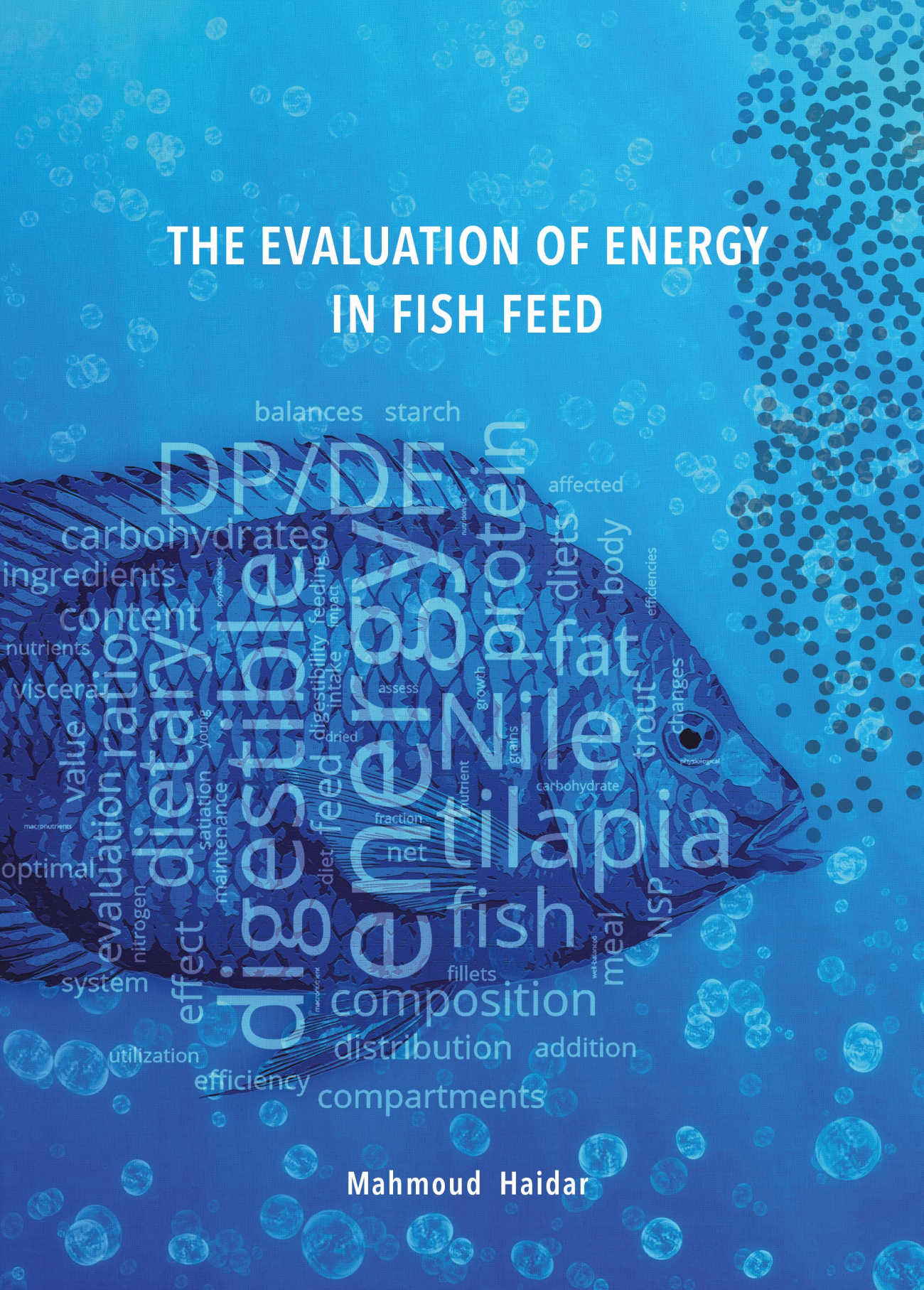
THE EVALUATION OF ENERGY IN FISH FEED



Mahmoud Haidar

Propositions

1. The energetic value of a diet or ingredient is dependent on the applied energy evaluation system.
   (this thesis)

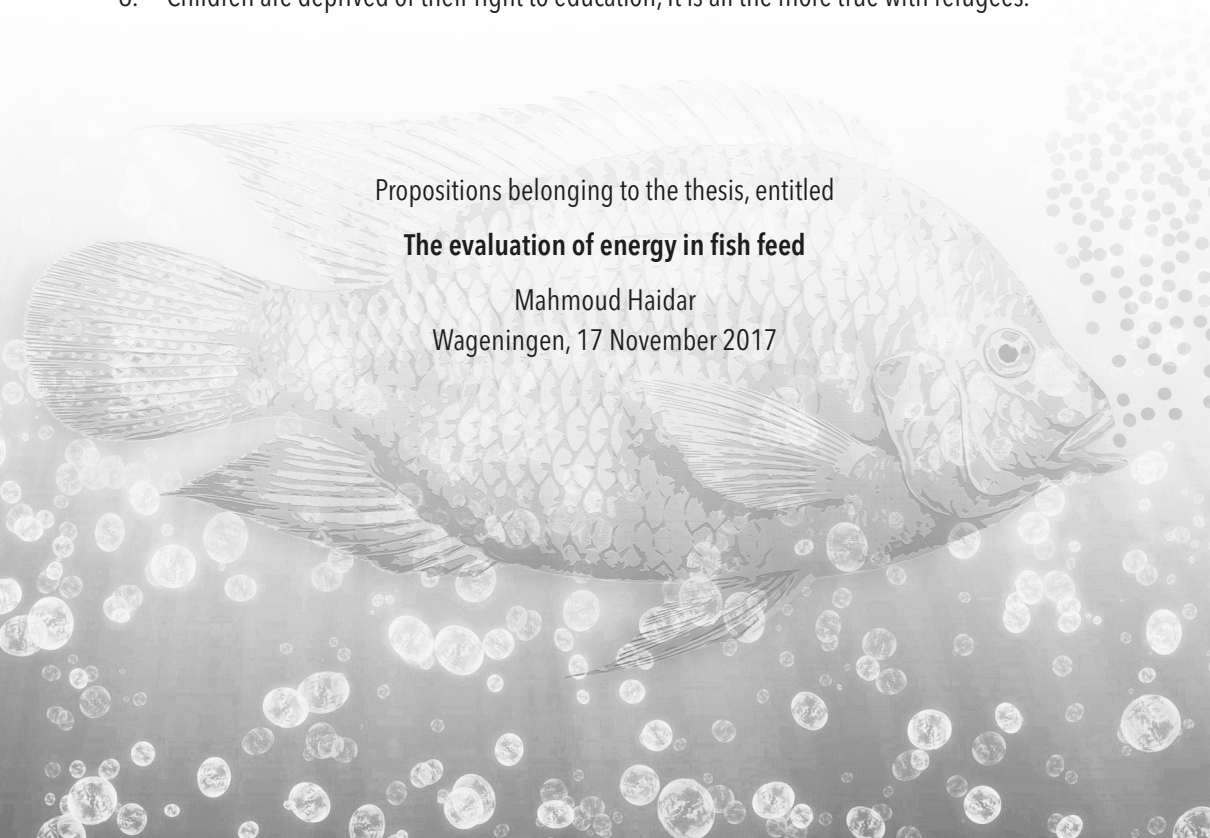
2. An optimal digestible protein to digestible energy ratio does not exist in Nile tilapia juveniles.
   (this thesis)

3. Organic farming is not the way to feed 8-9 billion people.

4. The finding by Wakefield et al. (1998) that MMR vaccines caused autism in young children is an example of how fraudulent scientific results have a devastating effect on public health.

5. In a sea of human beings, it is difficult to see the human as being.

6. Children are deprived of their right to education, It is all the more true with refugees.



Propositions belonging to the thesis, entitled

The evaluation of energy in fish feed

Mahmoud Haidar
Wageningen, 17 November 2017

The evaluation of energy in fish feed

Mahmoud Haidar

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The evaluation of energy in fish feed

Mahmoud Haidar

Thesis

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To my Parents

Abstract

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Wageningen University and Research, The Netherlands.

New and alternative plant ingredients are increasingly incorporated in fish feed due to the scarcity of captured fish and increased fishmeal and fish oil prices. As a result, current fish feeds are characterized by a highly variable ingredients composition, leading to a similar variability in the dietary macronutrients composition, especially the carbohydrates fraction. Appropriate formulation of the energy component in fish feeds requires information on nutrient digestibility, energy requirements for maintenance, and the efficiency of utilization of digestible energy for growth ($k_{g_{DE}}$). In fish feed formulation, the energy evaluation is based on digestible energy (DE) basis. The main assumptions of this DE system are that maintenance requirements and $k_{g_{DE}}$ are independent of dietary factors. The main objective of this thesis was to evaluate and improve the DE system for Nile tilapia. Data showed that, opposite to what is assumed in literature and irrespective of the feeding level applied, an optimal digestible protein to digestible energy ratio (DP/DE) for young Nile tilapia could not be detected. In addition, it was expected that Nile tilapia would show a maximal protein deposition in relation to a wide range of DP/DE ratios, however, this was either observed. Further investigations showed that different body compartments/organs responded differently in terms of protein and fat composition as a result of changes in the dietary DP/DE ratio. In tilapia, viscera and the "rest" fraction (head, skin, fins and bones) were the main site for fat retention. In addition, protein content of fillets seems to be constant (about 17%) and not affected by dietary factors in Nile tilapia. In addition, the effect of using new plant ingredients in Nile tilapia diets was also investigated. The results showed that the ingredients composition had an effect on the maintenance requirements of Nile tilapia. Further, changes in the ratio of starch vs non starch carbohydrates revealed that energy retention was lower when more dietary fibers were included. In addition, the net energy retention differed also when the levels of digestible protein, fat and carbohydrates changed in the diets. The latter results proved that $k_{g_{DE}}$ was not constant and was dependent on diet composition. All aforementioned results led us to calculate the energetic efficiencies of digestible protein, fat and carbohydrates for net energy retention. These estimated efficiencies were used to propose a net energy evaluation system being feasible for Nile tilapia.

Contents

Chapter 1

General Introduction

Worldwide, aquaculture represents about 44% of the total fish production (FAO, 2016) and it is estimated that about 70% of aquaculture production is leaning on commercial feed sources (Tacon and Metian, 2015). Aquatic food production has shifted through the years from capturing wild fish to farming various fish species. Both, the relative constant supply by capture fisheries and the increased demand for fish for human consumption have led to the intensification of aquaculture. This intensification was reflected in increased use of fish meal (FM) and fish oil from processed captured fisheries. In terms of protein supply, FM was and is an ideal protein source in finfish feeds because of its nutritional quality (digestibility, amino acid profile and palatability). However, due to the growth of the aquaculture sector, the demand for FM has increased so heavily that its supply from wild fisheries cannot meet this demand, resulting in increased and variable FM prices (FAO, 2016). As FM supplies are finite, it is essential to seek for alternative, new and sustainable protein ingredients. Nutritionally well balanced diets/feeds play an important role to ensure high/optimal fish performance, maintaining good health and minimizing waste production. Current and future fish diets are facing increased variability in ingredients composition due to e.g. FM and fish oil replacement. This will also increase the variation in dietary macronutrient content and inevitably increasing the carbohydrates fraction in fish diets. These current and future changes in ingredients use will make it more challenging to make well-balanced fish diets. For formulating well-balanced fish feeds, information is needed on the nutrient requirements of fish and on the potential of feed ingredients to supply these nutrients for optimal growth.

1.1 Protein to energy ratio requirements

In practice, the digestible protein to digestible energy ratio (DP/DE) is an important factor in fish feed formulation. Information on the optimal DP/DE ratio is derived: 1) from experimental studies on specific fish species assessing the growth response of fish in relation to varying dietary DP/DE ratios (NRC, 2011) ; or 2) by calculation using the factorial approach, which makes use of experimental studies on energy and protein partitioning for growth and maintenance requirements (Glencross, 2008; Glencross and Bermudes, 2012; Lupatsch et al., 2003a, 1998). Energy in the diet is not a single nutrient but the summation of the energy released from the feed when metabolic oxidation of proteins, fat and carbohydrates occur (NRC, 2011). In growing fish, new tissues are built and part of the energy supplied by the diet is stored as protein, lipid, and glycogen. Dietary amino acids are needed for protein synthesis but are also an important source of energy for fish, while they (i.e. dietary protein) are the most expensive dietary nutrients. Hence, for an efficient utilization of dietary protein for somatic tissue growth, sufficient dietary non-protein energy sources (fat and carbohydrates) are needed. When increasing the dietary DE content at the same DP content (lowering DP/DE) by either inclusion of fat or carbohydrates, proteins are spared from being used as energy source (protein sparing effect), thereby enhancing protein biosynthesis (Bureau et al., 2003; Kaushik, 1998). The benefit of using non-protein energy is that it increases the protein retention efficiency (i.e., the amount of protein deposited per unit of protein ingested). On the other hand, reducing the dietary DE at the same DP content (increasing DP/DE) will lower the protein biosynthesis as the amino acids instead of fatty acids (lipids) and glucose (carbohydrates) are used as energy source. Therefore, a good balance between dietary protein and dietary non-protein energy is crucial for efficient feed utilization, optimal growth, maximal protein retention efficiency and lowering nitrogen excretion into water.

Despite its practical relevance, few experimental studies have been done in Nile tilapia on the optimal DP/DE ratio. These few studies (Ali et al., 2008; El-Sayed and Teshima, 1992; Kaushik et al., 1995; Li et al., 2012) had inconclusive results and variable estimates due to various reasons like, only focusing on one criterion (growth or FCR) and using a limited range of DP/DE levels, etc. Current estimated DP/DE ratios in Nile tilapia are based on the factorial approach as was applied for Pangasius (*Pangasianodon hypothalamus*) by Glencross (2008) and for Gilthead seabream (Sparus aurata) by Lupatsch et al. (2003). This calculation approach assumes various assumptions like a fixed relationship between digestible energy intake and energy retention, a fixed (targeted) body composition, etc. It can however be questioned whether these assumptions are correct and if this give the physiological optimal DP/DE ratio for the fish.

As reported for many fish species in literature, the whole body protein content of fish is relatively constant but the fat content is variable and is influenced by both endogenous factors (such as genetics, fish size and growth rate) and exogenous factors such as diet composition and environment (Dumas et al., 2007; Lupatsch et al., 2003b; Shearer, 1994). Nutritional studies on the impacts on body composition have mainly focused on whole body nutrient content. Except for the study on Rainbow trout (Salze et al., 2014), in fish information on nutritional influences on the nutrient composition of the different compartments within the body (e.g., muscles, liver, gastrointestinal tract etc.) is scarce. In aquaculture fish are reared for their fillets (i.e., skeletal muscle). The skeletal muscle is the largest organ in fish. For many fish species the estimated fillet yield ranges between 25-35%. Thus, it would be of interest to investigate whether the optimal DP/DE ratio for fish is different when assessed on either whole fish performance or on different compartments such as fillet yield.

1.2 Diet macronutrient composition and energy retention

Proper diet formulation in terms of energy requires information on nutrient digestibility, energy requirements for maintenance (DEm), and the efficiency of utilization of digestible energy for growth (kg_{DE}). Therefore, when optimizing the dietary DP/DE ratio, it is required to estimate the DE part of this ratio used either for growth or for maintenance purposes. Historically, the differences in nutrient composition of fish diets were rather constant because these diets had mainly fat and protein originating from fishmeal and fish oil. The energy-yielding nutrients such as protein and lipids are catabolized to provide energy for metabolic processes. However, with increasing plant ingredients in the feeds, the amount of digestible carbohydrates is increasing and leads to sparing of lipid and protein as sources of energy. The optimization of the DE is usually done based on the retained energy (RE) in the fish body (Figure 1.1).

DEm is defined as the amount of DE required for fish to maintain zero energy balance (RE=0). The slope of the relation represents kg_{DE}. In fish few studies have addressed the impact of dietary composition on the relationship between DE and RE (Heinsbroek et al., 2007; Pfeffer et al., 1999; Schrama et al., 2012). As the composition of the diets changes, the slope of line can change and/or alter DEm. With increasing dietary carbohydrate inclusion, it is expected that Kg_{DE} decreases and coincides with an increase in DEm (see Figure 1). In general, dietary carbohydrates include low molecular sugars and starch being digested by the endogenous enzymes, and non-starch polysaccharides (NSP) (NRC, 2011). Mammals, birds and fish

lack endogenous enzymes to digest NSP (Choct and Kocher, 2000). Also the digestibility and the utilization of starch varies within and between fish species and depends on the composition of the sources, the processing of feeds and ingredients and the inclusion level (Krogdahl et al., 2005). In a study by Kaushik and de Oliva Teles (1985) the authors found that using gelatinized starch in rainbow trout diets lead to an increased protein and energy retention efficiency. On the other hand, Schrama et al. (2012) showed that replacing fat with starch in Nile tilapia's diet decreased energy utilization with increasing starch content of the diet. Therefore, it can be hypothesized that Kg_{DE} and DEm would not only be dependent on the level but also on the type of dietary carbohydrates (NSP vs. starch) in fish diets.

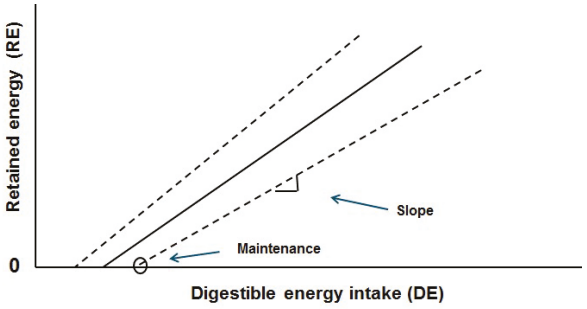

FIGURE 1.1 | The relation between digestible energy intake (DE) and retained energy (RE).

1.3 Energy evaluation of fish feed

The energy evaluation used in most fish feed formulations is on DE basis. Such DE systems do not consider the potential impact of diet composition on DEm and kg_{DE}. However, in a comparison between fish species, Schrama et al. (2012) showed that Kg_{DE} values altered when the dietary composition changed (especially fat to starch ratio). Furthermore, maintenance requirements vary depending on water temperature, dissolved oxygen concentration and stocking density (Glencross, 2009; Lupatsch et al., 2010; Lupatsch and Kissil, 2005). Information on dietary factors affecting these energy requirements for maintenance in fish is lacking. In contrast, in pig feeds, energy evaluation is done on the net energy (NE) basis (Noblet et al., 1994). In NE evaluation systems, the potential of diets/ingredients for energy retention is predicted. The specific energetic value for each type of digestible nutrient is used to calculate the NE content of a diet or feed ingredients (Figure 1.2). Until today, no estimation of energetic efficiencies of digestible nutrients (i.e., the specific energetic value per type of digestible nutrient) has been done to calculate the NE value of diets/ingredients in fish.

Therefore, based on the increased diversification of ingredients for fish diets, coupled with expected changes in Kg_{DE} induced by dietary factors, a transition of the energy evaluation system for fish diets/ingredients from a system based on DE to one based on NE would be worth to be investigated.

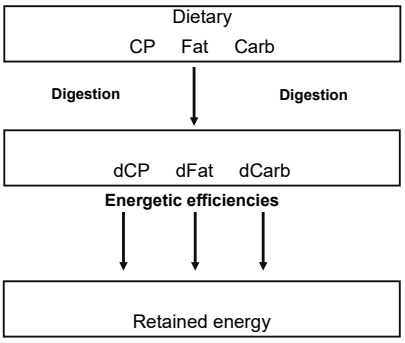

FIGURE 1.2 | Schematic presentation of the use of digestible crude protein (dCP), Crude fat (dFat) and carbohydrates (dCarb) for energy retention. (Modified from Rijnen et al., 2004).

1.4 Aim and outline of the thesis

Nile tilapia is one of the most widely used species in aquaculture and its distribution covers more than 50 countries over the continents (Fitzsimmons, 1997). Understanding and increasing the knowledge on the nutritional requirements would contribute to the welfare and sustainable production of this fish species. Formulation of balanced feeds for a fish species will require information on species-specific macronutrients requirements and the nutritional value of ingredients/feeds. The nutritional value of an ingredient is the result of its macronutrients content, digestibility and utilization efficiency by the fish species. The general aim of this thesis is to assess and improve the current energy evaluation of Nile tilapia feed. In **Chapter 2 and 3** the main objective was to quantify the optimal DP/DE ratio for Nile tilapia and to assess if the optimal DP/DE ratio differs between the physiological criterions used (e.g., growth, FCR, versus nitrogen and energy balance parameters). This was done by examining a wide range of DP/DE ratios first under restricted feeding (Chapter 2). Because an optimal DP/DE ratio was absent in Chapter 2, this was also tested for Nile tilapia that were fed to satiation (Chapter 3). In **Chapter 4** the objective was to investigate whether the impact of DP/DE on the nutrient composition and distribution within the body differed. For this objective, the effect of changing dietary DP/DE ratio on different body compartments (fillet, liver, gastrointestinal tract and "rest" fraction) was addressed. In **Chapter 5** the effect of changing dietary composition especially regarding the carbohydrates content on energy utilization was addressed. This was done to prove the incorrectness of the assumption that the utilization efficiency of DE is independent of dietary factors as is assumed in the DE evaluation systems. In **Chapter 6** it was studied whether changing dietary ingredient composition can affect the maintenance requirements of Nile tilapia. For this objective, different ingredients were tested. In this chapter, the effect of the ingredients composition on gut histology was also addressed. However, being a co-author in this chapter, I have only contributed to the part of the effect of dietary ingredients on the nitrogen and energy balances and the calculations of maintenance requirements by fish. In **Chapter 7** the energetic utilization efficiencies of different digestible nutrients (protein, fat and carbohydrates) was estimated and used to propose a new energy evaluation system on the basis of net energy for fish. Additionally, in this chapter a comparison was made between Nile tilapia and Rainbow trout; being fish with a different natural feeding pattern and a different capacity to metabolize glucose (herbivorous versus carnivorous). In the final chapter (**Chapter 8**) the main outcomes of the studies in this thesis are summarized and discussed within the context of the effect of current and future changes in dietary factors on feed energy evaluation of Nile tilapia.

Chapter 2

Effect of constant digestible protein intake and varying digestible energy levels on energy and protein utilization in Nile tilapia

This chapter is accepted for publication as:

M.N. Haidar, S. Bleeker, L.T.N. Heinsbroek, J.W. Schrama (2017). **Effect of constant digestible protein intake and varying digestible energy levels on energy and protein utilization in Nile tilapia.**

Abstract

In literature, the variability in the estimated optimal digestible protein to digestible energy ratio (DP/DE) is high. The present study aimed to estimate the optimal DP/DE ratio in Nile tilapia using different criteria (performance, energy and nitrogen balances parameters). Duplicate aquaria were randomly assigned to one of 16 diets. These diets had a wide range in DP/DE ratio (from 16.7 to 27 g MJ^{-1}). DP levels ranged between 36 and 50% and DE levels between 17.5 and 22 MJ kg^{-1}. Fish were fed restrictively based on a similar digestible protein amount at all 16 diets. An initial fish weight of 6.7g was used. Broken line analysis showed that no optimal DP/DE ratio was found in Nile tilapia within the DP/DE ratio range studied. Regression analysis showed that growth declined as DP/DE ratio increased and seemed to level off at high DP/DE ratio 25 g MJ^{-1}. FCR ranged between 0.8 and 1.1 and increased linearly with increasing DP/DE ratio. Decreasing the DP/DE ratio resulted in a linear increase in protein efficiency to a highest value of 53%. However, protein efficiency did not show a plateau or a maximum value. Moreover, decreasing the DP/DE ratio resulted in a very high fat content of the fish (over 16 %). In conclusion, an optimal DP/DE ratio in Nile tilapia seems to be absent or lower than 16 g MJ^{-1} when fed restrictively. A maximum protein deposition was not reached in Nile tilapia.

2.1 Introduction

The variability in dietary ingredient composition of fish feed is expected to increase, due to the limiting amount of fishmeal and fish oil; the growth of the aquaculture sector and the competition for ingredients for biofuel production and terrestrial animal feeds (Tacon et al., 2011). This increased variability will also coincide with a larger variability in digestibility of ingredients/nutrients. The ratio between digestible protein to digestible energy (DP/DE) is considered important for an optimal diet formulation. Excessive dietary level of protein will be catabolized and used as energy source. In several fish species, it has been shown that increasing the dietary non protein energy level minimizes the amount of protein used for energy and thereby increases protein efficiency (i.e., protein sparing effect) (Kaushik and de Oliva Teles, 1985; Kim and Kaushik, 1992; Tran-Duy et al., 2008). Moreover, this will increase growth and reduces nitrogen excretion (Kaushik, 1998) . In addition, too low DP/DE ratios are assumed to reduce growth and protein efficiency. However, most likely this effect is dependent on the feeding method (ad lib vs restricted). Ali and Jauncy (2005) suggested that too high dietary energy levels will reduce growth by reducing feed intake. Moreover, at low DP/DE ratio in combination with high feed intakes, growth might be limited by the genetic potential for protein gain, as has been demonstrated in pigs (Costa-Orvay et al., 2011). Such information for fish is scarce.

Many studies have addressed the importance of protein to energy ratio and its effect on growth and protein retention in various fish species such as, Rainbow trout *Oncorhynchus mykiss* (Kim and Kaushik, 1992; Lanari et al., 1995), African catfish *Clarias gariepinus* (Ali and Jauncey, 2005; Henken et al., 1986), Nile tilapia *Oreochromis niloticus* (Al Hafedh, 1999; Ali et al., 2008; El-Sayed and Teshima, 1992; Kaushik et al., 1995; Li et al., 2012; Shiau and Huang, 1990; Winfree and Stickney, 1981), Atlantic salmon *Salmo salar* (Einen and Roem, 1997; Hillestad and Johnsen, 1994), Carp *Cyprinus carpio* (Watanabe et al., 1987), Gilthead seabream *Sparus aurata* (Lupatsch et al., 2001). Comparison between these DP/DE studies is difficult because of the large variability between studies in factors like: fish weights; nutrients digestibility (diet quality, fishmeal vs. plant based diets); selected criteria for estimating the optimal ratio; feeding level (ad lib vs. restricted); experimental designs (e.g., number of DP/DE levels) and the range of protein and energy levels studied. The majority of the aforementioned studies were done at satiation feeding, resulting in the combined impact of both metabolism and feed intake on these estimates. In addition, the range of dietary DP/DE ratio tested was narrow using 4 to 9 levels and few studies included low DP/DE ratio (below 18 g/kJ). In some fish species studies, broken line analysis was performed to estimate optimal DP/DE ratio (Akpinar et al., 2012; Booth et al., 2007; Jena et al., 2012). In Nile tilapia, information on low DP/DE ratios is limited, the estimated range and number of DP/DE ratios are small and no broken line analysis has been applied.

The aims of the current study on Nile tilapia were: to determine the effect of DP/DE ratios on energy and nitrogen balances under restricted feeding; to assess the presence of a maximal protein deposition level in fish; to estimate the optimal DP/DE ratio by broken line analysis; and to assess whether DP/DE ratio is dependent on the selected criterion (growth, feed conversion ratio, protein efficiency, retained fat). In order to assess a maximal potential for protein deposition in Nile tilapia, fish were fed equal amount of digestible protein with varying digestible energy levels.

2.2 Materials and methods

2.2.1 Diets and feeding

Thirty two aquaria were randomly assigned to one of 16 experimental diets/treatments. Diets were formulated to cover a wide range of DP/DE based on predicted digestibility data. The realized DP/DE range, was from 16.6 to 27.4 g MJ^{-1} calculated from the measured digestibility (DP ranged from 364 to 483 g kg^{-1} and DE from17.5 and 22.2 MJ kg^{-1} on dry matter basis). The main ingredients used in the diets were: fish meal, soybean, sunflower meal, wheat, rapeseed oil (Table 2.1).

TABLE 2.1 | Ingredients composition of the test diets.

Ingriedents (%)	Mean	Min	Max
Fishmeal	12.9	9.5	22.8
Rape cake	6.7	2.4	10.0
Full Fat Soybean	4.7	3.4	8.1
Defatted Soybean Meal	26.3	5.8	40.0
Soya Protein concentration	23.2	3.8	30.0
Corn Gluten	8.2	2.8	12.0
Sunflower meal	1.5	1.5	1.5
Wheat	25.6	15.6	33.7
Wheat Gluten	4.5	2.8	7.0
Rapeseed oil	11.6	0.8	22.7
Premix	0.45	0.45	0.45
Methionine	0.31	0.10	0.40
Lysine	0.81	0.49	1.17
Threonine	0.18	0.02	0.29
MonoCalcium Phosphate	2.42	1.74	3.17
Yttrium oxide	0.10	0.10	0.10

All diets were formulated to provide the essential nutrients (amino acids; essential fatty acids; vitamins and minerals) required by Nile tilapia (NRC, 2011). Yttrium oxide (Y_2O_3) was added to both diets as an inert marker for digestibility measurements. The experimental feeds were extruded with pellet size of 2mm. Diets were produced by BioMar TechCenter (Brande, Denmark).

Fish were fed restrictively twice daily at 09:00 and at 15:00. The daily feed intake at the diet with the lowest DP/DE ratio was aimed to be 90% of the satiation level of Nile tilapia. All fish were fed an equal amount of digestible nitrogen intake and varying levels of digestible energy intake (Figure 2.1). The feed intake of all diets was equalized to ensure the same DP intake as at the lowest DP/DE diet. This calculation was based on the analysed crude protein content and predicted digestibility at all diets.

The daily feeding ration at the lowest DP/DE diet was calculated based on the mean initial fish weight, the feeding level of the treatment (in g $kg^{0.8}$ BW per d) and the expected growth of the fish. The daily growth of the feed ratio calculated was estimated from the expected feed: gain ratio (FCR).

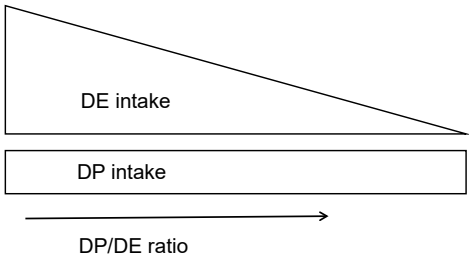

FIGURE 2.1 | Schematic illustration of the experimental setup. Fish were fed similar level of digestible protein (DP) and varying levels of digestible energy (DE).

2.2.2 Fish and housing

Mixed sex of Nile tilapia (*Oreochromis niloticus*) were obtained from the brood stock of the aquatic research facility (CARUS) in Wageningen University. The experiment was approved by the Ethical Committee judging Animal Experiments of Wageningen University, The Netherlands, and carried out according to the Dutch law on animal experiments. At the start of the experiment all fish were randomly divided over 32 tanks of 120L. The initial density was 60 fish per tank. All tanks were connected to the same recirculation system (comprising a common water reservoir, a lamella sedimentation unit for solids removal, a trickling filter for gas exchange and nitrification of NH_4^+). Water flow through each aquarium was kept constant at 7 l min^{-1} (expect for the first week, when water flow was 6 l min^{-1}). Each tank was supplied with an aeration stone in order to maintain the dissolved oxygen (DO) concentration above 4 mg l^{-1}. The measured outlet DO concentrations were above this level. Water quality was kept within the optimal range for tilapia (Tran-Duy et al., 2008) and was measured daily. A 12 h light–12 h dark photoperiod was maintained with daybreak set at 07.00 hours. The experiment lasted 6 weeks.

2.2.3 Measurements of nitrogen and energy balances

At the start of the experiment fish were weighed in groups of 20 fish and at the end of the 42 day (d) experimental period, individual fish were weighed after anaesthetizing fish with a phenoxy-ethanol solution (0, 2 ml l^{-1}). From weight measurements, mean initial body weight (BW0) and final mean body weight (BW42) were calculated per tank. Growth (g $fish^{-1}$) was calculated as (BW42-BW0). Specific growth rate (SGR) was calculated as (ln(BW42)-ln(BW0)/42) × 100. From the feed ration, uneaten feed and feed spillage, which were recorded daily, feed intake (g $fish^{-1}$) was calculated as FItot/n were FItot is total feed intake per tank during the experimental period corrected for dead fish and n is the final number of fish at the end of the experimental period . Feed spillage was recorded by counting the number of feed pellets trapped in the faeces collectors during the feeding period. The FCR was calculated as feed intake divided by growth (both in g $fish^{-1}$). A representative sample of each diet was taken and stored at 4°C and then was ground using a 1 mm-screen grinder for chemical analysis. Initial body composition was determined in 50 fish and final body composition in 10 randomly selected fish per tank. Fish were euthanized by an overdose of a phenoxy-ethanol solution (1.0 ml l^{-1}) and stored at - 20°C. Before chemical analysis, the sampled fish were cut into small pieces, homogenised by grinding in a mincing machine through a 4·5 mm-screen grinder two times and subsequently freeze-dried. Faeces were daily collected per aquarium during the last

4 weeks of the experiment, according to the procedure described by (Amirkolaie et al., 2006) using settling tanks. Daily faecal collection started about 15 min after the end of the feeding period. Faeces were only collected in the morning. Before starting morning feeding, faeces were collected, stored (daily) at -20°C and pooled per aquarium over the experimental period. Throughout the daily faecal collection period, the bottle trapping faeces was continuously submerged in ice water, to prevent bacterial decay. The collected faeces were freeze-dried and ground using a 1 mm-screen grinder.

Chemical analyses were done in triplicate for feed samples and in duplicate for the faeces samples. Dry matter (DM) was determined gravimetrically after drying at 103°C for 4, 4 and 24 hours (h) until constant weight, respectively, for feed, freeze-dried faeces and fish samples (ISO 6496, 1983); ash was determined after incineration at 550°C for 4 h (ISO 5984,1978). Crude protein (CP) (Nx6·25) was determined by the Kjeldahl method (ISO 5983, 1979). Fat was quantified after petroleum–diethyl ether extraction (ISO 6492, 1999). Before fat analysis, feed and faecal samples were hydrolysed by boiling for 1 h with 3M-HCl. Energy content was measured by direct combustion in an adiabatic bomb calorimeter (IKA-C-7000; IKA analysentechnik, Weitersheim, Germany). For feed and faeces, total carbohydrates (i.e., starch+free sugars+NSP) was calculated as DM-CP- fat-ash. Starch was enzymatically determined in feed and faecal samples by using amyloglucosidase with the ethanol extraction step and measuring glucose content as described by (Goelema et al., 1998). Apparent digestibility coefficients of nutrients were calculated for each aquarium as in (Amirkolaie et al., 2006), using Y_2O_3 as an inert marker. Energy and nitrogen (N) balance parameters were calculated per aquarium and expressed as, respectively, kJ fish^{-1} and mg fish^{-1}. N balance calculations were as follows:

Gross nitrogen intake (GN) = FI ×Nfeed, where FI = feed intake of the fish (g feed fish^{-1}), Nfeed = nitrogen content of the feed. Digestible nitrogen (DN) = (GN×ADCcp)/100, where GN = Gross nitrogen intake , ADCcp (%) = apparent digestibility coefficient of the crude protein in the feed. Faecal nitrogen losses = GN –DN. Branchial and urinary nitrogen loses (BUN) = DN-RN, where RN= retained nitrogen. RN = ((BWt×CP)/6.25)-((BW$_0$×CP)/6.25), where BWt = body weight of fish at the end of the experiment (kg), BW$_0$ = body weight of fish at the start of the experiment (kg), CP= crude protein content of the fish (g). Energy balance calculations were as follow: Gross Energy intake (GE) = FI × Efeed, where FI = feed intake of the fish (g feed/fish), Efeed =energy content of the feed. Digestible Energy (DE) = (GE×ADCE) /100, where ADCE (%) = apparent digestibility coefficient of the energy in the feed. Faecal energy losses (FE) = GE-DE. Metabolizable energy (ME) =DE-BUE where BUE = branchial and urinary energy losses. BUE = (BUN×24.9)/1000, where 24.9 kJ N g-1= energy concentration of NH_3-N calculated by (Bureau et al., 2003) and assuming that all N was excreted as (NH_3-N) . Retained energy (RE)= BWt×Et- BW$_0$×E$_0$, where Et = energy content of the fish at the end of the experiment, E$_0$ = energy content of the fish at the start of the experiment, BWt = body weight of fish at the end of the experiment, BW$_0$ = body weight of fish at the start of the experiment. Heat production (HP) = ME-RE.

2.2.4 Statistical analysis

Statistical analyses were performed using the statistical analysis system, statistical software package version 9.2 (SAS institute, Cary, NC, USA). All parameters were subjected to broken line analysis using the NLIN procedure of SAS (Robbins et al., 2006). Furthermore, the general linear model (GLM) procedure was used to fit a linear and quadratic regression model with DP/DE ratio as independent variable. The level of significant was set at 0.05.

2.3 Results

The analysed nutrient composition, digestible nutrients and DP/DE ratios are shown in table 2.2. The16 formulated diets showed a wide range in protein, fat, total carbohydrates and energy contents. Protein ranged from 37 to- 50%, fat from 3 to 27% and energy from 19.0 to 23.5 MJ kg^{-1}. Proximate composition combined with digestibility data resulted in a realized DP/DE ratio range from 16.6 to 27.4 g MJ^{-1}. The broken line analysis showed that no optimal DP/DE ratio was present for any of the measured/calculated variables in this study ($P>0.05$). Therefore, all parameters were subjected to regression analysis.

TABLE 2.2 | The analysed nutrients content of the test diets on dry matter basis.

Analysed nutrients g kg^{-1}	Mean	Min	Max
DM	924	906.2	954.2
Protein	431	377.0	498.2
Fat	137	28.9	266.8
Energy (kJ g^{-1})	21.2	19.3	23.5
Ash	78.8	76.7	83.5
Starch	175.1	139.0	233.3
Total carbohdrates	353.1	245.7	473.4
DP g kg^{-1}	417.7	364.4	482.8
DE MJ kg^{-1}	19.7	17.5	22.2
Phosphorous g kg^{-1}	12.9	12.3	14.6

DM, dry matter; DP, digestible protein; DE, digestible energy.

The design of the study aimed to have equal protein intake between diets that differed in DP/DE ratio. Therefore, feed intake decreased linearly with DP/DE ratio ($P<0.001$; Table 2.3). The fish growth was quadratic and related to the DP/DE ratio, despite similar protein intake, growth increased with decreasing DP/DE ratio ($P<0.05$; Table 2.3, Figure 2.2 a).

Growth seemed linearly at low DP/DE ratios. Growth declined with DP/DE ratio and started to level off at high DP/DE ratios (at about 25 g MJ^{-1}). FCR ranged between 0.8 and 1.1 and increased linearly with DP/DE ratio ($P<0.05$; Table 2.3, Figure 2.2 b).

TABLE 2.3 | The relation between DP/DE ratio and fish performance.

Variables	Mean	Range	SD	CV	Equation, X=DP/DE	R^2	Effect
Initial weight	6.8	6.5-6.7	0.08	1.14			
Final weight	44.2	35.8-54.5	4.69	10.61			
Feed intake (g $fish^{-1}$)	34.0	28.4-38.0	3.12	9.18	50.08(±2.96)-0.75(±0.14)X	0.500	L***
Growth (g $fish^{-1}$)	37.5	29.1-47.7	4.66	12.44	109.92(±19.08)-5.34(±1.76)X+0.09(±0.04)X^2	0.813	Q*
SGR (%day)	4.4	3.9-4.8	0.24	5.45	5.88(±0.15)-0.07(±0.001)X	0.772	L***
FCR	0.9	0.8-1.1	0.10	11.11	0.62(±0.13)+0.01(±0.01)X	0.15	L*

DP/DE, digestible protein to digestible energy (g MJ^{-1}); SGR, specific growth rate; FCR, feed conversion ratio. L, linear effect; Q, quadratic effect.* P<0.05,***P<0.001.

2

At the end of the experiment, all body composition parameters except ash content were linearly affected by DP/DE ratio (P<0.001; Table 2.4). Protein content increased with DP/DE ratio whereas fat content declined with DP/DE ratio.

In table 2.5 the relations between nitrogen and energy balances parameters and DP/DE ratio are shown. The experimental design aimed to have equal digestible protein intakes at all diets. However, the expected digestibility values slightly differed from the measured values. This resulted in a curvilinear response between DP/DE ratio and digestible nitrogen (i.e., protein) intake (P< 0.05). The numerical differences in nitrogen intake were small, indicated by the low CV (2.5%) for digestible nitrogen intake. The curvilinearity was predominantly caused by one diet with a DP/DE ratio of 21.1 g MJ^{-1}. The digestible nitrogen intake at this diet was 2245 mg $fish^{-1}$ compared to 2077 mg $fish^{-1}$ at the other diets. The nitrogen retention of tilapia was only linearly related to DP/DE (P<0.001; Table 2.5, Figure 2.3 c).

With decreasing DP/DE ratio (increasing energy intake), nitrogen retention increased. Parallel to nitrogen retention, the protein efficiency was linearly and negatively related to DP/DE ratio (P<0.001; Figure 2.3 d). At the lowest and highest dietary DP/DE ratio, the protein efficiency was 53 and 32%, respectively.

Regarding the energy balance, significant quadratic relations with DP/DE ratio were found for digestible energy intake, branchial and urinary losses, metabolizable energy, retained energy and retained energy as fat. However, gross energy intake, heat production and retained energy as protein were linearly related to DP/DE ratio. Branchial and urinary losses increased with DP/DE ratio and retained energy, retained energy as fat and protein increased with decreasing DP/DE ratio.

Fat balance is presented in table 2.6. All fat balance parameters showed a quadratic relation with DP/DE ratio (P<0.05), but the fat retention efficiency had a linear relationship (P<0.001). At the diets with a DP/DE ratio above 19 g MJ^{-1}, digestible fat intake was lower than the fat retention (Figure 2.4), which is also reflected by the fat retention efficiency being above 100%.
The difference between digestible fat intake and fat deposition increased with DP/DE ratio. Below the DP/DE ratio of about 19 mg/kJ the digestible fat intake was higher than the fat deposition.

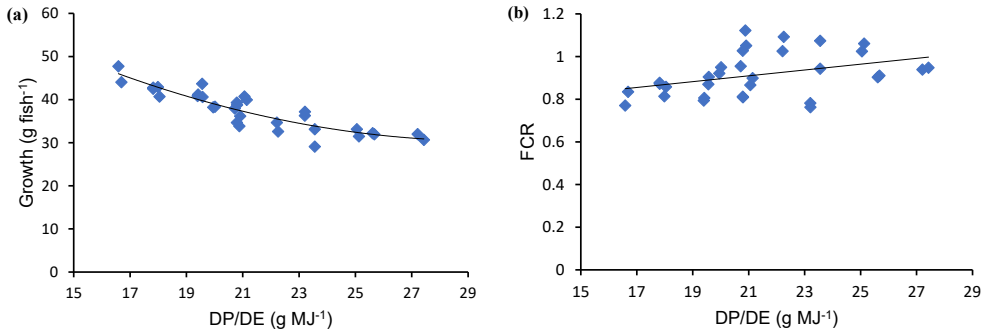

FIGURE 2.2 | The relation between DP/DE ratio, growth (a) and Feed conversion ratio (FCR, b) in Nile tilapia. Regression equations are in table 2.3.

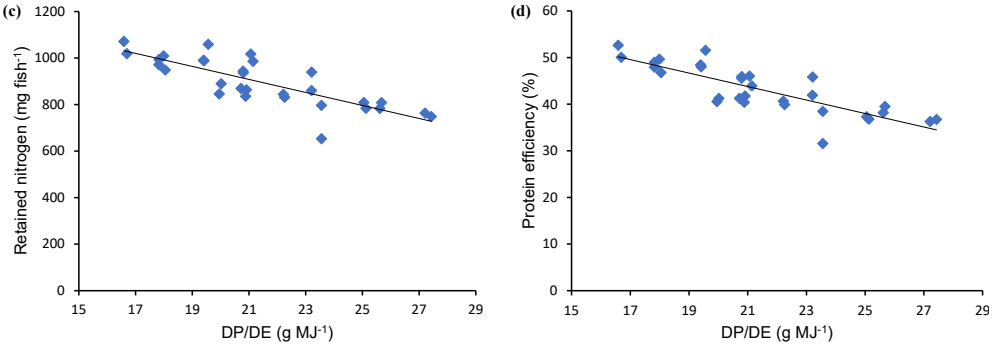

FIGURE 2.3 | The relation between DP/DE ratio, retained nitrogen (c) and protein efficiency (d) in Nile tilapia. Regression equations are in table 2.5.

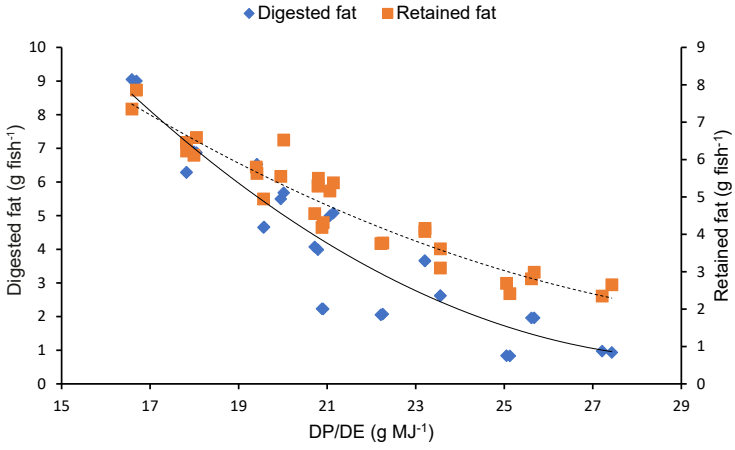

FIGURE 2.4 | The relation between DP/DE ratio, retained fat and digested fat in Nile tilapia. Regression equations are in table 2.6.

TABLE 2.4 | The relation between DP/DE ratio and final body composition (wet weight basis).

Variables	Mean	Range	SD	CV	Equation, X=DP/DE	R^2	Effect
Dry matter g kg^{-1}	304.1	264-345	20.69	6.8	434(±13.40)-6.06(±0.62)X	0.774	L***
Ash g kg^{-1}	32.7	30-35	1.23	3.8	31(±1.65)+0.081(±0.0.076)X	0.038	ns
Protein g kg^{-1}	149.0	139-157	4.63	3.1	133(±5.47)+0.78(±0.25)X	0.249	L**
Fat g kg^{-1}	119.7	77-167	23.61	19.7	274(±13.10)-7.18(±0.61)X	0.834	L***
Energy KJ g^{-1}	8.11	6.49-9.96	0.88	10.8	14(±0.46)-0.27(±0.02)X	0.851	L***

DP/DE, digestible protein to digestible energy (g MJ^{-1}); SD, standard deviation; CV, coefficient of variation. L, linear effect; Q, quadratic effect. *** P<0.01;***P<0.001;ns, not significant.

TABLE 2.5 | The relation between DP/DE ratio and nitrogen and energy balances.

Variables	Mean	Range	SD	CV	Equation, X=DP/DE	R^2	Effect
Nitrogen balance (mg $fish^{-1}$)							
Nitrogen intake	2146	2095-2315	53.22	2.48	1054(±465.23)+98.39(±42.84)X-2.17(±0.97)X^2	0.172	Q*
Digestible nitrogen intake	2077	2026-2245	52.85	2.54	1013(±458.20)+95.54(±42.19)X-2.10(±0.96)X^2	0.175	Q*
Branchial urinary nitrogen losses	1183	965-1417	121.32	10.26	-861(±651.60)+157.94(±60.10)X-2.87(±1.37)X^2	0.708	Q*
Retained nitrogen	895	653-1071	104.06	11.62	1495(±83.80)-27.95(±3.87)X	0.651	L***
Protein efficiency %	43	32-53	5.26	12.19	73.65(±3.88)-1.43(±0.18)X	0.69	L***
Energy balance (KJ $fish^{-1}$)							
Energy intake	664	509-811	80.43	11.99	1239(±23.55)-26.83(±1.09)X	0.953	L***
Digestible energy intake	618	464-767	79.08	12.80	1487(±132.79)-54.22(±12.23)X+0.63(±0.28)X^2	0.969	Q*
Branchial urinary energy losses	29	24-35	3.02	10.26	-22.39(±15.12)+4.05(±1.40)X-0.08(±0.03)X^2	0.708	Q*
Metabolisable energy intake	587	432-743	83.72	14.27	1496(±131.56)-56.90(±12.13)X+0.67(±0.28)X^2	0.974	Q*
Retained energy	315	205-459	73.42	23.28	1321(±196.51)-70.37(±18.12)X+1.08(±0.41)X^2	0.922	Q*
Heat production	271	223-302	23.05	8.50	367(±27.06)-4.43(±1.25)X	0.310	L**
Retained energy as protein	133	97-159	15.41	11.62	222(±12.41)-4.14(±0.57)X	0.651	L***
Retained energy as fat	183	91-308	60.25	32.97	1044(±155.04)-61.05(±14.30)X+0.96(±0.33)X^2	0.928	Q**

DP/DE, digestible protein to digestible energy (g MJ^{-1});SD, standard deviation. CV, coefficient of variance. L, linear effect; Q, quadratic effect.* P<0.05, **P<0.01, ***P<0.001.

TABLE 2.6 | The relation between DP/DE ratio and fat balance.

Variables (g $fish^{-1}$)	Mean	Range	SD	CV	Equation, X=DP/DE	R^2	Effect
Fat intake	4.34	0.86-9.20	2.36	54.24	42.26(±9.04)-2.81(±0.83)X+0.05(±0.02)X^2	0.836	Q*
Digested fat intake	4.27	0.83-9.05	2.33	54.61	41.38(±9.08)-2.74(±0.84)X+0.05(±0.02)X^2	0.831	Q*
Retained fat	4.73	2.35-7.86	1.53	32.37	24.97(±4.36)-1.40(±0.40)X+0.02(±0.01)X^2	0.912	Q*
Fat retention efficency (%)	140.41	81.26-320.37	66.43	47.31	-205(±62.12)+16.09(±2.87)X	0.529	L***

DP/DE, digestible protein to digestible energy (g MJ^{-1});SD, standard deviation. CV, coefficient of variance. L, linear effect; Q, quadratic effect. Fat retention efficiency= (Retained fat/digested fat)×100.
* P<0.05, ***P<0.001.

2.4 Discussion

The present study aimed to estimate the optimal digestible protein to digestible energy (DP/DE) ratio in Nile tilapia using various criteria (performance, energy and nitrogen balances parameters). Fish were fed diets with a wide range of DP/DE ratios (16.6-27.4 g MJ^{-1}). Restricted feeding was applied targeting to have an equal amount of digestible protein intake. Consequently, with decreasing dietary DP/DE ratio, the total DE intake increased (Figure 2.5). Broken line analysis revealed that no optimal DP/DE ratio was found in Nile tilapia within the studied range. Moreover, regression analysis showed that growth, retained nitrogen and protein efficiency confirmed the absence of an optimal DP/DE ratio. This suggests that the optimal DP/DE ratio is either not present or lower than 16 g MJ^{-1} for Nile tilapia.

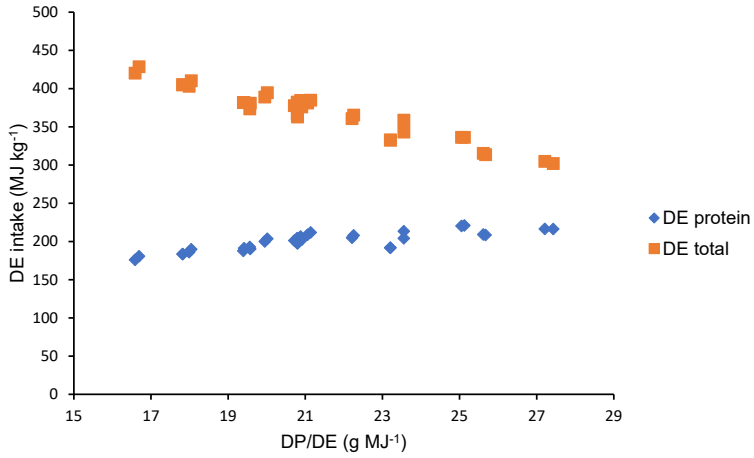

FIGURE 2.5 | The relation between DP/DE ratio and total digestible energy (DE) intake and energy intake as protein

The current finding of an optimal DP/DE ratio below 16 g MJ^{-1} is a very low estimate compared to other studies on Nile tilapia having reported optimal values between 18 and 26.3 g MJ^{-1} (Al Hafedh, 1999; Ali et al., 2008; El-Sayed and Teshima, 1992; Fernandes et al., 2016; Kaushik et al., 1995; Li et al., 2012; Van Trung et al., 2011). The value is also low compared the optimal DP/DE ratio reported for other fish species e.g.: Atlantic salmon 17-18 g MJ^{-1} (Einen and Roem, 1997); gilthead sea bream 19-23 g MJ^{-1} (Lupatsch et al., 2003b, 2001); fringe lipped carp 17.2-17.6 g MJ^{-1} (Jena et al., 2012). The differences in optimal DP/DE ratios within species and between species might be related to differences in body weight between studies. As fish grow, optimal DP/DE ratio declines (Glencross and Bermudes, 2012; Lupatsch et al., 2001). This is due to the change in body composition (i.e., higher fat content) and an increasing amount of the consumed DE being used for maintenance. The current study used relatively small Nile tilapia (initial weight 6g) compared to the other Nile tilapia and fish studies. Therefore, the body weight does not seem to explain the low or absence of an optimal DP/DE ratio. An alternative explanation might be differences in feeding level (restricted vs. satiation) between studies. Most studies assessing the impact of dietary protein to energy ratios are done at satiation feeding (e.g., tilapia (Ali et al., 2008; Fernandes et al., 2016; Kaushik et al., 1995) Salmon (Einen and Roem, 1997); gilthead sea bream (Lupatsch et al., 2001). The optimal

DP/DE ratio in various response criteria other than feed intake might still be a reflection of the impact of DP/DE ratio on the voluntary feed intake. At low DP/DE ratios, diets contained relatively high levels of fat and/or carbohydrates. High levels of fat may affect the energy intake (e.g., Rainbow trout , (Gélineau et al., 2002)). However, for many fish species, voluntary energy intake often is higher at higher dietary fat levels (e.g. Nile tilapia, (Saravanan et al., 2012); European sea bass, (Peres and Oliva-Teles, 1999)). If the DP/DE ratio is decreased by increasing the carbohydrates content this leads to low nutrient dense diets. Several studies have shown that dietary volume might restrict the feed intake (e.g., in Rainbow trout, (Saravanan et al., 2012)). Also, digestible carbohydrate content (i.e., starch content) has been found to result in a lower digestible energy intake in tilapia (Saravanan et al., 2012; Tran-Duy et al., 2008). Hence, in the case that energy intake is reduced with decreasing DP/DE ratio this might be the reason why studies done at satiation feeding are finding an optimal DP/DE ratio.

Less studies on fish have been performed using restrictive levels for estimating optimal DP/DE ratio, but in those studies reported in literature still optimal DP/DE ratios were found in the range of 19 to 28g MJ^{-1} (combining different fish species; (Ai et al., 2004; Ali and Jauncey, 2005; Garling and Wilson, 1976; Shiau and Huang, 1989, 1990; Takakuwa et al., 2006)). However, in most studies applying restricted feeding, fish were fed based on gram of feed per unit of body weight. As a consequence of altering the dietary DP/DE ratio, this implies that both the amount of non-protein energy as well as the amount of protein intake altered with varying the DP/DE ratio. In such a design, the protein intake increases with increasing dietary DP/DE ratio and none-protein energy intakes decreases with high DP/DE ratios. In such studies, it might be that the response found of optimal levels regarding e.g. protein efficiency are merely induced by differences in protein intake, induced by a too low protein intake at low DP/DE ratio diets. Since protein intake was kept constant between all diets (having different DP/DE ratio) in the current study (Figure 2.5), the absence of an optimum might be related to the prevention of having a limitation in protein intake.

In comparison to the other studies in Nile tilapia (> 6g) on optimal DP/DE estimation, the diets formulated in the current study had a high nutrient density. This is reflected in figure 2.6 where the crude protein and gross energy content of Nile tilapia studies are plotted. Except for the current study, all studies have a gross energy content lower than 18 MJ kg^{-1} . Moreover, the protein levels in our study did not include low levels of crude protein (all being above 38%). This graph also shows that in the studies of (Kaushik et al., 1995) and (Al Hafedh, 1999) the gross energy levels were kept constant and only protein content differed, ranging from 10 to 45%. The low nutrient density diets applied in most Nile tilapia studies, even when fed to satiation, may have led to absolute lower nutrients intake compared to the current study if dietary volume has been hampering feed intake. In general, the large differences in nutrient density may have been a source of variance between the studies.

Increasing the digestible non protein energy intake while keeping the DP intake constant (i.e. decreasing DP/DE ratio) resulted in our study in a linear increase in protein efficiency (Figure 2.3d). Opposite to the expectation, no maximum or plateau in protein efficiency was found when the digestible non-protein energy intake increased. Often in studies on optimal DP/DE, the protein efficiency ratio (PER) is used as a criterion for the estimation. Weaknesses of PER are that it also includes fat retention and it does not account

for differences in nutrient (protein) digestibility. Therefore, protein efficiency calculated as retained protein over digestible protein intake is a better criterion. In the current study, the maximal protein efficiency was about 53%. This values is comparable to reported and derived values from other Nile tilapia studies (Figueiredo-Silva et al., 2013; Saravanan et al., 2012; Schrama et al., 2012; Tran-Duy et al., 2008; Van Trung et al., 2011) and for carnivores fish (Booth et al., 2010; Grisdale-Helland et al., 2013; Hatlen et al., 2007; Lupatsch et al., 2001; Peres and Oliva-Teles, 1999) and values for pigs (Conde-Aguilera et al., 2011; Kyriazakis and Emmans, 1992) and poultry (Kong and Adeola, 2011). In mammals as well as in fish it is often hypothesised that animals under satiation feeding eat until they reach their maximum protein deposition capacity. E.g., (Geurden et al., 2006) suggested based on feed intake trails that rainbow trout regulate their feed intake to meet their maximum protein growth. Similarly in rats (Webster, 1993) found that they eat until they reach a maximum protein retention regardless of becoming obese. These hypotheses suggest the existence of a maximal protein deposition capacity in animals, however in juvenile fish this has not been proven. Also in our study, Nile tilapia did not show a maximum protein deposition capacity (i.e., no plateau was reached in protein retention) even though the fish became extremely fat. At the lowest DP/DE ratios body fat content was > 48% on DM basis (i.e., > 16% on fresh basis). In pigs it was postulated that the maximum protein retention is heritable (Whittemore et al., 1988), thus the maximum protein retention capacity is an animal related characteristics. In fish, such genetic predisposed maximum capacity for protein growth is not demonstrated as well as the existence of a maximal protein retention in juveniles. However, in rainbow trout, protein deposition increased linearly with body weight until fish reached 400 g body weight and after that the deposition of protein levelled off (Dumas et al., 2007). In juveniles, fish exhibit hyperplasia and hypertrophy as a means of muscle growth (Stickland, 1983). Across species, hyperplasia of muscle fibers disappears above around a body size of 45% of the maximal body size (Weatherley and Gill, 1985). Hence, in older fish muscle growth is only due to hypertrophy. The absence of a maximum protein deposition in the current study on 5-40g Nile tilapia might be due to the fact that at this age, hyperplasia is still occurring. However this hypothesis needs further testing.

Decreasing the DP/DE ratio in our study resulted in extremely high body fat contents for 40g Nile tilapia (>45% on DM basis). Similar high body fat have also been observed though in heavier tilapia (250g in (Saravanan et al., 2012)). Fish fed diets containing more dense energy levels often increase the rate of fat retention. In the current study, the higher fat retention still coincided with higher growth rates/protein retention rates when the DP/DE ratio declined. Despite the "obesity" of our young fish, feed intake at the low DP/DE ratio was high, 0.8 g $fish^{-1}$ day^{-1} and did not reach the satiation level. The realized feed intake at these DP/DE ratios was 20% of the ad lib intake reported in 40-200 g fish range (Saravanan et al., 2012; Tran-Duy et al., 2008). This probably indicates that in our study a further decrease in the DP/DE ratio below 16 g/MJ would enhance fat deposition. The current extreme high body content may be considered undesirable regarding potential negative health aspects in later life stage. This need further assessment. However, in the current study no health issues were observed at all experimental diets.

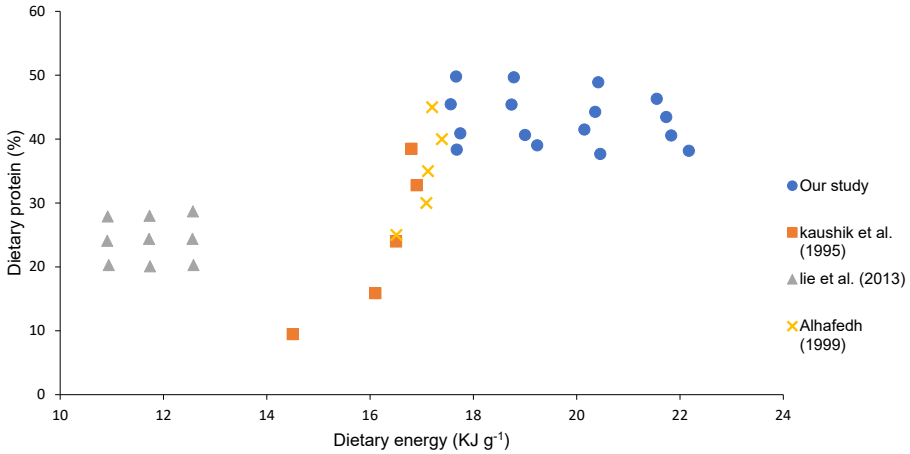

FIGURE 2.6 | Dietary energy and protein levels of different studies compared to our study.

One interesting observation from the current study is that retained fat was higher than fat digested by fish, this was reflected in fat retention efficiency over than 100 % (table 2.6; figure 2.4). This indicates that at a DP/DE ratio above 19 g MJ^{-1} *de novo* fat synthesis occurred. This has earlier been reported for Nile tilapia by (Saravanan et al., 2012; Schrama et al., 2012) and also in gilthead sea bream (Ekmann et al., 2013). When increasing the DP/DE ratio, part of the dietary protein was catabolized (i.e. lower protein retention efficiency) but at the same time was used for *de novo* fatty acid synthesis. The DP/DE ratio where retained and digested fat lines meet (no *de novo* lipid biosynthesis) is about 19 g/MJ. At this DP/DE ratio, theoretically the digested protein is retained as body protein and no *de novo* fatty acids took place. The DP/DE ratio value of 19 is similar to what was found for an optimal DP/DE ratio estimated in Nile tilapia (Ali et al., 2008; Kaushik et al., 1995). However, in the current study a DP/DE ratio value of 16.6 g/MJ has a better growth compared to 19 g/MJ.

2.5 Conclusion

In conclusion, the current study demonstrated that under restricted feeding and using regression analysis, an optimal DP/DE ratio for Nile tilapia cannot be found for the weight range (5-40 g). Moreover, a maximum protein deposition was not reached in Nile tilapia.

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Chapter 3

The effect of DP/DE ratios on energy and protein balances in Nile tilapia

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M.N. Haidar, W. van den Herik, F. Berkers, K. Hout, J.A.J.Verreth, J.W. Schrama (2017). **The effect of DP/DE ratios on energy and protein balances in Nile tilapia.**

Abstract

Among and within fish species, the reported optimal digestible protein to digestible energy ratio (DP/DE) is highly variable. For Nile tilapia the reported DP/DE ratios are not conclusive and also highly variable. The aim of this study was to estimate the optimal DP/DE ratio and to assess the effect of DP/DE ratio on Nile tilapia performance and energy and nitrogen balances under satiation feeding. Duplicate aquaria were randomly assigned to one of 8 experimental treatments. Dietary digestible protein (DP) ranged from 37 to 48 % and dietary digestible energy (DE) from 16 to 21 kJ g^{-1}. Consequently, the dietary DP/DE ratio of the 8 experimental diets ranged from 17 to 29 mg kJ^{-1}. Fish were fed to satiation. The initial average fish weight was 6.1g. In the current study broken line analysis revealed that no optimal DP/DE ratio could be found in Nile tilapia within the DP/DE ratio range studied. Regression analysis showed that growth decreased linearly with increasing DP/DE ratio. Feed intake had no relation with DP/DE ratio. Feed conversion ratio ranged between 0.9 and 1.2 and decreased linearly as DP/DE ratio decreased. The highest protein retention efficiency was about 44% and decreased linearly with increasing DP/DE ratio. A maximum value or even a plateaued relation between DP/DE ratio and protein retention efficiency was not observed. To conclude, an optimal DP/DE ratio in Nile tilapia seems to be absent or lower than 17 mg kJ^{-1} and a maximum protein deposition was not reached under satiation feeding. Changing DP/DE ratio in the current study had no effect on feed intake.

3.1 Introduction

The digestible protein to digestible energy ratio (DP/DE) is an important factor in diet formulation. To maximize protein utilization, the fraction of DE (i.e., non-protein energy source) in the DP/DE ratio should be high enough to spare dietary protein from being utilized as energy source. This protein sparing effect will decrease nitrogen excretion and enhance protein growth (Green and Hardy, 2008; Kaushik, 1998). However, if the DE fraction is in excess of the requirements, this may reduce feed intake and hence protein intake (Ali and Jauncey, 2005). The optimal DP/DE ratio is influenced by factors like fish size, feed intake and fish species (e.g., the growth potential of different fish species) (Einen and Roem, 1997; Glencross and Bermudes, 2012; Lupatsch et al., 2001).

The optimal dietary protein to energy ratio can be based on different criteria such as maximal growth rate, minimal feed conversion ratio (FCR), maximizing protein retention efficiency or minimizing fat retention. Numerous studies have investigated the optimal dietary protein to energy ratio in different fish species. A direct comparison of the optimal ratios between studies is difficult because of differences in experimental designs, diet composition, feeding methods, feeding levels applied, the expression of the dietary protein to energy ratio (on proximate versus digestible basis), etc. For example, most studies in Nile tilapia investigating the protein to energy ratio did not measure protein digestibility and the gross energy was calculated from the energetic values of protein, lipid and carbohydrates (Al Hafedh, 1999; Ali et al., 2008; El-Sayed and Teshima, 1992; Li et al., 2012; Shiau and Huang, 1990; Winfree and Stickney, 1981). By not measuring nutrient digestibilities, impacts of changes in diet composition on digestibility are omitted. Further, most studies on Nile tilapia were done at satiation feeding but did not apply a wide range of dietary energy and protein levels (Ali et al., 2008; El-Sayed and Teshima, 1992; Kaushik et al., 1995). In such experimental conditions, it is difficult to measure precise estimates the optimal protein to energy ratio by broken line analysis.

Fish in comparison to birds and mammals require high quality protein diets (Cowey, 1975). In the past, fish meal was used as the main protein source in fish diets. However, currently more plant protein ingredients are incorporated in fish diets. This increased the variability in dietary ingredients composition, thereby leadingto a larger variability in nutrient digestibility. This variability (among others) on protein digestibility has implications on the protein retention. For mammals and birds, maximal protein retention efficiency is about 50% (Kong and Adeola, 2011; Kyriazakis and Emmans, 1992). Also in carnivorouss fish species, about 50% of the protein intake can be retained as protein (Brett Glencross et al., 2008; Lupatsch et al., 2001; Peres and Oliva-Teles, 2005; Pirozzi et al., 2010a). In addition, Kyriazakis and Emmans (1992) and Webster (1993) found in mammals a maximum level of protein deposition. In herbivorous fish like Nile tilapia, however, information on protein retention efficiency and a maximal potential of protein deposition in relation to wide range of DP/DE ratio is limited.

In a recent study (Haidar et al., submitted) on Nile tilapia (average initial weight, 6 g), no optimal DP/DE ratio was found within the studied DP/DE ratios ranging from 16.7 to 27 mg kJ^{-1}. In that study the tilapia were fed restrictively with equal amounts of digestible protein intake. Feed intake can be affected by diet composition (Saravanan et al., 2012). At lower DP/DE ratios, feed intake might be restricted by the

maximal energy intake resulting in an even stronger reduction in DP intake (Saravanan et al., 2012). Hence it hypothesised that the restricted feeding level applied in the study of Haidar et al. (submitted) caused the absence of an optimal DP/DE ratio.

Therefore, we aimed in this study to determine the optimal DP/DE ratio by broken line analysis; to assess the existence of a maximal protein deposition level; to check whether DP/DE ratio has an impact on feed intake and to assess the effect of DP/DE ratios on nutrient digestibility. In the current study Nile tilapia was fed to satiation in order to investigate whether feeding level has an impact on the estimation of the optimal DP/DE ratio.

3.2 Materials and methods

3.2.1. Diets and feeding

Sixteen aquaria were randomly assigned to one of 8 experimental diets/treatments. The DP/DE ratios of these diets ranged from 17.2 to 28.9 mg kJ^{-1}. The DP/DE ratio was calculated from the measured digestibility. Between the diets, DP ranged from 368 to 477 mg g^{-1} and DE from 16.3 to 21.4 kJ g^{-1} on dry matter basis. The eight diets were selected among the 16 diets used in a previous study (Haidar et al., submitted). As selection criteria we used the largest range possible in DP/DE ratio among the 16 original diets and equally divided DP/DE ratios within this range. The DP/DE ratios in these 8 experimental diets were varied by altering the ingredient composition. The averaged and range of inclusion levels of ingredients used in the experimental diets are given in table 3.1.

TABLE 3.1 | Ingredients composition of the test diets.

Ingriedents (%)	Mean	Min	Max
Fishmeal	13.25	9.50	17.98
Rape cake	10.00	10.00	10.00
Full fat soybean	8.11	8.11	8.11
Defatted soybean meal	20.76	5.75	40.00
Soya protein concentration	26.90	9.51	30.00
Corn gluten	6.26	2.78	8.00
Wheat	24.22	20.15	33.46
Wheat gluten	4.16	2.79	7.00
Rapeseed oil	12.18	0.78	22.71
Premix	0.45	0.45	0.45
Methionine	0.30	0.20	0.37
Lysine	0.74	0.50	1.05
Threonine	0.12	0.02	0.26
MonoCalcium Phosphate	2.45	2.14	3.17
Yttrium oxide	0.05	0.05	0.05

All diets were formulated to provide the essential nutrients (amino acids; essential fatty acids; vitamins and minerals) required by Nile tilapia (NRC, 2011). Yttrium oxide (Y_2O_3) was added to all diets as an inert marker for digestibility measurements. The experimental feeds were extruded with pellet size of 2mm. Diets were produced by BioMar (BioMar TechCenter, Brande, Denmark). Fish were fed to apparent satiation by hand. Feeding was done twice daily for one hour (at 09:00 and 16:00h). After each feeding session, the amount of feed was recorded and the number of uneaten pellets (spilled from the tanks) were collected from the swirl separator and counted.

3.2.2. Fish and housing

All male Nile tilapia (*Oreochromis niloticus*) were obtained from Til-Aqua, The Netherlands. The experiment was approved by the Ethical Committee judging Animal Experiments of Wageningen University, The Netherlands, and carried out according to the Dutch law on animal experiments. At the start of the experiment all fish were randomly divided over 16 tanks of 120L. The initial density was 60 fish per tank. The initial average weight of the fish was 6.1g. All tanks were connected to the same recirculation system (comprising a common water reservoir, a lamella sedimentation unit for solids removal, a trickling filter for gas exchange and nitrification of ammonium ($NH4^+$). Water flow through each aquarium was kept constant at 7 l min^{-1} (expect for the first week, when water flow was 6 l min^{-1}). Each tank was supplied with an aeration stone in order to maintain the dissolved oxygen (DO) concentration above 4 mg l^{-1}. The measured outlet DO concentrations were above this level. Water quality was kept within the optimal range for tilapia (Tran-Duy et al., 2008) and was measured daily. Averaged over the total experimental period, the temperature was 27.5°C, pH was 6.5-7.9, N-NH_4^+ was 0.1 mg l^{-1}; N-NO_2^- was 0.1 mg l^{-1} ; N-NO_3^- 95 mg l^{-1} and DO was 6.7 mg l^{-1} at the outlet water. A 12 h light–12 h dark photoperiod was maintained with daybreak set at 07.00 hours. The experiment lasted 6 weeks.

3.2.3. Measurements of nitrogen and energy balances

At the start of the experiment fish were weighed in groups of 5 fish and at the end of the 42 day (d) experimental period, fish were weighed after anaesthetizing with a phenoxy-ethanol solution (0.2 ml l^{-1}). From weight measurements, mean initial body weight (BW0) and final mean body weight (BW42) were calculated per tank. Growth (g $fish^{-1}$) was calculated as (BW42-BW0). Specific growth rate (SGR) was calculated as (ln(BW42)-ln(BW0))/42*100. Growth rate per metabolic weight unit (in g $kg^{-0.8}$ BW d^{-1}) was calculated per tank as (BW42-BW0) x 42/MBWm, with MBWm being the mean metabolic BW during the experimental period (in $kg^{-0.8}$). From the feed given, daily uneaten feed and daily recorded feed spillage, feed intake in (f $fish^{-1}$) was calculated as FItot/**n** were FItot is total feed intake per tank during the experimental period corrected for dead fish and **n** is the final number of fish at the end of the experimental period. Feed intake per metabolic weight unit was expressed as g $kg^{-0.8}$ BW d^{-1} using MBWm. The FCR was calculated as feed intake divided by growth (both g $kg^{-0.8}$ BW d^{-1}). A representative sample of each diet was taken and stored at 4°C and then was ground using a 1 mm-screen grinder for chemical analysis. Initial body composition was determined in 50 fish and final body composition in 10 randomly selected fish per tank. Fish were

euthanized by an overdose of a phenoxy-ethanol solution (1.0 ml l^{-1}) and stored at - 20°C. Before chemical analysis, the sampled fish were cut into small pieces, homogenized by grinding in a mincing machine through a 4·5 mm-screen grinder two times and subsequently freeze-dried. Faeces were daily collected per aquarium during the last 4 weeks of the experiment, according to the procedure described by Amirkolaie et al. (2006) using settling tanks. 30 min prior to the morning feeding session, faeces were collected once a day in a detachable 250 ml glass bottle at the bottom of the swirl separator (44 cm in height, 24·5 cm in diameter; Aqua Optima AS, Trondheim, Norway). In order to minimise the bacterial decay of faeces, the glass bottles were kept in ice. Faeces were collected, stored (daily) at - 20°C and pooled per aquarium over the experimental period. The collected faeces were oven-dried and ground using a 1 mm-screen grinder.

Chemical analyses were done in triplicate on feed, fish and faeces samples. Dry matter (DM) was determined gravimetrically after drying at 103°C for 4, 4 and 24 hours (h) until constant weight, respectively, for feed, freeze-dried faeces and fish samples (ISO 6496, 1983); ash was determined after incineration at 550°C for 4 h (ISO 5984,1978). Crude protein (CP) (Nx6·25) was determined by the Kjeldahl method (ISO 5983, 1979). Fat was quantified after petroleum–diethyl ether extraction (ISO 6492, 1999). Before fat analysis, feed and faecal samples were hydrolysed by boiling for 1 h with 3 M-HCl. Energy content was measured by direct combustion in an adiabatic bomb calorimeter (IKA-C-7000; IKA analysentechnik, Weitersheim, Germany). The total carbohydrate content in feed and faeces was calculated as (DM-CP-fat-ash). Starch was enzymatically determined in feed and faecal samples by using amyloglucosidase with the ethanol extraction step and measuring glucose content as described by Goelema et al. (1998). The non-starch polysaccharides (NSP) content was calculated as (total carbohydrates-starch). Apparent digestibility coefficients of nutrients were calculated for each aquarium as in Amirkolaie et al. (2006), using Y_2O_3 as an inert marker. The yttrium content of feed and faeces was analysed using inductively coupled plasma-mass spectrometry (ICP-OES) according to the standard NEN 15510 (2007). Energy and nitrogen (N) balance parameters were calculated per aquarium and expressed as, respectively, kJ $kg^{-0.8}$ d^{-1} and mg $kg^{-0.8}$ d^{-1}. N balance calculations were as follows:

Gross nitrogen intake (GN) = FI ×Nfeed, where FI = feed intake of the fish (g feed $fish^{-1}$), Nfeed = nitrogen content of the feed. Digestible nitrogen (DN) = (GN×ADCcp)/100, where GN = Gross nitrogen intake, ADCcp (%) = apparent digestibility coefficient of the crude protein in the feed. Faecal nitrogen losses = GN –DN. Branchial and urinary nitrogen loses (BUN) = DN-RN, where RN= retained nitrogen. RN = ((BWt×CP)/6.25)-((BW_0×CP)/6.25), where BWt = body weight of fish at the end of the experiment (kg), BW_0 = body weight of fish at the start of the experiment (kg), CP= crude protein content of the fish (g). Energy balance calculations were as follow: Gross Energy intake (GE) = FI × Efeed, where FI = feed intake of the fish (g feed $fish^{-1}$), Efeed =energy content of the feed. Digestible Energy (DE) = (GE×ADCE) /100, where ADCE (%) = apparent digestibility coefficient of the energy in the feed. Faecal energy losses (FE) = GE-DE. Metabolizable energy (ME) =DE-BUE where BUE = branchial and urinary energy losses. BUE = (BUN×24.9)/1000, where 24.9 kJ N g-1= energy concentration of NH_3-N calculated by Bureau et al. (2003) and assuming that all N was excreted as (NH_3-N) . Retained energy (RE)= BWt×Et- BW_0×E_0, where Et = energy content of the fish at the end of the experiment, E_0 = energy content of the fish at the start of the experiment. Heat production (HP) = ME-RE.

3.2.4. Statistical analysis

Statistical analyses were performed using the statistical analysis system, statistical software package version 9.2 (SAS institute, Cary, NC, USA). All parameters were subjected to broken line analysis using the NLIN procedure of SAS (Robbins et al., 2006). Furthermore, the general linear model (GLM) procedure was used to fit a linear and quadratic regression model with DP/DE ratio as independent variable. The level of significancy was set at 0.05.

3.3 Results

The analysed nutrient composition, digestible nutrients and DP/DE ratios are shown in table 3.2. Dietary protein ranged from 39 to 50%, dietary fat from 4 to 26% and energy from 16 to 21.4 kJ g^{-1}. Using these analysed proximate compositions combined with digestibility data, the DP/DE ratio ranged from 17.3 to 28.9 mg kJ^{-1}. The broken line analysis showed that no optimal DP/DE ratio was present for any of the measured/calculated variables in this study ($P>0.05$). Therefore all parameters were subjected to regression analysis.

TABLE 3.2 | The analysed nutrients content of the test diets (on dry matter basis).

Analysed nutrients g kg^{-1}	Mean	Min	Max
DM	929	921	937
CP	444	393	501
Fat	136	36	257
Energy (MJ kg^{-1})	22	20	24
Starch	165	142	214
NSP	175	123	235
Ash	80	77	84
Total carbohydrates	341	270	448
DP (mg g^{-1})	421	368	477
DE (kJ g^{-1})	18.7	16.3	21.4
DP/DE ratio (mg kJ^{-1})	22.9	17.3	28.9

Min,minimum; Max,maximum; DP,digestible protein; DE,digestible energy; NSP,non starch polysaccharides. NSP was calculated as total carbohydrates-starch.

Performance data are shown in table 3.3. Feed intake expressed per metabolic body weight (in g $kg^{-0.8}$ d^{-1}) was not affected by the DP/DE ratio ($P>0.05$). All other performance parameters were linearly related with DP/DE ratio ($P<0.05$). Feed intake expressed in g/fish feed intake decreased linearly with the DP/DE ratio ($P<0.05$). Independent upon the unit of expression, growth linearly declined with increasing DP/DE ratio ($P<0.001$; Figure 3.1a). Feed conversion ratio (FCR) ranged between 0.9-1.2 and was linearly related with DP/DE ratio ($P<0.05$). The lowest FCR was observed in fish fed diets with low DP/DE ratio (Figure 3.1b).

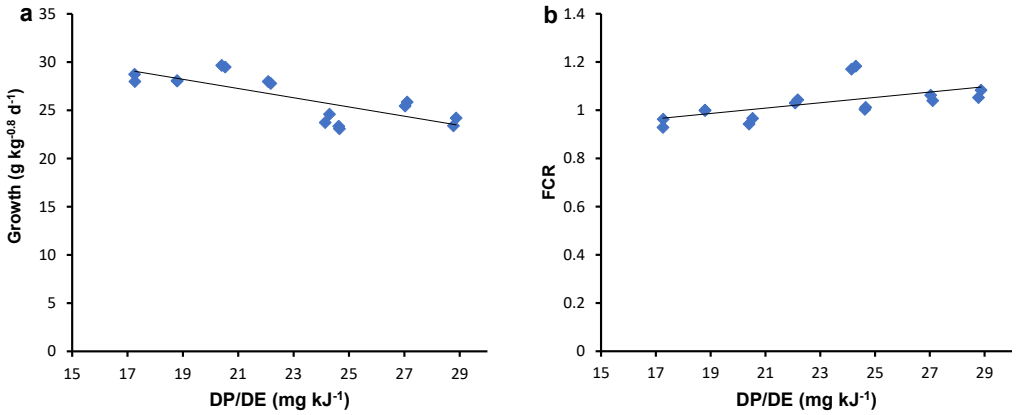

FIGURE 3.1 | The relation between digestible protein to digestible energy (DP/DE) ratio, growth (a) and feed conversion ratio (FCR, b) in Nile tilapia. The estimated regression equations are given in table 3.3.

TABLE 3.3 | The relation between DP/DE ratio and fish performance.

	Mean	Range	SD	CV (%)	Equation x=DP/DE	R^2	Effect
Initial BW (g $fish^{-1}$)	6.1	6.1-6.2	0.04	0.69			
Final BW (g $fish^{-1}$)	50	41.4-58.2	5.99	12.1			
Feed intake (g $fish^{-1}$)	45	35.7-49.5	4.60	10.3	61(±5.87)-0.72(±0.25)X	0.367	L*
Feed intake (g $kg^{-0.8}$ d^{-1})	27	23.4-29.1	1.83	6.8	31.4(±2.68)	-	ns
Growth (g $fish^{-1}$)	44	35.3-52.2	5.99	13.8	71.46(±5.93)-1.22(±0.26)X	0.619	L***
Growth (g $kg^{-0.8}$ d^{-1})	26	23.1-29.7	2.36	8.9	37.29(±2.36)-0.45(±0.10)X	0.612	L***
SGR (% d^{-1})	5.0	4.6-5.4	0.29	5.8	6.29(±0.29)-0.06(±0.01)X	0.606	L***
FCR	1.03	0.92-1.18	0.07	6.9	0.76(±0.09)+0.01(±0.01)X	0.368	L*

DP/DE, digestible protein to digestible energy ratio (in mg kJ^{-1}); SGR, specific growth rate; FCR, feed conversion ratio. L, linear effect; * P<0.05,***P<0.001; ns, not significant.

The experimental diets caused considerable differences in final body composition, which was indicated by the CV for all body composition parameters (table 3.4). Body fat content had the highest CV (15.8%). All final body composition parameters showed a significant linear relation (P<0.001) with DP/DE ratio (table 3.4). The protein as well as the ash content showed a positive linear relation with DP/DE ratio. Fat and also energy were negatively related with DP/DE ratio. Fish fed the diets with low DP/DE ratios were fatter and had a higher energy content (table 3.4).

The digestibility of starch, total carbohydrates and non-starch polysaccharides averaged over all diets was respectively 99.9%, 55.7% and 25.3%. The digestibility of these nutrients were not influenced by diet (i.e., DP/DE ratio; table 3.4). However, fat digestibility was curvilinearly related with DP/DE ratio (P<0.05; table 3.4). With increasing DP/DE ratio, fat digestibility first increased up to 97 % and then started to decrease at a DP/DE ratio above 20 mg kJ^{-1} (Figure 3.2). Also protein digestibility was affected by DP/DE in a linear relation(P<0.05). There was a tendency for a quadratic relation between protein digestibility and DP/DE ratio (P<0.1; table 3.4). Decreasing the DP/DE ratio resulted also in a linear increase in energy digestibility (P<0.001).

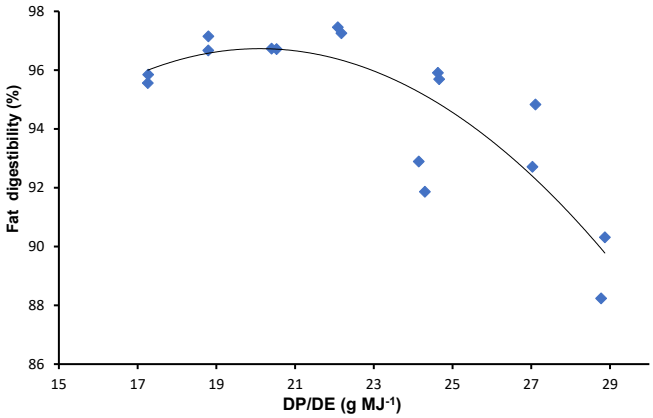

FIGURE 3.2 | The relation between digestible protein to digestible energy (DP/DE) ratio and fat digestibility in Nile tilapia. Regression equation is given in table 3.4.

TABLE 3.4 | The relation between DP/DE ratio, final body composition and digestibility of nutrients. (dry matter basis).

	Mean	Range	SD	CV (%)	Equation x=DP/DE	R^2	Effect
Final body content (g kg^{-1})							
Dry Matter	336	289-379	29.92	8.9	498(±19.33)-7.08(±0.83)X	0.838	L***
Crude Protein	453	378-533	56.37	12.5	147(±36.14)+13.34(±1.56)X	0.840	L***
Fat	440	327-533	69.57	15.8	829.23(±36.69)-16.97(±1.58)X	0.891	L***
Ash	103	79-131	15.87	15.4	15.89(±9.47)+3.81(±0.41)X	0.862	L***
Energy (kJ g^{-1})	28	26-31	1.60	5.7	36.92(±0.99)-0.38(±0.04)X	0.848	L***
Digestibilty (%)							
Dry matter	78.6	76.4-81.1	1.45	1.9	86.22(±1.08)-0.33(±0.05)X	0.787	L***
Crude protein	94.9	93.6-95.9	0.64	0.7	83.90(±4.54)+0.87(±0.40)X-0.02(±0.001)X^2	0.582	Q#
Energy	86.0	82.9-88.1	1.84	2.1	94.89(±1.72)-0.39(±0.07) X	0.662	L***
Fat	94.7	88.2-97.5	2.75	2.9	60.19(±15.65)+3.64(±1.39)X-0.09(±0.03)X^2	0.732	Q*
Ash	34.5	30.6-38.5	2.24	6.5	37.4(±3.50)	-	ns
Starch	99.9	99.8-99.9	0.06	0.1	99.87(±0.09)	-	ns
NSP	25.3	5.7-38.3	9.51	37.6	-13.84(±10.97)+1.71(±0.47) X	0.483	L**
Total carbohydrates	62.1	56.8-67.7	2.70	4.4	55.70(±3.98)	-	ns

DP/DE, digestible protein to digestible energy ratio (in mg kJ^{-1}); SD, standard deviation; CV, coefficient of variation; NSP, non starch polysaccharides; L, linear effect; Q, quadratic effect.* P<0.05, ***P<0.001, # P<0.1; ns, not significant.

The relation between nitrogen and energy balance parameters and DP/DE ratio is shown in table 3.5. The relation between retained nitrogen and DP/DE ratio tended to be linear (P<0.1). With decreasing DP/DE ratios in the diets, the nitrogen retention (i.e., protein gain) of the fish tended to increase (P<0.1). All other nitrogen balance parameters had a significant linear relation with DP/DE ratio (P<0.05). Digestible nitrogen intake increased with increasing DP/DE ratio. Despite this increased digestible nitrogen intake, the retained nitrogen tended to decline. Consequently, the protein efficiency was linearly and negatively related to DP/DE ratio (P<0.05;Figure 3.3). The protein efficiency did not show a plateau or reach a maximum and was 32 and 44%,respectively, at the highest and lowest dietary DP/DE ratio.

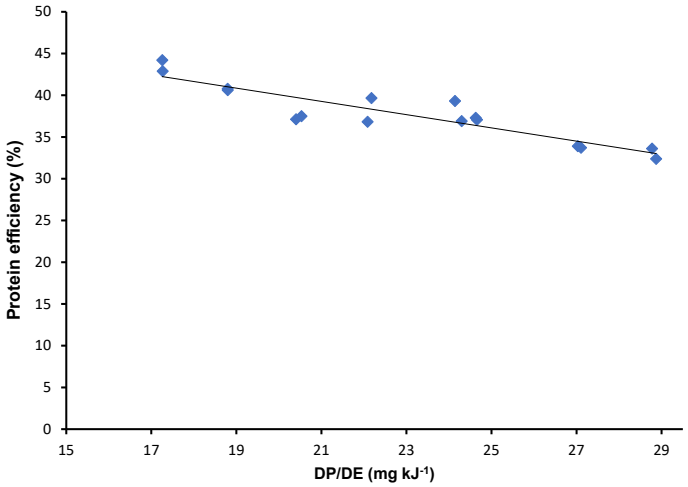

FIGURE 3.3 | The relation between digestible protein to digestible energy (DP/DE) ratio and protein efficiency in Nile tilapia. Regression equation is in table 3.5.

TABLE 3.5 | The relation between DP/DE ratio and nitrogen and energy balances.

	Mean	Range	SD	CV (%)	Equation x=DP/DE	R^2	Effect
Nitrogen balance (mg $kg^{-0.8}d^{-1}$)							
Nitrogen intake	1778	1566-2005	152.90	8.6	1302(±208.43)+20.83(±8.97)X	0.278	L*
Digestible nitrogen	1690	1466-1909	148.50	8.8	1192(±196.38)+21.69(±8.45)X	0.320	L*
Branchial urinary nitrogen losses	1055	818-1262	139.40	13.2	442(±149.72)+26.76(±6.44)X	0.552	L**
Retained nitrogen	634	558-716	40.70	6.4	750(±57.16)	0.233	L#
protein efficiency (%)	37.7	32-44	3.40	9	56(±2.25)-0.79(±0.10)X	0.827	L***
Energy balance (kJ $kg^{-0.8}$ d^{-1})							
Energy intake	545	447-620	65.70	12.1	876(±55.14)-14.45(±2.37)X	0.726	L***
Digestibale energy intake	470	376-543	64.70	13.8	803(±51.21)-14.55(±2.20)X	0.757	L***
Branchial urinary energy losses	26	20-31	3.50	13.2	11(±3.73)+0.67(±0.16)X	0.552	L**
Metabolisable energy	443	347-517	66.00	14.9	792(±47.58)-15.22(±2.05)X	0.798	L***
Retained energy	262	175-340	61.30	23.4	590(±42.59)-14.29(±1.83)X	0.813	L***
Heat production	181	167-200	9.40	5.2	202(±13.97)	_	ns
Retained energy as protein	63	55-71	4.30	6.9	76(±5.91)	0.269	#
Retained energy as fat	200	118-275	58.30	29.2	514(±38.61)-13.71(±1.66)X	0.829	L***

DP/DE, digestible protein to digestible energy ratio (in mg kJ^{-1}); SD, standard deviation. CV, coefficient of variance. L, linear effect; Q, * P<0.05; ** P<0.01; ***P<0.001, # P<0.1; ns, not significant.

Regarding the energy balance parameters, digestible energy intake, metabolizable energy intake and retained energy showed a significant negative linear relation with DP/DE ratio. All these parameters increased with decreasing the DP/DE ratio. Heat production was equal among all dietary treatments and showed no relation with DP/DE ratio (P>0.05). In line with nitrogen retention, retained protein energy tended to have a linear relation with DP/DE ratio (P<0.1).

3.4 Discussion

In this study protein retention efficiency (i.e., retained nitrogen/digested nitrogen) increased linearly with decreasing dietary DP/DE ratio (Figure 3.3) under the condition of satiation feeding. It was expected that protein retention efficiency would reach a maximum or even a constant value (i.e. a plateau) in the DP/DE range from 17 to 29 mg kJ^{-1}. The absence of an optimal DP/DE in this range is in line with the observation made by Haidar et al. (submitted) when Nile tilapia were fed restrictively equal amounts of digestible protein from diets differing in DP/DE ratio. Thus in this weight range of Nile tilapia (6-40g) feed intake is not determining the optimal DP/DE ratio.

The highest protein retention efficiency in the current study was 44%. This value is within the range of reported values for Nile tilapia (between 39 and 55%), but at the lower end of this literature range (Haidar et al., 2016; Kaushik et al., 1995b; Saravanan et al., 2012; Schrama et al., 2012; Tran-Duy et al., 2008; Van Trung et al., 2011). Some studies in Nile tilapia showed a protein retention efficiency above 51% (Figueiredo-Silva et al., 2013; Saravanan et al., 2012; Van Trung et al., 2011). The maximal value of protein retention efficiency in the current study is lower than values reported in carnivorous fish from 48 to70% (Booth et al., 2010; Brett Glencross et al., 2008; Grisdale-Helland et al., 2013; Hatlen et al., 2007; Lupatsch et al., 2001), or lower than found in pigs (about 52%) (Conde-Aguilera et al., 2011; van Milgen et al., 2001) and in poultry (about 63%) (Kong and Adeola, 2011). Differences between species might be related to e.g. sub optimal diet compositions. However, in a direct comparison between trout and salmon (Azevedo et al., 2005), it was shown that rainbow trout had a higher energetic efficiency for protein gain compared to Atlantic salmon. Since maximal protein retention efficiency is being compared, diet composition seems not a logic argument for the differences between animal species. Moreover, in chickens it was proposed that the rate of protein degradation was genetically determined and growth efficiency of chickens was higher due to lower protein degradation (Tomas et al., 1991).

Still the question remains why in the current study Nile tilapia did not show a maximum protein retention efficiency in relation to DP/DE ratio, even when fed to satiation. This absence in maximum protein retention efficiency might be related to the observation that neither in the current nor in a previous study with 6-40g tilapia (Haider et al. (submitted) with respectively satiation and restrictive feeding, no maximum level in protein deposition was reached . For farm animals it is commonly accepted that animals have a genetically determined maximum potential for protein deposition (e.g. in pigs, Kyriazakis and Emmans, 1992). For fish such a concept of a maximal genetically determined protein deposition level has not been demonstrated. Also in the current study, the nitrogen retention (i.e., protein retention) increased numerically from 560 to 715 mg $kg^{-0.8}$ d^{-1} when DP/DE declined. This observed range in retained nitrogen for 6-40g tilapia is much higher than previously reported values in heavier (40-200g) Nile tilapia fed to apparent satiation (425 mg $kg^{-0.8}$ d^{-1} in (Tran-Duy et al., 2008); 485 mg $kg^{-0.8}$ d^{-1}in (Saravanan et al., 2012) and restrictively fed 6-40g Nile tilapia (534 mg $kg^{-0.8}$ d^{-1} in Haidar et al. (submitted). This suggests that young Nile tilapia have a higher potential for protein deposition compared to older tilapia. In rainbow trout, Dumas et al. (2007) demonstrated that protein deposition increased with fish weight below 400g. Above this size, the increase in protein deposition levelled off with body weight. According to Weatherley and Gill (1985), below 40%

of the maximal fish length, muscles fibers grow because of combined hyperplasia and hypertrophy while above this body length it is solely due to hypertrophy. It can be hypothesised that the fish in the current study were so young that protein deposition was still the result of a combination of hyperplasia and hypertrophy. The ability to increase the number of muscle fibers might be an explanation for the absence of a maximal protein deposition in the current study. Hence, when Nile tilapia become older, it is possible that we could observe an optimal DP/DE ratio in relation to protein gain.

In general, the mean nutrient digestibility over all diets in the current study are well in line with reported values in literature with a tendency for being on the higher end of the reported range. Protein digestibility was on average 95%, being within the range of 88% to 95 % (Kaushik et al., 1995; Schneider et al., 2004; Amirkolaie et al., 2005; Amirkolaie et al., 2006; Leenhouwers et al., 2007; Schrama, Haidar et al., 2011). Literature data on starch digestibility shows clustering into two groups, either close to 100% (like in the current study) or values below 93% (Kaushik et al. 1995; Amirkoleai et al. 2005). Most likely this difference in starch digestibility between studies is due to feed processing. All studies with low starch digestibility were made by steam pelleting, resulting in possible differences in gelatinization degree. Using steam pelleting, Amirkolaie et al. (2008) demonstrated that gelatinized starch was better digested than native starch (99.3 vs 93.8%). In the current study, fat digestibility showed a curvilinear relation with DP/DE ratio. The diets with lower DP/DE ratios had a higher inclusion level of oils. Therefore the data might suggest that Nile tilapia have a maximum capacity to digest dietary fat. Fat digestibility was maximal (97%) at the DP/DE ratio of 20 mg kJ^{-1} in the current study. One might interpret this that this ratio is the optimal DP/DE ratio, but using nutrients digestibility as a criterion for estimating the optimal DP/DE ratio is not valid because differences in digestibility values are reflection of changes in diet composition.

The current study showed that feed intake in g $kg^{-0.8}$ BW d^{-1} showed no relation with DP/DE ratio. This suggests that DP/DE ratio has no impact on feed intake regulation in Nile tilapia. Averaged over all diets the feed intake was 27 g $kg^{-0.8}$ BW d^{-1}, which is higher compared to other studies measuring satiation feed intake in Nile tilapia (17.5 g $kg^{-0.8}$ BW d^{-1} in (Tran-Duy et al., 2008); 23g $kg^{-0.8}$ BW d^{-1} in (Saravanan et al., 2012) and 19.4 in(Haidar et al., 2016)). These differences in feed intake between studies could be attributed due to differences in diet composition (i.e., nutrient densities). In other words, gut volume in relation to nutrient concentration might explain differences in feed intake between studies. Moreover, the variation in satiation feed intake between studies might also be related to fish weight (age) being 6-40g in the current study versus 40-200g in the aforementioned studies. Historically, it has been often suggested that fish would control their feed intake aiming at equal digestible energy intake (Cho and Kaushik, 1990; Kaushik and Luquet, 2009; Lupatsch et al., 2001). However the current study demonstrates that 6-40g Nile tilapia did not equalize digestible energy intake, this is indicated by the negative linear relation between digestible energy and DP/DE ratio (Figure 3.4).

The current observation is in line with other studies showing differences in feed intake in fish fed different diets (Geurden et al., 2006; Saravanan et al., 2012). Another hypothesis for feed intake regulation is that heat production is involved in feed intake regulation. In Rainbow trout (Saravanan et al., 2012;) and Nile tilapia (Tran-Duy et al., 2008) it was observed that diets with different macro nutrient composition resulted

in differences in feed intake but in equal heat production. Also in the current study, changing the DP/DE ratio altered the nutrient intake (e.g., Fig 3.4) but heat production was equal between the diets (Table 3.5). This suggests that under the current experimental conditions heat production had a role in controlling feed intake. Other studies in fish suggested that feed intake could be controlled to reach a maximal protein gain (Azevedo et al., 2004; Geurden et al., 2006). Our results cannot rule out that Nile tilapia in the current study may have regulated their feed intake for achieving a similar protein gain (i.e. nitrogen retention). However, there was a tendency ($P<0.1$) for a linear relationship between DP/DE ratio and retained nitrogen. This would suggest that heat production most likely was the factor determining feed intake of juvenile Nile tilapia in the current study (Table 3.5).

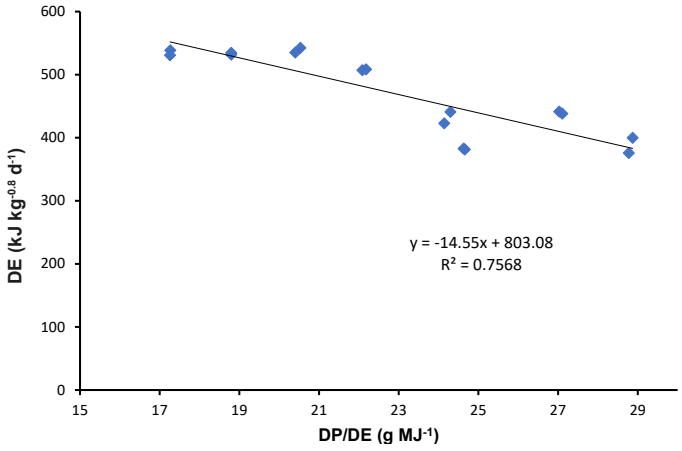

FIGURE 3.4 | The relation between digestible protein to digestible energy ratio (DP/DE) on digestible energy (DE) intake.

In conclusion, the effect of dietary digestible protein to digestible energy (DP/DE) ratio was assessed on performance, energy and nitrogen balance in Nile tilapia under satiation feeding. Within the studied DP/DE range (17-29 mg KJ^{-1}) no optimal DP/DE ratio was found for 6-40g Nile tilapia. This implies that either there is no optimal DP/DE ratio or the optimal DP/DE ratio is below 17 mg kJ^{-1} for Nile tilapia. Based on the current study it is hypothesed that juvenile Nile tilapia (<40g) do not have a maximal protein deposition level. For all criteria measured, differences in voluntary feed intake do not determine the optimal DP/DE ratio in Nile tilapia.

Acknowledgements

We thank Menno ter Veld and Ep Eding for their technical support in conducting the experiment and Ronald Booms and Tino Leffering for their assistance during the sampling and laboratory works. BioMar partially supported this research by producing and providing the experimental diets. The experimental work was funded by the Aquaculture and Fisheries Group of the Department of Animals Sciences, Wageningen University, The Netherlands.

Chapter 4

The effect of DP/DE ratios on body composition and carcass traits in Nile tilapia

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Abstract

Fish growth is usually defined as the increase in weight of the whole fish rather than looking at the growth and accretion of nutrients into different organs. This study aimed to assess the changes in the sizes and nutrients distribution within different body compartments of young Nile tilapia. These changes were studied under the effect of dietary digestible protein to digestible energy ratios (DP/DE). eight experimental diets differing in DP/DE ratio were fed to satiation to 16 groups Nile tilapia (mean initial weight was 6 g). The dietary DP/DE ratios ranged from 17 to 29 mg kJ^{-1}. At the end of 6 weeks experimental period, 10 fish from each group were sampled for measuring size and composition of the body compartments. Fish were partitioned into four main compartments: liver, viscera, fillets and the "rest" fraction. The results showed that fillet yield was maximal at a dietary DP/DE ratio ranging between 24-25 mg kJ^{-1}. About 10% of the fish weight represented by the viscera and between 22 to 29% comprised of fillets. The viscera and "rest" fraction contained the highest fat content being 30 and 60%, respectively. Protein content of the fillets was constant over the dietary treatments and was on average about 17%. Protein distribution within all body compartments was relatively constant and independent of the dietary DP/DE ratio. Whereas, fat distribution over body compartments was significantly affected by the dietary DP/DE ratios. In conclusion, Nile tilapia store fat mainly in the viscera and the "rest" fraction. Changes in dietary DP/DE ratio have clear effect on fat content but not protein content of body compartments.

4.1 Introduction

Fish growth rate is a major determinant for the profitability of aquaculture operations. Therefore numerous studies have addressed environmental, genetic and nutritional effects on fish growth. Most studies address fish growth at the whole body level; e.g. in determining nutrient requirements (NRC, 2011; Shearer, 1994). Fish whole body growth in terms of weight, energy and protein, like in all other animals, is the summation of the increase in size and composition of all organs/compartments (Weatherley, 1990). Next to high whole body growth rate, a high fillet yield is economically important for the aquaculture sector. Fillet yield differs between fish species, varying from f.ex., 35% in Nile tilapia (Rutten et al.,) to 44.5% in European sea bass (Lanari et al., 1999). Furthermore, within species variation exists due to genetic differences as well as due to nutritional factors. In Nile tilapia fillet yield was shown to be heritable (Rutten et al., 2004). Generalizing, nutritional studies on separate parts of the fish body often have only focussed on a small number of specific compartments (e.g., fillet, liver, gonads or viscera) and not on "all" compartments. Only recently Salze et al. (2014) assessed the impact of feeding level on different compartment size and composition in Rainbow trout. The impact of protein to energy ration (P/E) on composition and size of different compartments has been addressed e.g. in Sea bream (Santinha et al., 1999), Eurasian perch (Mathis et al., 2003), Atlantic salmon (Einen and Roem, 1997), Turbot (C. Regost et al., 2001), hybrid striped bass (Gummadi and Reigh, 2011) and channel catfish (Li et al., 1998; Li and Robinson, 1999). Between species the effect of P/E ratio on compartment size varies: e.g., hepatosomatic index (HSI) was negatively related to dietary P/E ratio in Hybrid striped bass but was unaffected in Atlantic salmon. Fillet yield increased with increasing dietary P/E ratio in Eurasian perch but did not alter in turbot. In Nile tilapia there is relatively little information on the impact of nutrition on the growth of different compartments/organs.

As reviewed by Shearer (1994), whole body chemical composition is highly dependent on the diet/nutrient intake, especially the dietary P/E ratio. However less information is present on the impact of diets on the different compartments. The scarce available data show large variability and contrasts in the response to dietary P/E ratio. E.g., in Cobia (*Rachycentron canadum*) increasing P/E decreased the fillet fat content but in Turbot no effect was observed (C. Regost et al., 2001; Wang et al., 2005). Part of this variability between studies may relate to species differences but also to fish age, dietary composition, feeding level and environmental conditions.

Most of the literature information regarding the body compartment size and composition have neglected the"rest" fraction left after gutting and filleting (i.e., head, skin, fins and the skeleton). It could be that this"rest" fraction can act as a major site for nutrients storage. In Nile tilapia there is no information on the effect of dietary P/E ratio on nutrients distribution over different compartments.

Therefore, this study assessed the effect of dietary digestible protein to digestible energy ratio (DP/DE) on the size of the different body compartments, protein and fat distribution over these compartments. In this study Nile tilapia was partitioned into four main compartments: liver, viscera, fillets and the "rest" fraction.

4.2 Materials and methods

4.2.1. General design

For assessing the impact of dietary digestible protein (DP) to digestible energy (DE) ratio on body compartments sizes and compositions extra fish were sampled from large experiment which was reported elsewhere (Haidar et al, submitted). In that study 8 experimental diets differing in DP/DE ratio were fed to satiation to 16 groups Nile tilapia (60 fish per group). At the end of the 6 week experimental period, 10 fish from each group were sampled for measuring size and composition of the defined compartments.

4.2.2. Diets and feeding

The measured DP and DE content of the 8 experimental diets ranged from 368 to 477 mg g^{-1} and from 16.3 to 21.4 kJ g^{-1}, respectively (on dry matter basis). Thus dietary DP/DE ratios ranged from 17.3 to 28.9 mg kJ^{-1}. For details on ingredient composition see Haidar et al., (submitted). The mean and range of dietary digestible nutrients contents are given in table 4.1.

TABLE 4.1 | Digestible nutrients contents of the test diets.

Digestible nutrinets (g kg^{-1})	Mean	Min	Max
Dry matter	730	706	756
Fat	130	32	246
Ash	27	24	30
Starch	154	132	199
NSP	59	17	104
Total carbohydrates	213	148	303
DP (mg g^{-1})	421	368	477
DE (kJ g^{-1})	18.7	16.3	21.4
DP/DE ratio (mg kJ^{-1})	22.9	17.3	28.9

Min,minimum; Max,maximum; DP,digestible protein; DE,digestible energy; NSP,non starch polysaccharides. NSP was calculated as total carbohydrates-starch.

All diets were formulated to provide the essential nutrients (amino acids; essential fatty acids; vitamins and minerals) required by Nile tilapia (NRC, 2011). The diets were extruded at a size of 2mm. Fish were fed to apparent satiation by hand. Fish were fed twice daily for one hour (at 09:00 and 16:00h). Feed intake was recorded.

4.2.3. Fish and housing

All male Nile tilapia (*Oreochromis niloticus*) were obtained from Til-Aqua, The Netherlands. The experiment was approved by the Ethical Committee judging Animal Experiments of Wageningen University, The Netherlands, and carried out according to the Dutch law on animal experiments. At the start of the experiment fish were randomly divided over 16 tanks of 120L. Mean initial weight was 6.1g. For details on housing condition see (Haidar et al, submitted).

4.2.4. Sampling and Data collections

4.2.4.1. Fish dissection

At the end of the 6 week experiment, 2 groups of each 10 fish were randomly selected per tank. The first group of 10 fish was used for whole body composition analysis and was reported by Haidar et al (submitted). The second group was used for the analysis of size, chemical composition of the four different body compartments. Fish were euthanized by an overdose of a phenoxy-ethanol solution (1.0 ml l^{-1}) and then whole body weight was recorded. Thereafter the dissection started for dividing the fish into 4 compartments: 1) liver (without gallbladder); 2) viscera (including gallbladder, spleen and gonads); 3) fillets; and 4) the"rest" fraction comprising of head, skin, bones and fines.

The dissection was done during two days. During the first sampling day, the fresh fish were eviscerated for the separate collection of liver and viscera (including abdominal fat, spleen, gonads and gallbladder). Prior to dissection all fish were kept on ice. First, the viscera together with peritoneal fat and internal organs were pulled out of the abdominal cavity by making an incision between the anus and the gills. Then, the liver was removed and separated from the gallbladder. The liver, viscera and the carcasses were pooled per tank and each pooled compartment samples per tank was weighed and thereafter stored at -20 °C until further analysis. The second sampling day was for filleting of the carcass samples. The frozen carcass samples were thawed in a water bath where they were kept under tap water (13-15°C) until the temperature of the carcass samples reached 1°C. The filleting was done by first peeling off the skin and then the fillets were carefully removed with a scalpel in an anterior-posterior direction to maximize the amount of fillets and minimize fillets damage. The"rest" fraction(skin, head, bones and fins) and the fillets were then pooled per tank, weighed and stored at -20 °C until further analysis. Filleting was done by the same people for all samples.

4.2.4.2. Body compartments samples preparation

Demi water (30 ml) was added to each pooled liver sample in order to increase the sample amount. These liver samples were blended and homogenised using a homogenizer for 30 seconds (Cat X1030, Ingenieursburo M. Zipper, Etzenbach, Germany). Viscera was homogenised without water addition. Prior to autoclaving, 50 ml of demi water was added to each fillet and "rest" fraction sample. These samples were autoclaved for 30 min at 120°C and thereafter homogenized for 90 seconds.

4.2.4.3. Chemical analysis

All body compartment samples were analysed in triplicates. Dry matter (DM) was determined gravimetrically after drying at 103°C for 4, 4 and 24 hours (h) until constant weight, respectively, for feed, oven-dried faeces and fish samples (ISO 6496, 1983); ash was determined after incineration at 550°C for 4 h (ISO 5984,1978) (except for liver and viscera sample). Crud protein (CP) (Nx6·25) was determined by the Kjeldahl method (ISO 5983, 1979). Fat was quantified after petroleum–diethyl ether extraction (ISO 6492, 1999). Energy content was measured by direct combustion in an adiabatic bomb calorimeter (IKA-C-7000; IKA analysentechnik, Weitersheim, Germany).

4.2.4.4. Calculations

Hepatosomatic index (HSI%) was calculated as: $W_{liver}/W_{fish} \times 100$, where W_{liver} is the wet weight of liver and W_{fish} is the final whole wet weight of the fish; viscerosomatic index (VSI%)=$W_{vis}/ W_{fish} \times 100$, where W_{vis} is the wet weight of viscera . Fillet yield (%) was calculated as: $(W_{fillets}/ W_{fish}) \times 100$, where $W_{fillets}$ is the wet weight of the fillet; carcass yield was calculated as: $W_{carcass}/ W_{fish} \times 100$, where $W_{carcass}$ is the wet weight of the carcass;"rest" fraction(%)=$W_{rest}/W_{fish} \times 100$, where W_{rest} is the wet weight of the "rest" fraction.

Nutrient distribution in the different body compartments was calculated as follows: $nutrient_{distribution}(\%) = ((BP_{nutrient} \times BP_{weight})/(\sum BPnutrient \times BPweight)) \times 100$,where $nutrient_{distribution}$ is nutrient distribution in body compartments; BP_{weight} is the weight of each body part; $BP_{nutrient}$ is the nutrient content of each body part.

4.2.5. Statistical analysis

Statistical analyses were performed using the statistical analysis system, statistical software package version 9.2 (SAS institute, Cary, NC, USA). The general linear model (GLM) procedure was used to fit a linear and quadratic regression model with DP/DE ratio as independent variable. In addition, GLM procedure was carried out to fit a linear and quadratic regression model between fat content as independent variable and moisture content of the different body compartments. Spearman's correlation coefficients were calculated between VSI, HIS, fillet yield and carcass ratio. The level of significant was set at 0.05.

4.3 Results

4.3.1. Fish performance

Performance, energy and nitrogen balance data of the total group of fish were presented by Haidar et al. (submitted). The realized digestible protein (DP) and energy (DE) intake of the Nile tilapia over the 42 d experimental period in relation to the dietary DP/DE ratio is shown in figure 4.1. DE intake declined with increasing dietary DP/DE ratio. At the low DP/DE ratios, DE intake was the highest around 22 kJ $fish^{-1}$ d^{-1}over the 42 d experimental period. DP intake was not clearly affected by the dietary DP/DE ratio; ranging between 0.34 and 0.46 mg $fish^{-1}$ d^{-1} during the experiment. Although at low DP/DE ratios DP intake seemed to be lower.

The final body weight of the fish on which body compartments analysis were done was linearly affected by the dietary DP/DE ratio. Final body weight increased with declining dietary DP/DE ratios (Figure 4.2).

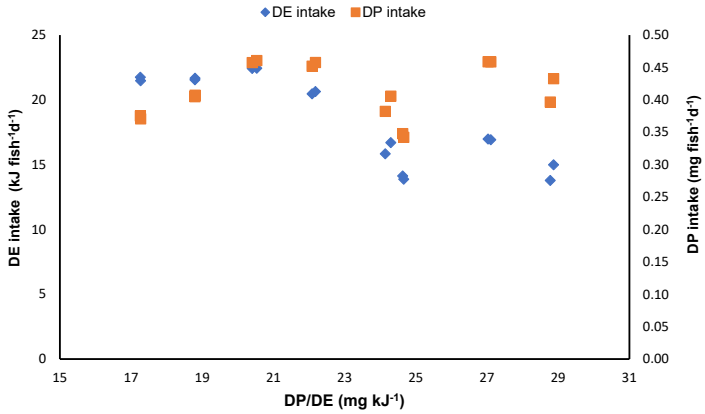

FIGURE 4.1 | The digestible nutrients intake of Nile tilapia over the 42 day experimental period in relation to the dietary digestible protein to digestible energy ratio (DP/DE).

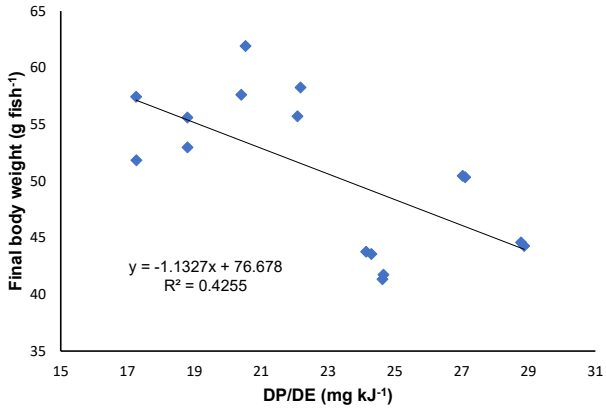

FIGURE 4.2 | The relation between dietary digestible protein to digestible energy (DP/DE) ratio and final body weight of Nile tilapia.

4.3.2. Carcass traits

Averaged over all diets VSI was 10% and showed a quadratic relation with the dietary DP/DE ratio ($P<0.05$; table 4.2). The weight of viscera decreased as the dietary DP/DE ratio increased and seemed to level off at DP/DE ratio of 28.8 mg kJ^{-1} (Figure 4.3). In contrast to VSI, HSI was not affected by the dietary DP/DE ratio ($P>0.05$).

Fillet yield in the current study ranged between 22 to 29%, was on average 27% and was influenced by the dietary DP/DE ratio. Fillet yield had a significant quadratic relation with DP/DE ratio. Fillet yield was maximal at a dietary DP/DE ratio ranging between 24-25 mg kJ^{-1} (Figure 4.3). Carcass yield was also affected by the dietary DP/DE ratio but had a linear relation with DP/DE ratio. Carcass yield was 86% on average over all treatments and declined with the DP/DE ratio. About 51% of the Nile tilapias comprised of head, bones, fins and skin ("rest fraction"). This"rest" fractionshowed a tendency towards a positive linear relationship with the dietary DP/DE ratio ($P<0.1$).

TABLE 4.2 | The effect of DP/DE ratio on carcass traits in Nile tilapia.

(%)	N	Mean	Range	SD	CV (%)	Equation[a]	R^2	P-value
HSI	16	2.1	1.7-2.5	0.26	12.4	Y=2.1(±0.06)	-	ns
VSI	15	9.9	7.1-13.1	2.14	21.6	Y=38(±7.02)-1.98(±0.62)x+0.03(±0.013)x^2	0.912	Q*
Carcass yield	16	85.9	82.7-89.7	2.28	2.7	Y=76(±2.41)+0.44(±0.10)x	0.576	L***
Fillet yield	16	26.5	21.8-29.2	1.78	6.7	Y=-17(±12.38)+3.65(±1.10)x-0.07(±0.02)x^2	0.600	Q**
"rest" fraction	16	51.9	44.9-59.8	3.21	6.2	Y=43.69(±4.65)+0.36(±0.20)x	0.186	L#

DP/DE, digestible protein to digestible energy ratio (in mg kJ^{-1}); SD, standard deviation; CV, coefficient of variation; HIS, hepatosomatic index, VSI viscerosomatic index; Rest,(head, bones,fins and skin) L, linear effect; Q, quadratic effect.
* P<0.05, ***P<0.001, ns, not significant.[a] X=DP/DE.

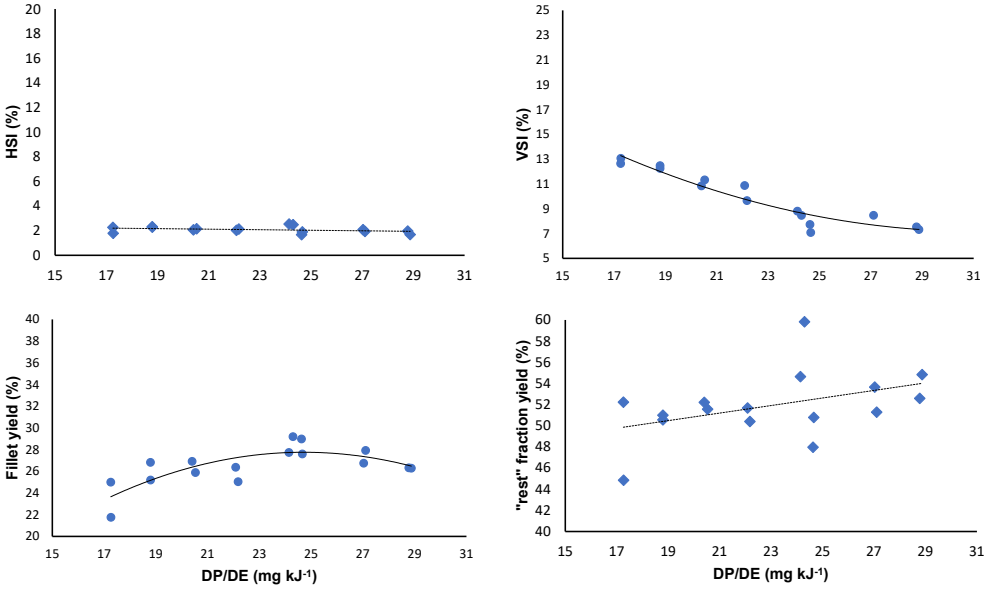

FIGURE 4.3 | The relationships between dietary digestible protein to digestible energy (DP/DE) ratio and carcass traits in Nile tilapia. Dotted lines indicate no significant relation and solid lines indicate significant relation with DP/DE ratio. The estimated regression equations are given in table 4.2.

4.3.3. Nutrients content in body compartments

In table 4.3, the proximate composition of the different body compartments is given. The protein content of fillets and"rest" fraction was unaffected by the dietary DP/DE ratio. Protein content of fillets was constant at about 17%. Protein content of liver tended to be linearly related to the dietary DP/DE ratio (P<0.1).

Of all 4 compartments studied, only the protein content of viscera had a linear relation with the dietary DP/DE ratio (Figure 4.4). The highest viscera protein content was about 8% at a dietary DP/DE ratio of 28.9 mg kJ^{-1} and decreased to 5% at a dietary DP/DE ratio of 17.3 mg kJ^{-1}.

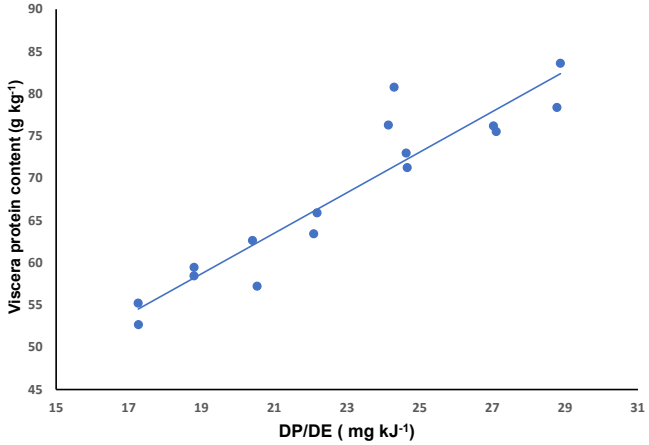

FIGURE 4.4 | The relation between dietary digestible protein to digestible energy (DP/DE) ratio and viscera protein content in Nile tilapia. The estimated regression equation is given in table 4.3.

TABLE 4.3 | The effect of DP/DE ratio on proximate composition of Nile tilapia body compartments (on fresh basis[1]).

	N	Mean	Range	SD	CV (%)	Equation x=DP/DE	R^2	P-value
protein (g kg^{-1})								
Liver	16	33	24-39	4.6	13.8	45.01(±6.68)-0.51(±0.287)X	0.183	L#
Viscera	16	68	53-84	9.9	14.6	13.15(±5.60)+2.40(±0.241)X	0.876	L***
Fillet	16	168	161-175	3.2	1.9	168(±0.79)	-	ns
"rest" fraction	15	127	124-129	1.5	1.2	127(±0.38)	-	ns
Whole fish	16	151	138-166	7.72	5.1	121(±9.38)+1.30(±0.40)X	0.427	L**
Fat (g kg^{-1})								
Liver	16	36	15-57	13.8	38.8	107.71(±10.52)-3.14(±0.452)X	0.775	L***
Viscera	16	323	162-479	117.6	36.40	995.27(±49.15)-29.33(±2.12)X	0.932	L***
Fillet	16	37	19-53	13.1	13.14	85.89(±16.41)-2.13(±0.71)X	0.394	L***
"rest" fraction	15	158	123-192	22.7	22.73	253.64(±24.86)-4.11(±1.07)X	0.513	L**
Whole fish	16	150	95-202	36.2	24.20	352(±19.36)-8.81(±0.83)X	0.889	L***
Energy (kJ kg^{-1})								
Liver	16	2.3	1-3	0.63	27.3	5.46(±0.56)-0.14(±0.024)X	0.696	L***
Viscera	16	14.8	9-21	4.40	29.7	39.97(±1.86)-1.097(±0.080)X	0.930	L***
Fillet	16	5.4	5-6	0.51	9.4	7.25(±0.647)-0.08(±0.027)X	0.371	L*
"rest" fraction	14	8.9	8-10	0.89	10.1	12.48(±1.03)-0.15(±0.04)X	0.464	L**
Whole fish	16	10	43046	1.35	14.2	17(±0.77)-0.33(±0.03)X	0.874	L***
Dry matter (g kg^{-1})								
Liver	16	82.1	54-111	17.88	21.8	167.98(±16.78)-3.75(±0.72)X	0.658	L***
Viscera	16	419.3	283-561	103.06	24.6	1009.26(±42.38)-25.73(±1.82)X	0.934	L***
Fillet	16	215.6	199-231	11.89	5.5	256(±15.61)-1.76(±0.67)X	0.329	L*
"rest" fraction	14	323.8	293-357	23.38	7.2	420.25(±26.41)-4.12(±1.14)X	0.484	L**
Whole fish	16	336	290-397	29.92	8.9	498(±19.33)-7.08(±0.83)X	0.838	L***

DP/DE, digestible protein to digestible energy ratio (in mg kJ^{-1}); SD, standard deviation; CV, coefficient of variation; Rest (head, bones and skin); L, linear effect; Q, quadratic effect.* P<0.05, ***P<0.001, #, P<0.1; ns, not significant.

[1] proximate composition of whole fish group expressed on dry matter basis was reported in (Haidar et al. submitted).

[2] X=DP/DE.

Fat content of all body compartments, had a negative linear relation with the dietary DP/DE ratio (P<0.001). Decreasing the DP/DE ratio resulted in higher fat content in all fish body compartments (table 4.3; Figure 4.5). The body compartment with the highest fat content was viscera, followed by the"rest" fraction and then liver and fillets; averaged over all dietary treatments the fat content was respectively 32%, 16%, 4% and 4%.

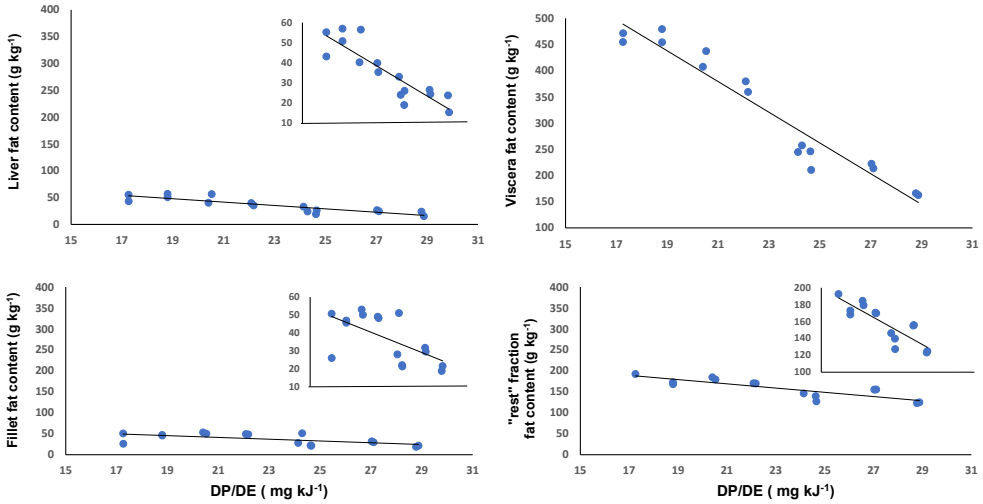

FIGURE 4.5 | The relation between dietary digestible protein to digestible energy (DP/DE) ratio and fat content in body compartments of Nile tilapia. Small graphs (zoom in) have the same units on the x axis, but have different y-axis scaling. The estimated regression equations are given in table 4.3.

The pattern in energy content in all body compartments paralleled the finding of the fat content. The energy content of all body compartments had a negative linear relationship with the dietary DP/DE ratio (table 4.3). The highest energy content was observed in the viscera and in "rest" fraction followed by fillets and liver. The dry matter content of different body compartments also showed a negative linear relationship with the dietary DP/DE ratio. The dry matter content of viscera was the highest among the body compartments and was on average 41%. The range of dry matter content of the fillets was from 19 to 22 %. In general, the highest dry matter content for all body compartments was found in Nile tilapia fed the lowest dietary DP/DE ratio (17.2 mg kJ^{-1}). Next to the composition of the compartments also the content of whole fish (reported on DM in Haidar et al. (submitted) is given in Table 4.3. The whole fish dry matter, fat, protein and energy contents were linearly related to the dietary DP/DE ratio (P<0.001). The whole fish protein content had a positive linear relationship with the dietary DP/DE ratio. The dry matter, fat and also energy content of whole fish were negatively related with DP/DE ratio. Fish fed the diets with a low DP/DE ratio were fatter and had a higher energy content (table 4.3).

4.3.4. Protein and fat distribution over body compartments

In relation to the dietary DP/DE ratio, protein content in body compartments expressed as a percentage of the total fish protein content (i.e., protein distribution) is shown in table 4.4. The protein distribution in the fillet and "rest" fraction was unaffected by the dietary DP/DE ratio (P>0.05). Averaged over all diets, 37%

and 55% of the protein in the whole fish was present in the fillet and "rest" fraction, respectively (Fig 4.6). Averaged over all diets, about 7% of the protein of the fish was present in the viscera. This percentage of protein present in viscera tended to have a quadratic relationship with the dietary DP/DE ratio (P<0.10).

TABLE 4.4 | The effect of dietary DP/DE ratio on protein and fat distribution in relation to total protein and fat content in body compartments of Nile tilapia.

	N	Mean	Range	SD	CV (%)	Equation[a]	R^2	P-value
protein (%)								
Liver	16	0.6	0.3-0.7	0.13	21.5	Y=1.02(±0.16)-0.02(±0.007)X	0.339	L*
Viscera	16	7.1	6.5-8.1	0.38	5.3	Y=15(±3.37)-0.67(±0.30)X+0.01(±0.006)X^2	0.346	Q#
Fillet	16	37.2	35.0-41.8	1.82	4.9	Y=37.2(±0.447)	-	ns
Rest	16	55.1	50.9-57.2	1.62	2.9	Y=55.1(±0.408)	-	ns
Fat (%)								
Liver	16	0.5	0.3-0.8	0.15	27.6	Y=1.08(±0.19)-0.02(±0.007)X	0.386	L*
Viscera	16	30.1	19.0-49.3	9.16	30.5	Y=140 (±33.52)-7.52(±2.97)X+0.12(±0.064)X^2	0.889	Q#
Fillet	16	7.1	3.7-9.5	1.39	19.6	Y=-27.4(±11.81)+3.08(±1.05)X-0.07(±0.02)X^2	0.402	Q*
Rest	16	62.3	46.5-74.6	9.01	14.5	Y=11.36(±4.28)+2.22(±0.184)X	0.912	L***

DP/DE, digestible protein to digestible energy ratio (in mg kJ^{-1}); SD, standard deviation; CV, coefficient of variation; Rest (head, bones and skin); L, linear effect; Q, quadratic effect.* P<0.05, ***P<0.001, #, P<0.1; ns, not significant. [a] X=DP/DE.

Only the percentage of protein present in the liver was affected by the dietary treatment. It had a negative linear relationship with the dietary DP/DE ratio (Figure 4.6). However, the difference in the amount of protein present in the liver between the lowest and highest DP/DE ratio was small (0.3 vs. 0.7%). In general, distribution of protein over the body compartments was minimally affected by the tested dietary DP/DE ratios.

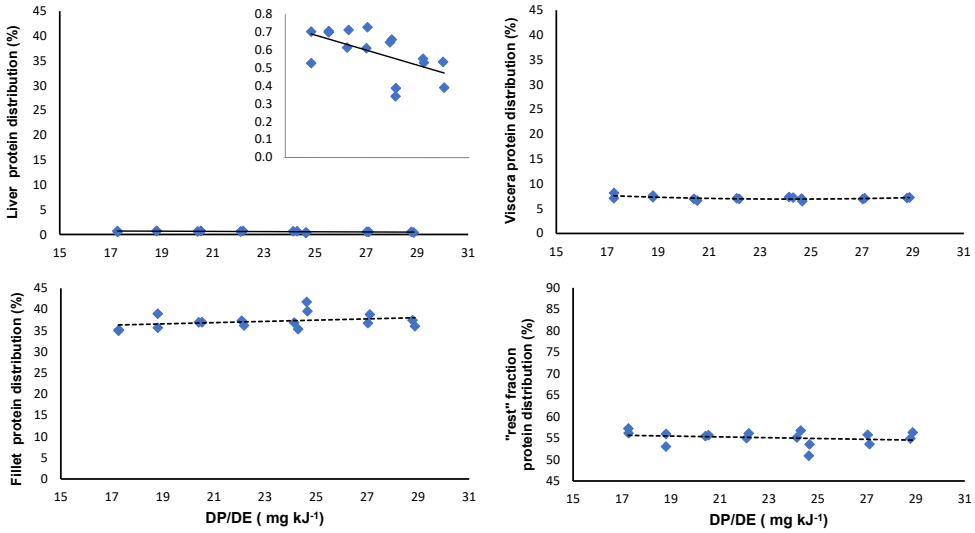

FIGURE 4.6 | The relation between dietary digestible protein to digestible energy (DP/DE) ratio and protein distribution over body compartments of Nile tilapia. Dotted lines indicate no significant relation (P>0.05) and solid lines indicate significant relation with DP/DE ratio (P<0.05). Small graph (zoom in) have the same units on the x-axis, but have different y-axis scaling. The estimated regression equations are given in table 4.4.

There was a clear effect of the dietary DP/DE ratio on fat distribution over the different body compartments. This was reflected by the significant linear relationship of DP/DE ratio with the percentage of fat present in the liver and the "rest" fraction and the significant quadratic relationship with the amount of fillet fat (Table 4.4 and Figure 4.7). The linear relationship of the percentage of visceral fat and the dietary DP/DE ratio was present ($P<0.001$), but also the quadratic relation with the dietary DP/DE ratio tended towards significance ($P<0.1$). With increasing DP/DE ratio the percentage of visceral fat declined. At the diet with the lowest DP/DE ratio about 49% of the total fat body was present in the viscera. The percentage of fat in the"rest" fractionwas opposite to that of the percentage of visceral fat, and it declined with increasing dietary DP/DE ratio (Figure 4.7). At the highest dietary DP/DE ratio (28.7 mg kJ^{-1}) 75% of the whole body fat was present in the ”rest” fraction. The amount of total body fat being present in the fillet had a weak curvilinear relationship with the dietary DP/DE ratio. Averaged over all diets about 7% of the body fat was present in the fillets (Figure 4.7).

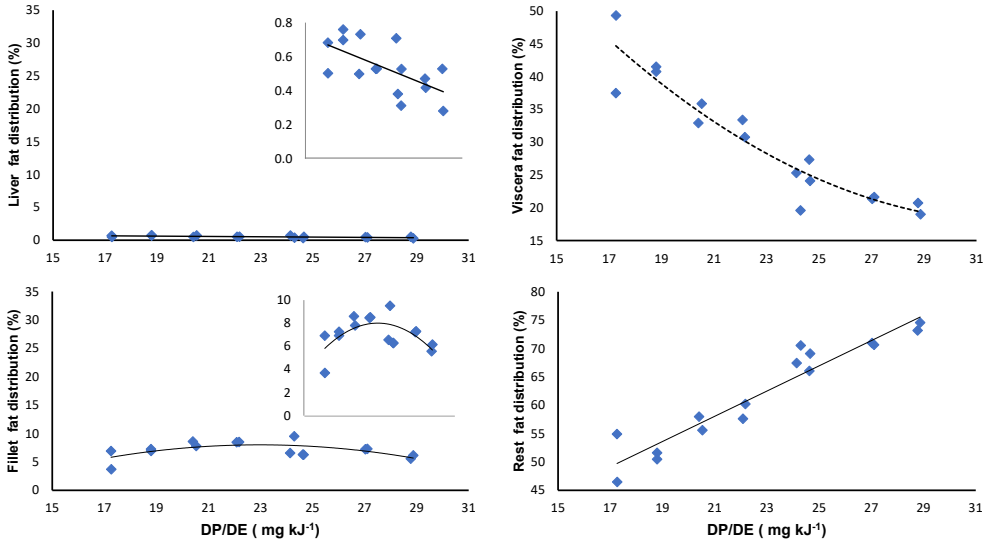

FIGURE 4.7 | The relation between digestible protein to digestible energy (DP/DE) ratio and fat distribution in body compartments of Nile tilapia. Dotted lines indicate no significant relation and black lines indicate significant relation with DP/DE ratio. Small graphs (zoom in) have the same units on the x-axis but different y-axis scaling. Regression equations are in table 4.4.

4.3.5. The correlation between carcass traits

The correlation coefficients between different carcass traits were given in Table 4.5. The VSI was negatively correlated with the fillet yield and "rest" fraction ($P<0.05$). HSI did not relate to any of the other carcass traits ($P>0.05$). Fillet yield was correlated with carcass yield ($P<0.01$), as fillet yield increased it was followed by an increase in carcass yield. In addition, the "rest" fraction was also positively correlated with carcass yield.

TABLE 4.5 | The correlation between carcass traits in Nile tilapia.

Traits (%)	HSI	Fillet yield	"rest" fraction	Carcass yield
VSI	0.316^{ns}	-0.612*	-0.554*	-0.831***
HSI		0.193^{ns}	-0.290^{ns}	-0.235^{ns}
Fillet yield			0.123^{ns}	0.677**
"rest" fraction				0.641*

HSI, hepatosomatic index; VSI, viscerosomatic index; *, P<0.05;**, P<0.01; ns, not significant.

4.4 Discussion

We explored the effect of changing digestible protein to digestible energy ratio (DP/DE) on the size and composition of the different body compartments in Nile tilapia. The whole fish was portioned into 4 body compartments: liver, viscera, fillets and "rest" fraction. The "rest" fraction contained the head, skin, fins and bones. About 10% of fish weight were viscera and 22% to 29% was present as fillet yield. Fat and dry matter content of all body compartments increased with decreasing dietary DP/DE ratio. The majority of the body fat was present in the viscera and "rest" fraction, respectively 30 and 62%. The distribution of protein over the different body compartments was relatively independent of the dietary DP/DE ratio, whereas the fat distribution was strongly affected by the applied dietary treatments. These findings suggest that the viscera and the "rest" fraction play the major role of fat storage in young Nile tilapia. Changes in dietary DP/DE ratio have clear effect on fat content but not protein content of body compartments of young Nile tilapia.

There was considerable variation in the proximate composition of whole fish between the different DP/DE ratio treatments in the current study (Table 4.3). Whole body proximate composition of fish can be affected by many factors such as genetics, environmental conditions and dietary factors (Shearer, 1994). In the current study the fish were genetically similar and also the environmental conditions were identical. Hence, the differences in whole body proximate composition were solely due to differences in the dietary DP/DE treatments. Whole body protein content of fish increased with increasing DP/DE, this is in line with other studies in Nile tilapia. However, in other tilapia species body protein content was constant and unaffected by the dietary protein to energy ratio (El-Dahhar and Lovell, 1995; Shiau and Huang, 1990; Winfree and Stickney, 1981). This suggests that the impact of dietary factors on the whole body protein content varies between tilapia species. Final whole body fat content increased when the DP/DE ratio decreased. This is in line with other studies on Nile tilapia (El-Sayed and Teshima, 1992; Hanley, 1991; Saravanan et al., 2012) and also in other species of tilapia (El-Dahhar and Lovell, 1995; Shiau and Huang, 1990; Winfree and Stickney, 1981). However two studies on Nile tilapia showed different results, e.g., no alteration in whole body fat content in relation to the DP/DE ratio (El-Saidy and Gaber, 2005; Schneider et al., 2004). The impact of dietary treatments in the current study was very large. The whole body fat content (7 g initial weight fish) in Nile tilapia at the lowest DP/DE ratio was 20% (on wet weight basis). This is comparable to 25% fat content found in 12 mg Nile tilapia (El-Sayed and Teshima, 1992). In a study by Saravanan et al. (2012) on heavier/older Nile tilapia (40-250 g), the authors found 16% whole body fat content at DP/DE ratios 14 and 15 mg kJ^{-1}.This indicates that Nile tilapia increases its fat content at low DP/DE ratios irrespective of age/size.

There is limited literature on the effect of dietary DP/DE ratio on carcass traits in Nile tilapia. In the current study carcass traits showed different responses to dietary DP/DE ratio. The viscerosomatic index (VSI) was on average 10%, which is similar to what was reported for Nile tilapia and hybrid red tilapia (Garduño-Lugo et al., 2003; Herath et al., 2016). On the other hand other studies on Nile tilapia reported lower VSI values (6.8% in (Kaushik et al., 1995) and (4.7% in (Fernandes et al., 2016)). In our study and in the study of Kaushik et al. (1995), decreasing the DP/DE ratio resulted in a higher VSI. This was also observed in other fish species such as Eurasian perch and Cobia (Mathis et al., 2003; Wang et al., 2005). However, in our study the relation between dietary DP/DE and VSI was quadratic and seemed to level off at DP/DE ratio higher than 28.8 mg kJ^{-1}. Moreover, at similar dietary DP/DE ratio (18 to 21 mg kJ^{-1}) our VSI values were double the values measured by Kaushik et al. (1995). This suggests that other factors than DP/DE alter VSI. The differences in VSI values between the current study and Kaushik et al. (1995) might relate to the absolute dietary fat level; ranging from 4 to 26% and from 11 to 12%, respectively. However, also differences in ingredient composition (fishmeal versus plant ingredients) might play a role. Higher dietary carbohydrate content especially non-starch polysaccharides might affect VSI (Rijnen, 2003). Hepatosomatic index (HSI) showed no relation with dietary DP/DE ratio in the current study and ranged between 1.7 to 2.5%. When looking at the result of Kaushik et al. (1995), HSI decreased with increasing DP/DE ratio and ranged between 1.6 to 5%. This higher HSI may be due to very high levels of dietary starch level in that study; ranging from 28 to 87 % compared to 14 to 21 % in the current study. As liver is a major site to store carbohydrates (Dabrowski and Guderley, 2002) as well as the production of fatty acids (Sargent et al., 2002) , the high amount of dietary starch may have been metabolized and deposited in the liver as glycogen and as fat. Consequently this may have caused the higher HSI values in their study.

Fillet yield in the current study showed a significant quadratic relation with DP/DE ratio. Fillet yield was 29% at maximum, which occurred between 24 to 25 mg kJ^{-1} (Figure 4.3). This suggests an optimal dietary DP/DE ratio around this value for young tilapia. In another study on Eurasian perch, it was found that fillet yield decreased with decreasing protein to energy ratio, however this relation was not quadratic (Mathis et al., 2003). Average fillet yield estimated in the current study (26.5%) is comparable to what was calculated by Clement and Lovell (1994) and Herath et al. (2016) but was lower than the reported range of 30 to 36% in other Nile tilapia studies (Fernandes et al., 2016; Furuya et al., 2004; Garduño-Lugo et al., 2003; M. Michelato et al., 2016; Mariana Michelato et al., 2016; Rutten et al., 2004). Most likely this is due to differences in body weight of the fish between studies. Most of these studies, which reported higher fillet yields, had higher body weight at slaughter ranging from 114 to 740 g, whereas the slaughter weight was around 60 g in the current study. However despite the higher slaughter weight (585 and 148g) the studies of Clement and Lovell (1994) and Herath et al. (2016) reported similar fillet yields as the current study. The discrepancies in reported fillet yield between studies can also be attributed to different filleting techniques and accuracies, fish sizes, and even to different experimental conditions and dietary factors.

Another observation in the current study is the negative correlation between VSI and fillet yield (Table 4.5). When the size of the viscera increased the fillet was affected and showed reduced yield, which is in line with other study on Eurasian perch (Mathis et al., 2003). On the other hand carcass yield in the current study increased linearly with increasing the dietary DP/DE ratio, which is line with a study on lake trout and Atlantic

salmon (Azevedo et al., 2004). On average carcass yield was 86%, this value is higher than estimated value of 51% for 585 g Nile tilapia (Clement and Lovell, 1994) and comparable to a value of 89% for 300 g European sea bass (Lanari et al., 1999), lake trout and Atlantic salmon (Azevedo et al., 2004). As the carcass is mainly composed of fillets and the "rest" fraction (bones, skin, head and fins), about 60% of the carcass in the current study was composed of "rest" fraction and about 30% fillets. In the aforementioned studies on Nile tilapia and sea bass, fillets represented about 50% of the carcass, but these studies unfortunately did not report the "rest" fraction. It could be possible that young Nile tilapia in the current study were not only maximizing fillet yield but also maximizing their growth through increased growth of "rest" fraction compartment. This was also seen from the positive correlation between carcass yield, fillet yield and "rest" fraction (Table 4.5). In mammals, it was stated that there is genetically predetermined minimum species specific organs size and that any subsequent increase in size is attributed to functional requirement (Goss, 1978). For young fish, the increase in "rest" fraction might be vital to enable the latter/concomitant growth of fillets due to the essence of having e.g. a proper sized skeleton and gill size. However, this hypothesis requires validation for fish.

In Nile tilapia there is no information on the effect of dietary DP/DE ratio on nutrient content in body compartments. However, for Nile tilapia there is few data available of the effect of changing the dietary fat and/or dietary protein content on chemical composition of fillets. Fillet protein content in the current study was on average 17% (on wet weight basis), which is within the reported range for Nile tilapia (16 to 20%) (Clement and Lovell, 1994; El-Saidy and Gaber, 2005; Garduño-Lugo et al., 2003; Herath et al., 2016; Michelato et al., 2016; Michelato et al., 2016). Our results (Table 4.3) showed that the fillet protein content was not affected by the dietary DP/DE, which is in agreement with other studies applying different experimental dietary treatments. The same trend is also observed in other fish species where protein content of the fillets was constant and independent of the dietary treatments applied (Einen and Roem, 1997; Hemre and Sandnes, 1999; Lanari et al., 1999; Li et al., 2001; Mathis et al., 2003; Robinson et al., 2004; Wang et al., 2005). It is interesting to observe that fish species (i.e. carnivorous, omnivorous and herbivorous) and irrespective of fish size are aiming to have a constant amount of protein in their muscles, which is possibly pre-determined genetically. In general averaged over literature sources across fish species, protein content in the fillets is about 18 and ranging between 16 to 20%%.

Decreasing the dietary DP/DE resulted in increased fat content of all body compartments in the current study. The amount of fat in the viscera was substantial and was on average 32% of the total amount of fat in the body. This value is much higher than what was reported in Nile tilapia (13% in (Fitzsimmons et al.,1997) and 15.3% in (Hanley, 1991)). In the study of Hanley (1991), also visceral fat content increased with decreasing protein to energy ratio, however it could be that the low dietary fat range (5-12%) did not promote visceral fat deposition when compared to 3-24% dietary fat range in the current study. Maximal visceral fat content of 48% of the whole body fat in the current study is comparable to what was found in Rainbow trout and Brown trout (55% by Gélineau et al. (2002) and 42% by Regost et al. (2001)) and even higher to what was found in Atlantic salmon (26% in Aursand et al. (1994)) and Gilthead seabream (37% in Santinha et al. (1999)) and Rainbow trout (31% in Jobling et al. (1998)). Compared to the other studies that used bigger fish, it was not expected that the young/small tilapia in our study would have deposited these

large amounts of fat in the viscera. Moreover, in current experiment fish deposited a considerable amount of fat in the "rest" fraction (a maximum of 19%). When calculating the fat distribution as a percentage of the total fat content in body compartments (table 4), on average 62 % of the fat was in rest, 30% in viscera, 7% in fillets and 0.5% in liver. In Atlantic salmon, Aursand et al. (1994) reported that about 43%,11.7% and 0.4% of the total fat content was in fillets, viscera and liver, respectively.

Fat distribution in the fillets in relation to dietary DP/DE ratio was quadratic and seemed to have a maximum of 7% at DP/DE of 22 mg kJ^{-1}. This trend was not expected as it is known from literature (e.g., (Gélineau et al., 2002; Shearer, 1994)). that increasing the fat/energy level would enhance fat deposition in the whole body, hence we would expect a negative linear relation with DP/DE ratio. When looking at some literature studies, some fish store their fat intramuscularly such as Atlantic salmon (Aursand et al., 1994; Einen and Roem, 1997) while other fish species store fat mainly in the viscera (e.g., (Regost et al., 2001),(Salze et al., 2014)). The results in the current study suggest that young Nile tilapia store less fat intramuscularly but mostly in the viscera and rest parts. Therefore, fish species that store fat in the viscera would be more prone to changes in dietary factors which would affect the relative sizes of their body compartments. When looking at the protein distribution in the body compartments (Table 4.4), only protein distribution in the liver showed a negative linear relation with DP/DE ratio. However, the difference between the lowest and highest protein distribution is relatively small. In addition protein distribution in the other body compartments is relatively constant and this is in line with what was stated in different studies that protein content in fish body is generally stable (e.g., (Shearer, 1994)).

4.5 Conclusion

The findings in our study showed that substantial amount of fat was stored in the viscera (30%) and rest part (60%) of the total fat content in young Nile tilapia. Furthermore, protein content in fillets was on average 17%, and it seems that protein content in fish fillets across many fish species is inherently predetermined and relatively constant. Changes in the dietary DP/DE ratio have clear effect on the fat distribution but not on the protein distribution in body compartments of young Nile tilapia.

Acknowledgements

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Chapter 5

The effect of type of carbohydrate (starch vs. non starch polysaccharides) on nutrients digestibility, energy retention and maintenance requirements in Nile tilapia

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Abstract

For Nile tilapia, the energetic value of non-starch polysaccharides (NSP) was compared to starch. It was assessed if carbohydrate type (NSP vs. starch) affected the energetic utilization for growth (kg_{DE}) and the energy requirements for maintenance (DEm). Eighteen groups of fish were assigned in 2x3 factorial design: two diets, with either a high NSP or high starch content; and three feeding levels (low, medium or satiation). The NSP diet contained 70% of the starch diet supplemented with 30% dried distillers grains with solubles. Nutrients digestibility, nitrogen and energy balances were measured. All nutrients digestibility decreased with increasing feeding level ($P<0.001$). Diet type (NSP vs. starch) affected the digestibility of all nutrients except for dry matter and fat. NSP of both diets were digested and the NSP digestibility ranged between 23% and 73%. Averaged over feeding levels, 5% and 17% of the total digestible energy originated from NSP at the starch and NSP diet, respectively. Although the digestible energy intake was similar, the contrast in type of carbohydrates between the diets resulted in lower energy retention with the NSP rich diet ($P<0.05$). Despite this impact on energy retention, both DEm and k_{gDE} were not significantly influence by diet. However, DEm was numerically higher (96 vs. 110 kJ $kg^{-0.8}$ BW d^{-1}) and kg_{DE} was numerically lower (65% vs. 58%) at the NSP diet compared to the starch diet. In conclusion, NSP are digested by Nile tilapia. Digested NSP are less well utilized for growth, which is reflected by a lower energy retention in fish and is due to the slightly higher DEm in combination with a slightly lower kg_{DE}.

5.1 Introduction

For all animals, nutrients are essential for growth, reproduction and sustaining their vital life processes (i.e., maintenance). Proper diet formulation in terms of energy requires information on nutrient digestibility, energy requirements for maintenance (DE_m), and the efficiency of utilization of digestible energy (kg_{DE}) for growth, both for fish and other animals (Schrama et al., 2012). Digested nutrients are used for ATP production or/and for anabolic processes (NRC, 2011). Energy requirements for both growth and maintenance can be met by the digested nutrients: protein, fat and/or carbohydrates. The relative importance of these digested nutrients for meeting energy requirements differs between species (e.g., herbivorous, omnivorous vs. carnivorous) (Halver and Hardy, 2002). Compared to terrestrial farm animals, in fish relatively little information (Anderson et al., 1984; Bergot, 1979; Erfanullah and Jafri, 1995; Hemre et al., 1989; Kim and Kaushik, 1992) is available on the potential of carbohydrates to meet the energy requirements.

The substitution of fishmeal in fish diets will result in more types and higher levels of plant ingredients (Glencross et al., 2007). Consequently this will enhance the variability in dietary nutrient composition, particularly regarding carbohydrates. Carbohydrates can be classified into: low molecular sugars and starch, being digested by endogenous enzymes; and non-starch polysaccharides (NSP) (NRC, 2011). Mammals, birds and fish lack endogenous enzymes to digest NSP (Choct and Kocher, 2000). Whereas, through the production of volatile fatty acids (VFA) by intestinal bacteria, fermentable NSP can still be a substantial energy source for humans and rats (Castiglia-Delavaud et al., 1998), pigs (Schrama et al., 1998) and poultry (Choct and Kocher, 2000). In fish, some studies showed qualitatively that fermentation of NSP occur in the intestine, which is indicated by the presence of VFA (Amirkolaie et al., 2006; Schrama et al., 2005). Some studies in fish have addressed the qualitative impact of NSP on digestion processes and their interference with the digestion of other nutrients (Leenhouwers et al., 2007a, 2007b, 2006; Refstie et al., 1999). However, quantitative data on NSP digestibility and the impact of digestible NSP on energy retention in fish is lacking.

Like in mammals (Pullar and Webster, 1977) and birds (Emmans, 1994), in fish it is shown that the energetic efficiency for growth depends on the composition of growth (fat to protein gain), with fat accretion being more efficient than protein (Lupatsch et al., 2003a; Pfeffer et al., 1999) and on the nutrient composition of the digested energy (protein, fat and carbohydrates) (Noblet et al., 1994; Schrama et al., 2012, 1998). In pigs and humans, NSP generate a lower energetic efficiency for growth (Noblet et al., 1994; Schrama et al., 1998, 1996) In fish, such information on the impact of the type of carbohydrates (NSP vs. starch) on kg_{DE} is absent.

In fish, the digestible energy requirement for maintenance (DEm) is dependent on environmental factors, such as: water temperature(Lupatsch and Kissil, 2005); water oxygen concentration(Glencross, 2009; Tran-Duy et al., 2012) and stocking density (Lupatsch et al., 2010). In Nile tilapia (*Oreochromis niloticus*) (Saravanan et al., 2013) and African catfish (*Clarias gariepinus*) (Dersjant-li et al., 2001; Dersjant-Li et al., 2000) dietary mineral composition affects also DEm. In various fish species, the impact of dietary macronutrients composition on DEm was often small and not consistent (Glencross et al., 2008, 2007; Pfeffer et al., 1999; Schrama et al., 2012). This might be due to the use of marine based experimental diets in

the past; i.e., low dietary carbohydrates content. In pigs, the type of carbohydrates (enzymatically digestible vs. fermentable) in a diet can alter maintenance requirements for energy through changes in physical activity (Schrama et al., 1998, 1996). Information on the impact of dietary NSP on DEm in fish is lacking.

In this paper, the energetic value of NSP for Nile tilapia is compared to starch. In other words, how does the type of carbohydrates influence the energy balances of Nile tilapia? It addresses the impact of the type of carbohydrates (NSP vs. starch) on: 1) the digestibility of nutrients; 2) the energetic utilization of digestible energy for growth (kg_{DE}); and 3) the energy requirement for maintenance (DEm).

5.2 Materials and methods

5.2.1. Diets and feeding

Eighteen aquaria were randomly assigned to one of six experimental treatments, which were arranged in a 2x3 factorial design: two diets (NSP vs. starch) and three feeding levels. The two diets were aimed to have an identical crude protein and fat content, but being different in the type of carbohydrate composition, i.e., NSP and starch content. Dried distillers grains with solubles of wheat origin (DDGS) was used as NSP source, because of the high NSP content and the expected high NSP digestibility. In pigs relatively high NSP digestibility of DGGS are reported (Jakobsen et al., 2015; Tanghe et al., 2015) The NSP diet was a mixture of 70% of the starch diet with 30% DGGS (Table 5.1). In order to keep crude protein and fat content similar between diets and based on the predicted protein and fat content of DDGS, the starch diet was formulated to have the same protein and fat content as NSP diet. Consequently inclusion of 30% DDGS resulted in two isonitrogenous and iso-lipidous experimental diets. The analysed chemical composition of diets in table 1 confirms this. When formulating the diets, both the starch and NSP diets were checked whether the amount of essential amino acids was meeting the requirements of Nile tilapia according to the recommendation of NRC, 1993.Both diets were supplemented with, lysine, methionine and threonine to ensure that none of the essential amino acids were limiting. Yttrium oxide (Y_2O_3) was used as inert marker to measure nutrient apparent digestibility coefficients. The experimental feeds were extruded and obtained from Research Diet Services B.V. (Wijk bij Duurstede, the Netherlands) and the pellets of both feeds were floating pellets (2mm). The dietary ingredients were mixed and hammer-milled (Condux LHM20/16; Hanau) through a 1mm screen. The diets were processed by extrusion using a Clextral BC45 laboratory scale twin-screw extruder (Clextral) with a 3mm die, resulting in about 2 mm pellet size. After extrusion, pellets were dried in a tray dryer at 70°C for 3 h and cooled to ambient temperature and then stored at in bags at 4°C.

TABLE 5.1 | Ingredients and analysed chemical composition of the experimental diets.

	Diets	
	Starch	**NSP**
Test ingredient (%)		
DDGS(from wheat)	---	30
Maize	23.5	16.0
Wheat	24.0	16.4
Wheat Bran	10.0	6.8
Wheat gluten	10.0	6.8
Fish meal*(CP>68%)	10.0	6.8
Soya bean meal (RC<50)	15.0	10.2
Fish oil†	2.0	1.4
Monocalciumphospate	0.78	0.78
L-Lysine HCl	0.3	0.3
DL-methionine	0.3	0.3
L-threonine	0.1	0.1
Ytrium oxide‡	0.02	0.02
Diamol§	2.00	2.00
Premix#	2.00	2.00
Chemical composition on dry matter (DM) basis (g kg^{-1})		
Dry matter (DM, g kg^{-1} diet)	959	962
Crude fat	73	73
Crude protein	333	328
Ash	69	72
Total carbohydrates	525	528
Starch	331	232
NSP	194	296
Gross energy (kJ/g DM)	19.7	19.9
Crude protein/gross energy (mg/kJ)	16.9	16.5
Digestible protein/digestible energy (mg/kJ)	18.0	17.9
Dietary viscousity (cP)	2.29	2.67

DDGS, dried distillers grains with solubles. Südzucker Bioethanol GmbH, Germany.
Total carbohydrate calculated as DM- crude protein – crude fat –ash content.
NSP,non-starch polysaccharides = 1000-crude protein- crude fat- ash - starch.
* Fishmeal LT (90% blue whiting and 10% sprat; crude protein content 72 %) Triple Nine Fish protein. Esbjerg, Denmark.
† Triple Nine Fish oil. Esbjerg, Denmark.
‡ Inert marker for calculation of apparent digestibility.
§ Diamol GM; Franz Bertram.
# Mineral and vitamin composition of premix identical to Tran-Duy et al.(Tran-Duy et al., 2008).

Three feeding levels were applied to create contrast in digestible energy intake (DE) in order to estimate the linear relation between DE and retained energy (RE). From the relation between RE and DE, the utilization efficiency of DE for growth (kg_{DE}) and the energy requirements for maintenance (DEm) was derived. The 3 applied feeding levels were: 2.0 times maintenance "low", 3.5 times maintenance "Mid" and apparent satiation "Sat" (being about 45%, 80% and 100% respectively of the ad libitum intake). The first two feeding levels are restrictive and were based on previous experiment with Nile tilapia (Schrama et al., 2012). The "Sat" treatments were hand-fed for one hour twice a day (at 9:00 and 16:00). The "Mid" treatments were fed

half of the calculated daily ration twice a day (at 9:00 and 16:00), "Low" treatments were fed the complete daily ration once a day (at 9:00). The daily feeding ration per aquarium was calculated based on the mean initial fish weight, the feeding level of the treatment (in g $kg^{0.8}$ BW d^{-1}) and the expected growth of the fish. The expected daily growth was estimated using an expected feed to gain ratio (FCR) of 1.5 which was assumed equal for all treatments.

5.2.2. Fish and housing

Male Nile tilapia of the Swansea red GMT (Genetically Male Tilapia) strain was obtained from a commercial breeder (Til-Aqua International, Velden, The Netherlands). The experiment was approved by the Ethical Committee judging Animal Experiments of Wageningen University, The Netherlands, and carried out according to the Dutch law on animal experiments. At the start of the experiment all male Nile Tilapia (*Oreochromis niloticus*) were randomly assigned to one of 18 tanks. The initial stocking density was 34 fish per 70-L tank, being equal to 21.3 kg m^3. All tanks were connected to the same recirculation system (comprising of a common water reservoir, a lamella sedimentation unit for solids removal, a trickling filter for gas exchange and nitrification of $NH_4^{+)}$. Water flow through each aquarium was kept constant at 7 L min^{-1} (expect for the first week, when water flow was 6 L min^{-1}). Each tank was equipped with an aeration stone in order to maintain the dissolved oxygen (DO) concentration in the water above 4 mg L^{-1}. The measured outlet DO concentrations were above this level. Water temperature was kept at 28°C. All water quality parameters were kept within the optimal range for tilapia(Tran-Duy et al., 2008)and were measured daily. A 12 h light–12 h dark photoperiod was maintained with daybreak set at 07.00 h. The experiment lasted 6 weeks.

5.2.3. Measurements of nitrogen and energy balances

At the start and end of the 42-day experimental period, individual fish weights were measured after anaesthetising fish with a phenoxy-ethanol solution (0.25 ml L^{-1}). From weight measurements, mean initial (BW_0) and final BW (BW_{42}) and the CV of BW_{42} were calculated per tank. Growth rate was calculated as growth rate per metabolic weight unit (in g $kg^{-0.8}$BW d^{-1}). It was calculated per tank as (BW_{42} - BW_0) x 42/MBWm, with MBWm being the mean metabolic BW during the experimental period (in $kg^{0.8}$). From the feed ration, unfed feed pellets and feed spillage, which were daily recorded, feed intake was calculated and expressed as g $kg^{-0.8}$BW d^{-1}. Unfed feed pellets were weighed after feeding and feed spillage was recorded by counting the number of feed pellets trapped in the faeces collectors during the feeding period. The FCR was calculated as feed intake divided by growth (both in g $kg^{-0.8}$BW d^{-1}). A representative sample of each diet was taken weekly, stored at 4°C and pooled per diet over the total experiment. Feed samples were ground using a 1 mm-screen grinder for chemical analysis. Initial body composition was determined in twenty fish and final body composition in ten randomly selected fish per tank. Fish were euthanized by an overdose of a phenoxy-ethanol solution (1.0 ml L^{-1}) and stored at - 20°C. Before chemical analysis, the sampled fish were cut into small pieces, homogenised by grinding in a mincing machine through a 4.5 mm-screen grinder two times and subsequently freeze-dried. Faeces were daily collected per aquarium during the last 4 weeks of the experiment using settling tanks, according to the procedure described by Amirkolaie et al.(2006). 15 min after the last feeding session, unfed feed were weighed. Furthermore, the number of pellets

spilled were collected and counted. Each tank was connected to a swirl separator (44 cm in height, 24·5 cm in diameter; Aqua Optima AS, Trondheim, Norway) to collect faeces for the determination of nutrient digestibility. 30 min prior to the morning feeding session faeces were collected once a day in a detachable 250 ml glass bottle at the bottom of the swirl separator. In order to minimise the bacterial decay of faeces, the glass bottles was kept in ice. Then, faeces were stored (daily) at - 20°C and pooled per aquarium over the experimental period. The collected faeces were freeze-dried and ground using a 1 mm-screen grinder.

Chemical analyses were done in triplicate. Dry matter (DM) was determined gravimetrically after drying at 103°C for 4, 4 and 24 hours (h) until constant weight, respectively, for feed, freeze-dried faeces and fish samples (ISO 6496, 1983); ash was determined after incineration at 550°C for 4 h (ISO 5984,1978). Crude protein (CP) (Nx6.25) was determined by the Kjeldahl method (ISO 5983, 1979). Fat was quantified after petroleum–diethyl ether extraction (ISO 6492, 1999). Before fat analysis, feed and faecal samples were hydrolysed by boiling for 1 h with 3 mol HCL. Energy content was measured by direct combustion in an adiabatic bomb calorimeter (IKA-C-7000; IKA analysentechnik, Weitersheim, Germany). For feed and faeces, total carbohydrate content being (starch + free sugars + NSP) was calculated as (DM-CP- fat-ash). Starch including free sugars was enzymatically determined in feed and faecal samples by using amyloglucosidase without the ethanol extraction step and measuring glucose content as described in (Goelema et al., 1998). NSP content was calculated as total carbohydrates – "Starch + free sugars". Apparent digestibility coefficients of nutrients were calculated for each aquarium as in Amirkolaie et al.(2006) using Y_2O_3 as an inert marker. The yttrium content of feed and faeces was analysed using inductively coupled plasma-mass spectrometry (ICP-OES) according to the standard NEN 15510 (2007). Energy and nitrogen (N) balance parameters were calculated per aquarium and expressed as, respectively, kJ $kg^{-0.8}$ BW d^{-1} and mg $kg^{0.8}$ BW d^{-1}. N balance calculations were as follow: Gross nitrogen intake (GN) = FI X Nfeed, where FI = feed intake of the fish (g feed/fish), Nfeed = nitrogen content of the feed. Digestible nitrogen (DN) = (GN*ADCcp)/100, where GN = Gross nitrogen intake , ADCcp (%) = apparent digestibility coefficient of the crude protein in the feed. Faecal nitrogen losses = GN –DN. Branchial and urinary nitrogen loses (BUN) = DN-RN, where RN= retained nitrogen. RN = ((BWt*CP)/6.25)-((BW0*CP)/6.25), where BWt = body weight of fish at the end of the experiment (kg), BW0 = body weight of fish at the start of the experiment (kg), CP= crude protein content of the fish (g). Energy balance calculations were as follow: Gross Energy intake (GE) = FI X Efeed, where FI = feed intake of the fish (g feed/fish), Efeed =energy content of the feed. Digestible Energy (DE) = (GE*ADCE) /100, where ADCE (%) = apparent digestibility coefficient of the energy in the feed. Faecal energy losses (FE) = GE-DE. Metabolizable energy (ME) =DE-BUE where BUE = branchial and urinary energy losses. BUE = (BUN*24.9)/1000, where 24.9 kj N g-1= energy concentration of NH3-N calculated by Bureau et al (2003) and assuming that all N was excreted as (NH3-N) . Retained energy (RE)= BWt*Et- BW0*E0, where Et = energy content of the fish at the end of the experiment, E0 = energy content of the fish at the start of the experiment, BWt = body weight of fish at the end of the experiment, BW0 = body weight of fish at the start of the experiment. Heat production (HP) = ME-RE.

5.2.4. Statistical analysis

The data were analysed using the Statistical Analysis Systems statistical software package version 9.1 (SAS Institute, Cary, NC, USA). All were analysed for the effect of diet, feeding level and their interaction by two-way ANOVA using the procedure GLM. When significant interaction found multiple comparison of means using Tukey's multiple range test were performed. DEm and kg_{DE} were estimated by linear regression of RE and DE intake values (both expressed as kJ $kg^{-0.8}$ BW d^{-1}) using the procedure GLM.

5.3 Results

Performance data are shown in table 5.2. FI was affected by the feeding level (P<0.001) but not by diet type. Growth was affected by the diet type (P< 0.05) and the feeding level (P< 0.001). Fish fed the starch diet showed a 15% increased growth rate (expressed in g $kg^{-0.8}$ BW d^{-1}) compared to fish fed the NSP diet. Increasing the feeding level resulted in higher growth rates of the fish. FCR was also different between diets and among the feeding levels (P=0.001). The higher FCR registered was at the low feeding level for both diets. FCR was affected by diet and was higher for the NSP diet (P<0.01). None of these performance parameters showed an interaction effect between diet and feeding level.

TABLE 5.2 | Effect of the experimental diets and feeding level on performance of Nile Tilapia (*Oreochromis niltoticus*). (Mean values with their standard errors)

	Diet							*P*-value		
	Starch			NSP						
	Low	Mid	Sat	Low	Mid	Sat	SEM†	Diet	Level	Diet*level
Growth period(d)	42	42	42	42	42	42	-	-	-	-
Tanks(n)	3	3	3	3	3	3	-	-	-	-
Fish per tank(n)	34	34	34	34	34	34	-	-	-	-
Survival(%)	97.1	100	97.1	98.5	100	100	-	-	-	-
Initial BW(g)	43.9	44.1	43.6	44.2	43.6	44.0	0.39	0.895	0.789	0.460
Final BW(g)	63.1	88.0	126.1	60.0	82.0	111.7	4.89	0.073	<.001	0.506
CV of final BW(%)	14.8	13.1	13.7	15.6	14.8	15.3	1.33	0.228	0.634	0.933
Feed intake ($gkg^{-0.8}BWd^{-1}$)	6.7	11.3	19.4	7.0	11.6	18.8	0.62	0.970	<.001	0.697
Growth ($gkg^{-0.8}BWd^{-1}$)	4.8	9.6	15.7	4.0	8.7	13.4	0.73	0.045	<.001	0.520
FCR	1.41	1.18	1.24	1.74	1.34	1.41	0.051	0.001	0.001	0.372

Low, low feeding level; Mid, medium feeding level; Sat, satiation feeding level; BW, body weight; d, day; CV, coefficient of variation; FCR, feed conversion ratio.

†n 3 per experimental treatment group.

Final body composition is presented in table 5.3. Feeding level affected the body composition of Nile tilapia at the end of the experiment (P< 0.001). Feeding level increased dry matter, fat, protein and energy content of fish, whereas, ash content decreased with feeding level. Regarding the effect of diet type (starch vs. NSP), final body composition differed between both experimental diets regarding ash (P< 0.001), fat (P< 0.05) and energy (P< 0.05) content. However, diet did not affect body protein content (P> 0.1). Averaged over feeding levels, the energy content of fish fed the starch diet was 3 % higher compared to fish fed the NSP diet and was due to the 6% higher body fat content in fish fed the starch diet. Feeding level affected the digestibility coefficients of all nutrients (P< 0.001). Increasing the feeding level resulted in decreased nutrient digestibility (Table 5.3). Diet

type significantly affected the digestibility of all nutrients except for DM and fat. Total carbohydrates content and energy digestibility were higher in the starch diet compared to NSP diet, whereas NSP and ash digestibility coefficients were higher in the NSP diet compared to the starch diet. Starch and protein digestibility were affected by the interaction effect between diet type and feeding level ($P< 0.05$). The difference in starch and protein digestibility between the starch and NSP diets increased with feeding level.

TABLE 5.3 | Effect of the experimental diets and feeding level on final body composition (on fresh weight basis) and apparent digestibility of nutrients of Nile tilapia (*Oreochromis niltoticus*).
(Mean values with their standard errors)

	Diet							*P*-value		
	starch			NSP						
	Low	Mid	Sat	Low	Mid	Sat	SEM*	Diet	Level	Diet*level
Final body composition† (gkg^{-1})										
Dry matter	241	263	288	243	260	283	3.86	0.489	<.001	0.581
Fat	66	84	104	62	79	98	2.89	0.047	<.001	0.885
Protein	142	150	152	144	155	159	4.23	0.189	0.035	0.889
Energy (KJg^{-1})	5.4	6.4	7.6	5.4	6.2	7.1	0.1	0.024	<.001	0.167
Ash	35	31	28	37	33.2	32.9	0.73	0.002	<.001	0.194
Apparent digestibility (%)										
Dry matter	86.5	83.1	76.0	86.9	79.9	73.6	1.01	0.058	<.001	0.208
FAT	96.8	94.9	91.2	96.7	94.0	90.8	0.41	0.193	<.001	0.551
Protein	92.9^{a}	92.1^{ab}	90.5^{d}	93.1^{a}	91.0^{bd}	88.9^{c}	0.30	0.007	<.001	0.036
Energy	89.8	87.0	80.8	89.6	83.6	77.9	0.87	0.010	<.001	0.193
Ash	49.3	42.4	24.1	59.8	46.1	34.5	3.14	0.008	<.001	0.483
Total Carbohydrates	85.0	80.3	70.4	84.6	74.6	65.7	1.31	0.006	<.001	0.147
Starch	99.6^{a}	99.4^{a}	98.6^{b}	99.2^{a}	98.4^{b}	97.5^{c}	0.12	<.001	<.001	0.023
NSP	60.4	47.8	22.5	73.2	56.0	40.8	2.93	0.000	<.001	0.265

Low, low feeding level; Mid, medium feeding level; Sat, satiation feeding level. NSP, non starch polysaccharides.

^{a,b,c,d} Mean values with unsimillar superscript letters within a row were significantly different ($P<0.05$).

*n 3 per experimental treatment group.

†Initial body composition on (fresh weight basis) was as follows: DM 272 g kg^{-1}; protein 151 g kg^{-1}; fat 84 g kg^{-1}; ash 31 g kg^{-1}; energy 7 kJ g^{-1}.

As planned with the experimental design, all parameters of the N and energy balances were strongly affected by the feeding level ($P<0.001$) (Table 5.4). Branchial urinary nitrogen loss was affected by the diet type ($P<0.05$); in fish fed the NSP diet this loss was higher. Moreover, nitrogen retention was affected by diet type ($P<0.05$), fish fed the starch diet had a higher nitrogen retention compared to NSP diet. Of all energy balance parameters, branchial and urinary energy losses, total energy retention, heat production and energy retention as protein were different between the two diets ($P<0.05$). Branchial and urinary losses and heat production were higher in fish fed the NSP diet, whereas total energy retention and energy retained as protein were higher in fish fed the starch diet. Energy retention as fat tended to be affected by diet type ($P<0.1$). The difference in metabolisable energy intake between diets (2 kJ kg$^{-0.8}$ BW d^{-1}) was not significant ($P=0.731$). Whereas, the difference in total energy retention between both experimental diets (14.7 kJ kg$^{-0.8}$ BW d^{-1}) was larger than the difference in metabolisable energy intake. Therefore, retained energy was significantly affected by dietary composition ($P <0.05$).

TABLE 5.4 | Effect of the experimental diets and feeding level on nitrogen and energy balances of Nile tilapia (*Oreochromis niltoticus*). (Mean values with their standard errors)

	Diet							*P*-value		
	Starch			NSP						
	Low	Mid	Sat	Low	Mid	Sat	SEM*	Diet	Level	Diet*level
N balance (mg kg$^{-0.8}$ BW d^{-1})										
N intake	330	577	963	342	586	945	36.9	0.973	<.001	0.910
Digestible N intake (DN)	307	532	871	318	534	840	31.2	0.819	<.001	0.776
Branchial urinary N losses (BUN)	207	287	463	243	328	511	22.3	0.043	<.001	0.959
Retained N (RN)	100	245	409	76	206	329	22.0	0.022	<.001	0.446
Energy balance (kJ kg$^{-0.8}$ BW d^{-1})										
Energy intake	122	214	357	130	222	358	13.8	0.631	<.001	0.966
Digestible energy intake	110	186	288	116	186	278	8.3	0.858	<.001	0.651
Branchial urinary energy losses	5	7	12	6	8	13	0.6	0.043	<.001	0.959
Metabolisable energy intake	105	179	276	110	178	266	7.9	0.731	<.001	0.613
Heat production	96	122	151	106	131	169	3.6	0.001	<.001	0.413
Retained energy	9	57	125	4	47	96	0.6	0.025	<.001	0.245
Retained energy as protein	15	36	61	11	31	49	3.3	0.022	<.001	0.446
Retained energy as fat	-6	21	64	-7	16	47	4.7	0.070	<.001	0.261

Low, low feeding level; Mid, medium feeding level; Sat, satiation feeding level.N, nitrogen. BW, body weight;d, day. *n 3 per experimental treatment group.

The linear relationship between digestible energy (DE) intake and total energy retention (RE) (both in kJ kg$^{-0.8}$ BW d^{-1}) was estimated per experimental diet (Fig 5.1), being at the starch diet;

$$RE = 0.6452 * DE\ (SE\ 0.028) - 62.022\ (SE\ 5.83)\ R^2 = 99.3\% \quad (1)$$

and at the NSP diet:

$$RE = 0.5843 * DE\ (SE\ 0.041) - 64.110\ (SE\ 8.55)\ R^2 = 97.2\% \quad (2)$$

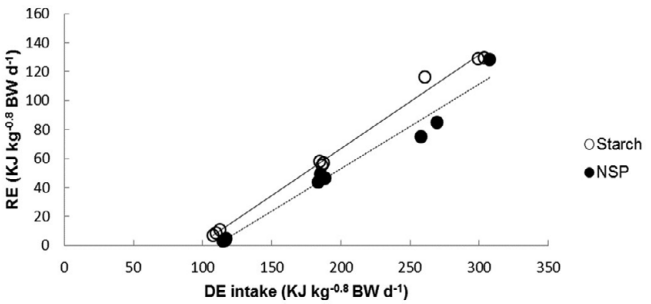

FIGURE 5.1 | The relationship between energy retention (RE) and digestible energy (DE) intake in Nile tilapia.Starch diet (○) and non starch polysaccharides (NSP) diet (●). The estimated regression lines at both diets are given in equations (1) and (2). BW, body weight.

Despite the impact of diet type on RE ($P<0.05$; Table 5.4) the linear relations between RE and DE intake were not significantly different between both diets. Both the intercept and slope of these relations were significantly different between diets, however numerical differences were present. From these equations, DEm were estimated as 96.1 and 109.7 kJ kg$^{-0.8}$ BW d^{-1} for the starch and NSP diets, respectively. kg_{DE} was 64.5% and 58.4% at the starch and NSP diet, respectively. kg_{DE} was 6.1% lower at the NSP diet compared to the starch diet.

5.4 Discussion

The present study investigated whether the nutritional value in terms of energy differed between types of carbohydrates in the feeds for Nile tilapia. Therefore, two diets were formulated having a similar fat and protein content but differing in type of carbohydrates (NSP vs. starch). NSP in both diets was not inert, indicated by the average NSP digestibility being larger than 56.7 %. Averaged over feeding levels, 5% and 17% of the digestible energy intake originated from NSP in fish fed the starch and NSP diets, respectively (Fig 5.2). The contrast in digested NSP resulted in lower energy retention with the NSP rich diet. However, this was only reflected in numerical differences in K_{gDE} and DEm when NSP was exchanged for starch.

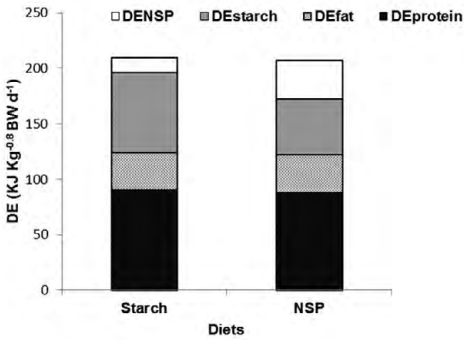

FIGURE 5.2 | Effect of diet composition on digestible energy (DE) in Nile tilapia. Fish were fed two diets: starch v. non starch polysaccharides (NSP).The bars show the amount of DE derived from the digestible protein, fat,starch and NSP. BW, body weight.

The observed NSP digestibility values in this study are higher than the scarce literature values in fish. In Nile tilapia, NSP digestibility ranged between 2 and 23% in diets enriched with purified NSP sources (guar gum and cellulose) (Amirkolaie et al., 2005) and between 13 and 24% in diets with different types of cereals (Leenhouwers et al., 2007a). These similar cereals tested in African catfish, showed NSP digestibility between 4 and 56% (Leenhouwers et al., 2007b). Our values with a maximum of 73% are well within the ranges of values found in pigs (Schrama et al., 1998), poultry (Jamroz et al., 2002), human and rats (Wisker et al., 1996). The variability found in NSP digestibility in fish can be explained by the type of NSP used in the experimental diets in the different studies. Amirkolaie et al. (2005) showed that cellulose is inert for Nile tilapia whereas guar gum is partially digested. Similarly in the studies on different types of cereals in Nile tilapia (Leenhouwers et al., 2007a) and African catfish (Leenhouwers et al., 2007b), digestibility of soluble NSP were always higher than that of insoluble NSP. Moreover, our higher NSP digestibility might also be related to pelleting conditions; we used extruded pellets whereas steam pelleting was used in previous studies (Amirkolaie et al., 2005; Leenhouwers et al., 2007a, 2007b) . Furthermore, the differences between studies might also be related to differences in feed intake. The current study showed that NSP digestibility was highest at the lowest feeding level (Table 5.3). Still the question remains how NSP is digested since fish like other mammals lack the endogenous enzymes to hydrolyse fibres. In tilapia the low pH in the stomach (pH< 2) (Saravanan et al., 2013) might be involved. However, fermentation in the distal part of the intestine is most likely. Moreover, it has been found that the VFA concentration increased towards the distal part of the intestine, indicating that fermentation took place (Amirkolaie et al., 2006). However, VFA concentration in chyme of fish strongly varies between species (highest in omnivores) and is much lower than in pigs, humans and rodents (Johan W. Schrama et al., 2005).

In the current study, the digestibility of all macro nutrients decreased with feeding level. In textbooks is often stated that fish feeding level has no or a minor impact on nutrient digestibility (Cho and Kaushik, 1990). However, several studies like the current study showed that with an increased feeding level, the digestibility of nutrients declined (Henken et al., 1985; Schrama et al., 2012; Windell et al., 1978). At the low feeding level in the current study, the differences in nutrients digestibility values were small, but when feeding level increased (relatively higher amounts of dietary NSP) the differences became larger. NSP can hold higher amount of water and form gum-like masses in the intestine, increasing the viscosity and hinder the digestive enzyme activity (Francis et al., 2001).

Moreover, nutrients digestibility of protein and starch were lower in the NSP rich diet which is in line with other studies on Nile tilapia and rainbow trout (Amirkolaie et al., 2005; Hossain et al., 2003; Storebakken, 1985) . On the other hand, two other studies on Atlantic salmon (Refstie et al., 1999) and Nile tilapia (Leenhouwers et al., 2007a) found no effect on starch digestibility. Most of these studies attributed the negative impact on nutrients digestibility to the ability of soluble NSP to increase the residence time of digesta in the intestine because of a higher viscosity and the subsequent reduced mixing of the digestive enzymes and substrates. In the present study, the difference in dietary viscosity was small between diets but still it was higher for the NSP diet (Table 5.1). It is possible that the degree of intestinal viscosity in relation to type and level of dietary NSP varies between fish species. In addition, in our study the amount of NSP included in the diets was higher than most of the mentioned studies which may explain the clear and high impact of NSP on other nutrients digestibility. The level of decline in digestibility values in our study is high and even higher than in the study of Schrama et al.(Schrama et al., 2012). The total carbohydrates content in our study is also higher than that in Schrama et al. (2012) which may explain the higher decline in nutrients digestibility. Moreover, in the present study the impact of feeding level on protein and starch digestibility seems to be dependent on the diet composition. In general the decline in nutrients digestibility is higher in the NSP diet.

The contrast in digestible NSP content between the diets resulted in a 14.7 kJ $kg^{-0.8}$ BW d^{-1} difference in energy retention which implies that the energetic utilization of carbohydrates (NSP v. starch) differed between the diets. However, this was only reflected in numerical differences in DEm and kg_{DE} between both diets. DEm increased numerically from 96.1 to 109.7 kJ $kg^{-0.8}$ BW d^{-1} when fish were fed the NSP diet. Moreover, the digestible energy intake was equal for the two treatments but the difference in DEm between the two treatments was about 13 kJ $kg^{-0.8}$ BW d^{-1} and may explain partially the difference in the energy retained.The higher DEm when feeding fish on a NSP diet can be attributed to more energy required for digestion along with the effect on the microbial balance in the intestine. In a recent study (Schrama et al., 2012), energy requirements for maintenance of different fish species were compared; it was found that these requirements ranged between 16 and 88 kJ $kg^{-0.8}$ BW d^{-1} . The authors attributed this large variability to differences in environmental and/or experimental conditions and to the dietary ingredient composition. Several studies reported an alteration in the gut anatomy and development due to soluble NSP inclusion in animal diets. Leenhouwers et al. (2006) showed that inclusion of guar gum in African catfish diets increased the weight of digestive organs. In broiler chicks, dietary NSP led to increased weight of the intestine and the mucosal morphology (Iji et al., 2001). It was reported that increasing viscosity due to dietary soluble NSP in Nile tilapia decreases transit time of the digesta and therefore increases intestinal VFA such as acetic acid, propionic

and butyric acid (Amirkolaie et al., 2006). These organic acids along with the delaying digesta transit time and effect of NSP may lower the pH of the intestine, decrease oxygen tension and therefore may affect or even change the gut micro flora (gut health) (Choct, 1997; Sinha et al., 2011). This disbalance may have a negative impact on fish health via increasing pathogens impact or toxins produced by anaerobic bacteria in the intestine (Carré et al., 1995).

The energy balance data in Table 5.4 showed that retained energy averaged over feeding levels was significantly lower at the NSP diet compared to the starch diet, despite the similar metabolizable energy intake (ME) at both diets. i.e., ME intake was unaffected by diet whereas retained energy was affected. This finding shows that NSP were less well utilized compared to starch. Part of this difference in retained energy was due to a numerical difference in k_{gDE} values (the slopes in figure 5.1), the kg_{DE} was about 6 % lower for the fish fed the NSP diet compared to the starch diet. In Nile tilapia (Schrama et al., 2012) kg_{DE} was altered when dietary macronutrient composition changed (exchanging fat by starch). One possible reason for this difference is that the contrast in dietary starch and NSP content was small in the current study. In pigs, the utilization efficiency of energy from fermentable carbohydrates is 30 % lower than enzymatically digested carbohydrates (Noblet et al., 1994). kg_{DE} of fish fed NSP diet (58.4 %) in the present study is comparable to what was found in sows(Rijnen et al., 2001) and in growing pigs (Noblet et al., 1994; Schrama et al., 1998). It is possible that due to NSP fermentation in the intestine some energy was lost through the breakdown of the polysaccharides by the bacteria. These losses resulted in a decrease in kg_{DE}.

In conclusion, the current study shows that NSP are not inert and that they are digested in Nile tilapia. However, energy balance data indicate that digested NSP are less well utilized for growth due to1) numerically higher DEm requirements and 2) numerically lower kg_{DE}.

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Chapter 6

Effects of feed ingredients on nutrient digestibility, nitrogen/energy balance and morphology changes in the intestine of Nile tilapia (*Oreochromis niloticus*)

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Abstract

The present study assessed the effect of different feed ingredients on nutrient apparent digestibility coefficients (ADC), nitrogen/energy balance and morphology changes in the intestine of Nile tilapia. Changes in intestinal morphology were correlated with nutrient digestibility and also nitrogen/energy balance. Seven diets, varying by different protein sources, were tested by a 1x7 factorial design. Test ingredients were hydrolysed feather meal (HFM), soybean meal (SBM), rice bran (RB), rapeseed meal (RM), sunflower meal (SFM) and dried distillers grains with solubles (DDGS). Six fish per treatment were sampled for intestinal morphology analysis at the end of week 1, 3 and 6. The proximal, middle and distal intestine was processed for quantitative histology counting the number of goblet cells (GC), and measuring the thickness of lamina propria (LP) and submucosa (SM). The study showed that the ADC of protein in raw materials were highest in SBM (92.2%), followed by SFM (90.2%), DDGS (89.2%), RM (87.8%), HFM (86.9%), and RB (84.0%). The nutrient ADCs had no correlation with intestinal morphology changes. Only the SBM diet caused noticeable changes in intestinal morphology such as an increase the thickness of SM and LP and the number of GC. The diet composition however altered the protein efficiency and the maintenance energy requirement. Protein retention efficiency was the lowest in fish fed HFM and the highest in RB. The highest maintenance energy requirements were observed in HFM and SBM treatments. However, neither of these changes in nitrogen/energy balance were correlated with the change in intestinal morphology.

6.1 Introduction

Over the last 20 years, aquaculture is growing more rapidly than all other animal food-production sectors (FAO, 2016). The expansion of aquaculture production has been accompanied by rapid growth of the aquafeed production. One of the challenges faced by the aquaculture industry is to identify alternatives to fish meal and fish oil on which many present aquafeeds are largely based. For many years, the aquafeed industry has recognised that a viable utilization of plant feedstuffs in formulated diets is an essential requirement for future development of aquaculture (reviewed by Gatlin et al., 2007). In the case of tilapia, a wealth of alternative protein sources are available and in use. For some of these ingredients, the apparent nutrient digestibility has been reported already, e.g. cottonseed meal and sunflower meal (El-Saidy and Gaber, 2003, Aanyu *et al.*, 2014), feather meal (Guimarães *et al.*, 2008a), dried distillers grains with solubles (DDGS) (Schaeffer *et al.*, 2010), rice bran (Guimarães *et al.*, 2008b), rapeseed (Borgeson *et al.*, 2006), and soybean meal (SBM) (Lin and Luo, 2011, Vidal *et al.*, 2015, Koch *et al.*, 2016). The substitution of fish meal coincides with an increased variability in dietary nutrient/ingredient composition. A larger variability in the nutrient digestibility is expected and hence the estimation of energy and nitrogen balance will also change. In a study by Schrama *et al.*, (2012) the authors stated that maintenance energy requirements of fish are dependent on the dietary macronutrient composition. For rainbow trout (*Oncorhynchus mykiss*), it was stated that changes in the dietary macronutrient composition and nutrient type (raw vs gelatinized starch) had an effect on energy and protein utilization (Kaushik and de Oliva Teles, 1985, Sanz *et al.*, 1994, Rodehutscord and Pfeffer, 1999). This indicates that any change in dietary ingredient/nutrient composition would have an effect on the energy balance. For Nile tilapia, information on the effect of different ingredients composition on energy and nitrogen balances is less investigated.

Plant ingredients contain often anti-nutritional factors such as protease inhibitors, phytates, glucosinates, saponins, tannins, non-starch polysaccharides, which can have negative impacts on the intestinal functions (Francis *et al.*, 2001). In the case of salmonids, SBM cause morphological and functional changes in the intestine such as widening of the lamina propria (Baeverfjord and Krogdahl, 1996), inducing enteritis in the distal intestine (Urán *et al.*, 2008b) and shortening villi and microvilli (van den Ingh *et al.*, 1991). These changes in intestinal morphology in fish fed SBM-based diets have been reported also for other species such as common carp (*Cyprinus carpio*) (Urán *et al.*, 2008a), rainbow trout (*Oncorhynchus mykiss*) (Nordrum *et al.*, 2000), gilthead sea bream (*Sparus aurata*) (Bonaldo *et al.*, 2008) and Nile tilapia (Mahmoud *et al.*, 2014, Ismaiel *et al.*, 2015). These changes in the intestinal morphology as a consequence of feeding SBM-based diets seem to be species specific, just as its ability to recover from these changes when fed a non-SBM-based diet. In Atlantic salmon, no signs of intestinal recovery of this SBM-induced enteritis occur with time (Urán *et al.*, 2009). On the other hand, a recovery of the distal intestinal epithelium was observed in carp starting from 4 weeks onwards after continuously being fed a SBM-based diet (Urán *et al.*, 2008a). Although most of the intestinal morphology studies were conducted using SBM or soybean co-products only, there is growing evidences that other plant ingredients may also affect the intestinal morphology. In an experiment with lupin products, ulcer-like lesson were observed in salmon fed lupins (Refstie *et al.*, 2006). Sitjà-Bobadilla *et al.* (2005) reported increased supranuclear protein droplets, lipidic vacuolisation

of enterocytes and hypertrophied submucosa with eosinophilic infiltration in the distal intestine of juvenile gilthead sea bream fed diets in which 50, 75 or 100% of the fish meal was replaced by a combination of plant proteins (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin). Therefore, measurement of the alterations in the intestinal morphology induced by plant ingredients is also an important step in the evaluation process of the potential value of an ingredient in diets for fish (e.g., tilapia).

Intestinal morphology, digestibility and nitrogen/energy balanced are rarely quantified in one single study. These measurements were combined in the present study in order to assess if alterations in intestinal morphology, induced by ingredients, do relate to nutrient digestibility and/or nitrogen/energy balance.

6.2 Materials and methods

6.2.1. General design

The experiment was conducted at the experimental facility "Carus", Wageningen University, The Netherlands, testing 7 diets in a 1x7 factorial design. It was conducted in accordance with the Dutch law on experimental animals and approved by the Wageningen University Animal Experimental Committee (20013003.b).

6.2.2. Feed ingredients and diet preparation

An extruded reference diet (Table 6.1) was formulated to meet the nutrient requirements of Nile tilapia (NRC, 2011). Yttrium oxide (Y_2O_3) was added to the reference diet at a concentration of 0.02%, and used as an inert marker. Test ingredients were obtained from Research Diet Services (Wijk bij Duurstede, The Netherlands), with the exception of hydrolysed feather meal that was supplied by Vionfood (Boxtel, The Netherlands).

TABLE 6.1 | Ingredient composition of the reference diets.

Ingredients (%)	Reference diet	Test diets
Fish meal	48.35	
Wheat	35.63	
Wheat bran	10	
Fish oil	2	
Soybean oil	2	
Vitamin and Mineral premix*	2	
Yttrium oxide	0.02	
Reference diet		70
Test ingredient**		30
Total	100	100

*Vitamin and mineral premix (per kg of feed): vitaimin A 6000 IU; D_3 2000 IU; E 100mg; C 100mg; K_3 10mg; $B_1$15mg; B_2 15mg; B_6 15mg; B_5 pantothenic acid 50mg; B_3 niacin 60mg; Biotine 0.2mg; B_{12} 0.025mg; Folic acid 3mg; Fe 50mg; Zn 100mg; Co 0.1 mg; Cu 10mg; Se 0.5 mg; Mn 20mg; Mg 500mg; Cr 1mg; I 2mg; Inositol 400mg; Choline 2000mg; Anti-oxidant 100mg; Calcium proprionate 1000mg.

**Test ingredients were hydrolysed feather meal, soybean meal (dehulled, solvent extracted), rice bran, rapeseed meal (rapeseed solvent extracted), sunflower meal (decorticated, solvent extracted) and dried distillers grains with solubles from wheat (DDGS).

The studied test ingredients, which are common in tilapia feed, were hydrolysed feather meal (HFM), soybean meal (SBM) (dehulled, solvent extracted), rice bran (RB), rapeseed meal (RM) (rapeseed solvent extracted), sunflower meal (SFM) (decorticated, solvent extracted) and dried distillers grains with solubles, wheat (DDGS). Six extruded test diets were formulated using 70% reference diet and 30% of each of the test ingredients as described by Foster (Forster, 1999). Proximate analysed nutrient composition of the test ingredients and diets are shown in table 6.2 and 6.3, respectively.

TABLE 6.2 | Nutrient composition of the ingredients used in the test diets.

Nutrient (% on dry matter)	Test ingredients					
	HFM	DDGS	SBM	RB	RM	SFM
Dry matter	96.8	90.5	89.02	89.22	89.94	91.29
Protein	87.21	33.38	55.34	15.22	39.52	37.68
Lipid	11.02	8.72	3.15	20.18	3.35	2.63
Ash	2.44	5.8	7.08	11.29	7.44	8.17
Phosphorous	0.37	0.8	0.76	2.65	1.31	1.37
Total Carbohydrate	-	52.1	34.42	53.31	49.69	51.52
Gross energy (kJ g^{-1})	25.01	21.26	19.86	21.48	19.61	19.12

HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

TABLE 6.3 | Nutrient composition of the reference and test diets

Nutrient (% on dry matter)	Test ingredients					
	HFM	DDGS	SBM	RB	RM	SFM
Dry matter	96.8	90.5	89.02	89.22	89.94	91.29
Protein	87.21	33.38	55.34	15.22	39.52	37.68
Lipid	11.02	8.72	3.15	20.18	3.35	2.63
Ash	2.44	5.8	7.08	11.29	7.44	8.17
Phosphorous	0.37	0.8	0.76	2.65	1.31	1.37
Total Carbohydrate	-	52.1	34.42	53.31	49.69	51.52
Gross energy (kJ g^{-1})	25.01	21.26	19.86	21.48	19.61	19.12

HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

6.2.3. Fish, housing conditions and feeding

735 unfed (feed-deprived for about 24h) juvenile Nile tilapia were individually weighed (under sedation 2-phenoxyethanol, 0.25 ml L^{-1} water) and randomly distributed among 21 aquaria (35 fish.$aquarium^{-1}$). Each aquarium was assigned randomly to one of the seven diets forming triplicates per diet. Throughout the experiment, the culture conditions and the water quality parameters (mean ± SD) were maintained at the optimal conditions for Nile tilapia; tank volume (70 L $aquarium^{-1}$), water flow over each aquarium (7 L min^{-1}), photoperiod 12h light:12h dark, water temperature (28 ±1^{0}C), pH (7.2 ± 0.2), dissolved oxygen (6.0 ±0.3 mg L^{-1}) and total ammonia nitrogen (<0.5 mg L^{-1}).

The fish were restrictively fed by hand at 3% of the body weight. The feeding ration was increased daily based on the measured fish weight per each tank at the start of the experiment and the predicted daily gain

assuming a FCR of 0.8 for all diets. Every second week, fish were weighed and these fish weigh data were used to calculated the feeding rations. Feeding was done twice daily starting at 900h and 1600h. During each feeding, the fixed feeding rate per tank was given in small portions by hand. This was done to ensure that feed pellets were quick consumed by the fish and no longer than 10 sec in the water. Tank by tank fish were fed and when the feeding response of the fish reduced, the fish caretaker started feeding the next tank. This clock wise procedure was done for maximally 1 hour. If present, the left over feed after 1 hour was weighed, however this did not occur during this experiment. Fifteen minutes after the end of feeding, the feed pellets that were flushed out the tanks, were collected per tank in the settling unit and counted. Weekly a sample of pellets of each diet was counted and weighted to determine the average pellet weight. From the feeding rate, the measured uneaten feed and the number of settled pellets, the actual daily feed intake was calculated.

To collect faeces for determination of nutrient digestibility, the outlet of each tank was connected to a swirl separator (44 cm height, 24.5 cm diameter; AquaOptima AS, Trondheim, Norway). The faeces were collected in a detachable 250 ml bottle placed at the bottom of each swirl separator. During faeces collection and in order to minimize the bacterial decomposition of faeces, the bottle was kept under ice. A different set of bottles was connected to the swirl separator during the feeding process to collect of uneaten feed pellets that were flushed out from the aquarium.

6.2.4. Sampling and measurements

During the last week of trial (week 6), faeces were collected per aquarium twice a day, 1 hour prior to feeding for measuring nutrient digestibility, and stored individually at -20^{0}C until be analysed. At the end of the experiment, and in order to determine the final biomass, fish from each aquarium were anaesthetized (2-phenoxyethanol, 0.25 ml L^{-1} water) and group weighed. To evaluate the intestinal morphology, at the end of week 1, 3 and 6, two fish from each aquarium (6 fish per treatment) were sampled. Fish were scooped gently out from each aquarium using hand dip net, and euthanized by an over dose of 2-phenoxyethanol, 1 ml L^{-1} water. Then, fish were weighted, dissected and the intestinal tract sampled for histological studies. The intestine was divided into three regions: proximal (from the pyloric part of the stomach to the spiral part of the intestine), mid (the spiral part of the intestine), and distal (from end spiral part of the intestine to 2 cm before anus) as described by Pirarat *et al*. (2011). One-cm portion of each of the three intestinal segments was fixed by immersion in Bouin's fixative solution. After fixation, the intestinal sample slides were prepared and analysed under a light microscope for SBM-induced enteritis following the method described in Tran-Ngoc *et al*. (2016). Briefly, the measurements were done on four random villi per slides and per intestinal segment for each fish. Three intestinal morphology parameters were assessed: a) the number of goblet cells (GC), b) the thickness of the lamina propria (LP) and c) the thickness of the sub-epithelia mucosa (SM) (Figure 6.1).

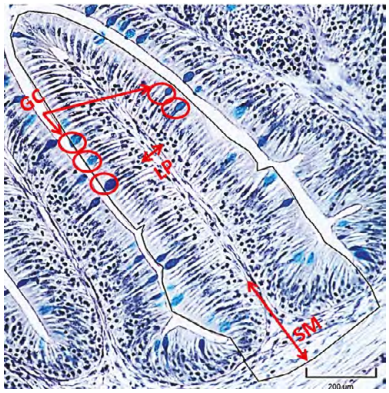

FIGURE 6.1 | Intestinal proximal morphology of Nile tilapia after 6 weeks cultured at a normal reference diet. The submucosa (SM) is a thin layer of connective tissue between base of folds and stratum compactum. The lamina propria (LP) is thin and delicate core of connective tissue in simple folds. The goblet cell (GC) is type of mucus-secreting in the epithelium and scattered among the enterocytes. Staining: Haematoxylin/Eosin and Alcian blue, x40.

6.2.5. Chemical analyses

Chemical analysis of the feed, and faeces were done in triplicate. Dry matter (DM) was determined gravimetrically after drying at 103^{0}C for 4h, 4h and 24h until constant weight, respectively (ISO 6496, 1983). Ash was determined after incineration at 550^{0}C for 4h (ISO 5984, 1978). Crude protein (CP) was determined by the Kjeldahl method (ISO 5983, 1979). Fat was quantified after petroleum-diethyl ether extraction (ISO 6492, 1999). Energy content was measured by an adiabatic bomb calorimeter (IKA-C-700; IKA analysentechnik, Weitersheim, Germany. Starch content was enzymatically determined in feed and faecal samples by using amyloglucosidase after ethanol extraction and measuring glucose content as described by Goelema *et al.* (1998). Fibre was analysed according to the standard NEN 5417 and ISO-standard 549 methods. The yttrium and phosphorous (P) content of feed and faeces was analysed using inductively coupled plasma-mass spectrometry (ICP-OES) according to the standard NEN 15510 (2007) method. Total carbohydrate was calculated as dry matter – crude protein – crude fat – ash content. Non-starch polysaccharides (NSP) was calculated based on DM - crude protein – crude fat – ash – starch.

6.2.6. Calculations

Specific growth rate was calculated as **SGR** (% bw d^{-1}) = [(lnW_f - lnW_i)/t] x 100, where W_f and W_i are the final and initial weight, respectively; t is the experimental duration in days. Feed intake (**FI_{bw}**) of fish was expressed as a percentage of body weight (in % bw d^{-1}) = FI / BW_{mean} x 100, where FI (g d^{-1}) is the average feed intake per fish per day and BW_{mean} is the mean body weight, which was calculated as BW_{mean} (g) = (W_f + W_i) / 2. Feed conversion rate (FCR) was calculated as **FCR** (g g^{-1})= FI_{tot} / (W_f - W_i), where FI_{tot} (g) is the total feed intake per fish during the experimental period. Apparent digestibility coefficients (ADC, in %) of dry matter, protein, lipid, ash, phosphorus, total carbohydrate, and energy of the test and reference diets were determined as described by Cho *et al.* (1982)

$$\mathbf{ADC_{test\ diet}\ (\%) = 100 - 100 \times (\%\ Y_{feed} / \%\ Y_{faeces}) \times (\%\ Nutrient_{faeces} / \%\ Nutrient_{feed})}$$

Where Y_{feed} and Y_{faeces} are the dietary and faecal yttrium oxide content and $Nutrient_{faeces}$ and $Nutrient_{feed}$ is the faecal and dietary nutrient content (all in % on dry matter basis).

Apparent digestibility coefficients of dry matter, protein, lipid, ash, phosphorus, total carbohydrate, and energy of the test ingredients using the equation propose by Forster (1999), mathematically simplified by Bureau and Hua (2006) and recently documented by the National Research Council (NRC, 2011).

$$\mathbf{ADC_{test\ ingredient}\ (\%) = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref.\ diet}) \times (0.7 \times D_{ref} / 0.3 \times D_{ingr})]}$$

Where D_{ref} = % nutrient (or kJ g^{-1} gross energy) of reference diet mash (as is); D_{ingr} = % nutrient (or kJ g^{-1} gross energy) in test ingredient (as is).

Energy and nitrogen (N) balance parameters were calculated per tank and expressed respectively as, kJ $kg^{-0.8}$. BW d^{-1} and mg $kg^{0.8}$ BW d^{-1}. N balance calculations were as follows: gross nitrogen intake (GN) = FI * N_{feed}, where FI = feed intake of the fish (g feed/fish), N_{feed} = nitrogen content of the feed. Digestible nitrogen (DN) = (GN x ADC_{cp}) / 100, where GN = gross nitrogen intake, ADC_{cp} (%) = apparent digestibility coefficient of the crude protein in the feed. Faecal nitrogen losses = GN – DN. Branchial and urinary nitrogen loses (BUN) = DN – RN, where RN = retained nitrogen. RN = ((BW_t x CP)/6.25) – ((BW_0 x CP)/6.25), where BW_t = body weight of fish at the end of the experiment (kg), CP = crude protein content of the fish (g). Energy balance were calculated as follows: gross energy intake (GE) = FI x E_{feed}, where FI = feed intake of the fish (g feed.$fish^{-1}$), E_{feed} = energy content of the feed. Digestible energy (DE) = (GE x ADCE) / 100, where ADCE (%) = apparent digestibility coefficient of the energy in the feed. Faecal energy losses (FE) = GE – DE.

Metabolizable energy (ME) = DE – BUE, where BUE = branchial and urinary energy losses. BUE = (BUN x 24.9) / 1000, where 24.9 kJ N g^{-1} = energy concentration of NH_3-N calculated by Bureau *et al.* (2003) and assuming that all N was excreted as (NH_3-N). Retained energy (RE) = (BW_tx E_t) – (BW_0 x E_0), where E_t = energy content of the fish at the end of the experiment, E_0 = energy content of the fish at the start of the experiment, BW_t = body weight of fish at the end of the experiment, BW_0 = body weight of fish at the start of the experiment. Heat production (HP) = ME – RE. Metabolizable energy for maintenance requirement (ME_{maint}) was estimated by ME_{maint} = ME – (RE_{pro}/0.54) – (RE_{lipid}/0.90); assuming k_P is 0.54 (cost of protein deposition) and k_L is 0.90 (cost of lipid deposition) (Lupatsch *et al.*, 2003). Retained energy as protein (kJ. $fish^{-1}$) was calculated as retained protein (retained N x 6.25) multiplied by 23.7 (Brafield, 1985). Retained energy as lipid (kJ.$fish^{-1}$) was calculated as the difference between total retained energy and the retained energy as protein, assuming total energy is equal to protein plus lipid.

6.2.7. Statistical procedure

Statistical analyses were performed using IBM SPSS 22 (SPSS Inc., Chicago, USA). Data were tested for normality and homogeneity (Shapiro Wilk and Levene test, respectively) and when necessary, transformed to achieve the required assumptions. Growth parameters and digestibility were subjected to one-way ANOVA in which tank was the experimental unit with three replicate per treatment. Histology data were analysed for effect of time and diet by two-way ANOVA in which fish was the experimental unit (2 fish per tank/ 6 fish per treatment). Pearson's correlation coefficients were calculated for the relationship between the enteritis symptoms and ADCs for nutrient and nutrient composition in diet. The results were considered statistically significant when *p-values* were below 0.05. When appropriate, the Tukey test was applied pair wise comparison of means.

6.3 Results

6.3.1. Fish performance

The effects on the different diets on growth are summarised in Table 6.4. Averaged over diets, the survival rate was 99% during the experimental period and was unaffected by dietary treatments.

TABLE 6.4 | Growth performance of Nile tilapia during the experimental period.

	Reference diet	Test diets (70% reference + 30% test ingredient)						
		HFM	DDGS	SBM	RB	RM	SFM	*P*-value
Experimental period (d)	42	42	42	42	42	42	42	
Tanks (n)	3	3	3	3	3	3	3	
Fish per tank (n)	35	35	35	35	35	35	35	
Survival (%)	100	98	100	98	99	100	100	
Initial BW (g)	10.83 ±0.2	10.93 ±0.4	10.73 ±0.3	11.13 ±0.5	11.17 ±0.4	10.9 ±0.3	11.0 ±0.2	ns
Final BW (g)	83.32 ±0.876^{b}	65.19 ±3.496^{a}	77.66 ±1.621^{b}	67.91 ±4.776^{a}	67.03 ±4.495^{a}	66.63 ±1.474^{a}	67.93 ±3.508^{a}	***
Feed intake (%bw.d^{-1})	3.23 ±0.062	3.29 ±0.197	3.32 ±0.110	3.35 ±0.172	3.41 ±0.168	3.44 ±0.133	3.42 ±0.221	ns
SGR (%bw.d^{-1})	4.87 ±0.058^{b}	4.27 ±0.058^{a}	4.70 ±0.110^{b}	4.30 ±0.100^{a}	4.27 ±0.252^{a}	4.30 ±0.000^{a}	4.33 ±0.115^{a}	***
FCR (g.g^{-1})	0.88 ±0.017	0.97 ±0.065	0.92 ±0.040	0.98 ±0.059	1.00 ±0.085	1.00 ±0.038	0.99 ±0.076	ns

Results are presented as mean ± SD (n=3). Values on the same row with different superscripts (a,b) are significantly different ($P<0.05$); ns, no significant difference: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal; BW, body weight; SGR, specific growth rate; FCR, feed conversion rate.

Feed intake was similar for all treatments, but final body weight and SGR were affected by diet ($P<0.001$), being higher for reference and DDGS diet treatments. Mean FCR ranged from 0.88 to 1.00; however no significant differences occurred among treatments.

6.3.2. Digestibility

Apparent digestibility coefficients (ADCs) of nutrient and energy in the experimental diets, and test ingredients for Nile tilapia are shown in Table 6.5 and 6.6, respectively.

In general, most of the ADCs values of nutrients and energy were affected by the composition of the test diets ($P<0.001$, table 6.5 and 6.6). Table 5 shows that protein and energy digestibility were similar for the reference and soybean diets and significantly higher than for the other diets. There was less variability in NSP digestibility for diets with high inclusion of animal products (reference and hydrolysed feather diets) (range -5.5 to -4.6%) than in NSP digestibility for diets in which plant products were included (range from 8 – 30%).

The ADCs of nutrients and energy differed between test ingredients: protein and fat digestibility were highest for soybean meal and lowest for rice bran and hydrolysed feather meal, respectively ($P<0.001$, table 6.5). Energy digestibility was significantly higher for hydrolysed feather and soybean meal and lowest for sunflower meal. The ADC of protein was general high for all ingredients (84 – 91%), while that for ash was low (21 – 52%).

TABLE 6.5 | Apparent digestibility of nutrients in the test diets.

% on dry matter	Reference diet	Test diets (70% reference + 30% test ingredient)						
		HFM	DDGS	SBM	RB	RM	SFM	*P*-value
Dry matter	80.1 ±0.1^{c}	78.1 ±0.2^{c}	73.7 ±0.9^{b}	78.4 ±1.7^{c}	71.5 ±0.9^{ab}	71.5 ±0.5^{ab}	69.2 ±2.1^{a}	***
Protein	92.3 ±0.5^{c}	87.4 ±0.5^{a}	89.9 ±0.8^{b}	92.2 ±1.0^{c}	87.4±1.0^{a}	88.8 ±0.4^{ab}	90.7 ±0.4^{bc}	***
Fat	94.1 ±1.0^{c}	84.8 ±2.4^{a}	92.0 ±0.9^{bc}	93.5 ±0.6^{bc}	86.9 ±1.3^{a}	90.5 ±0.4^{b}	91.3±0.5^{bc}	***
Ash	46.8 ±2.0^{bc}	32.3 ±8.3^{ab}	50.7 ±2.3^{c}	43.8±12.4^{abc}	29.2 ±0.9^{a}	37.1±1.8^{abc}	38.9±4.5^{abc}	**
Starch	99.6±0.1^{a}	99.9±0.1^{a}	99.7±0.2^{a}	99.9±0.8^{a}	99.6±0.3^{a}	99.9±0.1^{a}	99.9±0.2^{a}	ns
NSP	-5.5±1.7^{a}	-4.6±1.7^{a}	26.8±2.5^{cd}	30.0±3.2^{d}	17.2±3.4^{bc}	20.9±2.1^{cd}	8.1±7.2^{b}	***
Total Carbohydrate	68.6 ±0.6^{e}	66.5±0.6^{de}	58.2 ±1.5^{bc}	65.4±1.6^{de}	62.3±1.5^{cd}	56.7±1.2^{b}	49.4 ±3.9^{a}	***
Phosphorous	56.1 ±2.8^{d}	55.7 ±0.6^{d}	61.7 ±3.1^{d}	55.1 ±5.0^{cd}	37.0 ±1.3^{a}	46.5 ±0.6^{b}	47.7 ±3.1^{bc}	***
Energy	85.1 ±0.3^{d}	81.6 ±0.9^{c}	78.1 ±1.0^{b}	83.5 ±0.6^{cd}	77.1 ±1.2^{b}	77.0 ±0.7^{ab}	74.3 ±1.6^{a}	***

Results are presented as mean ± SD (n=3). Values on the same row with different superscripts (a,b,c) are significantly different (P<0.05); ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001; HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal; NSP, non-starch polysaccharide.

TABLE 6.6 | Apparent digestibility of nutrients in the test ingredients for Nile tilapia

% on dry matter	Test ingredients						
	HFM	DDGS	SBM	RB	RM	SFM	*P*-value
Dry matter	77.7 ±0.3^{b}	72.3 ±1.1^{b}	78.0 ±2.1^{b}	69.4 ±1.1^{ab}	69.5 ±0.6^{ab}	66.7 ±2.6^{a}	***
Protein	86.9 ±0.6^{b}	89.2 ±1.0^{bc}	92.2 ±1.1^{d}	84.0 ±1.7^{a}	87.8 ±0.4^{bc}	90.2 ±0.5^{cd}	***
Fat	82.6 ±2.9^{a}	91.3 ±1.2^{cd}	93.0 ±1.2^{d}	85.9 ±1.5^{ab}	87.4 ±0.7^{bc}	88.3 ±1.0^{bc}	***
Ash	21.1 ±14.6^{a}	52.1 ±3.1^{b}	42.9 ±15.9^{ab}	26.0 ±1.1^{a}	34.5 ±2.3^{ab}	37.0 ±6.1^{ab}	*
Total Carbohydrate	91.1 ±6.2^{d}	56.5 ±1.7^{bc}	64.6 ±2.0^{c}	61.2 ±1.7^{bc}	54.6 ±1.4^{ab}	46.3 ±4.5^{a}	***
Phosphorous	55.4 ±1.1^{cd}	63.9 ±4.2^{d}	54.6 ±7.1^{cd}	34.8 ±1.5^{a}	44.3 ±0.7^{ab}	45.8 ±3.7^{bc}	***
Energy	81.0 ±1.1^{c}	76.4 ±1.2^{b}	83.1 ±0.8^{c}	75.3 ±1.5^{b}	74.9 ±0.8^{ab}	71.5 ±2.0^{a}	***

Results are presented as mean ± SD (n=3). Values on the same row with different superscripts are significantly different (P<0.05). *p<0.05; **p<0.01; ***p<0.001; HFM, hydrolysed feather meal; DDGS, drain distiller grain with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

6.3.3. Nitrogen and energy balance

Nitrogen and energy balances are presented in table 6.7. All the nitrogen balance parameters were affected by diet (P<0.001). Gross nitrogen intake and digestible nitrogen intake reached the highest value in fish fed on hydrolysed feather meal diet while the highest value of retained nitrogen was reached in fish fed the reference and DDGS diet. All the other diets showed no significant difference in nitrogen retention. Protein retention efficiency ranged between 34 and 53% and was the lowest in fish fed the hydrolysed feather meal diet even though this diet showed the highest digestible nitrogen intake.

Regarding the energy balance data, all parameters were also significantly affected by test diets. Retained energy was different between diets caused by the differences in metabolized energy intake (P<0.001). Retained energy as protein followed the same trend as retained nitrogen and was the highest in the reference and DDGS diets. Maintenance requirements for fish fed hydrolysed feather diet was about 90

kJ $kg^{-0.8}$ BW d^{-1} and was significantly higher than maintenance requirements predicted for fish fed the rice bran and sunflower diets.

The correlation between nitrogen/energy balance and the changes in intestinal morphology were also addressed further. However, none of intestinal morphology parameters were correlated with any of the nitrogen/energy balance parameters (data not shown).

TABLE 6.7 | Nitrogen and energy balance in the reference and test diets for Nile tilapia.

	Reference diet	Test diets (70% reference + 30% test ingredient)							
		HFM	DDGS	SBM	RB	RM	SFM	SEM	*P*-value
N balance (mg $kg^{-0.8}$BW d^{-1})									
Gross nitrogen intake (GN)	1652^{c}	1943^{d}	1488^{b}	1599^{bc}	1242^{a}	1496^{b}	1501^{b}	43.1	***
Digestible nitrogen intake (DN)	1524^{d}	1699^{e}	1338^{b}	1474^{cd}	1085^{a}	1328^{b}	1361^{bc}	38.9	***
Branchial urinary nitrogen losses (BUN)	816^{bc}	1116^{d}	659^{ab}	853^{c}	514^{a}	711^{bc}	742^{bc}	15.9	***
Retained nitrogen (RN)	708^{b}	583^{a}	679^{b}	621^{a}	571^{a}	617^{a}	619^{a}	45.9	***
Protein efficiency (RN/DN)	47^{bc}	34^{a}	51^{cd}	42^{b}	53^{d}	47^{abc}	46^{bc}	1.8	***
Energy balance (kJ $kg^{-0.8}$ BWd^{-1})									
Energy intake (GE)	502^{c}	478^{abc}	497^{bc}	451^{a}	475^{abc}	458^{ab}	461^{abc}	12.3	**
Digestible energy intake (DE)	427^{d}	390^{c}	388^{c}	376^{bc}	366^{abc}	352^{ab}	343^{a}	9.5	***
Branchial urinary energy losses (BUE)	20^{bc}	28^{d}	16^{ab}	21^{c}	13^{a}	18^{bc}	18^{bc}	1.1	***
Metabolisable energy intake (ME)	407^{d}	362^{bc}	371^{c}	355^{bc}	354^{bc}	335^{ab}	324^{a}	8.5	***
Heat production (HP)	163^{ab}	175^{b}	144^{ab}	165^{ab}	132^{a}	146^{ab}	133^{a}	10.8	***
Retained energy (RE)	244^{b}	187^{a}	228^{b}	190^{a}	222^{b}	189^{a}	192^{a}	6.7	***
Retained energy as protein (RE_{prot})	105^{b}	86^{a}	101^{b}	92^{a}	85^{a}	91^{a}	92^{a}	2.4	***
Retained energy as fat (RE_{lipid})	139^{b}	101^{a}	127^{b}	98^{a}	137^{b}	98^{a}	100^{a}	4.8	**
Maintenance energy requirement (ME_{maint})	57.7^{ab}	90.5^{b}	43.7^{a}	76.3^{ab}	44.3^{a}	57.0^{ab}	43.3^{a}	12.5	*

Values on the same row with different superscripts are significantly different (n = 3; P<0.05). *p<0.05; **p<0.01; ***p<0.001; HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal; N, nitrogen; SEM, standard error; BW, body weight; d, day.

6.3.4. Intestinal morphology

Average values of the intestinal morphological parameters per diet and over time are described in table 8. The results show that the intestinal morphology of Nile tilapia was altered by the diets being fed. Soybean meal had a negative effect on the thickness of the submucosa (SM) showing this parameter reached the highest value in the proximal and middle part of the intestine (P<0.01; table 6.8). The effect of soybean meal on the thickness of the lamina propria (LP) (P<0.01) and on the number of goblet cells (GC) (P<0.05) followed a similar trend as that of the thickness of SM, with the highest values seen in the proximal and distal parts of the intestine. In contrast, the impacts on the intestinal morphology of the reference diet, hydrolysed feather meal and DDGS diets were neutral and showed minor differences between these diets. The other plant ingredient based diets (rice bran, rapeseed and sunflower) had an intermediate effect on the intestinal morphology (Table 6.8).

TABLE 6.8 | Effect of tested ingredients on morphological parameters at different sections of the intestine in Nile tilapia.

	Reference diet	Test diets (70% reference + 30% test ingredient							*P*-value		
		HFM	DDGS	SBM	RB	RM	SFM	SEM	Diet	Time	Diet x Time
Submucosa (µm)											
Proximal	376	353	434	458	365	363	421	23	**	***	ns
Middle	367	336	344	422	355	382	278	25	**	*	ns
Distal	432	468	414	428	418	400	422	41	ns	***	ns
Lamina propria (µm)											
Proximal	64^{ab}	61^{a}	61^{a}	79^{b}	74^{ab}	75^{ab}	67^{ab}	4	**	ns	**
Middle	97	91	94	108	99	93	86	9	ns	**	ns
Distal	115^{a}	116^{a}	144^{ab}	154^{b}	117^{a}	147^{ab}	127^{ab}	8	**	ns	***
Goblet cell (10^{-3} cells µm^{-1})											
Proximal	17.9^{a}	18.3^{a}	19.8^{ab}	23.2^{b}	21.5^{ab}	19.7^{ab}	20.1^{ab}	1	*	**	***
Middle	26.8	22.8	26.6	27.2	25.2	22.6	22.4	2	ns	ns	ns
Distal	20	18	24.8	28.2	26.1	27	27	2	*	ns	ns

Values on the same row with different superscripts are significantly different (n = 6; P<0.05); ns, no significantdifference, *p<0.05; **p<0.01; ***p<0.001; HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal; N, nitrogen; SEM, standard error.

In addition to the diet effect, many of the intestinal parameters were affected by time. The thickness of SM showed a significant time effect, whereas the time effect on the thickness of the LP and the number of GC was only observed in the middle and proximal intestine, respectively. The thickness of the SM increased with time in the proximal, middle and distal intestine (Figure 6.2) during the course of the experiment.

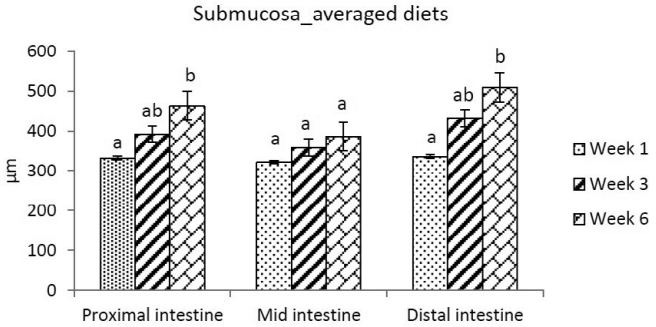

FIGURE 6.2 | The effect of time on the submucosa thickness average over all seven experimental diets in different parts of the intestine of Nile tilapia. Each bar shows overall mean the thickness of submucosa for each week with standard deviation represented by error bar. Bars within each region of intestine having no common letters are significantly different (n=42; p<0.05).

A significant interaction effect between diet and time was present for the parameters LP and GC. The effect of soybean meal on LP in the proximal intestine aggravated over time, while for the other diets that impact time was not present (Figure 6.3). The same trend was found for the thickness of LP in the distal intestine. On the other hand, the number of goblet cells in the proximate intestine significantly increased over time for fish fed plant protein diets with the exception for DGGS and rice bran (Figure 6.4). Fish fed the hydrolysed feather meal did not show the difference in GC over time and fish fed the reference diet showed an reduction in number of GC over time (Figure 6.4).

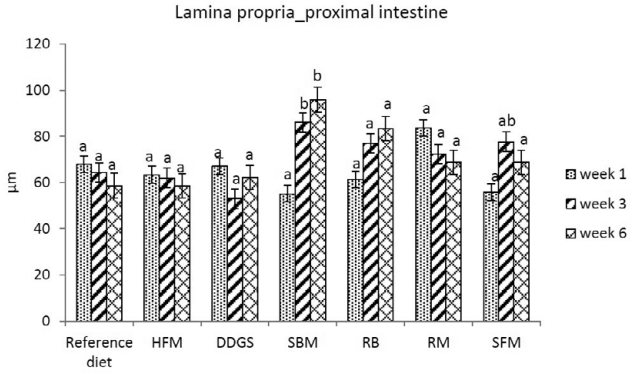

FIGURE 6.3 | Effect of diet composition on the thickness of lamina propria in proximal intestine of Nile tilapia over time. Each bar shows overall mean the time effect for each diet composition with standard deviation represented by error bar. Bars within diets lacking a common letter are significantly different (n=6; p<0.05). HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

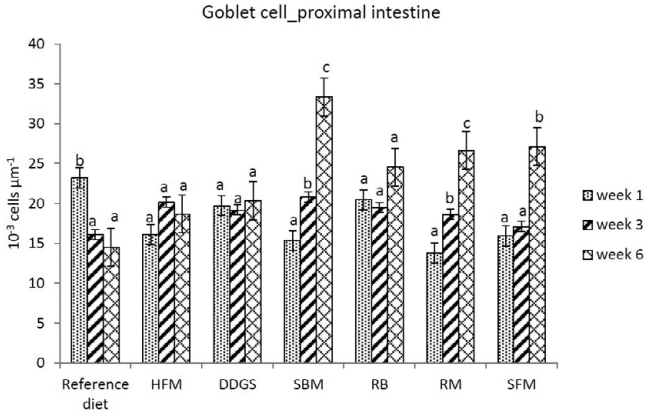

FIGURE 6.4 | Effect of diet composition on the number of goblet cell in proximal intestine of Nile tilapia over time. Each bar shows overall mean the time effect for each diet composition with standard deviation represented by error bar. Bars within diets lacking a common letter are significantly (n=6; p<0.05). HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

6.3.5. The correlation between intestinal morphology and digestibility of nutrient

Table 6.9 shows the correlation between the changes in intestinal morphology and the nutrient concentrations in the diet. With the exception of energy, there were no correlations between nutrient concentrations of the diet and intestinal morphology in the proximal and mid intestine. However, in the distal intestine, dietary NSP showed a positive relationship with LP and GC, whereas energy, protein and lipid content had a negative relationship.

TABLE 6.9 | Correlations between the intestinal morphology and the nutrient concentrations.

Nutrient composition in diet	Proximal intestine			Mid intestine			Distal intestine		
	SM	LP	GC	SM	LP	GC	SM	LP	GC
Protein	-0.14	0.26	-0.28	-0.03	-0.03	-0.16	0.21	-0.1	-0.47*
Lipid	-0.38	-0.19	-0.11	0.02	0.07	0.19	0.07	-0.53*	-0.34
Ash	0.14	0.42	0.3	0.1	0.1	0.21	-0.17	0.01	0.42
Phosphorous	-0.23	0.32	0.19	-0.07	0.02	0.01	-0.13	-0.27	0.27
Energy	-0.43	-0.48*	-0.38	-0.06	-0.03	-0.02	0.2	-0.48*	-0.65**
NSP	0.37	0.28	0.36	-0.1	-0.43	-0.34	-0.22	0.47*	0.64**

SM, submucosa; LP, lamina propria; GC, goblet cell; NSP, non-starch polysaccharides; *p<0.05; **p<0.01.

Furthermore we tested if intestinal morphological parameters correlated with the digestibility of the nutrients (Table 6.10). Most nutrients, digestibility did not correlate with morphological parameters. However, there was one exception, NSP digestibility, correlated strongly with morphological parameters changes in the proximal and distal intestine. In addition, the thickness of SM in the proximal intestine was positively related to the digestibility of protein and lipid.

TABLE 6.10 | Correlations between intestinal morphology and the digestibility of nutrients.

ADC	Proximal intestine			Mid intestine			Distal intestine		
	SM	LP	GC	SM	LP	GC	SM	LP	GC
Protein	0.52*	0.05	0.12	0.23	0.14	0.4	-0.15	0.3	0.17
Lipid	0.52*	0.12	0.13	0.25	0.14	0.33	-0.21	0.35	0.33
Ash	0.36	-0.19	-0.12	0.23	-0.21	0.25	-0.001	0.35	0.05
Phosphorous	0.3	-0.42	-0.23	0.13	-0.06	0.27	0.04	0.26	-0.22
Energy	0.07	-0.09	-0.08	0.50*	0.22	0.37	0.04	-0.07	-0.31
NSP	0.50*	0.44*	0.58**	0.37	0.15	0.19	-0.2	0.62**	0.63**

ADC, Apparent digestibility coefficients ; SM, submucosa; LP, lamina propria; GC, goblet cell; NSP, non-starch polysaccharides. *p<0.05; **p<0.01.

6.4 Discussion

6.4.1. Digestibility and nitrogen/energy balance

In general, the estimated nutrient ACDs of ingredients are in line with literature. The dry matter ADC provides a measure of the total quantity of ingredients (Fagbenro, 1999). The highest dry matter ADCs were observed for hydrolysed feather meal and soybean meal, which might related to the their low carbohydrates content. The protein ADCs of tested ingredients are in agreement with literature studies (Sklan *et al.*, 2004a, Guimarães *et al.*, 2008a, Tram *et al.*, 2011). In decreasing order, protein ADC was highest in soybean meal followed by sunflower meal, DDGS, rapeseed meal, hydrolysed feather meal, and rice bran. The high protein ADCs (>80%), especially soybean meal, show their potential as a protein source in tilapia feeds. Phytate bound phosphorous is unavailable for fish due to the lack of endogenous and microbial phytase in their intestine (Lall, 1991). With the exception for DDGS, phosphorous ADC in plant ingredients was inferior to animal ingredients. This agrees with results in hybrid tilapia (Zhou and Yue, 2012) and cobia (Zhou

et al., 2004). Despite the higher phosphorous content in rice bran, rapeseed and sunflower meal, their phosphorous ADCs were lower than those in hydrolysed feather meal, DDGS and soybean meal. This finding for a negative relation between phosphorus content and its ADC parallels the finding in hybrid tilapia (Zhou and Yue, 2012), rainbow trout (Burel *et al.*, 2000), and cobia (Zhou *et al.*, 2004). Energy ADCs were lower in plant compared to animal ingredients, excluding soybean meal. In general, this study confirms that nutrient digestibility of some specific plant ingredients can be higher than animal ingredients protein.

Hydrolysed feather meal had the lowest nitrogen retention despite the high protein ADC and consequently highest digestible nitrogen intake being reflected in the low protein efficiency (34%). Thus, protein in the hydrolysed feather meal diet was predominantly used as an energy source, which is also reflected in the high branchial and urinary nitrogen losses. Next to this oversupply of protein, also an imbalanced amino acids profile in feather meal (Lee, 2002, Guimarães *et al.*, 2008a) may explain the low nitrogen retention. Fish fed the rice bran diet had a similar nitrogen retention as the other diets, but had the highest protein efficiency. Except for hydrolysed feather meal, the protein efficiency values reported in the current study are comparable to other studies for Nile tilapia (Kaushik *et al.*, 1995, Tran-Duy *et al.*, 2008, Figueiredo-Silva *et al.*, 2013).

This study suggests that part of the differences in energy retention between diets is related to differences in maintenance energy requirements. Across fish species, energy maintenance requirements are affected by culture conditions like: water temperature (Lupatsch and Kissil, 2005, Pirozzi *et al.*, 2010); water dissolved oxygen content (Glencross, 2009); and stocking density (Lupatsch *et al.*, 2010). It is however less clear if dietary ingredient composition influences maintenance energy requirements. Glencross *et al.* (2008) found no effect of lupin kernel meal inclusion level on energy maintenance requirements in rainbow trout. In contrast, changing dietary mineral levels affected the maintenance requirements in Nile tilapia (Saravanan *et al.*, 2013) and African catfish (Dersjant-Li *et al.*, 2001). In pigs, dietary fibre content affected maintenance energy requirements by alterations in physical activity (Schrama *et al.*, 1998). In current study, fish fed hydrolysed feather meal and soybean meal had an increased energy maintenance requirements. The current study suggests that dietary ingredient composition can cause the differences in maintenance energy requirements.

Increasing energy maintenance requirements are suggested to be related to energy-demanding processes for vital life functions such as maintenance of the primary epithelia barrier as in the GI tract (Segner *et al.*, 2012). Changes in intestinal morphology induced by inflammation response are often considered an energy cost. However, in this study, correlation was present between intestinal morphology parameters and protein efficiency as well as energy maintenance requirements (data not shown).

6.4.2. Intestinal morphology

In the fish gut, food is digested and nutrients absorbed, while it also functions as primary barrier preventing translocation of harmful agents (Niklasson *et al.*, 2011). Feed ingredients can affect intestinal morphology (Baeverfjord and Krogdahl, 1996, Urán *et al.*, 2009). When replacing animal ingredients by plant-based ingredients, fish can get exposed to "foreign " components like starch and anti-nutritional factors that can interfere with the natural processes occurring in the intestine (Steiner and Encarnacão, 2010). Several

reviews addressed the fish meal replacement by plant protein in Nile tilapia diets (Tram *et al.*, 2011, Zhou and Yue, 2012, Vidal *et al.*, 2015, Figueiredo-Silva *et al.*, 2015). Recently, Tran-Ngoc *et al.* (2016) showed that soybean meal in combination with an environmental challenge affected the intestinal morphology of Nile tilapia, but information is lacking and how other plant-based ingredients alter intestinal morphology.

The cumulative effects of anti-nutritional factors by exposure of fish to plant ingredients can result in manifestation of pathological conditions (Krogdahl *et al.*, 2010). In the current study only soybean meal caused significant changes in intestinal morphology of Nile tilapia (widening of submucosa and lamina propria; more goblet cells). Soybean meal induces intestinal disorders in Atlantic salmon (*Salmo salar*) (van den Ingh *et al.*, 1991, Baeverfjord and Krogdahl, 1996, Urán *et al.*, 2009), rainbow trout (*Oncorhynchus mykiss*) (Heikkinen *et al.*, 2006, Venold *et al.*, 2012), and summer flounder (*P. dentatus*) (Bone, 2013). In Nile tilapia, the observed alterations in intestinal morphology were less severe than in salmonids. Moreover, these morphological alterations predominantly occur in the proximal intestine. In the proximal intestine of Nile tilapia, the mucosal villi are longer and more branched villi than the middle and distal intestine (Gargiulo *et al.*, 1998). This may make the proximal intestine more vulnerable for disorders.

The other plant ingredients in this study (rice bran, rapeseed, sunflower, DDGS), which also contain anti-nutritional factors (AFNs), did not induce severe morphological changes in the intestine. For salmonids, alcohol-soluble substances from soya (van den Ingh *et al.*, 1996, Francis *et al.*, 2001), especially soya saponins (Knudsen *et al.*, 2008, Knudsen *et al.*, 2007, Krogdahl *et al.*, 2010) are involved in the inducing of morphological changes. Bone (2013) suggested that soybean meal induced pathological changes in fish may be due to additive or synergistic impacts of several anti-nutritional factors. Saponin levels in soybean meal range between 5–7 g kg^{-1} (Knudsen *et al.*, 2006), but are very low or absent in other plant ingredients and thus insufficient to induce pathological changes in the intestine (Gatlin *et al.*, 2007). This is in line with Madalla (2008) that histopathological intestine were unaffected by morning leaf meal, cassava leaf meal and cassava root meal and Aanyu *et al.* (2014) that sunflower cake and cotton seed cake did not change intestinal fold length and numbers. Likewise whole cereal meal had no effect on intestinal morphology in gilthead seabream (*Sparus aurata*) (Couto *et al.*, 2016). Also in Atlantic salmon, intestinal morphology were unaffected by cellulose, native and extruded NSPs (Kraugerud *et al.*, 2007). In contrast, Sitjà-Bobadilla *et al.* (2005) observed hypertrophied intestinal submucosa in gilthead sea bream fed a plant proteins mixtures (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin). This effect on the submucosa in that study was similar to the current impact of soybean meal in Nile tilapia, but no other morphological changes were noted in gilthead sea bream. In line with literature this study confirms that aside from soybean meal, other plant ingredients have minor impact on fish's intestinal morphology.

6.4.3. The correlation between nutrient content, nutrient digestibility and intestinal morphology

In general, dietary nutrient content was unrelated to the intestinal morphology in the proximal and mid intestine, except for energy content. However, in the distal part, dietary nutrient content (protein, lipid and energy) were negatively correlated to number of GC and LP thickness. This might be related to the fact that a high dietary concentration of protein and lipid go together with low dietary carbohydrates levels

(especially NSP). Especially alteration in amounts of NSP in the distal intestine, might affected the intestinal epithelium integrity. Disturbances of the intestinal epithelium increases the mucus flow aim to remove pathogens from the intestine and thus preventing pathogens translocation via the damaged intestinal epithelium (van der Marel *et al.*, 2014). Facilitation of excretion of undigested material from the gut is another function of mucus (Sklan *et al.*, 2004b). The later may explain the current observation of a positive correlation between goblet cells number in the distal intestine and dietary NSP content.

In this study, nutrient digestibility was positively related to SM thickness in the proximal and mid intestine. A thick SM in proximal and mid intestine coincided with a better protein, lipid and energy digestibility. Also gilthead sea bream, a plant protein mixture increased SM thickness without impairment of feed conversion (Sitjà-Bobadilla *et al.*, 2005). Maybe a thicker SM increased the surface area for absorption. Furthermore, in the proximal intestine, the majority of protein/peptide are absorbed into the epithelium by pinocytosis and enclosed inside the cytoplasmatic vacuoles where they are further hydrolysed (Gargiulo *et al.*, 1998). This might also explain the correlation between nutrient digestibility and SM thickness in the proximal intestine. If this assumption is true, care should be taken in interpreting changes in the intestinal morphology.

Non-starch polysaccharides (NSPs) hamper digestion in fish (Sinha *et al.*, 2011). Enzymes such as ß-glucanase or ß-xylanases that digest NSPs are scarce or even non-existent in fish (Kuz'mina, 1996). Therefore, dietary NSPs remain indigestible and cannot be used as energy source. In Nile tilapia, a reduction in nutrient digestibility was associated with an increased digestion viscosity (Leenhouwers *et al.*, 2007a). The delay of digestion passage in the intestinal tract, as a result of increase in viscosity, may stimulate microbial fermentation of NSPs in the intestine with the production of volatile fatty acids (VFA) as an end product (Sinha *et al.*, 2011). Administration of NSPs in the diet of tilapia and African catfish (*Clarias gariepinus*) increase VFA levels in the intestinal tract such as those of acetic acid, propionic and butyric acid (Amirkolaie *et al.*, 2006, Leenhouwers *et al.*, 2007a, Leenhouwers *et al.*, 2007b). Scheppach (1994) showed that in domestic animal, VFAs stimulated the colonic sodium and fluid absorption and exerted a proliferative effect on the colonocytes. In fish, the beneficial effects of VFA resulted in strong antimicrobial activity, growth promotion (Elala and Ragaa, 2015, Koh *et al.*, 2016, Ng *et al.*, 2009, Zhou *et al.*, 2009) or improvement in villi development in the GI tract, leading to a better nutrient utilization (Robles *et al.*, 2013). Our study showed a positive correlation between NSP content and the widening of the lamina propria and the increase of the number of goblet cells. In addition, the better NSP were digested, the stronger were the alterations in intestinal morphology. These outcomes are opposite to the ones we would expect. At this moment, a plausible explanation is lacking.

6.4.4. Conclusion

This study demonstrates that feed ingredients have impact on the alteration in intestinal morphology parameters but also on the nutrient digestibility and the nitrogen/energy balance. Soybean meal caused the most obvious alteration in intestinal morphology although it was well digested. The digestibility of protein was highest in soybean meal followed by sunflower meal, DDGS, rapeseed meal, hydrolysed feather meal, and rice bran and in decreasing order. The lowest protein retention efficiency was found in fish fed the hydrolysed feather meal and the highest in rice bran. Hydrolysed feather meal and soybean meal resulted

in the highest predicted maintenance energy requirement. The alterations in intestinal morphology was not related to the nutrient digestibility nor to nitrogen/energy balance parameters. However, NSP digestibility was an exception, which was positively correlated with the intestinal morphology. The higher dietary NSP concentration was also related with the intestinal morphology.

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Chapter 7

Energy efficiency of digestible protein, fat and carbohydrates utilization for growth in rainbow trout and Nile tilapia

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Abstract

Currently, energy evaluation of fish feeds is done on digestible energy basis. In contrast to net energy (NE) evaluation systems, digestible energy evaluation systems do not differentiate between the different types of digested nutrients regarding their potential for growth. We aimed: 1) to develop a NE evaluation approach for fish by estimating the energy efficiency of digestible nutrients (protein, fat and carbohydrates); and 2) to assess if these efficiencies differ between Nile tilapia and versus rainbow trout. Two data sets were constructed. The Nile tilapia and rainbow trout data set contained respectively, 8 and 9 experiments in which respect for 23 and 45 different diets the digestibility of protein, fat and energy and the complete energy balances were measured. From these data the digestible protein (dCP), digestible fat (dFat) and digestible carbohydrate intake (dCarb) were calculated (expressed in $g/kg^{0.8}/d$). By multiple regression analysis, the retained energy (RE, in $kJ/kg^{0.8}/d$) was related to dCP, dFat and dCarb. In Nile tilapia, all digestible nutrients were linearly related to RE ($P<0.001$), giving the following NE formula: $NE = 11.5 \times dCP + 35.8 \times dFAT + 11.3 \times dCarb$. In rainbow trout RE was quadratically related to dCarb ($P<0.01$) and linearly to dCP and dFat ($P<0.001$), giving the following NE formula: $NE = 13.7 \times dCP + 33.2 \times dFAT + 34.2 \times dCarb - 3.78 \times d(Carb)^2$ (NE in $kJ/kg^{0.8}/d$; dCP, dFat and dCarb in g/kg0.8/d). In Nile tilapia the energy efficiency of dCP, dFat and dCarb were 49, 91 and 64% respectively, showing large similarity with pigs. Nile tilapia and trout had quit similar energy efficiencies of dCP and dFat, but differed regarding dCarb. In trout, the increase in NE value levels off with increasing dCarb intake, which indicates the limited capacity to handle starch/glucose by Rainbow trout.

7.1 Introduction

Various energy evaluation systems have been developed and used for animals and man. Systems with a net energy (NE) approach account for differences in utilization efficiencies of macronutrients. The impact of diet composition on the utilization efficiency of digestible (DE) and/or metabolizable energy (ME) is shown in man (see review (Elia and Cummings, 2007)), pigs (Labussière et al., 2011; Noblet et al., 1994; van Milgen et al., 2001) and various fish species (Bureau et al., 2003; Carter and Brafield, 1991; Heinsbroek et al., 2007; Lupatsch et al., 2003a; Pfeffer et al., 1999; Schrama et al., 2012). In net metabolizable energy systems for humans, the utilization efficiency for ATP production of fat and protein are respectively, 98% and 80% relative to the efficiency of glucose (Elia and Cummings, 2007). Most NE systems for animals were developed for growing and lactating animals (Just, 1982; Lofgreen and Garrett, 1968; Noblet et al., 1994). Consequently, in such NE systems for animals, the utilization efficiency of nutrients is a combination of the utilization efficiency for ATP production and the type of energy retained (protein or fat). In the Dutch NE system for growing pigs, the utilization efficiency for growth is 0.46, 0.92 and 0.77 for respectively, digestible protein, digestible fat and ileal digestible starch (CVB, 1993).

Energy evaluation for growing fish is predominantly done on DE basis (Bureau et al., 2003; NRC, 2011), thus assuming the utilization efficiency of DE for energy retention (kg_{DE}) being independent of dietary nutrient composition. Energy utilization efficiencies of digestible macronutrients, like those used in NE evaluation systems for pigs (*CVB*, 1993; Noblet et al., 1994) are not available for fish. Literature is inconsistent regarding fish-species differences in energy utilization efficiency. In an across fish species comparison, kg_{DE} was positively related to the trophic level, but could also be explained by difference in dietary proximate composition (Schrama et al., 2012). However, the energy efficiency of protein deposition was 9% higher in Atlantic salmon compared to Rainbow trout when fed the same experimental diets (Azevedo et al., 2005). Moreover, several fish species are believed to have a limited metabolic capacity for the utilization of dietary carbohydrates (Hemre et al., 2002; Kaushik, 1999; Moon, 2001). This would imply lower energy efficiencies of digestible carbohydrates in these fish species, like diabetes in human can affect food energetic values (Elia and Cummings, 2007).

The study objectives were: 1) to estimate the energy efficiency of digestible nutrients (protein, fat and carbohydrates) in fish; and 2) to assess if these efficiencies differ between fish species: Nile tilapia (a glucose tolerant fish) versus rainbow trout (a glucose "intolerant" fish). This was done by reexamining data of nine studies undertaken with rainbow trout (de Francesco et al., 2004; Dias, 1999; Martin et al., 2003; Panserat et al., 2000; Parisi et al., 2003; Richard, 2006; Vilhelmsson et al., 2004)(Geurden, unpublished data)and eight studies in Nile tilapia (Bone K, 2008; Duyster, 2004; Kallau M, 2009; Ramli N M, 2008; S. Saravanan et al., 2012; Schneider et al., 2004; Schrama et al., 2012; Tran-Duy et al., 2008).

7.2 Materials and methods

Experiments used for this study needed to have sufficient data to calculate energy retention (ER) and digestible macronutrient intake (crude protein, fat and total carbohydrates). Specific details on experiments included are given in Tables 7.1-7.4. Tilapia experiments were done at the experimental fish facilities (De Haar Vissen) of Wageningen University and all procedures involving fish were carried out according to the Dutch law on experimental animals and approved by the Wageningen University Animal Experimental Committee. The rainbow trout experiments were performed at the fish facilities of INRA (Donzacq or St Pée sur Nivelle), following the Guidelines of the National Legislation on Animal Care of the French Ministry of Research.

7.2.1. Fish, experimental unit and housing

All Nile tilapia (*Oreochromis niloticus*) experiments were done with male fish of the Swansea Silver GMT (Genetically Male Tilapia) strain. Initial body weight ranged between studies from 41 to 138 g (Table 7.1).

TABLE 7.1 | General aspects of the design and performance data of experiments included in the estimation of the net energy value equation for Nile tilapia (*Oreochromis niloticus*).

Experiment [a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Length experiment (d)	42	42	42	50	36	48	56	56
No. of diets	2	2	4	6	1	4	2	2
Feeding method [b]	R/1M[e]	R/2M	S/2M	R/4M	R/1M	S/2M	R/24B	R/24B
No. of tanks	12	4	16	12	6	12	3	3
No. of fish per tank	34	30	20	40	25	20	30	30
No. RAS used [c]	1	4	1	1	1	1	3	3
Temperature (°C)	27.7	27.9	27.9	26.9	27.7	27.7	28.1	28.3
Faeces collection method [d]	ST	ST	CC	ST	ST	ST	ST	ST
Initial body weight (g)	75	94	52	56	138	41	86	77
Feed intake (g DM/kg$^{0.8}$ BW per d)	7.6	10	16.3	12	11.3	23.2	10.7	10.7
Growth (g/kg$^{0.8}$ BW per d)	8.6	8.5	16.4	12.8	11	25.7	9.3	10.7

[a]Experiment number refers to the following sources: 1, Schrama et al. (2012); 2, Duyster (2004); 3, Tran-Duy et al. (2008); 4, Schneider et al. (2004); 5, Kallau (2009); 6, Saravanan et al. (2011); 7, Ramli (2008); 8, Bone (2008).

[b]Regarding feeding method abbreviation: S = feeding to apparent satiation; R= restrictive feeding; M with the preceding number is the number of meals fed per day; B with the preceding number is the number of hours using belt feeding.

[c]If number of RAS systems used in an experiment is 1, all tanks used are connected to the same system. If larger than 1 then each fish tank (experimental unit) was connected to a separate RAS system.

[d]Faeces collection method, ST is settling tanks and CC is Choubert collectors.

[e]In the study of Schrama et al. (2012) the two diets were fed at two levels: one close to maintenance and one at about 70% of the .maximal feeding level.

Tilapia were kept in groups of 20 to 40 fish per tank at a water temperature of 26.9 to 28.3°C. Tilapia tanks were connected to a recirculating system for water purification (gas exchange, solid removal and NH_4^+ removal by nitrification). All Rainbow trout (*Oncorhynchus mykiss*) experiments were done with diploid fish of mixed sex, having an initial body weight ranging between studies for 14 to 421 g (Table 7.2). These trout were reared at the experimental fish farm of the Institut National de la Recherche Agronomique in

Lées-Athas or in Donzacq (France). For trout, group size ranged from 45 to 100 fish per tank, water quality was maintained by flow-through and water temperature was constant within each experiment being either 8 or 18°C. For the current study, tank (i.e. group of fish) was used as experimental unit: 68 tilapia and 156 trout tanks.

TABLE 7.2 | General aspects of the design and performance data of experiments included in the estimation of the net energy value equation for rainbow trout (*Oncorhynchus mykiss*).

Experiment [a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Length experiment (d)	83	84	70	70	56	64	81	78	85
No. of diets	6	4	4	4	3	12	4	4	4
Feeding method [b]	S/2M	S/2M	S/2M	S/2M	S/2M	S/2M	S/2M	S/2M	S/2M
Temperature (°C)	18	18	19	8	17	17	18	18	18
No. of tanks	18	12	12	12	26	36	12	16	12
No. of fish per tank	100	100	55	55	45	55	75	75	50
Faeces collection method [c]	CC	CC	CC	CC	CC	CC	CC	CC	CC
Initial body weight (g)	73	75	76	86	268	53	14	19	421
Feed intake (g DM/kg$^{0.8}$ BW per d)	14.6	14.3	15.2	7.8	10.1	12.6	15.1	12.6	7.9
Growth (g/kg$^{0.8}$ BW per d)	12.2	11.6	11.5	5.4	11.7	12.2	15.3	16	8.9

[a]Experiment number refers to the following sources: 1, Dias (1999); 2, Dias (1999); 3, Panserat et al. (2000); 4, Panserat et al. (2000); 5, Geurden (unpublished data); 6, Geurden (unpublished data); 7, Martin et al. (2003); 8, different aspects of this experiment have been published by Vilhelmsson, et al. (2004) & de Francesco et al. (2004) & Parisi et al. (2003); 9, Richard (2006).

[b]Regarding feeding method abbreviation: S = feeding to apparent satiation; M with the preceding number is the number of meals fed per day.

[c]Faeces collection method CC is Choubert collectors.

7.2.2. Diets and feeding

In total 23 and 45 diets were used in the tilapia and trout experiments, respectively. A large range of ingredients were included in the diets (Table 7.3), being related to the specific aims of the various experiments. Consequently, also a large between diet variability in macronutrient composition was present (Table 7.4). Except for the large variability in macronutrients, all diets were formulated to be balanced for each fish species regarding vitamin, mineral and essential fatty acid content and amino acid profiles. In all trout and two of the tilapia experiments, fish were fed twice daily to apparent satiation. In the other seven tilapia experiments, fish were fed restrictively (Table 7.1 and 7.2). Feed intake on dry matter (DM) basis, ranged between experiments from 7.6 and 23.2 g/kg$^{0.8}$/d and from 7.8 to 15.2 g/kg$^{0.8}$/d, for respectively tilapia and trout.

7.2.3. Measurements

In tilapia, digestibility of nutrients and balances of energy and nitrogen were measured on the same fish (tanks), whereas digestibility of diets for trout was done in other fish than balance measurements. For digestibility measurements in tilapia and trout, respectively, acid-insoluble ash and chromium oxide were used as inert markers both being supplemented to the diets (Table 7.3). In seven of the tilapia experiments, faeces were collected by settling tanks and in all trout and 1 tilapia experiment by Choubert collectors (Table 7.1 and 7.2). For details on collections procedure by settling tank see Amirkolaie et al. (2006) and

by Choubert collectors see Choubert et al. (1982) for trout and Schneider et al. (2004) for tilapia. Faeces were daily stored at -20°C and pooled per tank. Both tilapia and trout faeces were freeze dried prior to analysis. Tilapia diets and faeces were analyzed for DM, crude protein (CP), fat, ash, acid-insoluble ash and energy content as described by Schrama et al. (2012). For tilapia, total carbohydrate (Carb) content of diet and faeces was calculated as DM minus CP minus fat minus ash. Trout diets and faeces were analyzed for DM, CP, fat, chromium oxide and energy content as described by Dias (1999). For trout ash was not analyzed in faeces. Consequently, Carb content was calculated from the measured energy content, CP and fat content, using 23.7, 39.5 and 17.6 kJ/g as the combustible energy content of CP, fat and carbohydrates, respectively. Analytical methods and procedures were identical between experiments within species. From daily feed intake, dietary macronutrient composition and apparent digestibility coefficients of nutrients, the daily intake of digestible protein (dCP), digestible fat (dFat) and digestible total carbohydrates (dCarb) was calculated.

TABLE 7.3 | Inclusion levels of ingredients in the diets (n=23) of the Nile tilapia and in the diets (n=45) of the rainbow trout experiments included in the dataset to estimate the net energy equation.

Ingredient	Nile tilapia			Rainbow trout		
	No. of diets	Mean level (%)	Maximum level (%)	No. of diets	Mean level (%)	Maximum level (%)
Fish meal	22	31.2	50	45	44.5	80.3
Fish oil	14	3.8	12.5	42	11.1	22.7
Wheat	9	21	43	15	15.3	35
Wheat gluten	10	12.6	22	6	11.2	20
Wheat bran	8	8.6	9.5	4	10.5	12
Maize	10	25.2	27.6	---	---	---
Maize gluten	--	---	---	14	14.9	42
Maize starch gelatinized	4	24.5	49.3	20	24.2	37.7
Maize starch native	--	---	---	2	23.5	28
Maize flour gelatinized	4	23.8	40	--	---	---
Soybean meal	12	21.4	30.3	14	13.3	33.1
Soy protein concentrate	6	13.1	30	2	45	45
Soy oil	7	3.2	7.6	--	---	---
Rapeseed mea	--	---	---	3	10.5	12
Rapeseed oil	4	7.6	14.1	1	22.7	22.7
Palm oil	4	4.8	7.6	--	---	---
Linseed oil	--	---	---	1	22.7	22.7
Olive oil	--	---	---	1	22.7	22.7
Pea protein concentrate	5	9.7	15	--	---	---
Extruded peas (dehulled)	--	---	---	15	22.2	36.4
Single cell protein	1	15	15	--	---	---
Cellulose	6	12.8	17.5	3	20	20
Zeolite	--	---	---	3	20	20
Guar gum	1	8	8	--	---	---
Pellet binder	10	1.2	2	39	1.5	5
Diamol (inert marker)	23	2	2.4	---	---	---
Synthetic amino acids	6	0.6	2.4	9	3.4	7.6
Minerals, vitamins and trace elements	23	2.2	6.1	46	2.7	6

TABLE 7.4 | Chemical characteristics and digestible nutrient contents of the diets (n=23) in the Nile tilapia and of the diets (n=45) in the rainbow trout experiments included in the dataset to estimate the net energy equation.

Item	Nile tilapia				Rainbow trout			
	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
Chemical composition, g/kg of DM								
Ash	98	18	73	134	---	---	---	---
Crude protein (CP)	415	74	295	541	409	92	275	574
Crude fat	109	51	36	232	186	56	75	278
Carbohydrates	378	94	197	558	304	93	94	516
Gross energy (GE), kJ/g of DM	20.5	1.4	18.6	23.2	22.4	1.9	15.7	25.5
CP/GE ratio, mg/kJ	20.3	3.2	12.8	26	18.3	4	11.6	28
Apparent digestibility coefficients, %								
Ash	46.9	6.3	38.2	62.6	---	---	---	---
Crude protein	90.9	3.4	83.3	95.7	91.2	2.1	86.5	94.9
Fat	93.2	5.5	78.9	98.6	90	8.71	66.2	96
Carbohydrates	68.1	17.3	12.2	92.9	60.8	17	33.1	90.9
Energy	84.8	5.7	69.3	94	83.2	7.61	66.5	93.6
Digestible nutrients, g/kg of DM								
Ash	46	11	29	67	---	---	---	---
Crude protein (DP)	379	76	272	515	374	89	240	533
Crude fat	101	44	30	209	165	46	68	249
Carbohydrates	264	100	27	460	182	65	59	325
Digestible energy (DE), kJ/g of DM	17.3	1.6	13.5	20.6	18.6	2.1	14	22.4
DP/DE ratio, mg/kJ	21.8	3.4	14.3	27.4	19.9	3.3	13.2	27.8

For both fish species, nitrogen (N) and energy balances were measured by the comparative carcass analyses technique. For number of fish sampled for initial and final body composition see Supplemental Table 1 and 2. DM, CP, fat and energy content of fish were determined as described by (Schrama et al., 2012) (for tilapia) and by (Dias, 1999) (for trout). Parameters of energy and N balance were calculated per tank as follows: N intake as feed intake times dietary N content; digestible N intake as N intake times N digestibility; N retention as final N body mass minus initial N body mass; N losses through branchia and urine as digestible N intake minus N retention; gross energy (GE) intake as feed intake times dietary energy content; DE intake as GE intake times energy digestibility; branchial urinary energy losses as N losses through branchia and in urine times the energy concentration of NH_3-N (24.9 kJ/g (Bureau et al., 2003), assuming all N being excreted as NH_3-N); ME intake as DE intake minus branchial urinary energy losses; RE as final minus initial body energy quantities; heat production as ME minus RE; energy retained as protein was calculated as N retention times 6.25 times 23.7 kJ/g; energy retained as fat was calculated as RE minus energy retained as protein. Additionally, fat retention efficiency was calculated as an indication for the extent of *de novo* synthesis of fat as follows; fat retained divided by dFat intake.

In order to account for differences in body weight between fish species and experiments, digestible nutrient intakes as well as N and energy balance parameters were expressed per unit of metabolic body weight; being calculated as geometric mean body weight (Wg) = ($\sqrt{Wi \times Wf}$), then expressed on mean metabolic body weight ($kg^{0.8}$) as $(Wg/1000)^{0.8}$ where Wi and Wf is the initial and final fish body weight in gram.

7.2.4. Statistical analysis

All statistics were done with Statistical Analysis Systems statistical software package version 9.1 (SAS Institute, Cary, NC, USA). Energetic efficiencies of digestible macronutrients were estimated separately for both species by multiple regression analysis of dCP, dFat and dCarb (in g/kg$^{0.8}$/d) on RE (in kJ/ kg$^{0.8}$/d):

$$RE_j = \mu + ß1 \times dCP_j + ß2 \times dFat_j + ß3 \times dCarb_j + e_j \quad (1)$$

Where μ is the intercept being a measure of energy mobilization at fasting (i.e., fasting heat production, FHP); ß1, ß2 and ß3 are respectively the energetic efficiency of dCP ($k_{NE;dCP}$) , dFat ($k_{NE;dFat}$) and dCarb ($k_{NE;dCarb}$) (in kJ/g digested nutrient intake); j= 1,.,n; n is 68 and 156 for tilapia and trout respectively. Using the approach of Noblet et al. (1994), NE was calculated in this paper from equation 1 as RE plus μ (i.e., NE = RE + FHP). Several studies in fish have demonstrated that environmental factors (e.g., water temperature, water oxygen level, stocking density) can strongly affect the DE requirements for maintenance (DE_m) without altering the energetic utilization of DE (or ME) (Glencross, 2009; Lupatsch et al., 2010; Lupatsch and Kissil, 2005; Pirozzi et al., 2010b). In a recent review (Schrama et al., 2012), a substantial between study variability in DE_m within several fish species was noted. Therefore, in order to get unbiased estimation of energetic efficiency, a fixed effect of experiment was included into the statistical model (equation 1). Residual analyses were performed if non included factors in the model (e.g., like initial body weight, body composition factors, ratio of protein to fat deposition) could explain part of the residual variation. Similarly, it was assessed if the relationships of dCP, dFat and dCarb with RE were polynomial. All aforementioned analyses were performed separately for rainbow trout and tilapia. Finally a combined mixed model was run (with linear components of dCP, dFat and dCarb) to test if the estimated energy efficiencies (ß1, ß2 and ß3; i.e., $k_{NE;dCP}$, $k_{NE;dFat}$, $k_{NE;dCarb}$) differed between Nile tilapia and rainbow trout.

7.3 Results

In the data set for estimating the energetic efficiency of digestible nutrients, mean dietary GE content was higher in the feeds for trout than in those for tilapia (22.4 vs. 20.5 kJ/g DM; Table 7.5). This was due to the higher fat content and lower carbohydrate content of trout diets. For both fish species, a substantial between diet variability in macronutrient composition was present. No large differences in mean apparent digestibility coefficient of nutrients over experimental diets were present between both fish species. Mean digestibility of CP and fat over diets was above 90%. Variability between diets regarding nutrient digestibility was comparable between trout and tilapia (Table 7.5). For both fish species, variability in digestibility was larger for carbohydrates compared to CP and fat. Carbohydrate apparent digestibility ranged from 12 to 93% and from 33 to 91% between tilapia diets and trout diets respectively. The higher GE content together with the similar energy digestibility resulted in a higher mean DE content for the trout diets than for the tilapia diets (17.3 vs. 18.6 kJ/g DM; Table 1). Averaged for all tilapia diets (n=23), 51, 23 and 26% of DE originated from respectively digestible CP (dCP), fat (dFat) and carbohydrates (dCarb) and the same for the trout diets (n=45) were 48, 35, and 17% respectively. The variability in digestible nutrient content between diets (table 7.4) within the tilapia as well as trout data sets was larger than the variability in digestible nutrient intake per unit metabolic body weight (table 7.5), due to the differences in feed intake between

experiments being included into the data sets. The coefficient of variance between diets in intake of dCP, dFat and dCarb was respectively, 43, 77 and 58% for tilapia and 29, 28 and 44% for trout.

Despite the fact that fish were fed to satiation in 2 of the 8 tilapia experiments (8 of 23 diets) and in all trout experiments, averaged GE, DE and ME intake over diets were slightly higher for tilapia (Table 7.5).

TABLE 7.5 | Digestible nutrient intake and energy balance of Nile tilapia and rainbow trout fed different diets (n=23 and n=45, respectively) in the experiments included in the dataset to estimate the net energy equation.

Item	Nile tilapia				Rainbow trout			
	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
Digestible nutrient intake, g/kg$^{0.8}$/d								
Ash	0.6	0.11	0.41	0.76	--	--	--	--
Crude protein (DP)	5.2	2.22	3.27	11.74	4.63	1.32	1.85	6.94
Crude fat	1.48	1.14	0.42	5.24	2	0.56	0.95	3.28
Carbohydrates	3.63	2.1	0.63	10.49	2.35	1.04	0.46	4.78
Energy balance parameters, kJ/kg$^{0.8}$/d								
GE intake	287	119	156	582	277	56	179	365
DE intake	242	99	141	468	230	49	120	294
Branchial urinary energy losses	11.8	6	6	30.5	10.9	3.5	4.3	18.7
ME intake	230	95	133	455	220	46	115	279
Heat production	119	27	86	180	101	23	59	137
Energy retention (total)	111	71	45	298	118	28	49	168
Energy retention as protein	53	21	30	96	45	12	15	72
Energy retention as fat	59	52	15	207	74	19	34	115
Fat retention efficiency, g/g	1.07	0.45	0.38	2.05	0.9	0.18	0.56	1.54

Although mean ME intake over diets was higher in tilapia, energy retention (RE) was lower in tilapia compared to trout. This lower RE was predominantly related to a larger part of the energy being retained as fat in trout than in tilapia (63% vs. 53%; Table 2). Averaged over diets, in tilapia a larger proportion of the ME intake was lost as heat compared to trout (52% vs. 46%). For all energy balance parameters, the variability between diets was larger in the tilapia than in the trout data set (Table 7.5). Averaged over diets, fat retention efficiency was 1.07 and 0.90 in the tilapia and trout data set respectively. At 10 of the 23 tilapia diets (4 of the 8 tilapia diets fed until apparent satiation) and at 7of the 45 trout diets, fat retention efficiency was above 1, indicating that *de novo* fat synthesis occurred.

By multiple linear regression of dCP, dFat and dCarb on RE (i.e., NE), the energetic efficiencies of dCP, dFat and dCarb were, respectively, 11.5 (49%), 35.8 (91%) and 11.3 kJ/g (64%) for tilapia and 15.2 (64%), 35.0 (89%) and 12.3 kJ/g (70%) for trout (Table 7.6; Eq 2 and 3). The energetic efficiency of dCP was significantly higher in trout than in tilapia ($P< 0.05$), whereas the energetic efficiency of dFat and dCarb were similar for trout and tilapia ($P> 0.05$). For both fish species, the fixed effect of experiment was significant in the multiple linear regression model ($P<0.001$; Eq. 1 and 2, Table 7.6), indicating that the intercepts (an indicator of FHP) differed between experiments within species. Using the mean dietary dCP, dFat and dCarb content within each species (Table 7.4) and the estimated fixed effect of experiment, the digestible

energy requirements for maintenance (DE_m) per experiment were calculated. Averaged over the 8 tilapia experiments DE_m was 71 kJ/kg$^{0.8}$/d and ranged from 56 to 89 kJ/kg$^{0.8}$/d. For 9 trout experiments, mean DE_m was 67 kJ/kg$^{0.8}$/d and DE_m ranged from 41 to 91 kJ/kg$^{0.8}$/d.

In Nile tilapia, all digestible nutrients were linearly related to RE in the multiple regression model (i.e., for none of the digestible nutrients a polynomial factor was significant; $P>0.05$). However, in trout the quadratic polynomial of dCarb was significant ($P<0.01$), whereas for dCP and dFat only the linear component was significant (Eq 3, Table 7.6). In Figure 7.1 A,B the relationship between dCarb and NE (corrected for zero dCP and dFat) in trout and tilapia is given.

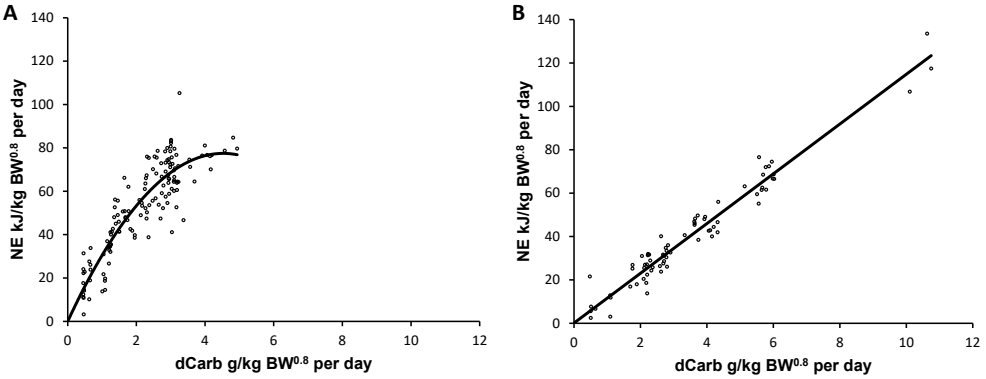

FIGURE 7.1A,B | Relationship between net energy (NE) and digestible carbohydrate (dCarb) intake for Rainbow trout (panel A) and Nile tilapia (panel B). The depicted NE values were corrected for variation in digestible crude protein intake (dCP) and digestible fat intake (dFat). This was done as follows: the measured retained energy value for each data point in the dataset was increased with the estimated fasting heat production to obtain the NE value which was then corrected towards zero dCP and dFat intake in order to have only the effect of dCarb on NE. For these calculation, equation (2) and (3) in Table 7.6 were respectively used for Nile tilapia and rainbow trout.

For tilapia NE increased linearly with dCarb (Figure 7.1B). In trout, NE also increased with increasing dCarb, but the increase in NE leveled off between an intake of dCarb of 3 to 4 g/kg$^{0.8}$/d. The linear relationships between NE and dCP and between NE and dFat in both fish species is given in figure 7.2.

TABLE 7.6 | Estimated net energy equation in Nile tilapia and rainbow trout in comparison to net energy formulas in pigs[1]

Source	Species	Equation[2]	Eq. number	R^2
Current study	Tilapia	NE = RE + 44 (±7) = 11.5 (±0.82) × dCP + 35.8 (±1.18) × dFat + 11.3 (±0.63) × dCarb	(2)	0.99
Current study	Trout	NE = RE + 50 (±9) = 15.2 (±1.18) × dCP + 35.0 (±2.00) × dFat + 12.3 (±2.00) × dCarb	(3)	0.91
Current study	Trout	NE = RE + 64 (±10) = 13.7 (±1.27) × dCP + 33.2 (±2.06) × dFat + 34.2 (±8.23) × dCarb - 3.78 (±1.38) × $(dCarb)^2$	(4)	0.92
Noblet et al. (1994)	Pigs	NE = 11.3 × dCP + 35.0 × dFat + 14.4 × ST + 12.1 × dRest	(5)	---
CVB, 1993	Pigs	NE = 10.8 × dCP + 36.1 × dFat + 13.5 × dSTe + 9.5 × dSTf + 9.5× dNSP	(6)	---

[1] In the estimated equation of the current study, net energy (NE) is expressed in kJ/(kg0.8·d) and digestible nutrient intakes (dCP, dFat and dCarb) in g/(kg0.8·d). Using the approach of Noblet et al. (2), NE in the current study was calculated as retained energy plus the fasting heat production (being the intercept, μ, from Eq. (1)). In the NE formulas for pigs, NE is expressed in MJ/kg feed and digestible nutrients in g/kg feed. dCarb, digestible carbohydrates (comprising of starch, sugars and non-starch polysaccharides); dCP, digestible protein; dFat, digestible fat; dNSP, digestible non-starch polysaccharides; dRest, the remaining dietary fraction being digestible dry matter minus dCP minus dFat minus ST and minus the digestible ash fraction (see Noblet et al. (1994); dSTe, enzymatically digestible starch; dSTf, the amount of starch that is digested after microbial fermentation; NE, net energy; RE, retained energy; ST, starch (both enzymatically and fermentable degradable).

[2] Values between brackets represent SEE.

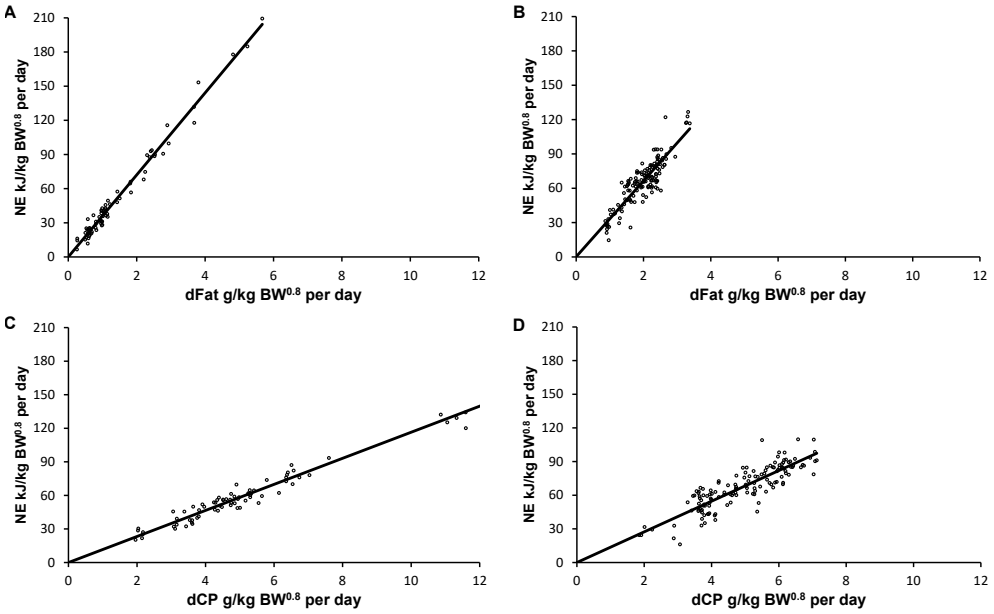

FIGURE 7.2A-D | Relationship between dFat and NE (A, Nile tilapia; B, Rainbow trout) and between dCP and NE (C, Nile tilapia; D, Rainbow trout). The NE values were corrected for variation in other digestible nutrient similarly as was done in Figure 1 for the relation between NE and dCarb. dCarb, digestible carbohydrate intake; dCP, digestible protein intake; dFat, digestible fat intake; NE, net energy NE.

7.4 Discussion

In aquaculture until now energy evaluation and feed formulation of feed are based on the digestible energy (DE) content of diets. This DE approach does not take into account the fact that utilization efficiency for energy retention might be affected by the nutrient composition of DE (i.e., the ratio between digestible protein, fat and carbohydrates; respectively dCP, dFAT and dCarb). In the Dutch (*CVB*, 1993) and French (Noblet et al., 1994) net energy evaluation (NE) system for pigs, the impact of the nutrient composition of DE is accounted for by relating/predicting the energy retention as multiple linear function of the different types of digestible nutrients intake (see table 7.6). In these NE approaches for pigs, linear relationships enable to estimate the NE value of a diet/ingredient independent of the feeding level (i.e., digestible nutrient intake). The current study demonstrated that for Nile tilapia all digestible nutrients were linearly affecting the energy retention, resulting in a NE formula being independent upon feeding level. However in trout this is not the case. The impact of dCarb on energy retention was curvilinear (Figure 7.1A), which has the implication that the NE value of a diet (ingredient) is dependent on the feed intake (i.e., dCarb intake) of the trout.

The curvilinear relation of dCarb with energy retention indicates that at higher intakes of carbohydrates the potential for energy retention (i.e., NE value) diminishes, which confirms the general consensus that trout (and more general carnivorous fish species) poorly metabolizes glucose (Glencross et al., 2017). In fish, the

absorption of glucose from the intestine originating from digested starch is highly efficient (Furuichi and Yone, 1981). Furthermore, increasing intakes of digestible carbohydrates result in elevated blood glucose levels in almost all species (Bergot, 1979; Furuichi and Yone, 1981). Coldwater fish like the rainbow trout exhibit a postprandial hyperglycemia and the clearance of the glucose from blood stream takes more time when compared to warm water fish (NRC, 2011). Excretion of glucose in the urine and through the gills has been observed in hyperglycemic fish (Bureau, 1997; Hemre et al., 2002) Due to this partial post-prandial excretion of glucose, (Bureau, 1997) stated that in rainbow trout the digestible starch reduced metabolizable energy value. In addition, it was initially hypothesized that limited glucose phosphorylation capacity would also limit metabolic utilization of glucose (Walton and Cowey, 1982) But, later studies have shown that almost all teleosts are capable of regulating glucose storage but there is a persistent high level of endogenous glucose production independent of carbohydrate intake level which may lead to a putative competition between exogenous (dietary) glucose and endogenous glucose as the source of energy, which may explain the poor dietary carbohydrate utilization in fish. Both aforementioned observations most likely explain the curvilinear relationship that was observed for trout in our study. Based on the curve (see figure 7.1A), it seems that at dietary dCarb intake of about 3-3.5 g $kg^{-0.8}$ d^{-1} trout cannot utilize any extra amount of digestible carbohydrates. In contrast, tilapia did not show any limit to utilize the digestible carbohydrates. It requires future assessment if this curvilinearity is representative for all salmonids/carnivorous fish species or whether this is specific for trout alone.

The efficiency of dCarb for energy retention ($k_{NE;dCarb}$) in Nile tilapia (66%) was lower than the efficiency for digested starch ($k_{NE;dStach}$) in pigs either using the French NE system (84%; Noblet et al., 1994) or the Dutch NE system (79%; (*CVB*, 1993)) (Table 7.6). The lower $k_{NE;dCarb}$ in tilapia compared to pigs might be due to an overestimation of measured digestibility coefficients of nutrients in fish due to issues of leaching of nutrients from both feed and faeces into the water (Hua and Bureau, 2009). This aspect of leaching might also have affected the energy efficiencies of the other nutrients (protein, $k_{NE;dCP}$; fat, $k_{NE;dFat}$). Most likely, the observed lower $k_{NE;dCarb}$ in tilapia may relate to the fact that in the current study no distinction was made between enzymatic digested carbohydrates versus fermentable carbohydrates (NSP). In pigs the energetic efficiency in the NE formulas are about 70% lower for carbohydrates that are fermented in comparison to the energetic efficiency of illeal digested starch ($k_{NE;dstarch}$; (*CVB*, 1993; Noblet et al., 1994) ; Table 7.6). Most likely also in fish the energetic efficiency of "digested"/fermented NSP is lower than $k_{NE;dStarch}$. However in most fish species, NSP is considered inert and the extent of microbial fermentation is marginal. However, this is most likely related to the water temperature (warm versus cold water fish species). In Nile tilapia, it was demonstrated that NSP fermentation is present based on positive digestibility coefficients for NSP and the increase in volatile fatty acids in the distal part of the intestine(Amirkolaie et al., 2006, 2005). In addition, in a diet rich in NSP originating from DDGS, about 17% of the digestible energy originated from fermented/digested NSP (Haidar et al., 2016). In data set on Nile tilapia used in the current study, on average 26% of the DE intake was coming from digested carbohydrates. Most likely also part of this carbohydrate related DE originated from NSP. Therefore, the estimated NE formula for tilapia might improve when the carbohydrate fraction is split into enzymatically digested and fermented components. This becomes more relevant when more NSP rich ingredients are included into fish diets. However, the lower $k_{NE;dCarb}$ might also be a reflection of a lower capacity of all fish species to metabolize glucose as is documented for salmonids (Polakof and Panserat,

2016). However, the data of the current study do not support such a glucose intolerance of tilapia, because the relationship between dCarb and retained energy remained linear over a wide range of dCarb intakes (Figure 7.1B). At the diets with highest levels of dCarb intake the fat retention efficiency was around 200% (Table 7.5), indicating a large *de novo* fat synthesis capacity in tilapia (Dias, 1999).

The energetic efficiency of dCP for energy retention ($k_{NE;dCP}$) of Nile tilapia estimated in this study is similar to the $k_{NE;dCP}$ used in NE formulas for pigs (Table 7.6). However the estimated $k_{NE;dCP}$ for Rainbow trout was dependent on how the dCarb fraction was included into to formula (linear versus quadratic; see Table 7.6). Using a linear relationship for dCarb, the $k_{NE;dCP}$ was significantly higher in trout compared to tilapia and thus also pigs. When including the quadratic component of dCarb, the estimated $k_{NE;dCP}$ was not (or less) different from tilapia and pigs. Ignoring the quadratic component for dCarb in trout thus introduces an artifact in the estimation of $k_{NE;dCP}$ which is related to the poor utilization of glucose by trout. In diets with a low a dCP content often coincide with a high dCarb content while at high dCP levels the reverse occurs. In the NE formula for trout where only linear components are used, the estimated $k_{NE;dCarb}$ was different from that of tilapia, this indicates that in such a "linear' NE formula for trout the energetic efficiency of digested protein ($k_{NE;dCP}$) is overestimated. This observation of biased estimations of the energetic efficiency of dCP might imply that in other fish species with limited ability to metabolize glucose, the estimates of energetic efficiency for protein might also be influenced. E.g., the higher energetic efficiency of DE (or ME) for protein deposition in salmon compared to trout might also related to differences in glucose tolerance (Azevedo et al., 2005).

The estimated $k_{NE;dCP}$ in tilapia and in trout (including the quadratic component of dCarb; equation 3, Table 7.6) is very much comparable to the values used for pigs (*CVB*, 1993; Noblet et al., 1994). This is opposite to the initial premise that terrestrial animals (like pigs) would have a lower $k_{NE;dCP}$ because of the differences in nitrogen excretion compared to fish; urea synthesis versus direct excretion of NH_4^+ via the gills (Walton and Cowey, 1982, 1977). We currently lack a good explanation for this observation. Diets with an imbalanced amino acid composition are known to have a reduced protein efficiency (e.g.,(Saravanan et al., 2013)). However, one could speculate that in all diets included in the dataset for tilapia as well as for trout imbalances were present in amino acid profile. But to our good knowledge, all studies included into the datasets were having a balanced amino acid profile according to (NRC, 2011). Applying the NE approach to other fish species would be interesting to validate if the observed equal $k_{NE;dCP}$ compared to pigs is universal for fish.

The energetic efficiencies of dFat ($k_{NE;dFat}$) estimated in the current study (table 7.6) indicate that both carnivorous (Rainbow trout) and omnivores (Nile tilapia) fish species can utilize dietary fat as efficient as terrestrial farm animals (ranging from 84 to 91%). This is again striking considering the large differences in trophic level between pigs, trout and tilapia. The observation in the current study for relatively constant utilization efficiencies for both digested protein and digested fat ($k_{NE;dCP}$; $k_{NE;dFat}$) confirms the hypothesis made that difference in energetic efficiency for growth in relation to trophic level is induced by difference in nutrient composition of the test diets (Schrama et al., 2012), which is also in line with the recent finding in Asian seabass (Glencross et al., 2017).

Up till now, for most fish species energy evaluation is based on a DE approach. In the DE approach of energy evaluation, it is assumed that the relationship between RE and DE intake is independent of diet composition. In other words, the efficiency of DE utilization for RE (kg_{DE}) is not altered by nutrient composition of DE (the

source of DE). The current estimated NE formulas for tilapia and trout show that fat is more efficiently used for NE in comparison to protein and carbohydrates. In fact the kg_{DE} of a diet can be calculated from the estimated NE formulas in combination to the dietary digestible nutrient composition/intake (i.e., for tilapia being a function of $k_{NE;dCP}$ $k_{NE;dFat}$ and $k_{NE;dCarb}$). In Figure 7.3A, the impact of digestible nutrient composition on kg_{DE} is depicted for Nile tilapia using the estimated NE formula (2) from table 7.6.

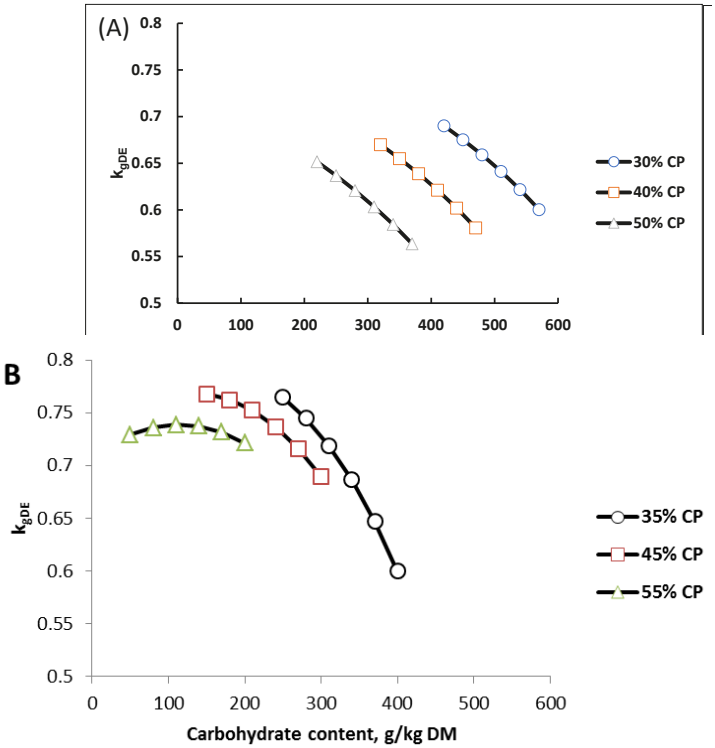

Figure 7.3 A,B | Impact of dietary composition on k_{gDE} derived from NE Eq. 2 for tilapia (A) and NE Eq. 4 for trout (B). NE equations are given in table 7.6. The exchange of fat by carbohydrates on weight basis in the diets is depicted for 3 dietary protein levels. In the calculations all diets contained 10% crude ash and the digestibility of protein, fat and carbohydrates was fixed at respectively 91, 93 and 67.5% for Nile tilapia and 91, 90 and 70% for rainbow trout. The NE equation for trout is dependent on the applied feeding level due to the fact that the quadratic component for digestible carbohydrates in the NE Eq. 4. The estimations of kg_{DE} for trout were done by calculating the increase in retained energy when the feed intake was increased from 13 to 13.1 g/($kg^{0.8}$·d). DM, dry matter; CP, crude protein kg_{DE}, utilization efficiency of digestible energy for energy retention; NE, net energy.

Because dCP, dFAT and dCarb are all linearly related to NE, the calculated kg_{DE} is independent upon the feeding level. The calculations demonstrate that increasing the digestible carbohydrate but also the digestible protein content results in a decline in kg_{DE} (figure 7.3A) and increasing the fat content increases kg_{DE} (data not shown). These findings are in line with observation of a reduction in kg_{DE} when dietary fat is exchanged by dietary starch (Glencross et al., 2017; Pfeffer et al., 1999; Schrama et al., 2012). In Figure 7.3B, the impact of digestible nutrient composition on kg_{DE} is depicted for Rainbow trout using the estimate NE formula (4) in which dCarb

is included as having a curvilinear response in NE from table 7.6. As a consequence of this quadratic response of dCarb, the NE of a diet and ingredient is dependent on the actual intake of dCarb. In other words the NE value is dependent on the feeding level (feed consumption) of the trout. This also implies that the kg_{DE} in trout is dependent on the feeding level (data not shown). With increasing DE intake, the NE value declines. This is also an explanation for the often observed curvilinear relationship between DE and RE in various fish species (Glencross et al., 2008, 2007; Glencross et al., 2011; Glencross, 2008). Figure 7.3B shows that in contrast to tilapia the impact of changes dietary nutrient composition on kg_{DE} are not linear. The impact of increasing the dietary dCarb content on kg_{DE} is dependent on both dCP and dFat content. At high protein levels the actual dCarb contents of the diets are lower and thus the change in dCarb has a smaller impact on kg_{DE}, whereas at diets with a low protein content the impact of increasing the carbohydrate content (starch content) is larger. The reduction of kg_{DE} due to higher dietary carbohydrates was also observed in a recent study on Asian seabass (*Lates calcarifer*)(Glencross et al., 2017) suggesting that this species also has a limited capacity to utilize dietary carbohydrates. The outcome of the current study for rainbow trout also demonstrates that the NE value of a starch rich ingredient is dependent upon the remaining composition of the diet and the actual inclusion level. The energetic value of starch rich ingredient will decline with the inclusion level more strongly at a low protein diet compared to a high protein diet (Figure 7.1A).

7.5 Conclusion

This study shows that the efficiency with which the digestible energy is used for energy retention is affected by the composition of the digestible energy, i.e., digestible protein (dCP), fat (dFat) and carbohydrates (dCarb). However, this effect of the composition of digestible nutrient intake on the energetic utilization efficiency was different between Nile tilapia and Rainbow trout. For Nile tilapia dCP, dFat and dCarb are linearly related to the energy retention. The estimated energetic efficiency of dCP, dFat and dCarb for net energy retention (NE) were 49, 91 and 64% respectively, showing large similarity with pigs. For trout dCP and dFat were linearly related to NE, but dCarb was not linearly but curvilinearly related to NE. With increasing dCarb intake the increase in NE leveled off, which indicates the limited capacity to handle starch/glucose by Rainbow trout (a carnivorous, glucose intolerant fish). In this study, NE formulas for Nile tilapia and Rainbow trout were derived to predict the potential for energy retention of diets/ingredients. The curvilinear relationship between dCarb and NE in trout implies that the actual NE value of a diet depends on the feeding level.

Acknowledgements

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Chapter 8

General Discussion

8.1 Introduction

For several decades many changes have occurred in the availability, quality and development of ingredients used in fish feeds. Optimal feed formulation is aimed to deliver the nutrients contained in these ingredients with the goal to meet the nutritional and production goals of the farmed species of concern. The cost of feed represents the highest proportion of the total cost of fish production, the energy component being the greatest proportion there in. Therefore, it is important to determine precisely both the energy requirements of the fish and the energetic value of the feeds. In this thesis, a series of studies was designed to critically assess the current digestible energy (DE) evaluation system of Nile tilapia feed. Nile tilapia, as a herbivorous species was chosen due to the high ability to tolerate plant ingredients in their diets. For this evaluation, energy requirements and factors affecting energy utilization were investigated. In this chapter, the main findings of the different studies are presented and discussed.

8.2 Energy utilization and maintenance requirements

A precise and correct evaluation of feed is important to supply the required nutrients for fish. Any feed evaluation system combines the desired requirements of fish with the available nutrients in the feed. In general, for fish and other farm animals there are several energy evaluation systems of animal feeds, e.g., the gross, digestible, metabolizable and net energy systems. The gross energy (GE) system calculates the total energy liberated from feed when burned. Fish however are not able to utilize all that energy and therefore another energy evaluation system is required. To cater for this purpose, the digestible energy (DE) system was introduced which accounts for energy losses that are not digested and absorbed in the gastro intestinal tract and excreted in feces. The next system is the metabolizable energy system which also accounts for energy losses in urine and combustible gases. By determining these energy losses, this system gives a better estimate of dietary energy available for the animal. However, direct determination of the metabolizable energy values for fish diets is difficult due to difficulties in measuring branchial and urinary losses released into water, thus this system is not used in fish. The net energy (NE) evaluation system calculates the energy available from the digestible nutrients and takes into account the utilization efficiencies of these digestible nutrients for energy retention. For pigs, this system has been applied for decades (*CVB*, 1993; Noblet et al., 1994) however in fish this system is still not applied. Historically, the main ingredients included in fish diets were fishmeal and fish oil, thus the variation in energy supplied by these ingredients was minimal. However, new alternative plant ingredients are included in fish diets resulting in higher variability in macronutrient content especially regarding carbohydrates content. Therefore, The NE system was not studied before in fish. Currently, for fish feed formulation, the DE evaluation system is commonly used and therefore is evaluated in this thesis. In fish nutrition, the DE system is based on three main assumptions: 1) the utilization efficiency of DE for growth (kg_{DE}) is constant and not affected by diet composition 2) maintenance energy requirements (DEm) are independent of diet composition 3) the nutrients digestibility coefficients are additive in nature.

In **Chapter 5**, we found a lower energy retention in the fish when the type of dietary carbohydrate (starch vs NSP) changed (Table 5.4). Although the dietary NSP contributed about 17% of the total DE intake for fish fed the NSP diet, the higher inclusion of NSP caused also a lower energetic efficiency of the dietary carbohydrates. In **Chapter 7,** we showed that changing the diet composition affected kg_{DE} and declined with increasing dietary protein and carbohydrate levels and oppositely, increased with increasing dietary fat levels (Fig 7.3A). These changes of kg_{DE} as a result of changing dietary macro-nutrient composition, are in line with results found in rainbow trout and European eel (Heinsbroek et al., 2007; Pfeffer et al., 1999). Further, in **Chapter 5** we also showed that the numerical difference in DEm between the fish fed the NSP and those fed the starch diet contributed to the lower energy retained in the NSP fed group. The DEm requirements as found in this thesis (96.1 and 109.7 on metabolic body weight for starch and NSP diet, respectively) are higher than what is reported in literature (between 53 to 88; (Meyer-Burgdorff et al., 1989; Saravanan et al., 2013; Schrama et al., 2012)) which can be attributed to differences in diet composition between and within the different studies. Saravanan et al. (2013) showed that the dietary electrolyte balance (i.e., dietary minerals composition) increased the maintenance requirements of Nile tilapia. In pigs, Schrama et al. (1998) reported that maintenance requirements altered with increasing dietary fiber content. In the current thesis, evidence was given that changing dietary ingredients altered DEm (**Chapter 6**, Table 6.7).

The DE system assumes that the digestibility coefficients are additive which means that the nutrient digestibility value of individual ingredients when added together equals the overall nutrient digestibility value of the diet. This assumption means that there are no interactions among dietary ingredients that would affect digestibility and that the inclusion level of an ingredient would not change its digestibility (Glencross et al., 2007). In **Chapter 5**, we showed decreased nutrients digestibility values for Nile tilapia when the intake of these nutrients increased (Table 5.3) in both diets, but the difference between both diets increased with the feeding level . In addition, the type of ingested dietary carbohydrate affected the digestibility value of total carbohydrates and other nutrients (Table 5.3). These observations indicate that digestible nutrients coefficients are not additive for Nile tilapia. This finding is in line with the observation that the starch type (raw vs. gelatinized) and the level (high vs low) had an effect on the digestibility coefficient of starch (e.g., Amirkolaie et al., 2006; Kim and Kaushik, 1992).

In comparison, **Chapter 7** shows that NE values for rainbow trout are not additive and only dependent on the absolute amount of digestible carbohydrate ingested, as indicated by the curvilinear relation (Fig 7.1A). This means that the NE value of an ingredient rich in starch (e.g., cassava) will be underestimated when included in rainbow trout diets that already have a high starch content. Therefore, we conclude that assumptions 1, 2 and 3 of the DE system, e.g., that kg_{DE} and DEm is constant and independent of diet composition and that nutrient digestibility coefficients are additive, are not true. In addition, the NE value is independent of the digestible nutrient intake for Nile tilapia, whereas for rainbow trout the NE value is dependent on the absolute amount of digestible energy being consumed. This implies that additivity of NE values of ingredients for trout are not valid.

8.3 Optimal DP/DE ratio

In general, protein is the most expensive nutrient in fish feed especially if it is used as energy source to supply fish with the required energy. Therefore, fish feed formulation is aimed to supply the minimum protein requirements coupled with an appropriate non-protein energy source to spare the use of proteins. Thus, the balance between dietary protein and these non-protein energy sources (i.e., DP/DE ratio) is essential for optimal/maximum growth. Any decrease in this ratio by reducing the dietary DP level at constant or increased DE would improve protein utilization and decrease nitrogenous losses (Cho and Kaushik, 1990). Therefore, the DP/DE ratio is considered an important factor in fish feed formulation. For pigs, it was suggested that this ratio has protein and energy dependent phases. When dietary energy is not limiting then increasing dietary protein intake would result in increased protein deposition until either dietary energy limits protein deposition or protein deposition reaches a maximum (PDmax) (Dumas et al., 2008). This relation between energy and protein deposition is usually described by a linear plateau model. The optimal DP/DE ratio would then be at PDmax (inflection point). Therefore, when dietary energy is provided in excess and PDmax is not reached yet, fat deposition is enhanced. In **Chapters 2 and 3**, a wide range of DP/DE ratios (16-27 mg kJ^{-1}) was used to test the existence of PDmax and the dependency of DP/DE ratio on other criteria under restricted and satiation feeding. In practical fish farming and also in most of the scientific literature, the main criteria to measure the effects of changes in dietary macro and micro nutrient composition are fish growth and feed conversion ratio (FCR) . However, in our study, we selected additional criteria to check whether the optimal DP/DE ratio would also be dependent on physiological responses. In **Chapter 2**, and assuming a linear plateau relation between DP and DE, we searched for the optimal DP/DE ratio by applying an equal amount of DP (i.e., restricted feeding) and varying the dietary DE level (Figure 2.1). In such a design it was expected that the optimal DP/DE ratio would be at the point where PDmax is reached. However, none of the criteria used succeeded to show an optimal DP/DE ratio. We hypothesized that feed intake could be the reason for these results and therefore the study was repeated with satiation feeding. Again no optimal DP/DE ratio was found for none of the criteria (**Chapter 3)**. These observations suggest that young Nile tilapia were not able to reach PDmax at a DP/DE ratio of 16 mg kJ^{-1}. Juvenile fish still exhibit hyperplasia and hypertrophy of muscle growth (Stickland, 1983). The hyperplasia of the muscle fibers disappears above a body size of 45% of the maximal body size (Weatherley and Gill, 1985). Thus, in adult fish muscle growth would be only due to hypertrophy. This would explain why in rainbow trout protein deposition increased linearly with body weight until fish reached 400 g body weight and after that, the deposition of protein levelled off (Dumas et al., 2007). In line with this observation in rainbow trout, it could be hypothesized that in young Nile tilapia , the muscles still display hyperplasia in this stage of development and thus maximum protein deposition was not reached. Therefore, it is expected that if this study was repeated for different higher weight classes, maximum protein deposition would occur.

In fact, when PDmax was not reached at the lowest DP/DE ratio, the excess energy was used to deposit substantial amounts of fat in Nile tilapia (16-20% of the body weight). This high fat retention was observed at the whole body model, therefore it was interesting to check how low DP/DE ratios would affect protein and

fat distribution in different body compartments. We tested this in **Chapter 4** for fillet, liver, gastrointestinal tract and a "rest" fraction. About 30% of the total fat content was stored in the viscera and about 60% was stored in the "rest" fraction. Therefore, lowering the DP/DE ratio below 16 mg kJ^{-1} would enhance fat accumulation. It could then be possible that a negative carryover of this high fat level would occur at later stages, reducing ultimately fish growth and final fish quality. On the other hand protein distribution over the different compartments was relatively constant suggesting that dietary factors (i.e., DP/DE ratio) would exert a bigger effect on body fat than on body protein which is in line with other studies in fish (Dumas et al., 2007; Lupatsch et al., 2003b; Shearer, 1994; Tibbetts et al., 2005).

In the general introduction of this thesis, we mentioned that the current way of calculating DP/DE ratio for fish assumes that the relation between DE intake and RE is constant and not affected by the dietary macronutrients composition. Our study clearly shows that this assumption is not valid (see **Chapters 5** and **7**). Therefore, we tried to estimate the optimal DP/DE ratio for Nile tilapia by the broken line method and a quadratic/linear regression analysis. However, neither these approaches enabled us to show an optimal DP/DE ratio. The results of the **Chapters 2, 3** and **4** show that, opposite to what is assumed in literature, an optimal DP/DE ratio for young Nile tilapia is not present. The wide range of DP/DE ratios tested in this thesis includes the reported ratios in literature and those recommended by NRC (2011) for different weight classes of Nile tilapia. However, due to the substantial amount of fat deposited it is not recommended to test lower ratios than what was tested in this thesis

8.4 Energy evaluation system for Nile tilapia

Fish require energy for maintenance, growth and reproduction. The available amount of energy in feed should be delivered to fish according to their energy requirements. Therefore, it is important that the energy value of feed ingredients is properly determined and is adjusted to the energy requirements of fish. In case of an inaccurate evaluation of feed ingredients this will result in variable fish performance responses once the dietary ingredients change. Usually the available energy in feeds is evaluated by either DE, ME or NE systems. Until today, in fish the available energy is based on DE which is usually calculated by subtracting the energy lost in the faeces from the gross energy. However, irrespective of the energy system used, the accuracy to predict true energy values of diets/ingredients remains difficult due to interactions between animals, environment, and ingredient characteristics. The main difference between DE or ME and NE is that DE and ME systems express potential energy, whereas the NE system reflects the energy that really can be used. It also gives an indication of the efficiency in which dietary nutrients are utilized. It is commonly accepted that the NE evaluation system used for pigs is currently the most accurate prediction of energy values of diets/ingredients for animals (Noblet, 2007).

In the NE evaluation system, the energy values derived from digestible protein (dCP), fat (DFat) and carbohydrates (dCarb) are used to calculate the NE content of diets/ingredients (*CVB*, 1993; Noblet et al., 1994). The superiority of the NE system over the DE system is that it takes into account differences in the utilization efficiency of DE for growth (kg_{DE}). In other words (kg_{DE}) is not a fixed value as assumed in the DE

evaluation system and can be altered by the nutrient composition of DE (the source of DE). The results of **Chapter 5** showed that kg_{DE} was numerically lower in fish fed the NSP diet compared to those fed a starch diet. This was further reflected in significantly lower energy retention (Table 5.4). It indicates that changing the dietary ingredient composition is a factor which affects the energy retention in Nile tilapia. In **Chapter 7**, kg_{DE} was different and dependent on dietary nutrients composition where dCarb and dCP reduced kg_{DE} in Nile tilapia and rainbow trout (Figure 7.3 A,B). These results confirmed the observation made by Schrama et al. (2012) and Glencross et al. (2017) that dietary carbohydrates caused a lower kg_{DE} in Nile tilapia and Asian seabass, respectively. Therefore, it seems that an energy evaluation based on DE is less favorable for the estimation of energy values of ingredients/diets for fish. We are convinced that a transition to an energy evaluation based on NE would be better for fish feed formulation, just as it has been applied in pigs. In **Chapter 7**, a new proposed NE evaluation system for fish is presented (table 7.6).

Using the NE equation 2 from table 7.6 for Nile tilapia, the relative energy values of different ingredients were calculated based on DE and NE system (Table 8.1). For these calculations the nutrients composition, and digestibility of different ingredients were used which were reported in **Chapter 6**.

TABLE 8.1 | Relative gross energy (GE)digestible energy (DE) and net energy (NE)values of ingredients for Nile tilapia. DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran;RM, rapeseed meal; SFM, sunflower meal, HFM, hydrolysed feather meal.

Ingredients	GE (kJ g^{-1})	DE (kJ g^{-1})	DE value as % of SBM	NE (kJ g^{-1})	NE value as % of SBM
DDGS	21.26	16.24	98.42	9.60	101.82
SBM	19.86	16.50	100.00	9.43	100.00
RB	21.48	16.17	98.01	11.36	120.51
RM	19.61	14.69	89.00	8.10	85.95
SFM	19.12	13.67	82.83	7.44	78.86
HFM	25.11	20.26	122.75	11.54	122.42

Noblet (2007) inferred that protein/fiber rich ingredients are overestimated and that fat rich ingredients are underestimated in the DE system. His argument was that differences in the digestion of these nutrients resulted in a different heat production. Table 8.1 shows the consequences of the feed evaluation system by either the NE or the DE system. When switching from a DE to a NE evaluation system, the ranking of energy value of ingredients is different between DE system and NE system. For instance, the energy value of SBM is comparable to RB in the DE system but contains 20% less NE. In the DE system, DDGS has a lower energy value compared to SBM but in the NE system it is higher. These differences between the NE and DE values express the advantage of the NE system above the DE system, providing a better estimate of the true energetic value of ingredients/diets. This implies that the cost of energy (per kJ) in the NE system will increase for protein/protein rich ingredients and decrease for fat/starch rich ingredients. The higher energy value of fat rich ingredients in the NE system allows higher inclusion levels of these ingredients without extra costs. Therefore, it is expected that fish feed formulation based on the NE system will include more fat and starch ingredients and less protein ingredients coupled with a reduction in dietary protein level. By applying the NE system, the fish feed sector will be able to utilize and include new low valued by-products

of the agro-food industry. In addition, the reduction of dietary protein level would reduce water and soil pollution by reduction in nitrogen excretion of fish farms.

In the current project, we tried to estimate the NE values of ingredients/diets first by calculating the energetic efficiencies of dCP ($k_{NE;dCP}$), dFat ($k_{NE;dFat}$) and dCarb ($k_{NE;dCarb}$) by multiple linear regression for both Nile tilapia and rainbow trout (**Chapter 7**, table 7.6). It is already established that the main roles of fat (i.e., fatty acids) is to generate free chemical energy in the form of Adenosine triphosphate (ATP) or to be the substrates for fat synthesis. Converting dietary fatty acids into body fatty acids is more efficient than the formation of fatty acids from amino acids (NRC, 2011). Although there are differences in the trophic level , it seems that the studied species share the same preference for dietary fat being the efficient source for energy retention. This was shown in **Chapter 7** where $k_{NE;dFat}$ was high and similar between fish species and pigs suggesting that fat is the preferred energy sources for energy retention. Regarding $k_{NE;dCarb}$, we saw that the value for Nile tilapia (66%) is lower than what is estimated for pigs (78 and 84%). We have discussed in **Chapter 7** that these differences might be related to overestimation of measured nutrients digestibility values in fish due to leaching of nutrients from both feed and faeces into the water. Currently, faeces collection in water using different systems is the most used method for the calculation of nutrients digestibility for most studies in literature. This aspect of nutrients leaching is still not fully addressed and quantified for fish. Therefore, improving the method of faeces collection may improve the estimation of digestibility values. This issue will not be only valid for Nile tilapia but is applied for all fish species. In addition, when we estimated $k_{NE;dCarb}$ we have only estimated the total carbohydrates and we did not make a distinction between enzymatically digested starch and fermentable carbohydrates as has been done in pigs. In the NE system $k_{NE;dCarb}$ is estimated for dietary starch, thus our estimated value of $k_{NE;dCarb}$ is lower. Therefore, it would be better to separate the total carbohydrates to digestible starch and fermentable carbohydrates to improve the NE evaluation equation proposed in this thesis.

The digestible nutrients in rainbow trout were not all linearly related to NE retntion. dCarb was curvilinearly related to NE (Figure 7.1A) indicating that the amount of energy retention derived from dCarb is dependent on the actual carbohydrate intake. This leads to the question whether a transition from the DE evaluation system to the NE system using equation 3 in table 7.6 is still valid for rainbow trout. In fact, for the latter species this dependency of the NE value on the intake level of dCarb is persistent, irrespective whether the DE or NE system is used. It is well documented that carbohydrates utilization as energy source is low for carnivorous fish species (Glencross et al., 2017). The complexity of carbohydrates included in rainbow trout diets, the dietary level and the processing of carbohydrates may have an effect on carbohydrates digestibility. These factors would also affect the prediction of the NE value of diets rich in carbohydrates. In addition, we indicated that intake digestible carbohydrate would affect the predictability of the NE value, thus using the NE system for rainbow trout should be used with caution. This is especially the case not only when formulating diets that include different sources of carbohydrates rich ingredients but also the feeding level applied. In farm animals it has been shown that NE system marginally improved the prediction of energy retention compared to pigs (Noblet, 2007). This species differences in farm animals may also valid among different fish species. Thus, it would be of interest to investigate whether a distinction between different fish species should be established.

On the other side, for Nile tilapia, the relation between all digestible nutrients and NE was linear (Figure 7.1 and 7.2) indicating that for this fish species, the NE evaluation system can improve the prediction of energy values derived from diets/ingredients. Therefore, we believe that the NE system can be used in feed formulation of Nile tilapia. In addition, applying the NE evaluation system would also be a valuable tool to minimize the amount of fat retention when the energy values of the different digestible nutrients can be estimated accurately.

Besides the fact that the NE system is used to improve the estimation of energy required for maximum growth, it can also be used to improve the fillet yield of fish. This point requires more investigation. The change from a DE to a NE evaluation system will coincide with changes in the composition of the fish diet, and this will be reflected in lower and higher dietary levels of protein and fat, respectively. Thus, the NE evaluation system will help to diversify the used ingredients. It can help to include lower quality ingredients and yet, keeping the aquafeeds well balanced. It is important to note that to further improve the estimation of energy derived from diets/ingredients for NE retention (i.e, growth), an accurate measurement of energy and nutrients digestibility is required.

8.5 Conclusions

From the results of the different studies carried out in this thesis, the following conclusions can be made:

1. For young Nile tilapia, an optimal (DP/DE) is absent and could not be quantified.
2. Changes in dietary DP/DE ratio have a clear effect on the fat distribution but not on the protein distribution in the body compartments of young Nile tilapia.
3. Dietary ingredients composition altered the maintenance requirements in Nile tilapia.
4. The utilization efficiency of DE for energy retention (kg_{DE}) is not constant and altered by the type of dietary carbohydrate.
5. The energetic efficiencies of dCP, dFat and dCarb for net energy retention (NE) were 49, 91 and 64% for Nile tilapia, respectively.
6. For Nile tilapia a NE evaluation system seems to be feasible but for rainbow trout this is not easy to implement because the NE value of a diet/ingredient depends on the feeding level (i.e., digestible starch intake).

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Summary

The optimization of fish feed formulation depends not only on an accurate estimation of the nutrients supplied by feed ingredients but also on quantifying the nutrients requirements of fish. As the aquaculture sector is rapidly growing, current and future fish diets are facing increased variability in ingredients composition due to fish meal and oil replacement. This will also increase the variation in dietary macronutrient composition especially regarding carbohydrates. Therefore, fish diets are becoming more complex and challenging to attain a well-balanced feed. The energetic value of ingredients/diets is the combined result of their macronutrients content, their digestibility and of the utilization efficiency by the fish. Today, nutritionists are using the digestible energy evaluation system to estimate the energetic value of ingredients/diets for fish. However, there is an indication that this system might not be the most precise and accurate method to determine the dietary energy value required for fish growth.

The goal of this thesis was to assess and improve the current digestible energy evaluation system used for Nile tilapia.

In **Chapters 2 and 3** we quantified the optimal digestible protein to digestible energy (DP/DE) ratio for Nile tilapia and assessed if the optimal DP/DE ratio differs between different physiological criteria including nitrogen and energy balances. We examined a wide range of DP/DE ratios from 16.7 to 29 g MJ^{-1}. In chapter 2 we have applied restricted feeding and in chapter 3 satiation feeding. The results of both chapters showed that neither a broken line analysis nor a regression analysis succeeded to estimate an optimal DP/DE ratio for Nile tilapia. In addition, protein retention efficiency was linearly related with DP/DE ratio and protein deposition did not reach a maximum. Moreover, decreasing the DP/DE ratio resulted in a very high fat content of the fish (from 16 to 20%).

In **Chapter 4** the objective was to investigate whether the impact of DP/DE on the nutrient composition and distribution differed within the body. Therefore, the effect of changing dietary DP/DE ratios on different body compartments was addressed. Eight experimental diets differing in DP/DE ratio were fed to satiation. The dietary DP/DE ratios ranged from 17 to 29 mg kJ^{-1}. Fish were partitioned into four main compartments: liver, viscera, fillets and the "rest" fraction. The viscera and "rest" fraction had a substantial amount of fat about (30 and 60%, respectively). The viscera and fillets accounted for about 10 and 29% of the total fish weight. A constant protein content of 17% was found in the fillets. Protein distribution over all body compartments was relatively constant and independent of the dietary DP/DE ratio. In contrast, the fat distribution over body compartments was significantly affected by the dietary DP/DE ratios. These results demonstrated that the viscera and the"rest" fractionwere the main site for fat storage in Nile tilapia. In addition, any changes in dietary DP/DE ratio affected fat content but not protein content of Nile tilapia.

In **Chapter 5** the objective was to assess the effect of changing the composition of the dietary carbohydrates on the energy utilization. In the digestible energy evaluation system, energy utilization is assumed constant and not affected by changes in dietary composition. Eighteen groups of fish were assigned in a 2x3 factorial design: two diets, with either a high non starch polysaccharides (NSP) or high starch content; and three feeding levels (low, medium or satiation). The NSP diet contained 70% of the starch diet supplemented with 30% dried distillers grains with solubles. About 5 and 17% of the total digestible energy originated

from NSP at the starch and NSP diet, respectively. This study demonstrated that digested NSP was less well utilized for growth which was reflected by a lower energy retention.

In **Chapter 6** the effect of six feed ingredients (hydrolysed feather meal (HFM), soybean meal (SBM), rice bran (RB), rapeseed meal (RM), sunflower meal (SFM) and dried distiller grains with solubles (DDGS)) were studied on nitrogen and energy balances (including maintenance requirements) in Nile tilapia. The study demonstrated that these feed ingredients did have an impact on nutrient digestibility and the nitrogen and energy balances. In addition, changing the dietary ingredient composition had a clear effect on the maintenance requirements of Nile tilapia.

In **Chapter 7** we aimed to estimate the energy efficiency of digestible nutrients (protein, fat and carbohydrates) to develop a net energy evaluation approach for fish. In addition we assessed if these estimated efficiencies differed between Nile tilapia and rainbow trout. For these objectives, the Nile tilapia and rainbow trout data sets contained respectively, 23 and 45 different diets in which the digestibility of protein, fat and energy and the complete energy balances were measured. The data of these diets allowed us to determine the digestible protein, digestible fat, and digestible carbohydrate intake. The energy efficiency in Nile tilapia was 49, 91 and 64% for digestible protein, digestible fat and digestible carbohydrates, respectively. Rainbow trout had quite similar energy efficiencies of digestible protein and digestible fat compared to Nile tilapia but differed regarding digestible carbohydrates. In trout, the increase in net energy value leveled off with increasing digestible carbohydrates intake. This study demonstrated that net energy evaluation is possible for Nile tilapia, however, for rainbow trout the net energy value depends on carbohydrate intake.

Finally in **Chapter 8** the main finding of the different studies in this thesis were summarized and discussed within the context of the effect of current and future changes in dietary factors on feed energy evaluation of Nile tilapia.

Overall the following conclusions can be drawn from this thesis:

1. For young Nile tilapia, an optimal (DP/DE) is absent and could not be quantified.
2. Changes in dietary DP/DE ratio have a clear effect on the fat distribution but not on the protein distribution in the body compartments of young Nile tilapia.
3. Dietary ingredients composition altered the maintenance requirements in Nile tilapia.
4. The utilization efficiency of digestible energy for energy retention (kg_{DE}) is not constant and altered by the type of dietary carbohydrate.
5. The energetic efficiencies of digestible protein, digestible fat and digestible carbohydrates for net energy retention were 49, 91 and 64% for Nile tilapia, respectively.
6. For Nile tilapia a NE evaluation system seems to be feasible but for rainbow trout this is not easy to implement because the NE value of a diet/ingredient depends on the feeding level (i.e., digestible starch intake).

Acknowledgements

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About the author

Training and Surpevision Plan

About the Author

Mahmoud Haidar Was born on January 22 of January 1978 in Damascus, Syria. After completing high school, he studied at the faculty of agriculture in Damascus University and graduated with a B.Sc. degree in 2003. During his study, he was specialized in the domain of animal production and poultry nutrition and his major thesis was on "The effect of dietary rapeseed meal inclusion level on the performance and carcass traits of broilers". After graduation, he joined the General Commission for Scientific Agricultural Research in Damscus and worked as supervisor of animal production and nutrition unit. In 2005, he moved to Aleppo University and worked at the faculty of agriculture as research and education assistant. In 2009, he started his master studies in the aquaculture and Fisheries program in Wageningen university and obtained his M.Sc. degree in 2011 with a major thesis entitled "Effect of dietary oxygen demand on feed intake in Nile tilapia". Also, he did a minor thesis entitled "The relation between pellet water stability characteristics and apparent digestibility coefficients in Nile tilapia". In 2011, he started his Ph.D. at the Aquaculture and Fisheries Chairgroup of Wageningen University. From January 2017, he is employed as the international product manager of the aquaculture department by de heus Animal Nutrition, The Netherlands.

Contact: Mahmoud.haidar@gmail.com

Training and Supervision Plan

EDUCATION AND TRAINING		
The Basic Package	**year**	**credits**
WIAS Introduction Course	**2013**	**1.5**
Course on philosophy of science and/or ethics.	**2012**	**1.5**
Subtotal Basic Package		**3**

Scientific Exposure	year	credits
International conferences		
EAS conference, AQUA (14-17 October) 2014 Spain	2014	1.2
EAS conference, AQUA (20-23 October) 2015 The Netherlands	2015	1.2
EAS conference, AQUA (20-23 September) 2016 Scotland	2016	1.2
Seminars and workshops		
symposium "Fibers in food and feed; Biological mechanisms of energy intake". Wageningen University, 31 October.	2013	0.3
WIAS Fiber Seminar, Wageningen University, 20 June 2014	2014	0.2
WIAS Science Day (Netherlands)	2014	0.3
WIAS Science Day (Netherlands)	2015	0.3
WIAS Science Day (Netherlands)	2016	0.3
Presentations		
Oral presentation at EAS conference, AQUA 2014 Spain	2014	1.0
Poster presentation at EAS conference, AQUA 2014 Spain	2014	1.0
Oral presentation at EAS conference, AQUA 2015 Rotterdam	2015	1.0
Poster presentations at EAS conference, AQUA 2015 Rotterdam	2015	1.0
Oral presentation at Science Day 2015	2015	1.0
Oral presentation at EAS conference, AQUA 2016 Scotland	2016	1.0
Subtotal Scientific Exposure		**11**

In-Depth Studies	year	credits
Disciplinary and interdisciplinary courses		
Recirculating Aquaculture Systems (RAS) Technology. Wageningen, The Netherlands.(22-25 April)	2013	1.3
Technology for novel fish feeds; Olhão, Algarve, Portugal. (26-29 October)	2014	1.0
Long Term Effects Of Low Fishmeal And Fish Oil Diets Across Life Stages. Las Palmas de Gran Canaria. (13-15 January)	2016	1.0
Advanced statistics courses		
Advanced statistics course: Design of Experiments (8-10 October)	2014	1.0
Statistics for Life Sciences (21-28 May)	2014	2.0
Subtotal In-Depth Studies		**6**

Statutory Courses	year	credits
Use of Laboratory Animals (February)	2014	3
Subtotal Statutory Courses		**3**

Professional Skills Support Courses	year	credits
Techniques for Writing and Presenting a Scientific Paper (1-4 July 2014)	2014	1.2
High-Impact Writing in Science (18-21 November)	2013	1.3
Project and Time Management courses	2014	1.5
Subtotal Professional Skills Support Courses		**4.0**

Research Skills Training	year	credits
Preparing own PhD research proposal	2013	6.0
External training period		
PhD knowledge exchange program, Japan	2016	1.5
Subtotal Research Skills Training		**8**

Didactic Skills Training	year	credits
Supervising practicals and excursions		
Nutrition, welfare and reproduction 2011	2011	0.4
Nutrition, welfare and reproduction 2012	2012	0.4
Life history of aquatic animals 2013	2013	0.2
Nutrition, welfare and reproduction 2014	2014	0.4
Aquaculture and fisheries 2014	2014	0.1
Nutrition, welfare and reproduction 2015	2015	0.9

Supervising theses		
MSc student (major), Wesley van den Herik	2014	2.0
MSc student (major), Frits berkers	2014	2.0
MSc student (major), Kevin Hout	2014	1.0
Subtotal Didactic Skills Training		**7**
Management Skills Training	year	credits
Organization of seminars and courses		
WIAS science day committee 2015	2015	2.0
Subtotal Management Skills Training		**2**
Education and Training Total		**44**

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