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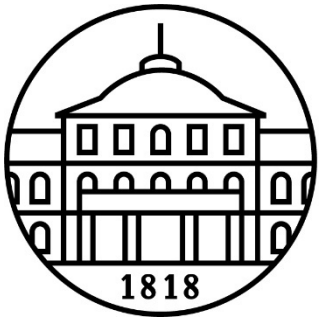

UNIVERSITY OF HOHENHEIM

Nutritional evaluation of oilseed press cakes in fish nutrition with emphasis on rainbow trout (*Oncorhynchus mykiss*, W.)

Dissertation

submitted in fulfilment of the requirements for the degree

”Doktor der Agrarwissenschaften”

(Dr. sc. agr. / PhD in Agricultural Sciences)

to the

Faculty of Agricultural Science

presented by

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born in Ostfildern/Ruit, Germany

2019

Die vorliegende Arbeit wurde am 13.03.2019 von der Fakultät Agrarwissenschaften der Universität Hohenheim als „Dissertation zur Erlangung des Grades eines Doktors der Agrarwissenschaften“ angenommen.

Tag der mündlichen Prüfung:	29.03.2019
Leitung des Kolloquiums:	Prof. Dr. Stefan Böttinger
Berichterstatter, 1. Prüfer:	Prof. Dr. M. Rodehutscord
Berichterstatter, 2. Prüfer:	Prof. Dr. W. Windisch
3. Prüfer:	Prof. Dr. J. Seifert

This project was in part supported by funds of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme.

"*Knowledge, like air, is vital to life. Like air, no one should be denied it.*"

- Alan Moore (1988): V for Vendetta. DC Comics, New York. USA

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List of abbreviations

(with the exception of abbreviations that were additionally used in Manuscripts 1-3 and for units defined by the International System of Units)

AA	Amino acid
ADC	Apparent digestibility coefficient
ANF	Antinutritional factor
CA	Crude ash
CF	Crude fibre
CL	Crude lipid
CP	Crude protein
FCR	Feed conversion ratio
HSI	Hepatosomatic index
InsP	Inositol phosphate
$InsP_6$	Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate)
NSP	Non-starch polysaccharide
P	Phosphorus

List of tables

(with the exception of tables presented in Manuscripts 1-3)

Chapter 1:
General Introduction

1. General Introduction

The average annual growth rate of global aquaculture production[1] was 5.9% between the years 2001-2015 (FAO 2017). The year 2015 marked a turning point of the sector. Global aquaculture production was estimated to be 76.6 Mt in the year 2015 and for the first time provided more fishery products for direct human consumption than capture production (72.4 Mt) (FAO 2017). This was in great part due to the steep increase of feed-based aquaculture production systems (FAO 2016). This in turn increases the demand for fish feed. Satisfying the demand for sources of dietary protein has since become one of the greatest challenges in sustaining the sector's growth (Tacon & Metian 2015).

Historically, fishmeal used to be the major protein source in fish feed. It was a widely available cheap raw material with many favourable nutritional characteristics. It is highly digestible, has a high crude protein (**CP**) concentration with a well-balanced amino acid (**AA**) profile, and contains valuable nutrients such as minerals like calcium and phosphorus (**P**) (NRC 2011). High quality fishmeal is commonly produced from whole fish that have a low economic value or are not suitable for direct human consumption. Fishmeal that is produced from fisheries and aquaculture by-products is considered of lesser quality due to its comparably low CP and high crude ash (**CA**) concentration.

The increasing fish feed demand, coupled with an increasing demand of fishmeal, has had a wide array of consequences. The share of globally available fishmeal that is used in aquaculture rose from 2% in 1960 to 74% in 2012 (Mallison 2013; Shepherd & Jackson 2013). An increased amount of fishmeal that can be made available from capture fisheries is not to be expected because the fishmeal production volume from capture fisheries has stagnated since the late 1980s and is not expected to increase. And the share of fish stocks fished within biologically sustainable levels has declined from 90% in 1970 to 69% in 2013 (FAO 2017). Consequently, the average price of fishmeal increased gradually (Indexmundi 2018). In addition, the global share of fishmeal produced from by-products has steadily increased and accounted for about 25% of the globally produced fishmeal in recent years (Ytrestøyl et al. 2015).

Research over the past decades has made great advances in counteracting the limited supply of fishmeal. The use of alternative proteins drastically decreased the proportion of fishmeal in fish feed and fishmeal has become a resource that is used rather strategically and selectively for specific stages of fish production (Tacon et al. 2009; FAO 2016). Furthermore, research on species-specific nutrient requirements and advances in feed production technology decreased the feed conversion ratio (**FCR**) for many species and enabled a more efficient use of feed resources (including fishmeal) (Naylor et al. 2009; NRC 2011).

[1] Unless otherwise specified aquaculture (production) and respective statistics referring to it do not include the production of aquatic plants.

Despite the pre-existing research efforts, owing to an estimated 360 farmed finfish species globally, a wide array of plant-derived feed ingredients, and a growing demand for fish feed, there is still much research needed to facilitate the sustainable growth of global aquaculture production. There are many feed ingredients that have been shown to, at least in part, replace fishmeal in fish feed (such as insect meal and meals made from various by-products of animal production) but the majority of protein in fish feed is plant-based and is for the most part made available from oilseeds in the form of solvent extracted oilseed meals (NRC 2011; Tacon et al. 2011).

However, most plant-based feed ingredients are nutritionally inferior to fishmeal in a few aspects. In comparison to fishmeal they: a) have an imbalanced AA profile (commonly lacking essential AAs such as lysine), b) can contain compounds which may act as antinutritional factors (**ANF**s), c) have a reduced palatability, and d) can contain minerals (especially P) in a form that has a limited availability in fish (Francis et al. 2001; Naylor et al. 2009).

Ample research effort has been made to evaluate the nutritional value of solvent extracted oilseed meals in fish nutrition. To the author's knowledge press cake[2] of oil crops as potential fishmeal substitute in fish feed has not been investigated to that extent. Press cake is made available by pressing (i.e. the mechanical extraction) of oilseed and thereby represents the primary by-product of vegetable oil production. It is comparably cheap, rich in CP and has a higher crude lipid (**CL**) content than its respective solvent extracted oilseed meal (Williams 2005). In addition, small scale oil pressing without a downstream solvent extraction is easily realised and makes press cake a resource with a decentralised and widespread availability (Kaltschmitt et al. 2009). In addition, its availability on the market, unlike fishmeal, has steadily increased in the past and is expected to further increase due to an increasing demand of vegetable food oil and biofuel (OECD/FAO 2017).

Numerous studies have evaluated the nutritional value of press cake in fish, but the majority was focused on herbivorous and omnivorous species reared in warm-water production systems (Hasan et al. 1997; Fagbenro 1998; Xie et al. 1998; Maina et al. 2002, 2003; Mohanta et al. 2007; Nyina-Wamwiza et al. 2010; Mazurkiewicz et al. 2011; Aanyu et al. 2014; Geremew et al. 2015). In comparison, few studies focused on press cake in salmonid nutrition (Nang Thu et al. 2011; Murray et al. 2014).

Salmonids (i.e. *salmonidae*) represent a taxonomic family of predominantly carnivorous (piscivorous) species that are commonly reared in cold-water production systems. The most dominantly produced salmonid species are salmon and trout whose share in world trade of aquaculture products has become the largest single commodity by 2013 (FAO 2016). Although

[2] In international literature the terms press cake, oilcake, oilseed cake, and oilseed meal are often used interchangeably. The differentiation between actual press-residue and solvent extracted material can sometimes only be made in dependence of the CL content of the material in question commonly ranging from higher than 6% and around 2% and lower, respectively.

salmonids only accounted for about 6% of total fish production in the year 2015, the production of salmonid feed used around one quarter of the total fishmeal used for aquaculture (Shepherd & Jackson 2013; FAO 2016, 2017). The substitution of fishmeal with press cake in salmonid feed could therefore, even at low levels, have a big impact on the economic and ecological sustainability of salmonid production.

Chapter 2:
Approach and Overview of Own Work

2. Approach and Overview of Own Work

In light of the potential that was outlined in the introduction, this thesis aims to add to pre-existing knowledge on the nutritive value of various press cakes in fish nutrition, with emphasis on rainbow trout (*Oncorhynchus mykiss* W.). The press cakes were chosen either because a vast amount of them can be made available owing to the amount of globally produced oilseed (press cake of rapeseed, sunflower seed, and soybean) or their appreciable crude nutrient composition (press cake of linseed, pumpkin seed, and walnut kernel). The focus was on rainbow trout because this species represents the most produced and economically most important freshwater salmonid on a global scale and in Germany (Brämick 2016; FAO 2016).

This chapter will provide an overview of the chosen approach and the conducted work. The three manuscripts that were written in line with the present thesis are provided in Chapter 3. Some press cakes did not meet the focus of the presented manuscripts and the results were not included therein. Because they were determined as part of the digestibility experiments presented in Manuscript 1 and 2 (using the same methodology described in the respective manuscript), the results will be additionally presented in Chapter 3. These results will briefly be discussed following the general discussion that intends to complement the discussions presented in the manuscripts (Chapter 4).

Manuscript 1: Utilization of unprocessed and fibre-reduced oilseed cakes of rapeseed and sunflower seed in rainbow trout (*Oncorhynchus mykiss* W.) nutrition – Evaluation of apparent digestibility and growth performance

Rapeseed (*Brassica napus* L.) and sunflower seed (*Helianthus annuus* L.) represent the oilseeds from which the most press cake can be made available in Europe. However, the potential of these press cakes as protein source for rainbow trout has not been evaluated. Therefore, the aim of a series of experiments was to determine the apparent digestibility coefficients (**ADC**s) of CP, CL, and energy of the respective press cakes in rainbow trout. Non-starch polysaccharides (**NSP**s) have been shown to act as ANFs but partial dehulling of the seeds prior to pressing has been shown to increase the digestibility of nutrients of press cakes in other species. Thus, the effect of dehulling on the ADCs of CP, CL, and energy of the press cakes in question have also been investigated. In addition, the effects on performance traits of rainbow trout were investigated in two experiments, in which dietary fishmeal was partially replaced by unprocessed and fibre-reduced press cakes on the basis of digestible CP.

As part of the digestibility experiments presented in this manuscript the ADCs of CP, CL, and energy of soybean and linseed cake were also determined (Chapter 3.4.1 and 3.4.3).

This manuscript was published in *Aquaculture Nutrition*.

Manuscript 2: Pumpkin seed cake as a fishmeal substitute in fish nutrition: effects on growth performance, morphological traits, and fillet colour of two freshwater salmonids and two catfish species

The seeds of the Styrian oilseed pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca*) have very thin outer hulls. The resulting press cake has a high CP and a low crude fibre (**CF**) concentration. This distinguishes this particular press cake and makes it an interesting press cake for the investigation as partial fishmeal substitute. To the authors' knowledge pumpkin seed cake of the Styrian oilseed pumpkin has only been investigated as partial fishmeal replacer in diets for Arctic charr (*Salvelinus alpinus*; Murray et al. 2014). Therefore, the primary aims of a series of experiments was to determine the ADCs of CP, CL, and energy in rainbow trout and to study the effects on rainbow trout performance traits when dietary fishmeal was partially replaced by pumpkin seed cake. Furthermore, the effect on morphological traits of rainbow trout resulting from the partial substitution of fishmeal with pumpkin seed cake in their diet were also evaluated. In addition, the effect of partial substitution of fishmeal with pumpkin seed cake on performance and morphological traits of brook trout (*Salvelinus fontinalis* M.), African sharptooth catfish (*Clarias gariepinus* B.), and wels catfish (*Silurus glanis* L.) were also investigated.

The ADCs of CP, CL, and energy of walnut kernel cake were also determined in rainbow trout as part of the digestibility experiment that is presented in this manuscript (Chapter 3.4.2).

This manuscript was published in *Archives of Animal Nutrition*.

Manuscript 3: Effects of phosphate and phytase supplementation on phytate degradation in rainbow trout (*Oncorhynchus mykiss* W.) and Atlantic salmon (*Salmo salar* L.)

Most P in fishmeal is associated with hydroxyapatite of bone, whereas the primary storage form of P in plant seeds are phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate; **$InsP_6$**) and its salts (phytate). However, unlike the P contained in fishmeal, most inositol-phosphate bound P (**InsP-P**) is not available to fish due to the (presumed) lack of the corresponding enzymes in the digestive tract. Therefore, the increasing replacement of fishmeal with plant-based proteins such as press cake has implications on the amount of available P in fish feed. Inorganic P supplements are commonly used to supply the dietary P requirements. But inorganic P is a very limited resource and will have to be increasingly used the more fishmeal is replaced with ingredients that have a limited P availability.

Increasing the availability of InsP-P in press cake can be an approach for a more sustainable replacement of fishmeal with press cake. It has been shown that the apparent digestibility (i.e. disappearance) of InsP-P can be increased by the supplementation of $InsP_6$-hydrolysing enzymes (i.e.

phytases) to fish feed. Phytases catalyse the hydrolytic cleavage of $InsP_6$ and its salts. To the authors' knowledge both the breakdown of $InsP_6$ and the appearance of specific lower inositol phosphates in the digestive tract were not investigated so far in fish. Therefore, the aims of two experiments were to compare rainbow trout and Atlantic salmon (*Salmo salar*) with regards to their capacity to hydrolyse $InsP_6$ in the digestive tract without and with a supplemented phytase, and to understand what the primarily formed lower InsPs are. Furthermore, the effect of supplemented mineral P on $InsP_6$ hydrolysis without and with a phytase supplement were compared between the two species.

This manuscript was published in *Aquaculture*.

Chapter 3:
Included Manuscripts and Additional Results

3. Included Manuscripts and Additional Results

3.1 Manuscript 1

Utilization of unprocessed and fibre-reduced oilseed cakes of rapeseed and sunflower seed in rainbow trout (*Oncorhynchus mykiss* W.) nutrition – Evaluation of apparent digestibility and growth performance

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Published in *Aquaculture Nutrition* (2018), 24:1133-1143.

DOI: https://doi.org/10.1111/anu.12652

Abstract:

Two digestibility trials were carried out to determine the apparent digestibility coefficients (ADCs) for crude protein (CP), crude lipid (CL) and energy of unprocessed and two differently processed fibre-reduced rapeseed and sunflower seed oil cakes (sieved oilseed cakes and oilseed cakes produced from partially dehulled seeds) in rainbow trout (40 fish per tank; four tanks per diet). Each trial was followed by a 63-day growth trial, wherein the respective oilseed cakes with the highest ADCs were evaluated as fishmeal substitutes, based on digestible CP. Oilseed cakes of rapeseeds and sunflower seeds had low nutrient ADCs (Trial 1). Nonetheless, the protein in rapeseed cake was able to replace up to 10% of the fishmeal protein in a diet without negatively affecting performance traits (Trial 2). Fibre reduction increased the ADCs of both types of oilseed cakes substantially (Trial 3). However, when fish were fed diets with 0%, 25% and 50% fishmeal protein replaced with dehulled rapeseed or dehulled sunflower seed cake protein, performance traits decreased with increasing substitution levels (Trial 4). Nonetheless, the amount of fishmeal needed per unit weight gain was lower for all fish fed the diets containing either one of the dehulled oilseed cakes than for the reference diets.

Keywords:

Antinutritional factors, digestibility, energy, fibre, growth performance, protein

Received: 21 June 2017 | Accepted: 11 November 2017

DOI: 10.1111/anu.12652

ORIGINAL ARTICLE

WILEY Aquaculture Nutrition

Utilization of unprocessed and fibre-reduced oilseed cakes of rapeseed and sunflower seed in rainbow trout (*Oncorhynchus mykiss* W.) nutrition—Evaluation of apparent digestibility and growth performance

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Funding information
Bundesministerium für Ernährung und Landwirtschaft, Grant/Award Number: 511-06.01-28-1-73.003-10

Abstract

Two digestibility trials were carried out to determine the apparent digestibility coefficients (ADCs) for crude protein (CP), crude lipid (CL) and energy of unprocessed and two differently processed fibre-reduced rapeseed and sunflower seed oil cakes (sieved oilseed cakes and oilseed cakes produced from partially dehulled seeds) in rainbow trout (40 fish per tank; four tanks per diet). Each trial was followed by a 63-day growth trial, wherein the respective oilseed cakes with the highest ADCs were evaluated as fishmeal substitutes, based on digestible CP. Oilseed cakes of rapeseeds and sunflower seeds had low nutrient ADCs (Trial 1). Nonetheless, the protein in rapeseed cake was able to replace up to 10% of the fishmeal protein in a diet without negatively affecting performance traits (Trial 2). Fibre reduction increased the ADCs of both types of oilseed cakes substantially (Trial 3). However, when fish were fed diets with 0%, 25% and 50% fishmeal protein replaced with dehulled rapeseed or dehulled sunflower seed cake protein, performance traits decreased with increasing substitution levels (Trial 4). Nonetheless, the amount of fishmeal needed per unit weight gain was lower for all fish fed the diets containing either one of the dehulled oilseed cakes than for the reference diets.

KEYWORDS
antinutritional factors, digestibility, energy, fibre, growth performance, protein

1 | INTRODUCTION

Fishmeal has seen continuous price increases in the past (Indexmundi, 2016), and this trend is expected to continue, given its high demand and limited availability. Salmonid feed usually includes a large share of fishmeal (Shepherd & Jackson, 2013), and is therefore, particularly prone to price hikes due to limited availability. Purified products of rapeseed (*Brassica napus* L.) and sunflower seed (*Helianthus annuus* L.), such as protein concentrates and protein isolates, have shown considerable promise as a fishmeal substitute in the diet of the rainbow trout (*Oncorhynchus mykiss* W.) (Mwachireya, Beames, Higgs, & Dosanjh, 1999; Slawski et al., 2013; Stickney et al., 1996). However, the production of purified proteins consumes high amounts of energy and resources, which is reflected in their prices, and thus limits their widespread accessibility.

The raw materials used for the production of the aforementioned protein-rich products are oilseed cakes (or oilcakes). They are a by-product resulting from the pressing, that is, the mechanical extraction, of oilseeds, and represent a widely available source of crude protein (CP) and crude lipid (CL). However, oilseed cakes contain relatively high amounts of an indigestible fibre fraction and lignin. Dehulling the seeds prior to oil pressing and sieving the oilseed cakes are viable methods for decreasing the dietary fibre fraction and lignin content of oilseed cakes and meals (Gill et al., 2006; Kracht et al., 2004). This

reduces the indigestible fibre fraction, potentially increasing the nutrient digestibility for rainbow trout, by decreasing the content of antinutritional factors (ANFs) associated with the fibre fraction, namely, non-starch polysaccharides (NSPs). In Europe, rapeseeds and sunflower seeds represent the oilseeds from which the largest amount of oilseed cakes are made available, as they are the predominantly grown oilseeds with a production quantity of approximately 29 Mt each in 2014 (FAOStat, 2017). To our knowledge, no study has evaluated the apparent digestibility coefficients (ADCs) of rapeseed and sunflower seed cakes in extruded diets for rainbow trout, the main freshwater salmonid species worldwide (FAO, 2012).

One objective of this study was to determine and compare the nutrient ADCs of unprocessed and two differently processed, fibre-reduced, rapeseed and sunflower seed oil cakes in rainbow trout. The second objective was to determine whether unprocessed oilseed cakes and oilseed cakes of partially dehulled rapeseeds and sunflower seeds could be a partial substitute for fishmeal in the diet for rainbow trout, when substituted on the basis of digestible CP. The experimental set-up consisted of four trials. In the first trial, the ADCs of CP, CL and energy were determined for rapeseed and sunflower seed cakes. In the second trial, performance traits of rainbow trout were assessed following the replacement, based on digestible CP, of 0, 50 and 100 g/kg of the fishmeal protein in a reference diet with rapeseed cake protein. In the third trial, the ADCs of CP, CL and energy for two differently processed, fibre-reduced rapeseed and sunflower seed cakes were determined. In the fourth trial, performance traits of rainbow trout were assessed following the replacement of 0%, 25% and 50% of the fishmeal protein in a reference diet with either partially dehulled rapeseed cake or partially dehulled sunflower seed cake protein, based on their respective CP ADCs.

2 | MATERIALS AND METHODS

2.1 | Oilseed cakes and their processing

In trials 1 and 2, commercially available (i.e., unprocessed) rapeseed and sunflower seed cakes were used. They were obtained from a commercial oil mill (Marbacher Ölmühle GmbH, Marbach, Germany).

In Trial 3, sieved rapeseed and sunflower seed cakes, that were prepared by the Fraunhofer Institute for Process engineering and Packaging IVV, Freising, Germany, were used. The sieved oilseed cakes were prepared by processing the rapeseed and sunflower seed cakes used in trials 1 and 2, as explained below. The oilseed cakes were ground using an impact mill equipped with a 2-mm mesh (Hosokawa Alpine AG, Augsburg, Germany). The ground oilseed cakes were then sieved for 10 min using a horizontal sieve shaker equipped with a 250-µm mesh (AS 400 control, Retsch GmbH, Haan, Germany). The horizontal circular motion was set to 300 rpm, and its operating direction was inverted every 30 s. The resulting fine yields, that is, sieved oilseed cakes, were used in Trial 3.

In trials 3 and 4, oilseed cakes of partially dehulled oilseeds, obtained from a commercial oil mill (Teutoburger Ölmühle GmbH, Ibbenbüren, Germany), were used. Here, prior to oil pressing, the respective oilseeds were cracked and their hulls were partially separated by air classification.

The chemical composition of all the oilseed cakes used in this study is presented in Table 1. The amino acid and fatty acid profiles of the unprocessed oilseed cakes of rapeseeds and sunflower seeds are presented in Table S1 (supplement available in online).

2.2 | Diet preparation

The diets were manufactured using a pilot-scale, co-rotating, intermeshing twin-screw extruder (ZSK 26, Coperion GmbH, Stuttgart, Germany), at an average temperature of 106°C and an exposure time of approximately 59 s across the five extruder barrels. The exit temperature of the feed dough at the extruder die averaged at 104°C and 112°C for the digestibility diets (trials 1 and 3) and the growth performance diets (Trial 2), respectively. The pellet diameter was 4.5 mm for the digestibility diets and 3 mm for the growth performance diets, and all diets were dried at 90°C, until the moisture content dropped below 100 g/kg. The resulting feeds were then vacuum-coated with the respective amount of fish oil. The diets were prepared by the Fraunhofer Institute for Process Engineering and Packaging IVV in Freising, Germany. The growth performance diets used in Trial 4 had a pellet diameter of 4.5 mm and were produced by Aller Aqua, Christiansfeld, Denmark.

For the digestibility trials, a fishmeal-based reference diet was produced. The experimental diets for the digestibility trials contained 750 g/kg of the reference diet and 250 g/kg of the respective oilseed cake on a dry matter basis (Table 2). Titanium(IV) oxide (Merck KGA, Darmstadt, Germany) served as an inert marker. The deviation from the 70:30 ratio commonly applied in digestibility studies was due to the potentially adverse effects on animal health that could result from a higher inclusion of rapeseed cake (Trial 1), given its glucosinolate content (Burel et al., 2001). The digestibility diets used in Trial 3 were then produced accordingly, in order to allow a comparison between the different oilseed cakes due to similar feed processing parameters.

The growth performance diets, with regard to fishmeal replacement, were calculated based on the CP ADCs obtained in the respective digestibility trials. In Trial 2, 0%, 5% and 10% of the fishmeal protein in the reference diet were replaced with rapeseed cake protein based on the determined CP ADC in Trial 1. In Trial 4, 0%, 25% and 50% of the fishmeal protein in the reference diet were replaced by the protein of either partially dehulled rapeseed cake or partially dehulled sunflower seed cake based on the determined CP ADCs of the respective oilseed cakes in Trial 3. To balance the nutrient composition, the wheat and rapeseed oil contents of the growth performance diets were adjusted accordingly, and they were formulated to contain a similar CP:energy ratio on a digestible basis (Table 3).

2.3 | Experimental conditions

The trials were carried out in the indoor facility of the Institute for Fisheries of the Bavarian State Research Center for Agriculture, Starnberg (Germany), using the house strain of rainbow trout. The

TABLE 1 Characterization of oilseed cakes used in the four trials with rainbow trout

	Rapeseed cake	Rapeseed cake (sieved)[a]	Rapeseed cake (dehulled)[b]	Sunflower seed cake	Sunflower seed cake (sieved)[a]	Sunflower seed cake (dehulled)[b]
Chemical composition [g/kg DM]						
Dry matter	926	935	932	926	932	928
Crude protein	294	372	384	224	323	347
Crude lipid	203	178	137	152	128	128
Crude fibre	197	83	107	318	226	195
Crude ash	55	67	67	67	64	67
NfE[c]	252	300	305	240	259	263
Gross energy [MJ/kg DM]	21.6	22.7	21.7	22.3	21.6	21.6
Phosphorus and phytate [g/kg DM]						
Total phosphorus	3.9	10.8	1.50	4.9	n.a.	n.a.
Phytate phosphorus	2.6	9.84	0.97	3.9	n.a.	n.a.
Selected antinutritional factors						
Tannins [g/kg DM]	13.0	20.3	17.2	n.a.	n.a.	n.a.
Glucosinolates [g/kg DM]	5.38	12.1	7.3	n.a.	n.a.	n.a.
Progoitrin [g/kg DM]	2.56	4.69	3.08	n.a.	n.a.	n.a.
Volatile mustard oils [mg/kg DM]	21.6	3.74	<3.0	n.a.	n.a.	n.a.

[a]Respective unprocessed oilseed cakes were sieved; oilseed cakes were provided by Marbacher Ölmühle GmbH, Germany.
[b]Provided by Teutoburger Ölmühle GmbH, Germany.
[c]NfE (nitrogen free extracts) = 1,000 g − (crude protein [g] + crude fat [g] + crude fibre [g] + crude ash [g]).
n.a., not analysed.

digestibility trials were approved by the Bavarian State Animal Welfare Commission (ref. no. 55.2-1.54.2532.38.2016). The growth trials were exempt from the aforementioned directives on the basis of the results of the respective digestibility trials. Prior to the trials, the fish received a commercial feed (BioMar INICIO 917, Biomar A/S, Brande, Denmark). In each trial, the diets were fed to the fish in four tanks (n = 4), in a completely randomized design. All the fish were carefully handfed to avoid feed losses, and feed intake was recorded daily. An overview of the experimental conditions is provided in Table 4.

In the digestibility trials, the fish were fed the diets for 8 days prior to faecal collection. Faecal samples were collected in accordance with the method described by Kinzinger (1992), by manually stripping all the fish of each tank 2 hr postfeeding, after sedation using MS-222. The fish were carefully dried with a moist towel to remove the surface water before applying gentle pressure that started above the pelvic fin and followed through to the anus. Care was taken to ensure that the faeces were not contaminated with urine or mucus. After stripping, each fish was placed in fresh water until the sedation wore off and then returned to its respective tank. The faecal samples of all the fish in each tank were pooled and immediately frozen at −17°C after the sampling of each tank was completed. To obtain adequate sample sizes, the fish were fed the digestibility diets for another 8-day period, after which faecal samples were collected once more as described above. The faecal samples collected from each tank were pooled across the two sampling days.

2.4 | Calculations

The ADCs of the nutrients in the diets and of the oilseed cakes were calculated according to the following equations, as suggested by NRC (2011):

$$\text{ADC nutrients diet} = (1 - (\text{Marker concentration in feed})/(\text{Marker concentration in faeces}) \times (\text{Nutrient concentration in faeces})/(\text{Nutrient concentration in feed})) \times 100$$

$$\text{ADC nutrients oilseed cake} = \text{ADC digestibility diet} + ((\text{ADC digestibility diet} - \text{ADC ref. diet}) \times (0.75 \times D\,\text{ref.}/0.25 \times D\,\text{ingredient}))$$

where D ref. and D ingredient are the corresponding nutrient or energy contents in the reference diet and the respective oilseed cake, respectively.

The following equations were used to assess the performance of the fish over the course of the growth trials:

$$\text{WG: weight gain[g]} = \text{final average weight [g]} - \text{initial average weight [g]}$$

TABLE 2 Ingredients and chemical composition of diets used in the digestibility trials

	Trial 1			Trial 3				
	REF	RS	SF	REF	RS_S	RS_D	SF_S	SF_D
Ingredients [g/kg DM]								
Fishmeal (anchovy)[a]	601	450	450	601	450	450	450	450
Fish oil[a]	133.6	100	100	133.6	100	100	100	100
Wheat[a]	153.6	115	115	153.6	115	115	115	115
Wheat gluten[a]	106.8	80	80	106.8	80	80	80	80
Rapeseed cake[b]	–	250	–	–	–	–	–	–
Sunflower seed cake[b]	–	–	250	–	–	–	–	–
Rapeseed cake (sieved)[c]	–	–	–	–	250	–	–	–
Rapeseed cake (dehulled)[d]	–	–		–	–	250	–	–
Sunflower seed cake (sieved)[c]	–	–	–	–	–	–	250	–
Sunflower seed cake (dehulled)[d]	–	–	–	–	–	–	–	250
Titanium(IV) oxide	5	5	5	5	5	5	5	5
Chemical composition analysed [g/kg DM]								
Dry matter	977	972	980	919	905	908	928	927
Crude protein	541	480	458	567	488	493	489	484
Crude lipid	198	193	191	172	203	186	187	179
Crude fibre	2	32	58	9.2	33	34	52	56
Crude ash	108	99	104	111	96	99	97	97
Gross Energy [MJ/kg DM]	23.6	23.1	23.2	22.9	22.9	23.0	22.9	22.9

REF = reference diet (a separate reference diet was produced for each of the trials); RS = rapeseed cake diet; SF = sunflower seed cake diet; RS_S = rapeseed cake diet (sieved cake); RS_D = rapeseed cake diet (dehulled cake); SF_S = sunflower seed cake diet (sieved cake); SF_D = sunflower seed cake diet (dehulled cake).

[a]Provided by Emsland Aller Aqua GmbH, Germany.

[b]Provided by Marbacher Ölmühle GmbH, Germany.

[c]Same as [b] sieved by the Fraunhofer Institute for Process engineering and Packaging IVV, Germany.

[d]Provided by Teutoburger Ölmühle GmbH, Germany.

$$\text{FI: feed intake [g DM]} = \frac{\text{total feed intake [g DM]}}{\text{number of fish per tank}}$$

$$\text{FCR: feed conversion ratio} = \frac{\text{FI}}{\text{WG}}$$

2.5 | Chemical analyses

Crude nutrient concentrations of oilseed cakes, feed and faeces were analysed according to VDLUFA (2007): dry matter (DM; method 3.1), crude ash (CA; method 8.1), crude protein (CP; method 4.1.1), crude lipid (CL; sample treated with HCl and extracted with petroleum ether, method 5.1.1b) and crude fibre (CF; method 6.1.1; Fibretherm, Fa. C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Gross energy (GE) was determined using a bomb calorimeter (C 200; Ika-Werke GmbH & Co.KG, Staufen, Germany). Titanium was analysed as described in detail by Boguhn, Baumgärtel, Dieckmann, and Rodehutscord (2009). Phosphorus and phytate contents were determined using a Megazyme kit (K-PHYT, Megazyme, Bray, Ireland). Glucosinolate, volatile mustard oil and progoitrin contents of the rapeseed cakes were analysed in accordance with ISO (1992). Tannin content of the rapeseed cakes was determined according to AOAC (1970). All samples were analysed in duplicate.

2.6 | Statistical analysis

Unless stated otherwise, mean values are reported along with their respective standard deviations. The comparison of the means was performed using one-way ANOVA (SAS 9.3, SAS Institute Inc., Cary, NC, USA), after normality and homogeneity were confirmed. Statistical pairwise differences between means were determined using Tukey's HSD test at $p < .05$ significance level.

TABLE 3 Ingredients and chemical composition of diets used in the growth trials

	Trial 2			Trial 4				
	REF[a]	R05[a]	R10[a]	REF[b]	R25[b]	R50[b]	S25[b]	S50[b]
Ingredients [g/kg DM]								
Fish oil[c]	110	110	110	110	110	110	110	110
Soybean meal[c]	247.4	247.4	247.4	235	235	235	235	235
Blood meal[c]	90	90	90	100	100	100	100	100
Wheat gluten[c]	90	90	90	97.4	97.4	97.4	97.4	97.4
Fishmeal (anchovy)[c,d]	200	190	180	160	120	80	120	80
Rapeseed cake[e]	–	51.5	103	–	–	–	–	–
Rapeseed cake (dehulled)[f]	–	–	–	–	78	156	–	–
Sunflower seed cake (dehulled)[f]	–	–	–	–	–	–	92.2	184.5
Rapeseed oil[c]	50	55	60	75	77.5	80	67.8	60.5
Wheat[c]	200	153.5	107	210	170	129	165	120
L–Lysine·HCL[c]	2	2	2	2	2	2	2	2
DL-Methionine[c]	5.6	5.6	5.6	2.6	2.6	2.6	2.6	2.6
Monocalcium phosphate[c]	3	3	3	3	3	3	3	3
Vit–min premix[g]	2	2	2	5	5	5	5	5
Chemical composition analysed [g/kg DM]								
Dry matter	942	951	935	927	922	918	920	924
Crude protein	474	470	482	461	466	454	445	443
Crude lipid	172	209	206	226	222	228	227	233
Crude fibre	14.5	23.6	31.2	11	17	32	42	56
Crude ash	62.7	61.1	61.7	54	54	51	52	52
Gross energy [MJ/kg DM]	22.3	21.7	22.1	24.7	24.4	24.7	24.6	24.7

[a]REF = reference diet; R05 and R10 = diets in which 5% and 10% of fishmeal of REF were replaced by rapeseed cake (R) on the basis of digestible crude protein.
[b]REF = reference diet; R25; R50; S25; and S50 = diets in which 25% and 50% of fishmeal of REF were replaced by either partially dehulled rapeseed cake (R) or partially dehulled sunflower seed cake (S) on the basis of digestible crude protein.
[c]Provided by Emsland Aller Aqua GmbH, Germany.
[d]Protein digestibility assumed to be 97% according to Gaylord and Barrows (2008).
[e]Provided by Marbacher Ölmühle GmbH, Germany.
[f]Provided by Teutoburger Ölmühle GmbH, Germany.
[g]Vit–min premix included (IU/kg or g/kg of premix): vitamin A, 0.8 MIU; vitamin D3, 0.5 MIU; vitamin E, 40 g; vitamin K3, 2 g; vitamin C, 30 g; biotin, 0.16 g; folic acid 1.6 g; vitamin B1, 3 g; vitamin B2, 4 g; vitamin B6, 3 g; vitamin B12, 0.01 g; nicotinamide, 30 g; inositol, 80 g; betaine, 84 g; ferrous sulphate, 30 g; copper sulphate, 0.6 g; manganese sulphate, 5 g; zinc sulphate, 12 g; calcium iodate 0.8 g; sodium selenite 0.04 g.

3 | RESULTS

3.1 | Digestibility trials

The digestibility diets were readily accepted by the fish. The ADCs of the experimental diets and those of the oilseed cakes for trials 1 and 3 are summarized in Table 5.

In Trial 1, no significant differences were detected in CL ADCs, either among the ingredients or among the experimental diets, owing to large standard deviations. However, the ADCs of CP differed significantly across diets and oilseed cakes. In comparison with the CP ADC of rapeseed cake (42%), the CP ADC of sunflower seed cake was significantly lower (25%). However, it should be noted that the faeces collected from the fish fed the sunflower seed cake diet were very watery, as opposed to the firm faecal matter collected from the fish that were fed the other digestibility diets.

In Trial 3, there were significant differences between the ADCs of the oilseed cakes. The dehulled rapeseed cake had the highest overall ADCs, with 87% for CP, 105% for CL and 72% for energy, whereas the sieved rapeseed cake had the lowest ADCs at 67%, 60% and 40%, respectively. The CL ADC of the sieved sunflower seed cake did not differ significantly from dehulled rapeseed cake (at 94%) and was significantly higher than the CL ADC of the dehulled sunflower seed cake (75%).

3.2 | Growth trials

When 0%, 5% and 10% of fishmeal protein in the reference diet were replaced with rapeseed cake protein (Trial 2), the weight gain of fish

TABLE 4 Experimental conditions of the four trials with rainbow trout

Variable	Trial 1	Trial 2	Trial 3	Trial 4
Type of trial	Digestibility	Growth	Digestibility	Growth
Initial average weight [g]	300	50.2	302	191
Final average weight [g]	–	153	–	401
Number of fish per tank	40	100	40	40
Stocking density [kg/m^3]	26.7	11.2	26.8	17.0
Number of tanks per diet	4	4	4	4
Type of tank	Fibreglass	Fibreglass	Fibreglass	Fibreglass
Water volume [L/tank]	450	450	450	450
Water source	Aerated spring	Aerated spring	Aerated spring	Aerated spring
Water flow rate [L/s]	0.32	0.32	0.31	0.31
Average water temperature [°C][a]	10.2	10.1	10.1	10.1
Average dissolved oxygen [mg/L][a]	8.79	8.64	8.57	8.58
Feeding level	Apparent satiation	Apparent satiation	Apparent satiation	Apparent satiation
Feeding frequency	Once daily	Once daily	Once daily	Once daily
Duration of trial (feeding days)	2 × 8[b]	63	2 × 8[b]	63
Photoperiod (light:dark)	12:12	12:12	12:12	12:12

[a]Recorded daily (Oxi 325, Xylem Analytics Germany Sales GmbH & Co. KG, WTW, Weilheim, Germany);
[b]Fish were fed the experimental diet for 8 days after which faeces were obtained by the stripping method; this procedure was repeated to obtain enough sample, and the faecal samples of each tank were pooled across the two periods.

was not significantly different and the average weight of all fish tripled from 50.2 ± 2.57 g to 153 ± 2.2 g over the course of the 63-day trial (Table 6). Furthermore, there were no significant differences among the treatments in terms of feed intake (FI; 79.4 ± 2.28 g DM) and feed conversion ratios (FCR; 0.78 ± 0.01 g).

An overview of the performance traits obtained from the growth trial, wherein 0%, 25% and 50% of the fishmeal protein in a reference diet were replaced by the protein of either partially dehulled rapeseed cake (R25 and R50) or partially dehulled sunflower seed cake (S25 and S50) (Trial 4), is presented in Table 7. Performance traits of the fish fed the S25 diet were not significantly different from the fish that received the reference diet. However, the fish fed the other oilseed cake-containing diets had a significantly lower performance than the fish that received the reference diet. The fish fed the R50 diet had the lowest overall performance.

4 | DISCUSSION

4.1 | Methodology

Faecal stripping is regarded as a conservative method that generally achieves lower ADCs in rainbow trout than do the other faecal collection methods (Vandenberg & de la Noüe, 2001; Weatherup & McCracken, 1998). While stripping is not the only factor that could have contributed to the low ADCs obtained in the present study, it might have had a pronounced effect on the ADCs of the sunflower seed cake. Using the subjective scale for rating faecal integrity, suggested by Glencross et al. (2005), we assigned the faeces obtained from fish fed sunflower seed cake a score of 2 of 5 (1: liquid; 5: firm and dryish). The watery integrity of the faeces suggests that more than just the distal part of the intestine was emptied during faecal collection. This could have resulted in the collection of substantial amounts of undigested feed, leading to low ADCs. This represents a limitation of the faecal stripping procedure and questions the validity of the reported ADCs for unprocessed sunflower seed cake. However, this would not explain the relatively low ADCs of unprocessed rapeseed cake, as the feed resulted in firm faecal material.

4.2 | Effects of processing on the nutritive value of oilseed cakes

With the exception of the CL ADC of sieved rapeseed cake, all fibre-reduced oilseed cakes had substantially higher CP, CL and energy ADCs compared with the ADCs of the respective unprocessed oilseed cakes. The increased energy ADCs of the fibre-reduced oilseed cakes can be explained by an increased content of digestible organic matter. Rainbow trout are unable to digest cellulose (Bergot & Breque, 1981), and there are no studies indicating that rainbow trout are able to digest other non-starch polysaccharides (NSPs) or lignin. The positive effect of fibre reduction on energy ADC was also observed in tilapia (*Oreochromis niloticus*) (Amirkolaie, Leenhouwers, Verreth, & Schrama, 2005; Maina et al., 2002) and Australian silver perch (*Bidyanus bidyanus*) (Booth, Allan, Frances, & Parkinson, 2001). An increase in the digestibility of organic matter with decreasing amounts of cellulose was also observed in rainbow trout; however, an inclusion of cellulose of up to 300 g/kg in rainbow trout diets did not affect the CP ADCs (Glencross, 2009; Hansen & Storebakken, 2007). The degree to which non-cellulosic NSPs influence the ADCs of nutrients in rainbow trout depends much

TABLE 5 Apparent digestibility coefficients (ADCs) for crude protein (CP), crude lipid (CL) and energy of the experimental diets and the oilseed cakes in the digestibility trials (trials 1 and 3)

	ADCs		
	CP [%]	CL [%]	Energy [%]
Trial 1			
Diets			
Reference diet	88.4 ± 0.65^{a}	83.8 ± 4.13	82.7 ± 2.00^{a}
Rapeseed cake diet	81.3 ± 0.81^{b}	84.8 ± 4.72	70.6 ± 0.80^{c}
Sunflower seed cake diet	79.9 ± 0.76^{c}	79.4 ± 4.76	68.5 ± 1.81^{c}
Oilseed cakes			
Rapeseed cake	42.3 ± 5.26^{a}	87.6 ± 18.6	30.9 ± 3.44
Sunflower seed cake	25.0 ± 5.64^{b}	62.4 ± 22.9	24.2 ± 7.47
Trial 3			
Diets			
Reference diet	90.1 ± 0.14^{a}	86.7 ± 1.64^{bc}	86.0 ± 0.66^{a}
Rapeseed cake (partially dehulled) diet	89.4 ± 0.49^{ab}	90.4 ± 1.45^{a}	82.7 ± 0.92^{b}
Rapeseed cake (sieved) diet	86.0 ± 0.91^{d}	79.7 ± 1.64^{d}	74.5 ± 1.22^{d}
Sunflower seed cake (partially dehulled) diet	88.5 ± 0.53^{bc}	84.3 ± 0.53^{c}	77.2 ± 0.29^{c}
Sunflower seed cake (sieved) diet	87.6 ± 0.38^{c}	88.1 ± 1.56^{ab}	77.3 ± 0.79^{c}
Oilseed cakes			
Rapeseed cake (partially dehulled)	86.6 ± 2.64^{a}	104.8 ± 7.10^{a}	72.3 ± 3.81^{a}
Rapeseed cake (sieved)	67.1 ± 5.09^{c}	59.6 ± 6.41^{c}	39.7 ± 4.92^{c}
Sunflower seed cake (partially dehulled)	81.0 ± 3.13^{ab}	74.8 ± 2.66^{b}	49.2 ± 1.23^{b}
Sunflower seed cake (sieved)	74.6 ± 2.36^{b}	93.7 ± 7.86^{a}	49.9 ± 3.30^{b}

Values are reported as the mean of four replicates (n = 4) with their respective standard deviation.
Different superscripts within columns of the respective trial indicate significant differences (p < .05), between either the diets or the ingredients.

on the NSPs' origin, their ability to disperse in water, and their inclusion level (Glencross, 2009; Glencross, Boujard, & Kaushik, 2003; Glencross, Rutherford, & Bourne, 2012; Storebakken & Austreng, 1987).

For evaluating the effects of fibre reduction on CP ADCs in oilseed cakes, the fibre-reduced oilseed cakes were compared with their unprocessed counterparts, considering that the unprocessed oilseed cakes were used to produce the sieved oilseed cakes. We assume that the low CP ADCs of unprocessed oilseed cakes and the watery integrity of the faeces collected from fish fed the unprocessed sunflower seed cake resulted from its high NSP content. NSPs have shown to act as ANFs, as they negatively affect the CP ADCs (Glencross, 2009; Storebakken, 1985) and influence the digesta viscosity in fish (Sinha, Kumar, Makkar, De Boeck, & Becker, 2011), as fish lack the respective digestive enzymes to hydrolyse the β-glycosidic bonds of NSPs (Rust, 2002). There were no noticeable differences in the faecal integrity among all collected faecal samples from the fish fed either of the fibre-reduced oilseed cakes, and no watery faecal samples were observed. Therefore, it can be assumed that the fibre reduction reduced the NSP content of sunflower seed cake to a level that did not noticeably influence the faecal integrity at a dietary concentration of 25%. This would have had the most dominant positive effect on their CP ADCs, as an undefinable collection of undigested faecal samples during the stripping procedure could be avoided.

An increase in the CP ADCs as a result of fibre reduction in plant-derived feed ingredients has been previously observed for rainbow trout (Glencross et al., 2007) and Australian silver perch (Booth et al., 2001). However, the extent to which CP ADCs are influenced by NSPs strongly depends on the specific NSPs and their dietary concentration (Glencross, 2009; Storebakken & Austreng, 1987). When the rapeseed cake and the sieved rapeseed cake are compared in terms of their CP ADCs (42% and 67%, respectively), it becomes apparent that reducing the CF content of rapeseed cake had a clearly positive effect on its CP ADC, despite an increase in other ANFs.

The comparison of rapeseed cake and sieved rapeseed cake in terms of fibre reduction and ANFs illustrates the inevitable increase in ANFs that are more concentrated in the seeds as opposed to the hulls. This explains the elevated levels of phytate, glucosinolates and tannins in the sieved rapeseed cake, as these compounds are the least concentrated in rapeseed hulls (Kracht et al., 2004; Liu, Wu, Pu, Li, & Hu, 2012; Matthäus, 1998). However, when the ADCs of the two fibre-reduced rapeseed cakes are compared with each other, it becomes apparent that the comparably low ADCs of the sieved cake cannot be attributed exclusively to NSPs, as, despite its lower CF content, it had significantly lower ADCs than the oilseed cake of partially dehulled rapeseeds. It is likely that, apart from a potential individual variation in NSP composition found in rapeseed cakes (Bach Knudsen,

1997), other rapeseed ANFs negatively influenced the ADCs of the sieved rapeseed cake, compared with the oilseed cake of partially dehulled rapeseeds. However, it should be noted that the different ANF concentrations of the two fibre-reduced oilseed cakes in the present study are likely the result of different batches of rapeseed used for their production and not the result of differences in the fibre reduction processes.

It is unclear why, despite its higher CP ADC, the CL ADC of sieved rapeseed cake was lower than the CL ADC of its unprocessed counterpart. Interpreting the CL ADCs of the various oilseed cakes used in the present study is difficult, due to the high standard deviations of the CL ADCs of the unprocessed oilseed cakes. Furthermore, the diet substitution method can yield ADCs > 100% (Gaylord & Barrows, 2008; Glencross et al., 2007). Although it might seem that these values lack biological plausibility, they were not corrected. They potentially indicate an interaction between the ingredients of the respective diets.

4.3 | Effects of fishmeal replacement by oilseed cakes on performance traits

In spite of the relatively low nutrient ADCs found for rapeseed cake, Trial 2 demonstrated that a partial substitution of up to 10% of fishmeal protein by rapeseed cake protein on the basis of CP ADCs is possible without exerting a negative influence on the performance traits of rainbow trout (Table 6). Furthermore, rapeseed cake did not significantly affect the feed intake at a dietary inclusion of up to 10%. This shows that despite a wide array of ANFs present in untreated rapeseed cake, it can serve as a readily available feed ingredient for rainbow trout, when included at a moderate level.

In Trial 4, wherein 0%, 25% and 50% of fishmeal protein in the reference diet were replaced by the protein of either partially dehulled rapeseed cake or partially dehulled sunflower seed cake, an increase in the FCR of the fish fed the oilseed cake diets was expected, given the higher content of indigestible nutrients in these diets. Therefore, it was peculiar that the FCR of the fish fed the sunflower seed cake diets was less negatively affected, despite the fact that the sunflower seed cake diets had a higher CF content for both levels of fishmeal replacement. In part, this could be the result of varying NSP compositions between the oilseed cakes of rapeseed and sunflower seed. Sunflower seed cake has a higher cellulose fraction, relative to other NSPs, than rapeseed cake (Bach Knudsen, 1997). Cellulose is "merely" indigestible, while other NSPs can negatively influence feed digestibility. Furthermore, rapeseed glucosinolates have been shown to negatively affect feed utilization in rainbow trout (Burel et al., 2001). Glucosinolates could be partly responsible for the increased FCR observed in the present study for the fish fed the rapeseed cake diets, as Burel et al. (2001) observed a decreased feed efficiency ratio at a glucosinolate concentration as low as the theoretical glucosinolate concentration of the R25 diet.

A decrease in feed intake of rainbow trout has been observed in studies investigating plant protein sources as potential fishmeal substitutes at high or total fishmeal replacement levels (Gomes, Rema, & Kaushik, 1995; Stickney et al., 1996). This can be caused by a lack of dietary essential amino acids (Rodehutscord, Becker, Pack, & Pfeffer, 1997; Rodehutscord, Jacobs, Pack, & Pfeffer, 1995), as their

TABLE 6 Performance traits of fish obtained in Trial 2 (growth trial with rapeseed cake) over the duration of 63 days

Diet[a]	Initial weight [g]	Final weight [g]	WG [g]	FI [g DM]	FCR [FI/WG]
REF	50.1 ± 0.22	154 ± 1.6	103 ± 1.8	81.7 ± 1.44	0.79 ± 0.01
RS_5	50.3 ± 0.23	153 ± 1.8	103 ± 2.0	78.7 ± 1.33	0.77 ± 0.01
RS_10	50.3 ± 0.12	152 ± 3.2	101 ± 3.2	77.8 ± 2.47	0.77 ± 0.00

[a]REF = reference diet; RS_5 and RS_10 = diets where 5% and 10% of fishmeal of REF were replaced by rapeseed cake on the basis of digestible protein.
WG, weight gain; FI, feed intake (dry matter); FCR, feed conversion ratio.
Values are reported as the mean of four replicates (n = 4) with their respective standard deviation.

TABLE 7 Performance traits of fish obtained in Trial 4 (growth trial with partially dehulled oilseed cakes) over the duration of 63 days

Diet[a]	Initial weight [g]	Final weight [g]	WG [g]	FI [g DM]	FCR [FI/WG]
REF	192 ± 1.0^{a}	424 ± 4.1^{a}	231 ± 4.7^{a}	199 ± 2.7^{a}	0.86 ± 0.01^{c}
R25	191 ± 0.9^{a}	386 ± 9.2^{bc}	196 ± 8.9^{bc}	180 ± 6.8^{bc}	0.92 ± 0.01^{ab}
R50	191 ± 1.2^{a}	363 ± 13.5^{c}	172 ± 13.4^{c}	162 ± 9.5^{c}	0.94 ± 0.03^{a}
S25	191 ± 1.2^{a}	406 ± 18.0^{ab}	215 ± 17.1^{ab}	190 ± 12.5^{ab}	0.88 ± 0.01^{bc}
S50	191 ± 0.6^{a}	390 ± 9.9^{b}	199 ± 10.4^{b}	181 ± 6.6^{b}	0.91 ± 0.03^{ab}

[a]REF = reference diet; R25; R50; S25 and S50 = diets where 25% and 50% of fishmeal of REF were replaced by either dehulled rapeseed cake (R) or dehulled sunflower seed cake (S) on the basis of digestible crude protein (R25; R50; S25 and S50, respectively).
WG, weight gain; FI, feed intake (dry matter); FCR, feed conversion ratio.
Values are reported as the mean of four replicates (n = 4) with their respective standard deviation.
Different superscripts within columns indicate significant differences (p < .05).

concentrations in proteins of plant origin are often lower than in fishmeal (NRC, 2011). Therefore, the amino acid concentrations for lysine, methionine, histidine, valine, leucine and isoleucine of the growth trial diets were estimated (oilseed cakes: Table S1, other feed ingredients: NRC 2011). However, their theoretical concentrations were all found to be higher than needed to achieve a 95% feed intake in rainbow trout (Rodehutscord et al., 1995, 1997). Therefore, a lack of any of the aforementioned amino acids is unlikely to have caused the decrease in feed intake observed for the fish fed the oilseed cake diets, in the growth trial of the present study. However, the extrusion process may have reduced the bioavailability of amino acids in rainbow trout, through the formation of biologically unavailable amino acid–carbohydrate complexes as a result of the Maillard reaction (Plakas, Lee, & Wolke, 1988).

A factor that could account for the significantly affected feed intake in Trial 4 is feed processing (i.e., extrusion time and temperature), as different extrusion parameters have been shown to influence feed intake in rainbow trout (Barrows, Stone, & Hardy, 2007). While, according to the manufacturer, the growth performance diets were all produced under the same conditions, they were most likely produced under different conditions than the diets used for the digestibility trial (Trial 3). In the present study, the dietary concentration of glucosinolates in the diets containing rapeseed cake of partially dehulled rapeseeds could have been affected by different feed processing parameters. A glucosinolate concentration corresponding to the R50 diet in the present study has been shown to negatively affect the feed intake in rainbow trout (Burel et al., 2000). Furthermore, Collins et al. (2013) suggested that glucosinolates negatively influence performance mainly by reducing the feed intake. Although glucosinolates and their hydrolysing enzyme, myrosinase, are readily destroyed by heat, the extent to which they are destroyed strongly depends on their exposure time to the respective heat treatment (Kozlowska, Nowak, & Nowak, 1983). A shorter extrusion time and/or lower extrusion temperatures of the growth performance diets containing rapeseed cake of partially dehulled rapeseeds, when compared with the respective digestibility trial diets, could therefore have resulted in a higher remaining concentration of both glucosinolates and myrosinase. This could explain a negative effect on feed intake in the growth performance diets R25 and R50, as a decreased feed intake was not observed for any of the rapeseed cake-containing digestibility diets, despite a higher rapeseed cake content compared with the rapeseed cake-containing diets used in the growth trials (unpubl. observation). This could further explain the more pronounced negative effect of the rapeseed cake diets on feed intake, compared with the sunflower seed cake diets, observed in Trial 4.

5 | CONCLUSION

Fishmeal is often regarded as a "gold standard," against which potential alternative ingredients are commonly compared based on various criteria. In the present study, only the replacement of 25% fishmeal protein by sunflower seed cake protein of partially dehulled sunflower seeds (basis: CP ADCs) resulted in a similar performance compared with the fish fed the reference diet. Neither the two growth performance diets containing rapeseed cake of partially dehulled rapeseeds, nor the diet with 50% fishmeal protein substitution by sunflower seed cake of partially dehulled sunflower seeds, resulted in a statistically similar performance when compared with the reference diet. From this point of view, dehulled oilseed cakes of rapeseed and sunflower seed do not seem to be adequate substitutes, as their inclusion results in a higher overall feed input and the requirement of longer production cycles per unit of produced rainbow trout biomass.

However, when fishmeal is considered a finite resource, which is bound to become scarcer over the coming years, the dehulled oilseed cakes of rapeseed and sunflower seed have proven to be viable alternatives. With either of the growth trial diets of the present study, irrespective of the fishmeal replacement level or the type of oilseed cake, it was possible to produce healthy rainbow trout using less fishmeal per unit weight gain, compared with the respective reference diets. Based on the obtained results, the above observation not only holds true for the duration of the observed trial, but also when a prolonged production period coupled with an increased feed input is considered, for reaching the same final weight as the fish fed the respective reference diets.

The explicit reason for the significantly reduced feed intake of the oilseed cake diets remains unclear. It could be the result of a reduced palatability, due to various ANFs. It could also be the result of a decreased retention time of the chyme, due to the faecal bulking properties of the fibre fractions of the oilseed cakes, or a reflection of the lower weights of the respective fish due to a reduced growth. However, this could not be inferred with certainty using this experimental set-up and would require further investigation.

ACKNOWLEDGEMENTS

The project was supported by funds of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme. We would like to thank Bernhard Baier, Oxana Tyapkova and Christian Zacherl from the Fraunhofer Institute for Process engineering and Packaging IVV (Freising, Germany) and Hanno Slawski from Aller Aqua (Christiansfeld, Denmark) for the preparation of the experimental feeds and for constructive discussions regarding fish feed formulations and production.

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SUPPORTING INFORMATION

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How to cite this article: Greiling AM, Reiter R, Rodehutscord M. Utilization of unprocessed and fibre-reduced oilseed cakes of rapeseed and sunflower seed in rainbow trout (*Oncorhynchus mykiss* W.) nutrition—Evaluation of apparent digestibility and growth performance. *Aquacult Nutr*. 2018;24:1133–1143. https://doi.org/10.1111/anu.12652

3.2 Manuscript 2

Pumpkin seed cake as a fishmeal substitute in fish nutrition: effects on growth performance, morphological traits and fillet colour of two freshwater salmonids and two catfish species

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Published in *Archives of Animal Nutrition* (2018), 72 (3):239-259.
DOI: https://doi.org/10.1080/1745039X.2018.1464713

Abstract:

The objectives of this study were to investigate the digestibility of pumpkin seed cake (PSC) for the rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), and effects on performance and product quality traits of four different fish species when PSC partially replaced fishmeal in extruded diets. A digestibility trial was carried out to determine apparent digestibility coefficients (ADC) for crude protein (CP), ether extract (EE) and gross energy (GE) of PSC fed to rainbow trout. In subsequent growth trials, effects on performance and morphological traits and fillet colour values of four different fish species [rainbow trout; brook trout, *Salvelinus fontinalis* (Mitchill, 1814); African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822); and wels catfish, *Silurus glanis* (Linnaeus, 1758)] were evaluated when 60% of fishmeal protein of a reference diet was replaced by PSC protein (based on digestible CP). Nutrient ADC of PSC were high (CP: 89%, EE: 88% and GE: 84%). No significant effects on growth and only minor effects on fillet colour were detected in the trials. However, replacing fishmeal with PSC at the chosen level affected morphological traits and feed conversion in all four species to different extents. Replacement effects of PSC should be tested at lower levels of inclusion before conclusions are drawn on its suitability in fish diets.

Keywords:

African sharptooth catfish, brook trout, oilseed cakes, performance, pumpkinseed, rainbow trout, wels catfish

3.3 Manuscript 3

Effects of phosphate and phytase supplementation on phytate degradation in rainbow trout (*Oncorhynchus mykiss* W.) and Atlantic salmon (*Salmo salar* L.)

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Published in *Aquaculture* (2019), 503:467-474

DOI: https://doi.org/10.1016/j.aquaculture.2019.01.035

Abstract:

The objectives of the present study were to investigate the single and interactive effects of mineral phosphorus (P) (monoammonium phosphate; MAP; 1 g P/kg DM of diet) and *Aspergillus oryzae* 6-phytase (2800 FTU/kg DM diet) supplements on the disappearance of phytic acid ($InsP_6$) and its lower inositol phosphate isomers (InsPs) and related P digestibility effects in Atlantic salmon (*S. salar*) and rainbow trout (*O. mykiss*). For each species, one experiment was conducted applying species-specific conditions but using the same diets in both experiments. Faeces were obtained by stripping the fish.

The faecal $InsP_6$ disappearance was similar in Atlantic salmon (8.6%) and rainbow trout (8.1%) when diets did not contain the MAP or phytase supplement. Phytase supplementation significantly increased $InsP_6$ disappearance in both species, but to a larger extent in rainbow trout. The hydrolysis of lower inositol phosphate isomers progressed to a greater extent in rainbow trout. Supplementation of MAP had no significant effect on $InsP_6$ disappearance in rainbow trout but slightly decreased $InsP_6$ disappearance in Atlantic salmon. No significant interactive effect of the supplements on $InsP_6$ disappearance was detected in both experiments. The analysis of InsPs in the faeces of the two species suggested that the degradation pathway of $InsP_6$ differed between the two species. In both species, each supplement increased the amount of digested P. This effect was additive in rainbow trout but not in Atlantic salmon. In Atlantic salmon, the MAP supplementation decreased the efficacy of phytase by reducing the amount of digested InsP-P to negligible amounts. However, no significant interactive effect of MAP and phytase supplementation on P digestibility, digested P, and digested InsP-P was detected in either species.

The present study provided insight into details of $InsP_6$ degradation in the digestive tract and revealed similarities and differences between the two fish species. The effects of phytase in Atlantic salmon when using low-P diets may not be the same for commercial diets that contain more inorganic P.

Keywords:

Atlantic salmon, rainbow trout, phytate degradation, inositol phosphate, phosphorus, phytase

Aquaculture 503 (2019) 467–474

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Effects of phosphate and phytase supplementation on phytate degradation in rainbow trout (*Oncorhynchus mykiss* W.) and Atlantic salmon (*Salmo salar* L.)

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ARTICLE INFO

Keywords:
Atlantic salmon
Rainbow trout
Phytate degradation
Inositol phosphate
Phosphorus
Phytase

ABSTRACT

The objectives of the present study were to investigate the single and interactive effects of mineral phosphorus (P) (monoammonium phosphate; MAP; 1 g P/kg DM of diet) and *Aspergillus oryzae* 6-phytase (2800 FTU/kg DM diet) supplements on the disappearance of phytic acid ($InsP_6$) and its lower inositol phosphate isomers (InsPs) and related P digestibility effects in Atlantic salmon (*S. salar*) and rainbow trout (*O. mykiss*). For each species, one experiment was conducted applying species-specific conditions but using the same diets in both experiments. Faeces were obtained by stripping the fish. The faecal $InsP_6$ disappearance was similar in Atlantic salmon (8.6%) and rainbow trout (8.1%) when diets did not contain the MAP or phytase supplement. Phytase supplementation significantly increased $InsP_6$ disappearance in both species, but to a larger extent in rainbow trout. The hydrolysis of lower inositol phosphate isomers progressed to a greater extent in rainbow trout. Supplementation of MAP had no significant effect on $InsP_6$ disappearance in rainbow trout but slightly decreased $InsP_6$ disappearance in Atlantic salmon. No significant interactive effect of the supplements on $InsP_6$ disappearance was detected in both experiments. The analysis of InsPs in the faeces of the two species suggested that the degradation pathway of $InsP_6$ differed between the two species. In both species, each supplement increased the amount of digested P. This effect was additive in rainbow trout but not in Atlantic salmon. In Atlantic salmon, the MAP supplementation decreased the efficacy of phytase by reducing the amount of digested InsP-P to negligible amounts. However, no significant interactive effect of MAP and phytase supplementation on P digestibility, digested P, and digested InsP-P was detected in either species. The present study provided insight into details of $InsP_6$ degradation in the digestive tract and revealed similarities and differences between the two fish species. The effects of phytase in Atlantic salmon when using low-P diets may not be the same for commercial diets that contain more inorganic P.

1. Introduction

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate; $InsP_6$) and its salts (phytate) are the primary storage form of phosphorus (P) in plant seeds and feed raw materials originating from plant seeds (Eeckhout and De Paepe, 1994; Rodehutscord et al., 2016). When plant proteins are used in compound fish feed instead of fishmeal, they carry substantial amounts of $InsP_6$, particularly oil seed meals or press cakes. Utilisation of $InsP_6$-bound P ($InsP_6$-P) by fishes depends on $InsP_6$ hydrolysis in the digestive tract because P absorption is assumed to occur mainly as orthophosphate (Lall, 2002). $InsP_6$-hydrolysing enzymes, such as phytases (myo-inositol hexakisphosphate phosphohydrolases) and other phosphatases, catalyse the hydrolytic cleavage of $InsP_6$ and its salts via several phosphorylated intermediary products to myo-inositol (Greiner and Konietzny, 2010). Owing to the (presumed) lack of these enzymes in the fish's digestive tract, $InsP_6$-P has limited availability. Hua and Bureau (2010) concluded that cyprinids and salmonids are unable to digest $InsP_6$-P, whereas tilapias appeared to be able to digest it to a certain extent. The same authors had previously shown that the digestibility of various P sources is different in rainbow trout (Hua and Bureau, 2006). Furthermore, there is evidence that the digestibility of P differs between rainbow trout and Atlantic salmon when they are provided with the same diet, and even when they are reared in similar environments (Glencross et al., 2004;

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https://doi.org/10.1016/j.aquaculture.2019.01.035
Received 2 December 2018; Received in revised form 12 January 2019; Accepted 15 January 2019
Available online 17 January 2019
0044-8486/

Refstie et al., 2000). While the underlying reasons for these observations can be manifold, they have not been investigated in detail.

The P digestibility from plant-based diets improved when the feed was supplemented with phytases of microbial origin in rainbow trout (*Oncorhynchus mykiss* W.) (Rodehutscord and Pfeffer, 1995) and Atlantic salmon (*Salmo salar* L.) (Sajjadi and Carter, 2004). This implies that microbial phytases are active under the physiological conditions of the digestive tract (pH value, temperature, etc.) and can partially hydrolyse $InsP_6$. Several in vitro studies have shown that the position of initial dephosphorylation on the inositol ring is specific to origin of the phytases. Accordingly, they were classified as 3-phytases (E.C.3.1.3.8), 4-/6-phytases (E.C.3.1.3.26), and 5-phytases (E.C.3.1.3.72) (IUPAC-IUB Commission on Biochemical Nomenclature (CBN), 1976). Depending on the type of phytase, the appearance of different positional isomers of pentakis-, tetrakis-, and trisphosphate was also different. Recent studies with broiler chickens showed that these differences, originally detected using in vitro assays, also exist in the digestive tract (Zeller et al., 2015). However, it was also shown that disappearance of $InsP_6$ in the digestive tract does not mean the degradation process is complete, as lower inositol phosphates (InsPs) remain undegraded. Studies with broiler chickens also showed that gastrointestinal $InsP_6$ hydrolysis is reduced when the diet contains a P supplement, indicating interactions between the phosphatases and phosphate in the gut (Shastak et al., 2014; Sommerfeld et al., 2018). In line with this observation, Sajjadi and Carter (2004) found in Atlantic salmon that a phytase supplement significantly increased P digestibility when the diet did not contain a P supplement, but had no effect when P was also supplemented. Whether this was owing to interactive effects in regard to $InsP_6$ hydrolysis or an excess in P intake of the fish was unclear.

$InsP_6$ can form $InsP_6$-protein complexes with incomplete solubility (Cheryan and Rackis, 1980). If a phytase supplement could reduce the formation of these complexes, this would potentially increase protein digestibility. The extent to which phytase supplementation to the diet affects protein digestibility has been studied in various fish species yielding ambiguous results depending on dosage and specific phytase supplement used (Cao et al., 2007).

To the best of our knowledge, the breakdown of $InsP_6$ and appearance of specific lower inositol phosphates in the digestive tract and their dependence on mineral P supplements have not been investigated previously in fishes. Therefore, the objectives of this research were to:

- study the capacity of rainbow trout and Atlantic salmon to hydrolyse $InsP_6$ in the digestive tract with and without supplemented phytase;
- study the effect of supplemented mineral P on $InsP_6$ hydrolysis with and without a phytase supplement;
- describe the primarily formed lower InsPs;
- compare rainbow trout and Atlantic salmon in their specific environment using identical diets.

Our hypotheses were that, similarly to broiler chickens, there are interactions between phytase and phosphate supplements on $InsP_6$ disappearance and that $InsP_6$ degradation is different between rainbow trout and Atlantic salmon. Furthermore, the Atlantic salmon trial also investigated effects on amino acid digestibility.

2. Materials and methods

2.1. Experimental diets

Two fishmeal-free and $InsP_6$-rich experimental diets were prepared. One diet did not contain a P supplement (basal diet), whereas the other was supplemented with 3.89 g monoammonium phosphate (MAP)/kg of diet (Table 1). The dry ingredients were mixed and conditioned (86 °C) prior to extrusion (107 °C and 20–25 bar at die, 11 min). Both diets were divided into two equal parts of which one part of each diet was top-coated with *Aspergillus oryzae* 6-phytase (Ronozyme®P (L), DSM Nutritional Products, Basel, Switzerland) at an intended activity of 2800 phytase units (FTU)/kg of diet). The diets contained an yttrium oxide containing premix as an inert marker for the digestibility determination at a concentration of 1 g/kg of diet. The pellets (4 mm diameter) were refrigerated and stored at 4 °C until the start of the respective experiments.

Table 1
Ingredients and chemical composition of diets used in experiments with rainbow trout and Atlantic salmon.

	MAP-	MAP-	MAP +	MAP +
	Phy-	Phy +	Phy-	Phy +
Ingredients [g/kg DM]				
Soy protein concentrate	300	300	300	300
Wheat gluten	200	200	200	200
Faba beans (whole)	117	112	117	112
Corn gluten	100	100	100	100
Rapeseed oil	85.4	85.4	85.4	85.4
Fish oil	85.4	85.4	85.4	85.4
Sunflower meal	50	50	50	50
Rapeseed meal	50	50	50	50
MAP	–	–	3.89	3.89
Vitamin, mineral and other premixes	11.4	11.8	11.4	11.8
Yttrium oxide 10% premix	1	1	1	1
Analysed chemical composition [g/kg DM]				
Dry matter	927	924	930	927
Crude protein	512	516	515	529
Crude lipid	222	216	223	209
Crude ash	55	55	52	52
Total P	6.07	5.96	6.93	7.10
Phytase activity [FTU/kg]	–	2767	–	2810
Analysed inositol phosphate isomers [µmol/g DM][a]				
$InsP_6$	16.6	17.1	16.8	16.7
$Ins(1,2,3,4,5)P_5$	0.98	1.02	0.98	1.02
$Ins(1,2,3,4,6)P_5$	0.49	0.38	0.38	0.45
$Ins(1,2,4,5,6)P_5$	1.72	1.67	1.71	1.67
$Ins(1,2,5,6)P_4$	0.12	0.12	0.12	0.12
Analysed amino acid composition [g/kg CP]				
Alanine	44.4	44.4	44.4	44.7
Arginine	56.9	56.4	56.2	56.4
Asx[b]	80.8	80.4	80.7	80.6
Cysteine	16.5	16.6	16.5	16.5
Glx[c]	244	244	245	245
Glycine	40.0	40.0	39.7	39.7
Histidine	25.8	25.6	26.1	26.0
Isoleucine	39.4	40.6	38.9	38.9
Leucine	84.7	85.2	85.0	85.1
Lysine	55.3	54.5	54.7	55.1
Methionine	19.1	18.9	18.7	18.7
Phenylalanine	51.8	50.2	50.7	50.2
Proline	77.0	79.4	80.4	79.9
Serine	52.4	51.8	52.6	52.5
Threonine	35.4	35.2	35.2	35.2
Tyrosine	34.2	33.2	33.4	33.4
Valine	41.7	43.3	41.8	42.0

MAP: monoammonium phosphate; Phy: phytase; − without supplementation; + with supplementation.
[a] If the isomer concentration of a sample was between the detection and quantification limits, the mean concentration is reported;
[b] Aspartic acid and asparagine together;
[c] Glutamic acid and glutamine together.

2.2. Experimental conditions

One experiment was conducted with rainbow trout and one with Atlantic salmon. In both experiments, three replicated tanks were used per diet. Experimental conditions were species-specific and summarized in Table 2.

The experiment with Atlantic salmon was carried out at Lerang

Table 2
Experimental conditions of experiments using Atlantic salmon and rainbow trout.

	Atlantic salmon	Rainbow trout
Fish		
No. of fish per tank	35[a]	25
Initial average weight [g][b]	234 ± 7	202 ± 5
Rearing conditions		
Type of tank	Fibreglass	Fibreglass
Water source	Sea water	Fresh water
Water volume [l/tank]	450	600
System type	Flow-through	Flow-through
Acclimation time [days]	21	–
Duration [feeding days]	15	33
Photoperiod (light:dark) [h]	24:0	24:0
Water characteristics		
Water flow rate [l/s]	0.11	0.58
Water temperature [°C][c]	12.0	15.4
Dissolved oxygen [mg/l][c]	8.4	7.6
Salinity [g/kg]	34	0
Feed		
Number of tanks per diet	3	3
Feeding level	Excess	Excess
Feeding frequency	Thrice daily	Twice daily
Feeding method	Belt feeder	By hand or belt feeder

[a] Faecal samples of 20 fish per tank were pooled.
[b] Data presented as means (n = 12 tanks, ± standard deviation).
[c] Measured twice a week.

Research Station (Skretting ARC, Forsand, Norway). The experiment with rainbow trout was carried out at Skretting Aquaculture Research Centre Mozzecane, Italy.

Prior to the start of the experiments, all tanks were bulk weighed. The day after the last feeding day the fish were sedated and euthanized with MS-222 (240 mg/l Triacine; Pharmaq Ltd., Hampshire, UK) and were then stripped for faeces collection in accordance with Method II described by Austreng (1978). The faecal samples from the fish in a tank (n = 20 Atlantic salmon, n = 25 rainbow trout) were pooled and after removing blood, yellowish faeces, urine and water they were mixed with a spatula. The pooled faeces samples were immediately stored at −20 °C until further processing.

Uneaten feed was not quantified during the experiments, and therefore feed related performance traits were not calculated.

2.3. Chemical analyses

Prior to analyses, all samples were freeze dried (Gamma 1–20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) and stored at −30 °C until analysis. Unless otherwise stated, all samples were analysed in duplicate.

Crude nutrient concentrations of feed were analysed according to the official methods (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA), 2007): dry matter (DM; method 3.1), crude ash (CA; method 8.1), crude protein (CP; method 4.1.1), crude lipid (CL; sample treated with HCl and extracted with petroleum ether, method 5.1.1b), and crude fibre (CF; method 6.1.1; Fibretherm, Fa. C. Gerhardt GmbH & Co. KG, Königswinter, Germany). The yttrium concentration in feed and faeces was measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Eurofins Environment Testing Norway AS, Moss, Norway). Phytase activity was determined following phytase extraction from feed samples using an acetate buffer solution, pH 5.5, and its incubation with sodium phytate for 60 min at 37 ± 3 °C and a pH of 5.5 ± 0.05 (Engelen et al., 1994). The optical density of the formed vanadomolybdophosphorus complex was then measured using photo spectrometry at a wavelength of 415 nm. Phytase activity is expressed in Phytase Units (FTU), where a unit is defined as the amount of phytase required to release 1 μmol of inorganic phosphate per minute as determined under the aforementioned assay conditions.

Defatted feed samples were used for the analyses of P and $InsP_6$. The P content of the faeces and defatted feed samples was analysed using the wet digestion method described by Boguhn et al. (2009) followed by subsequent measurement using an inductively coupled plasma optical emission spectrometry (ICP-OES, VISTA PRO, Varian, Palo Alto, Australia) at a wavelength of 213.618 nm according to Shastak et al. (2012). The concentration of $InsP_6$ and lower InsPs in faeces and defatted feed samples was determined according to Sommerfeld et al. (2018).

Amino acid analysis of feed and faecal samples was performed according to Rodehutscord et al. (2004). Cysteine and methionine were determined as cysteic acid and methionine sulphone following a sample oxidation step. As the amide residue in the side group of asparagine and glutamine is lost during acid hydrolysis and aspartic acid and glutamic acid are formed (Fontaine, 2003), these AA were determined together with aspartic acid and glutamic acid, respectively. The concentrations of histidine, phenylalanine, and tyrosine may be affected to some extent by the oxidation procedure (Mason et al., 1980).

2.4. Calculations and statistical analysis

The apparent digestibility coefficient (ADC) of P, the disappearance of $InsP_6$ and the ADCs of individual AAs was calculated according to the following equation (NRC, 2011):

$$\mathrm{ADC} = \left(1 - \left(\frac{\text{marker in diet}}{\text{marker in faeces}}\right) * \left(\frac{\text{nutrient in faeces}}{\text{nutrient in diet}}\right)\right) * 100$$

where, ADC: apparent digestibility coefficient in %; marker in diet and faeces: yttrium concentration in the diet and faeces in g/kg DM; nutrient in diet and faeces: nutrient concentration in the diet and faeces in g/kg DM.

Digested P was calculated as follows:

$$\text{Digested P} = \frac{(\text{P digestibility} * \text{P content in feed})}{100.}$$

where Digested P is in g/kg DM; P digestibility is in %; and P content in feed is in g/kg DM. Digested InsP-P was calculated accordingly.

The statistical analysis was performed using R (Version 3.3.2; R Foundation for Statistical Computing, Vienna, Austria) with an RStudio interface (Version 1.0.136, RStudio Inc., Boston, USA). Prior to analysis, the studentised residuals were tested for deviations from the normal distribution and the data were tested for homogeneity of variances. When assumptions for a parametric comparison of means were violated, data was transformed to meet the requirements. Mean comparison was performed using either a two-way ANOVA with MAP and phytase as factors (chosen model: $Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij}$) or ANOVA with MAP as a factor (chosen model: $Y_i = \mu + \alpha_i + \varepsilon_i$), where Y = response variable, μ = overall mean, α_i = effect of MAP addition, β_j = effect of phytase addition, $(\alpha\beta)_{ij}$ = possible interactions between MAP and phytase supplementation, and ε = residual error. Replicates (= tanks) were considered as random effects. Statistical analysis of InsP concentration was performed only if the InsP concentration was quantifiable in the majority of samples. Pairwise differences between the means were determined using Tukey's HSD test and were considered significant at $p < .05$. The concentration of individual InsPs that were detected (> LOD), but below the quantification limit (< LOQ), was considered as the mean concentration between the detection and quantification limit. They are presented accordingly.

Table 3
Disappearance of inositol-6-phosphate ($InsP_6$), digestibility of phosphorus (P), digested P, and digested inositol phosphate-P (InsP-P) in experiments using Atlantic salmon and rainbow trout.

Treatment		$InsP_6$ disappearance		P digestibility		Digested P		Digested InsP-P	
		[%]		[%]		[mg/kg DM]		[mg/kg DM]	
		Atlantic salmon	Rainbow trout[¶]	Atlantic salmon	rainbow trout	Atlantic salmon	rainbow trout	Atlantic salmon	Rainbow trout[¶]
MAP-	Phy-	8.61^{b}	8.14^{b}	16.8^{b}	33.2^{b}	898^{c}	1870^{d}	199^{ab}	250^{b}
MAP-	Phy +	32.1^{a}	81.7^{a}	24.3^{a}	69.9^{a}	1276^{bc}	3852^{b}	628^{a}	2379^{a}
MAP +	Phy-	4.39^{b}	15.4^{b}	24.2^{a}	41.8^{b}	1508^{ab}	2697^{c}	44^{b}	520^{b}
MAP +	Phy +	22.1^{a}	79.9^{a}	30.4^{a}	73.9^{a}	1908^{a}	4860^{a}	51^{b}	2383^{a}
Pooled SEM		3.61	4.97	2.20	3.06	131	178	160	209
P-values	MAP	0.024	0.283	0.003	0.020	< 0.001	< 0.001	0.012	0.241
	Phytase	< 0.001	< 0.001	0.002	< 0.001	0.003	< 0.001	0.0903	< 0.001
	Interaction	0.291	0.194	0.670	0.313	0.910	0.456	0.100	0.249

Data presented as treatment means ($n = 3$); MAP: monoammonium phosphate; Phy: phytase; − without supplementation; + with supplementation.
Different superscripts within column of respective species indicate significant differences between treatments ($p < .05$).
[¶] Data were subjected to sqrt-transformation prior to statistical analysis.

3. Results

3.1. $InsP_6$ disappearance

$InsP_6$ of the basal diet disappeared in the digestive process to a similar extent in Atlantic salmon and rainbow trout (8.6 and 8.1%, respectively; Table 3). Phytase supplementation significantly increased $InsP_6$ disappearance in both species ($p < .001$), but to a much greater extent in rainbow trout than Atlantic salmon. The MAP supplementation had no significant effect on $InsP_6$ disappearance in rainbow trout ($p = .283$), but slightly decreased $InsP_6$ disappearance in Atlantic salmon ($p = .024$). No significant interactive effect of MAP and phytase supplementation on $InsP_6$ disappearance was detected in both experiments.

3.2. P digestibility and digested P in Atlantic salmon

In Atlantic salmon, supplementation of either MAP or phytase increased P digestibility significantly from 17% to about 24% ($p = .003$ and $p = .002$, respectively, Table 3). The amount of digested P from the basal diet was 0.9 g/kg DM. The amount of digested P was significantly affected by phytase supplementation ($p = .003$) and increased by 0.4 g/kg DM diet, irrespective of MAP presence. The supplementation of MAP significantly increased digested P by 0.6 g/kg DM diet ($p < .001$), irrespective of phytase presence. The increase in the amount of digested P was almost exclusively caused by MAP supplementation, as the amount of digested InsP-P was reduced to negligible amounts in the presence of MAP ($p = .012$). No significant interactive effect of MAP and phytase supplementation on P digestibility, digested P, and digested InsP-P was detected in Atlantic salmon.

3.3. P digestibility and digested P in rainbow trout

In rainbow trout, the P digestibility was also significantly affected by both MAP and phytase supplementation ($p = .003$ and $p = .020$, respectively, Table 3), but not by their interaction. However, only phytase supplementation significantly increased P digestibility from 33% to 70%, whereas the increase of P digestibility to 42% owing to MAP supplementation was not significant. The amount of digested P from the basal diet was 1.9 g/kg DM. Phytase supplementation significantly increased the amount of digested P by about 2 g/kg DM diet ($p < .001$), irrespective of MAP presence. The MAP supplementation significantly increased the amount of digested P ($p < .001$) by 0.8–1 g/kg DM diet, without and with phytase supplementation, respectively. No significant interactive effect of MAP and phytase supplementation on P digestibility, digested P, and digested InsP-P was detected in rainbow trout.

3.4. Lower inositol phosphate isomers in Atlantic salmon

Phytase supplementation had a more pronounced effect on the concentration of InsP isomers in the faeces of Atlantic salmon than MAP supplementation (Table 4). Phytase significantly decreased the concentration of $InsP_6$ and increased the concentration of $Ins(1,2,3,4,5)P_5$.

Table 4
Concentrations of different inositol phosphate (InsP) isomers (μmol/g DM) in the faeces of Atlantic salmon.

Treatment		$InsP_{3x}$[¶]	$Ins(1,2,3,4)P_4$	$Ins(1,2,4,5)P_4$	$Ins(1,2,5,6)P_4$	$Ins(1,2,3,4,6)P_5$	$Ins(1,2,3,4,5)P_5$	$Ins(1,2,4,5,6)P_5$	$Ins(1,3,4,5,6)P_5$	$InsP_6$
MAP-	Phy-	0.4^{b}	0.3^{c}	n.d[#]	0.5^{b}	1.1	2.7^{b}	4.3	0.2[†]	37.4^{a}
MAP-	Phy +	1.5^{a}	1.2^{b}	0.9	0.7^{b}	1.1	4.9^{a}	4.3	0.2[†]	28.3^{b}
MAP +	Phy-	0.4^{b}	0.3^{c}	n.d.	0.5^{b}	1.3	2.7^{b}	4.2	0.2[†]	36.7^{a}
MAP +	Phy +	2.0^{a}	1.7^{a}	1.2	1.0^{a}	1.3	6.1^{a}	4.9	0.2[†]	30.5^{b}
Pooled SEM		0.165	0.139	0.145	0.071	0.133	0.402	0.283	–	1.545
P-values	MAP	0.081	0.023	0.084[‡]	0.029	0.115	0.052	0.277	–	0.521
	Phytase	< 0.001	< 0.001	–	< 0.001	0.733	< 0.001	0.172	–	< 0.001
	Interaction	0.052	0.019	–	0.029	1	0.062	0.104	–	0.216

Data presented as treatment means ($n = 3$); MAP: monoammonium phosphate; Phy: phytase; − without supplementation; + with supplementation.
Different superscripts within columns indicate significant differences ($p < .05$).
[¶] At least one of the following isomers: $Ins(1,2,6)P_3$, $Ins(1,4,5)P_3$, $Ins(2,4,5)P_3$;
[#] n.d. = not detected;
[†] If the isomer concentration of a sample was between the detection and quantification limits, the mean concentration is presented; as the majority of samples could not be quantified, they were excluded from statistical evaluation;
[‡] ANOVA computed for effect of MAP in Phy + Diets;

Table 5
Concentrations of different inositol phosphate (InsP) isomers (μmol/g DM) in the faeces of rainbow trout.

Treatment		Ins(1,2)P_2	$InsP_{3x}$[¶]	Ins(1,2,3,4)P_4	Ins(1,2,5,6)P_4	Ins(1,2,3,4,6)P_5	Ins(1,2,3,4,5)P_5	Ins(1,2,4,5,6)P_5	Ins(1,3,4,5,6)P_5	$InsP_6$
MAP-	Phy-	n.d[#]	0.4[†]	0.4[b]	0.7[ab]	1.3	2.6	5.5[a]	0.5[†]	54.9[a]
MAP-	Phy +	10.2	2.8	1.9[a]	0.5[b]	0.4[†]	2.2	1.7[b]	n.d.	10.8[b]
MAP +	Phy-	n.d.	0.4[†]	0.6[b]	0.8[a]	1.3	2.5	5.4[a]	0.6[†]	50.8[a]
MAP +	Phy +	9.3	3.2	2.1[a]	0.6[ab]	0.5[†]	2.5	2.0[b]	n.d.	12.1[b]
Pooled SEM		0.994	1.395	0.329	0.081	0.058	0.398	0.306	–	2.0
P-values	MAP	0.401[‡]	0.452[‡]	0.459	0.08	1[◆]	0.689	0.652	–	0.365
	Phytase	–	–	< 0.001	0.013	–	0.533	< 0.001	–	< 0.001
	Interaction	–	–	0.827	0.8	–	0.463	0.457	–	0.105

Data presented as treatment means ($n = 3$); MAP: monoammonium phosphate; Phy: phytase; − without supplementation; + with supplementation.
Different superscripts within columns indicate significant differences ($p < .05$).
[¶] At least one of the following isomers: Ins(1,2,6)P_3, Ins(1,4,5)P_3, Ins(2,4,5)P_3;
[#] n.d. = not detected.
[†] if the isomer concentration of a sample was between the detection and quantification limits, the mean concentration is presented; as the majority of samples could not be quantified, they were excluded from statistical evaluation.
[‡] ANOVA computed for effect of MAP in Phy + Diets.
[◆] ANOVA computed for effect of MAP in Phy- Diets.

Other $InsP_5$ were not significantly affected by phytase. No significant effect of MAP supplementation and no significant interactive effect of MAP and phytase supplementation was detected for the concentration of $InsP_5$ isomers. Regarding $InsP_4$ isomer concentrations, a significant effect was detected both for MAP and phytase supplementation and their interaction. Phytase supplementation increased the concentration of Ins(1,2,3,4)P_4 and Ins(1,2,5,6)P_4 in faeces. The Ins(1,2,4,5)P_4 isomer was detectable in faeces only in the presence of phytase. The concentration of $InsP_3$ isomers in Atlantic salmon faeces also was significantly increased by phytase supplementation, whereas no significant effect of MAP supplementation was detected.

3.5. Lower inositol phosphate isomers in rainbow trout

Phytase supplementation had a more pronounced effect on the concentration of InsP isomers in rainbow trout faeces than MAP supplementation (Table 5). Phytase significantly decreased $InsP_6$ and $InsP_5$ concentrations. Phytase increased $InsP_4$, $InsP_{3x}$, and $InsP_2$ concentrations. No significant effect of MAP supplementation and no significant interactive effect between MAP and phytase supplementation was detected for $InsP_6$ concentrations and lower InsP isomers in rainbow trout faeces. The effect of phytase and its interaction with MAP could not be statistically evaluated for all lower InsP isomers. However, the effect of phytase on these InsP isomers was still apparent, as some of them were only quantifiable in the presence of phytase.

3.6. Amino acid digestibility in Atlantic salmon

The ADC of individual amino acids was generally relatively high and ranged between 73% and 95% (Table 6). The ADCs of individual amino acids could not be statistically evaluated, as there was not enough sample from one tank left for amino acid analysis. However, despite marginally different individual ADCs, the ADC of the sum of all amino acids did not differ substantially between the treatments and ranged between 89% and 91%.

4. Discussion

One hypothesis was that there are interactions between phytase and phosphate supplements with the disappearance of $InsP_6$. Furthermore, we hypothesised that $InsP_6$ degradation is different between rainbow trout and Atlantic salmon. In the present study, no significant interaction effects between the two supplements were determined on the disappearance of $InsP_6$ in either species, but $InsP_6$ degradation differed between the two species. The effects of phytase and MAP supplementation on $InsP_6$ disappearance and its degradation will be discussed,

Table 6
Values of amino acid digestibility of the four diets fed to Atlantic salmon (%).

	MAP-	MAP-	MAP +	MAP +
	Phy-	Phy +	Phy-[a]	Phy +
Essential amino acids				
Arg	94 ± 0.1	94 ± 0.2	93 ± 0.1	94 ± 0.1
His	87 ± 0.2	86 ± 0.8	85 ± 0.1	85 ± 0.4
Ile	90 ± 0.3	90 ± 0.7	88 ± 0.2	89 ± 0.1
Leu	92 ± 0.2	91 ± 0.3	91 ± 0.1	91 ± 0.1
Meth	93 ± 0.2	92 ± 0.1	92 ± 0.2	92 ± 0.1
Phe	93 ± 0.1	92 ± 0.3	91 ± 0.1	92 ± 0.1
Lys	91 ± 0.1	90 ± 0.6	89 ± 0.0	90 ± 0.1
Thre	84 ± 0.4	82 ± 0.8	81 ± 0.1	82 ± 0.3
Val	90 ± 0.2	89 ± 0.7	88 ± 0.4	88 ± 0.2
Non-essential amino acids				
Ala	90 ± 0.1	89 ± 0.5	88 ± 0.2	89 ± 0.3
Asx[b]	80 ± 0.4	79 ± 1.3	76 ± 0.1	78 ± 0.4
Cys	78 ± 1.0	76 ± 0.3	73 ± 0.5	74 ± 0.8
Glx[c]	95 ± 0.2	94 ± 0.2	94 ± 0.1	94 ± 0.1
Gly	84 ± 0.4	83 ± 0.8	81 ± 0.1	82 ± 0.4
Pro	93 ± 0.3	92 ± 0.2	92 ± 0.0	92 ± 0.2
Ser	89 ± 0.2	88 ± 0.4	87 ± 0.1	88 ± 0.1
Tyr	92 ± 0.2	91 ± 0.5	90 ± 0.1	91 ± 0.2
Σ all amino acids	91 ± 0.2	90 ± 0.5	89 ± 0.1	89 ± 0.1

Data presented as treatment means ± standard deviation ($n = 3$); MAP: monoammonium phosphate; Phy: phytase; − without supplementation; + with supplementation;
[a] $n = 2$, data presented as treatment means ± mean absolute deviation;
[b] Aspartic acid and asparagine together;
[c] Glutamic acid and glutamine together.

after which its effect on P digestibility will be highlighted in the following sections.

4.1. $InsP_6$ disappearance and degradation

Upon phytase addition to the feed, the main phytase activity was observed in the stomach of fish (Dersjant-Li et al., 2015). A lower pH in the stomach of rainbow trout is a possible reason for the higher $InsP_6$ disappearance in rainbow trout than in Atlantic salmon in the presence of phytase. Freshwater fish have been shown to have a lower pH in the stomach than saltwater fish (Márquez et al., 2012). The pH in the stomach and intestine of rainbow trout is 2.5 and 8.5, respectively (Bucking and Wood, 2009), whereas a pH of 3.5–5.6 and around 8–8.5 has been determined in the stomach and intestine of Atlantic salmon reared in seawater, respectively (Austreng et al., 2000; Krogdahl et al., 2015; Nordrum et al., 2000). Most phytases exhibit a pH optimum

between 4 and 6 (Greiner and Konietzny, 2010). The phytase used in the present study has a pH optimum of about 4-4.5 (Lei et al., 2013). There are two aspects that could have favoured a higher dephosphorylation of $InsP_6$ and its formed lower InsP isomers in rainbow trout despite the pH in the stomach of Atlantic salmon being closer to the optimum phytase pH. For one, cation-phytate complexes are less strong under acidic conditions (Maenz, 2001). This is further supported by the higher concentration of lower InsPs found in faeces of rainbow trout than in the faeces of Atlantic salmon. In addition, some phytases have exhibited their second highest activity around a pH of 2.5, which is similar to the pH found in the stomach of rainbow trout (Cao et al., 2007; Morales et al., 2011).

As fish are ectotherms, the temperature of the rearing water is important to consider. Temperature-dependent activity of phytase might also be responsible for observed differences in $InsP_6$ disappearance between the two species. Morales et al. (2011) have reported temperature-dependent effects of phytase on in vitro $InsP_6$ de-phosphorylation simulating the gastric conditions of rainbow trout using a crude enzymatic extract of the rainbow trout stomach. It was found that a higher temperature (16 vs. 6 °C) resulted in an increased liberation of orthophosphate from $InsP_6$. In the present study, different rearing temperatures were used for Atlantic salmon (12 °C) and rainbow trout (15 °C). This could have favoured the $InsP_6$ disappearance in rainbow trout in the present study. However, this is a speculation, as to our knowledge there are no other detailed studies on temperature-dependent effects of phytase on $InsP_6$ dephosphorylation in any fish species reared at different temperatures in vivo.

Increased $InsP_6$ disappearance owing to phytase supplementation is consistent with previous findings in Atlantic salmon (Denstadli et al., 2006; Denstadli et al., 2007; Sajjadi and Carter, 2004) and rainbow trout (Forster et al., 1999). In comparison with other studies, the $InsP_6$ disappearance of the basal diet in the respective species is relatively low. For example, in diets devoid of supplemental phytase, apparent $InsP_6$ disappearance values between 5% (Forster et al., 1999) and 22% (Morales et al., 2016a) were reported for rainbow trout. In Atlantic salmon, an $InsP_6$ disappearance of 15% has been reported for the basal diet (Denstadli et al., 2006). However, as the phytase activity of the control diets were not quantified in the present study, any species-specific intrinsic phytase activity could not be quantified and is therefore difficult to account for. However, in light of the discussed aspects, making assumptions based on other findings would be too speculative. The effects of mineral P or phytase supplements on $InsP_6$ disappearance seem to be heavily dependent on a wide array of physiochemical parameters (species-specific stomach pH, rearing temperature, etc.), feed composition, and feed production parameters as reviewed by Cao et al. (2007), Kumar et al. (2012), Lemos and Tacon (2017), and Morales et al. (2016b). In addition, the phytase supplements that were used differed in their specific pH and temperature optima. The development of second generation phytases, i.e., phytases that are more active at lower pH ranges and more resistant to proteolysis (Dersjant-Li et al., 2015), further diminishes the direct comparability between studies when different phytase supplements are used.

To our knowledge, the present study is the first that directly compares $InsP_6$ degradation pathways between two fish species using the same diet in vivo. Although the same diets were used in both experiments, it seems that the main $InsP_6$ degradation products differed between the two fish species:

$$\text{Atlantic salmon: } InsP_6 -> Ins(1,2,3,4,5)P_5 -> Ins(1,2,3,4)P_4 -> InsP_{3x}$$

$$\text{Rainbow trout: } InsP_6 -> Ins(1,2,4,5,6)P_5 -> Ins(1,2,5,6)P_4 -> InsP_{3x}$$

In Atlantic salmon, the degradation followed a pattern that was expected from a 6-phytase, such as the one used in the present study. However, in rainbow trout, the observed main degradation products were similar to those that are characteristic for a 3-phytase. Phytases are generally categorized according to the stereospecificity of $InsP_6$ hydrolysis products (IUPAC-IUB Commission on Biochemical Nomenclature (CBN), 1976). However, this classification refers to the major, but not exclusive, position of the initial de-phosphorylation of $InsP_6$ in vitro. $InsP_6$ degradation pathways have been shown to differ depending on the pH (Greiner and Konietzny, 2010). Furthermore, it has been shown that 6-phytases can exhibit a low 3-phytase activity and the preferred initiation site seemed to be even less specific at pH values outside their respective optimum pH range (Pontoppidan et al., 2007). The more acidic conditions of the rainbow trout stomach could have therefore influenced the degradation pathway, as the pH optimum of the used enzyme is 4 to 4.5 (Lei et al., 2013). As the stomach pH was not quantified in the context of this study, it can only be speculated about its potential involvement in the investigated mechanisms.

4.2. P digestibility

Increased P digestibility owing to supplementation of inorganic P or phytase has been shown for both Atlantic salmon (Sajjadi and Carter, 2004) and rainbow trout (Cheng and Hardy, 2002; Forster et al., 1999), as well as in other fish species (Cao et al., 2007). The higher P digestibility in rainbow trout can be explained by the lower stomach pH than that of Atlantic salmon, as P of various sources has been shown to be rendered more soluble at lower stomach pH values (Sugiura et al., 2006; Vielma et al., 2001). This could explain the higher digestibility of P in rainbow trout compared with Atlantic salmon when they were offered the same diet (Glencross et al., 2004; Refstie et al., 2000).

The effect of either MAP or phytase supplementation on InsP-P digestibility differed in their extents between the two species in the present study. The aforementioned possibility of a more effective dephosphorylation in rainbow trout owing to its lower stomach pH is supported by the fact that a higher amount of digested InsP-P occurs in rainbow trout in the presence of phytase. The observation made in Atlantic salmon in the present study is in line with the observation made by Sajjadi and Carter (2004), where a phytase supplement did not increase P digestibility when inorganic P was also supplemented. However, whether this was the consequence of interactive effects on $InsP_6$ hydrolysis, or simply of an excessive P intake is unknown. This observation concurs with the mechanisms of an end-product inhibition of phytase owing to P release by $InsP_6$ hydrolysis as described by Shieh et al. (1969) and Liu et al. (1998). The decreased $InsP_6$ disappearance owing to MAP supplementation observed in the present study also concur with an end product inhibition.

The present study revealed a disproportion between the increased $InsP_6$ disappearance and amount of digested InsP-P in Atlantic salmon when both phytase and MAP were supplemented. This can be explained by an accumulation of $InsP_5$ and $InsP_4$ isomers in Atlantic salmon faeces as indicated by either a significant or indicative increase of their concentrations owing to MAP supplementation. This could explain the findings of Sajjadi and Carter (2004). It remains unclear why this was only observed in Atlantic salmon and not in rainbow trout. Apart from the overall diet digestibility, different water chemistry, and the species-specific digestive physiology, all other factors could be eliminated owing to the experimental design, reducing the factors that would possibly be responsible for different InsP-P utilisation in the presence of MAP that was observed between the two species.

The observed differences between the two species could have various explanations. The species-specific differences regarding stomach physiology could have favoured the accumulation of $InsP_5$ and $InsP_4$ in Atlantic salmon and not in rainbow trout. A more efficient absorption, i.e., removal of phosphate by rainbow trout is plausible, as various fish species have been shown to differ considerably with respect to phosphate transporter expression, distribution, regulation, and function (Sugiura, 2009). It has been suggested that the high concentration of divalent cations in seawater may interfere with P bioavailability owing

to the formation of insoluble phosphate compounds (Bakke et al., 2010). It is possible that this reduced the phosphate absorption in Atlantic salmon. Phosphate absorption in fishes occurs in the intestine, but there are no studies describing $InsP_6$ hydrolysis along the fish digestive tracts. A detailed investigation of this could further reveal details and possible explanations for the observed differences between the two species of the present study.

4.3. Amino acids

The effects of MAP and phytase supplementation on AA digestibility in Atlantic salmon could not be statistically evaluated in the present study owing to insufficient sample size. But the effects of either supplement or their combination seem to have been marginal. This is consistent with the study of Morales et al. (2016a), who found that phytase supplementation to a diet high in plant protein increased growth, feed conversion, N retention efficiency, and $InsP_6$ digestibility but not CP digestibility in rainbow trout. There are biochemical reactions explaining how $InsP_6$ can bind to proteins, thereby rendering them less accessible to absorption or digestive enzymes. However, the reported magnitude of the effects of phytase supplements on CP digestibility varies greatly. In Atlantic salmon, Denstadli et al. (2006) found that $InsP_6$ has a marginal effect on CP digestibility, albeit significant. Another investigation revealed no significant effects of phytase supplementation on CP digestibility in Atlantic salmon (Denstadli et al., 2007). The ADC of AA in the basal diet of the present study was at a very high level, resulting an increasing effect of phytase appear less probable.

5. Conclusions

In rainbow trout, MAP and phytase supplements can be combined without affecting each other. However, considering the limited resources of mineral phosphate, in Atlantic salmon using a combination of the two is not recommended, as the effect of phytase is rendered obsolete in the presence of MAP. However, this interaction between phytase and phosphate supplements may be different with phytase and phosphate products other than those used in the present work.

The detailed analysis of $InsP_6$ and its specific degradation products provides better insight into the mode of action of phytase, potential bottlenecks in phytase effectiveness, and amount of P rendered available for absorption. Especially when considering the results obtained in the experiment with Atlantic salmon, more studies are needed to further evaluate this effect in vivo along the digestive tract of fish.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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3.4 Additional results

3.4.1 Soybean cake

The nutrient digestibility of soybean cake was determined as part of the digestibility experiment with unprocessed press cakes that was performed and described as part of Manuscript 1 using the same materials and methods.

Soybean (*Glycine max* (L.) Merr.), is the most produced oilseed (335 Mt in the year 2016) (FAOStat 2018). It represents the oilseed from which the most press cake can be globally made available. Soybean cake has a favourable nutrient composition owing to its high CP concentration, coupled with a low CF concentration (434, 93, and 112 g/kg DM, respectively; Table 1). Its ADCs for CP, CL, and energy were 77%, 102%, and 63%, respectively (Table 2). This represents the second highest CP and energy ADCs and the highest CL ADC of all unprocessed press cakes investigated as part of the present thesis. Consequently, out of all press cakes used in this thesis, soybean cake represents the press cake from which the most CP can be made available for rainbow trout.

Table 1: Analysed nutrient composition of the unprocessed press cakes included in the present thesis in comparison to fishmeal (g/kg DM, unless otherwise specified)

	FM [1]	LSC	PSC	RSC	SBC	SFC	WKC
Dry matter [g/kg]	920	910	921	926	916	926	926
Crude protein	654	406	610	294	434	224	514
Crude lipid	76	132	137	203	93	152	196
Crude fibre	10	129	29	197	112	318	12
Crude ash	143	47	80	55	58	67	97
NfE [2]	117	287	144	252	304	240	182
Gross energy [MJ/kg DM]	-	n.a.[3]	22.7	21.6	21.9	22.3	22.8

FM = fishmeal (anchovy), LSC = linseed cake, PSC = pumpkin seed cake, RSC = rapeseed cake, SBC = soybean cake, SFC = sunflower seed cake, WKC = walnut kernel cake; [1] data adapted from NRC (2011); [2] NfE (nitrogen free extracts) = 1000 g - (crude protein [g] + crude fat [g] + crude fibre [g] + crude ash [g]); [3] n.a. = not analysed.

Table 2: Apparent digestibility coefficients (ADCs) for crude protein (CP), crude lipid (CL) and energy of the press cakes included in the present thesis in comparison to fishmeal

	ADCs [1]		
	CP [%]	CL [%]	Energy [%]
Fishmeal [2]	86-97	97	99
Linseed cake [3]	-	-	-
Pumpkin seed cake [4]	88.5	75.8	74.5
Rapeseed cake [5]	42.3	87.6	30.9
Rapeseed cake (sieved) [5]	67.1	59.6	39.7
Rapeseed cake (partially dehulled prior to pressing) [5]	86.6	105 [6]	72.3
Soybean cake	76.8	102 [6]	63.0
Sunflower seed cake [5]	25.0	62.4	24.2
Sunflower seed cake (sieved) [5]	74.6	93.7	49.9
Sunflower seed cake (partially dehulled prior to pressing) [5]	81.0	74.8	49.2
Walnut kernel cake	49.6	95.7	49.0

[1] Values (except for fishmeal) are reported as the mean of four replicates (n = 4); [2] data adapted from NRC (2011); [3] Could not be determined because feed was refused by the fish, refer to section '3.4.3 Linseed cake' for further details; [4] data adapted from Manuscript 2; [5] data adapted from Manuscript 1; [6] The diet substitution method may yield ADCs > 100%, refer to Chapter 4 for details.

3.4.2 Walnut kernel cake

The nutrient digestibility of walnut kernel cake was determined as part of the digestibility experiment that was performed and described as part of Manuscript 2 using the same materials and methods. To the authors knowledge, this is the first time the nutrient digestibility of any walnut product has been investigated in rainbow trout.

The global walnut (*Juglans regia*, L.) production in the year 2016 amounted to 3.7 Mt (FAOStat 2018). The walnut kernel makes up for about 23-64% of the nut weight and contains between 620-740 g/kg CL with a moisture content of about 4%, depending on the cultivar and cultivation conditions (Martínez et al. 2010; McNeil 2013). The walnut kernel press cake had the second highest CP and CL concentration and the lowest CF concentration of all investigated press cakes of the present thesis (514, 196, and 12 g/kg DM, respectively; Table 1). Furthermore, walnut oil contains a high proportion of polyunsaturated fatty acids and a low, i.e. favourable, n6/n3 ratio (Pereira et al. 2008; Vingering et al. 2010; Robbins et al. 2011). The ADCs for CP, CL, and energy of walnut kernel cake were 50%, 96%, and 49%, respectively (Table 2). This represents the second highest CL ADC determined for

unprocessed press cakes of the present thesis. However, its CP and energy ADCs are low when compared with the other press cakes that were investigated as part of this thesis.

3.4.3 Linseed cake

Linseed cake was investigated in the digestibility experiment with unprocessed press cakes that was performed and described as part of Manuscript 1 using the same materials and methods.

The global linseed (*Linum usitatissimum* L.) production in the year 2016 was 2.9 Mt, which is very little when compared with other oilseeds (FAOStat 2018). It was made part of the digestibility evaluation owing to its appreciable concentration of CP and CL and a comparably low concentration of CF (406, 132, and 129 g/kg DM, respectively; Table 1). However, the nutrient digestibility could not be determined for linseed cake because the fish refused to eat the diet towards the end of the first 8-day-period of the digestibility trial. In addition, whitish mucilaginous particles were observed from the third day onwards in the water of the respective tanks.

Chapter 4:
General Discussion and Conclusions

4. General Discussion

The evaluation of a feed ingredient commonly begins with its detailed chemical characterisation and the determination of its nutrient digestibility. The palatability of a feed ingredient, interpreted as the effect on overall feed intake, is also an important criterion that is assessed. In the course of digestibility experiments some indications regarding the palatability of an ingredient can be obtained. However, as the composition of diets used in digestibility experiments commonly does not resemble practical diets, conclusions about ingredient palatability should not be drawn exclusively based on these observations. However, should the preliminary evaluation yield promising results, the experimental follow up includes the evaluation of the ingredients' effects on feed intake, nutrient utilisation, and product quality. In the following chapters the results of the experiments of the present thesis will be discussed accordingly, following the commonly applied approach of feed ingredient evaluation. It should be noted that some cited references refer to the rainbow trout as *Salmo gairdneri* because its scientific name was changed to *Oncorhynchus mykiss* in the late 1980s based on osteological evidence (Billard 1989).

4.1 Chemical characterisation of press cake

A detailed chemical characterisation is the first step in evaluating the potential nutritional value of a feed ingredient. It has been shown that the chemical composition of press cake depends much on the oilseed itself (i.e. species, its respective cultivar, the applied agronomic practises, and environmental conditions) and its processing conditions (Seigler 1998; Copeland & McDonald 2001; Kaltschmitt et al. 2009). Accordingly, the press cakes investigated in the present thesis differed much in their nutrient composition (Table 1).

A high CP and a low CF concentration are desired characteristics of products sought to replace fishmeal in rainbow trout feed, because there is no evidence that rainbow trout are able to digest the constituents that are analytically determined as CF (Bergot & Breque 1981; Morales et al. 1999). The analysis of the AA profile should also be considered, as plant-derived proteins often have an imbalanced AA profile when compared to fishmeal (Naylor et al. 2009). In addition, plant-based feed ingredients may contain ANFs which have been shown to adversely affect their nutritional quality or animal health and should therefore also be considered (Francis et al. 2001). The analysed AA concentrations of the press cakes investigated as part of the present thesis are compared to fishmeal in Table 3. The AA profile of walnut kernel cake was not analysed and is therefore not presented.

Table 3: Analysed amino acid profile and selected antinutritional factors of the unprocessed press cakes included in the present thesis in comparison to fishmeal (g/kg CP)

	FM [1]	LSC	PSC	RSC	SBC	SFC
essential amino acids						
Arginine	56	86	140	53	61	68
Histidine	24	19	28	24	24	23
Isoleucine	47	38	34	35	41	38
Leucine	76	52	69	61	66	58
Lysine	78	34	32	56	51	39
Methionine	30	15	18	18	12	21
Phenylalanine	41	44	49	38	44	43
Threonine	43	33	30	42	35	36
Valine	54	45	43	47	43	48
non-essential amino acids						
Alanine	n.d.[2]	41	44	41	39	41
Aspartate	n.d.	84	87	64	99	82
Cysteine	9	16	11	24	15	17
Glycine	n.d.	183	54	155	161	169
Glutamate	n.d.	52	173	47	39	56
Proline	n.d.	31	35	53	44	38
Serine	n.d.	41	51	40	44	39
Tyrosine	33	22	29	27	30	22

FM = fishmeal, LSC = linseed cake, PSC = pumpkin seed cake, RSC = rapeseed cake, SBC = soybean cake, SFC = sunflower seed cake; [1] fishmeal data adapted from NRC 2011; [2] n.d. = no data available.

Across all press cakes pumpkin seed cake had the highest CP and lowest CF concentration (Table 1). And although it seems to have a comparably good AA profile (highest concentration of 4 out of 9 essential AAs), it had the lowest concentration of lysine which is considered the first limiting AA in many plant-based fish feed ingredients (Gaylord & Barrows 2009).

A detailed analysis of the amino acid profile of all used feed ingredients in animal nutrition experiments is desirable. However, it is not always possible because often compromises are made for a number of reasons (e.g. financial, timely, comparability, etc.).

4.2 Nutrient digestibility

The determined ADCs for CP, CL, and energy of all press cakes investigated as part of the present thesis is presented in Table 2. However, there are many factors that influence the nutrient digestibility and its determination in rainbow trout. The first part (4.2.1) of this section focusses on the applied methodology and experimental design. The second part (4.2.2) is specifically focussed on the chemical constituents of press cake and their potential influence on nutrient digestibility. The last part of this section (4.2.3) focuses on press cakes whose nutrient digestibility was determined as part of the digestibility experiments presented in the Manuscripts 1 and 2, despite not being included in the final manuscripts.

4.2.1 Methodological considerations

There are two ways to determine nutrient digestibility, the direct and the indirect method (NRC 2011). The direct method requires quantifying all consumed feed and its resulting faeces. The feed intake could theoretically be quantified, even accounting for nutrient leaching of uneaten pellets (Helland et al. 1996). However, the precise quantification of faeces is rather difficult in experiments with fish without drawbacks that may render the obtained results questionable (NRC 1993). Therefore, in the digestibility experiments of the present thesis the **indirect, or marker based, method was applied** to determine the ADCs of CP, CL, and energy of the press cakes.

Irrespective of the applied method, the **apparent digestibility of nutrients** is calculated based on the determined concentration of crude nutrients or specific compounds in feed and faeces. This has some drawbacks. It may lead to an underestimation of CP and energy digestibility because endogenous losses (shed intestinal cells, enzymes, and microbial matter) are contained in faeces. As this factor cannot be quantified a CP rich reference diet was used to minimise the proportion of CP resulting from endogenous losses in the faeces. The ADCs of CP, CL, and energy for the press cakes were calculated according to the equation recommended by NRC (2011).

There are two common approaches to formulate diets for digestibility experiments when evaluating a single feed ingredient, the diet replacement and the ingredient replacement method (Aksnes et al. 1996). The ingredient replacement method requires a well-defined reference ingredient that will be replaced by the test ingredient. Since the present work lacked the capacity and time to evaluate the digestibility of a reference ingredient under the specific experimental conditions, the **diet substitution method was applied** in the digestibility experiments of the present thesis. A standardised recommendation for inclusion levels of test ingredients does not exist, probably owing to the wide array of different ingredients and experimental designs. The inclusion rate of the press cakes in the digestibility diets of the present thesis was 250 g/kg DM. This concentration was chosen based on the analysed glucosinolate content of rapeseed cake because a higher dietary glucosinolate concentration

has been shown to negatively affect rainbow trout health (Burel et al. 2001). A higher dietary inclusion of rapeseed cake was therefore deemed unsuitable. The other press cakes were included at the same dietary level, so the feed extrusion parameters did not have to be changed markedly. It should be noted that the diet substitution method can result in calculated ADCs >100% (Glencross et al. 2007; Gaylord & Barrows 2008). In the present experiments this was the case for the CL ADC of partially dehulled rapeseed cake (105%) and soybean cake (102%) (Table 2). While these results may not be biologically plausible, they were not corrected, as it has been suggested that this could indicate an interaction between the ingredients of the respective diets (Glencross et al. 2007).

Various types of **markers**, both external (added to diet) and internal (naturally part of diet), have been investigated in digestibility experiments with rainbow trout (Tacon & Rodrigues 1984; Morales et al. 1999; Austreng et al. 2000). The recovery of the commonly used chromic oxide has been shown to be inconsistent compared with other metal oxides (Austreng et al. 2000). However, titanium dioxide was found to be a suitable external marker in many digestibility studies with different animal species that has been shown to be determinable with a high analytical accuracy and has been shown to have a high recovery rate in various sample matrices (Boguhn et al. 2009). This could explain the fact that titanium dioxide has been shown to yield higher ADCs than chromic oxide in rainbow trout (Vandenberg & de la Noüe 2001). It could also explain that the deviation of determined ADCs was smaller across various faecal collection methods when titanium dioxide was compared to chromic oxide (Vandenberg & de la Noüe 2001). Therefore, it is assumed that the use of titanium dioxide as external marker will yield more reliable results. Thus, titanium dioxide was used as a marker in the diets for the digestibility experiments of the present thesis.

There are three commonly applied methods of **obtaining faecal samples** for analysis and determination of ADCs, namely dissection, collection of voided faeces (by sedimentation or sieving), and stripping. Dissection of fish is a useful method when digestive processes along different segments of the gastrointestinal tract are evaluated (Austreng 1978). However, this method is very laborious and does not allow for repeated sampling of the same individuals. The collection of faeces from the water by either sedimentation or sieving allows for the continuous collection of faecal samples from fish without physically having to handle them. The effort for collection of faecal samples is independent from the number of individuals which enables pooled sampling of many individuals thereby reducing the effect of individual variation. The biggest drawback of the collection of voided faeces from water is the leaching of nutrients from the faecal sample, which can lead to the overestimation of nutrient ADCs. The manual collection of faeces directly from the, commonly sedated, individual (i.e. stripping) is considered a conservative method that may underestimate nutrient ADCs due to a potentially incomplete digestion of the feed. Stripping of faeces has been shown to result in lower ADCs compared with the settlement faecal collection in numerous experiments with rainbow trout (Windell et al. 1978;

Vens-Cappell 1985; Storebakken et al. 1998; Weatherup & McCracken 1998; Vandenberg & de la Noüe 2001). Therefore, the different methods of faecal collection must be considered when ADCs of feed ingredients are compared across various publications. Furthermore, it has been shown that the nutrient digestibility can be affected in dependence of the starting point of stripping because CP, CL, and energy ADCs were found to be highest at the posterior part of the hindgut in rainbow trout (Austreng 1978). It has been shown that the CP, CL, and energy ADCs were, respectively, 1%, 4%, and 4% higher when determined in faecal samples from the posterior part of the hindgut compared with samples obtained from the anterior part of the hindgut of rainbow trout (Austreng 1978). Owing to the lack of descriptive statistics and information regarding the statistical significance of the observed differences conclusions regarding the impact of these results on calculated ADCs of the respective nutrients cannot be drawn. To minimise the effect of incomplete nutrient digestion and to eliminate possible nutrient leaching through faecal settlement in water, the stripping method (starting from the ventral fins) described by Austreng (1978) was applied in all presented digestibility experiments.

The digestibility of CP and energy can vary between **strains of rainbow trout**. The CP digestibility has been shown to differ up to about 9% between strains in dependence of the diets' CP content (Austreng & Refstie 1979). The energy digestibility has been shown to differ up to about 19% between strains in dependence of the diets' percentage of metabolizable energy made available from carbohydrates (Refstie & Austreng 1981). However, the strain used for the experiments of the present thesis has not been characterised using molecular markers and its digestibility characteristics have not been compared with other strains. Therefore, it would be speculative to rank the strain used based on the observed nutrient digestibility. However, literature suggests that there may be strains which could be found to have a higher press cake digestibility than the strain used in the presented press cake digestibility experiments of Manuscript 1 and 2. However, in all digestibility experiments with press cake in the present thesis the same strain of rainbow trout was used, thereby minimising the variability of this factor on the determined nutrient digestibility and increasing the comparability between these results.

Much effort has been made to assess the effects of **rearing conditions**, such as rearing **temperature and feeding regime**, on the digestibility of nutrients in rainbow trout (Bergot 1979; Storebakken & Austreng 1987a; Cho & Kaushik 1990; Azevedo et al. 1998; Bolliet et al. 2000; Yamamoto et al. 2001; Ng et al. 2003; Yamamoto et al. 2007). However, these studies yielded controversial results. The discrepancies are probably attributable to different diet formulations and ingredient compositions as well as different methodological approaches across the experiments. As fish are ectothermic, their metabolism and the activity of digestive enzymes is directly linked to the surrounding temperature. Across all digestibility experiments with press cake and rainbow trout in the present thesis the water temperature was similar, and their feeding regime was maintained (hand fed,

once daily to apparent satiation, 2 h after lights were turned on). Therefore, the effects of water temperature and feeding regime will not have markedly influenced the comparability within and between the presented digestibility experiments with press cake in rainbow trout.

Extrusion parameters of fish feed has been shown to exert only minor effects on the digestibility of nutrients in rainbow trout when the same diet was processed under varying conditions (Pfeffer et al. 1991; Sørensen et al. 2002, 2005; Barrows et al. 2007). This was attributed to the short residence time of diets in the extruder in which the feed was exposed to different processing parameters. Nonetheless, the extrusion parameters were maintained similar during the production of the digestibility diets in the press cake experiments of the present thesis in order to minimise potential effects on nutrient digestibility.

The **particle size** of feed ingredients has not been shown to have an effect on nutrient digestibility in rainbow trout (Zhu et al. 2001). However, a maximum particle size for ingredients (<0.5 mm) is recommended for the production of a homogeneous diet (NRC 1993). As press cake is commonly a very heterogeneous product, the press cakes used in the digestibility experiments were ground through a 250 µm mesh.

The determination of a feed ingredient's nutrient digestibility is meaningful for gathering information on its nutritive value. There is a wide array of assay details that have been shown to influence nutrient digestibility of feed ingredients as well as its determination in rainbow trout and fish in general. In an effort to increase the comparability between the respective experiments with rainbow trout care was taken to reduce possible sources of error by applying the same methodology and experimental design. Therefore, the comparability between the presented nutrient ADCs for the press cakes is believed to be high owing to similar rearing conditions, feed preparation and the stringent methodology that was applied for their determination.

4.2.2 Influence of chemical constituents of press cake on nutrient digestibility

The potential effects of press cake specific ANFs on nutrient digestibility has been discussed in the respective manuscripts (Manuscript 1 and 2) and will therefore not be repeated here. There are however some aspects regarding nutrient digestibility of press cake in rainbow trout that should generally be considered. They are presented in the following chapter along with information specific to the press cakes that were not included in the manuscripts.

Fibre fractions

Press cake can contain considerable concentrations of fibre fractions, namely **NSPs and lignin**, which have been shown to affect the digestibility of nutrients. NSPs include cellulose, hemicellulose, β-glucans, pectins, and an array of gums, and carnivorous fish have not demonstrated to be able to

utilise either lignin or NSPs (Stone 2003; Krogdahl 2005). In addition to being indigestible, NSPs have been shown to negatively affect CP digestibility in rainbow trout, thereby acting as ANFs (Storebakken 1985; Glencross et al. 2003; Glencross et al. 2012). Thereby, the digestibility of organic matter and energy are prone to fibre fractions. The digestibility of organic matter and energy generally increases with decreasing concentrations of fibre fractions as has been shown in rainbow trout (Hansen & Storebakken 2007; Glencross 2009b). In line with this observation, an increased CP digestibility has been shown for the press cake of rapeseed and sunflower seed (Manuscript 1). When fibre-reduced press cake was compared to its respective unprocessed counterpart the CP digestibility increased from 42% to 67% (rapeseed cake) and 25% to 75% (sunflower seed cake). Both the composition and the concentration of NSPs varies between oilseeds and subsequently their respective press cake (Bach Knudsen 1997). The extent to which NSPs affect CP digestibility in rainbow trout seems to depend much on the specific NSP and their dietary concentration (Storebakken & Hansen 1987; Glencross 2009b). There is little available data on the underlying mechanisms, and which specific carbohydrate is responsible for the depressed nutrient digestibility observed in the aforementioned studies. It has been suggested to be related to the effects of NSPs on chyme/ digesta viscosity, gastric evacuation, and their potential interference with digestive enzymes (Storebakken 1985; Bach Knudsen 2001; Glencross et al. 2012). Based on the available data it is difficult to estimate the effects of fibre fractions on nutrient digestibility. Likely, the low concentration of CF in pumpkin seed cake will, in part, have enabled its high digestibility determined in rainbow trout (Tables 1 and 2). However, considering the CF concentration of walnut kernel cake and its comparably low nutrient digestibility, it becomes clear that factors other than the CF content can have a great impact on press cakes' nutrient digestibility.

It is difficult drawing conclusions on the effects of fibre fractions on nutrient digestibility based only on the comparison of fibre-reduced ingredients and their unprocessed counterparts. Fibre-reduction proportionally increases the concentration of constituents that are not removed by this process. It was found that for rapeseed cake the concentration of ANFs associated with the seed, rather than its hulls, increased when it was sieved (Manuscript 1). These ANFs have been shown to have detrimental effects on rainbow trout health or to reduce CP digestibility (Francis et al. 2001). Therefore fibre-reduction is likely to have influenced the nutrient digestibility. However, it cannot be defined with certainty how big this effect is.

Phytic acid and phytate

Replacing fishmeal with plant-based ingredients, such as press cake, has implications regarding dietary P. It is an essential mineral that plays an important role in bone formation, serves as a structural component of cell membranes, represents the main source of energy for metabolic processes (in the

form of adenosine triphosphate), and is a component of nucleic acids (Lall 2002). The primary storage form of P in plant seeds and plant seed derived feed ingredients such as press cakes are $InsP_6$ and its salts (phytate) (Eeckhout & De Paepe 1994; Rodehutscord et al. 2016). Analysis revealed that phytate bound P amounted to more than half of the total P in the respective press cake (Table 4).

Table 4: Total phosphorus, phytate phosphorus, and phytic acid concentration of press cakes (g/kg DM)

	LSC [1]	PSC [2]	RSC [1]	SBC [1]	SFC [1]
Total phosphorus	4.0	n.a. [3]	3.9	3.1	4.9
Phytate phosphorus	2.4	9.8 [4]	2.6	1.9	3.9
Phytic acid	8.7	34.8	9.2	6.6	13.7

LSC = linseed cake, PSC = pumpkin seed cake, RSC = rapeseed cake, SBC = soybean cake, SFC = sunflower seed cake; [1] determined by Masterlab (Netherlands) using Phytic Acid/Total Phosphorus Assay Kit (K-PHYT 12/12, Megazyme, Bray, Ireland); [2] data from Manuscript 2; [3] n.a. = not analysed; [4] calculated based on phytic acid content.

Therefore, replacing fishmeal protein with plant-based proteins inadvertently replaces a highly digestible inorganic P source with $InsP_6$ and its salts which have a limited digestibility in fish (Rodehutscord & Pfeffer 1995; Francis et al. 2001; Naylor et al. 2009). In addition, phytate is often regarded as an ANF, as it has been shown to form phytate-mineral complexes and sparingly soluble phytate-protein complexes that can reduce the availability of mineral cations and dietary proteins (Cheryan & Rackis 1980). It has also been shown to have detrimental effects on the epithelial layer of the pyloric caecae in chinook salmon (*O. tshawytscha*) and common carp (*C. carpio*), thereby directly impairing nutrient uptake (Francis et al. 2001). Rainbow trout, like most fish species, have not been shown to exhibit activity of $InsP_6$ hydrolysing enzymes, such as phytases (myo-inositol hexakisphosphate phosphohydrolases) and other phosphatases in their gastrointestinal tract (Lall 1991; Hua & Bureau 2010). Therefore, nutrient digestibility of ingredients in rainbow trout is prone to dietary phytate concentrations. In addition, pumpkin seed cake also has the highest phytic acid concentration (Table 5), a compound commonly associated with having antinutritional properties (Francis et al. 2001).

From available literature it becomes apparent that fish species differ much regarding their physiological responses to dietary phytate, phytase supplementation, and the type of mineral supplement used to counteract the decreased P availability (Cao et al. 2007; Kumar et al. 2012). These species-specific differences also became apparent when the breakdown of $InsP_6$, the appearance of specific lower inositol phosphates in the digestive tract, and their dependence on mineral P supplements were compared between rainbow trout and Atlantic salmon (Manuscript 3). The addition of phytase (2,800 FTU/kg feed) has been shown to increase the de-phosphorylation of $InsP_6$ to a much

higher extent in rainbow trout than in Atlantic salmon and that more $InsP_6$-P can be made available in rainbow trout. In addition, it has been shown that the supplementation of inorganic P (in the form of monoammonium phosphate) decreased the phytase efficacy in Atlantic salmon but not in rainbow trout. The results suggest that, when reared in their respective environments, the use of press cake in feed for rainbow trout would be more advantageous than in feed for Atlantic salmon with regards to a more sustainable use of P resources. Furthermore, it suggests that the negative effects that $InsP_6$ has been shown to exhibit in fish, such as decreasing CP digestibility, are expected to be of a lower magnitude in rainbow trout compared with Atlantic salmon.

It appears most of the research that is focussed on $InsP_6$ neglects its potential as valuable source of P and myo-inositol. However, the finite mineral P resources should increase research on using $InsP_6$ as a phosphate source in the near future. Furthermore, the complete dephosphorylation of $InsP_6$ yields myo-inositol, a water-soluble vitamin and at the same time the only optically active inositol form with biological activity (NRC 2011). It is commonly added to commercial diets as part of the vitamin premix as most fish species, unlike mammals, depend on an exogenous source of myo-inositol (Woodward 1994). However, while it is acknowledged to be an essential nutrient for rainbow trout, there are very few recent studies regarding the effects of myo-inositol supplementation in trout nutrition (Woodward 1994, Barrows et al. 2010).

Comparing performance traits or physiological parameters resulting from the dietary supplementation of an enzyme, like phytase, between different fish species often poses a challenge. When the effects are investigated using the species' respective common rearing conditions the magnitude of the observed effects will likely reflect the effects to be expected under common rearing conditions. However, the observed effects may not be entirely attributed to the supplement but could in part be attributed to the different environmental conditions. If it is decided to maintain similar rearing conditions for the different species, the observed effects are more likely to represent differences between the species, but the validity of the results may be challenged. Because few fish species share the same optimal rearing conditions, the magnitude of the effects of the supplement may be compromised.

Antinutritional factors

Antinutritional factors have been shown to affect nutrient digestibility in fish (Francis et al. 2001). Selected antinutritional factors of some of the press cakes that were investigated are presented in Table 5. However, the list of analysed ANFs is incomplete because only a limited number of analyses could be performed. The analyses were therefore focused on the ANFs known to be present in rapeseed, as it represents the press cake from which the most CP can be made available in Germany.

Table 5: Selected antinutritional factors of linseed cake (LSC), rapeseed cake (RSC), and soybean cake (SBC) [1]

	LSC	RSC	SBC
Cyanide formation potential [mg/100g DM] [2]	31.8	n.a.	n.a.
Glucosinolates [g/kg DM]	n.a.[4]	5.4	n.a.
- Progoitrin [g/kg DM]	n.a.	2.6	n.a.
Lectins [g/kg DM] [3]	n.a.	n.a.	0.1
Tannins [g/kg DM]	n.a.	13.0	n.a.
Volatile mustard oils [mg/kg DM]	n.a.	21.6	n.a.

[1] References provided only for methods not described in the manuscripts; [2] analysed in accordance with method described by Nahrstedt (1977); [3] analysed in accordance with method described by Vretblad (1976); [4] n.a. = not analysed.

A detailed analysis of all antinutritional factors of feed ingredients is rarely presented as these analyses are still very costly. Accordingly, values are often assumed based on available literature. This could be a source of potential errors in interpretation, considering the variability of the chemical composition of plant-based feed ingredients, even when they are derived from the same raw material. For example, the rapeseed press cake used in the experiments presented in Manuscript 1 had a glucosinolate content of 11.2 µmol/g DM (5 g/kg DM). It has been shown that the glucosinolate content of defatted rapeseed meal from the same variety (*B. napus*) ranges from 10 to 188 µmol/g DM (Mailer et al. 2008). Owing to this, conclusions drawn regarding their involvement must be considered speculative.

The inferior characteristics of plant derived products as fishmeal replacers were often in part attributed to their ANF content. However, in most cases the diets are formulated by replacing increasing amounts fishmeal with an increasing concentration of the respective ingredients which can attribute the observed effects to a wide array of factors (e.g. AA imbalances). While this may yield representative results, it does not narrow down the effect that specific ANFs exhibit on investigated traits. It is therefore recommended to combine these experiments with dose-response data of extracted ANFs of the respective ingredient to better understand the role of specific ANFs.

4.2.3 Nutrient digestibility of press cake not included in the presented manuscripts

In addition to the general discussion, the results of the press cakes that are not included in the manuscripts are briefly discussed in the following. Only additional information on specific key aspects that could have influenced their nutrient digestibility in rainbow trout is highlighted.

Soybean cake

The nutrient digestibility of soybean cake is low compared with solvent-extracted soybean meal and other processed soybean products (Glencross et al. 2005). Soybean contains ANFs that have been shown to decrease nutrient digestibility in rainbow trout (Francis et al. 2001). The higher nutrient digestibility of solvent-extracted soybean meal compared with soybean cake can have a number of reasons. The solvent extraction process (including the desolventisation) represents a process that can improve the nutritive value of soybean cake. The applied heat during this processing step can reduce the concentration of heat-labile ANFs, like trypsin and chymotrypsin inhibitors as well as lectins, which have been shown to negatively influence nutrient digestibility of soybean products in rainbow trout (Krogdahl et al. 1994; Anderson & Wolf 1995; Francis et al. 2001). The increased nutrient digestibility of soybean concentrate and soybean isolate compared with soybean meal can also be the result of specific solvent extraction processes which decrease the concentration of fibre fractions and saponins (Drew et al. 2007). Both fibre fractions and saponins have been shown to negatively affect nutrient digestibility, either directly by reducing enzymatic accessibility to nutrients (fibre fractions) or indirectly by damaging the intestinal mucosa as has been shown in rainbow trout (Bureau et al. 1998).

Walnut kernel cake

The ADCs of CP and energy were comparably low, especially when considering the low amount of fibre fractions that can be expected from the low concentrations of CF and nitrogen-free extracts. One reason that could account for the comparably low CP digestibility of walnut kernel cake is its high content of phenolic compounds, in particular tannins (Martínez et al. 2010). Tannins have been shown to reduce CP digestibility in fish by binding to either proteins or enzymes, both of which can reduce CP digestion (Francis et al. 2001). The total phenolic content of walnut cake was not determined. Herein literature suggests that it is high compared with many other oil-crops (nuts and oilseeds) (Naczk et al. 1998; Sze-Tao et al. 2001; Pereira et al. 2008; Arcan & Yemenicioğlu 2009; Terpinc et al. 2012; Thiyam-Holländer & Schwarz 2013). It should be noted that the solvents used to extract tannins differed between some of the aforementioned studies and this can influence the determined total phenolics yield of the respective extracts.

Linseed cake

There is a wide array of biologically active compounds in linseed that have been shown to act as ANF for fish (Francis et al. 2001; Shim et al. 2014). Linseed stands out among the oilseeds investigated as part of the present thesis regarding its fibre fraction composition. It contains a very high concentration of soluble NSPs (Bach Knudsen 1997; Brenes et al. 2004). Linseed meal and linseed hulls have been shown to absorb 8 and 13-fold of their weight in water, respectively (Bhatty & Cherdkiatgumchai 1990). This could explain the significantly increased chyme viscosity and the decreased nutrient absorption observed in broiler chickens when linseed was included at 8–16% in the diet (Alzueta et al. 2003) as well as the decreased nutrient absorption observed in pigs and rats when offered diets that contained linseed (Kiarie et al. 2007; Kristensen et al. 2013). The positive effect of reducing linseed mucilage on rainbow trout performance has been shown by Thiessen (2004), where the aqueous extraction of linseed resulted in significantly increased performance traits than those seen in response to untreated linseed at the same dietary inclusion level. However, the dietary inclusion level of linseed used in the present thesis (25%) was more than twice as high as in the aforementioned study. This suggests that the high soluble NSP content of linseed cake, coupled with the high dietary inclusion rate in the present study, were key factors associated with the refusal of the diet by the fish. This is supported by the observation of whitish mucilaginous particles that accumulated in the respective tanks which could be indicative of a digestive disorder.

Owing to the discussed limitations of digestibility determinations for feed ingredients the applied methodology can only serve as an initial evaluation as it: a) used diets with little practical relevance, b) cannot reveal long term effects on performance traits, and c) cannot provide information on nutrient partitioning and retention. The following chapter focuses on the effects on performance and morphological traits of rainbow trout when fishmeal was partially replaced by press cake.

4.3 Press cake as fishmeal substitute in rainbow trout diets – effect on performance and morphological traits

The complementation of digestibility data with an assessment of performance and morphological traits of fish is key to the evaluation of feed ingredients. Rapeseed and sunflower seed cake have been shown to decrease performance traits in rainbow trout when they replaced 50% of fishmeal on the basis of digestibile CP (Manuscript 1). An assessment of the effects on morphological traits was therefore deemed unnecessary.

In contrast, pumpkin seed cake has not decreased performance traits of rainbow trout when it replaced 60% of fishmeal on the basis of digestibile CP (Manuscript 2). But it has been shown that the maximum weight gain does not necessarily reflect maximum protein gain in rainbow trout

(Rodehutscord et al. 1997; Encarnação et al. 2004). Therefore, the effects on morphological traits of rainbow trout, when fishmeal was in part replaced with pumpkin seed cake, were also investigated, as in this case further information on its suitability as potential fishmeal replacer can be revealed. It has been shown that, except for resulting in a significantly decreased hepatosomatic index (**HSI**), no significant differences across the other morphological traits were found between rainbow trout that were fed with the basal diets and the fish that received the pumpkin seed cake diets.

There are factors that can affect the performance and morphological traits of rainbow trout and their determination which should be considered when interpreting the results of the presented experiments. The following sections are focused on methodological considerations with the intention to complement the discussions of the presented manuscripts in which press cake was used as a partial fishmeal replacer.

4.3.1 Methodological considerations - experimental diets

When evaluating the effects of feed ingredients on performance traits and morphological traits of fish much attention must be given to the **composition** of experimental diets used. The substitution of fishmeal with alternative protein sources, especially press cake, may have both nutritional and practical implications that can influence the fishes' performance. In the experiments with press cake the CP of fishmeal was replaced based on the determined CP digestibility of the respective press cake. The formulation of the experimental diets based on the determined ADCs could be a source of error because the determined ADCs are subject to a degree of uncertainty as discussed in Section 4.2. In addition, diets used in growth experiments commonly have a different formulation than diets for digestibility experiments and often more ingredients are used to resemble diets with a certain degree of practical relevance. A potential **interaction** between the press cake and the ingredients of the growth diets diet could therefore not be accounted for in the context of the experiments of the present thesis. This type of interaction between ingredients was thought to be a reason for a calculated nutrient digestibility that can be higher than 100% as was calculated for the CL ADC of sieved rapeseed cake (105%; Manuscript 1) and soybean cake (102%) (Glencross et al. 2007).

Substituting fishmeal with press cake based on digestible CP has implications regarding the overall nutrient composition which is best seen in the experiment in which unprocessed rapeseed cake was used (Manuscript 1). In order to substitute 10 g/kg DM fishmeal 51.5 g/kg DM of rapeseed cake had to be included in the diet owing to its low CP content coupled with its low CP digestibility. The **energy density** will then in turn have to be balanced by adjustment of the concentration of other feed ingredients because rainbow trout have been shown to be able to adapt their feed intake in dependence of the digestible energy content of the feed (Hilton et al. 1983; Boujard & Médale 1994; Yamamoto et al. 2002). An imbalanced energy density of the feed could therefore mask the effects of

press cake inclusion on feed intake. There are different approaches applied in such cases when investigating a single ingredient. In some studies, the reference diet contains an inert filler (e.g. an indigestible fibre fraction like cellulose) which is then replaced by the test feed ingredient (Sanz et al. 1994). In dependence of the type of filler and its dietary concentration this may interfere with nutrient digestibility and intestinal passage rate of the feed (NRC 2011). In other cases, digestible carbohydrates like wheat or maize are replaced in favour of the included feed ingredient in question (Burel et al. 2000; Refstie et al. 2000; Nang Thu 2011). And in some cases, the experimental diets are formulated combining the two methods (Collins et al. 2012). In the diets of the growth experiments of the present thesis press cake was included at the expense of wheat. To balance the energy density of the diet the oil concentration was increased to compensate the digestible energy lost owing to the decreased content of digestible carbohydrates.

Lipids have a higher gross energy density than carbohydrates and are well digested by rainbow trout (NRC 2011). They are therefore well suited to balance the energy density of compound feed when press cake is included in the diets because press cake inclusion can introduce substantial amounts of indigestible components to the feed. The balancing of the energy density could also have influenced the morphological traits of the fish as observed in Manuscript 2. The **significantly decreased HSI** of the fish that received the diet in which fishmeal was replaced with pumpkin seed cake was explained by the decreased amount of digestible carbohydrate in the pumpkin seed cake diet. Other studies that came to the same conclusion were found in addition to the literature cited in Manuscript 2. It has been shown that the HSI of rainbow trout and other fish increased when the proportion of metabolizable energy supplied as carbohydrates increased, owing to an increased hepatic glycogen deposition (Austreng et al. 1977; Wilson 1994; Kamalam et al. 2017). Therefore, it seems likely that the form of digestible energy which was supplied to the fish is in part responsible for the lower HSI determined for the fish fed the pumpkin seed cake diets.

The dietary concentration of carbohydrates can influence the **physical parameters** of fish feed (e.g. water stability) which have been found to affect the gastric evacuation time, the digestibility of nutrients, and feed intake in rainbow trout (Hansen & Storebakken 2007; Sørensen 2012). In the growth experiments of the present thesis the physical parameters of the feed were not evaluated and therefore no conclusions can be drawn regarding this aspect. But, the feed for the growth experiments was produced under similar conditions within each respective experiment because it has been shown that feed extrusion parameters influence feed intake in rainbow trout (Barrows et al. 2007). But the composition and concentration fibre fractions will have varied owing to the inclusion of different press cakes. Therefore, it is possible that the physical properties of the feed used in each respective experiment differed from one another despite similar feed production parameters. However, the

extent to which performance traits of fish are affected by the physical pellet properties is unclear due to little available data and divergent observations among the few available studies.

In most studies, as well as in the growth experiments of the present thesis, a vitamin-mineral premix and commercially available AAs are supplemented to the reference diets to match recommended requirements (if available) of the respective species based on data obtained by chemical analysis. It should be noted that the published requirements of AAs vary in dependence of the applied methodology (Bureau & Encarnação 2006). Furthermore, the balancing of AAs, as well as micro- and macronutrients often proves difficult owing to the **discrepancy between the analysed nutrient composition and the nutrient's digestibility or availability**. It has been shown that the digestibility of specific AAs in rainbow trout can vary in dependence of the feed ingredient and in the case of some essential AAs was found to be significantly lower for oilseed meals than fishmeal (Gaylord et al. 2010). In addition, it has been shown that the utilisation of lysine can be affected by the diet composition (Rodehutscord 2000; Encarnação et al. 2004). Furthermore, a limited availability of essential amino acids has been shown to decrease feed intake in rainbow trout (Rodehutscord et al. 1995; 1997). The P availability in rainbow trout has been shown to depend much on the feed ingredient in question, commonly being lower in plant-based than in animal-derived ingredients (Riche & Brown 1996; Sugiura et al. 1998). It is possible that the AAs and P of fibre-reduced rapeseed cake and pumpkin seed cake differ in their digestibility and availability in rainbow trout. This could in part explain the significantly affected performance traits of rainbow trout observed for fibre-reduced rapeseed cake in Manuscript 1 and not for pumpkin seed cake in Manuscript 2 despite using a similar reference diet. However, owing to a lack of data this is difficult to account for.

Some performance traits are often difficult to interpret in the context of growth experiments. There are many indices which are used to evaluate performance traits that can be found in literature. Interpreting the effects of a feed ingredient on feed intake requires much attention, as it must not always be directly related to the feed ingredient in question. The term 'palatability' is often used to describe the quality of a feed or feed ingredient, and often in connection to ANFs. However, it cannot always be concluded with certainty if the observed effects are related to "taste" per se. The term palatability may therefore be misleading, and it would be better to refer to the observed effects as apparent palatability or simply to refer to feed intake.

4.3.2 Methodological considerations - experimental design

To minimise potential errors the experimental conditions of the performance experiments were kept similar to the conditions under which nutrient digestibility of the press cakes was determined. Nonetheless, there are many aspects regarding the experimental design that have been shown to affect performance traits of rainbow trout. However, within each growth experiment with

press cake of the present thesis these factors were accounted for. They did not deviate between treatments to an extent that is believed to have markedly influenced the determined performance traits. As this can have implications regarding the comparability between results of the presented experiments and other studies they will be discussed in the following.

Rearing conditions have been shown to influence the performance traits of rainbow trout. Feed intake of rainbow trout has been shown to increase with increasing **water temperature** without affecting feed efficiency (Azevedo et al. 1998). However, a higher water temperature could have favoured the $InsP_6$ disappearance in rainbow trout compared with Atlantic salmon (Manuscript 3). Temperature-dependent effects of phytase on in vitro $InsP_6$ de-phosphorylation simulating the gastric conditions of rainbow trout have been reported and have been shown to be higher at 16°C than at 6°C (Morales et al. 2011). **Dissolved oxygen** may affect feed intake when it is below a critical level (Glencross 2009a). Other **environmental factors** such as dissolved nitrogenous compounds and water pH have also been shown to influence feed intake in fish (Kestemont & Baras 2001). Prolonged **lighting** periods have been shown to increase feed intake in rainbow trout resulting in higher weight gain owing to longer feeding activity, without affecting feed conversion (Ergün et al. 2003; Taylor et al. 2005). The **feeding regime** (i.e. feeding time(s), frequency, and allocated level) has been shown to affect both performance and morphological traits of rainbow trout (Storebakken & Austreng 1987a; Storebakken et al. 1991; Bolliet et al. 2000).

Fish related parameters have also been shown to influence performance traits of rainbow trout. Both the FCR and the condition factor have been shown to depend on the **size** of rainbow trout. Whereas the FCR was found to be lower in smaller fish than in larger fish, the condition factor increased with increasing fish size (Rønsholdt 1995). The performance traits of different rainbow trout **strains** have been shown to differ (Edwards et al. 1977; Austreng & Refstie 1979). Morphological traits have also been shown to differ between trout strains (Refstie & Austreng 1981; Smith et al. 1988). It has been shown that the relative rank of performance of different strains in direct comparison remains the same irrespective of the proportion of plant protein in the diet (Reinitz et al. 1978). A more recent study demonstrated the possibilities of selective breeding for a better utilisation of plant-based proteins in rainbow trout (Overturf et al. 2013).

Feed was offered by hand to apparent satiation and uneaten feed could not be recovered. This can be a source of **personal error**. This approach presumes that the person feeding the fish adapted the offered amount to the actual intake and reduced feed losses to a similar extent each feeding day and tank. In order to minimise potential errors during each trial the same person was responsible for feeding the fish and the experimental diets were anonymised to eliminate potential bias. Nonetheless, the feed intake and performance traits that are calculated based on the feed intake are prone to a degree of uncertainty. This personal error can also influence determined morphological traits. For the

determination of morphological traits (Manuscript 2) each "station" (dissecting, weighing, etc.) was assigned to one person that did not change during sampling.

Drawing conclusions based on the comparison of performance and morphological traits determined across publications from different workgroups is difficult owing to the wide array of factors that can affect the obtained results. However, across the experiments presented in Manuscript 1 and 2 care was taken to minimise potential errors for each respective type trial. For further reading on this subject the reviews of Shearer (1994) and Rasmussen (2001) are recommended.

4.4 Conclusions

Based on the results obtained from the experiments which are presented in this thesis the following conclusions are drawn:

- The press cake of various oilseeds has different potential as partial fishmeal replacer in rainbow trout owing to differing nutritional value.
- Of all investigated press cakes, pumpkin seed cake has been shown to have the highest potential to partially replace fishmeal CP in diets for rainbow trout owing to its high CP content coupled with a high CP digestibility.
- The complex chemical composition of press cake is influenced by many factors. There is a lack of knowledge on the effects of many press cake specific constituents on their nutritive value in rainbow trout. A more detailed chemical characterisation of press cake can generate data regarding the variability between different sources.
- Press cake can contain a high concentration of fibre fractions which are indigestible in rainbow trout and most other fish species. Irrespective of the interaction of fibre fractions with press cake nutrients and their digestibility, the concentration of indigestible fibre fractions can determine or limit their potential inclusion in high performance diets for rainbow trout.
- The determination of ADCs is influenced by different factors, which can compromise a direct comparison between results of different workgroups. Maintaining the same methodology has increased the comparability between the determined ADCs within and between the presented digestibility experiments in which press cake was investigated.

4.5 Perspectives for future research

There is a wide array of species raised in feed-based aquaculture production systems that have been shown to digest and utilize nutrients from feed ingredients differently (NRC 2011). Evaluating the nutritive value of different types of press cakes in **other species** would help in identifying its most suitable application and could improve the strategic use of press cake as plant-based protein source.

One of the major constraints of using plant-based feed ingredients in fish feed are ANFs. In most cases there is biochemical evidence of their mode of action. However, there are only few dose-response studies that investigated the nutritional effects of ANFs. Commonly their dietary concentration is increased indirectly with the increasing concentration of an ANF containing feed ingredients, which brings about many factors that can obscure the specific effects of individual ANFs. The extent to which ANFs can affect specific traits depends on many factors (e.g. diet formulation, water chemistry, etc.). Detailed information on dose dependent effects would help in narrowing down **ANF specific dietary thresholds**. This could facilitate an estimation of maximum dietary inclusion levels of plant-based feed ingredients, such as press cake, and enable their increased use as fish feed ingredients.

The composition of press cake depends much on the processing characteristics in the oil mills. While much research has focused on the effects of feed processing parameters on the nutritive value of plant-based feed ingredients, little is known about the effects that the **oil pressing conditions** may have. The screw-press operations commonly used to press oil can have different feed extrusion parameters (e.g. temperature, exposure time, single vs. double pressing, etc.). While they are unlikely to be modified by the oil mills to improve the nutritive value of their by-product, this information could help to identify possibilities of increasing the nutritive value of press cake, preferably without negatively affecting oil production.

Evaluating the **techno-functional properties** (e.g. impact of press cake inclusion on physical feed properties and the extrusion process) of press cake would be complementary to the evaluation of its nutritive value. It could further help to better estimate their practical value as potential fish feed ingredients. Investigations that could be of interest are not only related to feed extrusion and processing, but also to logistics. As press cake can contain considerable amounts of CL, some attention should be given to the potential oxidation of fatty acids during transport, storage, and the storage of feed, as it may have nutritional implications.

The experiments that investigated **$InsP_6$ degradation in rainbow trout and Atlantic salmon** provided insight on the faecal appearance of specific lower inositol phosphates. However, only one mineral supplement and one specific phytase were investigated at one level of dietary concentration. More research is needed to be able to gain a deeper understanding of the described mechanisms and the observed effects (e.g. different phytases at varying concentrations, mineral supplementation at different concentrations, etc.).

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Chapter 5:
Summary

5. Summary

Fishmeal is a valuable, protein rich ingredient for fish feed. It is a source of highly digestible crude protein (**CP**) with a balanced amino acid (**AA**) profile, well digestible inorganic phosphorus (**P**), and a highly digestible energy content. However, its availability is decreasing owing to an increasing demand that is driven by the increased production of fish in feed-based production systems. Research has made great advances in counteracting the limited supply of fishmeal. As a result, the majority of dietary CP in fish feed is made available from oilseeds and their processed by-products. Despite the pre-existing research efforts, the continuous evaluation of feed ingredients in search for alternatives to fishmeal is key to facilitate a sustainable growth of feed-based fish production.

Oilseed press cake represents a widely available source of CP. While numerous studies have evaluated the nutritional value of press cake in fish feed, the majority focused on species reared in warmwater production systems. Thus, the objective of this thesis was to add to pre-existing knowledge on press cake and its potential to replace fishmeal in fish feed, with special emphasis on rainbow trout (*Oncorhynchus mykiss* W.).

Initially the nutrient digestibility of various press cakes (linseed, pumpkin seed, rapeseed, soybean, sunflower seed, and walnut kernel cake) was determined in rainbow trout. Two experiments were conducted in which groups of 40 fish (Experiment 1: initial average weight = 300 g) or 45 fish (Experiment 2, initial average weight = 224 g) in four replicates each were fed with the experimental diets. The diet substitution technique was applied whereby 25% of a basal diet were replaced with press cake. Titanium dioxide was used as external marker and faecal samples were obtained by stripping the fish. It was shown (Manuscript 1 and 2), that the unprocessed press cakes differed greatly in their digestibility of crude nutrients, with CP digestibility ranging from as low as 25% (sunflower seed cake) up to 88% (pumpkin seed cake).

To evaluate the effect of fibre fractions on press cake digestibility another digestibility experiment was conducted (Experiment 3: initial average weight = 302 g; n = 4). The fibre fractions of rapeseed cake and sunflower seed cake were reduced using two different processing methods (sieving and dehulling of seeds prior to pressing). The digestibility of the resulting press cakes was determined using the same methodology that was applied to determine the digestibility of their unprocessed counterparts. The fibre-reduced press cake of rapeseed and sunflower seed cake had a substantially higher CP digestibility than their unprocessed counterpart (Manuscript 1). The CP digestibility of fibre-reduced rapeseed cake were 67% and 87% (unprocessed: 42%) and the fibre-reduced sunflower seed cake had a CP digestibility of 75% and 81% for the sieved and dehulled cake respectively. The different nutrient digestibility that was found between the processed and unprocessed press cakes was

discussed to be mainly attributed to the variation in AA content and availability, indigestible fibre fractions, and antinutritional factors.

Three growth experiments were conducted to study the effect of partial replacement of fishmeal with press cake on performance traits (weight gain, feed intake, and feed conversion ratio (**FCR**)) of rainbow trout. In all growth trials groups of rainbow trout (four replicates each) were fed with either a basal diet or diets in which fishmeal CP was in part replaced by press cake based on its CP digestibility that was determined in the preceding digestibility experiments. The fish were fed once daily to apparent satiation for a duration of 63 days in each respective trial, at the end of which the fish that received the basal diet had at least doubled their individual average weight in all experiments. In the first growth experiment (100 fish per experimental unit; initial average weight = 50 g) no significant differences were found across performance traits between the fish that received the basal diet and the fish that received diets in which either 5% or 10% of fishmeal CP was replaced with rapeseed cake CP (Manuscript 1). In the second growth experiment (40 fish per experimental unit; initial average weight = 191 g), different performance traits were observed between the fish that received the basal diet (FCR = 0.86) and the fish that received diets in which either 25% or 50% of fishmeal CP was replaced with CP of fibre-reduced press cake of either rapeseed (FCR = 0.92 and 0.94, respectively) or sunflower seed cake (FCR = 0.88 and 0.91, respectively; Manuscript 1). Only the performance traits of the fish that received the diets in which 25% of fishmeal CP was replaced by sunflower seed cake CP were found not to be significantly lower than the performance traits of the fish that received the basal diet. The reduction of performance traits was discussed to result from an increased dietary content of indigestible fibre fractions and antinutritional factors owing to the inclusion of press cake.

In the third growth experiment (50 fish per experimental unit; initial average weight = 84 g) no significant differences were found across performance traits between the fish that received the basal diet and the fish that received the diet in which 60% of fishmeal CP was replaced with pumpkin seed cake CP (Manuscript 2). The low concentration of fibre fractions and a low content of antinutritional factors were thought to be the reason for its comparably high nutrient digestibility and the observed performance traits. Furthermore, no significant differences were found between the morphological traits of rainbow trout that received the reference diet and the fish that were fed with the pumpkin seed cake diet, when pumpkin seed cake replaced 60% of fishmeal CP except for a significantly decreased hepatosomatic index (1.69 and 1.39, respectively). The decreased hepatosomatic index of the fish that received the pumpkin seed cake diets was discussed to be likely caused by substituting digestible carbohydrates of the basal diets with lipids in the pumpkin seed cake diets to balance the energy density of the diets.

The use of press cake in fish feed introduces substantial amounts of a form of dietary P that has a limited availability in fish, namely phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, $InsP_6$) and its salts (phytate). Therefore, mineral P is commonly supplemented to fish feed, owing to its higher P digestibility. However, global P resources are limited. The supplementation of phytase has been shown to increase the amount of $InsP_6$-P that can be made available in fish, thereby reducing the required amount of mineral P supplements and decreasing the P discharge into effluent water.

To gain further insights regarding the potential utilisation of InsP-P and the formation of inositol phosphate isomers in fish two experiments were conducted. The single and interactive effects of a mineral P supplement (monoammonium phosphate; **MAP**; 1 g P/kg DM of diet) and an $InsP_6$ hydrolysing enzyme (*Aspergillus oryzae* 6-phytase; 2800 FTU/kg DM diet) were compared between rainbow trout and Atlantic salmon (*Salmo salar*). For each species a digestibility experiment was conducted applying common rearing conditions of each species but using the same diets. Four diets (basal diet, basal diet + MAP, basal diet + phytase, and basal diet + MAP + phytase) were offered to triplicate groups of fish (25 rainbow trout or 35 Atlantic salmon per experimental unit) in each experiment. Titanium dioxide was used as external marker and faecal samples were obtained by stripping the fish.

The faecal disappearance of $InsP_6$ was generally low (approximately 8%) but similar between the species when the diets were devoid of either supplement. The supplementation of phytase significantly increased $InsP_6$ disappearance in both species, but the effect was found to be more pronounced in rainbow trout. The analysis of lower inositol phosphate isomers revealed that their hydrolysis progressed to a greater extent in rainbow trout and it suggested that $InsP_6$ is subject to a different degradation pathway in the two species. While no significant interactive effects on $InsP_6$ disappearance were found between the two supplements for either species, the MAP supplementation slightly decreased $InsP_6$ disappearance in Atlantic salmon but not in rainbow trout. The experiments provide an insight into the breakdown of $InsP_6$ and the faecal appearance of specific lower inositol phosphates and suggest that more $InsP_6$-P can be made available in rainbow trout. Therefore, the use of press cake in feed for rainbow trout seems to be more beneficial than in feed for Atlantic salmon with regards to a more sustainable use of P resources. However, more experiments are recommended to complement these initial findings to gain a better understanding of $InsP_6$ hydrolysis in fish.

Chapter 6:
Zusammenfassung

6. Zusammenfassung

Fischmehl ist ein wertvoller Proteinträger für die Herstellung von Fischfuttermitteln. Es beinhaltet hochverdauliches Rohprotein (**XP**) mit einem ausgewogenen Aminosäuremuster, gut verdaulichem anorganischen Phosphor (**P**) und hat einen hochverdaulichen Bruttoenergiegehalt. Durch den ansteigenden Bedarf von Fischfuttermitteln und stagnierenden Wildfangmengen sinkt jedoch die Verfügbarkeit des Fischmehls. Dank intensiver Forschung wird bereits der größte Teil des benötigten XP in Fischfuttermitteln in Form von pflanzlichem XP von Ölsaaten und deren Koppelprodukten bereitgestellt. Das Evaluieren verschiedenster XP-Quellen ist jedoch weiterhin notwendig, um den Bedarf an XP für die steigende Fischfuttermittelnachfrage nachhaltig decken zu können.

(Öl-)Presskuchen sind eine gut verfügbare XP-Quelle. Die ernährungsphysiologischen Eigenschaften von Presskuchen wurden, bislang, größtenteils mit Warmwasserspezies evaluiert. Ziel dieser Doktorarbeit war es, zusätzliche Daten über Presskuchen und deren Potenzial als Fischmehlsubstitut in Fischfuttermitteln, insbesondere für die in Kaltwasseranlagen produzierte Regenbogenforelle (*Oncorhynchus mykiss* W.), zu gewinnen.

Es wurde hierfür zunächst die Nährstoffverdaulichkeit verschiedener Presskuchen (Lein-, Kürbiskern-, Raps-, Sonnenblumenkern-, Soja- und Walnusskernpresskuchen) in Regenbogenforellen bestimmt. Hierzu wurden zwei, als Differenzversuche konzipierte, Verdaulichkeitsversuche durchgeführt in denen Gruppen von 40 Fischen (Verdaulichkeitsversuch 1: anfängliches Durchschnittsgewicht = 300 g) oder 45 Fischen (Verdaulichkeitsversuch 2: anfängliches Durchschnittsgewicht = 224 g), in jeweils vierfacher Wiederholung, mit den Futtermitteln gefüttert wurden. Als Futtermittel wurden jeweils eine Basalration und presskuchen-haltige Futtermittel, in denen die Basalration anteilig zu 25% durch Presskuchen ausgetauscht wurde, eingesetzt. Titandioxid diente jeweils als Indikator. Die Kotproben wurden durch Abstreifen gewonnen und zu einer Sammelprobe je Versuchseinheit vereint. Die XP-Verdaulichkeit der Presskuchen wies starke Unterschiede auf (Manuskript 1 und 2) und variierte von 25% (Sonnenblumenpresskuchen) bis zu 88% (Kürbiskernpresskuchen).

Um den Effekt der Faserfraktionen auf die Nährstoffverdaulichkeit von Presskuchen zu untersuchen wurde ein weiterer Verdaulichkeitsversuch durchgeführt (Verdaulichkeitsversuch 3: 40 Fische je Versuchseinheit, anfängliches Durchschnittsgewicht = 302 g; n = 4). Hierbei wurde die Nährstoffverdaulichkeit von Presskuchen von Rapssaat und Sonnenblumenkernen, deren Faserfraktionen entweder durch Sieben der Presskuchen oder Schälen der Saat vor dem Pressen reduziert wurden, untersucht. Es wurde die gleiche Methodik eingesetzt wie in den vorangegangen Verdaulichkeitsversuchen. Die faser-reduzierten Presskuchen von Rapssaat und Sonnenblumenkernen

hatten eine deutlich höhere XP-Verdaulichkeit als die unbehandelten Presskuchen (Manuskript 1). Die unterschiedliche Verdaulichkeit der unbehandelten und der behandelten Presskuchen wurde auf sich unterscheidende AS-Gehalte und deren Verdaulichkeit, den Gehalt und die Zusammensetzung der verschiedenen Faserfraktionen und auf antinutritive Faktoren zurückgeführt.

In drei Wachstumsversuchen wurde evaluiert inwiefern sich Presskuchen eignet Fischmehl in Futtermitteln für Regenbogenforellen zu ersetzen. Hierbei wurde der Fischmehlanteil in Basalrationen zu verschiedenen Anteilen mit verschiedenen Presskuchen, jeweils auf Basis der XP-Verdaulichkeit, ersetzt und erfasste Leistungsparameter (Zuwachs, Futteraufnahme und Futterquotient (**FQ**)) der Fische miteinander verglichen. In allen Wachstumsversuchen wurden Gruppen von Regenbogenforellen (jeweils vier Wiederholungen) einmal täglich bis zur augenscheinlichen Sättigung über einen Zeitraum von 63 Tagen mit den Versuchsfuttermitteln gefüttert. In allen Versuchen konnte bei den Fischen, welche die Basalrationen erhielten mindestens eine Gewichtsverdoppelung des durchschnittlichen Anfangsgewichts in dem Versuchszeitraum erreicht werden. In dem ersten Wachstumsversuch (100 Fische je Versuchseinheit; anfängliches Durchschnittsgewicht = 50 g) wurde das XP des Fischmehlanteils der Basalration zu 5% und 10% durch XP von unbehandeltem Rapspresskuchen ersetzt. Es konnten keine signifikanten Unterschiede der Leistungsparameter zwischen den verschiedenen Behandlungen festgestellt werden. In dem zweiten Wachstumsversuch (40 Fische je Versuchseinheit; anfängliches Durchschnittsgewicht = 191 g) wurde das XP des Fischmehlanteils der Basalration zu 25% und 50% durch XP von faser-reduzierten Raps- oder Sonnenblumenkernpresskuchen ersetzt. Es wurden unterschiedliche Leistungsparameter zwischen den Fischen welche die Basalration erhielten (FQ = 0,86) und den Behandlungen in denen das XP des Fischmehls zu 25% und 50% durch XP von faser-reduzierten Raps- (entsprechend: FQ = 0,92 und 0,94) oder Sonnenblumenkernpresskuchen (entsprechend: FQ = 0,88 und 0,91) ersetzt wurde festgestellt. Lediglich die Leistungsparameter der Fische die das Futter bekamen in dem 25% des Fischmehls durch faser-reduzierten Sonnenblumenkernpresskuchen ersetzt wurde unterschieden sich hierbei nicht signifikant von den Leistungsparametern der Fische, welche die Basalration erhielten. Die geringere Leistung wird auf den gestiegenen Anteil unverdaulicher Faserfraktionen der Futtermittel und antinutritive Faktoren der Presskuchen zurückgeführt.

In dem dritten Wachstumsversuch (50 Fische je Versuchseinheit; anfängliches Durchschnittsgewicht = 84 g) wurde das XP des Fischmehlanteils der Basalration zu 60% durch XP des Kürbiskernpresskuchens ersetzt. Es wurden keine signifikanten Unterschiede der erfassten Leistungsparameter zwischen den Behandlungen bei Regenbogenforellen festgestellt. Dies wird auf den niedrigen Fasergehalt des Kürbiskernpresskuchens und auf dessen geringen Gehalt an antinutritiven Faktoren zurückgeführt. Ferner, wurden fast keine signifikanten Unterschiede der morphologischen Merkmale zwischen den Behandlungen festgestellt. Lediglich ein signifikant

geringerer hepatosomatischer Index wurde bei den Fischen, welche die Kürbiskernpresskuchenration erhielten festgestellt. Dies wird darauf zurückgeführt, dass verdauliche Kohlenhydrate der Basalration durch Lipide ersetzt wurden, um die Energiedichte des Kürbiskernfuttermittels an die Energiedichte der Basalration anzupassen.

In Pflanzensamen, und somit auch in Presskuchen, liegt P überwiegend in Form von Myo-Inositol 1,2,3,4,5,6-hexakisdihydrogenphosphat; **$InsP_6$**) oder dessen Salzen (Phytat) vor. Da $InsP_6$ eine für Fische nur geringfügig hydrolysierbare Form von P ist, wird Futtermitteln mit hohen Anteilen pflanzlicher Komponenten gewöhnlich mineralischer P zugegeben. Mineralischer P ist für Fische besser verfügbar, dessen Ressourcen sind jedoch begrenzt. Es hat sich gezeigt, dass der Einsatz von Phytasen die $InsP_6$-P Verfügbarkeit in Fischen erhöht, womit sowohl die endlichen mineralischen P-Ressourcen entlastet als auch die in die Umwelt emittierte P-Menge aus Aquakultursystemen verringert werden kann. Um das Potenzial von InsP-P als P-Quelle und dessen Abbau in Fischen näher zu untersuchen wurden zwei Experimente durchgeführt. Die Effekte und Interaktionen der Zulage eines mineralischen P-Supplements (Monoammoniumphosphat; **MAP**; 1 g P/kg TM) und eines $InsP_6$-hydrolysierenden Enzyms (*Aspergillus oryzae* 6-Phytase; 2800 FTU/kg TM) wurden zwischen Regenbogenforellen und atlantischen Lachsen (*Salmo salar*) verglichen. Es wurde für jede Spezies ein Verdaulichkeitsversuch unter jeweils gewöhnlichen Haltungsbedingungen durchgeführt. In beiden Versuchen wurden dieselben vier Futtermittel (Basalration, Basalration + MAP, Basalration + Phytase und Basalration + MAP + Phytase) eingesetzt (n = 3; jeweils 25 Regenbogenforellen oder 35 Atlantische Lachse je Versuchseinheit). Titandioxid diente jeweils als Indikator. Die Kotproben wurde durch Abstreifen gewonnen und zu einer Sammelprobe je Versuchseinheit vereint.

Der native Abbau des $InsP_6$ war in beiden Fällen vergleichbar gering (ca. 8%). Die Zulage der Phytase erhöhte den $InsP_6$-Abbau signifikant, in Regenbogenforellen jedoch zu einem höheren Ausmaß. Die Analyse der niederen Inositolphosphatisomere deutete darauf hin, dass sich die Abbauwege des $InsP_6$ zwischen den Spezies zu unterscheiden scheinen. Ferner war der $InsP_6$-Abbau in Regenbogenforellen weiter fortgeschritten als in atlantischen Lachsen. Es wurden in beiden Spezies keine signifikanten interaktiven Effekte der beiden Supplemente auf den $InsP_6$-Abbau festgestellt. Die MAP-Zulage verringerte jedoch den $InsP_6$ -Abbau bei atlantischen Lachsen leicht. Diese Versuche liefern erste Ergebnisse bezüglich des $InsP_6$-Abbaus und der Formation niederer Inositolphosphatisomere im Kot von Fischen. Die Ergebnisse zeigen, dass in Regenbogenforellen eine höhere Menge $InsP_6$-P verfügbar gemacht werden kann und der Einsatz pflanzlicher Proteinträger, wie Presskuchen, in Bezug der P-Verwertung in Forellenfuttermitteln aussichtsreicher erscheint als in Futtermitteln für atlantische Lachse. Weitere Versuche müssen jedoch unternommen werden, um die präsentierten Informationen zu ergänzen und den $InsP_6$-Abbau in Fischen besser nachzuvollziehen.

Acknowledgements

I would like to express my sincerest gratitude to my supervisor Prof. Dr. Markus Rodehutscord without whose support and encouragement this thesis could not have been realised. His excellent supervision and relentless efforts through all stages of this project were truly amazing and I thank him for sharing his expertise every step along the way. His professionalism and wits never ceased to amaze me, and I am convinced that his guidance did not exclusively affect my scientific skills, for which I am truly grateful. And I cannot forget to thank him for the laughs we shared, as they are often underrated, but nevertheless an important part of the process. Thank you.

I would also like to thank my supervisor Dr. Reinhard Reiter and Dr. Helmut Wedekind from the Institute for Fisheries for giving me the opportunity to work with them and the flexibility to fulfil my tasks within my own schedule. I am grateful for the support that they have offered me and the valuable lessons I learned while working with them.

I thank all external partners that were involved in realising this thesis for their cooperation, especially the Staff from the Fraunhofer IVV in Freising and the staff from the TTE of the University of Natural Resources in Vienna. I am very thankful for your involvement in this thesis, for your fruitful input, and the open dialog.

I thank everyone at the Institute of Animal Sciences for always welcoming me with open arms and for your support on every level imaginable. From laboratory analysis, administration, and IT, to inspiring discussions and logistics, you were always there for me. This type of generosity and kindness across the board is not only hard to find but is overwhelming. I hereby wish to express my deepest gratitude to each and every one of you and wanted to let you know how happy and proud I was to be part of your team.

I would also like to thank everyone at the Institute for Fisheries who offered me their assistance and has helped me realise this project. I highly appreciate your involvement, especially with administration of the projects, technical assistance, and the help with the experiments. I could not have done it without you.

Many thanks go out to the staff from the former aquaculture department who have successfully managed not to scare me off, but instead have increased my interest in the subject area. I am thankful for you sharing your expertise with me and for all fun we had together over the years.

I would also like to thank my dear friends Johannes T., Manuel G., Manuel T., Timo S., Andreas E., Verena E., Marion L., Dorian K., Daniela K., Tobias Z., Karin Z., Gero S., Lena S., Goran G., Jochen K., Wolfgang S., Markus G., Johannes P., Mario E. G. C., Dennis R., Adam R. L., Marcus Z., Christian S.,

Christian V., and Maxim T. for all the great times we had and everything that I was able to learn from you. You have helped me make this (at times very stressful) project a truly wonderful experience that I will never forget.

Finally, I wish to extend a special thanks to my family. For my parents, Jürgen and Alemtsehai, I have nothing but the outmost respect and appreciation. They have always led by example and had continuously gone out of their way to support me, even when times were rough. I thank them for and being there for me whenever I needed you. Moreover, I thank my brother Johannes for being the best brother one could wish for and for always being there for me (even at very odd hours). I am thankful for all the laughs we shared both when I needed cheering up and when things were just simply hilarious. I will never forget how you believed in me when I found it hard to believe in myself and, at times, even helped me with my trials. It is my family's unconditional love and support that has helped me get where I am and for which I can only consider myself to be truly lucky.

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Stuttgart, 15th January 2019 _____ (Alexander Michael Greiling)

Fun Page

(inspired by Andre M.)

Word Search:

Try to find 10 hidden words in the following:

- Hint: All words refer to something or someone indispensable for the realisation of this work.

P R E S S C A K E O J Z F T F
W K D Y D D T F Z K O U E E I
I S X L Q U C Y H W W A T D S
N G O I E A T M T N M W R W H
F M O M V B Y P U W A O V U F
X I S A H F J G O E C Z F J E
R W S F N W A R R S E M Y X E
H R Q H E N K E T A X F B Q D
N Z G Z M X L U W C Z T F A M
S Z B L Z E H B O Q S G Y O R
A R J T F E A H B G K Y M E C
V L S T D M K L N H Y L T E F
K N V O S D N E I R F A V F G
I J R V C Z G X A W W T W K Q
B K U S O A T U R P D Y S L E

The number of letters of the hidden words are indicated below:

a. _ _ _ _ _

b. _ _ _ _ _ _

c. _ _ _ _ _ _

d. _ _ _ _ _ _ _

e. _ _ _ _ _ _ _ _

f. _ _ _ _ _ _ _ _

g. _ _ _ _ _ _ _ _

h. _ _ _ _ _ _ _ _ _

i. _ _ _ _ _ _ _ _ _ _ _ _

j. _ _ _ _ _ _ _ _ _ _ _ _