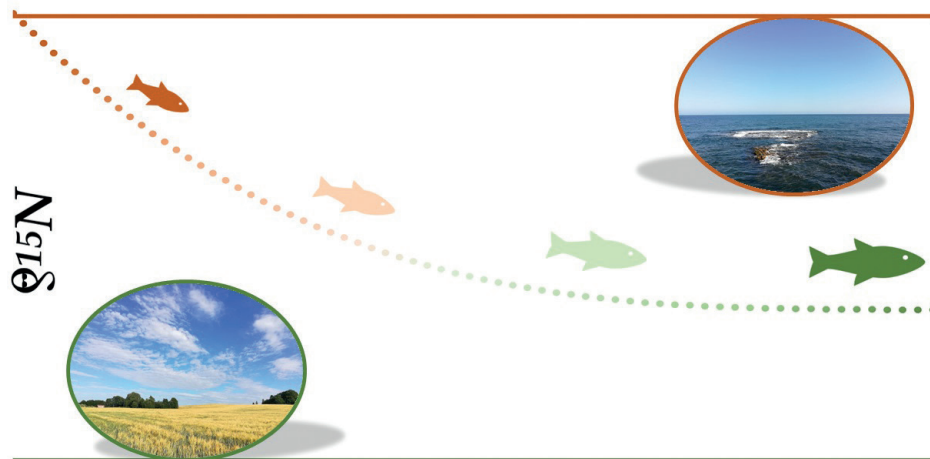


Gerile Siqin

# Complementary Approaches for Assessing Protein Sources in Fish Feed

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JYU DISSERTATIONS 45

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**Gerile Siqin**

# **Complementary Approaches for Assessing Protein Sources in Fish Feed**

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella  
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## ABSTRACT

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Yhteenveto: Vaihtoehtoiset menetelmät kalanrehujen proteiinilähteiden arvioimiseksi

Diss.

Fluctuating fishmeal (FM) prices and declining marine fish stocks have led to the current trend of replacing dietary FM with plant proteins (PP) in fish feed. PPs offer challenges both with respect to nutritional quality and the occurrence of antinutrients. The assessment of utilisation efficiency of PPs for growth is important for the sustainable production of fish feed. The overall aim of this thesis was to investigate the applicability of stable isotope analysis in combination with respirometry in the evaluation of dietary PPs for juvenile rainbow trout (*Oncorhynchus mykiss*). The dietary PPs were corn gluten meal (CGM), rapeseed protein concentrate (RPC) and potato protein (PoP). Complete replacement of FM with CGM was feasible without adversely affecting the metabolic oxygen consumption ( $MO_2$ ) of the trout. However, the growth of fish and apparent digestibility coefficient (ADC) of CGM and/or RPC decreased with their increasing dietary proportion through which the nutritional quality of the diets was compromised. The growth reduction may be caused by lower nutritional quality and antinutrients of the PPs. ADC of CGM and/or RPC was estimated from the faecal nitrogen isotope ratio ( $\delta^{15}N$ ). There was a strong negative correlation between diet-tissue shift ( $\Delta^{15}N$ ) of  $\delta^{15}N$  values and dietary protein quality, and these correlations observed in muscle, liver, mucus and faeces were similar. Carbon and nitrogen turnover in muscle and liver of fish fed with corn and potato diet were estimated using stable isotope mixing model. This thesis only suggests the potential applications of stable isotope analysis and respirometry in fish nutrition research instead of providing an exact replacement level of FM. Stable isotope analysis cannot replace conventional assessment of feed ingredients and feeds, but it will bring new insight into the field of fish nutrition research provided the preconditions for using stable isotope analysis are met. In addition, development of non-lethal approach is possible by measuring the isotope ratio of faeces and mucus.

Keywords: Diet-tissue shift; digestibility; fishmeal; metabolic oxygen consumption; plant protein; rainbow trout; stable isotope mixing model.

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## LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which will be referred to in the text by their Roman numerals I-IV.

- I Siqin G., Laine L., Vielma J., Kiljunen M., Storebakken T. & Pirhonen J. 2018. Dietary fishmeal replacement by plant protein sources decreases growth and induces a shift in  $\delta^{15}\text{N}$  in rainbow trout, *Oncorhynchus mykiss*. Manuscript.
- II Siqin G., Laine L., Kiljunen M., Vielma J. & Pirhonen J. 2018. Estimation of apparent digestibility coefficient of plant proteins with nitrogen stable isotope ratio in juvenile rainbow trout (*Oncorhynchus mykiss*). Manuscript.
- III Siqin G., Kiljunen M., Vielma J., Fernandes R. & Pirhonen J. 2018. Bayesian quantification of carbon and nitrogen turnover in rainbow trout (*Oncorhynchus mykiss*) fed plant-based diet. Manuscript.
- IV Siqin G. & Pirhonen J. 2017. Replacement of fishmeal with corn gluten meal in feeds for juvenile rainbow trout (*Oncorhynchus mykiss*) does not affect oxygen consumption during forced swimming. *Aquaculture* 479: 616–618.

The contribution of Gerile Siqin as well as other authors to preparation of article I-IV are presented in Table 1.

TABLE 1 The contribution of different authors to articles I-IV (GS = Gerile Siqin, LL = Lily Laine, JP = Juhani Pirhonen, MK = Mikko Kiljunen, JV = Jouni Vielma, TS = Trond Storebakken, RF = Ricardo Fernandes).

	I	II	III	IV
Original idea	MK, JV, LL, JP	GS, JP	GS, MK, JP, JV	JP
Data collection	LL, JP	LL, JP	GS, JP, JV	GS
Data analysis	GS, MK, JV, JP	GS, MK, JP	GS, MK, JP, JV, RF	GS, JP
Writing	GS, MK, JV, JP, TS	GS, JP	GS, MK, JP, RF	GS, JP

## ABBREVIATIONS

AA	amino acid
ADC	apparent digestibility coefficient
ANF	antinutritional factor
C	carbon
CF	condition factor
CGM	corn gluten meal
Corn diet	diet based on corn gluten meal
CP	crude protein
$\Delta^{13}\text{C}$	diet-tissue shift of carbon isotope ratio
$\Delta$	diet-tissue shift in general
D <sub>1-6</sub>	diet 1 to diet 6 in the second feeding trial
$\Delta^{15}\text{N}$	diet-tissue shift of nitrogen isotope ratio
EAA	essential amino acid
FW	final weight
FCR	feed conversion ratio
FM	fishmeal
IW	initial weight
Mixed diet	diet containing more than 1 protein source
MO <sub>2</sub>	metabolic oxygen consumption
N	nitrogen
P	phosphorus
PoP	potato protein
Potato diet	diet based on potato protein
PP	plant protein
PP:FM ratio	plant protein to fishmeal ratio in the diet
PRE	protein retention efficiency
RPC	rapeseed protein concentrate
SGR	specific growth rate
Single diet	diet containing only 1 protein source
WG	wet weight gain
$\delta^{13}\text{C}$	carbon isotope ratio
$\delta^{15}\text{N}$	nitrogen isotope ratio



# 1 INTRODUCTION

## 1.1 Current trends in aquaculture

The world aquaculture production increased from 32 million tonnes in 2000 to 77 million tonnes in 2015 with an average annual growth rate of 6 %, while the cost of feed represents about 50–80 % of its production cost (Anon. 2017). Protein is the most expensive ingredient in fish feed. Fishmeal (FM) has been largely used in fish feed for its high nutritional quality. Salmonids, such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), are the preferred farming species in temperate regions, and FM has traditionally been the major protein source in the feed for these high trophic level species. The use of FM exerts pressure on marine fish stocks (Naylor *et al.* 2000), as FM is mainly produced from the small pelagic fish which could be directly used as human food (Cashion *et al.* 2017). In addition, the price of FM is increasing (Anon. 2018), although the marine protein dependency of salmon feed decreased dramatically since 1990 (Ytrestøyl *et al.* 2015). Hence, the replacement of FM with alternative protein sources, such as proteins derived from plants, microalgae, yeast, bacteria and insects, therefore, is becoming paramount. In Finland, rainbow trout accounted for over 90 % of total food fish production in 2017. The use of locally produced plant protein (PP) sources, such as rapeseed and potato, might provide means to produce fish feed in an economically and environmentally viable way.

## 1.2 Plant proteins in fish feed

The demand for PP, as an alternative protein source for replacing FM in fish feed, appears to increase further (Hardy 2010). The nutritional quality of PP, including meals derived from legumes, grains and oilseeds, is inferior to that of FM in diet of carnivorous fish for several reasons. First, PPs are deficient in some essential amino acids (EAA), especially methionine and lysine (Gatlin *et*

*al.* 2007), and some physiologically important compounds such as a sulphur-containing amino acid-like compound known as taurine (Salze and Davis 2015). PPs typically contain several antinutritional factors (ANF), for example non-starch polysaccharides (Kokou and Fountoulaki 2018), alkaloids and protease inhibitor (Francis *et al.* 2001). The presence of ANFs, such as glycoalkaloid, seems to have adverse effect on the palatability of feed thereby reducing dietary feed intake (Tusche *et al.* 2011a). The presence of ANFs is also associated with the reduced digestibility of PPs (Francis *et al.* 2001). Furthermore, most of the phosphorus (P) in plant protein present in the form of phytate which is not digestible for humans and monogastric animals, including fish (Raboy 2001), thereby increasing the P discharge into water bodies. The P and nitrogen (N) excreted with waste materials are the major nutrients contributing to the eutrophication of water bodies. There is also concern for increasing amount of genetically modified plant products in the market (Flachowsky *et al.* 2005), and the presence of genetically modified PPs is also an issue under debate, particularly in Europe (Pusztai and Bardocz 2006).

However, there are some solutions to those limitations mentioned above. The amino acid (AA) profile of compound feed can be improved by mixing various protein sources, through which the AA profile of the final feed can be balanced. For example, post-smolt Atlantic salmon were successfully raised on FM free diet without compromising growth, in which the FM free diet consisted of mixture of PP and poultry meal (Davidson *et al.* 2016). The use of PP concentrates with crystalline AA supplements will further improve the AA profile of the feed, although crystalline AAs are expensive. The combination of various PPs can mutually compensate for the AA deficiency in a specific PP (Gatlin *et al.* 2007, Hardy 2010). The substitution of 50–95 % of dietary FM with mixture of PPs is possible for some carnivorous species, including turbot (*Psetta maxima*), European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), Senegalese sole (*Solea senegalensis*), Atlantic salmon and rainbow trout, without depressing growth and feed utilisation (Burel *et al.* 2000, Kaushik *et al.* 2004, Dias *et al.* 2009, Cabral *et al.* 2011, Johnsen *et al.* 2011, Zhang *et al.* 2012). In addition, modern processing technologies could produce the oilseed by-products with improved nutrient composition and AA profile, and reduced ANFs (Duodu *et al.* 2018). Feed attractants could also compensate the adverse effect of ANFs on dietary feed intake. There are also several solutions for reducing the P waste in the effluent water from aquaculture. First, the availability of P in fish feed could be increased via supplementation of the microbial enzyme phytase. The reduction of P content in plant seeds is also possible by knocking out a specific gene in the node to reduce the allocation of P to seeds (Yamaji *et al.* 2016). With all these pros and cons, PPs have been widely used in fish feed. However, the effect of FM replacement with PP on feed, feed processing, fish and environment need to be investigated further to maximize utilisation efficiency, and to reduce the cost of feed production as well as the environmental impact of aquaculture. In addition, the potential effect of FM substitution with PP on the proportion of the long-chain polyunsaturated fatty acids in the products should be examined carefully since

those fatty acids, especially eicosopentaenoic acid and decosohexaenoic acid, are intertwined with human nutrition and health. For instance, the fatty acid composition in the fillet of gilthead seabream was slightly altered by the 75 % of dietary PP (Francesco *et al.* 2007), while the fatty acid composition in the fillet of Atlantic salmon was not affected by the inclusion of PP up to 95 % in the diet (Pratoomyot *et al.* 2010).

### 1.3 Protein assessment with stable isotopes and respirometry

#### 1.3.1 Isotopic difference among protein sources

Isotopes are the atoms of the same chemical element with same numbers of protons but different numbers of neutrons in their nuclei. Stable isotopes are natural parts of an organism and they do not decay over time. The heavier forms of stable isotopes, such as  $^{13}\text{C}$  and  $^{15}\text{N}$ , have an extra neutron in their nucleus. As a consequence, the heavier and lighter forms of same element are differentially assimilated into the tissue of plants and animals, because of the physical and chemical fractionation driven by the extra neutron during complex biological processes (Fry 2006). The carboxylation enzymes in the chloroplast of plants have differing fractionation effect against  $\text{CO}_2$  incorporation during photosynthesis, leading to different photosynthetic pathways, *i.e.*  $\text{C}_3$ ,  $\text{C}_4$  and CAM (Crassulacean Acid Metabolism), in plants (Farquhar *et al.* 1989). Consequently, variation in the carbon (C) isotope ratio ( $\delta^{13}\text{C}$ ) is seen among  $\text{C}_3$  and  $\text{C}_4$  plants. The  $\delta^{13}\text{C}$  value is near  $-28\text{‰}$  and  $-14\text{‰}$  in  $\text{C}_3$  and  $\text{C}_4$  plants, respectively (Farquhar *et al.* 1989). The isotope ratio of  $\text{C}_3$  and  $\text{C}_4$  plants is reflected further in that of the consumer tissue. The heavier forms of the stable isotopes are preferentially assimilated into the animal tissue during biological reactions such as assimilation, and therefore upper trophic level consumers have higher values of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  compared to those at the lower trophic level. The enrichment of heavier isotopes between trophic levels is stepwise. For example, the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values increase about 1 and 3.2 ‰, respectively, at each unit of increase in trophic level (Le Vay and Gamboa-Delgado 2011), depending on food quality, life stages, temperature and species. Consequently, the  $\delta^{15}\text{N}$  of the protein sources derived from animals, such as FM, is higher than that in the PPs. These isotopic differences between different sources, both in terms of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, allow researchers to mathematically estimate the contribution of a particular source relative the others in a mixture of different sources. In ecological research,  $\delta^{13}\text{C}$  values are typically used to identify the origin of different sources of C within a food web (Jones *et al.* 1998). The trophic position of the consumers within a food web also can be estimated based on the combination of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values (Post 2002, Quezada-Romegialli *et al.* 2018). Animal tissues is a record of the isotope ratio present in its diet. For example, European eels (*Anguilla anguilla*) from habitats with different salinity were differentiated based on the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values present in their muscle tissue

(Harrod *et al.* 2005). In mammalian research, the  $\delta^{13}\text{C}$  value of tooth enamel from fossil equids (horses) has been applied to uncover the  $\text{C}_3/\text{C}_4$  composition in their diet (Cerling *et al.* 1997).

### 1.3.2 Isotopic difference between animal and its diet

Animal consumers asymptotically reach isotopic equilibrium following a dietary change, if the same diet is constantly available (Gamboa-Delgado *et al.* 2008, Martínez del Rio and Carleton 2012). It takes time for animal tissues to eventually come to isotopic equilibrium with their new diet (Fry and Arnold 1982). A 6- to 8-fold increase in weight is required to reach isotopic equilibrium according to Herzka (2005). Juvenile rainbow trout reached isotopic equilibrium with a 15-fold increase in weight over an 80-d feeding trial (Badillo *et al.* 2014). At the equilibrium stage, the isotopic difference between the diet consumed and animal tissues, *i.e.* the diet-tissue shift ( $\Delta$ ), becomes constant (Fry and Arnold 1982).

At this point, 3 main questions appear to be critical. Namely, why there is an isotopic difference between animal and its diet, and when does this isotopic difference becomes constant, and what are the main factors influencing the magnitude of this isotopic difference.  $\Delta$  is a net result of the multiple changes associated with series of biological reactions (Sutka *et al.* 2008) during which the heavier isotopes (e.g.  $^{13}\text{C}$  and  $^{15}\text{N}$ ) are preferentially incorporated into the tissues, and therefore it is difficult to predict both mathematically and experimentally. For instance, the  $^{15}\text{N}$ -enrichment of consumer tissue over its diet is potentially related to the transamination and oxidative deamination of AAs (Martínez del Rio and Wolf 2005, Barreto-Curiel *et al.* 2018).

The magnitude of  $\Delta$  is potentially affected by various factors such as feeding level, diet quality, water temperature, species and tissue type. Juvenile organisms are preferred in experiments using stable isotopes since they have potential to grow fast and reach isotopic equilibrium in a relatively short period of time, and also space requirement is less with juvenile organisms. The  $\Delta$  following a dietary change has been considered as a combined effect of accretion of new tissue (growth) and metabolic turnover of existing tissue, and their relative contribution can be separated using an exponential model (Hesslein *et al.* 1993). In rapidly growing juvenile organisms, it seems that  $\Delta$  is largely driven by growth (Vander Zanden *et al.* 2015), which depends on diet quality in controlled laboratory feeding experiments (Gamboa-Delgado and Le Vay 2009a, Zhou *et al.* 2016). Previous studies on Pacific white shrimp (*Litopenaeus vannamei*) showed that the magnitude of  $\Delta$  increases with the decreasing nutritional quality of dietary protein (Gamboa-Delgado and Le Vay 2009a, Gamboa-Delgado *et al.* 2013, 2014, 2016).

### 1.3.3 Stable isotope mixing models

Stable isotope analysis has been used to evaluate the movement of chemicals in various ecosystems. The proportional contributions of different potential

sources towards a biological mixture can be estimated using stable isotope mixing models. For example, the relative contribution of potential prey fish (sources) to the diet of European catfish (*Silurus glanis*) population has been estimated using a 2-source mixing model in the study of river ecosystem (Syväranta *et al.* 2009). Stable isotope mixing models are mathematical tools that incorporate the isotopic composition of a mixture and its potential sources to determine the proportions of sources in that mixture. In nutritional experiments with fish, the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of the protein sources and fish tissue can be used to estimate the proportional contribution of dietary proteins to the tissue of fish raised on diet containing more than 1 protein source (mixed diet) using mixing models (Gamboa-Delgado *et al.* 2008, Badillo *et al.* 2014, Filbrun and Culver 2014, Zapata *et al.* 2016). A diet containing individual test protein source (single diet) is formulated to determine the  $\Delta$  for specific test protein. At the equilibrium stage, the isotope ratios of test proteins are corrected for  $\Delta$  before incorporating them into mixing model with the isotope ratio of the fish reared on mixed diet. The determination of  $\Delta$  for each test protein is necessary as  $\Delta$  might differ substantially between protein sources (*e.g.* PP vs. FM).

Compound feed contains various ingredients such as proteins, fish oil and starch. In experiments using stable isotopes, the single diet must contain 1 protein source to determine the  $\Delta$  for that specific protein. However, it is impossible to produce a diet with adequate physical and nutritional quality using only 1 protein source. For example, carnivorous fishes, such as rainbow trout, do not grow well on the single diet containing only 1 type of PP, primarily due to ANFs, AA imbalance, poor palatability and digestibility. The metabolic stress induced by the poor nutritional quality of PP in the single diet prevents the fish from reaching isotopic equilibrium, and it is difficult to determine the  $\Delta$  of PP at isotopic equilibrium in the experiment with carnivorous fish. Nonetheless, it is important to uncover how the dietary PP is utilized in carnivorous fish raised on single diet, because this type of experiment may provide new insight into the difficulties to achieve isotopic equilibrium.

Various types of mixing models have been increasingly used in ecological research (Phillips *et al.* 2014). The simplest version of mixing model is based on the linear interpolation between 2 known points (Focken 2004). However, biological mixing is far more complex than linear interpolation, especially in an ecosystem. The measured isotope ratio of the biological mixture does not always linearly respond to that of the sources due to various factors such as difference in elemental concentration and digestibility. In addition, the magnitude of  $\Delta$  used to correct the isotope ratio of sources depends on various factors such as diet quality and tissue type. In this context, it is not always valid to assume a constant  $\Delta$  for different diets or tissues. Furthermore, variation in the isotope ratio of sources is propagated into the uncertainties of mixing model outputs. There are also many potential food sources in an ecosystem compared to the limited numbers of stable isotope tracers. In order to cope with the problems mentioned above, more sophisticated mixing models were developed (Phillips *et al.* 2014) based on the previously developed simple linear models,

including some Bayesian mixing models such as IsoSource (Phillips and Gregg 2003), SIAR (Parnell *et al.* 2010), MixSIAR (Stock *et al.* 2018) and FRUITS (Fernandes *et al.* 2014). These Bayesian models take the different sources of uncertainties related to the simple linear models into account to provide more robust estimation (Phillips *et al.* 2014).

However, mixing models have been less frequently used in fish nutrition research compared to their application in food-web studies (Phillips *et al.* 2014). A few studies have used mixing models, most of them simple linear models such as IsoError (Phillips and Gregg 2001) (Table 2). The IsoError model considers the variation in the isotope ratio of both sources and mixture but it does not address the significant difference in elemental concentration. The IsoConc (Phillips and Koch 2002) model does consider the significant difference in elemental concentration, but it neglects the uncertainties associated with the variation in isotope ratios.

TABLE 2 Applications of stable isotope mixing models in fish nutrition research.

Species	Tracers	Models	References
<i>Oreochromis niloticus</i>	$\delta^{13}\text{C}$	IsoError	Focken 2004
<i>Cyprinus carpio</i>	$\delta^{13}\text{C}$	IsoError	Schlechtriem <i>et al.</i> 2004
<i>Solea senegalensis</i>	$\delta^{13}\text{C}$	IsoError	Gamboa-Delgado <i>et al.</i> 2008
<i>Clarias gariepinus</i>	$\delta^{13}\text{C}$ & $\delta^{15}\text{N}$	SIAR	Enyidi <i>et al.</i> 2013
<i>Oncorhynchus mykiss</i>	$\delta^{15}\text{N}$	IsoConc	Badillo <i>et al.</i> 2014
<i>Ictalurus punctatus</i>	$\delta^{13}\text{C}$ & $\delta^{15}\text{N}$	MixSIR	Filbrun and Culver 2014
<i>Totoaba macdonaldi</i>	$\delta^{15}\text{N}$	2-source, 1-isotope	Zapata <i>et al.</i> 2016
<i>Rachycentron canadum</i>	$\delta^{15}\text{N}$	IsoError	Zhou <i>et al.</i> 2016

### 1.3.4 Metabolic oxygen consumption

With the increasing level of FM replacement with PPs in modern aquaculture, the potential effect of growing fish on low FM diet has been studied from different perspectives, including growth, fatty acid composition of the tissue and gene expression (Terova *et al.* 2013, Rimoldi *et al.* 2015, Lazzarotto *et al.* 2018). Changes in dietary composition, e.g. dietary proteins, have certain effect on the oxygen consumption of fish, which is potentially associated with changes in metabolism (Kaczanowski and Beamish 1996, Saravanan *et al.* 2012, 2013a, b). Change in oxygen consumption is indirectly associated with both metabolic rate and physiological state of fish. However, there has been little published data on metabolic oxygen consumption ( $\text{MO}_2$ ,  $\text{mg kg}^{-1} \text{h}^{-1}$ ) and swimming capacity of fish raised on different protein sources such as FM and PPs.

Indirect calorimetry, also known as respirometry, is the conventional method for measuring metabolism (heat production) in fish. The  $\text{MO}_2$  of the

fish can be easily determined with respirometry in a respirometer. The oxygen demand of the fish can be affected by various factors such as fish size and water temperature. In a study with Atlantic salmon, there were no significant differences between treatments in terms of  $MO_2$ , swimming performance and recovery ability from swimming exercise, regardless of the dietary sources of lipids (fish oil, poultry fat and vegetable oil) and proteins (FM and poultry by-product meal) (Wilson *et al.* 2007). Previous studies have shown that the increasing level of dietary PPs in the diet of carnivorous fish could adversely affect the growth performance (Panserat *et al.* 2009, Rimoldi *et al.* 2015) which also may lead to further change in the composition of muscle tissue. Information associated with metabolic rate and physiological state of fish raised on PP-based diet is critical for establishing an appropriate growing condition in practice. However, a systematic understanding of how the increasing level of PPs affects the  $MO_2$  and swimming performance of carnivorous fish is still lacking.

## 1.4 Protein assessment with other methods

The use of PPs in fish feed have been studied using various approaches from different perspectives, including the effects on extrusion parameters, physical quality and digestibility of the feed produced (Draganovic *et al.* 2011, Gong *et al.* 2017), as well as the growth performance,  $MO_2$ , and both gastrointestinal and immune system of the fish (Wilson *et al.* 2007, Urán *et al.* 2008, Storebakken *et al.* 2015, Zhou *et al.* 2017). In the following chapters, the standard methods used in fish nutrition studies are briefly summarized.

### 1.4.1 Growth performance and production parameters

In general, a growth trial is conducted to observe the relevant responses of the fish to the dietary treatments which contain graded levels of test nutrient such as FM. The growth of the fish depends on the genetic strain, life stage, rearing conditions, sex, diet and feeding level. Growth parameters, such as weight gain (WG), are measured and compared among different dietary groups. If the initial weight (IW) of the experimental fish is similar, specific growth rate (SGR) also can be expressed and compared between treatments, based on the natural logarithm of initial and final weight (FW) of dietary treatment. In addition, the feed conversion ratio (FCR), *i.e.* the weight of feed fed to the fish for obtaining a unit of WG, is also a very important parameter to evaluate the capacity of feed formulation to support fish growth. Further, the nutrient components of both feed and fish consumed can be analysed via proximate analysis (Fig. 1), and the retention efficiency of a specific nutrient (nutrient gain/nutrient intake), for example protein retention efficiency (PRE), can be quantified.

With proximate analysis, the crude protein (CP) content is determined by Kjeldahl method (McDonald *et al.* 2011). However, the Kjeldahl method

requires toxic chemicals, such as  $H_2SO_4$  and  $NaOH$ , in order to quantify the N content (McDonald *et al.* 2011). The chemical waste produced during the analyses requires further treatment. Compared to Kjeldahl method, the N content of a sample can be rapidly determined using Dumas method (Marcó *et al.* 2002) without using hazardous chemicals for sample digestion (McDonald *et al.* 2011). The N content can be readily determined during the measurement of isotope composition of a c. 0.6 mg of sample with mass spectrometer coupled with elemental analyser as well, and the N content can be further converted into CP using a proper conversion factor (*e.g.* 6.25).

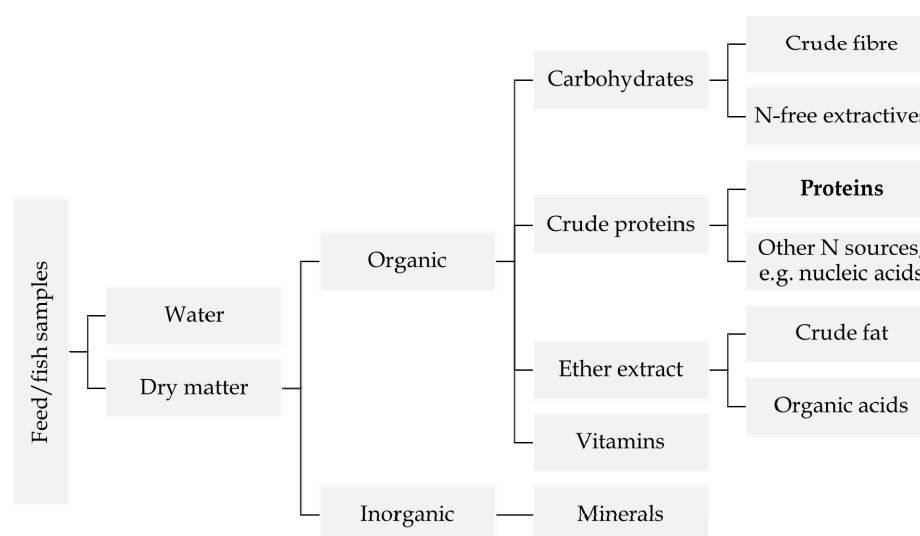


FIGURE 1 Proximate analysis for nutrient components of feed and fish samples (adapted and redrawn from McDonald *et al.* 2011).

However, the CP content is determined on the basis of total N using Kjeldahl method, Dumas method and stable isotope analysis, through which the nonprotein N sources cannot be separated, and therefore these methods may lead to unexpected overestimation of CP content. Recently, some advanced technologies have been proposed and discussed to improve the reliability and specificity of the previous methods (Moore *et al.* 2010, Otter 2012, Cubero-Leon *et al.* 2014).

#### 1.4.2 Digestibility experiment

Digestion is the first step after a consumer ingests food. Digestibility is a measure to determine the proportion of digestible nutrients after the digestion processes. The protein digestibility in fish feed has been studied using both *in vivo* and *in vitro* methods. However, the *in vitro* method cannot closely simulate the digestive processes of a living fish (Carvalho *et al.* 2016). Thus, the *in vivo* method has been commonly used.  $\delta^{13}C$  values have also been used to estimate the digestibility of  $C_3$  and  $C_4$  plants in the diets for tambaqui (*Colossoma macropomum*) (Oliveira *et al.* 2007), but there is limited information about using stable isotope analysis in digestibility trial with fish.



The measurement of true digestibility is impossible due to contamination from endogenous cells and nutrient leaching of the faeces collected, and therefore the term “apparent digestibility coefficient” (ADC) has been applied in fish nutrition research (Bureau *et al.* 2003). The straightforward way to estimate ADC of a nutrient is to quantify the concentration of nutrient both in feed consumed and faeces produced during the entire feeding trial, but it is extremely difficult to quantify the total amount of feed consumed and faeces produced accurately due to the aqueous environment (Bureau *et al.* 2003). Therefore, the ADC has been conventionally estimated indirectly by introducing inert, indigestible digestion markers into experimental diets (Austreng *et al.* 2000). The digestion marker (*e.g.* chromic oxide) needs to be homogeneously mixed into the diet. The sampling of a representative faecal material, however, is important for determining ADC. The fish require some time to adapt to the experimental diet, and the faeces should be sampled after this acclimation period. Chemical analyses are further carried out to determine the nutrient and marker concentrations both in faeces and corresponding diet, so that the ADC of a specific nutrient can be estimated based on the nutrient and marker concentration (Bureau *et al.* 2003). In digestibility experiment with fish, sampling of faeces is challenging, and underestimation or overestimation of ADC of the nutrients occurs (Belal 2005) depending on the sampling method applied (Fig. 2).

Conducting a digestibility trial in juvenile fish is technically demanding. Firstly, it is challenging to collect enough faeces for the chemical analyses. Further, the use of active collection method induces too much stress on juvenile fish, and it may also increase the contamination from endogenous cells (Fig. 2). The passive collection of faeces from the effluent water, therefore, could be an appropriate option for juvenile fish (Storebakken *et al.* 1998), although nutrient leaching can occur in the water (Fig. 2). Compared to the mixing of digestion marker at physical level, stable isotopes are bound chemically with the ambient molecules in the faeces, and therefore the aqueous environment might have less impact on the isotope ratio of faeces. To my knowledge, there has been no detailed investigation of ADC estimation in fish nutrition research using stable isotopes.

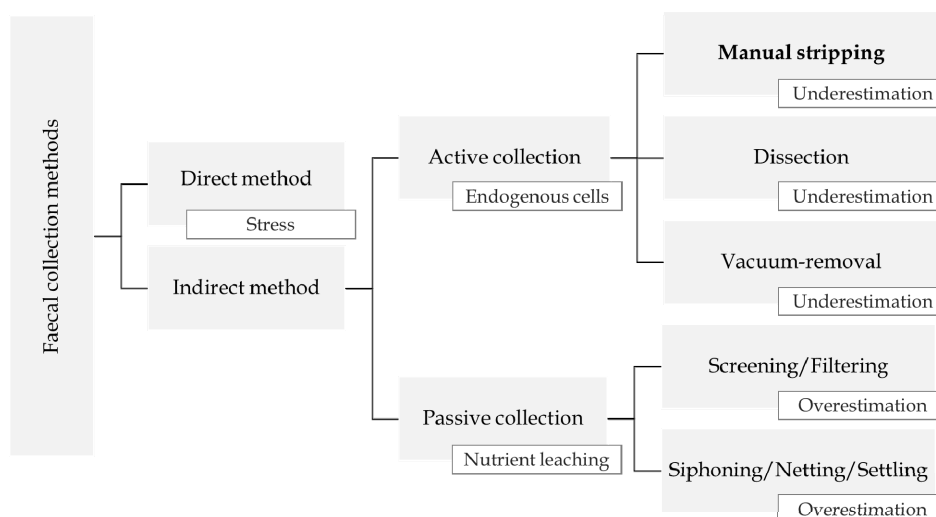


FIGURE 2 The methods for sampling faeces for determining apparent digestibility coefficient (ADC) of nutrients and notes on their likely biases to experimental conclusions (illustration based on the narration by Bureau *et al.* 2003).

### 1.4.3 The use of omic technologies

There are constant interactions among the genome, RNA, proteins and metabolites inside a cell, and the change in diet formulation may affect the interactions between them (Vera *et al.* 2017). In order to unfold the molecular picture of how fish metabolism responds to external factors such as dietary proteins, more advanced technologies have been applied in fish nutrition research (Fig. 3), including genomics, transcriptomics, proteomics and metabolomics (Rodrigues *et al.* 2012, Tacchi *et al.* 2012, Jobling 2016).

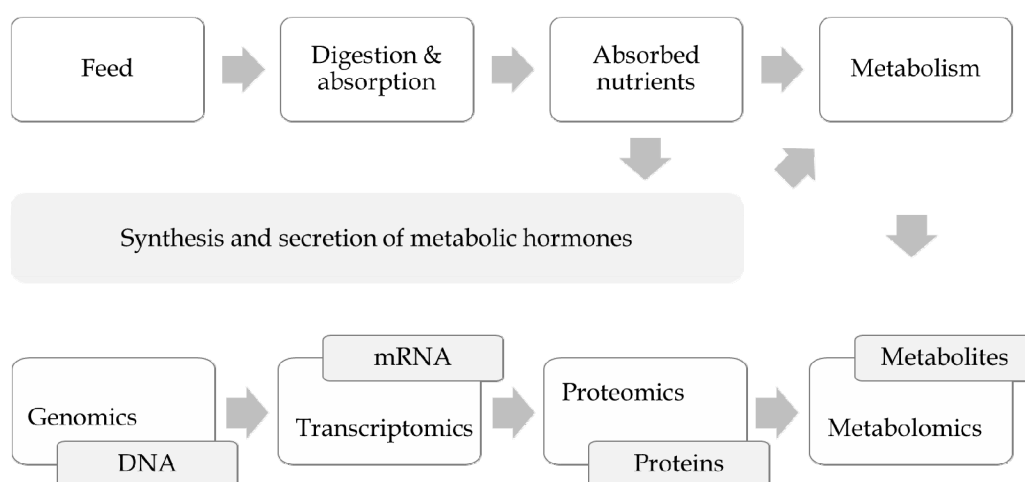


FIGURE 3 The change in feed formulation and the consequent change in the metabolic pool of absorbed nutrients and their metabolic pathways; the effect of feed formulation on the metabolism of fish can be studied at molecular level using multiomics approach (adapted and redrawn from Jobling 2016).

For example, some proteins are involved in transcription and enzymatic activity. The interactions are complex inside a living organism, and therefore the combined use of different approaches, *i.e.* multiomics, might give a broader insight into how the change in diet compositions alters the cellular processes and interactions among them.

## 1.5 Aims of the study

The main objectives of this study were a) to examine the potential applications of stable isotope analysis in fish nutrition research, comparing the results from stable isotope analysis with the growth and production parameters, and b) to assess the utilisation of PPs in the compound feed for juvenile rainbow trout, using stable isotope analysis with the measurement of growth and production parameters including WG, FCR, SGR and PRE. The specific research objectives of the articles I-IV were:

- I To examine the relationship between growth parameters (WG, FCR and PRE) and  $\Delta$  of mucus, muscle and liver tissue of juvenile rainbow trout; to examine the relationship between  $\Delta$  and PP:FM ratio in the diet.
- II To estimate the ADC of corn gluten meal (CGM), rapeseed protein concentrate (RPC) and their mixture using  $\delta^{15}\text{N}$  value of faeces sampled from juvenile rainbow trout fed extruded feed containing graded level of FM.
- III To quantify the turnover of C and N in the muscle and liver tissue of juvenile rainbow trout fed potato protein (PoP)- and CGM-based diets using Bayesian mixing model; to assess the assimilation of N derived from PoP and CGM in the muscle and liver tissue of juvenile rainbow trout.
- IV To assess the potential effect of dietary FM and CGM on the  $\text{MO}_2$  and swimming capacity of juvenile rainbow trout using a swim-tunnel respirometer.

## 2 MATERIALS AND METHODS

### 2.1 Feeding experiment in a flow-through system

A large sample (Table 3) of all-female juvenile diploid rainbow trout were transferred to the laboratory of the University of Jyväskylä from a commercial fish farm (Hanka-Taimen Ltd., Finland). The fish were maintained on the commercial diet from the farm until start of the feeding trials. The fish were randomly distributed into the experimental aquaria (Table 3). Each aquarium (15 l) represented 1 experimental unit, and each of them was an independent flow-through system with common header tank. The system water was filtered from a bore-hole using a 3-step filtration system (Viqua AWP110-2 sediment filter with 5  $\mu\text{m}$  mesh size, Pentair Structural water softener and a limestone column).

All the experiments were carried out under the permit ESAVI/10807/04.10.07/2013 from the Animal Experiment Board in Finland.

TABLE 3 The experimental set-up of the 2 feeding trials. Initial weight is expressed as mean  $\pm$  SD.

Article	Number of fish	Initial mass g	Acclimation period d	Fish/aquarium	Treatments	Replicates /treatment
I and II	270	6.3 $\pm$ 0.1	28	15	6	3
III	153	6.6 $\pm$ 0.2	11	17	3	3

## 2.2 Diet formulation and production

Experimental diets were produced at Laukaa fish farm of the Natural Resources Institute, Finland, using a twin-screw Creusot-Loire (Clextal) extruder. The isotope composition of the ingredients was measured before designing the feed formulation, and the protein sources with distinct isotope ratio were selected to produce the diets with distinct isotope ratio (Fig. 4). Prior to processing the feeds, the isotope ratio of the final diets was estimated with mass balance mixing equation (Fry 2006).

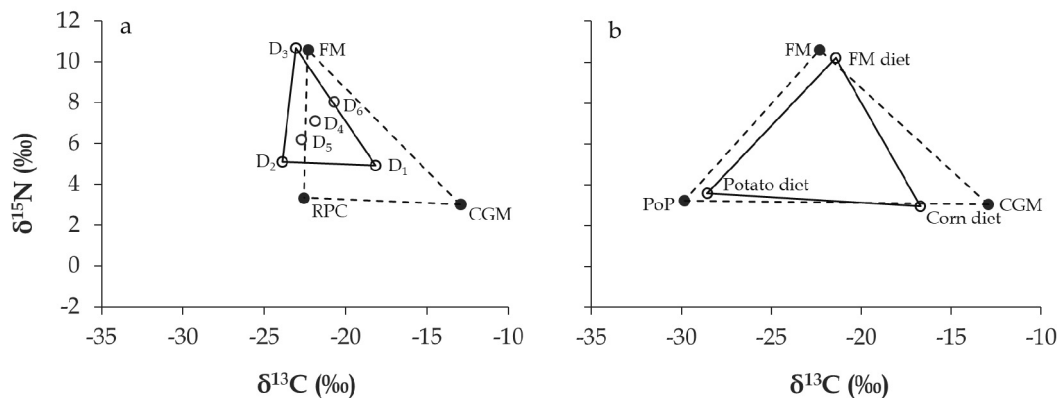


FIGURE 4 Isotopic composition of the protein sources (solid circles) and experimental diets (empty circles). a) First feeding trial (I and II): 6 diets (D<sub>1</sub>–D<sub>6</sub>) were produced using fishmeal (FM), corn gluten meal (CGM) and rapeseed protein concentrate (RPC) as protein sources. b) Second feeding trial (III and IV): FM diet, corn diet and potato diet were produced using FM, CGM and potato protein (PoP) as protein sources. The data points were connected using dash (proteins) and solid (diets) lines to make the illustration more graphic.

Homogenously mixed ingredients were conditioned with 20 % boiling water, and 3 % of the fish oil was added and mixed to produce a malleable mash prior to the extrusion process. The mash was pressed through the extruder barrel at 130–140 °C, using a 2 mm die. The pellets were dried at 40 °C and crumbled, sieved and coated with fish oil. The completed feeds were kept in airtight plastic bags at -20 °C until use. The proximate composition of final feeds was analysed via proximate analysis at the Finnish Food Safety Authority (I and II) and Eurofins Scientific Finland Ltd. (FM and corn diet used in III and IV), using standard methods. The standard conversion factors for carbohydrate (17 kJ g<sup>-1</sup>), protein (27 kJ g<sup>-1</sup>) and lipids (39 kJ g<sup>-1</sup>) were applied to estimate the approximate gross energy content of the FM diet and corn diet (Jobling 1994). The isotope composition of final feeds was also measured.

## 2.3 Feeding and sample collection

In both feeding experiments, fish were fed to apparent satiation twice per weekday (9–10 AM, 15–16 PM), and faeces, mucus and uneaten pellets were removed c. 20 min before feeding. The fish were reared at the conditions presented in Table 4. The fish were sampled after c. 65 h feed depletion over the weekends in order to reduce the effect of stomach content on estimation of mass and the effect of this fasting period on growth was assumed negligible based on the results from a previous study (Nikki *et al.* 2004). Sampled fish were frozen at  $-20\text{ }^{\circ}\text{C}$  until the measurement of isotope ratio. Scales, skin and gall bladder were separated carefully from the mucus, dorsal muscle and liver samples, respectively.

TABLE 4 Rearing conditions during the first (I and II) and second (III and IV) feeding trial. Data are expressed as mean  $\pm$  SD. In IV, the range of water temperature and dissolved  $\text{O}_2$  is provided.

Article	Temp. $^{\circ}\text{C}$	Dissolved $\text{O}_2$ $\text{mg l}^{-1}$	Conductivity $\mu\text{S cm}^{-1}$	pH	Photoperiod D:L ratio	Flow $\text{l min}^{-1}$
I and II	$16.4 \pm 0.1$	$8.1 \pm 0.1$			12:12	0.7
III	$15.3 \pm 0.2$	$9.1 \pm 0.2$	$217.4 \pm 8.7$	$6.9 \pm 0.1$	12:12	$0.6 \pm 0.03$
IV	14.8–15.2	8.5–9.5			12:12	

Prior to the first feeding trial, 1 fish from each aquarium was sampled for measuring the initial isotope ratio of different tissues. The first feeding trial continued 6 weeks, and 1 fish from each aquarium was sampled for stable isotope analysis at each sampling point (sampling points: day 5, 10, 15, 22, 32 and 42). From each sampled fish, the external mucus was collected using previously developed method (Church *et al.* 2009), and after the sampling of mucus, the faeces were sampled by stripping method (Austreng 1978). During handling, fish were anaesthetised with clove oil (dissolved in ethanol at 1:10).

Before the start of second feeding trial, 10 fish were sampled to determine the initial isotope ratio of muscle and liver tissue. During the second feeding trial, fish were fed with FM, potato and corn diet over 7 weeks in aquaria, and 1 fish from each aquarium was sampled for stable isotope analysis each week. After 7 weeks, the fish from the 3 replicates of each dietary treatment were combined and transferred into 3 different 500 l flow-through tanks where the feeding trial continued with the same diet, but with a larger pellet size. Including the 7 weeks in aquarium, the fish were fed with FM, potato and corn diet 87, 107 and 128 d, respectively. At the end of second feeding trial, 5 fish from each treatment were sampled for stable isotope analysis.

## 2.4 Respirometer experiment

Ten fish from each of FM and corn treatment were selected for respirometer experiment. The experiments were conducted in a 10 l swim-tunnel respirometer, connected to an optical oxygen meter (Loligo Systems, Viborg, Denmark). As stated by the manufacturer, the 10 l swim-tunnel respirometer is appropriate for fish from 50 to 150 g. To avoid the confounding effect associated with size differences, the respirometer experiment of fish fed corn diet was carried out after day 128 since the wet weight of those fish was not similar with that of fish fed on FM diet at day 87. The average weight of the fish from 2 treatments was between 74 to 81 g. However, the feeding of potato treatment was ended on day 107 due to mortality, and the average weight was c. 18 g, and therefore the potato treatment was excluded from the respirometer experiment.

The intermittent test of each fish consisted of 18 loops, 210 s for each loop (Table 5). Each loop contained 3 steps, *i.e.* 90 s of flushing (oxygenated water pumped into the measurement chamber from the ambient bath), 60 s of waiting (chamber was closed to reach equilibrium in O<sub>2</sub> concentration) and 60 s of measuring (chamber was closed) step. The MO<sub>2</sub> of each fish was calculated by the AutoResp software (Loligo Systems, Viborg, Denmark) on the basis of the decline in dissolved oxygen concentration over the 60 s of measuring step.

Before the test of each fish, water flow was adjusted to be constant based on fish total body length (BL). Each test consisted of acclimation, exercise and recovery period (Table 5). During the acclimation period, the fish were allowed to recover from handling (netting, measuring weight and length) over the 6 loops at water flow of 0.5 BL s<sup>-1</sup>. In the exercise period, water flow was adjusted from 1 to 2.5 BL s<sup>-1</sup>, and there was a recovery loop (0.5 BL s<sup>-1</sup>) after each increase in water flow by 0.5 BL s<sup>-1</sup>. There were 2 consecutive loops at 2.5 BL s<sup>-1</sup>. After the exercise period, the fish were allowed to recover from the exercise over a 4 consecutive loops at 0.5 BL s<sup>-1</sup>.

TABLE 5 Water flow (BL s<sup>-1</sup>) during each measurement consisting of 18 loops (250 s loop<sup>-1</sup>) over 3 different periods.

Period	Acclimation period						Exercise period								Recovery period			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Flow	0.5	0.5	0.5	0.5	0.5	0.5	1.0	0.5	1.5	0.5	2.0	0.5	2.5	2.5	0.5	0.5	0.5	0.5

## 2.5 Calculation of production parameters

At each sampling point, the fish biomass in each aquarium was weighed. The wet weight and length of each sampled fish were also measured. The proximate

composition of fish was analysed via proximate analysis at Finnish Food Safety Authority (I and II) using standard methods. Wet weight gain (WG), feed conversion ratio (FCR), protein retention efficiency (PRE), specific growth rate (SGR) and PP:FM ratio (see I) and Fulton condition factor (CF) (III) were calculated.

## 2.6 Measurement of isotope composition

Samples for stable isotope analysis were freeze-dried in glass vials, and pulverized with a sharpened nickle spatula following drying. The measurements of isotope ratio were carried out at the University of Jyväskylä, Finland. The pulverized homogenous samples were weighed to c. 0.1–0.6 mg and loaded into tin capsules to measure the isotope composition with a Carlo Erba Flash EA 1112 elemental analyser interfaced with Thermo Finnigan DELTA<sub>plus</sub> Advantage continuous-flow stable isotope ratio mass spectrometer (CF-IRMS). Each sample was analysed in duplicate in the first feeding trial (I and II) and once in second feeding trial (III). The dried white muscle of pike *Esox lucius* was analysed as internal standard in duplicate, after every 10 measurements in each sequence.

The isotope values of C and N were expressed in the delta notation ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) to describe the deviation from that of Vienna Pee Dee Belemnite (VPDB, Eq. 1) and atmospheric N (Eq. 1 in I) in parts per mil (‰). There are various terms to describe the isotopic difference between diet consumed and animal tissues. In this thesis, the term “diet-tissue shift” was used to avoid confusing interpretation (Auerswald *et al.* 2010). For clarity, faeces were considered as tissue throughout this thesis. The  $\Delta$  of  $\delta^{13}\text{C}$  (Eq. 2) and  $\delta^{15}\text{N}$  (Eq. 2 in I) value was calculated by subtracting the isotope ratio of the diet from that of the corresponding tissue.

$$\delta^{13}\text{C} = \left[ \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{VPDB}}} - 1 \right] \times 1000 \quad \text{Eq. 1}$$

$$\Delta^{13}\text{C} = \delta^{13}\text{C}_{\text{tissue}} - \delta^{13}\text{C}_{\text{diet}} \quad \text{Eq. 2}$$

## 2.7 Analysis of stable isotope data

The  $\Delta^{15}\text{N}$  values were compared among the 6 different dietary treatments within the tissue and among 3 different tissues (mucus, muscle and liver) within a dietary treatment. The relationship of  $\Delta^{15}\text{N}$  values with WG, PRE, FCR and PP:FM ratio was estimated (I).



The N in the faeces was assumed as a mixture of N from undigested FM and PP (CGM and/or RPC), and the undigested fraction of N from PP (Eq. 1 in II) and FM (Eq. 2 in II) was estimated using a previously developed 2-source (FM and PP), 1-isotope ( $\delta^{15}\text{N}$ ) mixing model (Phillips 2012). The ADC of the CGM (in diet D<sub>1</sub> and D<sub>6</sub>), RPC (in diet D<sub>2</sub>) and their mixture (in diet D<sub>4</sub> and D<sub>5</sub>) was estimated using the equation (Eq. 3 in II) modified from the previous studies (Oliveira *et al.* 2007, Gong *et al.* 2017). Both estimated proportion of undigested PP and FM ( $f_{\text{PP}}$  and  $f_{\text{FM}}$ ) and dietary proportion of PP and FM ( $d_{\text{PP}}$  and  $d_{\text{FM}}$ ) were incorporated into the modified equation (Eq. 3 in II). The  $\delta^{15}\text{N}$  value of the faeces from fish fed complete PP-based diet was estimated from the negative correlation established between the dietary inclusion level of PP and the  $\delta^{15}\text{N}$  value of faeces (II).

The original tissue (muscle and liver) of fish raised on a single diet was assumed as a contributing source of N and C over time, and another source was the dietary PP. Based on this context, a 2-source (original tissue vs. dietary PP), 1-isotope ( $\delta^{15}\text{N}$  or  $\delta^{13}\text{C}$ ), concentration-dependent mixing model was defined in the Bayesian mixing model FRUITS (food reconstruction using isotopic transferred signals, Fernandes *et al.* 2014) in order to estimate the C and N turnover in the muscle and liver tissue of fish fed potato and corn diet. Prior to estimation in FRUITS, the  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of the sources were corrected using the  $\Delta^{15}\text{N}$  and  $\Delta^{13}\text{C}$  values calculated at the end of each feeding experiment. FRUITS incorporates all the features of previous mixing models and it provides credible intervals and posterior probability distributions (III).

## 2.8 Statistical analyses

Statistical analyses were conducted using IBM SPSS statistics 24.0 software package (I, III and IV) and R 3.5.1 (Anon. 2018) (II). Homogeneity of variances and normality of dependent variables were checked using Levene's test and Shapiro-Wilk test, respectively. One-way ANOVA was used to determine the overall difference among treatments followed by LSD (I) and Tukey HSD (III and II) test as *post hoc* comparison. When data did not support the assumptions of homogeneity of variances and normality, the Kruskal-Wallis H test followed by Dunn's nonparametric comparison (FCR in I), and Welch ANOVA followed by Games-Howell *post hoc* test (FCR and CF in III) were applied to test the statistical significance of the difference among different treatments. The relationships of both  $\Delta^{13}\text{C}$  and  $\Delta^{15}\text{N}$  values with growth parameters, dietary PP:FM ratio, as well as dietary PP were estimated by linear regression (I and II). Repeated measures ANOVA was used to detect the overall differences between fish raised on corn and FM diet in terms of  $\text{MO}_2$  over time during 3 consecutive test periods (IV). The  $\text{MO}_2$  at highest speed (2.5 BL  $\text{s}^{-1}$ ) was compared between fish raised on corn and FM diet using Mann-Whitney U test (IV). All statistical tests were carried out at a significance level of 0.05.

## 3 RESULTS AND DISCUSSION

### 3.1 Isotopic difference between tissue and diet (I, II)

There are various factors affecting  $\Delta$ , for example growth which also changes depending on the diet quality and water temperature. Most of the potential factors influencing growth were controlled in the current feeding experiment. In the present case, the difference in dietary protein quality is the major factor influencing the level of  $\Delta^{15}\text{N}$  value in a specific tissue (I). The nutritional quality of a specific protein source is mainly determined by its level of ANFs, balance between essential and non-essential AAs and digestibility. In previous studies, the presence of ANFs in RPC has interfered with the digestibility and palatability of the diet (Teskeredžić *et al.* 1995, Slawski *et al.* 2012), and this might be the reason for the difference in FI among the treatments in the present experiment (I). Compared to FM, CGM is limited in some EAAs such as lysine and arginine (Pereira and Oliva-Teles 2003), and this could further affect the growth.

In the present study, the FCR significantly increased with the level of CGM and RPC while the FI decreased (I). These observed differences in both FCR and FI were further reflected in the growth parameters of the fish, including WG, PRE and SGR. The growth of the trout significantly decreased with increasing dietary PPs which also lowered the PRE. Consistent with previous studies (Gamboa-Delgado and Le Vay 2009a, Gamboa-Delgado *et al.* 2013, 2014, 2016),  $\Delta^{15}\text{N}$  value increased with the level of dietary PPs since the growth was compromised due to the reduced FI and nutritional quality of the diets (Fig. 5).

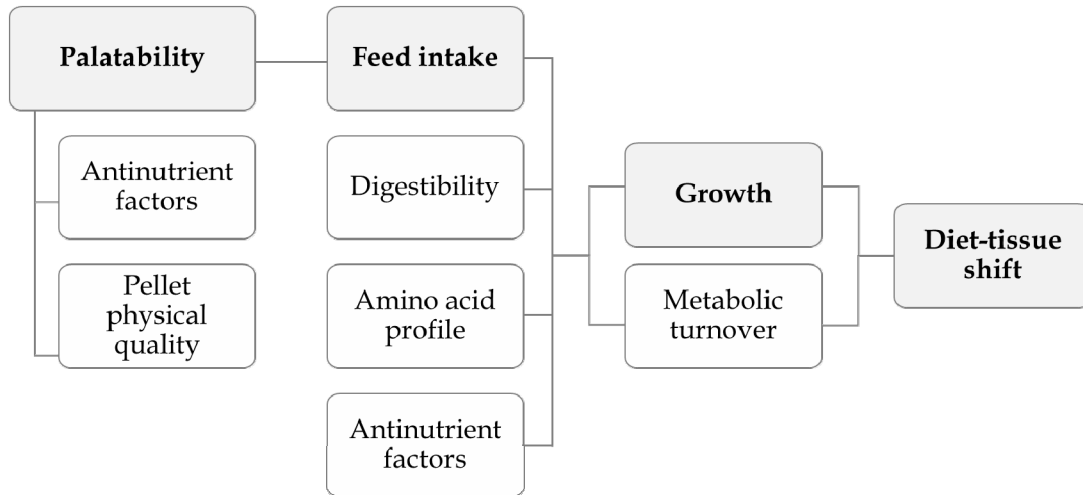


FIGURE 5 Potential mechanism associated with the variations in the isotopic difference between animal and its diet, *i.e.* the diet-tissue shift ( $\Delta$ ).

Among the treatments within a tissue,  $\Delta^{15}\text{N}$  values increased significantly ( $R^2 > 0.9$ ,  $P < 0.01$ ) with the level of PPs (I). Diets  $D_1$  and  $D_2$  contained mostly CGM and RPC, respectively, and the highest  $\Delta^{15}\text{N}$  values were observed in these 2 treatments, while it was lowest in treatment  $D_3$  containing only FM as protein source (I). Treatments  $D_4$ ,  $D_5$  and  $D_6$  contained intermediate levels of PP and intermediate  $\Delta^{15}\text{N}$  values were observed in these treatments (I). The  $\delta^{15}\text{N}$  value of the faeces was lower than the diet in  $D_3$  treatment (Fig. 6g), and therefore the  $\Delta^{15}\text{N}$  value was negative (Table 6). The difference in  $\Delta^{15}\text{N}$  values induced by dietary treatment is associated with both growth and metabolic turnover, in which growth played a dominant role in the present study (I).

Among the tissues within a diet, the  $\Delta^{15}\text{N}$  values also differed significantly ( $P < 0.05$ ), except those fish fed with diet  $D_3$  (I). The significantly highest  $\Delta^{15}\text{N}$  values were observed in the mucus (Fig. 6e, f) followed by muscle (Fig. 6a, b) and liver (Fig. 6c, d) tissue. The  $\Delta^{15}\text{N}$  values were significantly different among tissues (mucus, muscle and liver) in fish fed mostly CGM-based diet ( $D_1$ ) while there was no difference among tissues of those fed FM-based diet ( $D_3$ ), and this indicates that the faster growth in fish fed diet  $D_3$  accelerated the assimilation of dietary N into the tissue compared to the slower growth in those fed with diet  $D_1$  (I). The faeces (Fig. 6g, h) were lower in  $\Delta^{15}\text{N}$  value (Table 6), compared to mucus, muscle and liver (I). Compared to the observations of mucus, muscle and liver (I), and faeces (II), fat tissue was very low in  $\delta^{13}\text{C}$  values (Table 6). A specific dietary element (*e.g.* N) could be differentially incorporated into different tissues at different speed (Gannes *et al.* 1998), through which the isotopic difference among different tissues might be achieved in the present study (I).

TABLE 6 Mean  $\delta^{13}\text{C}$  ( $\pm$  SD,  $n = 3$ ) value and its diet-tissue shift ( $\Delta^{13}\text{C}$ ) in fat and faeces (II) sampled at the end of feeding trial from juvenile rainbow trout fed 6 experimental diets over 42 d.

	D <sub>1</sub>	D <sub>2</sub>	D <sub>3</sub>	D <sub>4</sub>	D <sub>5</sub>	D <sub>6</sub>
<b>Fat</b>						
$\delta^{13}\text{C}$	$-27.5 \pm 0.10$	$-30.1 \pm 0.04$	$-30.3 \pm 0.02$	$-28.8 \pm 0.48$	$-29.7 \pm 0.04$	$-28.8 \pm 0.03$
$\Delta^{13}\text{C}$	$-9.4 \pm 0.10$	$-6.3 \pm 0.04$	$-7.3 \pm 0.02$	$-7.0 \pm 0.48$	$-7.0 \pm 0.04$	$-8.1 \pm 0.03$
<b>Faeces</b>						
$\delta^{13}\text{C}$	$-22.8 \pm 0.46$	$-27.9 \pm 0.35$	$-27.0 \pm 0.13$	$-26.5 \pm 0.17$	$-27.1 \pm 0.14$	$-24.8 \pm 0.29$
$\Delta^{13}\text{C}$	$-4.6 \pm 0.46$	$-4.0 \pm 0.35$	$-4.0 \pm 0.13$	$-4.6 \pm 0.17$	$-4.4 \pm 0.14$	$-4.1 \pm 0.29$
$\delta^{15}\text{N}$	$7.0 \pm 0.28$	$7.2 \pm 0.61$	$10.1 \pm 0.24$	$8.6 \pm 0.35$	$7.8 \pm 0.28$	$9.0 \pm 0.08$
$\Delta^{15}\text{N}$	$2.0 \pm 0.28$	$2.1 \pm 0.61$	$-0.5 \pm 0.24$	$1.5 \pm 0.35$	$1.6 \pm 0.28$	$0.9 \pm 0.08$

After a dietary change, the fish start to synthesise new tissue using the nutrients derived from the new diet, and the original tissue will be gradually replaced (or diluted) by the newly synthesised tissue. In a feeding experiment, the duration of experiment must be long enough to ensure that the responses induced by dietary treatments are sufficient for the final comparison among treatments before stopping the feeding trial. A WG up to 1000 % (10 times) of IW was recommended to be achieved in rapidly growing juvenile organisms before stopping the feeding trial, although a 300 % (3 times of IW) increase is a frequently used standard (Anon. 2011). A 6- to 8-fold increase in weight was suggested for reaching isotopic equilibrium with the diet consumed (Herzka 2005). On the other hand, isotopic equilibrium was observed when the juvenile rainbow trout obtained 15-fold increase in weight during an 80-d feeding trial (Badillo *et al.* 2014). Therefore, stable isotope analysis is not necessarily a quick alternative to conventional feeding experiments. In the present feeding experiment (I, II), a 4.4- to 7.1-fold increase in weight was observed after 42 d of feeding, and the  $\Delta^{15}\text{N}$  values were calculated using the  $\delta^{15}\text{N}$  values in tissues sampled at day 42 (Fig. 6). It seems that the fish did not fully reach an isotopic equilibrium with their diets in this experiment (I). However, it was assumed that the detected responses, in terms of  $\Delta^{15}\text{N}$  value, were sufficient for indicating the growth differences induced by the dietary treatments (I).

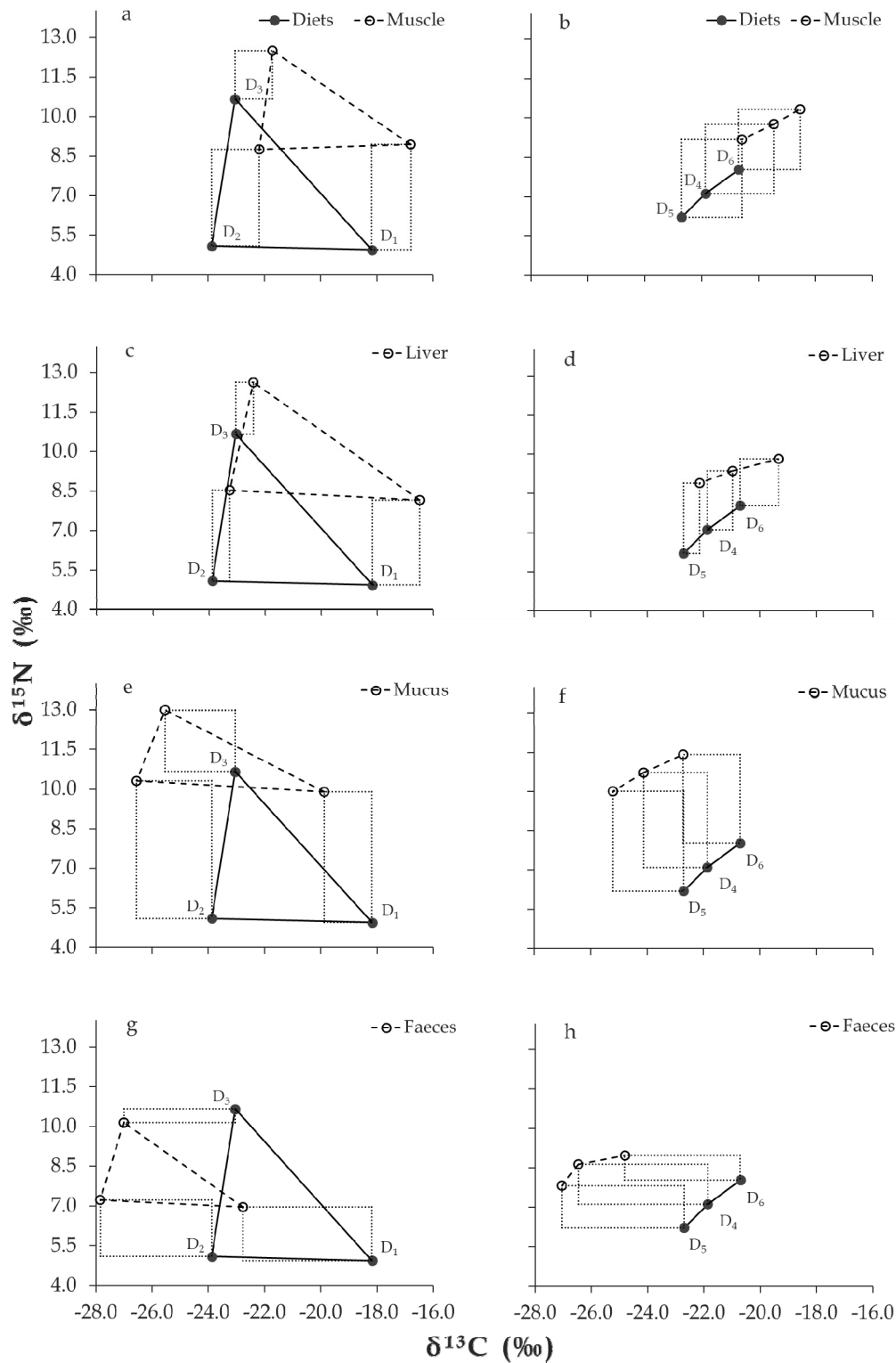


FIGURE 6 Diet-tissue shifts ( $\Delta^{13}\text{C}$  and  $\Delta^{15}\text{N}$ ) of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in muscle (a, b), liver (c, d), mucus (e, f) and faeces (g, h, in II) of juvenile rainbow trout fed 6 different diets ( $D_1$ - $D_6$ ) over the 42 d of feeding experiment. The solid circles at the vertices of solid line triangles represent the mean  $\delta^{15}\text{N}$  value of experimental diets, and the empty circles at the vertices of dash line triangles represent that of the tissues of fish fed respective diets. The horizontal lines between solid circles (diets) and empty circles (tissues) indicate the  $\Delta^{13}\text{C}$  values and the vertical lines indicate the  $\Delta^{15}\text{N}$  values.

With the increasing concern for animal welfare, the development of non-lethal method is critical in nutritional studies. Both faeces and external mucus can be sampled without killing the fish. Most importantly, the difference in dietary protein quality was also indicated by the  $\Delta^{15}\text{N}$  values observed in faeces (II) and mucus (I) in the current feeding experiment. In experiments using stable isotopes, the repeated sampling of faeces and mucus from same individual during the entire feeding trial could be an option for preventing the variations of isotope ratios measured from different individuals. In addition, the repeated sampling further reduces the effect of sampling individual fish at different sampling points during the feeding on the calculation of growth parameters such as WG.

### 3.2 Apparent digestibility coefficient of PPs (II)

During the 42 d of the feeding trial, the fish fed mostly CGM-based diet (D<sub>1</sub>) had a lowest WG while there was no significant difference among the other treatments (D<sub>2</sub>, D<sub>4</sub>, D<sub>5</sub> and D<sub>6</sub>). The ADC of crude protein in the CGM was 49.5 % in treatment D<sub>1</sub> while it was 79.7 % in treatment D<sub>6</sub>. The ADC of crude protein in the CGM, RPC and their mixture decreased with the level of FM in the diet of juvenile rainbow trout, which was also reflected in the growth reduction caused by the high level of PPs. The ADC values were also positively correlated with the SGR and PRE.

In the present study, treatments D<sub>4</sub> and D<sub>5</sub> contained mixtures of CGM and RPC as protein sources in each. As suggested by Lupatsch *et al.* (2003), the estimated ADC in treatment D<sub>4</sub> and D<sub>5</sub> is sum of the ADC of crude protein in CGM and RPC. The published values of ADC for CGM and RPC were 92–97 % (Anon. 2011) and 89–98 % (Teskeredžić *et al.* 1995), respectively, which are considerably higher than the values found in this experiment. It is practical to replace up to 18 % of FM with CGM in the diet for rainbow trout (Stone *et al.* 2005, Saez *et al.* 2014). The 15 % replacement of dietary FM with CGM led to the decrease in protein metabolism in Japanese seabass (*Lateolabrax japonicas*) (Men *et al.* 2014). In the present study, the dietary level of CGM was 80.1 and 41.8 % in treatment D<sub>1</sub> and D<sub>6</sub>, respectively. The high level of PPs led to decreased ADC of protein in CGM and RPC for rainbow trout, and therefore it seems that the high inclusion level of PPs also interfered with the digestion process of protein before the absorption and utilisation by the fish in the present case.

The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in the faeces gradually become stable during the 42 d of feeding. At day 42, all faecal samples were very low in  $\delta^{13}\text{C}$  value, and the  $\delta^{15}\text{N}$  value of faeces from fish fed D<sub>3</sub> was lower than that in the diet. As concluded in article I, the  $\Delta^{15}\text{N}$  values were negatively correlated with the WG and also with ADC of protein in the PPs (Fig. 7a, b).

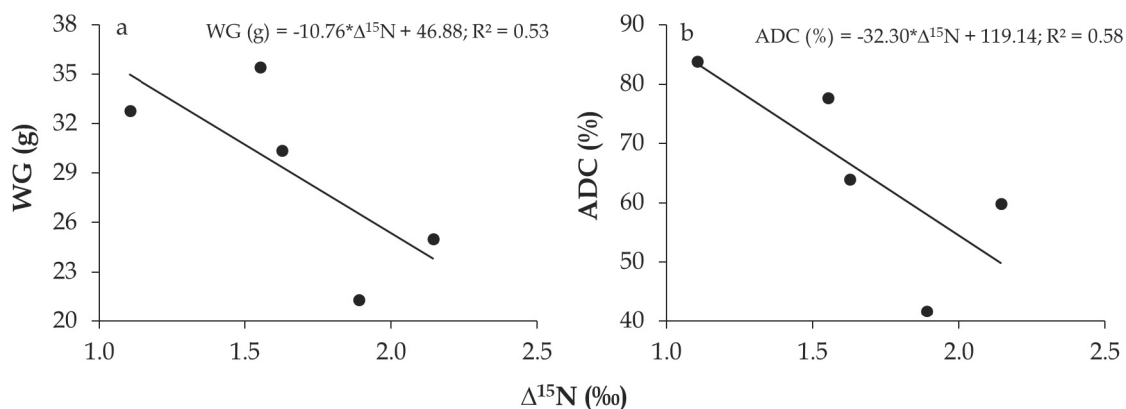


FIGURE 7 Linear regression between diet-faeces shift ( $\Delta^{15}\text{N}$ ) of  $\delta^{15}\text{N}$  value and both weight gain (WG, a) and apparent digestibility coefficient (ADC, b) of protein in CGM, RPC and their mixture (II). Each of black solid circles represents an ADC value estimated from mean value of  $\delta^{15}\text{N}$  in faeces from the 3 replicates.

In a previous study, the ADC of C<sub>3</sub> (white rice and soybean meal) and C<sub>4</sub> (cornmeal and corn gluten) plant ingredients in diet for tambaqui was estimated using  $\delta^{13}\text{C}$  value of both faeces and diet (Oliveira *et al.* 2007), and the equation used in their study is not applicable for present case. First, there were some other C sources, such as fish oil and starch, in all diets, and this might be responsible for the very low  $\delta^{13}\text{C}$  values in the faeces. In addition, RPC and FM are very similar in  $\delta^{13}\text{C}$  value, thereby preventing the use of  $\delta^{13}\text{C}$  values to estimate ADC of PPs. In terms of  $\delta^{15}\text{N}$  values, the RPC and CGM are very close. However, both RPC and CGM are PPs and therefore, they can be grouped as a mixture of PPs (Phillips *et al.* 2005). The possible interaction between C<sub>3</sub> and C<sub>4</sub> plants during the digestion process may have also affected the result of this study as mentioned by Focken (2004).

With the stable isotope approach, the effect of nutrient leaching on ADC estimation (Watanabe *et al.* 1996) might be reduced since the aqueous environment might have less effect on the isotope ratio of faeces. However, the effect of different sampling methods on the isotope ratio of faeces requires further investigation. Most importantly, a tiny amount (0.1–0.6 mg) of dried faecal sample is required for stable isotope analysis, and therefore, it is convenient for conducting digestibility trial with small fish, which is normally considered challenging with the conventional approach using digestion marker. Even with the stable isotope method, it is impossible to quantify the share of endogenous N in the faeces. In the present case, it is possible that the contamination from endogenous N source was partly responsible for the lower ADC of protein in CGM and RPC as it is difficult to sample faeces from small fish using the stripping method. In addition, the stable isotope approach is practical provided there is a clear isotopic difference between protein sources, and this limitation can be improved through grouping of sources with similar characteristics (Phillips *et al.* 2005). As described in this study, there is a need for determining the faecal isotope ratio of fish fed single diet, but it is very challenging to grow carnivorous fish on PP-based single diet. In the present

study, the faecal  $\delta^{15}\text{N}$  value of fish fed PP-based single diet was estimated using the relationship found between the faecal  $\delta^{15}\text{N}$  value and dietary PP content.

### 3.3 Turnover of carbon and nitrogen (I, III and IV)

During the entire feeding period (III, IV), the fish from FM and corn treatment reached similar weight at day 87 and 128, respectively, while the mortality increased from day 107 in potato treatment.

Previous studies suggested that the palatability of the feed could be negatively influenced by the heat stable glycoalkaloid in PoP concentrate (Tusche *et al.* 2011a, b, 2013). In the present case, PoP is the only protein source in the potato diet, and the mortality seen in the potato treatment was most likely caused by the presence of ANFs such as glycoalkaloid which could interfere with both ingestion and digestion of the feed (III). Dietary FM was replaced up to 60 % using PoP concentrate without compromising growth of rainbow trout with IW of 52 g (Tusche *et al.* 2013). Previous studies suggested that AA imbalance and lower nutrient and energy digestibility of CGM could be the main factors for growth retardation (Regost *et al.* 1999, Pereira and Oliva-Teles 2003, Kikuchi 2007). In the present study, the slow growth of the fish fed corn diet was probably associated with the lower nutritional quality of the CGM compared to FM (III, IV).

The 11- to 13-fold increase in weight indicated that the muscle and liver tissue of fish from the FM and corn treatments were likely close to the isotopic equilibrium (III, IV). In contrast, those fish in the potato treatment only gained c. 3-fold increase in weight, and interestingly the isotope ratio present in tissues also gradually approached that of potato diet (III). It has been suggested that the share of metabolic rate in existing tissue increases when growth decreases (Maruyama *et al.* 2001, Sakano *et al.* 2005). Therefore, it is possible that the isotope ratio of fish in potato treatment approached towards that of potato diet through the contribution of increasing metabolic rate in existing tissues (III).

Higher growth accelerated the incorporation of dietary N into tissues (I), and similarly, the fish fed with the FM diet had a faster growth which led to the faster assimilation of C and N in this study (III). The type of tissue also influences the rate of change in isotope ratio (Buchheister and Latour 2010, Xia *et al.* 2013). In the present study, the change in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  value was faster in liver than muscle tissue (III). The isotopic difference between muscle and liver tissue decreased towards the end of the feeding trial. At the end the isotope ratio of muscle and liver tissue was close to overlap in FM and corn treatment (III).

Both in muscle and liver, the  $\Delta^{13}\text{C}$  and  $\Delta^{15}\text{N}$  values decreased towards the end of feeding experiment. As observed in the first experiment (I), the  $\Delta^{15}\text{N}$  values of muscle and liver were negatively correlated with dietary FM content and SGR. The highest  $\Delta^{15}\text{N}$  value was observed in potato treatment followed by corn and FM treatment (III).



The turnover of C and N increased towards the end of the feeding trial. C and N turnover in liver tissue was faster than muscle in the corn treatment. In all treatments, N turnover was faster than C, in both muscle and liver tissue. In the muscle tissue from potato treatment, C turnover was c. 52 % at day 43 while it was c. 31 % in corn treatment. At day 22, N turnover was over 50 % in the muscle and liver tissue of fish from corn treatment while it was still below 50 % in potato treatment. At the last sampling point, C and N turnover were c. 82 % and c. 90 %, respectively, in the muscle and liver tissue of fish fed potato diet. C turnover of potato treatment was higher than corn treatment while N turnover was opposite, both in muscle and liver tissue. The effect of lipid extraction on the model output was negligible (III).

In previous study, the estimated proportional contributions from the mixing model was compared with the established proportion of proteins in the diet (Gamboa-Delgado and Le Vay 2009b), and this could be true only if the proteins were assimilated at the ratio as included in the diet. However, the isotope ratio present in animal tissue is only representative of the assimilated fraction (Phillips 2012). Therefore, protein digestibility and FI should be considered when the outputs of mixing models are compared with the dietary proportion of protein sources. In the present study, the difference in FI and digestibility might have certain effect on the amount and bioavailability of the AAs in the metabolic pool, through which the difference in C and N turnover between the corn and potato treatment was achieved (III).

### **3.4 Effects of PPs on $MO_2$ and swimming capacity (III, IV)**

Cereal grains and their by-products are limited in some EAAs such as lysine and methionine (McDonald *et al.* 2011). The corn diet was supplemented with AAs and fish protein hydrolysate to avoid the potential growth retardation due to EAA deficiency (lysine and methionine) and palatability deterioration. During the initial 7 weeks of feeding (III), the average individual FI ( $\pm$  SD) was  $217.2 \pm 3.8$  g and  $113.5 \pm 8.0$  g for FM and corn treatment, respectively, and it was 47.0 g (at 39 d) and 65.6 g (at 79 d) during the extended feeding period (IV). In addition, for the fish raised on corn diet it took 128 d to reach the testing size (74.1 g) while the fish in FM treatment reached 80.7 g after 87 d, and this was also reflected in the slower SGR of corn treatment (Table 7). Further, the FCR of FM diet was lower than corn diet (III). There was no significant difference between FM and corn treatment in terms of CF (IV).

In a previous study, the effect of age, ration size and swimming speed on the fatty acid profile of muscle tissue from chinook salmon (*Oncorhynchus tshawytscha*) has been compared during a 300-d experiment (Kiessling *et al.* 2005), and the muscle fatty acid composition was largely affected by age followed by ration size and swimming speed. In the present experiment, the fish in FM treatment had a higher FI during the initial 7 weeks of feeding, and the FI in corn treatment was higher after 7 weeks. Therefore, according to the

previous study (Kiessling *et al.* 2005), the difference in FI between treatments may cause changes in the muscle fatty acid profile which may affect the metabolism in muscle tissue further.

We expected that the fish from corn treatment will be lower in the capacity of coping with energy demand for swimming exercise and consume more oxygen compared to FM treatment. However, there was no statistically significant difference found between the rainbow trout from the FM and corn treatments in terms of  $MO_2$  at 15 °C (IV). The  $MO_2$  increased during the exercise period compared to the acclimation and recovery period (Table 7). In a previous study, difference in protein and lipid sources in the feed for Atlantic salmon (*Salmo salar*) did not have a significant effect on  $MO_2$  and swimming performance of the fish at 9 °C (Wilson *et al.* 2007). The results of the present study suggest that total replacement of FM with CGM does not affect the  $MO_2$  and swimming capacity of rainbow trout, even though growth was retarded due to high inclusion level of CGM (IV). CGM could be considered as a potential alternative protein source for substituting dietary FM without affecting the  $MO_2$ , but growth of the fish was compromised with corn diet in the present case. Therefore, the cost associated with extra amount of corn diet fed to the fish to achieve similar body weight should be examined further.

TABLE 7 Initial weight (IW), final weight (FW), wet weight gain (WG), specific growth rate (SGR) and metabolic oxygen consumption ( $MO_2$ , during the acclimation, exercise and recovery period, n = 10) of fish raised on FM and corn diet (IV). Data were expressed as mean  $\pm$  SD. The range of minimum and maximum value for  $MO_2$  is provided.

	IW, g	FW, g	WG	SGR	$MO_2$ , mg kg <sup>-1</sup> h <sup>-1</sup> (min-max)		
					Acclimation	Exercise	Recovery
FM	6.3 $\pm$ 0.1	80.7 $\pm$ 12.1	74.4	2.0	225.1-267.9	187.5-404.3	190.3-248.5
CGM	6.6 $\pm$ 0.1	74.1 $\pm$ 12.3	67.5	1.9	213.9-254.8	201.4-427.1	181.5-220.0

The present study only applies to rainbow trout raised on CGM diet at 15 °C (IV). Hence, further research is also required for different fish species and different PPs at different rearing temperatures in order to have a more comprehensive understanding of the potential effect of PPs on  $MO_2$  and swimming capacity of fish.

## 4 CONCLUSIONS

Regarding the objective of evaluating the applicability of stable isotope analysis in fish nutrition research and of the use of PPs (CGM, RPC and PoP) to substitute FM in feed for juvenile rainbow trout, it can be concluded that:

Growth of rainbow trout decreased with increasing level of PPs in 2 feeding experiments (I-IV). The ADC of crude protein in PPs decreased with increased substitution level of FM (II), and growth of the trout might be adversely affected by this decrease in protein ADC throughout the 2 feeding trials. After a dietary change, animals start to assimilate dietary C and N, and the assimilation rate is growth dependent. In the second feeding trial, FM group showed the highest growth followed by corn and potato treatment (III, IV). There was less change in the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values present in the muscle and liver tissue from FM treatment during the 2 feeding trials (I, III), which indicated that faster growth of the fish in FM treatment accelerated the incorporation of dietary C and N into their tissues. The  $\Delta^{15}\text{N}$  values increased with the level of dietary PPs, and the growth differences induced by different levels of PPs were clearly indicated by the differences in  $\Delta^{15}\text{N}$  values among dietary treatments within a tissue (I, II and III). Therefore, the  $\Delta^{15}\text{N}$  value could be applied in fish nutrition research as an indicator for nutritional quality of dietary proteins.

The  $\delta^{15}\text{N}$  value of the faeces was used to calculate the undigested fraction of PPs in the faeces, from which the ADC of PPs in the diet was estimated (II). However, the ADC estimation with stable isotopes should be further checked using digestion markers (*e.g.* chromic oxide). In previous studies, single diets, *i.e.* diets containing only 1 protein source, have been used to determine  $\Delta$  for a specific protein source (Gamboa-Delgado *et al.* 2008, 2014, 2016). In the present study, the isotopic difference between the original tissue of fish and the dietary PP allowed the use of stable isotope mixing models to quantify C and N turnover in muscle and liver tissue (III). In addition, complete replacement of FM with CGM did not adversely affect the  $\text{MO}_2$  and swimming performance of juvenile rainbow trout raised at 15 °C, during the swimming test in swim-tunnel respirometer (IV). However, further research is required to examine

thoroughly the potential effect of FM replacement with other PPs on  $MO_2$  and swimming capacity of different carnivorous fish at different rearing temperatures.

The development of non-lethal approaches is very important from the perspective of both animal welfare and nutritional studies. In the present studies, similar patterns were observed from muscle and liver tissue (I) as seen from external mucus (I) and faeces (II) in terms of  $\Delta^{15}N$  values. Faeces also could be used for determining ADC of dietary proteins. Most importantly, both mucus and faeces could be sampled without killing the experimental fish, and this could also prevent experimental error resulted from sampling of individual fish at each sampling point to check the time series change in isotope ratios.

Application of stable isotope analysis is limited to protein sources which have distinct difference in isotope ratios. Most of the PPs used in fish feed are  $C_3$  plants which have very similar  $\delta^{13}C$  values, and both of  $C_3$  and  $C_4$  plants are very close in terms of  $\delta^{15}N$  value. However, the isotope ratios of the protein sources with similar isotope ratio could be grouped together using a simple mass balance mixing equation (Fry 2006). The PP-based single diets are not nutritionally adequate for carnivorous fish. In the present study, the  $\delta^{15}N$  value of faeces from those fish raised on PP-based single diet was estimated using the negative correlation established between  $\delta^{15}N$  value and dietary PP content (II). In experiments with stable isotopes, the most convenient way is to use nutritionally adequate protein sources which have distinct difference in terms of their natural abundance of isotope ratio. With all these pros and cons, stable isotope technique cannot replace conventional assessment of feed ingredients and feeds but can provide additional information provided the preconditions for using stable isotope analysis are met.

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## YHTEENVETO (RÉSUMÉ IN FINNISH)

### Vaihtoehtoiset menetelmät kalanrehujen proteiinilähteiden arvioimiseksi

Kalanrehujen koostumus on muuttunut merkittävästi viime vuosikymmeninä. Aikaisemmin suurin osa rehusta oli kalajauhoa, joka tuotettiin yleensä mm. pelagisista valtamerikaloista. Kalajauhon hinnan nousu ja kalanviljelytuotannon globaali kasvu ovat johtaneet siihen, että nykyrehuissa suurin osa kalajauhosta on korvattu erityisesti kasvisperäisillä, mutta myös muilla elintarviketeollisuuden sivutuotevirrasta ylijäävillä proteiineilla. Nämä korvaavat proteiinilähteet eivät ole sellaisenaan ravitsemuksellisesti optimaalisia varsinkaan petokaloille. Niille soveltuvien rehujen kehittäminen vaatii siis vielä paljon tutkimusta ja ravitsemuskokeiden menetelmien kehittämistä.

Ekologisessa tutkimuksessa on jo pitkään käytetty hiilen (C) ja typen (N) vakaita isotooppeja tutkittaessa aineen kulkeutumista ravintoverkossa trofia- tasolta toiselle. Kun mahdollisten ravintolajien isotooppisuhteet tunnetaan, kuluttajan isotooppisuhteista voidaan arvioida kunkin ravintolajin osuus ravinnossa. Tässä tutkimuksessa sovellettiin samaa periaatetta: kun tiedetään rehun eri raaka-aineiden isotooppikoostumukset ja määrät rehussa, voidaan arvioida, missä määrin kala on hyödyntänyt kasvuunsa kutakin proteiinilähdettä. Tässä työssä tutkittiin mahdollisuutta käyttää vakaita C- ja N- isotooppeja kirjolohen (*Oncorhynchus mykiss*) ravitsemustutkimuksessa. Lisäksi uintirasitustestin avulla tutkittiin rehun korkean kasviproteiiniosuuden vaikutusta kirjolohen suorituskykyyn.

Työssä käytettiin rypsi- ja perunaproteiinituotteita, rypsi- ja perunaproteiineja tutkittiin niiden kotimaisuuden vuoksi, mutta maissigluteenia käytettiin, koska sen C-isotooppikoostumus poikkeaa kaikista suomalaisista kasviproteiineista. Poikkeava isotooppikoostumus mahdollistaa koeasetelman, jossa voidaan vertailla samanaikaisesti useita proteiinilähteitä ja näiden seoksia. Rehun eri proteiinilähteiden hyödyntämistä mitattiin C- ja N- isotooppien avulla kalojen lihaksesta, maksasta, pintalimasta ja ulosteesta. Kaloja ei tarvitse lopettaa, kun mittaukset tehdään pintalimasta tai ulosteesta.

Tutkimuksessa selvisi, että kalajauhon korvaaminen kasviproteiineilla heikentää kalojen kasvua mutta ei vaikuta merkittävästi kalojen suorituskykyyn tai hapenkulutukseen uintirasituksessa. Tuloksissa näkyivät erilaisten koerehujen aiheuttamat isotooppimuutokset tutkimuksissa kudoksissa ja se, että muutokset tapahtuvat eri nopeudella eri kudoksissa. Vakaiden isotooppien käyttö kokeellisessa ravitsemustutkimuksessa on haastavaa, eikä menetelmä korvaa perinteisiä mittausten menetelmiä, mutta isotooppimittaukset täydentävät perinteisemmillä mittareilla saatavia tuloksia. Lisäksi osa näytteistä voidaan ottaa kaloja tappamatta, mikä mahdollistaa sen, että näytteitä voidaan ottaa useita kertoja samasta kalasta.

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## ORIGINAL PAPERS

### I

#### DIETARY FISHMEAL REPLACEMENT BY PLANT PROTEIN SOURCES DECREASES GROWTH AND INDUCES A SHIFT IN $\delta^{15}\text{N}$ IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

by

Gerile Siqin, Lily Laine, Jouni Vielma, Mikko Kiljunen, Trond Storebakken & Juhani

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## II

# ESTIMATION OF APPARENT DIGESTIBILITY COEFFICIENT OF PLANT PROTEINS WITH NITROGEN STABLE ISOTOPE RATIO IN JUVENILE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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### III

## **BAYESIAN QUANTIFICATION OF CARBON AND NITROGEN TURNOVER IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FED PLANT-BASED DIET**

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## IV

**REPLACEMENT OF FISHMEAL WITH CORN GLUTEN MEAL  
IN FEEDS FOR JUVENILE RAINBOW TROUT  
(*ONCORHYNCHUS MYKISS*) DOES NOT AFFECT OXYGEN  
CONSUMPTION DURING FORCED SWIMMING**

by

Gerile Siqin & Juhani Pirhonen 2017

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## Short communication

# Replacement of fishmeal with corn gluten meal in feeds for juvenile rainbow trout (*Oncorhynchus mykiss*) does not affect oxygen consumption during forced swimming



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Protein sources

## ABSTRACT

We compared oxygen consumption ( $MO_2$ , mg/kg/h) of c. 80 g rainbow trout (*Oncorhynchus mykiss*) in an intermittent-flow swim respirometer at 15 °C. Before the tests the fish were grown in flow through tanks (15 °C) with either fishmeal (FM) or corn gluten meal (CGM) based diets (c. 52% protein) for a period of 3–4.5 months. Ten individuals from both treatment groups were fasted for 48 h before the swim test, which consisted of 18 loops of 210 s over three different periods: acclimation period (6 loops at 0.5 body lengths per s, BL/s), exercise period (8 loops at increased speed from 1 to 2.5 BL/s with recovery loops at 0.5 BL/s), and a recovery period (four loops at 0.5 BL/s). We did not observe significant differences in  $MO_2$  between the two groups at any of the three measurement periods (repeated measures-Anova). The maximum (mean  $\pm$  SE)  $MO_2$  values, measured during the last exercise period at 2.5 BL/s, did not differ significantly between the treatments:  $404 \pm 18.7$  mg/kg/h and  $427 \pm 50.6$  mg/kg/h in FM and CGM groups, respectively. Our result supports an earlier finding that origin of the protein does not affect  $MO_2$  during swimming in salmonids. This is the first report of the effect of a plant protein on  $MO_2$  of a carnivorous fish during forced swimming, and these data lend support to further development of sustainable diets to replace fishmeal with plant proteins.

## 1. Introduction

Traditionally the feeds for carnivorous fishes have contained fishmeal (FM) as protein source. However, there is a high demand for the development of fish feeds with alternative protein sources, firstly due to dwindling marine fish stocks and a consequent rise with wide temporal variation in FM market price (Indexmundi, 2017) and secondly due to the need for more sustainable aquaculture production. The use of FM alternatives is likely to increase even more in the coming decades (Engle et al., 2017). FM in fish feeds is typically replaced with plant proteins, and the most common alternative is soybean meal but also corn gluten meal (CGM) has been used widely (Gatlin et al., 2007).

Metabolism (heat production) in fish has conventionally been estimated indirectly by measuring oxygen consumption in a respirometer. To the best of our knowledge there are no reports of experiments for swimming metabolism, i.e.  $O_2$  consumption ( $MO_2$ , mg/kg/h) during forced swimming (Cech, 1990), between the fish fed with either FM or plant protein based diets. Wilson et al. (2007) measured  $MO_2$ , swimming capacity and recovery from swimming of Atlantic salmon (*Salmo salar*) fed diets differing in lipid (fish oil, poultry fat and vegetable oils)

and protein (FM and poultry by-product meal) source and found no significant differences between the dietary groups.

As the use of plant proteins in feeds for carnivorous fish is increasing, it would be important to know whether their use affects the swimming performance and  $MO_2$  of fish. In this experiment, we measured swimming metabolism of juvenile rainbow trout (*Oncorhynchus mykiss*) which had been reared on two extreme experimental diets: one with FM and the other one with CGM as the only protein source. The fish fed with CGM diet did not grow to the swim test size as fast the fish fed FM diet, which may have led to changes in muscle tissues and consequently decreased the swimming capacity (Kiessling et al., 1989; Pelletier et al., 1993; Weber et al., 2016). Our hypothesis was that the performance (lower capability to maintain high swimming speed and higher  $MO_2$  during exercise and recovery) of the fish fed CGM diet would be inferior to that of the fish fed FM diet.

## 2. Materials and methods

The measurements were conducted in the wet laboratory of the University of Jyväskylä, Finland. The all-female rainbow trout

Abbreviations: BL, body lengths; CF, condition factor; CGM, corn gluten meal; FM, fishmeal;  $MO_2$ , oxygen consumption (mg/kg/h)

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**Table 1**

Formulation and composition of experimental diets (CGM = corn gluten meal based diet, FM = fishmeal based diet) fed to juvenile rainbow trout before tests in the swim respirometer.

Ingredient	CGM (g/kg)	FM (g/kg)
Fish meal	0	755
Corn gluten meal	730	0
Cellulose	42	65
Corn starch	40	58
Wheat starch	0.0	20
Fish oil	82	82
Fish protein hydrolysate	10	0
Vitamin premix <sup>a</sup>	20	20
Methionine	5	0
Lysine	25	0
Threonine	10	0
Monocalcium phosphate	36	0
Proximate composition		
Protein (g/kg)	534 ± 53	515 ± 52
Fat (g/kg)	164 ± 16	161 ± 16
Ash (g/kg)	57 ± 9	105 ± 16
Fiber (g/kg)	< 10	< 10
Moisture (%)	5.0	6.8
Gross energy (kJ/g) <sup>b</sup>	22.4	21.0

<sup>a</sup> Vitamins added to supply the following (kg<sup>-1</sup> diet): retinol acetate, 8000 IU; cholecalciferol, 3000 IU; all-race- $\alpha$ -tocopherol acetate, 300 IU; menadione sodium bisulfite, 10 mg; thiamine HCl, 20 mg; riboflavin, 30 mg; calcium D-pantothenate, 90 mg; biotin, 0.3 mg; folic acid, 6 mg; vitamin B12, 0.04 mg; niacin, 120 mg; pyridoxine HCl, 20 mg; ascorbic acid (Stay C), 300 mg; inositol, 200 mg. Minerals added to supply the following (mg kg<sup>-1</sup> diet): zinc, 150; manganese, 60; iodine, 4.

<sup>b</sup> Gross energy was calculated based on energy content of protein (24 kJ/g), lipids (39 kJ/g) and carbohydrates (17 kJ/g). See text for details of calculation.

**Table 2**

The average ± SE (n = 10) weight, total length, and condition factor of juvenile rainbow trout fed experimental diets (CGM = corn gluten meal based diet, FM = fishmeal based diet) and used in the swim respirometer test.

	CGM	FM
Length, cm	19.1 ± 0.3	19.2 ± 0.3
Weight, g	74.1 ± 3.9	80.7 ± 3.8
Condition factor	1.05 ± 0.03	1.13 ± 0.03

There were no statistically significant differences ( $P > 0.05$ , *t*-test) between the treatments in these variables.

originated from a commercial fish farm (Venekoski farm, Hanka-Taimen Ltd.) and they were brought to our facilities on 16 June 2016 at the size of c. 6.1 g. The fish were used in a feeding trial where they were fed the extruded FM and CGM diets (Table 1) for seven weeks. Then, fish from the replicate tanks of each dietary treatment (FM and CGM) were combined and held in two aerated 500 L flow-through tanks (temperature 14.8–15.2 °C, oxygen 8.5–9.5 mg/L) where they were fed the same feed formulations but at larger pellet sizes than previously. The fish fed FM grew faster than those given CGM, and in an attempt to avoid the confounding effects of fish size on oxygen consumption the swimming tests were carried out at different times on the two groups of fish. The fish fed FM were tested on 19–23 September, and the fish fed CGM were tested on 31 October–4 November 2016. There were no significant differences (*t*-test,  $n = 10$ ) between the fish in weight, total length or condition factor ( $CF = \text{weight (g)} / \text{length (cm)}^3 \times 100$ ) at the time of testing (Table 2). Before the swimming tests and measurements of oxygen consumption, the fish were held individually without food for c. 48 h in 15 L flow-through aquaria at 15 °C to reduce the effect of food digestion and processing on oxygen consumption (SDA) (Jobling, 1994). Water in the laboratory was filtered water from a bore-hole, and photoperiod was 12L:12D.

The experimental diets were analysed for dry matter and moisture (ISO 6496-M), crude protein (EN ISO 5983-2), crude fat (EC Dir 98/64;

EU DIR 71/393), crude fiber (ISO 5498) and ash (ISO 5984) at Eurofins Scientific Finland Ltd. Carbohydrates were calculated by decreasing the sum of analysed components from 100 (McDonald et al., 2011); fiber was not included in this calculation because it was under the detection limit. The gross energy content of the diets was proximately calculated using standard conversion factors for carbohydrates (17 kJ/g), protein (24 kJ/g) and lipids (39 kJ/g) (Jobling, 1994) (Table 1).

The respirometer experiments were carried out using a 10 L swim tunnel respirometer, connected to an OXY-4 oxygen meter with an optical sensor (system manufacturer: Loligo Systems, Viborg, Denmark). According to the manufacturer, the 10 L swim respirometer is suitable for fishes in the range of 50 to 150 g. Before placing the fish into the swim chamber, it was netted out from the aquarium, weighed (to 0.1 g) in a bucket with c. 3 L of water, and the total length was taken (to 1 mm). Immediately after these measurements, the fish was placed into the respirometer chamber, and the computer-assisted intermittent measurement was started. Water temperature during the measurements varied between 14.6 and 15.4 °C.

Each measurement in the respirometer consisted of 18 loops, 210 s each. As the measurement was intermittent, each of the loops was set to contain three stages: 1) flush period of 90 s (oxygenated water pumped into the chamber from the surrounding water bath), 2) wait period of 60 s (flush pump was stopped *i.e.* chamber was closed, and O<sub>2</sub> concentration was let to equilibrate) and 3) a 60 s measurement period (chamber closed). Based on the decrease of O<sub>2</sub> concentration during the 60 s measurement period, the AutoResp software (Loligo Systems, Viborg, Denmark) calculated MO<sub>2</sub> for each loop.

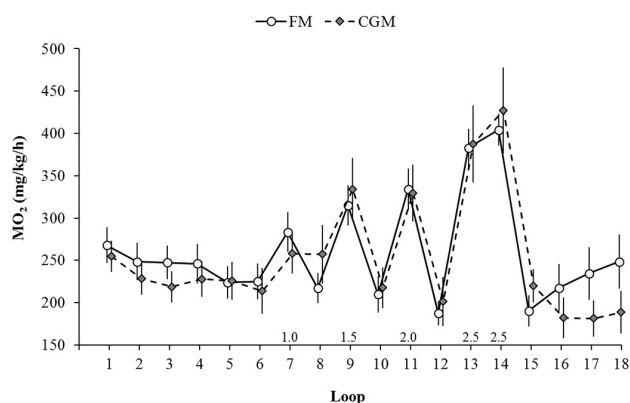
Water speed in the respirometer was adjusted to match the fish total length. At the start of the measurement period the fish was let to recover from handling for a period of six loops at the water speed of 0.5 BL/s. After the recovery period, water speed was increased by 0.5 BL/s steps up to 2.5 BL/s, and by giving the fish a recovery loop (at 0.5 BL/s) after each exercise loop, *i.e.* 1.0, 0.5, 1.5, 0.5, 2.0, 0.5 BL/s. Finally, the speed was increased to 2.5 BL/s for two consecutive loops, after which the fish was let to recover from the exercise for a period of four loops. After the measurement was ended, the fish was netted out of the chamber and killed with a sharp blow on the head.

The rear of the chamber was equipped with an electric grid (DC 3.5 V), which would have been turned on in the case the fish started to lean with the tail against it, but this did not happen in our experiment. The experiment was done with the permission ESAVI/10412/04.10.07/2015 from the Animal Experiment Board of Finland.

The possible difference in MO<sub>2</sub> between the two treatments was tested by repeated measures Anova using IBM SPSS statistics 24.0 software. We tested the possible difference between the two treatments separately for the whole experimental period (18 loops), for the acclimation period (first 6 loops), for the exercise period (8 loops after acclimation) and for the final recovery period (last 4 loops). We also compared the possible difference in MO<sub>2</sub> at the highest test velocity (the second 2.5 BL/s loop) using Mann-Whitney *U* test. Each individual was used as an observation, *i.e.*  $n = 10$ , and  $P < 0.05$  was set as the level for a statistically significant difference.

### 3. Results and discussion

Rainbow trout of the FM and CGM groups performed equally in terms of MO<sub>2</sub> (Fig. 1). There were no significant differences in their MO<sub>2</sub> when calculated for the whole experimental period ( $P = 0.795$ ), for the acclimation period ( $P = 0.514$ ), for the exercise period ( $P = 0.795$ ) and for the final recovery period ( $P = 0.366$ ). The maximum (mean ± SE) MO<sub>2</sub> during the second 2.5 BL/s velocity (loop 14) were 404 ± 18.7 mg/kg/h and 427 ± 50.6 mg/kg/h in FM and CGM groups, respectively, without being significantly different ( $P = 0.257$ ) (Fig. 1). As such, we conclude that feeding a diet based on CGM as the only protein source does not affect oxygen consumption in rainbow trout when swimming.



**Fig. 1.** The mean ( $\pm$  SE,  $n = 10$ ) oxygen consumption ( $MO_2$ ) of juvenile rainbow trout fed diets containing fishmeal (FM) or corn gluten meal (CGM) during the 18-loop swim respirometry test. Each loop lasted for 210 s. Swimming speed was 0.5 BL/s except during the loops 7, 9, 11, 13 and 14, as indicated by the values above the x-axis. There were no statistically significant differences ( $P > 0.05$ , RM Anova) between the treatments.

It must be noted that the feeds differed in terms of their composition: only the CGM diet contained added amino acids and fish protein hydrolysate. The lysine and methionine are considered as limited essential amino acids both in cereal grains and in their by-products (McDonald et al., 2011). In the present study the amino acids and fish protein hydrolysate were supplemented to avoid the typical deficiency of certain amino acids of plant proteins, especially lysine in CGM (Gatlin et al., 2007), and the decrease in feed palatability. Our unpublished experiments have shown low palatability of CGM based diets as such.

The absence of difference in oxygen consumption in rainbow trout fed FM or CGM diets concurs with the results of Wilson et al. (2007). They grew Atlantic salmon (from 84 g to c. 0.5 kg) with feeds containing different sources for lipids and proteins and then tested the fish for oxygen consumption, swimming performance and recovery from exhaustive exercise at 9 °C without finding any significant differences between the dietary treatments. The result of the current experiment and those of Wilson et al. (2007) suggest that swimming performance, and  $MO_2$  during exercise and recovery from swimming of 24 h (Wilson

et al., 2007) or 48 h (the present experiment) starved fish would not be affected by the protein source.

This is likely the first experiment looking at the potential effects of plant proteins on swimming performance and  $MO_2$  in fish. Despite the fish fed CGM grew slower than the fish fed FM based diet, the current result regarding fish performance during forced swimming is promising for the feed industry and for further development of more sustainable fish feeds. However, it must be noted in the present experiment we tested only the effects of one plant protein (corn gluten meal) on oxygen consumption during forced swimming in juvenile rainbow trout at c. 15 °C. Thus, a more comprehensive research is needed to confirm if our finding is universal, i.e. whether it applies to other plant proteins and other carnivorous fish, also at sub- and supraoptimal temperatures.

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