <sup>2</sup>	0	0.004	0	0.04
Phytic acid (%) <sup>2</sup>	0	0.14	0	0.20
<b>Amino acid composition (% DM)<sup>2, 3</sup></b>				
Arginine	3.7	3.7	2.7	3.1
Histidine	1.2	1.2	1.9	2.1
Isoleucine	2.2	2.2	1.4	1.5
Leucine	4.0	4.1	4.3	4.6
Lysine	2.9	2.6	3.4	3.1
Methionine	0.7	0.7	0.8	0.7
Cysteine	0.5	0.5	0.4	0.7
Phenylalanine	2.3	2.4	2.4	2.5
Threonine	1.8	1.7	1.7	1.8
Valine	2.5	2.5	3.0	3.2

<sup>1</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash); <sup>2</sup> based on analysis of feed ingredients; <sup>3</sup> contents of all amino acids that were available in Slawski (2012) are displayed.

If effects from ANFs were non-significant in this experiment, protein and amino acid composition might be the decisive factors impairing feed conversion and feed intake. Literature suggests that voluntary feed intake might depend on an interaction between feed evaluation via olfaction and gustation (Hara, 2006; Kasumyan, 2019; Kasumyan & Sidorov, 2012) and subsequent feed intake regulation via pre- and/or post-absorptive signaling pathways (also referred to as pre- and post-absorptive mechanisms or effects; Comesaña et al., 2018a; Comesaña et al., 2018b; Comesaña et al., 2020). This interaction might be majorly affected by amino acid and/or protein composition as both initial feed evaluation (Comesaña et al., 2018b; Hara, 2006; Jones, 1989; Kasumyan & Sidorov, 2012; Kohbara & Caprio, 2001; Marui et al., 1983) and subsequent feed regulation (Comesaña et al., 2018a; Comesaña et al., 2018b; Comesaña et al., 2020) were shown to interact with specific amino acids, depending on species. A limited range of amino acids affected feed intake of rainbow trout in multiple



studies (Comesaña et al., 2018b; Jones, 1989; Kasumyan & Sidorov, 2012; Kohbara & Caprio, 2001; Marui et al., 1983; Tusche, 2012) emphasizing the potential importance of amino acid compositions for regulation of feed intake. The application of capsules leads to the effect, that fish ingest diets based on post-prandial signals rather than taste (Brännäs & Strand, 2015). Fat encapsulation of diets led to a significant reduction in DFI in this study, demonstrated by the significantly reduced DFI in encapsulated diets in comparison with their non-encapsulated counter parts (table 3.7), although differences between RI100 and RI100 C were not significant. Results suggest that a capsule mainly consisting of stearic acid reduces palatability of diets compared to similar diets without fat encapsulation. However, this effect should not compromise the capsulation-effect of the stearic acid and fish oil as they formed a solid and continuous capsule on the diets. Additionally, fat encapsulation prevents the loss of flavor-carrying compounds if these compounds do not diffuse through the fat capsules (Reineccius, 1989). DFI was significantly reduced due to an increasing exchange rate of fishmeal without a significant interaction between exchange rate of fishmeal and effects from encapsulation. Hence, post-absorptive effects could affect feed intake regulation independently from pre-absorptive effects. This would also be in line with observations from Comesaña et al. (2018b), who found that the homeostatic regulation of feed intake could be independent of putatively palatable nutrients. Different amino acids caused expressions of neuropeptides of varying magnitudes involved in feed intake regulation in the hypothalamus subsequent to intracerebroventricular (ICV) or intraperitoneal (IP) treatment respectively in rainbow trout (Comesaña et al., 2018a; Comesaña et al., 2018b). These observations give rise to the assumption that the post-absorptive composition of amino acids could affect regulation of voluntary feed intake. This was also hypothesized in earlier literature (de la Higuera, 2001). Since post-prandial amino acid composition is significantly dependent on dietary amino acid composition (Rolland et al., 2015), differences in dietary amino acid composition (table 3.4) caused differences in feed intake regulation between treatments with different levels of fishmeal exchange rate. Previous studies showed that increasing essential amino acid (EAA) availability improves feed intake at least until requirement levels are reached (de la Higuera, 2001). Levels of lysine for instance, were above minimum requirement levels for growth in treatment RI100 (National Research Council, 2011), but might have been below optimal levels for feed intake stimulation in comparison to CD FM. Although supplementation with amino acids can improve feed intake in some fish species when alternative protein sources are being

used (de la Higuera, 2001), treatment RI100 AA did not change DFI significantly in comparison with RI100 despite having the same amino acid composition as CD FM. This limited effect could be due to different or even reduced effects that free amino acids could have on palatability in comparison with protein bound amino acids (Jones, 1989). Additionally, pre-absorptive feed intake regulation might have played an important role in non-capsulated diets as it was shown that certain amino acids have pre-absorptive effects on feed intake in rainbow trout (Comesaña et al., 2020; Hara, 2006). The exclusion of glycine from a dietary amino acid mix for example, led to a significantly reduced feed intake in rainbow trout compared with the control diet, while the same dietary amino acid mix with glycine (1.8 % of diet in DM) led to a similar feed intake compared to control (Gaylord & Barrows, 2009). Additionally, the existence of a hedonic feed regulation system in fish has been speculated (Comesaña et al., 2018b; Mueller & Wullimann, 2009; O'Connell et al., 2011). Since RI100 AA was not capsulated, pre-absorptive feed intake regulation could have reduced DFI due to inclusion or lack of certain amino acids. Further studies will be necessary to improve our understanding of the interactive regulation of feed intake and the exact role of free and protein bound amino acids involved in the process. Effects on DFI from different factors other than dietary amino acid composition cannot be excluded with results from this study, since supplementation with amino acids did not increase feed intake significantly. However, given the high purification level of the rapeseed protein isolate used in this study and the potentially different effects on DFI from free and protein bound amino acids, an effect from protein or amino acid composition seems plausible.

Although differences in FCR could have been mainly caused by varying feed intake between groups due to the commonly known relationship between FCR and DFI, protein utilization could depend on protein and amino acid composition. Furthermore, as mentioned in 4.1, inclusion of fiber and NfE can impair nutrient digestibility. The increasing amount of cellulose in diets containing rapeseed protein could also have reduced digestibility of nutrients to some extent, therefore reducing FCR.

As previously mentioned, post-prandial amino acid composition is significantly dependent on dietary amino acid composition (Rolland et al., 2015). If the absorbed amino acids do not match the required amino acid composition to a high degree, excessive amino acids could be oxidized (Carter et al., 2001). This oxidation will happen because at least one essential amino acid will become limiting at some point in time, resulting in other amino acids to be oxidized

(de la Higuera, 2001). Moreover, protein utilization was shown to depend on dietary amino acid ratios (Kaushik & Seiliez, 2010; National Research Council, 2011). The ratio between essential and non-essential amino acids might be of major importance as well as the ratio between lysine and sulfur amino acids (Kaushik & Seiliez, 2010). Additionally, lysine content in RI66, RI66 C, (both 2.67 % of diet in DM), RI100 and RI100 C (both 2.63 % of diet in DM) was above the requirement level for growth (National Research Council, 2011) but might have been below the optimal level for protein deposition (2.77 % of diet in DM; Rodehutscord et al., 1997). Hence, differences in dietary amino acid profiles led to differences in protein utilization, affecting PER, PRE and subsequently FCR due to the strong association between live weight gain and protein deposition (Dumas et al., 2007; Shearer, 1994). Imitation of the amino acid profile of control group via supplementation with free amino acids was effective in elevating PRE of RI100 AA to a level comparable to CD FM. A similar improvement of PRE was observed in rainbow trout in previous literature (Gaylord & Barrows, 2009). Consequently, imitation of the amino acid profile of control diet was an efficient method to compensate for a non-optimal dietary amino acid composition in terms of protein utilization to a certain extent, however, due to significantly reduced DFI and its previously mentioned effect on FCR, growth performance could not have been improved significantly as well. These results are in accordance with previously mentioned observations from literature and demonstrate the importance of the dietary amino acid composition for protein utilization in diets containing purified rapeseed protein products as alternatives to fishmeal.

Masking of pre-absorptive effects with fat encapsulation of diets is not sufficient to compensate for impairment of palatability due to high dietary inclusion with rapeseed protein isolate. Non-optimal dietary amino acid or protein composition could determine feed intake via post-absorptive effects from amino acids independently from pre-absorptive effects. Major supplementation with crystalline amino acids also proved to be inefficient in overcoming negative effects from dietary rapeseed protein isolate inclusion on growth performance despite improving protein utilization. For a successful total replacement of fishmeal in diets for rainbow trout, it might be essential to optimize the dietary amino acid composition in replacement diets, especially in regards to branched-chain amino acids (BCAA) as they could have the most significant effect on hypothalamic feed intake regulation (Comesaña et al., 2018b). When using highly purified protein sources, post-prandial plasma amino acid profiles are probably dependent on dietary content and not on protein origin

(Rolland et al., 2015). This should enable using high-quality plant proteins, like rapeseed protein isolates, in combination with other protein products to replace fishmeal successfully. Altering proportions between cruciferin and napin could also improve rapeseed protein products, as this would change the amino acid profile. Since content of all analyzed amino acids differs between fishmeal and protein isolates of this study and Slawski (2012), no tangible conclusion can be drawn on which specific amino acids could be responsible for impairment of growth performance. The considerable lower content of lysine in rapeseed protein isolate of this study in comparison with fishmeal could be of importance as it is the only amino acid with a lower content in the rapeseed protein product compared with fishmeal (table 3.1). Additionally, further research should investigate if other less studied factors present in rapeseed protein products could potentially limit feed intake.

#### 4.3 Body composition

The reduced crude ash content of RI100 AA and RI100 C could indicate a deficiency in phosphorus either for optimal growth or for optimal bone development (Ketola & Richmond, 1994; Skonberg et al., 1997). However, due to inclusion of high amounts of  $\text{Ca}_2\text{PO}_4$  (table 3.3), available phosphorus levels should not be at a deficient level for optimal growth in rainbow trout (Ketola & Richmond, 1994; Skonberg et al., 1997; Sugiura et al., 2000). The lowered amounts of available phosphorus from rapeseed protein isolate and free amino acids in comparison with fishmeal could have led to sub-optimal levels of dietary phosphorus for optimal bone development. This could have reduced crude ash content of treatments RI100 AA and RI100 C. Other minerals which are crucial for bone development such as calcium can be absorbed from the surrounding water via the gills and should therefore not have been limiting (National Research Council, 2011).

The increased crude protein content in whole body of CD FM C could be caused by the increased protein utilization in comparison with RI66 C (table 3.7).

#### 4.4 Health parameters

HSI is being elevated with increasing dietary inclusion of rapeseed protein isolate, although differences are not significant. This would be in line with observations from Panserat et al. (2009), showing an over-expression of genes affecting lipid biosynthesis in the liver of rainbow trout after receiving a plant-based diet. Usage of diets with non-optimal amino acid profiles cannot only lead to increased fat deposition in liver, but also in fillet and peritoneal cavity (Gaylord & Barrows, 2009). The addition of free amino acids might lead to increased fat deposition in the muscle instead of the liver or peritoneal cavity (Gaylord & Barrows, 2009). Latter observation could explain the significantly decreased HSI in RI100 AA in comparison with RI66 and RI100 (table 3.9).

## 5. Conclusion

In accordance with previous literature, nutrient digestibility of rapeseed protein products was optimized by reduction of ash, fiber and NfE to minimal amounts while a minor content of phytic acid might be negligible. Fat encapsulation of experimental diets was ineffective in improving growth performance of fish fed diets containing rapeseed protein isolate and showed that feed intake regulation might depend on post-absorptive factors. Supplementation with substantial amounts of free amino acids was an effective method in improving protein utilization, however, significantly reduced DFI still led to impaired growth performance. Dietary amino acid composition could be important for feed intake regulation, however, effects from other factors like lesser-known ANFs could not have been excluded. Imitation of the amino acid profile of fishmeal based diets could improve the effectiveness of rapeseed protein products at least in terms of protein utilization. Altering the protein composition of rapeseed protein products could improve the amino acid composition in regards to its similarity to fishmeal. This could be an effective method for improving already purified rapeseed protein products containing limited amounts of ANFs.

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## General Discussion

### Digestibility

Previous literature showed varying crude protein digestibility for different rapeseed protein products in rainbow trout (table GD 1). Although different faeces collection methods were applied and results could therefore vary to some extent, overall results strengthen the hypothesis that phytic acid might play a minor role in crude protein digestibility of highly purified rapeseed protein products as mentioned in chapter 3. Despite phytic acid content of 4.2 % in a rapeseed meal, crude protein digestibility comparable to fishmeal was achieved (Burel et al., 2000b). Results of chapter 3 suggest, that phytic acid levels of 1.3 % in rapeseed protein products have no significant influence on crude protein digestibility. This would be in line with Teskeredžić et al. (1995), who stated that the endogenous form of phytic acid as it occurs in rapeseed protein concentrates might have an insignificant effect on protein availability in salmonids. This could be due to the circumstance that phytic acid mostly occurs in rapeseed as salts of Ca, Mg and K (Mills & Chong, 1977; Thompson, 1990). Moreover, if endogenous phytic acid is already bound to other substances, it is unable to bind with dietary substances (Rickard & Thompson, 1997).

Additional to phytic acid, other ANFs present in rapeseed like tannins and trypsin inhibitors can potentially impair digestibility of proteins in fish (Francis et al., 2001). Literature already suggested that the content of lastly mentioned ANFs in purified rapeseed protein products could be insignificant in regards to protein digestibility. Adem (2014) stated that dietary content of tannins in processed protein products should not have a significant impact on growth performance of fish, which was also demonstrated by results from Mwachireya et al. (1999), showing that content of phenolic compounds and tannins in commercial rapeseed meal is not sufficient to significantly reduce nutrient digestibility. Most fish can compensate for effects from protease inhibitors like trypsin inhibitors on availability of proteins by increasing trypsin secretion to a certain extend (Francis et al., 2001). Hence, remaining content of tannins and protease inhibitors in purified rapeseed protein products is most likely not sufficient to cause a significant impairment of protein digestibility. In comparison to other purified rapeseed protein products (table GD 1), reduction of ash, fiber and NfE to minimal amounts was probably responsible for reaching the highest crude protein digestibility of a rapeseed protein product to ever be recorded in rainbow trout using the stripping method. As

demonstrated by Greiling et al. (2018), content of fiber could be of higher importance in comparison with other carbohydrates in terms of its influence on nutrient digestibility. Due to the indigestible nature of cellulose and lignin for rainbow trout, both substances can negatively influence nutrient digestibility (Greiling et al., 2018). However, the influence of non-starch polysaccharides (NSPs) on nutrient digestibility in rainbow trout highly depends on the type of NSP and its dietary concentration (Glencross, 2009; Storebakken & Austreng, 1987). These observations could explain differences in digestibility between different rapeseed protein products (table GD 1). Conclusively, crude protein digestibility comparable to that of fishmeal can be achieved at relatively high content of ash, fiber, NfE and phytic acid (table GD 1), while fiber could determine crude protein digestibility to a greater extent than other carbohydrates. Effects from ANFs like phytic acid on crude protein digestibility of purified rapeseed protein products in rainbow trout could be of lesser importance. To attain the highest crude protein digestibility possible, ash, fiber and NfE should be excluded from rapeseed protein products and content of phytic acid should be kept under 1.3 %.

Table GD 1 Crude ash, crude protein, NfE + crude fiber and phytic acid content of different rapeseed protein products (and fishmeal used in chapter 3) and their crude protein digestibility determined in rainbow trout.

<b>Protein product</b>	<b>Crude protein content of protein product (% DM)</b>	<b>Crude ash content of protein product (% DM)</b>	<b>NfE + crude fiber content of protein product (% DM)<sup>1</sup></b>	<b>Phytic acid content of protein product (% DM)</b>	<b>Crude protein digestibility of protein product (%)</b>	<b>Source</b>
<b>Rapeseed cake</b>	29.4	5.5	44.9	Not provided	42.3	Greiling et al. (2018)
<b>Albumin concentrate</b>	70.1	20.8	8.7	2.0	77.7 (albumin diet)	Slawski (2012)
<b>Canola isolate</b>	81.2	2.7	15.0	0.7	84.6 (canola diet)	Slawski (2012)
<b>Fishmeal</b>	72.4	15.8	0.0	0.0	84.7	Chapter 3
<b>Rapeseed cake (partially dehulled)</b>	38.4	6.7	41.2	Not provided	86.6	Greiling et al. (2018)
<b>Canola meal</b>	36.3	11.0	45.8	2.6	88.1	Mwachireya et al. (1999)
<b>Globulin concentrate</b>	56.3	0.9	29.5	1.5	88.8 (globulin diet)	Slawski (2012)
<b>Rapeseed meal (solvent-extracted)</b>	43.1	7.9	44.2	4.2	90.9	Burel et al. (2000b)
<b>Rapeseed isolate</b>	93.9	4.6	0.1	1.3	95.2	Chapter 2
<b>Canola isolate</b>	90.8	5.5	0.4	0.2	97.6	Mwachireya et al. (1999)
<b>Rapeseed isolate</b>	98.7	0.4	0.0	1.3	99.8	Chapter 3

<sup>1</sup> NfE (nitrogen-free extract) + crude fiber = 100 – (crude protein + crude lipid + crude ash).



**Growth performance: Feed intake**

Replacement of fishmeal with rapeseed protein products in diets fed to rainbow trout led to reduced feed intake in previous literature (Greiling et al., 2018; Hernández et al., 2013; Hilton & Slinger, 1986; Slawski, 2012), while other studies observed no negative effects on feed ingestion even at complete replacement of fishmeal (Slawski, 2012; Teskeredžić et al., 1995; table GD 2). Anti-nutritive factors like glucosinolates, which can exude bitter taste, were made responsible for reduced feed intake when rapeseed protein products were incorporated in diets for different animals, including fish (Hilton & Slinger, 1986; Mawson et al., 1994a, b; Slawski, 2012). Results of chapter 1 suggested that effects from glucosinolates on voluntary feed intake are insignificant at dietary inclusion levels of up to 1.7  $\mu\text{mol/g}$ . Feed intake might even be unaffected by dietary glucosinolate levels of up to 19.3  $\mu\text{mol/g}$  (Burel et al., 2000a; table GD 2). These observations lead to the assumption that rainbow trout and possibly other species of fish (Hossain & Jauncey, 1988; von Danwitz & Schulz, 2020) could be less sensitive to dietary glucosinolates than terrestrial animals in terms of its effect on feed intake. However, specific circumstances under which glucosinolates are being broken down and/or the activity of myrosinase could determine effects from glucosinolates and have to be considered when addressing threshold concentrations of this group of ANFs (chapter 1). Deactivation of myrosinase via conditioning of seeds could be a key factor for reducing impairment of feed intake and growth performance of fish due to dietary inclusion of glucosinolates since most negative effects on fish growth are being associated with brake down products of glucosinolates (Francis et al., 2001). Effects on feed intake of rainbow trout due to dietary inclusion of glucosinolates or glucosinolate brake down products could potentially vary. If myrosinase has been deactivated, voluntary feed intake should be unaffected by dietary inclusion of glucosinolates of 1.7  $\mu\text{mol/g}$  or less (chapter 1).

Results of chapter 1 showed a significant effect from phytic acid on feed intake at a dietary supplementation level of 2.1 %. This is in line with observations of different studies investigating effects from supplemented phytic acid and phytate respectively on grass carp and Atlantic salmon (Denstadli et al., 2006; Liu et al., 2018; Zhong et al., 2019; table GD 3). However, significant effects occurred at lower dietary inclusion of phytic acid in grass carp in comparison with rainbow trout. Other studies investigating effects from supplemented phytic acid observed no significant alterations in feed intake (Riche & Garling, 2004; Richardson et al., 1985; Spinelli et al., 1983; von Danwitz & Schulz, 2020; table GD 3). Sensitivity of different

species of fish towards dietary content of phytic acid could therefore vary to some extent which would be in line with the highly species specific taste preferences among fish species (Morais, 2017). Effects from supplemented phytic acid on feed intake could, however, vary to some extent depending on diet formulation (chapter 1). Results from chapter 1 should therefore be confirmed by additional research. Feed intake of rainbow trout was unaffected in studies with dietary endogenous phytic acid content of up to 1.3 % (table GD 2).

Most studies observing significant negative effects on feed intake of rainbow trout due to dietary inclusion of rapeseed protein products were below previously mentioned thresholds for glucosinolates and phytic acid or did not provide information about inclusion levels (table GD 2). Significantly reduced feed intake in diets devoid of fishmeal was also observed in chapters 2 and 3 despite insignificant inclusion of glucosinolates ( $<0.02 \mu\text{mol/g}$ ) and phytic acid ( $<0.3\%$ ). Slawski (2012) did not observe a significant impact on feed intake at comparable or even higher dietary inclusion of both ANFs in comparison with rapeseed protein isolates used in chapter 2 and 3 (table GD 2). Aforementioned observations demonstrate that other substances could be of greater importance for impairment of diet palatability at these inclusion levels. Other ANFs like non-starch polysaccharides (NSPs) or sinapine and sinapic acid present in rapeseed can potentially limit feed intake as well (Francis et al., 2001; von Danwitz & Schulz, 2020). Due to the high level of purification, inclusion levels of NSPs in rapeseed protein isolates of chapters 2 and 3 (NfE + crude fiber content: 0.08 and 0.00 % of DM) are most likely insignificant. Dietary inclusion of cellulose as a filler substance might have had an impact on feed intake, however, given the minor differences in cellulose levels between RI66 and RI100 (chapter 2 and 3), a significant impact on feed intake seems unlikely. Sinapic acid could negatively affect diet palatability, however, von Danwitz and Schulz (2020) demonstrated that dietary inclusion of sinapic acid in levels commonly found in processed rapeseed protein products did not affect feed intake of turbot significantly. Additionally, effects from glucosinolates are allegedly more severe towards feed intake in comparison with sinapine (Josefsson & Uppström, 1976) and processing methods applied in this study have been demonstrated to be effective in removal of sinapine (Adem, 2014). Other potentially deterrent substances present in rapeseed whose effects on feed intake were not yet investigated adequately in literature could also influence palatability of rapeseed protein products. These substances could for example include Kaempferol 3-O- (2''-O-Sinapoyl- $\beta$ -sophoroside) as it was discovered to contribute to the bitter taste of rapeseed protein isolates

(Hald et al., 2018). Effects on feed intake from multiple substances that are of bitter taste in humans were investigated in different fish species (Hara, 2006; Kasumyan & Döving, 2003; Oike et al., 2007; Yamashita et al., 2006) and one of these substances (Q-HCl) impaired feed intake in rainbow trout (Hara, 2006). Interestingly, content of Kaempferol 3-O-(2''''-O-Sinapoyl- $\beta$ -sophoroside) is more than tenfold higher in cruciferin in comparison with napin (Hald et al., 2018). Consequently, an analysis of the Kaempferol derivate content in rapeseed protein isolates of chapter 2 and 3 by the Technical University of Munich, Freising, Germany (according to methods applied in Hald et al., 2018) revealed that the isolate of chapter 3 contained about five times more of this putatively ANF than the isolate used in chapter 2. As DFI of dietary groups with the same level of fishmeal exchange rate in chapter 2 and 3 were almost identical, significant effects from Kaempferol 3-O-(2''''-O-Sinapoyl- $\beta$ -sophoroside) on DFI in this study seem unlikely but cannot be excluded. More research is needed on effects from lesser-known substances on palatability of rapeseed protein products. If dietary content of ANFs, including lesser known substances, is insignificant in regards to their effect on feed intake due to a high level of purification, palatability of proteins and amino acids could be a key factor for replacement of fishmeal with rapeseed protein products. Comesaña et al. (2018a); Comesaña et al. (2018b) and Comesaña et al. (2020) demonstrated that pre- and post-absorptive detection of different amino acids can have varying effects on mechanisms regulating feed intake in rainbow trout. Pre- and post-absorptive compositions of amino acids could therefore determine feed intake at least in this species of fish. Results of chapter 3 suggest that feed intake could be dependent on post-prandial effects which is in line with Comesaña et al. (2018b), who stated that feed intake is mainly regulated by homeostatic pathways and could be independent from putatively palatable amino acids. It could be speculated that feed intake regulation acts as a guiding system to achieve optimal blood amino acid compositions for protein utilization. However, pre-absorptive effects from diets on feed intake also play a role in feed intake regulation (Comesaña et al., 2020) and the existence of a hedonic feed intake regulation system cannot be excluded (Comesaña et al., 2018b; Mueller & Wullimann, 2009; O'Connell et al., 2011). The absolute dietary content of certain amino acids as well as their ratio towards other amino acids could be of importance as both are essential for protein utilization (National Research Council, 2011). Slawski (2012) was able to replace 100% of fishmeal with a rapeseed protein concentrate as well as with an isolate. As an example, lysine content of replacement diets was close to control and at least

2.8 % of dry matter in both studies by Slawski (2012; table GD 2). Lysine content of 100 % fishmeal exchange diets in chapters 2 and 3 (2.65 % and 2.63 % of DM) might have been at a deficient level for optimal feed intake compared to control. Dietary amounts of other amino acids could have been at non-optimal levels of inclusion as well. Addition of feed attractants (blood meal and/or crustacean meal) in experiments from Slawski (2012) could therefore actually have affected feed intake due to alteration of post-prandial amino acid composition (or additional to changes in pre-absorptive effects). This would also be in line with a preliminary analysis from de la Higuera (2001), demonstrating that deficiencies in single amino acids (arginine, leucine, lysine, methionine) can reduce feed intake. Further research will be necessary to investigate whether selective changes in amino acid compositions can improve palatability of rapeseed protein products and what amount of amino acids determines regulatory mechanisms. As demonstrated by results of chapter 3, supplementation with substantial amounts of free amino acids could be ineffective in compensating for reduced feed intake. Findings from Comesaña et al. (2018a); Comesaña et al. (2018b) and Comesaña et al. (2020) demonstrated in parts how the feed intake regulation in fish due to dietary amino acid composition could generally work. However, at the current state of literature it can only be speculated why amino acid supplementation was ineffective in improving feed intake. Protein bound amino acids have to be broken down before being absorbed by mucosal cells (National Research Council, 2011). This leads to a delay in postprandial blood/plasma free amino acid peaks from protein bound amino acids in comparison with free dietary amino acids (Cowey & Walton, 1988; Rolland et al., 2015). Furthermore, free amino acids could also be oxidized to a high degree after their rapid absorption, leading to a relative deficiency in the post-prandial amino acid pattern (de la Higuera, 2001). Amino acids originating from the free amino acid mix used in chapter 3 could have led to an increase in certain amino acids reaching the amino acid sensing mechanisms in the hypothalamus prior to the remaining dietary protein bound amino acids. Additionally, fish are able to distinguish between various amino acids via their pronounced sense of taste (Kasumyan, 2019; Morais, 2017) and certain amino acids were shown to be more palatable for rainbow trout than others (Comesaña et al., 2018b; Jones, 1989; Kasumyan & Sidorov, 2012; Kohbara & Caprio, 2001; Marui et al., 1983; Tusche, 2012; table GD 4). Moreover, some free amino acids were found to act as dietary attractants while others can be feeding deterrents (de la Higuera, 2001). Consequently, while imitation of the amino acid profile of the fishmeal diet via dietary supplementation with free amino acids

(chapter 3) could theoretically mimic the optimal amino acid composition, effects from certain free amino acids or a combination of multiple free amino acids might have a more deterrent impact. Additionally, non-essential amino acids could be metabolized more rapidly after ingestion than essential amino acids and the uptake of amino acids to certain tissues was demonstrated to happen more readily in comparison with other tissues (Carter et al., 2001). To avoid complications due to the use of substantial amounts of dietary free amino acids, amino acid composition of diets which contain rapeseed protein products and are devoid of fishmeal should be altered by using high-quality protein sources. Alternatively, amino acid composition of rapeseed protein products could be changed. Albumin (napin) and globulin (cruciferin) possess different amino acid compositions (Slawski, 2012) and their ratio could be altered to improve palatability of rapeseed protein products. It might be advisable to resemble the amino acid composition of fishmeal as closely as possible, as this might be essential to exchange fishmeal successfully with alternate protein sources (Kaushik & Seiliez, 2010). Additionally, the optimal ratio between albumin and globulin could be dependent on species. However, findings in this study could not conclusively exclude effects from other substances than amino acids present in rapeseed on feed intake. Lesser-known compounds and their influence on feed intake should be further investigated in future studies.

Additionally, gastric emptying rates are known to influence feed intake in fish (Jia et al., 2021) and the physical quality of pellets can affect these emptying rates (Aas et al., 2011). Hence, physical feed properties should be considered when testing new feed ingredients (Aas et al., 2011). Changes in the water stability of diets used in chapter 3 were observed (see appendix for applied methods) due to inclusion of rapeseed protein products. Water absorption of RI100 was significantly higher in comparison with CD FM and loss of dry matter to surrounding water was significantly lower after 240 min of shaking from RI100 and RI66 compared to CD FM (table GD 5), indicating lower water stability of CD FM (Aas et al., 2011; Baeverfjord et al., 2006). Pellets with lower water stability resulted in increased feed intake in rainbow trout compared to pellets with higher water stability (Aas et al., 2011). However, there are currently no established methods available to measure the physical feed quality that specifies the expected feed intake in fish and available data on effects from physical properties of diets on the nutritional response of fish are limited and conflicting (Aas et al., 2020). When defined measurements for the physical feed quality are available, effects from gastric emptying rates on feed intake should be taken into consideration in future research.

Table GD 2 Rapeseed protein products used in trials with rainbow trout and their effects on feed conversion and feed intake.

Protein product	Glucosinolate content in diets without fishmeal (DM)	Phytic acid content in diets without fishmeal (% of diet DM)	Ratio fishmeal (FM)/ rapeseed protein product (RPP)	Effect from rapeseed protein product on feed intake	Effect from rapeseed protein product on feed conversion	Lysine content of control diet (% of diet DM)	Lysine content of test diets (% of diet DM)	Initial fish weight (g)	Source
<b>Rapeseed cake</b>	Not provided	Not provided	5.3; 2.4; 1.5	Sign. negative at highest inclusion level	Sign. negative at all inclusion levels	Not provided	Not provided	37	Hernández et al. (2013)
<b>Rapeseed cake</b>	0.06; 0.12 %	Not provided	3.7; 1.7	-	-	Not provided	Not provided	50	Greiling et al. (2018)
<b>Rapeseed cake (partially dehulled)</b>	0.06; 0.11 %	Not provided	1.5; 0.5	Sign. negative at both inclusion levels	Sign. negative at both inclusion levels	Not provided	Not provided	191	Greiling et al. (2018)
<b>Rapeseed meals</b>	1.4-19.3 µmol/g	Not provided	1.2; 0.4	-	Sign. negative at all inclusion levels	2.2	2.4	20	Burel et al. (2000a)
<b>Canola meal</b>	Not provided	Not provided	5.5; 2.8; 2.0; 1.5	-	Sign. negative correlation	2.9	2.9	235	Collins et al. (2012)
<b>Canola protein concentrate</b>	Not provided	Not provided	5.4; 2.7; 1.8; 1.4	-	Not sign.	2.9	2.9	235	Collins et al. (2012)

Table GD 2 continued.

Product	Glucosinolate content in diets without fishmeal (DM)	Phytic acid content in diets without fishmeal (% of diet DM)	Ratio fishmeal (FM)/ rapeseed protein product (RPP)	Effect from rapeseed protein on feed intake	Effect from rapeseed protein on conversion	Lysine content of control diet (% of diet DM)	Lysine content of test diets (% of diet DM)	Initial fish weight (g)	Source
<b>Rapeseed protein concentrates</b>	Not provided	0.1-1.3	1.8; 0.4; 0	-	Sign. negative at highest inclusion level	Not provided	Not provided	4.2-4.4	Teskeredžić et al. (1995)
<b>Canola Protein concentrate</b>	Not provided	Not provided	1.0; 0.3; 0	- (feed intake in g feed/fish)	Sign. negative at highest inclusion level	Not provided	Not provided	15	Drew et al. (2007)
<b>Canola protein concentrate</b>	Not provided	Not provided	1.9; 0.6	- (Fixed ration)	Sign. negative at both inclusion levels	Not provided	Not provided	12	Stickney et al. (1996)
<b>Canola protein concentrate</b>	Not provided	Not provided	0.9; 0.3	-	Not sign.	Not provided	Not provided	28	Thiessen et al. (2004)
<b>Rapeseed protein concentrate</b>	0.3; 0.4 µmol/g	0.4; 0.5	0.5; 0	-	Sign. negative at FM to RPP ratio of 0.5	2.8	2.8; 2.8	38	Slawski (2012)

Table GD 2 continued.

<b>Rapeseed protein product</b>	<b>Glucosinolate content in diets without fishmeal (DM)</b>	<b>Phytic acid content in diets without fishmeal (% of diet DM)</b>	<b>Ratio fishmeal (FM)/ rapeseed protein product (RPP)</b>	<b>Effect from rapeseed protein product on feed intake</b>	<b>Effect from rapeseed protein product on feed conversion</b>	<b>Lysine content of control diet (% of diet DM)</b>	<b>Lysine content of test diets (% of diet DM)</b>	<b>Initial fish weight (g)</b>	<b>Source</b>
<b>Albumin concentrate</b>	Under detection minimum	0.4; 0.6; 0.8	0.9; 0.3; 0	Sign. negative at FM to RPP ratio of 0.3 and 0	-	3.4	3.8; 3.8; 3.9	32	Slawski (2012)
<b>Globulin concentrate</b>	0.2; 0.5; 0.6 $\mu\text{mol/g}$	0.3; 0.5; 0.6	0.8; 0.3; 0	Sign. negative at all inclusion levels	Sign. negative at FM to RPP ratio of 0.3 and 0	3.4	3.3; 3.3; 3.3	32	Slawski (2012)
<b>Rapeseed protein isolate</b>	<0.05 $\mu\text{mol/g}$	0.1; 0.1; 0.2; 0.2	3.3; 1.1; 0.4; 0	-	Sign. positive effect at FM to RPP ratio of 0.4	3.4	3.3; 3.1; 33.3; 3.1	32	Slawski (2012)
<b>Rapeseed protein isolate</b>	<0.02 $\mu\text{mol/g}$	0.1; 0.1; 0.2	2.9; 0.7; 0	Sign. negative at highest inclusion level	-	2.9	2.8; 2.7; 2.7	78	Chapter 2
<b>Rapeseed protein isolate</b>	<0.005 $\mu\text{mol/g}$	0.1; 0.1	0.8; 0	Sign. negative at highest inclusion level	Sign. negative at both inclusion levels	2.9	2.7; 2.6	24	Chapter 3



Table GD 3 Results from studies investigating effects from supplemented phytic acid on different species of fish.

Form of phytic acid	Species of fish	Main source of protein	Supplemented levels of phytic acid (% of diet)	Supplemented levels of phytic acid with significant effect (% of diet)	Significant negative effect on	Source
Casein-phytate complex	Rainbow trout	Casein	0.5	0.5	FCR	Spinelli et al. (1983)
Phytic acid	Grass carp	Casein, fishmeal	0.5 - 4.0	0.5	DFI; PER	Liu et al. (2018)
Phytic acid	Turbot	Casein	1.1	1.1	FCR	von Danwitz and Schulz (2020)
Sodium phytate	Atlantic salmon	Fishmeal	0.1 - 2.1	1.0 and 2.1	2.1 % dietary inclusion on DFI and FCR	Denstadli et al. (2006)
Phytic acid	Grass carp	Casein	0.8 - 4.0	1.6 and 3.2	1.6 % dietary inclusion on DFI; 3.2 % dietary inclusion on feed efficiency and DFI	Zhong et al. (2019)
Sodium phytate	Chinook salmon	Casein	0.2; 2.6	2.6	FCR; PER	Richardson et al. (1985)
Sodium phytate	Nile tilapia	Fishmeal	0.3 - 2.6	2.6	SGR (FE; PER)	Riche and Garling (2004)
Phytic acid	Rainbow trout	Rapeseed protein concentrate, fishmeal	1.0 - 2.3	2.1	DFI	Chapter 1
Sodium phytate	Atlantic salmon	Casein and fishmeal	0.1	-	-	Sajjadhi and Carter (2004)

Table GD 4 Palatable free amino acids for rainbow trout according to literature.

	<b>Marui et al. (1983)</b>	<b>Jones (1989)</b>	<b>Kohbara and Caprio (2001)</b>	<b>Tusche (2012)</b>	<b>Kasumyan and Sidorov (2012)</b>	<b>Comesaña et al. (2018b)</b>
<b>Amino acids</b>	Pro, Ala, Leu, Phe, Bet <sup>1</sup>	Pro, Leu, Glu, Ile, Asp, Gly, Ala, Tau, Bet, Arg <sup>1</sup>	AGPA, Pro	Ala, Gly, Bet <sup>2</sup>	Ala	Pro, Leu

<sup>1</sup> amino acids in order of their effectiveness in triggering feeding behavior, from highest effect to least effective; <sup>2</sup> authors used a mixture of all three amino acids.

Table GD 5 Results from water stability test (mean  $\pm$  SD, n=3).

<b>Treatment</b>	<b>CD FM</b>	<b>RI66</b>	<b>RI100</b>
<b>Initial wet weight (g)</b>	3.2 $\pm$ 0.01	3.2 $\pm$ 0.00	3.2 $\pm$ 0.01
<b>Initial dry matter content (%)</b>	93.9 $\pm$ 0.02	93.7 $\pm$ 0.04	93.2 $\pm$ 0.00
<b>Wet weight post-shaking (% of initial)</b>	42.6 $\pm$ 1.9 <sup>a</sup>	144.5 $\pm$ 54.5 <sup>a</sup>	307.6 $\pm$ 38.5 <sup>b</sup>
<b>Remaining dry matter post-shaking (% of initial)</b>	12.7 $\pm$ 3.4 <sup>a</sup>	19.2 $\pm$ 0.5 <sup>b</sup>	21.3 $\pm$ 0.6 <sup>b</sup>

Values in the same row with different superscript letters are significantly different ( $P < .05$ ).

**Growth performance: Feed and protein conversion**

Most studies investigating effects from inclusion of rapeseed protein products in diets for rainbow trout observed significant impairment of feed and protein conversion (table GD 2). Similar to effects on feed intake, ANFs present in rapeseed were made responsible for inferior feed conversion of diets. One major factor was the reduced nutrient digestibility in different species of fish due to the ability of phytic acid to bind with bivalent and trivalent mineral ions and form protein-phytate complexes lowering the availability of those substances for fish (Burel et al., 2000b; Francis et al., 2001; Mwachireya et al., 1999). As previously mentioned, effects from endogenous phytic acid present in highly purified rapeseed protein products on digestibility of proteins might be negligible and rainbow trout are most likely not dependent on a mineral supply from rapeseed protein products in diets including a mineral premix, especially when considering their ability to absorb minerals from the surrounding water (National Research Council, 2011). Results of chapter 1 even suggested that supplementation with 2.3 % phytic acid could be unable to significantly affect feed conversion or protein availability. However, determination of exact threshold concentrations of dietary endogenous phytic acid without significant effects on feed conversion via supplementation with phytic acid or phytate could be more complicated than previously assumed. Previous literature demonstrated that dietary supplementation with phytic acid could negatively influence feed conversion at inclusion levels of 0.5 % in rainbow trout (Spinelli et al., 1983) and 1.1 % in turbot (von Danwitz & Schulz, 2020) due to reduced protein digestibility. Supplemented phytic acid is highly reactive and can form complexes with other dietary components like minerals or proteins (Rickard & Thompson, 1997). The nature of these reactions and the preference of phytic acid to bind to other substances is dependent on different factors, including pH (Adeola & Sands, 2003; Dendougui & Schwedt, 2004; Rickard & Thompson, 1997). Formation of protein-phytate complexes, for example is dependent on the isoelectric pH of the proteins (Rickard & Thompson, 1997). Hence, effects from supplemented phytic acid could be dependent on diet formulation and factors like dietary pH. While endogenous phytic acid could limit availability of substances present in rapeseed, supplemented phytic acid could limit availability of different substances in the diet. While dietary supplementation with phytic acid could therefore demonstrate general effects from endogenous phytic acid, threshold concentrations could vary to some extent, depending on species and factors like diet composition and dietary pH. Furthermore, supplemented phytic acid could have additional

effects on dietary factors like phosphorus availability or pH due to its chemical attributes (chapter 1). These numerous variables associated with supplemented phytic acid could influence effects on feed and protein conversion as well as on feed intake. Spinelli et al. (1983) demonstrated that phytic acid can reduce protein digestibility in rainbow trout by dietary supplementation with a casein-phytate complex, however, results were most likely not intended to represent a threshold concentration for negative effects on FCR from endogenous phytic acid as phytic acid was specifically bound to proteins. Additionally, casein is a putatively preferred substrate for binding with phytic acid (Denstadli et al., 2006). This could lead to increased formation of protein-phytate complexes when phytic acid is supplemented to diets including casein as the main source of protein, which could at least partly explain differences between effects on FCR and protein utilization in different studies (table GD 3). However, due to the numerous factors affecting binding preferences of phytic acid in diets, preferred substrates for phytic acid cannot be accurately predicted with current findings from literature. Hence, threshold concentrations determined in chapter 1 could be viable, but more research should be conducted to confirm the results. Nevertheless, results of chapter 3 demonstrated that protein digestibility is most likely unaffected by an endogenous phytic acid content of 1.3 % in rapeseed protein isolate. This could be a provisional and conservative threshold concentration for effects from endogenous phytic acid on protein utilization via reduced digestibility until further research is conducted.

If content of ash, fiber and NfE is practically removed and levels of endogenous phytic acid are at 1.3 % or below in a purified rapeseed protein product, protein digestibility can be close to 100% (chapter 3). However, protein utilization was impaired in chapters 2 and 3, despite using rapeseed protein products with very limited ANF content as alternatives for fishmeal. Previous studies demonstrated that protein utilization is dependent on dietary ratios among amino acids and content of single amino acids like lysine (Kaushik & Seiliez, 2010; National Research Council, 2011; Rodehutsord et al., 1997) since the dietary content of amino acids significantly influences the postprandial amino acid composition (Rolland et al., 2015). If at least one essential amino acid becomes limiting, others are being used as sources for energy and will consequently be oxidized (de la Higuera, 2001). Hence, non-optimal dietary content of amino acids will reduce protein utilization. This conclusion, together with effects on feed intake could explain differences in success with replacing fishmeal with different purified rapeseed protein products (table GD 2). Lysine can again be used as an example. Slawski (2012) reached

comparatively high dietary lysine contents in diets devoid of fishmeal (2.8 and 3.1 %) and no significant effect on feed or protein conversion was observed in both experiments. Amino acid ratios and content of single amino acids could have been sufficiently similar to compositions of control diets to successfully replace fishmeal with rapeseed protein concentrate or isolate respectively. These results indicate that the dietary amino acid composition is of greater importance for protein utilization in comparison with small amounts of dietary phytic acid. Results from chapter 3 suggest that high inclusion of crystalline amino acids is able to compensate for non-optimal dietary amino acid composition in terms of protein utilization to a certain extent, emphasizing previously mentioned connection between dietary amino acid composition and protein metabolism. Improvement of protein utilization in rainbow trout due to supplementation with free amino acids has also been demonstrated in earlier research (Gaylord & Barrows, 2009), however, results of chapter 3 still show a trend towards reduced PRE in the dietary group given amino acid supplemented diets compared to control ( $P = .09$ ). This reduced protein utilization could also be caused by the aforementioned delay in uptake time between free and protein bound amino acids (Kaushik & Seiliez, 2010). Imitation of the amino acid profile of control via protein bound amino acids might have been more effective in increasing PRE by avoiding a delay in amino acid absorption. Alternatively, encapsulation of dietary free amino acids with agar was found to reduce differences in uptake time between free amino acids and protein bound amino acids (Kaushik & Seiliez, 2010).

To improve overall growth performance of diets devoid of fishmeal, it could be of major importance to alter the dietary amino acid composition in comparison to experimental diets in chapter 2 and 3 as this could determine feed intake and protein utilization of purified rapeseed protein products containing low amounts of ANFs, including ash, fiber and NfE. Alteration of amino acid profiles of purified rapeseed protein products could further improve their application as fishmeal alternatives since postprandial amino acid composition is dependent on dietary content and not protein origin (Rolland et al., 2015). Content of branched-chain amino acids (Comesaña et al., 2018b) and lysine could be of special importance. It might be necessary to combine rapeseed protein products with other protein sources to achieve the required amino acid composition for total replacement of fishmeal (Kaushik & Seiliez, 2010).

### **Body composition**

Phosphorus availability from rapeseed protein products is limited in comparison to fishmeal, since the main form of storage in rapeseed for this substance is phytate (Mills & Chong, 1977; Reddy et al., 1982; Thompson, 1990), which is not digestible for non-ruminants (Liener, 1989). Consequently, exchange of fishmeal for rapeseed protein products can lead to reduced whole body ash content, when requirements for optimal growth or bone development are not met (Ketola & Richmond, 1994; Skonberg et al., 1997). Dietary inclusion of other sources for phosphorus, like dicalcium phosphate can compensate for deficiencies in growth, however, supplementation with inorganic phosphorus can have a negative impact on the environment by increasing phosphorus content of surrounding waters (Bonsdorff et al., 1997). Supplementation of diets with phytase can increase phosphorus digestibility of plant protein products in different species of fish (Forster et al., 1999; Sugiura et al., 2001; Vandenberg et al., 2012; Vielma et al., 2004; von Danwitz et al., 2016). However, the effectiveness of phytase might highly depend on different factors like species of fish and husbandry conditions (Rodehutschord & Pfeffer, 1995; Sugiura et al., 2001). Advances in the effective application of phytase at low temperatures have been made recently (Lee et al., 2020) and could lead to a reliable availability of phosphorus from plant protein products in the future.

### **Health parameters**

Different health issues are being associated with the inclusion of rapeseed protein products in diets for carnivorous fish. Breakdown products from glucosinolates can cause adverse effects on thyroid functions by impairing the activity of thyroid follicles and competing with iodine for thyroidal transport (Burel et al., 2001; Fenwick & Heaney, 1983; Mawson et al., 1994b). However, breakdown of glucosinolates is dependent on the presence and activity of the enzyme myrosinase (Mawson et al., 1993). Additionally, factors like pH can influence the composition of breakdown products. Results of chapter 1 suggest that inclusion levels of up to 1.7  $\mu\text{mol/g}$  do not affect thyroid activity significantly after 38 days of feeding. Effects on thyroid might have been prevented by denaturation of myrosinase during processing by exceeding a temperature of 60 °C (Björkman & Lönnerdal, 1973). A longer experimental duration could be used to confirm results from chapter 1 in the future.

Additional to effects on thyroid activity, detoxification of glucosinolates in liver and kidney can trigger hypertrophy, hyperplasia and necrosis of cells in these organs (Woyengo et al., 2017).

These effects are, however, again dependent on multiple factors like formation of glucosinolate breakdown products and species (Mawson et al., 1994b). No significant differences were observed in histological analysis of liver, intestine and spleen in chapter 1, suggesting that up to 1.7  $\mu\text{mol/g}$  of dietary glucosinolates do not cause histopathological changes in these organs under applied processing conditions after 38 days of feeding.

Dietary inclusion of phytic acid in chapter 1 (3.0 %) did not cause histopathological changes in either of the examined organs. Effects from phytic acid on organ health might be species-specific, as different thresholds were found in literature for grass carp and Atlantic salmon (Zhong et al., 2019).

Additional to effects on organ health and thyroid activity, high inclusion of plant proteins can change lipid biosynthesis in rainbow trout (Panserat et al., 2009). A non-optimal profile of dietary amino acids can cause elevated fat deposition in liver, fillet and peritoneal cavity (Gaylord & Barrows, 2009). Although elevation of HSI was insignificant at 66 and 100% exchange of fishmeal with rapeseed protein isolate in comparison to control (chapter 3), results still emphasize that excessive fat deposition is a supplementary reason for optimization of amino acid profiles in plant protein based diets.

**Overall conclusion**

This thesis showed that further reduction of glucosinolates and phytic acid content in processed rapeseed protein products is not an effective method to improve the nutritional value of these products compared to other factors. Effects from glucosinolates on growth performance and health status of fish could be more dependent on the activity of myrosinase than the dietary content. Content of ash, fiber and NfE in rapeseed protein products seems to be more important for nutrient digestibility in comparison to small amounts of endogenous phytic acid and the dietary amino acid profile could affect feed intake and protein utilization to a greater extent than limited contents of glucosinolates and phytic acid. The nutritional value of processed rapeseed protein products containing limited amounts of known ANFs should be improved by optimization of the amino acid profile. Additionally, changes in physical feed properties due to dietary inclusion of rapeseed protein products and lesser-known substances present in rapeseed protein products need to be investigated since both factors could potentially affect feed intake in rainbow trout.



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## Summary

Due to a growing aquaculture sector, stagnating prices for fishmeal and an increasing prioritization of a sustainable global food supply, needs for alternative protein sources for fish nutrition have never been more urgent. While protein products from rapeseed are considered to be valuable candidates for replacing fishmeal in diets for different species of fish, nutritive value of rapeseed protein products appears to be inferior to fishmeal. This impairment is considered to be caused by anti-nutritive factors (ANFs) like glucosinolates and phytic acid present in rapeseed. Various processing techniques have been applied in the past to reduce effects from ANFs. The present study investigated the potential of new purification methods to improve the effectiveness of rapeseed protein products as alternative protein sources for fishmeal in diets for carnivorous fish.

In the first experiment effects from supplemented glucosinolates and phytic acid were tested on growth performance and health status of juvenile rainbow trout. Triplicate groups of fish were fed eight identical basal diets which were supplemented with graded levels of either glucosinolates (0.5; 1.0; 1.7  $\mu\text{mol/g}$ ) or phytic acid (1.0; 1.3; 1.9; 2.1; 2.3 %). A ninth basal diet served as control. After 38 days of feeding, results showed that up to 1.7  $\mu\text{mol/g}$  of glucosinolates do not impair growth performance or health status of rainbow trout significantly. A significantly affected growth performance was observed after 56 days of feeding with 2.3 % dietary phytic acid supplementation. Deactivation of the enzyme myrosinase could be a key factor in reducing impairment of growth performance and health of fish from glucosinolates as most negative effects are associated with break down products of different glucosinolates. Inclusion levels of phytic acid with negative effects on growth performance could be species-specific and could depend on feed formulation. Additionally, effects from these ANFs could depend on multiple factors like the interaction with other substances and they are therefore in need of further research.

The potential of a highly purified rapeseed protein isolate as an alternative protein source for fishmeal was tested in the second experiment. Nutrient digestibility of the rapeseed protein isolate was tested for that purpose and triplicate groups of juvenile rainbow trout were fed isonitrogenous and isoenergetic diets with increasing dietary levels of rapeseed protein isolate (0; 33; 66; 100 % of digestible dietary fishmeal protein). Crude protein digestibility was exceptionally high ( $95.2 \pm 1.7$  %) compared to other rapeseed protein products. Feeding

rainbow trout to apparent satiation twice per day for 56 days showed that 66 % of dietary fishmeal can be replaced with the rapeseed protein isolate without significantly affecting growth performance or health status of rainbow trout. When dietary fishmeal was replaced by rapeseed protein isolate to 100 %, DFI was significantly decreased and overall growth performance was subsequently impaired. Reduction of fiber and NfE as well as of phytic acid content was made responsible for the increased nutrient digestibility as previously reported in literature. Due to high levels of purification, significant effects from ANFs present in the rapeseed protein isolate on growth performance and health status of fish appeared to be unlikely. The dietary amino acid composition could be a major factor influencing feed intake as it was shown to affect voluntary feed intake regulation in previous literature.

In the third and last experiment, a different rapeseed protein isolate, mainly consisting of cruciferin was tested as an alternative protein source for fishmeal in diets for rainbow trout. Nutrient digestibility of the rapeseed protein isolate was measured as well. Juvenile rainbow trout were fed seven isonitrogenous and isoenergetic diets in triplicate groups. In three of those diets the dietary inclusion of 19 % fishmeal was exchanged to 0, 66 and 100 % of digestible protein with rapeseed protein isolate. Duplicates of these diets with reduced content of rapeseed oil were encapsulated with stearic acid and fish oil. A portion of the rapeseed protein isolate in the 100 % exchange diet without fat encapsulation was replaced by a mixture of amino acids to achieve the seventh diet. The highest crude protein digestibility of a rapeseed protein product in rainbow trout was measured using the stripping method ( $99.8 \pm 1.6$  %). After 56 days of feeding, results revealed that both exchange levels of fishmeal reduced feed intake, feed conversion and subsequently overall growth performance of rainbow trout significantly compared with control in encapsulated and non-encapsulated diets. Hence, fat encapsulation could not prevent impairment of feed intake due to high dietary inclusion of the rapeseed protein isolate. Supplementation with free amino acids was not an effective method for increasing feed intake significantly, however, protein utilization was increased and comparable to that of the control diet. Reduction of ash, fiber and NfE to minimal amounts could be the decisive factor in reaching the highest crude protein digestibility of rapeseed protein products in rainbow trout while a limited content of phytic acid might be negligible. Observations made in the last experiment also suggest that post-absorptive effects on feed intake from rapeseed protein products could be independent from pre-absorptive effects. The negative impact from highly purified rapeseed protein products

on voluntary feed intake could be dependent on post-absorptive amino acid composition. Further improvement of highly purified rapeseed protein products containing limited amounts of ANFs could therefore be achieved by alteration of the amino acid profile of the protein products.

## Zusammenfassung

Aufgrund der wachsenden Aquakulturbranche, stagnierender Preise für Fischmehl und der zunehmenden Priorisierung eine nachhaltige weltweite Lebensmittelversorgung zu schaffen, war der Bedarf an alternativen Proteinquellen für die Fischernährung noch nie so hoch. Obwohl Proteinprodukte aus Raps als vielversprechende Alternativen für Fischmehl im Futter verschiedener Fischarten gelten, scheinen Rapsproteinprodukte im Vergleich zu Fischmehl einen verringerten nutritiven Wert zu haben. Diese Einschränkung wurde anti-nutritiven Stoffen (ANS), wie Glucosinolaten und Phytinsäure, die im Raps enthaltenen sind, zugeschrieben. In der Vergangenheit wurden verschiedene Prozessverfahren entwickelt um die Effekte von ANS zu reduzieren. Die aktuelle Studie diente der Untersuchung des Potentials neuartiger Prozessverfahren für die Verbesserung von Rapsproteinprodukten als Proteinquelle in Fischfuttermitteln für karnivore Fischarten.

Im ersten Experiment wurden die Effekte von Glucosinolaten und Phytinsäure auf das Wachstum und die Gesundheit von juvenilen Regenbogenforellen untersucht. In Triplikaten vorliegende Gruppen von Fischen wurden mit 8 identischen Basisdiäten gefüttert, die ansteigende Gehalte an Glucosinolaten (0,5; 1,0; 1,7  $\mu\text{mol/g}$ ) und Phytinsäure (1,0; 1,3; 1,9; 2,1; 2,3 %) beinhalteten. Eine neunte Basisdiät diente als Kontrolle. Nach 38 Fütterungstagen konnte gezeigt werden, dass ein Glucosinolat-Gehalt von bis zu 1,7  $\mu\text{mol/g}$  weder das Wachstum noch die Gesundheit von Regenbogenforellen signifikant beeinflusst. Bei einem Phytinsäure-Zusatz von 2,3 % konnte nach 56 Fütterungstagen ein signifikant beeinflusstes Wachstum festgestellt werden. Die Deaktivierung des Enzyms Myrosinase könnte ein Schlüsselfaktor für die Reduzierung der negativen Effekte auf das Wachstum und die Gesundheit der Fische durch Glucosinolate sein, da die meisten dieser negativen Effekte mit den Abbauprodukten der Glucosinolate in Verbindung gebracht werden. Die Gehalte an Phytinsäure in Futtermitteln mit negativen Auswirkungen auf das Wachstum von Fischen könnte von der Fischart und der Futterzusammensetzung abhängen. Zusätzlich könnten die Effekte beider ANS von mehreren Faktoren, wie beispielsweise der Interaktion mit anderen Stoffen, abhängen und dadurch bedürfen sie weiterer Untersuchungen.

Im zweiten Experiment wurde das Potential eines hochreinen Rapsproteinisolates als eine alternative Proteinquelle zu Fischmehl untersucht. Die Nährstoffverdaulichkeit des Rapsproteinisolates wurde dafür getestet und triplikate Gruppen von juvenilen Regenbogenforellen wurden mit isonitrogenen und isoenergetischen Futtermitteln gefüttert,

die einen ansteigenden Anteil an Rapsproteinisolat aufwiesen (0; 33; 66 und 100 % des verdaulichen Fischmehlproteins im Futter). Die Rohproteinverdaulichkeit war im Vergleich mit anderen Rapsproteinprodukten sehr hoch ( $95,2 \pm 1,7$  %). Nach 56 Fütterungstagen bei zweimaliger scheinbarer Sättigung pro Tag konnte gezeigt werden, dass 66 % des Fischmehls im Futter durch das Rapsproteinisolat ersetzt werden kann ohne das Wachstum oder die Gesundheit der Regenbogenforellen signifikant einzuschränken. Der Austausch von Fischmehl im Futter zu 100 % senkte die Futtermittelaufnahme und in Folge dessen auch die Wachstumsrate signifikant. Die Reduzierung von stickstofffreien Bestandteilen, Fasern und Phytinsäure wurde für das Erreichen einer sehr hohen Rohproteinverdaulichkeit verantwortlich gemacht. Durch die hohe Reinheit des Rapsproteinisolates erschien ein signifikanter Effekt der ANS auf das Wachstum und die Gesundheit der Fische unwahrscheinlich. Die Aminosäurezusammensetzung im Futter könnte ein Hauptfaktor für die Futtermittelaufnahme darstellen, da in der Literatur gezeigt wurde, dass diese die Futtermittelaufnahme regulieren kann.

Im dritten und letzten Versuch wurde ein anderes Rapsproteinisolat als Alternative zu Fischmehl im Fischfutter für Regenbogenforellen getestet, welches hauptsächlich aus Cruziferin bestand. Auch hier wurde wieder die Nährstoffverdaulichkeit gemessen. Triplikate Gruppen von juvenilen Regenbogenforellen wurden mit sieben isonitrogenen und isoenergetischen Diäten gefüttert. In drei dieser Diäten wurde der Fischmehlanteil von 19 % im Futter zu 0, 66 und 100 % des verdaulichen Proteins gegen das Rapsproteinisolat ausgetauscht. Duplikate dieser Diäten mit reduziertem Anteil an Rapsöl wurden mit einer Mischung aus Stearinsäure und Fischöl eingekapselt. Für die siebte Diät wurde ein Teil des Rapsproteinisolats in der nicht eingekapselten Diät ohne Fischmehl durch eine Mischung aus Aminosäuren ersetzt. Die höchste Rohproteinverdaulichkeit eines Rapsproteinprodukts, die je in Regenbogenforellen unter Einsatz der Stripping-Methode gemessen wurde, konnte erreicht werden ( $99,8 \pm 1,6$  %). Nach 56 Fütterungstagen zeigten die Ergebnisse bei einem Vergleich zwischen den Kontrolldiäten und beiden Austauschstufen vom Fischmehl in verkapselten und nicht-verkapselten Diäten, dass die Futtermittelaufnahme, der Futterumsatz und schlussendlich die allgemeine Wachstumsleistung signifikant reduziert wurden. Das Verkapseln der Diäten mit Fetten konnte eine Reduzierung der Futtermittelaufnahme durch hohe Anteile von Rapsproteinisolat im Futter nicht verhindern. Die Zugabe von Aminosäuren in das Futter war keine effektive Methode um die Futtermittelaufnahme zu erhöhen, es konnte allerdings den

Proteinumsatz auf das Niveau der Kontrollgruppe erhöhen. Die Reduzierung von Asche, Fasern und stickstofffreien Bestandteilen auf minimale Anteile könnte der entscheidende Faktor dafür gewesen sein, dass die höchste Rohproteinverdaulichkeit eines Rapsproteinprodukts in Regenbogenforellen erreicht wurde, während limitierte Phytinsäureanteile eine untergeordnete Rolle gespielt haben könnten. Beobachtungen im letzten Versuch haben außerdem gezeigt, dass Effekte, die nach der Futteraufnahme auftreten, unabhängig von Effekten, die vor der Futteraufnahme auftreten, auf die Futteraufnahmeregulation wirken könnten. Der negative Einfluss von hochreinen Rapsproteinprodukten auf die Futteraufnahme könnte von der körpereigenen Aminosäurezusammensetzung abhängen. Die weitere Verbesserung von hochreinen Rapsproteinprodukten, die einen geringen Anteil an ANS aufweisen, könnte daher über die Veränderung des Aminosäureprofils der Proteinprodukte erfolgen.

## Appendix

### Nutrient and amino acid composition

Used in chapter 1-3

Analysis of amino acid concentration and macronutrients of raw materials as well as macronutrients analysis of diets, faeces and whole body was carried out according to the European Commission Regulation (EC) No 152/2009 (European Commission, 2009).

Macronutrient composition of raw materials, diets, faeces and whole body was determined at the laboratory of the Gesellschaft für Marine Aquakultur, Büsum, Germany. This analysis was conducted to determine content of dry matter (DM), crude protein, crude lipid and ash. Additionally, gross energy content was determined. Whole body homogenates were freeze-dried (alpha 1-4 LSC; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) before analysis until a constant weight was achieved. Homogenization of samples was subsequently conducted using a knife mill (Grindomix, Retsch, Haan, Germany). Macronutrient composition analysis of raw materials, diets and homogenized whole bodies was done in duplicates. Determination of crude lipid content was done according to the Soxhlet-protocol. Prior to extraction with petroleum ether (SOX THERM 416, Multistat/SX PC; C. Gerhardt GmbH & Co. KG, Königswinter, Germany), samples (2 g) were hydrolyzed with hydrochloric acid (HYDROTHERM HT 6; C. Gerhardt GmbH & Co. KG, Königswinter, Germany) and subsequently dried. Standard Kjeldahl methods were used to analyze crude protein content. These methods include digestion of samples (0.5 g) with sulphuric acid (KjelDigester K-449 and Scrubber K-415; BÜCHI Labortechnik GmbH, Essen, Germany), titration (877 Titrino plus; Deutsche METROHM GmbH & Co. KG, Filderstadt, Germany) using a nitrogen to protein coefficient of 6.25 and distillation (KjelFlex 360; BÜCHI Labortechnik GmbH) with sodium hydroxide and boric acid. Samples of 2 g were dried at 103 °C for 4 hours (ED 53, Binder GmbH, Tuttlingen, Germany) until mass was constant and subsequently weighted for determination of dry matter. Following determination of dry matter, same samples were combusted at 550 °C for 12 hours using a combustion oven (P300, Nabertherm, Lilienthal, Germany). Bomb calorimetry was used to determine gross energy content (MJ/kg) of samples (0.5 g) subsequent to calibration of the bomb calorimeter (C 200; IKA-Werke GmbH & Co. KG, Staufen, Germany). Nitrogen-free extracts (NfE) plus crude fiber content was defined as remaining portion of macronutrients in raw material, diets and whole body (NfE + crude fiber = 100 – (crude protein + crude lipid + crude ash)).

For the analysis of amino acid composition, methods according to European Commission Regulation (EC) No 152/2009, III, F: 2009-02 (European Commission, 2009) and European Commission Regulation (EC) No 152/2009, III, G: 2009-02 (European Commission, 2009) were used by AGROLAB LUFA GmbH, Kiel, Germany. All amino acids displayed during this thesis except tryptophan (European Commission Regulation (EC) No 152/2009, III, F: 2009-02; European Commission, 2009) were extracted with hydrochloric acid and the resulting solution is set at a pH value of 2.2. The same amino acids are then being separated by ion exchange chromatography and reacted with ninhydrin. Finally, amino acids are being identified by photometric analysis at 570 nm. Content of tryptophan is being determined according to European Commission Regulation (EC) No 152/2009, III, G: 2009-02 (European Commission, 2009). Samples are being hydrolyzed with a saturated barium hydroxide solution under alkaline conditions and heated to 110 °C for 20 hours. Subsequent to hydrolysis, an internal standard is added. To determine tryptophan content, samples are being extracted under acidic conditions. Tryptophan and the internal standard in the hydrolysate are being determined by high performance liquid chromatography (HPLC) using a fluorescence detector.

### **Analysis of titanium and calculation of apparent digestibility coefficient (ADC)**

Used in chapter 2 and 3

Content of titanium in faeces was determined according to DIN EN ISO 11885:2009 (DIN EN ISO 11885:2009-09, 2009; AGROLAB LUFA GmbH, Kiel, Germany). This method is based on the principle of inductively coupled plasma optical emission spectrometry. Ionized argon gas is used to create inductively coupled plasma for the production of excited atoms and ions. These atoms and ions emit electromagnetic radiation at specific wavelengths. Titanium is then determined at a wavelength of 336.121 nm. Since the concentration of the element is proportional to the intensity of the emission, the titanium concentration in the sample can be measured.

Apparent digestibility coefficients (ADC) of different nutrients in diets were calculated according to Maynard and Loosli (1979) subsequent to faeces and diet nutrient and marker analysis:

$$\text{ADC}_{\text{Nutrient, Diet}} (\%) = 100 * (1 - (\text{Marker}_{\text{Diet}} / \text{Marker}_{\text{Faeces}}) * (\text{Nutrient concentration}_{\text{Faeces}} / \text{Nutrient concentration}_{\text{Diet}}))$$



The formula for calculation of ADC of test substance was derived from Bureau and Hua (2006):

$$\text{ADC}_{\text{Test substance}} = (\text{ADC}_{\text{Test diet}} - \text{ADC}_{\text{Base diet}}) * ((30 * \text{Nutrient concentration}_{\text{Test diet}} + 70 * \text{Nutrient concentration}_{\text{Base diet}}) / 30 * \text{Nutrient concentration}_{\text{Test diet}})$$

### **Anti-nutritive factor content**

Used in chapter 1-3

Methods applied in Sashidhar et al. (2020) were used to measure phytic acid content (Christian-Albrechts-University, Kiel, Germany). The enzyme test as described by Heaney et al. (1988) and HPLC analysis after Fiebig and Arens (1992) were used for determination of glucosinolate content (Christian-Albrechts-University, Kiel, Germany).

The extraction process for phytic acid was done with three replicates of 50 mg freeze-dried samples which were grounded into a fine powder. Extraction followed the procedure described in Matthäus et al. (1995) with minor modifications. The samples were defatted with 1 ml n-hexane overnight in a rotator and the supernatant was subsequently washed in 1 ml n-hexane, mixed and centrifuged. The residue was dried for 30 min at 40 °C in a SpeedVac (Thermo Fischer Scientific Inc., Waltham, USA). Phytic acid was extracted by adding 1 ml 0.5 M HCl for 4 hours at 30 °C in a Bioer shaker (Hangzhou Bioer Technology Co., Ltd, Hangzhou, China). 800 µl supernatant is subsequently centrifuged and stored at -18 °C. Prior to injection in HPLC column, HCl was removed from extract by freeze-drying and addition of 1 ml bidistilled water.

Phytic acid content was determined using a gradient anion-exchange HPLC with a simple post-column detection system according to Rounds and Nielsen (1993). Calibration was done using a phytic acid standard (Sigma P-8810, Sigma-Aldrich, St. Louis, USA). Dowex 1x2 Cl<sup>-</sup> (Dow Chemical Company, Midland, USA) was used as an anion exchanger. The gel was prepared by washing in 500 ml water and repeated removal of fines. After removal of fines, gel was washed in 500 ml 96 % ethanol and twice in 250 ml 2 N HCl. After washing, gel was heated to 100 °C in 2 N HCl until supernatant was clear. The columns were then prepared with 4 ml of the Dowex 1x2 Cl<sup>-</sup> and washed twice with 5 ml bidistilled water. 50 µl of extract were injected to HPLC column after preparation.

Extraction of glucosinolates was done with freeze-dried and milled samples which were pre-treated for 2 min at 75 °C to inactivate myrosinase. Samples were subsequently extracted three times with 2 ml hot 70 % methanol for 10 minutes at 75 °C while stirring occasionally. Resulting extract was centrifuged and supernatants were collected. Supernatants were then reduced in a SpeedVac (Thermo Fischer Scientific Inc., Waltham, USA) to 1 ml. After the reduction, bidistilled water is added to achieve a final volume of 10 ml. The extract was stored at -18 °C.

Gel preparation for HPLC was done by suspending 20 g of DEAE-Sephadex A-25 in 2 N HAc. This gel suspension was filled with 2 N HAc to double volume of gel bed. Column was prepared with 0.5 ml gel suspension. Then, column was washed twice with 1 ml 6 M imidazole formiat solution and twice with 1 ml bidistilled water. After preparation, 1 ml extract was applied to the column and washed twice with 1 ml 0.02 M NaAc solution at pH 4. 500 µl of 1 mg/ml sulfatase solution in water was added. This solution was left at room temperature to react for 18 hours. Resulting desulfoglucosinolates were eluted twice with 1 ml bidistilled water. For HPLC, column temperature was 30 °C and detection wave length was at 229 nm. 10-50 µl of extract were injected into HPLC column.

For the enzymatic determination of glucosinolates, 20 g of DEAE-Sephadex A-25 was suspended in 0.5 M pyridine acetate buffer at pH 5. Suspension was subsequently washed twice with 0.02 M pyridine acetate buffer at pH 5 and filled with 0.02 M pyridine acetate buffer to double volume of gel bed. 1 ml of the gel suspension is then pipetted to the column and washed twice with 0.5 ml bidistilled water. After this column preparation, 4 ml crude extract was applied to column and washed twice with 0.5 bidistilled water und 0.5 ml 0.02 M pyridine acetate buffer at pH 5. 250 µl of 4 mg/ml myrosinase solution in 0.02 pyridine acetate was added to the column afterwards. Solution reacted at room temperature for 8 hours and was then eluted twice with 0.5 bidistilled water. Finally, solution was shock-frozen and freeze-dried. Before enzyme test was used, solution was dissolved in 100 µl bidistilled water. 50 µl of the solution was put in GOPOD Glc test (Megazyme, Co. Wicklow, Ireland).

### **Processing of rapeseed protein isolates**

Used in chapter 2 and 3

For the processing of both rapeseed protein isolates, rapeseed meal was used as the starting material. The seeds ("Raffiness") were conditioned at 70-80 °C for 20 min in a vacuum dryer and the oil is subsequently extracted by oil pressing at 60-65 °C and hexane extraction at 50-60 °C. Following desolventising of hexane, rapeseed seeds were crushed to a particle size of 0.2-0.5 mm (chapter 2) or 0.2 mm (chapter 3) respectively. In contrast to previous processing methods (Adem, 2014; chapter 1; Slawski, 2012), ethanol extraction was omitted to increase the protein dispersibility index (PDI) of the resulting rapeseed meal. This enhances the extractability of the rapeseed protein.

In chapter 2, protein from the rapeseed meal was extracted at 50 °C with a 0.5 M salt solution. After decantation, suspension was filtrated (UF/MF-Laboranlage LTH Dresden, Dresden, Germany). The membrane size was 10 kDa and filtration ended at a conductivity of 2 mS/cm<sup>2</sup> (Multi 3320, WTW GmbH, Weilheim, Germany). Resulting solution was spray dried (Laborsprührockner, NUBILOSIA Molekularzerstäubung Dipl.-Ing. G. Ladisch GmbH & Co., Koblenz, Germany).

In chapter 3, protein extraction was carried out without salt and the protein was obtained from the extraction solution by precipitation instead of filtration. Extraction solution was precipitated with citric acid and the resulting solution was centrifuged (Beckman Avanti JXN-26, Beckman Coulter GmbH, Krefeld, Germany) and freeze-dried (Zirbus Sublimator 3x4x5, ZIRBUS technology GmbH, Bad Grund, Germany).

### **Water stability test**

Used in general discussion

Methods used for the water stability measurement of diets from chapter 3 were based on methods applied in Baeverfjord et al. (2006) with minor adjustments. Triplicate samples (3.2 g) were taken from diets CD FM, RI66 and RI100 and placed in pre-weighted, custom made wire netting baskets with 1 mm mesh size, a diameter of 2.5 cm and a length of 12 cm. Netting baskets were placed in glass beakers filled with 300 ml tap water. Beakers were subsequently shaken (100 rpm) for 240 min at room temperature (PSU-20i Multi-functional Orbital Shaker, SIA Biosan, Riga, Latvia). After the shaking process was terminated, baskets were gently dried with paper tissues and weighted. To determine remaining dry matter content, netting baskets

were dried at 103 °C for 20 hours (ED 53, Binder GmbH, Tuttlingen, Germany) and subsequently weighted. The statistical program SPSS (version 20) was used for data analysis of the water stability test. Prior to application of Kruskal-Wallis test or one-way analysis of variances (ANOVA), Kolmogorov-Smirnov and Levene test were applied to determine normal distribution and homogeneity of variances. Tukey's HSD test was used for multiple comparisons when differences among groups were identified. The aggregate type I error was defined at 5 % ( $p < .05$ ) for each set of comparisons to determine statistical significance.

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## Curriculum Vitae

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März 2017 bis November 2017 Praktikum bei der Gesellschaft für Marine Aquakultur mbH in Büsum

Oktober 2014 bis Februar 2017 Master of Science  
Studium der Aquakultur und Searanching, Masterarbeit:  
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Universität Rostock  
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Oktober 2011 bis April 2014 Bachelor of Science  
Studium der Biologie, Bachelorarbeit:  
„Fressverhalten von Raubfischen in der Fulda bei Hann. Münden“

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Juli 2010 Allgemeine Hochschulzugangsberechtigung (Abitur)  
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