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Differences in energy utilisation efficiencies of digestible macronutrients in common carp (*Cyprinus carpio*) and barramundi (*Lates calcarifer*)

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Abstract

This study aimed to assess macronutrient-specific energy utilisation efficiency (*i.e.*, protein, lipid and carbohydrate) for growth in common carp (an omnivorous species) and barramundi (a carnivorous species) and to assess if species-specific differences exist in energy efficiency of digestible protein (dCP), digestible fat (dFat) and digestible carbohydrates (dCarb). This was achieved by conducting a feeding trial experiment on common carp and by re-analysing data of a recent study on barramundi. A total of four diets were formulated following a 2×2 factorial design with 2 dCP-to-dFat ratios and 2 dCP-to-dCarb ratios. For carp, 2 feeding levels were applied such that the overall experimental design was a 2×2×2 factorial design, however for barramundi, three feeding levels were applied (satiation, 80% initial satiation and 60% initial satiation), resulting in a 2×2×3 factorial design. For each fish species, multiple regression of retained energy (RE) as a function of dCP, dFat and dCarb (in $g.kg^{-0.8}.d^{-1}$) was applied to estimate the energy utilization efficiency of each digestible macronutrient. For carp, dCP, dFat and dCarb show linear relationships to RE, however for barramundi, dCP and dFat were linearly related to RE, but dCarb was curvilinearly related to RE. The estimated energy efficiencies of dCP, dFat and dCarb (respectively, $k_{NE;dCP}$, $k_{NE;dFat}$, and $k_{NE;dCarb}$) for energy retention were 47, 86 and 60%, respectively, showing large degree of similarity with Nile tilapia and pigs. Carp and barramundi had similar $k_{NE;dFat}$ (86 *vs.* 94%), but different $k_{NE;dCP}$ (47 *vs.* 64%) and $k_{NE;dCarb}$ (60 *vs.* 18%). The net energy equations were NE = 11.2 x dCP + 34.1 x dFat + 10.4 x dCarb for carp, and NE = 15.9 x dCP + 35.2 x dFat + 9.4 x dCarb − 1.9 x $(dCarb)^2$ for barramundi.

Key words: Energy evaluation; Energy metabolism; Bioenergetics: Net energy; Energy efficiency; Digestible nutrients; *Cyprinus carpio*; *Lates calcarifer*.

Introduction

Growth in fish, like other animals, requires amino acids, essential fatty acids and minerals, but also energy. Fish needs to consume energy for the accretion of fish biomass (*i.e.*, protein, fat and bone structures) and for maintenance processes. Dietary energy is provided in the form of lipids, carbohydrates and proteins. Each of these macronutrients is metabolised using different biochemical processes to yield energy (NRC, 2011). Differences in these metabolic pathways lead to distinct values of efficiency in deriving energy from digested proteins, lipids, and carbohydrates. The respective proportions of each dietary macronutrient therefore affect the overall energy efficiency of fish feeds. The effect of dietary macronutrient composition on the energy utilisation values have been shown in Nile tilapia (Schrama *et al.*, 2018), rainbow trout (Saravanan *et al.*, 2012) and barramundi (Glencross *et al.*, 2014, Glencross *et al.*, 2017). Observations of the energy utilisation of these macronutrients by these fish species for maintenance and growth, appears to be species-specific and/or trophic level-specific (*i.e.,* herbivorous, omnivorous or carnivorous fish) (Schrama *et al.*, 2012).

In the evaluation of animal feed, various systems have been used to estimate dietary energy availability after being ingested, ranging from digestible (DE) and metabolisable (ME) to net energy (NE) systems (NRC, 1981). The DE-based factorial approach has been widely applied to estimate fish dietary energy requirements (Glencross, 2006, Williams *et al.*, 2003, Williams *et al.*, 2006, Glencross, 2008, Glencross and Bermudes, 2012). In such factorial approaches (*i.e.,* DE approach), the efficiency of digestible energy utilisation for growth (k_{gDE}) is given by the regression slope of retained energy and DE intake. This k_{gDE} is assumed to be independent of feed composition and thus the composition of DE (*i.e.*, digested protein, fat and carbohydrates). However, the k_{gDE} varies in barramundi (Glencross *et al.*, 2017), Nile tilapia (Schrama *et al.*, 2012) and rainbow trout (Rodehutscord and Pfeffer, 1999) when fed diets with different macronutrient profiles. This indicates that k_{gDE} is affected by dietary macronutrient profile. Moreover, Schrama *et al.* (2012) found k_{gDE} to be correlated to species' trophic level, although this might be related to variation in dietary nutrient content. This variability in k_{gDE} demonstrates the limitation of DE feed evaluation systems. With the diversification of ingredients used in fish feeds, also the composition of the digestible macronutrient profile will become more variable. Consequently, in practical feed formulation using DE evaluations will introduce a potential bias due to this variability in k_{gDE}. Others have addressed the necessity for alternative energy evaluation of fish feeds (Glencross *et al.*, 2014,

Hua *et al.*, 2010, Azevedo *et al.*, 2005). A NE approach might be such an alternative. NE evaluation has been applied for pig feed for several decades. In NE evaluation systems, each macronutrient (*i.e.*, protein, lipid and starch) has its own partial efficiency for growth, which is estimated by multiple regression between retained energy and digested protein, lipid and starch respectively (Noblet *et al.*, 1994). In other words, k_{gDE} is considered to be a function of the underlying specific energy utilization efficiencies of each type of digestible macronutrients.

The first steps to develop a NE evaluation for fish feed were recently undertaken for Nile tilapia and rainbow trout using a meta-analysis approach (Schrama *et al.*, 2018). The estimated energy utilisation efficiencies for growth of digestible protein (dCP; $k_{NE;dCP}$), digestible fat (dFat; $k_{NE;dFat}$) and digestible carbohydrate (dCarb; $k_{NE;dCarb}$) were respectively, 49%, 91%, 66% for Nile tilapia and 64%, 89% and 70% for rainbow trout (Schrama *et al.*, 2018). Digestible protein was utilised for energy retention more efficiently in rainbow trout (64%) than in Nile tilapia (49%) suggesting that the energy efficiency of digestible protein ($k_{NE;dCP}$) is also dependent on the trophic level in the NE approach (Schrama *et al.*, 2018). Species-specific effect of individual macronutrient inclusion level was also shown by the curvilinear relationship found between retained energy and digestible carbohydrate intake in rainbow trout (Schrama *et al.*, 2018).

Aims of this study were: (1) to assess macronutrient-specific energy utilisation efficiency (*i.e.*, protein, lipid and carbohydrate) for growth in common carp (an omnivorous species) and barramundi (a carnivorous species); and (2) to assess if species-specific differences exist in energy efficiency of dCP, dFat and dCarb. This was achieved by conducting a feed trial experiment on common carp and by re-analysing data of a recent study on barramundi (Glencross *et al*., 2017), which had the similar setup but different data analysis method from that of the study on common carp.

Materials and methods

Carp experiment

Experimental diets. A total of four diets were used in the carp feed trial, with different proportions of crude protein (28.5 – 52.9%), crude lipid (7.1 -25.8%) and carbohydrates (23.4 -52.8%). This large variability in dietary macronutrient composition was created using a wide range of ingredients (Table 1). Despite this large variability, diets were formulated to meet requirements for vitamins, minerals, essential fatty acids and amino acids of common carp.

Despite the differing levels of dietary protein, amino acid ratios were kept constant meeting the ideal ratios based on the available knowledge (NRC, 2011). The analysed amino acid composition of the experimental diets are given in Supplementary Table S1.

The triangle approach (Raubenheimer, 2011) was applied to create a wide range of macronutrient (*i.e.*, crude protein, lipid and total carbohydrates) inclusion levels in the four experimental diets (Table 1). Diets were formulated following a 2×2 factorial design with 2 dCP-to-dFat ratios and 2 dCP-to-dCarb ratios. For each diet, 2 feeding levels were applied such that the overall experimental design was a 2×2×2 factorial design with a total of 8 treatments. This design was required to achieve large contrasts in digestible macronutrient intake among the 4 diets. This facilitated multiple regression analysis of energy retention (*i.e.*, growth response) as a function of dCP, dFat and dCarb intake.

Fish handling. The experiment started December 2014. It was approved by the Ethical Committee judging Animal Experiments of Wageningen University, The Netherlands (DECnr 2014109b) and carried out according to the Dutch law on animal experiments.

A total of 840 common carps (*Cyprinus carpio)*, with a mean body weight (BW) of 28.9 g (SE 0.25) were obtained from the carp population (Strain: R3R8 F12, Breed: FxM, mixed sex) of Wageningen Aquatic Research Facility (CARUS-ARF, Wageningen, the Netherlands). The experiment was conducted at the aquatic respiration unit of Wageningen Aquatic Research Facility (CARUS-ARF, Wageningen, the Netherlands), which includes a total of twelve 200-L tanks with a water flow of 7 L/min. Water temperature was maintained at $23 \pm 0.5^{\circ}C$ and the dissolved oxygen level of inlet water ranged from 8 to 11 ppm. At the start of the experiment, groups of thirty five fish were batch-weighed and randomly assigned to one of the twelve tanks.

Carp were hand-fed one of the four diets and one of the two feeding levels of approximately 12 and 20 $g.kg^{-0.8}.d^{-1}$. Fish were fed twice daily for 28 days from 09:00 to 10:00 hours and from 16:00 to 17:00 hours. To obtain 3 replicates per treatment (*i.e.*, 24 tanks in total), 2 consecutive trials were run in the 12 tanks aquatic respiration unit under identical conditions.

Sample preparation and chemical analysis. At the beginning of each trial, ten fish from the initial population were euthanized by overdose of 2-phenoxyethanol for the analysis of initial body composition. At the end of each trial, ten fish from each tank were euthanized in the same way to determine final body composition. The fish were then frozen at $-20^{\circ}C$. The

samples were prepared for chemical analysis according to the methods reported by Saravanan *et al.* (2012).

After sample collection, fish were sawn into slices and minced to ensure sample homogeneity. Fresh fish samples were used for dry matter (DM), ash and crude protein (CP) analysis whereas fish samples for fat and gross energy (GE) analyses were first freeze dried. Diet and oven-dried (70 °C) faecal samples were analysed for DM, yttrium, Ca, P, CP, fat, starch and gross energy contents.

Proximate composition of fish, feed and faeces were determined according to ISO-standard analysis for determination of dry matter (DM; ISO 6496, 1983), crude ash (ISO 5984, 1978), crude fat (ISO 6492, 1999), crude protein (ISO 5983, 1997, crude protein = Kjeldahl-N $\times$ 6.25), energy (ISO 9831,1998), and starch (NEN/ISO 15914) (Meriac *et al.*, 2014). Total carbohydrates content of feed and faeces was calculated as DM minus crude protein minus crude ash minus crude fat.

Nutrient digestibility measurement. Yttrium oxide was added as an inert marker to experimental diets at 0.02% (as-is). Each of the twelve tanks was connected to a separate faeces settling unit. Settling columns were equipped with an ice-cooled glass bottle at the bottom to prevent bacterial degradation of faecal nutrients. Faeces settled in the column overnight were collected daily prior to the morning feeding during the last 2 weeks of the experiment and pooled per tank. The procedure of faeces collection was identical to the study of Meriac *et al.* (2014).

Apparent nutrient digestibility coefficients ($ADC_{nutrient}$) of the diets were calculated using the following equation:

$$ADC_{nutrient} = (1 - (Y_{diet}/ Y_{faeces}) \times (Nutrient_{faeces}/Nutrient_{diet})) \times 100\%,$$

where Y_{diet} and Y_{faeces} are the yttrium oxide concentration of the diet and faeces, respectively, and $Nutrient_{diet}$ and $Nutrient_{faeces}$ are the DM, protein, fat, carbohydrates or energy content of diet and faeces, respectively.

Nutrient balances calculations. To standardise for differences in body weight and digestible macronutrient intake, nitrogen and energy balance parameters were expressed per unit of metabolic body weight. Metabolic body weight was calculated as the average of initial and final metabolic body weight (calculated as BW/1000)$^{0.8}$)). The calculations of energy and nitrogen balances were based on those described by Saravanan *et al.* (2012). Intake of each

nutrient on a gross basis was determined by multiplying the averaged feed intake for each treatment by the nutrient inclusion level in the diet. Digestible nutrient intake was determined by multiplying gross nutrient intake with the diet-specific nutrient digestibility coefficient. Energy and nutrient retention rates were determined from net gain, calculated by difference between initial and final whole-body content. Branchial and urinary N losses (BUN) were calculated based on difference between digestible N intake N and N retention. Branchial and urinary energy (BUE) was measured by multiplying BUN by 24.85, which is the energy content (in kJ) of 1 g excreted nitrogen with the assumption that NH_3-N is the only form of this excretion (Bureau *et al.*, 2003). Metabolisable energy intake was determined by difference between digestible energy intake and BUE. Heat production was measured by deducting ME from retained energy.

Barramundi data set

The barramundi data set was derived from a study by Glencross *et al.* (2017), which assessed the impact of dietary macronutrient composition on energy, nitrogen and fat balances in juvenile barramundi weighing 69.6 g (SD 0.75). The unit and measurement of body weight, digestible macronutrient intake, nitrogen and energy balance parameters in the barramundi dataset are identical to those employed in the analysis of the carp experiment. The design of the barramundi study was similar to the study on carp, where four diets were formulated having contrasting protein, fat and starch levels. However, in the barramundi study, three feeding levels were applied (satiation, 80% initial satiation and 60% initial satiation), resulting in a 2x2×3 factorial design. All treatments were duplicated using 24 tanks (Glencross *et al.*, 2017). Digestibility measurements were based on faeces collected after the growth experiment. Only fish fed to satiation during the growth experiment (8 tanks) were used to collect faeces. Faecal collection was conducted by manual stripping once a day at about 6 hours post-feeding (Glencross *et al.*, 2017). Since crude ash content was not measured in faeces, the carbohydrates content of both feed and faeces was calculated from the measured energy, crude protein and crude fat content as described by Schrama *et al.* (2018). In this calculation, 23.6, 39.5 and 17.2 $kJ.g^{-1}$ were used as the combustible energy content of CP, fat and carbohydrates, respectively (NRC, 2011).

Data analysis

Statistical analysis systems (SAS Institute) statistical software package version 9.1 was used to conduct data analysis. For carp, the effect of diet, feeding level and their interaction

on digestibility, growth performance, nitrogen and energy balances data were tested by two-way ANOVA. For the barramundi dataset, no ANOVA analyses were done as the data are published elsewhere (Glencross *et al.*, 2017).

For each fish species, multiple regression of retained energy (RE) as a function of dCP, dFat and dCarb (in g.kg$^{-0.8}$.d^{-1}) was applied to estimate the energy utilization efficiency of each digestible macronutrient using the following model:

$$RE_i = \mu + \beta_1 \times dCP_i + \beta_2 \times dFat_i + \beta_3 \times dCarb_i + e_i \quad \text{(Eq. 1)}$$

where μ is the intercept, being an estimate for fasting heat production (FHP); β_1, β_2, β_3 are the energy utilisation efficiencies of dCP ($k_{NE;dCP}$), dFat ($k_{NE;dFat}$) and dCarb ($k_{NE;dCarb}$); e_i is error term and i =1,..., n with n = 24 for both carp and barramundi data. The linearity and curve-linearity were checked in the relationship of RE with dCP, dFat and dCarb. Analyses were implemented separately for each species. To assess differences in β_1, β_2 and β_3 (*i.e.*, $k_{NE;dCP}$, $k_{NE;dFat}$, $k_{NE;dCarb}$) between carp and barramundi, a combined mixed model was used with the inclusion of a fixed effect of species and 2-way interaction of species with each type of digestible macronutrient intake (dCP, dFat or dCarb). Significance was set at *P*<0.05.

Results

Carp experiment

Overall growth performance was good in the carp experiment with daily weight gain ranging from 9.9 to 22.4 g.kg$^{-0.8}$.d^{-1} (Supplementary Table S2). Both feeding level and diet significantly (*P*<0.001) influenced daily digestible nutrient intake (Table 2 and Supplementary table S3) and ultimately final BW and daily body weight gain (Supplementary Table S2). Information on the impact of feeding level and diet on N balance parameters and body composition is given in Supplementary Table S4 and S5.

Overall average ADCs (*i.e.*, regardless of treatment) were 86% for DM, 90% for energy, 95% for CP, 91% for fat, 81% for carbohydrates, 99% for starch, 49% for NSP and 33% for ash. The carbohydrates fraction showed the largest between-diets variability with ADCs ranging from 68 to 91% in diets 1 (high FL) and 4 (low FL) respectively. Averaged over both feeding levels, protein ADC was lowest for the 2 carbohydrates-supplemented diets (Diet 2 and 4), while fat ADC was lowest for the 2 fat-supplemented diets (Diet 3 and 4). Total carbohydrates ADC was highest in the 2 carbohydrates-supplemented diets (Diet 2 and 4)

(Table 3). Except for ash and total phosphorus, all nutrient ADCs were affected by both diet, feeding level and their interaction ($P<0.001$; Table 3). Overall, nutrients ADC declined when the feeding level was raised. However, the significant interaction effect between diet and feeding level indicated that this decline with feeding level differed between diets. The decline in protein, total carbohydrates and starch ADC with feeding level was largest for Diet 1 (high protein content, no starch neither fat supplementation). In contrast, the decline in fat ADC with feeding level was largest for Diet 3 (fat-supplemented diet) (Table 3).

NE equations

The main aim of this paper was to assess differences in energy utilisation efficiency for growth (*i.e.*, quantifying NE equations) among digested macronutrients (*i.e.*, protein, lipid and carbohydrates). Energy and nitrogen balances were calculated based on digestible nutrient intake (dCP, dFat and dCarb) for both the carp and barramundi experiments. Energy and nitrogen balances are reported for the carp experiment in Supplementary table 2 and 3 respectively. These were reported by Glencross *et al.* (2017) for the barramundi dataset. The mean as well as the range of dCP and dFat daily intake (in $g.kg^{-0.8}.d^{-1}$) were comparable between carp and barramundi. However, the mean daily dCarb intake was much lower in barramundi compared to carp (0.94 *vs.* 4.22 $g.kg^{-0.8}.d^{-1}$). For both species, the large variability in digestible nutrient intake resulted in a large range in energy retention (RE). RE ranged from 86 to 207 $kJ.kg^{-0.8}.d^{-1}$ in carp and from 45 to 209 $kJ.kg^{-0.8}.d^{-1}$ in barramundi (Table 2).

The relationship between DE intake and RE in carp is given in Supplementary Fig. S1 and by Glencross *et al.* (2017) for barramundi. The average energy utilisation efficiency of DE for growth (k_{gDE}) was lower in carp (0.62), ranging from 0.59 to 0.66 than in barramundi (0.68), ranging from 0.55 to 0.79 (Supplementary Fig S1).

Multiple linear regression of RE (*i.e.*, NE) as a function of dCP, dFat and dCarb yielded the following equations for carp:

RE = - 22 (SE 5) + 11.2 (SE 0.8) dCP + 34.1 (SE 1.2) dFat + 10.4 (SE 0.5) dCarb (Eq. 2)

and for barramundi:

RE = - 18 (SE 3) + 15.2 (SE 0.9) dCP + 37.1 (SE 3.4) dFat + 3.1 (SE 1.2) dCarb (Eq. 3).

The energy utilisation efficiencies of dCP, dFat and dCarb (respectively, $k_{NE;dCP}$, $k_{NE;dFat}$, and $k_{NE;dCarb}$) were 15.2 $kJ.g^{-1}$ (64%), 37.1 $kJ.g^{-1}$ (94%) and 3.1 $kJ.g^{-1}$ (18%) for barramundi and 11.2 $kJ.g^{-1}$ (47%), 34.1 $kJ.g^{-1}$ (86%), 10.4 $kJ.g^{-1}$ (60%) for carp, respectively. Barramundi had a 36% higher energy utilization efficiency of dCP for growth than carp ($P=0.002$). Conversely, carp had a 235% higher energy utilization efficiency of dCarb for growth than

barramundi ($P<0.001$). The energy utilisation efficiency of dFat ($k_{NE;dFat}$) did not differ between the two species ($P>0.05$).

In carp, dCP, dFat and dCarb were all linearly related to RE (*i.e.*, no significant polynomial effect, $P>0.05$). Conversely, in barramundi, a significant quadratic component was found for dCarb ($P<0.01$), but not for dCP and dFat. Inclusion of the quadratic component for dCarb for barramundi resulted in the following relationship between RE and digestible nutrient intake:

RE = - 22 (SE 3) + 15.9 (SE 0.87) dCP + 35.2 (SE 3.11) dFat + 9.4 (SE 2.71) dCarb − 1.9 (SE 0.74) (dCarb)^2 (Eq. 4)

Inclusion of the quadratic component for dCarb into the equation had only a minor impact on the absolute values of $k_{NE;dCP}$ and $k_{NE;dFat}$ (Eq. 3 and 4; Table 4).

In Fig. 1, the relationship between NE (corrected to zero dCP and dFat intake) and dCarb intake is given. For barramundi, inclusion of dCarb into the diet raised NE, but the increase in NE started to level off when dCarb intakes reached about 1.5 to 2.0 g.kg$^{-0.8}$.d^{-1}. For common carp, NE increased linearly over the full range of increasing dCarb from 2 to 8 g.kg$^{-0.8}$.d^{-1}. The linear relationships between NE and dFat and between NE and dCP in both common carp and barramundi are shown in Supplementary Fig. S2.

Discussion

Feed formulation in aquaculture is currently based on the energy requirements of fish species and requires information on (1) nutrient digestibility of ingredients, (2) energy requirements for maintenance and (3) utilization efficiency of digestible energy (DE) or metabolizable energy (ME) for growth (k_{gDE} and k_{gME} respectively). Factors which can influence the evaluation of dietary energy are environmental conditions, choices of ingredients, nutrient digestibility and utilisation of digested nutrients. In this study, environmental conditions were identical across treatments for each species, therefore, this did not affect the feed evaluation.

The nutrient digestibility of raw materials are commonly used in feed formulation, assuming that these are additive. However, all macronutrient ADCs observed in the carp study (except ash and phosphorus) were affected by the interaction effect between diet and feeding level. This suggests that respective ingredients' nutrient ADCs are not additive, which is in contrast to Cho and Kaushik (1990). This is possibly due to the higher quality of fish diets used in the

past (*e.g.*, fishmeal-rich), which may have diminished the effects of feeding level on nutrient ADCs.

The protein-rich diet (Diet 1) for carp was diluted with maize starch and/or a vegetable oil blend, both containing minimal protein. If ingredient nutrient ADC were additive, the ADC of protein in all 4 diets would be equal. However, ADC of protein differed among diets (Table 3). At the low feeding level, the negative effect of dietary carbohydrate level on ADC of protein is in line with a previous study (Takeuchi *et al.*, 1979). This was also found in African catfish possibly due to the increased chyme viscosity in the stomach (Harter *et al.*, 2015). Averaged over all diets, raising feeding level declined the protein ADC in carp (Table 3), which was in agreement with studies on African catfish (Henken *et al.*, 1985) and Nile tilapia (Haidar *et al.*, 2016). Decreasing dietary protein significantly increased protein digestibility only at the high feeding level, which was also observed at high feeding levels on mirror carp (Ufodike and Matty, 1983). In the current study, the largest protein ADC decline in the protein-rich diet may reflect an upper limit for protein digestion but may also be due to a larger fraction of the endogenous faecal nitrogen loss.

In the present study, the negative effect of dietary fat level on fat digestibility (Table 3) is in agreement with a previous finding on common carp (Yamamoto *et al.*, 2007). However it is in contrast to previous findings in various fish species such as Nile tilapia (Schrama *et al.*, 2012), African catfish (Harter *et al.*, 2015) and Atlantic salmon (Bendiksen *et al.*, 2003). This might reflect a lower capacity for fat digestion in common carp compared to other fish species. The lowered fat digestibility observed at the high feeding level in the present study, especially in fat-rich diet, indicating carp maximal fat digestion capacity reached.

Increasing crude fibre in the carbohydrate fraction reduced total carbohydrates digestibility while increasing the starch contribution leads to the opposite (Kirchgessner *et al.*, 1986). This is illustrated by the low NSP and high starch digestibility observed in the present study. Digestibility of NSP was increased by the addition of starch to Diet 2, but not by the addition of both starch and lipids to Diet 4. Digestion of NSP is assumed to result mainly from intestinal bacterial fermentation, as was suggested for Nile tilapia (Haidar *et al.*, 2016). The high digestibility of NSP in starch-rich diet (Diet 2) was thus most likely caused by a higher intestinal fermentation activity (Yamamoto *et al.*, 2007). However, NSP ADC did not

improve in Diet 4 rich in starch and fat. Dietary fat inhibits intestinal microbial activities, which could have led to reduced NSP digestibility (Heinritz *et al.*, 2016).

The utilization efficiency of DE or ME for growth (k_{gDE} and k_{gME}) are commonly based on linear regression of RE with DE or ME intake (*i.e.*, the slope of the linear regression). This approach does not account for the possible effect of dietary macronutrient composition on k_{gDE} and k_{gME}. In NE approach (*i.e.*, multiple regression between RE and intake of digested macronutrients including dCP, dFat, dCarb), a differentiation in energy utilization efficiency of digested protein ($k_{NE,dCP}$), fat ($k_{NE,dFat}$) and carbohydrates ($k_{NE,dCarb}$) is made. In the current study, such a NE approach was applied in two experiments with common carp and barramundi. The large contrast among digestible nutrients intake created in both experiments (Table 2) facilitated the multiple regression between RE and dCP, dFat and dCarb intake. This allowed to assess energy utilisation efficiencies for growth for each type of digested macronutrient (respectively, $k_{NE;dCP}$, $k_{NE;dFat}$, and $k_{NE;dCarb}$). In Table 4, a species comparison of NE formulas (*i.e.*, $k_{NE;dCP}$, $k_{NE;dFat}$, and $k_{NE;dCarb}$) is made among barramundi and carp (this study), Nile tilapia and trout (Schrama *et al*., 2018) and pigs (CVB, 1993; Noblet *et al*., 1994). Table 4 shows that for all fish species studied, of all the nutrients digested, fat has the highest energy efficiency ($k_{NE;dFat}$), in line with results obtained for pigs. When considering the linear regression equations only, $k_{NE;dFat}$ ranged from 86 to 94%, while $k_{NE;dCP}$ ranged from 46 to 64% and $k_{NE;dCarb}$ from 18 to 84%. This also shows that the variability in energy efficiency was lowest for dFat and highest for dCarb. The lowest $k_{NE;dFat}$ was observed for carp (this study), which is in line with the general statement that carp are less able to utilise dietary fat (NRC, 2011). However, although lower, the energy efficiency of fat did not decrease with increasing fat intake (linear relationship between NE and dFat (Supplementary Fig. S2). Even at high dietary fat levels ($> 28\%$, Diet 3 and 4), RE was not reduced with increased dFat intake. This suggests that carps are not less able to handle fat at high dietary levels but that their overall efficiency of fat utilisation is lower than that of other fish species. However, the smaller variability in $k_{NE;dFat}$ between fish species (Table 4) seems to demonstrate that the energy efficiency of dFat is more conserved among fish species than that of dCP and dCarb.

Schrama *et al.* (2018) reported that the estimated energy efficiency of protein ($k_{NE;dCP}$) in rainbow trout was reduced when the quadratic component of dCarb was included in the multiple regression analysis. The estimated $k_{NE;dCP}$ was then closer to that observed in Nile tilapia and pigs. In the current study, the relation of dCarb with NE was curvilinear for

barramundi. Inclusion of the quadratic component in the NE formula (Eq. 3 and 4; Table 4) for barramundi did not strongly affect the estimated $k_{NE;dFat}$ (35.2 vs. 37.1 kJ.g^{-1}) and $k_{NE, dCP}$ (15.9 vs. 15.2 kJ.g^{-1}) . This absence of inference is probably due to the low value of $k_{NE;dCarb}$ and the relatively low dCarb intake. When including the quadratic component into the equation for both barramundi and trout, it appears that the estimated $k_{NE, dCP}$ is higher than the estimates for $k_{NE;dCP}$ in common carp and Nile tilapia. This suggests that the energy efficiency of digested protein might be different between fish species. This might suggest a higher $k_{NE;dCP}$ for fish having a higher trophic level (barramundi and trout vs. common carp and Nile tilapia). Among carnivorous species, there seems to be a difference in $k_{NE;dCP}$ as the efficiency was higher for barramundi than for rainbow trout (64 vs. 57%). This difference could be related to differences in glucose tolerance among carnivorous fish. Barramundi appeared to be less capable of handling hyperglycaemia than rainbow trout (Palmer and Ryman, 1972, Stone, 2003), although both of them are glucose intolerant. The limited capacity of barramundi to handle digested dietary glucose is confirmed by the extremely lower $k_{NE;dCarb}$, compared to other fish species (carp, trout, tilapia) and pigs (Table 4). This may cause protein and lipid to be used more efficiently by barramundi to compensate for the low energy efficiency of carbohydrate in this study. Estimation of NE equations in other carnivorous fish species with low glucose tolerance would help better understanding the potential influence that glucose tolerance has on the estimation of $k_{NE;dCP}$ in carnivorous fish.

The linearity in the pig NE evaluation system facilitates estimation of diets NE value since feeding level does not affect the energy efficiency of macronutrients. This also applies to evaluation of dietary NE values for Nile tilapia (Schrama $et\ al$., 2018) and carp (present study). Conversely, estimation of diet NE value for barramundi is feeding level-dependent because of the curvilinear relation observed between dCarb intake and diet NE value. Curvilinearity in relationship between retained energy and dCarb shows that raising carbohydrate intake decreases the potential retention of energy ($i.e$., NE value of diet), which illustrates that carnivorous fish have difficulties to handle carbohydrates (Glencross $et\ al$., 2017). The potential use of carbohydrates, of which only sugars and starch are nutritionally available to fish (Stone, 2003, Kaushik, 2001), is dependent on the key enzymes involved in digesting starch, metabolising (Enes $et\ al$., 2009, Krogdahl $et\ al$., 2005) and transporting glucose (Krasnov $et\ al$., 2001, Planas $et\ al$., 2000, Teerijoki $et\ al$., 2000) and inducible glucokinase (Panserat $et\ al$., 2001a). Fish can efficiently absorb starch-derived glucose through the intestine (Furuichi and Yone, 1981). As a consequence of increasing dCarb

intake, glucose levels increase in the blood stream in most species (Furuichi and Yone, 1981, Bergot, 1979a). If carbohydrate utilisation efficiency is assessed based on the rate of glucose distribution from absorption in digestive system to clearance in blood stream, carnivorous fish seems to be poor carbohydrate users (NRC, 2011). The rate of delivering glucose, its peak concentration in blood and clearing rate depend on species as well as carbohydrate sources and dietary inclusion levels (Stone, 2003, Bergot, 1979b, Hemre and Hansen, 1998, Wilson and Poe, 1987). In vertebrates, the role of liver in monitoring glucose homeostasis by being both consumer and producer of glucose is important. Several enzymes can be either turned on or off to dispose glucose, synthesize glycogen and lipid from glucose when the blood glucose pool increases, or to initiate de novo glucose synthesis and release glucose from glycogen when blood glucose decreases in order to meet fish glucose demand (Kamalam *et al.*, 2017). In fish, when blood glucose levels are over the threshold of glycaemia, glucose is released through urine and gills (Deng *et al.*, 2001, Hemre and Kahrs, 1997). Therefore, blood glucose concentration is dependent on the glucose flux as a result of producing and removing glucose simultaneously (Pilkis and Granner, 1992, Postic *et al.*, 2004). In carnivorous species, like rainbow trout, the liver is not able to downregulate the production of glucose in response to high dietary carbohydrates levels (Panserat *et al.*, 2001b). This contrasts with herbivorous and omnivorous species like carp and seabream (Panserat *et al.*, 2002). In the present study, carp also did not indicate any problems to handle dCarb. Barramundi on the other hand seems to be unable to handle any excess amount of digestible carbohydrate over $1.5 - 2.0$ g.kg$^{-0.8}$.d^{-1}, which is lower than that of rainbow trout ($3.0 - 3.5$ g.kg$^{-0.8}$.d^{-1}) (Schrama *et al.*, 2018). This can be because the peak concentration of blood glucose when challenged with glucose input is lower in barramundi than in rainbow trout, indicating that barramundi is less tolerant to glucose than rainbow trout (Stone, 2003, Legate *et al.*, 2001). This observation validates that carnivorous fish have difficulties to handle carbohydrate-rich feeds. Carbohydrates were used less efficiently in carp (60%) than in tilapia (66%) (Schrama *et al.*, 2018). These $k_{NE,dCarb}$ were lower than that of dStarch in pigs using either French NE approach (84%) (Noblet *et al.*, 1994) or Dutch NE approach (78%) (CVB, 1993).

By using DE approach, the energy utilisation efficiency of barramundi and carp were determined based on the slope in the linear regression of RE as a function of DE intake. Though variations appeared in the slopes among diets between two species (Supplementary Fig S1) partly because of the diversification in protein, lipid and carbohydrates sources included in the feed formulation, the DE approach (*i.e.,* factorial approach) is unable to

specify the differences and quantify energy efficiency values of digested protein ($k_{NE,dCP}$), fat ($k_{NE,dFat}$) and carbohydrates ($k_{NE,dCarb}$). By using the NE approach (*i.e.*, the multiple regression between RE and digested macronutrients), these values however can be assessed for each species and significant differences in the energy utilisation efficiency of digested protein and carbohydrates between these two species can be clarified.

Conclusion

This study proves that the dietary energy utilisation efficiency of fish is affected by the relative composition of dietary digestible macronutrients, which are dCP, dFat and dCarb. This effect on the energy utilisation efficiency was distinct between carp and barramundi. For carp, dCP, dFat and dCarb show linear relationships to the energy retention. The estimated energy efficiencies of dCP, dFat and dCarb for energy retention were 47, 86 and 60%, respectively, showing large degree of similarity with Nile tilapia and pigs. However, for barramundi, dCP and dFat were linearly related to NE, but dCarb was curvilinearly related to NE. Increasing dCarb intake results in an inflexion of dietary NE towards a plateau, illustrating the limited capacity of barramundi, a carnivorous, glucose-intolerant fish, to handle dietary starch/glucose. In this study, NE equations for carp and barramundi c were estimated to predict the potential for energy retention of diets/ingredients. The linearity in relationship between RE and intake of dCP, dFat and dCarb in carp implies that assessing the feed NE value for carp is applicable, regardless of feed intake. Conversely, the curvilinear relationship found between dCarb and NE in barramundi indicates that barramundi diet NE value depends on daily carbohydrate intake. Therefore, NE evaluation of barramundi feeds requires estimates of the feed intake, dietary carbohydrate content and digestibility.

Supplementary data to this article can be found online at ...

Acknowledgement

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Table 1
Formulation and composition in each of four experimental diets for carp

	Diet 1	Diet 2	Diet 3	Diet 4
	"Protein"	"Protein"	"Protein"	"Protein"
		+ Carb	+ Fat	+Carb +Fat
Diet formulation (g.kg^{-1}, as-is)				
Gelatinised Maize Starch	-	342.9	-	300
Oil blend[*]	-	-	178.6	125
Fish meal (CP>680)	156.5	102.9	128.6	90
Wheat gluten	156.5	102.9	128.6	90
Pea protein concentrate	156.5	102.9	128.6	90
Soya protein concentrate	156.5	102.9	128.6	90
Wheat	234.8	154.3	192.9	135
Fish oil	34.8	22.9	28.6	20
Soy oil	34.8	22.9	28.6	20
Monocalciumphosphate	34.8	22.9	28.6	20
Lime (CaCO$_3$)	8.7	5.7	7.1	5
L-Lysine sulphate	3.68	1.79	3.00	2.05
DL-Methionine	4.69	2.89	3.92	2.90
Premix	17.4	11.4	14.3	10
Yttrium oxide	0.2	0.2	0.2	0.2
Nutrient composition (g.kg^{-1}, DM)				
DM	926	887	940	918
Crude protein	529	339	430	285
Digestible protein	506	319	412	273
Total lipid	110	71	258	186
Digestible lipid	103	65	231	168
Total carbohydrate	265	528	234	474
Digestible carbohydrate	191	471	172	428
Total starch	152	330	126	391
Digestible starch	149	329	125	390
Gross energy (kJ.g^{-1})	21.20	19.64	24.92	22.39
Digestible energy (kJ.g^{-1})	19.06	17.92	22.24	20.46
Ash	96	62.4	78.7	54.8
Phosphorus (total)	16.8	10.9	13.5	9.3

Diet 1, high protein diet; Diet 2, supplemental starch diet; Diet 3, supplemental lipid diet; Diet 4, supplemental starch and lipid diet; Carb, Carbohydrates. DM, dry matter.
[*]Equal amount of rapeseed, soya and palm oil.

Table 2

Digestible nutrient intake and energy balance of carp (n =3) and barramundi (n=2), fed 4 different diets (mean values and standard deviations)

Variables	Carp				Barramundi			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Digestible nutrient intake (g.kg$^{-0.8}$.d^{-1})								
dCP	4.95	1.56	2.82	7.96	5.08	2.56	2.59	9.63
dFat	1.86	0.93	0.66	3.59	1.12	0.65	0.39	2.60
dCarb	4.22	2.24	1.71	7.88	0.94	1.15	0.04	4.16
Energy balance parameters (kJ.kg$^{-0.8}$.d^{-1})								
GE intake	291	71	204	399	224	113	120	407
DE intake	263	62	186	352	180	90	98	328
Branchial urinary energy losses	11.4	3.8	6.4	19.2	9.9	4.8	5.1	19.3
ME intake	251	60	178	341	170	86	92	309
Heat production	111	21	86	139	67	28	43	137
Energy retention (total)	140	41	86	207	103	61	45	209
Energy retention as protein	49	15	27	79	61	33	29	115
Energy retention as fat	91	35	37	152	44	29	12	98
Fat retention efficiency (% of digestible intake)	1.27	0.5	0.77	2.35	0.94	0.31	0.45	1.59

Min, minimum; Max, maximum; dCP, digestible protein intake; dFat, digestible fat intake; dCarb, digestible carbohydrate intake; GE, gross energy; DE, digestible energy; ME, metabolisable energy.

Table 3

Apparent digestibility coefficient (ADC) (%) of dietary nutrients in carp (n=3) fed 4 diets at 2 feeding levels (FL) over 28 days

Nutrient	FL	Diet 1 "protein"	Diet 2 "protein" +Carb	Diet 3 "protein" +Fat	Diet 4 "protein" +Carb+Fat	SEM	*P* values Diet	FL	Diet x FL
DM						0.625	<.0001	<.0001	<.0001
	Low	85.2[bcd]	88.0[ab]	84.7[cd]	89.0[a]				
	High	81.4[e]	87.6[abc]	83.3[de]	88.6[a]				
Energy						0.423	0.0002	0.0002	0.0002
	Low	91.7[a]	91.6[a]	90.2[ab]	91.7[a]				
	High	88.1[c]	90.9[a]	88.2[bc]	91.0[a]				
Protein						0.255	<.0001	<.0001	<.0001
	Low	96.4[a]	94.3[cd]	96.3[a]	95.5[abc]				
	High	94.7[bcd]	94[d]	95.3[abc]	95.7[ab]				
Fat						0.616	<.0001	<.0001	<.0001
	Low	95.1[a]	92.2[ab]	91.5[bc]	91.4[bc]				
	High	92.9[ab]	91.3[bc]	87.8[d]	88.8[cd]				
Carbohydrates						0.942	<.0001	<.0001	<.0001
	Low	76.4[b]	89.7[a]	73.6[b]	90.5[a]				
	High	67.9[c]	88.8[a]	73.2[b]	90.3[a]				
Starch						0.258	<.0001	<.0001	<.0001
	Low	99.6[a]	99.7[a]	99.5[a]	99.9[a]				
	High	96.7[b]	99.3[a]	98.6[a]	99.8[a]				
NSP						2.988	<.0001	0.0457	0.0474
	Low	45.3[b]	73.0[a]	43.5[bc]	45.8[b]				
	High	29.3[c]	71.1[a]	43.6[bc]	45.3[b]				
Ash						2.631	0.532	0.532	0.532
	Low	36.6	33.8	31.6	33.9				
	High	32.0	38.2	32.8	36.3				
Phosphorus						2.203	0.1255	0.1255	0.126
	Low	47.5	46.3	48.5	49.8				
	High	42.9	49.8	49.5	51.7				

Diet 1, high protein diet; Diet 2, supplemental starch diet; Diet 3, supplemental lipid diet; Diet 4, supplemental starch and lipid diet; carb, carbohydrates; DM, dry matter; NSP, non-starch polysaccharides

[abc]If interaction effect is significant, means lacking a common superscript differ significantly (*P*<0.05)

Table 4

Estimated net energy equations in common carp, barramundi, Nile tilapia, rainbow trout and pigs

| Species	Equation*	R^2	References	
Carp	NE = 11.2 dCP + 34.1 dFat + 10.4 dCarb	0.99	Present study	(2)
Barramundi	NE = 15.2 dCP + 37.1 dFat + 3.1 dCarb	0.99	Present study	(3)
Barramundi	NE = 15.9 dCP + 35.2 dFat + 9.4 dCarb − 1.9 (dCarb)^2	0.99	Present study	(4)
Trout	NE = 15.1 dCP + 35.0 dFat + 12.1 dCarb	0.91	Schrama *et al.*	(5)
Trout	NE = 13.5 dCP + 33.0 dFat + 34.0 dCarb − 3.64 (dCarb)^2	0.92	Schrama *et al.*	(6)
Tilapia	NE = 11.5 dCP + 35.8 dFat + 11.3 dCarb	0.99	Schrama *et al.*	(7)
Pig	NE = 11.3 dCp + 35.0 dFat + 14.4 ST + 12.1 dRest		Noblet *et al.*	(8)
Pig	NE = 10.8 dCp + 36.1 dFat + 13.5 dST$_e$ + 9.5 dST$_f$ + 9.5 dNSP		CVB	(9)

NE, net energy; RE, retained energy; dCP, digestible protein; dFat, digestible fat; dCarb, digestible carbohydrates (comprising of starch, sugars and NSP); dRest, the remaining dietary fraction being digestible (dRest = DM − dCP − dFat − ST − digestible ash) (see Noblet *et al.*); dST$_e$, enzymatically digestible starch; dST$_f$, the amount of starch that is digested after microbial fermentation; ST, starch (both enzymatically and fermentable degradable); dNSP, dgestible NSP.

*In the estimated equation of the present study, NE is expressed in $kJ.kg^{-0.8}.d^{-1}$ and digestible nutrient intakes (dCP, dFat and dCarb) in $g.kg^{-0.8}.d^{-1}$. In the NE equations for pigs, NE is expressed in $MJ.kg^{-1}$ feed and digestible nutrients in $g.kg^{-1}$ feed.
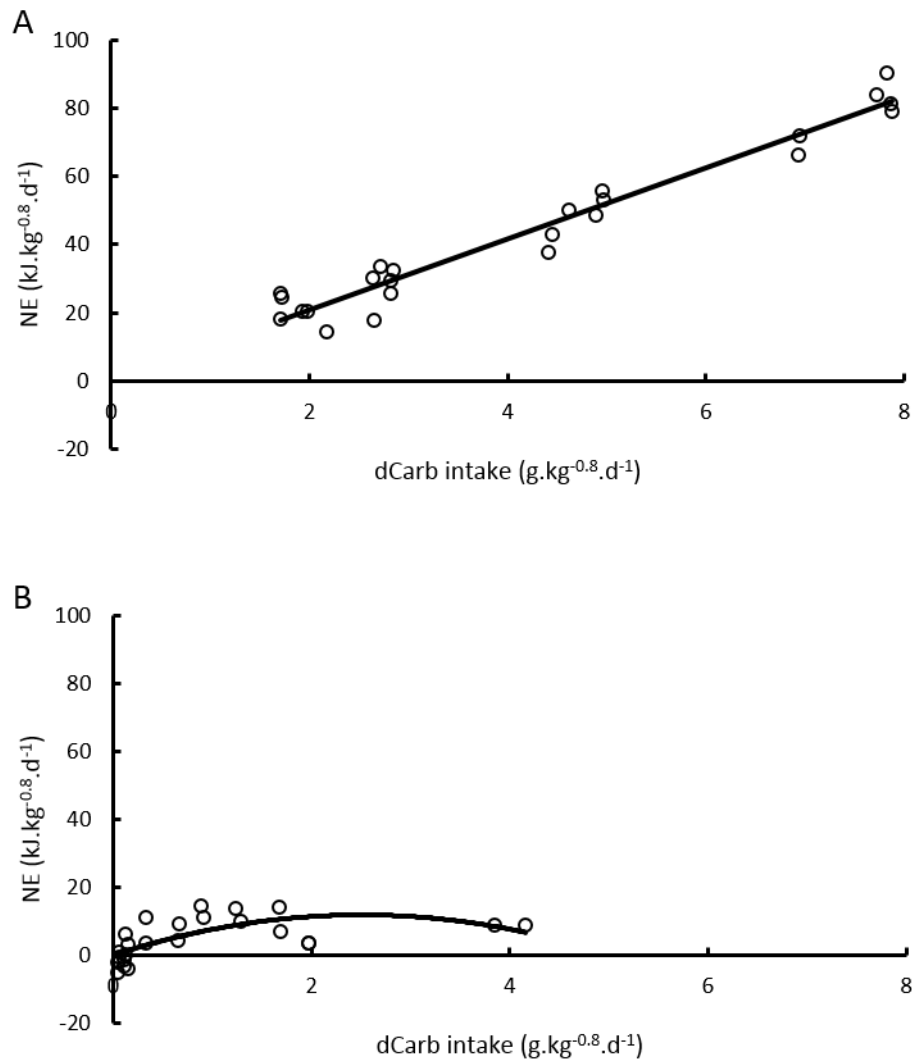
Fig. 1

Fig 1. Relationship between net energy (NE) and digestible carbohydrate (dCarb) intake for carp (a) and barramundi (b). The NE values are corrected for variation in digestible protein (dCP) and digestible fat (dFat). This was performed as follows: the measured retained energy value for each data point in the data set 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. This was conducted using Equation (2) for Carp and Equation (4) for Barramundi (Table 4).

Highlights

Formulating balanced fish diets requires a precise energy evaluation system.
Net energy equations were estimated for common carp and barramundi.
Energy utilisation efficiency differs between digested macronutrients.
Especially energy utilisation efficiency of carbohydrate differs between carp and barramundi