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Original Research Article

Differences in utilisation of digestible macronutrients between two different size classes of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The aim of this study was to investigate whether fish size has an effect on the utilisation efficiency of digestible protein, digestible fat and digestible carbohydrates (Carb) for energy gain in rainbow trout. Four different diets ranging in protein, fat and Carb were fed to two size classes of rainbow trout, 30 to 75 g and 92 to 214 g, at three different feeding levels (12, 8 and 4 g/kg$^{0.8}$ per day). This led to 24 treatments with 2 replicates (tanks). Tanks contained 36 fish for the 30 to 75 g fish and 24 fish for the 92 to 214 g fish. Faeces was collected for the determination of the digestibility of protein, fat and Carb. Energy retention was determined from the initial and final body composition. The design of this trial allowed for multiple regression analysis to determine the utilisation efficiency of the different digestible macronutrients for energy gain. By doing so, it was also possible to establish specific net energy (NE) formulae for 30 to 75 g and 92 to 214 g rainbow trout. The results showed that there were differences between the two size classes in the estimated NE formulae and the corresponding partial efficiencies of digestible protein, fat and Carb for NE ($k_{gNE,CP}$, $k_{gNE,Fat}$ and $k_{gNE,Carb}$). The $k_{gNE,CP}$ was not different ($P = 0.586$) between the two size classes (on average 76%), but the $k_{gNE,Fat}$ decreased ($P = 0.005$) from 82% to 65% in the bigger fish and the $k_{gNE,Carb}$ showed a tendency ($P = 0.077$) to increase from 55% to 73% in the bigger fish. The findings of this study showed that fish size has an effect on the estimation of net energy in feeds for rainbow trout.

1. Introduction

A detailed understanding of dietary energy utilisation is crucial for accurate feed evaluation. Whereas in the past feed formulation was predominantly based on digestible energy (DE), it is now shifting towards a net energy (NE) approach. The main advantage of this approach is that it accounts for differences in the utilisation efficiencies of digestible protein, digestible fat and digestible carbohydrates (Carb). This system has already been applied within pig nutrition for many years (Blok et al., 2015; Noblet et al., 1994; van Milgen et al., 2001). The first NE model for rainbow trout was developed in 2018 (Schrama et al., 2018), and a recent article demonstrated that the net energy approach enhances the accuracy of feed evaluation when compared to the digestible energy approach (Groot et al., 2021).

Recent research has shown that the efficiency of digestible energy use for growth (k_{gDE}) in rainbow trout can also be affected by the composition of growth of different genetic strains. More specifically, a higher potential for fat deposition in some strains can lead to a higher k_{gDE} (Groot et al., 2022). This higher k_{gDE} may be related to the lower energetic cost of depositing fat versus that of depositing protein (energetic cost of protein deposition is 1.79 to 1.90 kJ per kJ protein energy deposited compared to 1.10 to 1.31 kJ per kJ fat deposited (Lupatsch et al., 2003)). This suggests that both the dietary macronutrient composition and the genetic background

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of the fish need to be taken into account when moving to a NE system in this species.

The composition of growth is however not only different between different genetic strains but is also known to be related to fish size. Bigger fish have a higher fat to protein gain than smaller fish (Dumas et al., 2007) which therefore could potentially also influence the k_{gDE}.. The studies of Azevedo et al. (2004) and Einen and Roem (1997), have observed that the gross energy utilisation efficiency in other salmonids (lake trout and salmon) decreases with increasing fish size which was suggested to be related to an increase in maintenance energy requirement of bigger fish. However, these studies only focused on the gross energy utilisation efficiency and did not separate the energy used for maintenance which excluded the possibility of investigating specific effects of fish size on k_{gDE}. The study of Glencross (2008) is one of the only studies that did look at the effect of fish size on the k_{gDE} in barramundi and here they did saw that the k_{gDE} increased as the fish became more energy dense.

The current study aimed to further assess the robustness of a NE approach for feed evaluation in rainbow trout. More specifically, we investigated whether fish size influences the utilisation of different digestible macronutrients for energy gain in a NE approach for rainbow trout. The same batch of four diets with different ranges of protein, fat and Carb were fed to two different size classes of rainbow trout which were selected based on their expected differences in composition of growth (protein-to-fat gain ratio). These two size classes included a “small” group which ranged from 30 to 75 g on average and a “big” group which ranged from 92 to 214 g on average. Both groups came from the same batch of fish, but reflected different growing phases. The study was divided into two experimental periods. The first experimental period included the 30 g fish, whilst the remaining fish from the original batch of fish was on-grown to 92 g for use in the second experimental period. Three different feeding levels were used which permitted a multiple regression analysis to determine the utilisation efficiency of each specific macronutrient for energy gain whilst separating the maintenance energy requirement. The experiment thus aimed to assess whether specific NE formulae are required for different size classes of rainbow trout.

2. Materials and methods

2.1. Animal ethics statement

This experiment was part of project number AVD23-30020197264 and was conducted in accordance with the Dutch law on the use of experimental animals (Act on Animal Experiments) approved by the Central Animal Experiments Committee (CCD) of The Netherlands. The experiment was performed in the experimental facilities of the Alltech Coppens Aqua Centre (Leende, The Netherlands), and fish were managed and handled in agreement with the current EU-legislation on maintaining experimental animals.

2.2. Aquaria system design

The experiment was performed in a system with twenty-four 200 L Guelph-style metabolic tanks. These Guelph-style tanks were constructed according to the original Guelph type system (Cho et al., 1982) with the exception of one settling tank per fish tank. All fish tanks were connected into one recirculating system with a water purification unit and oxygenating reactor. Within this system, solids not collected by the settling unit of each tank were removed by a drum filter and NH_4^+ by nitrification in a bio filter. Furthermore, a protein skimmer was present, and bacteria were controlled by ozonisation and UV disinfection. Fresh well water was added to the system daily (approximately 15%/d). Total NO_2^- and NH_4^+ were measured twice a week in the outlet water of the tank using the MQuant Ammonium test and MColortest Nitrite test (both from Supelco). Averaged over both experimental periods, mean NO_2^- and NH_4^+ were 0.1 (SD 0.0) and 0.0 mg/L (SD 0.0), respectively. Water pH, redox potential, temperature and oxygen content were monitored continuously with a SCADA system (OxyGuard Pacific Monitoring Units). Average pH, redox potential and temperature values were 8.1 (SD 0.1), 225.4 mV (SD 11.8) and 15.9 °C (SD 0.2) respectively. The outlet water oxygen saturation remained between 95% and 105%.

2.3. Experimental design and diets

The experiment consisted of a 2 × 4 × 3 factorial design: two size classes (30 to 75 g and 92 to 214 g fish), four diets (protein, Carb, fat and Carb + fat) and three feeding levels. This led to 24 treatments in total with 2 replictes (tanks). The experiment was divided into two experimental periods, the first started with 30 g fish and the second started with 92 g fish. All fish for both experimental periods originated from a single batch, where one cohort was used for the first experimental period, whilst a second cohort was on-grown to 92 g for use in the second experimental period. Both size classes were fed with the same 4 diets on three feeding levels to allow for multiple regression analysis between digestible protein, fat and Carb intake on retained energy (RE). The first experimental period lasted for 6 weeks whereas the second experimental period lasted for 8 weeks. This increase in length of the second period was chosen as bigger fish tend to have a slower relative growth than smaller fish, and the aim was to at least double the average body weight in both experimental periods. The three feeding levels were defined as feed intake per metabolic bodyweight per day. This allowed to keep the three feeding levels equal across the different periods for the two size classes. The experimental diets were formulated using Bestmix Feed (Adifo, Industrielaan 11B 9990 Maldegem, Belgium). Diets were formulated to create a wide contrast in macronutrient composition which was achieved by adding gelatinized potato starch and rapeseed oil on the same iso-energetic basis to the basal diet to form the Carb and fat diet. For the Carb + fat diet, both gelatinized potato starch and rapeseed oil were added to the basal diet. All diets were formulated to meet the vitamin, mineral, essential fatty acid and amino acid requirements of rainbow trout (NRC, 2011). The diet formulae and the analysed macronutrient content are displayed in Table 1. Yttrium oxide was added to all diets as an indigestible marker to permit the measurement of apparent nutrient digestibility coefficients.

The experimental diets were produced by SPAROS LDA (Olhão, Portugal). All powder ingredients (excluding both rapeseed and fish oil) were mixed according to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 400 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets with a pellet size of 3 mm were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. The extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France) for approximately 12 min with a temperature gradient ranging from 120 °C in the first section and 70 °C at the exit. After cooling, the remaining oils were added to the feed by vacuum coating (700 mbar, for approximately 90 s) (model PG-10VCLAB, Dinnissen, The Netherlands). Immediately after coating, diets were packed in sealed plastic buckets and shipped to the research facilities of Alltech Coppens.

Table 1
Formulation and analysed nutrient composition of experimental diets.

Item	Protein	Carb	Fat	Carb + Fat
Ingredients, g/kg on as is basis				
Fishmeal[1]	420.6	276.3	346.1	241.8
Potato starch	0.0	343.0	0.0	300.0
Wheat	266.7	175.1	219.4	153.2
Rapeseed oil	0.0	0.0	177.0	125.0
Wheat gluten[2]	100.0	65.7	82.3	57.5
Soya protein concentrate[3]	100.0	65.7	82.3	57.5
Fish oil	88.0	57.8	72.4	50.6
Mineral and vitamin premix[4]	13.2	8.7	10.8	7.6
Monocalciumphosphate	7.0	4.6	5.7	4.0
Choline chloride	4.4	2.9	3.6	2.5
Yttrium	0.2	0.2	0.2	0.2
Nutrient composition, g/kg dry matter				
Dry matter, g/kg as is	952	915	945	947
Crude protein	514	346	419	297
Crude fat	161	100	317	225
Crude ash	82	59	67	50
Carb[5]	243	496	198	428
NFE[6]	231	478	187	415
Starch	187	412	154	363
NSP[7]	56	84	44	66
Crude fiber	12	18	10	13
Gross energy, MJ/kg	22.0	21.0	24.8	23.2

[1] Fish meal from Capeline (*Mallotus villosus*), supplied by Köster Marine Proteins, Germany.
[2] Wheat gluten supplied by Beneo, Belgium.
[3] Soya protein concentrate supplied by Cefetra Feed Service, Netherlands.
[4] One kilogram of premix provide the following: zinc chelate of protein hydrolysates 100,000 mg; manganese chelate of protein hydrolysates 16,700 mg; copper (II) chelate of protein hydrolysates 4200 mg; calcium iodate, anhydrous 800 mg; vitamin A 3,333,333 IU; vitamin E 66,667 mg; vitamin K_3 8333 mg; vitamin B_1 3333 mg; vitamin B_2 6666 mg; vitamin B_6 5000 mg; vitamin B_{12} 13.33 mg; biotin 333 mg; niacinamide 50,000 mg; pantothenic acid 13,333 mg; folic acid 2666 mg; inositol 50,000 mg; sodium calcium ascorbyl phosphate 100,000 mg.
[5] Carbohydrate (Carb) content equals dry matter minus the sum of protein, fat and ash.
[6] NFE = nitrogen free extract, equals dry matter minus the sum of protein, fat, ash and crude fibre.
[7] NSP = non-starch polysaccharides, equals dry matter minus the sum of protein, fat, ash and starch.

2.4. Animal management

The two size classes of rainbow trout (*Oncorhynchus mykiss*) used in the experiment were selected based on contrasting levels of body fat content and thus in composition of growth. The analysed levels of initial body fat content on a fresh weight basis was 120 g/kg for the 30 to 75 g fish and 146 g/kg for the 92 to 214 g fish. The rainbow trout used in the experiment were all female and obtained from FREA A/S (Kibæk, Denmark). All fish were fasted for one day before being bulk weighed. After weighing, 36 fish were randomly assigned to each tank in the first experimental period whereas 24 fish were randomly assigned to each tank in the second experimental period, as these fish were bigger. The 24 tanks in each experimental period were randomly assigned to the 12 treatments for each size class. An additional 10 fish in each experimental period were euthanized with a lethal dose of phenoxyethanol (1 mL/L water) and stored at −20 °C for the determination of initial whole-body nutrient composition. These 10 fish were selected with the condition that they were ±10% of the mean weight of each size class.

The fish were fed twice daily for one week before the start of the experiment in order to acclimate them to the test diets. Three feeding levels were used, which were 12, 8 and 4 g/kg$^{0.8}$ per day, respectively. These levels were chosen as the highest level was close to satiation feeding and the lowest level was just above maintenance. Two thirds of the daily ration was hand fed at 07:00 with the remainder being fed at 14:30. Uneaten pellets were counted after each feeding period and multiplied by the average weight per pellet to calculate the amount of uneaten feed in grams. Faeces samples were collected in chilled bottles below each of the individual Guelph tanks during week 2, 4 and 6 (and 8 in the second experimental period tanks) respectively. Each faecal collection period lasted 7 days. Faecal material was recovered every day before each feeding period and stored at −20 °C. All samples were pooled per fish tank prior to freeze drying and nutrient analysis.

At the end of the experiment the fish were fasted for one day before the final sampling. The final sampling consisted of weighing all fish. Six fish per tank within ±10% of the mean final weight of the tank were randomly selected and euthanized with a lethal dose of phenoxyethanol and were stored at −20 °C prior to analysis of whole-body nutrient composition.

2.5. Sample analysis

All chemical analyses were performed in duplicate by Nutricontrol BV (Ncb Laan 52, 5462 GE Veghel). Upon completion of the trial, both faecal and fish samples were homogenized and then freeze-dried prior to analysis. The analysis of feed, faecal and fish samples consisted of the following determinations; dry matter (DM) content by drying at 103 °C until constant weight for 4 and 24 h respectively (ISO 6496, 1999); ash content after incineration at 550 °C for 4 h (ISO 5984, 2002); crude protein (CP) based on nitrogen × 6.25 using the Kjeldahl method (ISO 5983, 2005); fat after an initial acid-hydrolysis step followed by a petroleum-diethyl ether extraction (ISO 6492, 1999); phosphorus by an internal method using an optical spectroscopic technique based on NEN-EN 15510:2017; gross energy content with the adiabatic bomb calorimeter method (ISO 9831, 1998); starch after an enzymatic degradation using the hexokinase method (ISO 6493, 2000); crude fibre by an internal method and determined as the fat-free organic substance which is insoluble in acid and alkaline media (underlying method as in Commission Regulation [EC] No 152/2009).

2.6. Calculations

The calculations of the different parameters assessed are displayed in Table 2. Furthermore, the utilisation efficiencies of both digestible nitrogen (k_{kDN}) and energy (k_{gDE}) were estimated as the slope of the linear regression analysis of digestible nitrogen versus retained nitrogen (both as mg/kg$^{0.8}$ per day) and digestible energy versus retained energy (both as kJ/kg$^{0.8}$ per day). The requirement of both digestible nitrogen and energy for maintenance (DN_m and DE_m) was also estimated from these regressions as the point where the line crosses the x-axis at $y = 0$. The utilisation efficiency of the digestible macronutrients for retained energy were estimated as the k factors in the multiple regression analysis with digestible crude protein ($k_{NE,CP}$), fat ($k_{NE,Fat}$) and Carb ($k_{NE,Carb}$) (all as g/kg$^{0.8}$ per day) on retained energy (as kJ/kg$^{0.8}$ per day). Unfortunately, there was insufficient fecal material available to perform the moisture and energy analysis in of one of the two protein diet groups at the low feeding level for the small fish. Therefore $n = 23$ for the following parameters in the small fish: dry matter, Carb, non-starch polysaccharides (NSP) and energy digestibility, and digestible and metabolizable energy intake, and heat production. For all other parameters $n = 24$, but because of this difference in observations it was decided to report the pooled SEM for these parameters.

2.7. Statistics

The individual tanks were designated as the experimental unit in all statistical analysis, which was performed using the program

Table 2
Calculations of the different parameters used in this study.

Parameter	Calculation
MBWm, $kg^{0.8}$	$\{[\text{Initial BW (kg)} + \text{Final BW (kg)}]/2\}^{0.8}$
Growth, $g/kg^{0.8}$ per day	{[Final BW (g) − Initial BW (g)]/MBWm ($kg^{0.8}$)}/Days
Feed intake, $g/kg^{0.8}$ per day	[Feed consumed (g)/MBWm ($kg^{0.8}$)]/Days
FCR	Feed intake ($g/kg^{0.8}$ per day)/Growth ($g/kg^{0.8}$ per day)
Condition factor, g/cm^3	100 × BW (g)/Body length $(cm)^3$
HSI, %	Liver weight (g)/Body weight (g)
VSI, %	Visceral weight (g)/Body weight (g)
Corrected VSI, %	[Visceral weight (g) − Liver weight (g)]/Body weight (g)
ADC, %	1 − [Yttrium feed (g)/Yttrium faeces (g)] × [Nutrient faeces (g)/Nutrient feed (g)]
N intake, $mg/kg^{0.8}$ per day	Feed intake ($g/kg^{0.8}$ per day) × Dietary N content (mg/g)
Digestible N intake, $mg/kg^{0.8}$ per day	N intake ($mg/kg^{0.8}$ per day) × N digestibility coefficient (%)
Retained N, $mg/kg^{0.8}$ per day	{[Final N concentration in the body (mg/g) − Initial N concentration in the body (mg/g)]/MBWm ($kg^{0.8}$)}/Days
BUN losses, $mg/kg^{0.8}$ per day	Digestible N intake ($mg/kg^{0.8}$ per day) − Retained N ($mg/kg^{0.8}$ per day)
Gross energy intake, $kJ/kg^{0.8}$ per day	Feed intake ($g/kg^{0.8}$ per day) × Gross energy content of diet (kJ/g)
DE intake, $kJ/kg^{0.8}$ per day	Gross energy intake ($kJ/kg^{0.8}$ per day) × Energy digestibility coefficient (%)
BUE losses, $kJ/kg^{0.8}$ per day	BUN losses ($mg/kg^{0.8}$ per day) × Energy concentration of NH_3-N (24.9 kJ N/g)[1]
ME intake, $kJ/kg^{0.8}$ per day	DE intake ($kJ/kg^{0.8}$ per day) - BUE losses ($kJ/kg^{0.8}$ per day)
RE, $kJ/kg^{0.8}$ per day	{[Final energy concentration in the body (kJ/g) − Initial energy concentration in the body (kJ/g)]/MBWm ($kg^{0.8}$)}/days
Heat production, $kJ/kg^{0.8}$ per day	ME intake ($kJ/kg^{0.8}$ per day) − RE ($kJ/kg^{0.8}$ per day)

MBWm = mean metabolic body weight; BW = body weight; FCR = feed conversion ratio; HSI = hepatosomatic index; VSI = visceral somatic index; ADC = apparent digestibility coefficient; N = nitrogen; BUN = branchial urinary nitrogen; DE = digestible energy; BUE = branchial urinary energy; ME = metabolizable energy; RE = retained energy.

[1] Assuming that all N will be excreted as NH_3-N.

SPSS statistics 20, (IBM Statistics Inc., USA). Growth performance, digestibility coefficients, body composition, and nitrogen and energy balance parameters were analyzed using a three-way ANOVA for the effect of size, diet, feeding level and all interaction terms. Analysis was performed with a confidence interval of 0.05. Regression analysis on retained energy was performed with digestible energy intake as covariate and size and diet as fixed factors which was possible as no curvilinearity was detected. For this, the following model was used:

$$RE_i = \mu + \beta \times DE + e_i,$$

where μ is the intercept, β is the digestible energy utilisation efficiency; e_i is error term and $i = 1, \ldots,$ with $n = 6$ per diet per size.

However, in the case of digestible nitrogen, curvilinearity was observed for the protein diet in both size classes. The curvilinearity here made it impossible to include size and diet as fixed factors and therefore regression analysis on retained nitrogen was only performed using digestible nitrogen as covariate. For this, the following model was used:

$$RN_i = \mu + \beta \times RN + e_{i,}$$

where μ is the intercept, β is the digestible protein utilisation efficiency; e_i is error term and $i = 1, \ldots,$ with $n = 6$ per diet per size.

Additionally, multiple regression analysis was performed on retained energy using digestible protein intake, digestible fat intake and digestible Carb intake as covariate and size as fixed factor. This was done using the following the model:

$$RE_i = \mu + \beta_1 \times dCP_i + \beta_2 \times dFat_i + \beta_3 \times dCarb_i + e_i\ ,$$

where RE is retained energy; μ is the intercept, being an estimate for fasting heat production (FHP); dCP is digestible protein intake; dFat is digestible fat intake; dCarb is digestible Carb intake; β_1, β_2, β_3 are the energy utilisation efficiency of dCP ($k_{NE;dCP}$), dFat ($k_{NE;dFat}$) and dCarb ($k_{NE;dCarb}$); e_i is error term and $i = 1, \ldots, 48$. Also in this multiple regression analysis, curvilinearity was checked for dCP, dFat and dCarb intake but was not present in this study. NE was calculated from these relationships as retained energy plus the intercept which reflects the fasting heat production.

3. Results

In this study four diets and three feeding levels were used to allow multiple regression analysis of digestible protein, fat and Carb intake against RE. This multiple regression was used to estimate the utilisation efficiency of each specific macronutrient for energy gain. However, the effect of diet and feeding level on growth, body composition and digestibility were not a goal to address in this study. Therefore, only the main effect of size is shown for these parameters. An overview of the average values for every treatment and the full outcome of the three-way ANOVA with effect of size (small and big), diet (protein, Carb, fat and Carb + fat) and feeding level (low, middle and high) can be found in Tables S1–S5 in the supplementary data.

Performance data are shown in Table 3. Feed intake per $kg^{0.8}$ was affected by size ($P < 0.001$), but numerically the differences in feed intake were minor. Averaged over the feeding levels and diets, the fish more than doubled their weight during each experimental period. The difference in start and final biomass between the groups was inherent to the design of the trial, but there was also an effect of size on growth of the fish ($P < 0.001$). The small fish had a higher growth and lower FCR as compared to the bigger fish ($P < 0.001$). The bigger fish were also observed to have a lower HSI ($P = 0.009$), but a higher visceral somatic index (VSI) when corrected for liver weight ($P < 0.001$) than the small fish.

Table 3
Main effect of size (small versus big) on performance parameters and body indexes.

Item	Small	Big	SEM	*P*-value
Growth period, d	46	60	–	–
No. of fish per tank	36	24	–	–
Survival, %	99.1	99.5	0.27	0.296
Start biomass per fish, g	29.9	91.8	0.19	<0.001
Final biomass per fish, g	75.3	214.0	0.48	<0.001
Feed intake, $g/kg^{0.8}$ per day	8.0	8.1	0.02	<0.001
Growth, $g/kg^{0.8}$ per day	9.7	8.6	0.03	<0.001
FCR	0.84	0.96	0.004	<0.001
Condition factor, g/cm^3	1.36	1.35	0.018	0.858
HSI, %	2.61	2.35	0.066	0.009
VSI, %	12.70	13.07	0.128	0.051
Corrected VSI for liver weight, %	10.08	10.72	0.114	<0.001

SEM = standard error of the mean; FCR = feed conversion ratio; HSI = hepatosomatic index; VSI = visceral somatic index.

All parameters of the body composition were affected by size ($P < 0.05$) (Table 4). Final mean body protein content increased slightly from 159 to 164 g/kg, but the mean body fat content even increased from 119 to 141 g/kg in the bigger fish. The body dry matter and energy content followed the same pattern in body fat content for the effect of size.

Most of the apparent digestibility coefficients (ADC) were different between both size classes (Table 5). Dry matter, crude protein, crude ash and starch were all lower ($P < 0.05$) for the bigger fish, whereas crude fat, Carb and energy were unaffected ($P > 0.05$) by size. On the other hand, ADC of NSP tended to be higher ($P = 0.081$) in the bigger fish but was negative in both size classes.

The differences in nitrogen and energy intake were statistically different ($P < 0.05$), but numerically small between the two size classes (Table 6) which reflected the difference in overall feed intake (Table 4). Branchial urinary nitrogen (BUN) was higher ($P = 0.006$) for the bigger fish and resulted in a lower retained nitrogen in the bigger fish ($P = 0.021$). Branchial urinary energy (BUE) and heat production were also higher for the bigger fish ($P < 0.05$) and resulted in a tendency for a lower retained energy ($P = 0.078$). The lower retained energy in the bigger fish seemed to be mainly related to a lower retained energy as crude protein which was also lower for the bigger fish ($P = 0.021$). Retained energy as fat was not different ($P = 0.466$) between the two size classes. Protein-to-fat gain ratio (as g/g and kJ/kJ, respectively) was not different ($P > 0.05$) between the two size classes.

The relationships between retained nitrogen, retained energy, digestible nitrogen intake and digestible energy intake are shown in Figs. 1 and 2, respectively, and are described in Tables 7 and 8. The slope of these lines reflect the partial efficiency of digestible nitrogen and energy intake for growth (k_{gDN} and k_{gDE}). Digestible nitrogen intake was linearly related to retained nitrogen in most diets. However, a curvilinear relationship between digestible nitrogen intake and retained nitrogen was found for the protein diet in both size classes (R^2 ranges from 0.979 to 0.999). Because of this curvilinearity in two of the treatments it was not possible to investigate the effect of size and diet on this relationship in a general linear model. Numerically, the slopes of these relationships are generally lower for the bigger fish. The relationship between digestible energy intake and retained energy were all only linearly related to each other. Statistical analysis showed a significant effect of diet ($P = 0.008$) on k_{gDE} whereas size only showed a tendency for an effect ($P = 0.091$) on the k_{gDE}. Differences in the k_{gDE} between diets were more pronounced in the smaller size class. On average, the k_{gDE} decreased from 77% to 73% in the bigger fish indicating that the bigger fish were less efficient in converting digested energy into energy gain. Although the interaction effect was not significant ($P = 0.142$), numerically some differences were observed between k_{gDE} depending on size. For the small fish the highest k_{gDE} was found for the fat diet (84%), whereas for the bigger fish the k_{gDE} was highest for the protein diet (79%). The intercept of the lines, which reflects the fasting heat production, were similar ($P = 0.376$). This indicated that the DE_m was not different between the two size classes.

Table 4
Main effect of size (small versus big) on body composition.

Item	Small	Big	SEM	*P*-value
Final body composition (on fresh basis)[1]				
Dry matter, g/kg	300	326	1.3	<0.001
Protein, g/kg	159	164	0.8	<0.001
Fat, g/kg	119	141	1.6	<0.001
Gross energy, MJ/kg	8.3	9.2	0.06	<0.001

SEM = standard error of the mean.

[1] Initial body composition on (on fresh weight basis) was as follows for the small fish: dry matter 290 g/kg; protein 150 g/kg; fat 120 g/kg; gross energy 8.1 MJ/kg; and for the big fish: dry matter 312 g/kg; protein 144 g/kg; fat 146 g/kg; gross energy 9.1 MJ/kg.

Table 5
Main effect of size (small versus big) on apparent digestibility coefficients (%).

Item	Small	Big	SEM	*P*-value
Dry matter	84.0	83.4	0.14[3]	0.011
Crude protein	93.1	92.4	0.07	<0.001
Crude fat	94.9	94.9	0.10	0.982
Crude ash	63.2	60.1	0.22	<0.001
Carb[1]	70.9	70.5	0.28[3]	0.313
Starch	94.4	93.4	0.19	<0.001
NSP [2]	−29.5	−27.0	0.96[3]	0.081
Energy	87.7	87.4	0.17[3]	0.365

SEM = standard error of the mean.

[1] Carbohydrates (Carb) equals dry matter minus the sum of protein, fat and ash.

[2] NSP = non-starch polysaccharides, equals dry matter minus the sum of protein, fat, ash and starch.

[3] This was calculated as pooled SEM due to missing value in the small group.

Table 9 shows the net energy formulae derived from the multiple regression analysis between retained energy and digestible protein intake, digestible fat intake and digestible Carb intake. The k factors in these formulae reflect the net energy value of each macronutrient in kJ/kg$^{0.8}$ per day whereas the intercept reflects the fasting heat production. Dividing the k factors in these formulae by the combustible energy content of the different macronutrients (23.6 kJ/g for protein, 39.5 kJ/g for fat and 17.2 kJ/g for Carb) results in the partial efficiencies of these macronutrients for net energy ($k_{gNE,CP}$, $k_{gNE,Fat}$ and $k_{gNE,Carb}$). The $k_{gNE,CP}$ was not significantly different ($P = 0.586$) between size classes with an average value of 76%. An increase in size decreased the $k_{gNE,Fat}$ from 82% to 65% ($P = 0.005$) and showed a tendency to increase the $k_{gNE,Carb}$ from 55% to 73% ($P = 0.077$). The intercepts and thus the fasting heat production were similar between size classes ($P = 0.588$). No curvilinearity was detected for each of the tested parameters in the model.

4. Discussion

The current study investigated whether fish size has an effect on the utilisation of different digestible macronutrients for energy gain in a NE approach for rainbow trout in order to establish whether specific NE formulae are required for different size classes within this fish species. Two size classes were selected (small and big) based on their expected differences in composition of growth (protein-to-fat gain ratio). The fish in both size classes originated from the same batch of fish (same genetic background) and were fed with the same batch of the different diets.

Apparent digestibility coefficients of most nutrients were lower for the bigger fish which was unexpected as the gastrointestinal system was expected to be better developed and established in the bigger fish. Although differences in nutrient digestibility between the size classes were statistically different, numerically the differences were small (<1%). Literature on the effect of fish size on digestibility is both limited and conflicting, with contrasting trends being described in different studies. Studies with milkfish and Gibel carp reported that digestibility increases with size (Ferraris et al., 1986; Liu et al., 2016), whereas the study of Windell et al. (1978) showed limited differences in protein digestibility with only a significantly lower in protein digestibility in rainbow trout of 18 g at 7°. On the other hand, in a study with African catfish the effect of size seemed to be dependent upon the protein source (Usmani and Jafri, 2002). The limited impact of size on digestibility in the current study could also mean that the size difference between the two

Table 6
Main effect of size (small versus big) on nitrogen and energy balances.

Item	Small	Big	SEM	*P*-value
N intake, mg/kg$^{0.8}$ per day	472	478	1.0	<0.001
Digestible N intake, mg/kg$^{0.8}$ per day	438	441	1.0	0.028
BUN, mg/kg$^{0.8}$ per day	182	192	2.5	0.006
Retained N, mg/kg$^{0.8}$ per day	256	249	2.1	0.021
GE intake, kJ/kg$^{0.8}$ per day	171	173	0.4	<0.001
DE intake, kJ/kg$^{0.8}$ per day	149	151	0.4[1]	0.006
BUE, kJ/kg$^{0.8}$ per day	4.5	4.8	0.06	0.006
ME intake, kJ/kg$^{0.8}$ per day	144	146	0.4[1]	0.013
Heat production, kJ/kg$^{0.8}$ per day	60	64	1.2[1]	0.025
Retained energy, kJ/kg$^{0.8}$ per day	84	82	1.0	0.078
Retained energy as fat, kJ/kg$^{0.8}$ per day	47	48	1.1	0.466
Retained energy as CP, kJ/kg$^{0.8}$ per day	38	37	0.3	0.021
Protein-to-fat gain ratio, g/g	2.4	2.2	0.47	0.813
Retained energy as CP/retained energy as fat, kJ/kJ	1.4	1.3	0.40	0.813

SEM = standard error of the mean; N = nitrogen; BUN = branchial and urinary nitrogen losses; GE = gross energy; DE = digestible energy; BUE = branchial and urinary energy losses; ME = metabolizable energy; CP = crude protein.

[1] SEM was calculated as pooled SEM due to missing value in the small group.

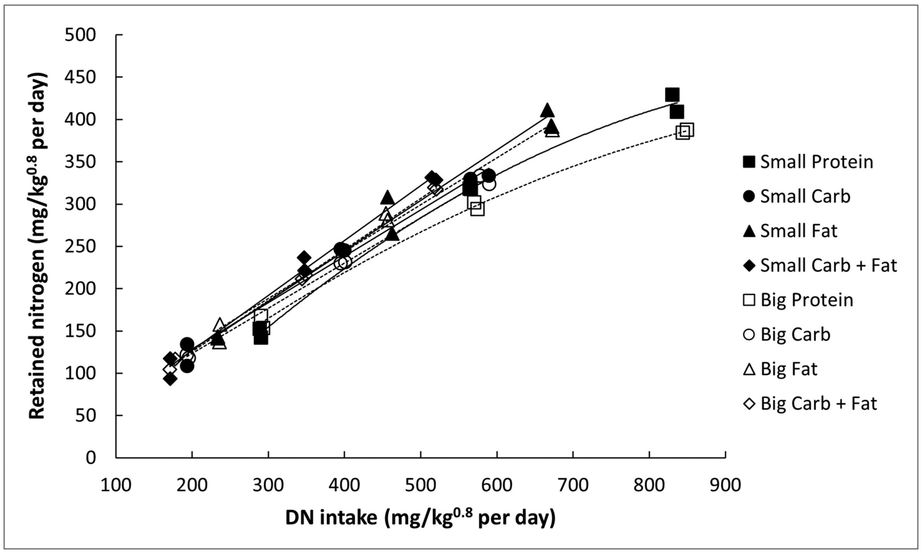

Fig. 1. The relationship between digestible nitrogen (DN) intake (mg/kg$^{0.8}$ per day) and retained nitrogen (mg/kg$^{0.8}$ per day) for two size classes of rainbow trout (small, solid lines and solid symbols; big, dashed lines and open symbols) fed four different diets (protein, Carb, fat and Carb + fat). The estimated regression lines are presented in Table 7. Carb = carbohydrates.

groups was insufficient to detect appreciable effects. More specifically, it could be inferred that there were no differences in the development of the intestinal tract between the two size classes and as such any differences in digestibility between size classes were therefore minimal. NSP digestibility showed negative values in the current study which was most probably related to the fact that NSP is a calculated value and is dependent on the analysis of all other nutrients in the feed and faeces. Therefore, the value is also influenced by the allowed analysis error of these other nutrients. The fact that it is negative implies that NSP was not digested by rainbow trout in the current study and that the digestible Carb fraction consisted primarily of digested starch. This finding was consistent with previous studies of the authors (Groot et al., 2021, 2022).

Body protein and fat were both higher in the bigger fish, which is in agreement with the described differences in growth composition reported in literature (Dumas et al., 2007; Kaushik and Schrama, 2022). However, even though mean body fat content was higher in the bigger fish (142 versus 119 g/kg) the average calculated protein-to-fat gain ratio was only slightly lower and not significantly affected by size in this study. This finding was contradictory to both the author's expectation and the published literature and could again mean that the size difference in the current study was insufficient to find this difference. However, a significantly higher VSI (especially when corrected for the liver weight) was measured in the larger size class, meaning that the bigger fish apportioned a relatively higher amount of energy towards the viscera. Another reason for the limited difference in protein-to-fat gain ratio could also be due to a limit in fat deposition in the bigger fish. Body fat content in the small fish was related to digestible fat intake in a linear way, however, in the bigger fish this relationship was curvilinear (Fig. 3 and Table 10). The curvilinearity in this relationship could suggest a limit in fat deposition and that the trout in the current study did not deposit additional fat when the final body fat content is 170 to 180 g/kg. Additionally, the dry matter and energy content of the fish were observed to be affected by the interaction effect between size and diet (Table S3 in the supplementary data) indicating the size classes indeed reacted differently on the diets depending on size for these parameters. A tendency for the same effect was also found for body fat content

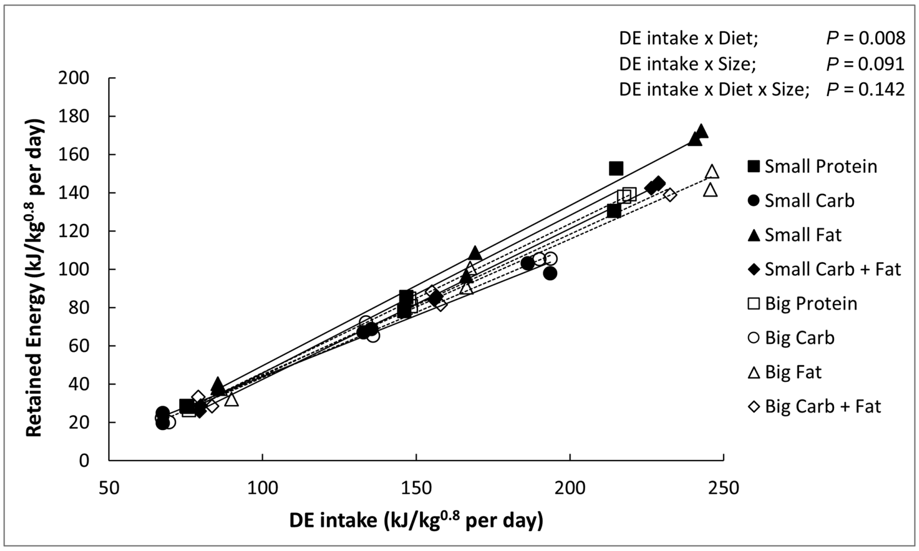

Fig. 2. The relationship between digestible energy (DE) intake (kJ/kg$^{0.8}$ per day) and retained energy (kJ/kg$^{0.8}$ per day) for two size classes of rainbow trout (small, solid lines and solid symbols; big, dashed lines and open symbols) fed four different diets (protein, Carb, fat and Carb + fat). The estimated regression lines are presented in Table 8. Carb = carbohydrates.

Table 7
The relationships between DN intake and RN for two size classes of rainbow trout (small and big) fed four different diets (protein, Carb, fat and Carb + fat).

Size	Diet	Regression line	R^2	DN$_m$, mg/kg$^{0.8}$ per day
Small	Protein	RN = −106.5 ± 33.08 + (1.005 ± 0.133) × DN intake - (0.0004 ± 0.0001) × DN intake^2	0.996	111
Small	Carb	RN = 19.1 ± 13.22 + (0.548 ± 0.031) × DN intake	0.987	−35
Small	Fat	RN = 6.5 ± 21.19 + (0.596 ± 0.043) × DN intake	0.979	−11
Small	Carb + Fat	RN = −2.6 ±12.57 + (0.649 ± 0.034) × DN intake	0.989	4
Big	Protein	RN = −33.6 ± 24.41 + (0.753 ± 0.097) × DN intake - (0.0003 ± 0.0001) × DN intake^2	0.997	45
Big	Carb	RN = 16.4 ± 6.79 + (0.535 ± 0.016) × DN intake	0.996	−31
Big	Fat	RN = 21.4 ± 13.57 + (0.555 ± 0.028) × DN intake	0.990	−39
Big	Carb + Fat	RN = 4.8 ± 3.37 + (0.604 ± 0.009) × DN intake	0.999	−8

Carb = carbohydrates; DN = digestible nitrogen, mg/kg$^{0.8}$ per day; RN = retained nitrogen, mg/kg$^{0.8}$ per day; DN$_m$ = requirement of digestible nitrogen for maintenance.

Table 8
The relationships between DE intake and RE for two size classes of rainbow trout (small and big) fed four different diets (protein, Carb, fat and Carb + fat).

Size	Diet	Regression line	R^2	DE$_{m,}$ kJ/kg$^{0.8}$ per day
Small	Protein	RE = −36.4 ± 13.84 + (0.823 ± 0.082) × DE intake	0.971	44
Small	Carb	RE = −19.7 ± 4.93 + (0.638 ± 0.035) × DE intake	0.988	31
Small	Fat	RE = −34.1 ± 5.50 + (0.838 ± 0.031) × DE intake	0.995	41
Small	Carb + Fat	RE = −36.2 ± 2.04 + (0.787 ± 0.012) × DE intake	0.999	46
Big	Protein	RE = −31.5 ± 2.18 + (0.776 ± 0.014) × DE intake	0.999	41
Big	Carb	RE = −24.8 ± 4.13 + (0.682 ± 0.029) × DE intake	0.993	36
Big	Fat	RE = −25.5 ± 6.91 + (0.706 ± 0.039) × DE intake	0.988	36
Big	Carb + Fat	RE = −29.6 ± 5.99 + (0.740 ± 0.036) × DE intake	0.991	40

DE = digestible energy, kJ/kg$^{0.8}$ per day; RE = retained energy, kJ/kg$^{0.8}$ per day; Carb = carbohydrates; DE$_m$ = requirement of digestible energy for maintenance.

Table 9
The estimated NE[1] formulae derived from the linear relationships between RE and dCP, dFat, and dCarb intake for two size classes of rainbow trout (small and big).

Size	Equation	R^2
Small	NE = RE + 29.9 ± 3.35 = (18.5 ± 1.24) × dCP + (32.2 ± 1.69) × dFat + (9.7 ± 1.37) × dCarb	0.989
Big	NE = RE + 27.6 ± 2.66 = (17.6 ± 1.03) × dCP + (25.8 ± 1.39) × dFat + (12.9 ± 1.11) × dCarb	0.991
Combined	NE = RE + 28.7 ± 2.58 = (18.0 ± 0.98) × dCP + (29.0 ± 1.32) × dFat + (11.3 ± 1.06) × dCarb	0.983
Schrama et al. (2018)	NE = RE + 50 ± 9 = (15.1 ± 1.18) × dCP + (35.0 ± 2.00) × dFat + (12.1 ± 1.98) × dCarb	0.910

NE = net energy; RE = retained energy, kJ/kg$^{0.8}$ per day; dCP = digestible crude protein, g/kg$^{0.8}$ per day; dFat = digestible fat, g/kg$^{0.8}$ per day; dCarb = digestible carbohydrate, mg/kg$^{0.8}$ per day.

[1] NE was calculated as retained energy plus the fasting heat production (being the intercept of the model).

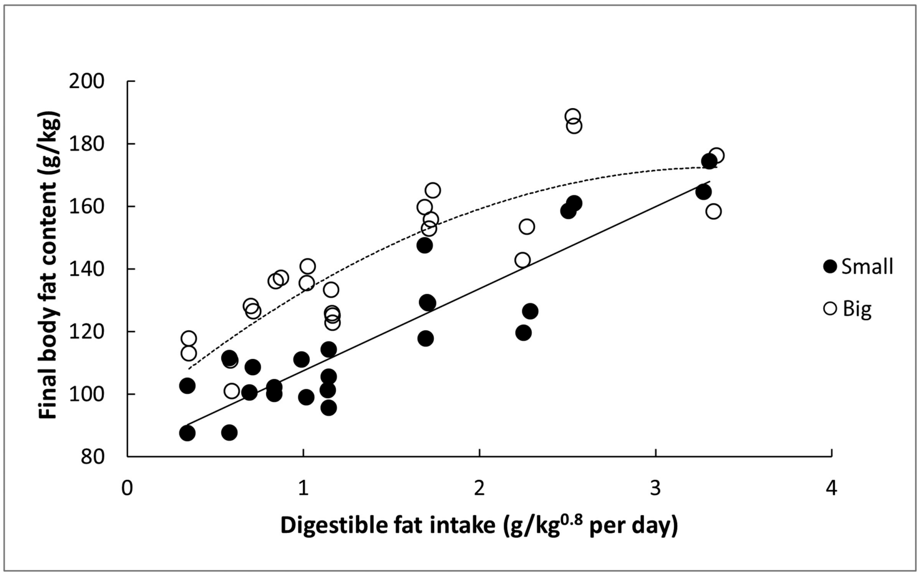

Fig. 3. The relationship between digestible fat intake (g/kg$^{0.8}$ per day) and final body fat content (g/kg) for two size classes of rainbow trout (small, solid lines and solid symbols; big, dashed lines and open symbols). The estimated regression lines are presented in Table 10.

Table 10
The relationships between digestible fat (dFat) intake (g/kg$^{0.8}$ per day) and final body fat content (g/kg) for two size classes of rainbow trout (small and big).

Size	Equation	R^2
Small	Final body fat content = 81.3 ± 4.35 + (26.2 ± 2.61) × dFat	0.821
Big	Final body fat content = 92.3 ± 9.07 + (47.6 ± 12.01) × dFat − (7.05 ± 3.27) × dFat^2	0.758

"±" sign refers to the standard error.

which could support the suggestion for a limit in fat deposition at a higher body fat content in the current study.

Retained nitrogen expressed per metabolic bodyweight was lower for the bigger fish and was caused by a higher BUN in these fish. A reduced nitrogen retention with increased fish size has been found before in other studies with salmonids (Azevedo et al., 2004; Einen and Roem, 1997). The authors of these studies suggested that the lower level of nitrogen retention in bigger fish is related to a higher maintenance requirement for energy leaving less energy to be used for protein deposition. The lower nitrogen retention was also reflected in the k_{gDN}, where in general lower values were observed for the bigger fish. However, for the k_{gDN} it was also observed that in the protein diet for both sizes the relationship was curvilinear which might indicate a maximum protein deposition. This maximum protein deposition could be linked to the high digestible protein intake with this diet, combined with a high protein-to-energy ratio leading to a situation where part of the protein is used as an energy source rather then used for protein deposition. The concept of maximum protein deposition is not uncommon and has previously been suggested in rainbow trout (Bureau et al., 2006; Glencross et al., 2007, 2008, 2009).

Retained energy tended to be lower in the bigger fish and could at least partly explain the lower retained nitrogen as this indicated that less energy was available to support body protein deposition. The lower retained energy was partly related to a higher BUE, but the biggest difference was reflected in a higher heat production per unit growth. This effect was also reflected in the k_{gDE} which tended to be lower in the bigger fish and decreased on average from 77% to 73%. A potential reason for this lower k_{gDE} could be related to the fate of protein, with a larger portion of the protein being used as an energy source rather than for protein deposition in the bigger fish. The efficiency of ATP production from protein is lower as compared to glucose or fat (Blaxter, 1989). Additionally, also the use of dietary protein for lipid deposition is in theory energetically less efficient than when being used to deposit body protein (Blaxter, 1989). The lower k_{gDE} was in contrast to the finding of Glencross (2008) which did see that in barramundi the k_{gDE} increased in relation to a higher energy density with increasing fish size. Even though the bigger fish in this study were also energy denser, it was already discussed that the protein-to-fat gain ratio was not significantly different which could explain the difference with the study of Glencross (2008). The k_{gDE} was found to be affected by diet with the biggest change in the fat diet and less for the protein and carb diets. No difference in the DE_m was found between the two size classes or the different diets. This is in contrast to the suggestion from literature that a decrease in energy retention with increasing fish size may be related to a higher maintenance requirement for energy (Azevedo et al., 2004; Einen and Roem, 1997).

The main focus of the current study was on the NE formulae derived from the multiple regression analysis on retained energy and digestible macronutrient intake. The derived NE formulae for both size classes combined led to the following estimated utilisation efficiencies: 76% for the $k_{gNE,CP}$, 73% for the $k_{gNE,Fat}$ and 64% for the $k_{gNE,Carb}$. When comparing the $k_{gNE,CP}$, $k_{gNE,Fat}$ and $k_{gNE,Carb}$ to the estimated values from the meta-analysis of Schrama et al. (2018), the average $k_{gNE,CP}$ measured in the present study is higher (76% versus 64%), the $k_{gNE,Fat}$ is lower (73% versus 89%) and the $k_{gNE,Carb}$ is somewhat lower but comparable (64% versus 69%). The $k_{gNE,CP}$ was not only higher when compared to the meta-analysis, it is also markedly higher when compared to values found for most other species (Tilapia, Carp, Barramundi, Striped catfish, snakehead) which range from 47% to 64% (Phan et al., 2019, 2021a,b; Schrama et al., 2018). The only exception here is in African Catfish where the value was also comparably high, at 86% (Phan et al., 2022). The high $k_{gNE,CP}$ reported in African catfish was reported to be potentially caused by an underestimation of the protein ADC, which would result in an underestimation of digestible protein and thereby in an overestimation of the $k_{gNE,CP}$. However, when comparing protein utilisation efficiencies between fish species, it is also important to consider the role of trophic level. Carnivorous species like rainbow trout could for example have a higher efficiency for protein in general. This is also reflected in the high values for the k_{gDN} in the current study which reached a level of 65%. Even still, the value for the $k_{gNE,CP}$ in the current study is also appreciably higher than in the meta-analysis from Schrama et al. (2018). This difference could be explained by a different factor. First of all, there could be a difference in the k_{gDN} between studies and a higher k_{gDN} could potentially lead to a higher $k_{gNE,CP}$. Secondly, the study of Schrama et al. (2018) was a meta-analysis versus a direct estimation in the current study. The $k_{gNE,Fat}$ on the other hand was, at 73%, lower than the values estimated for all other fish species thus far which range from 78% to 95%. This could partly be explained by similar reasons as those discussed for the higher $k_{gNE,CP}$, but may also be attributed to whether digestible fat was used for ATP production or deposited as body fat. The $k_{gNE,Carb}$ was, at 64%, lower than the $k_{gNE,Fat}$ which was in line with both the expectation at the outset of the experiment and earlier findings that indicate that digestible Carb are used less efficiently as an energy source when compared to digestible fat. In the current study digestible Carb were only linearly related to retained energy. This was contradictory to the meta-analysis of Schrama et al. (2018) which observed a curvilinear relationship for digestible Carb indicating that digestible Carb could only be used efficiently up to a certain maximum point. In the present study there was no indication for a limited $k_{gNE,Carb}$ at higher intake level of digestible Carb. This was surprising as trout is often reported to have a limited capacity to regulate blood glucose (Dai et al., 2014; Enes et al., 2009; Hemre et al., 1990; Kirchner et al., 2008; Moon, 2001; Panserat et al., 2001). The potential role of genetics should not be discounted in this finding. Several different studies have reported a higher Carb utilisation in some genetic strains. This is often related to a higher lipogenic potential which is also considered as one of the key factors that leads to high muscle fat content in fat trout strains (Jin et al., 2014; Kolditz et al., 2008; Skiba-Cassy et al., 2009; Song et al., 2018). An earlier study of this group also found a similar effect where the k_{gDE} was higher for a fat strain as compared to a lean strain using a high Carb diet (Groot et al., 2022).

A significant effect of fish size was observed within the estimated NE formulae derived from the present study. This indicated that the utilisation of digestible macronutrients for energy gain did depend on fish size. More specifically it was observed that even though the $k_{gNE,CP}$ was not different (on average 76%), the $k_{gNE,Fat}$ was significantly lower for the bigger fish (65% versus 82%) and the $k_{gNE,Carb}$ showed a tendency to be higher in the bigger fish (73% versus 55%). These differences in the $k_{gNE,Fat}$ and the $k_{gNE,Carb}$ between both sizes could partly be related to a different way of using these macronutrients in the body. It could for example suggest a higher use of glucose for ATP production in the bigger fish. The lower $k_{gNE,Fat}$ of 65% in the bigger fish could also be related to the limit in fat deposition at a higher body fat content in the current study as discussed before. The $k_{gNE,Fat}$ of 82% in the small fish was more in line with the values reported in other species which range from 78% to 95%. The tendency for a higher $k_{gNE,Carb}$ for the bigger fish indicated that the utilisation of Carb increases with increasing fish size. In a study with tilapia, bigger fish were reported to possess an improved glucose tolerance compared to small fish (Pen-Hsing and Shi-Yen, 1993) which might be related to a more developed metabolism. The study of Sundby et al. (1991) found higher plasma insulin with increased body weight in different salmonid species which could play a role here even though insulin has been discussed to poorly regulate Carb utilisation in salmonids (Enes et al., 2009; Moon, 2001). Although the NE formulae were affected by size for the $k_{gNE,Fat}$ and the $k_{gNE,Carb}$, no differences were found in the fasting heat production which is reflected by the intercept of the model.

5. Conclusion

This study showed that NE formulae for rainbow trout are affected by fish size which was related to differences in the partial efficiencies of digestible macronutrients for NE. Future lines of research could be aimed at further examining this effect of fish size in other fish species or could be aimed at further evaluation of the robustness of the NE formulae for rainbow trout.

Credit Author Statement

Ruben Groot: Conceptualization, Methodology, Formal analysis, Investigation, Project administration, Writing – Original Draft, Visualization. **Philip Lyons:** Conceptualization, Resources, Writing – Review & Editing. **J.W. Schrama:** Conceptualization, Methodology, Investigation, Writing – Review & Editing Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: the research project is funded by Alltech Inc., Dunboyne, Ireland, and Ruben Groot and Philip Lyons are currently employers of the company.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.05.014.

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