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Interactive effects of protein and energy intake on nutrient partitioning and growth in Nile tilapia

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ABSTRACT

Studies of fish growth response to changes in dietary protein and energy content are often conducted with fish fed to apparent satiation or at fixed percentages of their body mass. Such designs result in simultaneous changes in protein and non-protein energy intake, thereby failing to distinguish their separate effects on nutrient partitioning and growth. The present study was designed to address this limitation and test the existence of distinct protein- and non-protein energy-dependent growth phases in Nile tilapia (*Oreochromis niloticus*). All-male Nile tilapia (63 g, SD = 1.3) were subjected to an 8 × 2 factorial design consisting of eight levels of digestible protein (**DP**) intake (0.44–1.25 g/day) and two levels of non-protein digestible energy (**NPDE**) intake (16.0 and 22.4 kJ/day). Fish (n = 960) were housed in 60-litre tanks with two replicates per treatment and hand-fed twice a day for 42 days. Nutrient balances were calculated from changes in body mass, analysed body composition and digestible nutrient intake. Linear regression models were compared to linear-plateau regression models to determine whether protein gain followed distinct protein- and non-protein energy-dependent phases or not. Body mass gain increased linearly with increasing DP intake and was significantly higher (2.6 vs 2.3 g/d, $P < 0.05$) in fish receiving a high NPDE intake. This increase mainly reflected a higher mean fat gain (0.29 vs 0.20 g/d) rather than a higher protein gain (0.42 vs 0.39 g/d) in fish fed a high vs low level of NPDE intake. The comparison of linear and linear-plateau models did not give clear support for the presence of distinct protein and non-protein energy-dependent phases in protein gain. These results indicate that non-protein energy intake has a modest protein-sparing potential, and that protein gain is simultaneously limited by protein and energy intake in Nile tilapia.

Implications

Farmed fish require a balanced supply of protein and energy to grow. In practice, fish feeds are often formulated to meet an optimal dietary protein-to-energy ratio. This ratio, which maximises growth per unit of feed consumed, is often assumed to ensure an optimal use of dietary protein too. This experimental study shows that it is not the case, and that protein gain is simultaneously limited by protein and energy intake in male Nile tilapia. Our results provide quantitative information useful to the prediction of Nile tilapia growth response to changes in the protein and energy content of fish feeds.

Introduction

Fish obtain the energy they need to live and grow by oxidising amino acids, fatty acids and monosaccharides obtained from the digestion and absorption of dietary proteins, lipids and carbohydrates, respectively (Bureau et al., 2002). In intensive and semi-intensive aquaculture systems, feed composition dictates the relative contributions of proteins, lipids and carbohydrates to meeting fish energy needs for maintenance and growth. A practical consequence of this is that fish nutritionists can alter diet composition to optimise macronutrient utilisation of farmed species. This is particularly relevant to the utilisation of dietary protein for skeletal muscle (*i.e.*, fillet) growth. Increasing the dietary content of non-protein energy substrates (*i.e.*, lipids and carbohydrates) causes the dietary digestible protein-to-digestible energy ratio (**DP/DE**)

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to decline. This spares amino acids from being used as energy substrates (Beamish and Medland, 1986; De Silva et al., 1991; Shiau and Peng, 1993), thereby increasing their use as building blocks for protein synthesis (Cho and Bureau, 2001; Kaushik and Seiliez, 2010). For example, increasing DP/DE of Nile tilapia feeds from 16.6 to 27.4 g/MJ led to a reduction in the proportion of digested protein retained (*i.e.*, protein retention efficiency) from 53 to 32% (Haidar et al., 2018). The motivations for improving protein retention in farmed fish are primarily economic: protein-rich feed ingredients are usually the most expensive (El-Sayed, 1999; Montoya-Camacho et al., 2019; Adéyèmi et al., 2020), while protein is a major constituent of fish fillets, especially in lean species like Nile tilapia (Karl et al., 2014; Haidar, 2017). Improving dietary protein retention is also motivated by environmental considerations: oxidised amino acids are not retained by the fish but excreted as ammonia which can contribute to eutrophication of natural water bodies (Cho and Bureau, 2001; Cao et al., 2007; Luo et al., 2018).

Balancing the dietary DP/DE of fish feeds is among the most effective way of increasing protein utilisation efficiency on fish farms (Cho and Bureau, 2001; Kaushik and Seiliez, 2010). From the perspective of nutrient partitioning, finding an optimal balance between dietary digestible protein and energy (*i.e.*, an optimal DP/DE) follows the assumptions that the energetic cost of protein deposition is proportional to the amount of protein being deposited, and that absorbed amino acids are preferentially used for body protein synthesis, rather than oxidative catabolism. Under these hypotheses, deviations from the optimal DP/DE should lead to inefficient use of available amino acids (above the optimum) or energy (below the optimum). This has been extensively tested in terrestrial farmed animal species. Studies on lambs (*Ovis aries*, Black and Griffiths, 1975), pigs (*Sus domesticus*, Campbell et al., 1985; Kyriazakis and Emmans, 1992) and chickens (*Gallus gallus domesticus*, Gous et al., 2018) reported that, under restricted energy intake conditions, protein gain increases linearly with protein intake at low levels of protein intake (protein-dependent phase, Fig. 1). At higher levels of protein intake, non-protein energy intake becomes limiting (energy-dependent phase), which causes a shift in amino acid partitioning, from protein gain to oxidative catabolism, and thus a plateau in protein gain (Fig. 1). In practice, the existence of distinct protein- and energy-dependent growth phases means that the utilisation of dietary protein for growth can be maximised by adjusting the dietary DP/DE. If so, the optimal dietary DP/DE for growth (or protein gain) is the one that is realised at the transition from one phase to the other (Fig. 1). To date, there is no literature indicating that fish respond to increasing protein and non-protein energy intake with distinct protein- and non-protein energy-dependent growth phases as depicted in Fig. 1. This may be due to several physiological differences existing between fish and terrestrial animals. Fish are often considered to rely more on dietary protein as an energy substrate than terrestrial animals do, owing to their lower maintenance requirements (poikilothermy) and to their ability to derive more energy from absorbed proteins (ammoniotelism) (Kaushik and Seiliez, 2010). In addition, the non-protein energy is not equally available to all fish species. In comparison with lower trophic level species (*e.g.*, common carp *Cyprinus carpio*), some high trophic level species (*e.g.*, barramundi *Lates calcarifer* or snakehead *Channa striata*) show limited ability to derive energy from carbohydrates (Phan et al., 2019, 2021). Aspects of experimental design may also explain the absence of distinct protein- and energy-dependent growth phases in fish. Various approaches have been employed to test the interactive effects of protein and energy intake on fish growth, and ultimately to determine an optimal dietary DP/DE. These included the distribution of non-isoenergetic formulated diets in which protein was gradually replaced by lipids and/or carbohydrates (Ogino et al., 1976), or the distribution of isoenergetic diets varying in their protein content for a single (Jauncey, 1982) or multiple levels of dietary energy (Lee and Putnam, 1973; Pirozzi et al., 2010). In terms of feeding, most studies were done with fish fed either to apparent satiation (Kaushik et al., 1995; Lupatsch et al., 2001; Booth et al., 2007; Li et al., 2013) or at fixed percentages of their body mass (Jauncey, 1982; Azevedo et al., 2002; Allan and Booth, 2004). Alternative approaches including separate feeding of restricted amounts of protein and non-restricted amounts of non-protein energy (Kaushik et al., 1981; Kaushik and Luquet, 1984) and free-choice experiments (Yamamoto et al., 2000; Vivas et al., 2006; Fortes-Silva and Sánchez-Vázquez, 2012) showed that fish can regulate their energy intake in response to protein restriction and dilution. In most of these experiments, feeding conditions have led to simultaneous changes in protein and non-protein energy intake, thereby preventing the distinction of their separate effects on growth, as already pointed out by Haidar et al. (2018). In Nile tilapia, Haidar et al. (2018) observed that fish fed a fixed amount of protein (*i.e.*, under restricted feeding) increased their protein gain linearly with increasing non-protein intake. The authors made the same observation in a parallel experiment testing the same diets fed to apparent satiation (Haidar, 2017). These results contradict the presence of distinct protein- and energy-dependent phases of protein gain, and thus the existence of an optimal DP/DE within the range tested by the authors (17–27 g/MJ).

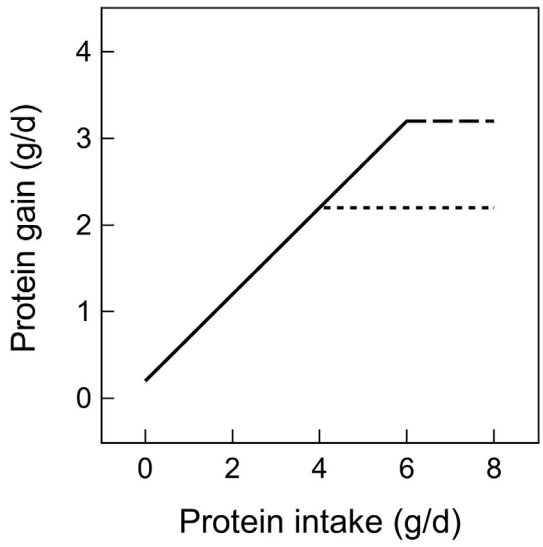

Fig. 1. In pigs (Campbell et al., 1985), lambs (Black and Griffiths, 1975) and poultry (Gous et al., 2018), distinct protein- and energy-dependent growth phases are seen in response to increasing protein intake. In the protein-dependent phase (—), protein gain is solely limited by protein intake. In the energy-dependent phases, protein gain is only limited by energy intake and levels-off at distinct levels in animals receiving low (- - -) and high (– – –) energy intake.

Published estimates of the optimal protein-to-energy ratio of Nile tilapia feeds vary from 13.3 to 26.3 g/MJ (El-Sayed and Teshima, 1992; Sweilum et al., 2005). The lack of a physiological basis for this concept (*i.e.*, the absence of distinct protein- and energy-dependent phases) may explain part of the variability. The present study was designed to test if growth follows distinct protein- and energy-dependent phases in Nile tilapia, and thus, if a physiological optimum exists for the DP/DE of Nile tilapia feeds. This was tested in an *in vivo* factorial trial involving two levels of non-protein energy intake and eight levels of protein intake. If distinct protein- and energy-dependent growth phases exist, the relationship between protein intake and protein gain should be non-linear. In this case, the transition from one phase to the other should occur at a lower level of DP intake (but a similar DP/DE) in fish fed a lower level of non-protein energy.

Material and methods

Experimental design: diets and feeding strategy

The experiment consisted of a 42-day balance trial, using a 2 × 8 factorial design, with two levels of non-protein digestible energy (**NPDE**) daily intake and eight equidistant levels of digestible protein (**DP**) daily intake. Contrasts in NPDE and DP intake were achieved by feeding 16 diets at fixed restricted levels. The 16 diets were formulated by gradually mixing a protein mix and a non-protein energy mix, while including a vitamin and mineral premix at a level which would ensure equal daily intake among all treatments. This was done to ensure that, at each of the two levels of NPDE intake, the only variation in daily nutrient intake would be in DP. Table 1 shows the ingredient content of the three ingredient mixes. Table 2 shows the feeding level, the analysed nutrient content and the digestible nutrient content of four of the 16 experimental diets, based on feed and faecal analyses. These are the diets fed to the fish receiving the combinations of the lowest (D1 and D9) and highest (D8 and D16) levels of DP intake and the lowest (D1 and D8) and highest (D9 and D16) levels of NPDE intake. The same information is given for all diets (D1–D16) in Supplementary Table S1. The protein mix was formulated with purified protein sources (Table 1) to minimise its contribution to the NPDE fraction of the experimental diets. Maize starch was included at 9.5% in the protein mix (Table 1) to guarantee pellet stability at the highest protein mix inclusion level (diet D8, Table 2). Wheat bran was included at 20% in the energy mix (Table 1) to ensure faecal stability, thereby facilitating their collection for apparent digestibility determination. Although this caused the energy mix to contain protein, the effect on the protein content of experimental diets was minor, owing to the low protein content of wheat bran (173 g/kg of DM according to Heuzé et al. (2015)) and its relatively low inclusion level in the experimental diets (ranging from 4.0 to 11.6%). Yttrium oxide was used as indigestible marker and included in the premix. The three ingredients' mixes were each produced in a single batch and consecutively mixed in varying proportions to make the 16 experimental diets. Research Diet Services (Wijk bij Duurstede, the Netherlands) produced the ingredient mixes and the experimental diets, in the form of 2 mm extruded pellets. Table 3 shows the ranges of digestible amino acid content, expressed per 100 g of DP for the four most contrasting diets (*i.e.*, those presented in Table 2). The absence of a large variation in amino acid profile across these four diets shows that dietary protein was of equal quality for all treatments. Unlike their relative content, the absolute content of each amino acid (in g/kg DM) varied largely across diets, just as CP and DP content (Table 2). This information is given in Supplementary Table S2.

The feeding strategy was to have eight equidistant levels of daily DP intake for each of the two levels of NPDE intake. Because

Table 1
Ingredient composition of the protein mix, energy mix and premix used to formulate the 16 experimental diets fed to Nile tilapia.

Ingredients (g/kg, as is)	Protein mix	Energy mix	Premix
Fish meal	225	–	–
Casein	225	–	–
Soy protein concentrate	225	–	–
Pea protein concentrate	225	–	–
DL-Methionine	5	–	–
Maize starch	95	600	–
Wheat bran	–	200	–
Soybean oil	–	70	–
Rapeseed oil	–	70	–
Fish oil	–	60	–
Freshwater fish premix[1]	–	–	200
$CaCO_3$	–	–	200
Monocalcium phosphate	–	–	597
Yttrium oxide	–	–	3

[1] Freshwater fish premix composition: Vitamins (mg or IU/100 g of premix): vit. A palmitate, 30 000 IU; vit. B1, 100; vit. B2, 100; vit. B3, 200; vit. B5, 400; vit. B6, 100; vit. B8, 2; vit. B9, 20; vit. B12, 0.15; vit. C phosphate, 1 000; vit. D3-500, 24 000 IU; vit. E, 1 000 IU; vit. K3 K-menadione sodium bisulphite (51%), 100; inositol, 4 000; choline, 15 000; Minerals (mg/100 g of premix): iron (as $FeSO_4 \cdot 7H_2O$), 500; zinc (as $ZnSO_4 \cdot 7H_2O$), 300; cobalt (as $CoSO_4 \cdot 7H_2O$), 1; copper (as $CuSO_4 \cdot 5H_2O$), 100; selenium (as Na_2SeO_3), 5; manganese (as $MnSO_4 \cdot 4H_2O$), 200; magnesium (as $MgSO_4 \cdot 7H_2O$), 5 000; chromium (as $CrCl_3 \cdot 6H_2O$), 10; iodine (as $CaIO_3 \cdot 6H_2O$), 20; Others (mg/100 g of premix): butylated hydroxytoluene (antioxidant E300–321), 1 000; calcium propionate, 10 000.

Table 2
Feeding level, ingredient mixes inclusion level and analysed nutrient content of the four most contrasting diets (*i.e.*, those fed to fish allocated to the lowest (1) and highest (8) levels of digestible protein (DP) intake for each of the two levels of non-protein digestible energy (NPDE) intake (Low and High). Realised digestible intake of Nile tilapia are given on the last rows. The same information is given for all 16 experimental diets in Supplementary Table S1.

	Low NPDE intake		High NPDE intake	
DP intake level	1	8	1	8
Diet	D1	D8	D9	D16
Feeding level (g/day)[1]	1.51	2.46	1.85	2.83
Ingredient mix[2] (g/kg)				
Protein mix	427	743	347	650
Energy mix	480	200	578	300
Premix	93	57	75	50
Analysed nutrient content (g/kg DM)				
DM (g/kg as is)	938	956	957	948
CP (N × 6.25)	329	548	273	484
Crude fat	129	79	147	98
Total carbohydrates[3]	441	283	496	338
Starch	352	204	398	256
Sugars	16	12	9	12
NSP[4]	74	67	89	71
Ash	101	90	84	79
Calcium	21.7	17.0	17.6	15.9
Phosphorus	19.1	17.3	14.8	15.3
GE (MJ/kg DM)	20.4	20.9	20.9	21.1
Non-protein GE (MJ/kg DM)	12.6	8.0	14.5	9.7
CP/GE (g/MJ)	16.2	26.2	13.0	22.9
Digestible nutrient content (g/kg DM)				
DM (g/kg as is)	791	840	812	826
Protein (N × 6.25)	306	531	253	467
Fat	124	76	142	94
Total carbohydrates[3]	361	220	408	267
Starch + sugars[5]	367	216	406	267
Ash	50	51	44	43
Calcium	8.5	6.9	8.2	5.3
Phosphorus	11.4	11.6	10.5	10.1
DE (MJ/kg DM)	18.3	19.3	18.7	19.3
NPDE (MJ/kg DM)	11.0	6.7	12.7	8.3
DP/DE (g/MJ)	16.8	27.5	13.5	24.2
Digestible nutrient intake (g/d)[6]				
DM	1.20	2.06	1.50	2.34
Protein (N × 6.25)	0.44	1.25	0.45	1.25
Fat	0.18	0.18	0.25	0.25
Total carbohydrate	0.44	0.43	0.66	0.59
NPDE (kJ/d)	15.7	15.8	22.5	22.1

Abbreviations: CP/GE = CP-to-gross energy ratio; DE = digestible energy; DP/DE = digestible protein-to-digestible energy ratio; N = nitrogen; NPDE = non-protein digestible energy; NSP = non-starch polysaccharides.

[1] Averaged over the whole experimental period (*i.e.*, total feed intake/42 days).

[2] Ingredient composition of protein mix, energy mix and premix is reported in Table 1.

[3] Calculated on DM basis as: 1 000 − (CP + fat + ash).

[4] Calculated on DM basis as: total carbohydrate − (starch + sugars).

[5] No separate digestibility coefficients were calculated for starch sugars because these were analysed together in faecal samples.

[6] Calculated as: Feeding level (g/d) × DM content (g/kg) × digestible nutrient content (g/kg DM). For example, the digestible protein intake (g/d) of diet D9 = 1.85 × 0.957 × 0.253 = 0.45 g/d. Rounding of the values reported in the table may lead to small differences between the realised digestible nutrient intake (value reported in the table) and recalculated values.

Table 3
Ranges of dietary digestible amino acid content, expressed per 100 g of digestible protein (DP). Digestible amino acid content was calculated based on apparent digestibility coefficients measured in Nile tilapia during the trial. Minimal and maximal values are given for the four most contrasting diets (*i.e.*, those fed to the fish allocated to the lowest (1) and highest (8) levels of DP intake, for each of the two levels of non-protein energy intake (Low and High)).

Amino acid	Digestible dietary content (g/100 g DP)	
	Min	Max
Alanine	4.3	4.4
Arginine	6.2	6.5
Aspartic acid	9.7	9.9
Cysteine	0.87	0.93
Glutamic acid	18	19
Glycine	3.9	4.0
Histidine	2.5	2.6
Isoleucine	4.6	4.7
Leucine	8.4	8.6
Lysine	7.2	7.5
Methionine	2.8	2.8
Phenylalanine	4.9	5.1
Proline	6.2	6.4
Serine	5.0	5.1
Threonine	3.9	4.1
Valine	5.4	5.5
All[1]	*94*	*97*

[1] Sum of all amino acids (without ammonia).

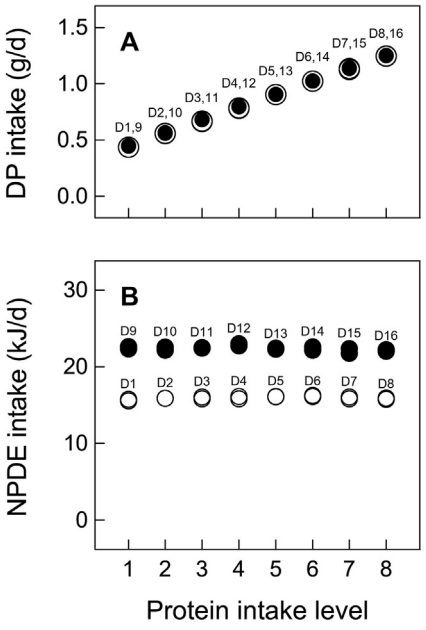

Fig. 2. Average daily intake of digestible protein (DP, panel **A**) and non-protein digestible energy (NPDE, panel **B**) of Nile tilapia (n = 32 tanks) receiving a low (○, diets D1–D8) or high (●, diets D9 – D16) level of NPDE intake for 42 days.

diets differed in their nutrient composition (Table 2), this design was achieved by applying 16 treatment-specific feeding levels. A pair-feeding scheme was implemented in which the feeding level of the fish receiving the combination of the highest levels of DP and NPDE (*i.e.*, diet D16 in Table 2) and the DP and NPDE content of each determined the feeding level of all other treatments. At the start of the experiment, the daily individual feeding level of fish fed diet D16 was set at 20 g/kg$^{0.8}$/d, close to their expected maximal voluntary intake capacity. On the first day of the experiment, the daily feed intake of fish fed diet D16 was calculated based on the overall average initial metabolic body mass (in kg$^{0.8}$) and, during the rest of the experiment, on calculated individual body mass, assuming a feed conversion ratio (**FCR**) of 1.1. The daily feed intake of all other treatments (D1–D15) were calculated based on their respective protein contents. Due to differences in protein content between the 16 diets (Table 2), the intended daily feeding levels differed between treatments. At the start of the experiment, these equalled 10.8, 11.7, 12.7, 13.7, 14.6, 15.6, 16.5 and 17.5 g/kg$^{0.8}$/d for the low NPDE intake series (Diet D1 to D8, respectively) and 13.3, 14.2, 15.2, 16.2, 17.1, 18.1, 19.0 and 20.0 g/kg$^{0.8}$/d for the high NPDE intake series (Diet D9–D16, respectively). During the experiment, these levels were adapted, based on the observed feed intake capacity of fish fed the combination of the highest levels of both DP and NPDE intake (diet D16, Fig. 2). The combination of contrasts in dietary DP and NPDE content and treatment-specific feeding levels resulted in two levels of individual daily NPDE intake (15.9 (SD = 0.19) and 22.4 (SD = 0.30) kJ/d) at each of the eight levels of individual daily DP intake (ranging from 0.44 to 1.20 g/d, Table 2). Fig. 2 shows daily DP (panel A) and NPDE (panel B) intake averaged over the whole experimental period, for diets D1 to D16. Each experimental diet was distributed to duplicate tanks by hand, two times a day (at 0900 and 1500), for 30 minutes each time. After each feeding and for each tank, the number of uneaten pellets was recorded to estimate the corresponding uneaten feed mass.

Fish and housing conditions

All-male Nile tilapia juveniles with an average body mass of 0.2 g were provided by Til-Aqua International (Someren, the Netherlands). These were obtained from a brood stock consisting of males and females carrying two Y and two X chromosomes, respectively. Selection of the YY-male parents was achieved through temperature-induced changes in the phenotypic sex of the grandparents (*i.e.*, without using hormones). Fish were grown at the Aquatic Research Facility of Wageningen University (Wageningen, the Netherlands) until the start of the experiment. During this pre-experimental period (13 weeks), fish were housed in 120-litre glass tanks connected to a recirculating aquaculture system consisting of a pump, a trickling filter, a moving bed filter and a sump. Until the start of the experiment, fish were fed restrictively a commercial starter diet containing 550 and 150 g/kg of protein and lipid, respectively (Skretting France, Fontaine-les-Vervins, France). At the start of the experiment, a total of 960 size-sorted fish with an average body mass of 63.4 g (SD = 1.29 g) were randomly allocated to one of 32 60-litre glass tanks, at a density of 30 individuals per tank. At the same moment, 20 additional fish were randomly selected, euthanised by an overdose of 2-phenoxyethanol and stored at −20 °C until analysis of initial body composition. Likewise, 10 fish per tank were sampled at the end of the experiment for final body composition analyses. All tanks were connected to the same recirculating aquaculture system as the one used during the pre-experimental period. Water flow was monitored once a week and maintained at 7 l/min. Water quality parameters were regularly monitored during the experiment and maintained within optimal ranges: mean water temperature = 27.6 °C (SD = 0.24), pH = 7.2 (SD = 0.40), conductivity = 2990 mS/m (SD = 340.0), dissolved oxygen concentration at tank outlet = 4.9 mg/l (SD = 0.53), NH_4-N = 0.5 mg/l (SD = 0.33), NO_2-N = 0.6 mg/l (SD = 0.23), NO_3-N = 79.1 mg/l (SD = 31.96). A light: dark regime of 12:12 hours was maintained during the experiment.

Biomass recording and sampling procedures

Fish were counted and batch-weighed per tank on the first and last day of the experiment to determine average individual initial and final body mass. This followed a 24-hour fast to prevent undigested feed from contributing to recorded body mass and analysed

body composition. Feed samples were collected weekly during the experiment, pooled per dietary treatment and stored at 4 °C until analyses. Faecal samples were collected overnight during the 2nd, 4th and 6th week of the experiment, using swirl separators connected to individual tank's outflow as settling faecal collection units. These were equipped with glass bottles submerged in ice-cooled water to minimise faecal degradation during the collection period. Faecal samples were pooled per week and stored at −20 °C until analyses.

Analytical methods

Prior to analyses, frozen faecal samples were oven-dried at 60 °C, pooled per tank and ground through a 1 mm mesh size screen, using a mixer mill set at 12 000 RPM (Retsch, Haan, DE; model MM2000). Frozen whole-fish were ground and homogenised twice using a meat mincer with a 4.5 mm die. Nutrient and gross energy content of feed pellets, ground faecal samples and ground fish were determined in duplicate. Fat and energy analyses of ground fish were done on freeze-dried samples while DM, ash and nitrogen analyses were done on fresh samples thawed at ambient temperature. Samples were dried at 103 °C until constant mass (ISO 6496:1999) to determine DM content. Ash content was determined after 4-hours of incineration at 550 °C (ISO 5984:2002). Nitrogen content was determined by the Kjeldahl method (ISO 5983-2:2009) and multiplied by Jones factor (6.25) to calculate CP content. Fat content was analysed by petroleum-diethyl ether extraction (ISO 6492:1999). Total carbohydrate content was calculated by difference, as Total carbohydrate = 1 000 − (CP + Fat + Ash). Gross energy content was determined by direct combustion in an adiabatic bomb calorimeter (ISO 9381:1998). Dietary, faecal and fish body calcium, magnesium and phosphorus contents, as well as dietary and faecal yttrium contents were determined in duplicates by inductively coupled plasma optical emission spectrometry, following Dutch analytical standards (NEN 15510:2017). Dietary and faecal starch content was determined after enzymatic digestion by amyloglucosidase and 40%-ethanol extraction performed by Nutricontrol BV (Veghel, the Netherlands). The 40%-ethanol washing step excludes sugars up to a chain length of 10 glucose units from the analysis. To allow calculation of dietary sugar content by difference, feed samples were analysed with and without the ethanol washing step. Faecal samples were analysed without the ethanol washing step only. Thus, pooled starch and sugar content was used for the calculation of apparent starch digestibility. Dietary and faecal amino acid content was analysed by wet chemistry (AMINOLab®, Hanau, Germany) for the 4 diets fed to the fish receiving the lowest and highest levels of DP and NPDE intake (*i.e.*, diets D1, 8, 9 and 16, Table 2). Proteins were hydrolysed with 6 mol/l HCl for 24 hours at 110 °C to liberate amino acids. Individual amino acid content was quantified using ion-exchange chromatography with postcolumn derivatisation with ninhydrin. Absorption of reaction products was measured at 570 nm.

Calculations

Individual daily body mass gain was calculated as the difference between final and initial body mass, divided by the duration of the experiment (d = 42 days). Individual daily feed intake was expressed on DM basis and corrected for fish mortality and uneaten pellet counts. The FCR was calculated as the ratio of total individual feed intake (on DM basis) to body mass gain. Nutrient apparent digestibility coefficients ($ADC_{Nutrient}$) were calculated as:

$$ADC_{Nutrient}\ (\%) = 100 \times \left[1 - \left(\frac{Y_{diet}}{Y_{faeces}} \times \frac{Nutrient_{faeces}}{Nutrient_{diet}}\right)\right] \quad (1)$$

where Y_{diet} and Y_{faeces} are the dietary and faecal yttrium oxide content, respectively, and $Nutrient_{diet}$ and $Nutrient_{faeces}$ are the dietary and faecal nutrient content, respectively.

Nitrogen and energy balances were expressed per fish in mg/d and kJ/d, respectively. For each nutrient (*i.e.*, nitrogen and energy), daily gross intake was calculated as the product of individual daily feed intake (g/d) and dietary nutrient content (mg or kJ/g). Daily digestible intake was calculated as the product of daily gross nutrient intake (mg or kJ/d) and apparent nutrient digestibility coefficient (%). Daily nutrient retention was calculated as the difference between final and initial body nutrient mass, divided by the duration of the experiment (mg or kJ/d). Retention efficiencies were calculated as the ratio of nutrient retention (mg or kJ/d) to digestible nutrient intake (mg or kJ/d). In this manuscript, the protein retention efficiency (*i.e.*, nitrogen retention efficiency) is calculated as retained protein divided by digestible protein intake. Protein retention efficiency should be distinguished from the protein efficiency ratio (**PER**), often used in the fish nutrition literature. The PER, calculated as the ratio of body mass gain to CP intake, was not considered in this manuscript. Daily branchial and urinary nitrogen losses were calculated as the difference between digestible nitrogen intake (mg/d) and retained nitrogen (mg/d). In the energy balance, daily branchial and urinary nitrogen losses were converted to kJ/d, using a multiplication factor of 24.9 kJ/g (Cho and Kaushik, 1990). Daily metabolisable energy intake was calculated as the difference between digestible energy intake (kJ/d) and branchial and urinary energy losses (kJ/d). Daily heat production was calculated as the difference between retained energy (kJ/d) and metabolisable energy intake (kJ/d). Retained energy as protein and fat were calculated by multiplying daily protein and fat gain (in g/d) by their expected energy content (23.6 and 39.5 kJ/g, respectively).

Statistical analyses

All statistical analyses were performed using the Statistical Analysis Systems software package version 9.4 (SAS Institute Inc., Cary, NC, USA). Tank was the experimental unit of all statistical analyses. General linear models were fitted using SAS GLM procedure (Supplementary Material S1). Regardless of quality of fit, linear models were fitted to all response variables to test the interactive effect of daily DP intake (DP_j, continuous variable, in g/d) and NPDE intake ($NPDE_i$, discrete variable, "low" or "high"), using the following model:

$$Y_{ij} = \mu + NPDE_i + \beta_1 \times (DP_j - \bar{DP}) + \beta_2 \times \left[(DP_j - \bar{DP}) \times NPDE_i\right] + \varepsilon_{ij} \quad (2)$$

where Y_{ij} is the response variable to the i^{th} level of NPDE intake (i = "low" or "high") of the j^{th} duplicate within each treatment (j = 1, 2), μ is the intercept, β_1 is the regression coefficient of DP_j, $\bar{DP}$ is the mean digestible protein intake of all treatments, β_2 is the interaction effect between DP_j and $NPDE_i$ and ε_{ij} is the model's residual. Linear-plateau models were fitted to daily body mass, protein and fat gain, as well as protein retention efficiency with SAS NLMIXED procedure (Robbins et al., 2006), using the Gauss optimisation method to solve the non-linear least-square problem (Supplementary Material S2). The corrected Akaike information criterion (**AICc**) (Hurvich and Tsai, 1989) and the residual sum of squares were used to compare the quality of fit between linear and linear-plateau models. In addition, a two-way ANOVA with interaction was conducted using the GLM procedure (Supplementary Material S3) and by adapting Eq. (2) as:

$$Y_{ijk} = \mu + NPDE_i + DP_j + (DP \times NPDE)_{ij} + \varepsilon_{ijk} \quad (3)$$

where Y_{jkl} is the response variable for the i^{th} level of NPDE intake (i = 1, 2) and j^{th} level of DP intake (j = 1, …, 8) of the k^{th} replicate within each dietary treatment (k = 1, 2), μ is the intercept and ε_{ijk} is the model's residual.

Results

This section focusses on the regression analyses and estimated linear and non-linear relationships of selected response variables with DP intake. The mean growth performance, apparent digestibility coefficients of macronutrients and amino acids, body nutrient composition and nitrogen and energy balances of each of the 16 dietary treatments, and the results of their two-way ANOVA analyses, are given in Supplementary Tables S3–S8.

Growth performance

Initial body mass did not differ across treatments (P > 0.1). As intended, eight levels of protein intake (0.43–1.25 g/d) were achieved – as illustrated by the overlapping black and white circles in Fig. 2A – at each of the two levels of daily NPDE intake (16.0–22.4 kJ/d, Fig. 2B). This was obtained by a linear increase in feed intake (Table 4), which, expressed relatively to the fish geometric mean metabolic body mass, ranging from 9.7 to 16.0 g/kg$^{0.8}$/day (on DM basis) at the lowest and highest DP-NPDE level combinations, respectively. Both NPDE and DP intake affected body mass gain (Table 4 and Fig. 3). Averaged over the two levels of NPDE intake, daily body mass gain was increased by 1.95 g/d per gram of increase in DP intake (*i.e.*, slope of the lines in Table 4 and Fig. 3). The slope of the linear relationship between DP intake and daily body mass gain was similar at both levels of NPDE intake (P > 0.1, Table 4). Averaged over all levels of DP intake, fish fed a high NPDE intake gained 0.28 g more per day than fish fed a low NPDE intake. At both levels of NPDE intake, the FCR declined with increasing DP intake (P < 0.001, Table 4). During the 42-d experimental period, only one fish died.

Body composition

Daily DP and NPDE intake affected the body content of all nutrients (P < 0.05, Table 5), except for calcium which did not differ between fish fed low and high levels of NPDE intake. Fish fed a high NPDE intake had lower body protein, water, ash and mineral content but higher body fat and energy content than their low NPDE-fed counterparts (Table 5). Of all nutrients, body fat was the most impacted by changes in NPDE and DP intake (Table 5), especially in fish receiving a high NPDE intake, for which body fat content decreased from 121 to 85 g/kg per gram across the range of DP intake tested (Fig. 4).

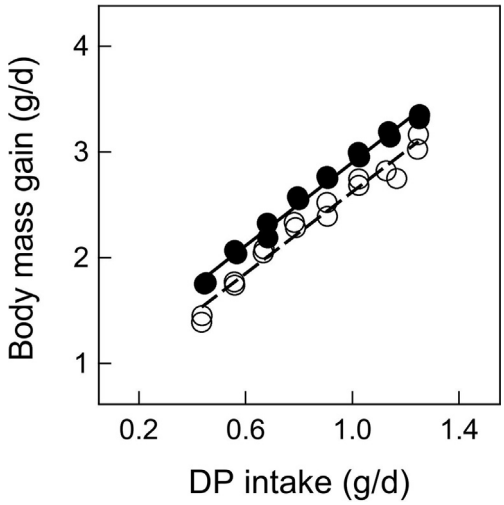

Fig. 3. Body mass gain increased linearly with digestible protein (DP) intake in Nile tilapia (n = 32 tanks) fed low (○) and high (●) levels of non-protein digestible energy intake. Dash and solid lines represent significant linear relationships obtained for the low and high levels of non-protein digestible energy intake, respectively (Table 4).

Energy and nitrogen balances

Complete nitrogen and energy balances were calculated to estimate the composition of growth and the partitioning of digested nutrients. Nitrogen balance parameters were all affected by both DP and NPDE intake, but the magnitude of the DP intake effect was much larger than that of NPDE intake. Retained nitrogen increased with DP intake (P < 0.01, Table 6), at a rate which did not differ (Interaction P > 0.05, Table 6) between low and high levels of NPDE intake. This is shown in Fig. 5, in which the almost overlapping lines (linear relationships) illustrate the significant (P < 0.05) but minor impact of NPDE intake on protein (*i.e.*, nitrogen) retention. When expressed as CP gain (N × 6.25), the slopes of the lines (53 and 60 mg/g, Table 6) equal 0.33 and 0.37 g/g. These can be regarded as "marginal" protein retention efficiencies (*i.e.*, the proportion of extra intake that is retained by the fish) of 33 and 37%, respectively. Branchial and urinary nitrogen losses also increased with DP intake (P < 0.05, Table 6), at rates close to twice as high as that of retained nitrogen. Consequently, nitrogen retention efficiency (*i.e.*, protein retention efficiency) decreased with

Table 4
Linear effect of digestible protein intake (DPI, g/d), at each of two non-protein digestible energy intake levels (NPDE, low vs high) on the growth performances of Nile tilapia (n = 2 tanks/treatment). Fish were fed one of eight levels of DPI and one of two levels of NPDE intake, during a 42-d balance trial.

Dependent variable (Y)	NPDE	Equation	R^2	P		
				DPI	NPDE[1]	DPI × NPDE[1]
Final body mass (g)	Low	Y = 90 (SE = 3.3) + 83.5 (SE = 3.14) × *DPI*	0.98	<0.001	<0.001	0.95
	High	Y = 102 (SE = 3.3) + 83.8 (SE = 3.17) × *DPI*				
Body mass gain (g/d)	Low	Y = 0.7 (SE = 0.07) + 1.9 (SE = 0.07) × *DPI*	0.98	<0.001	<0.001	0.88
	High	Y = 0.9 (SE = 0.07) + 2.0 (SE = 0.07) × *DPI*				
DM intake (g/d)	Low	Y = 0.9 (SE = 0.01) + 1.1 (SE = 0.01) × *DPI*	1.00	<0.001	<0.001	0.21
	High	Y = 1.3 (SE = 0.01) + 1.1 (SE = 0.01) × *DPI*				
Feed conversion ratio[2]	Low	Y = 1.0 (SE = 0.04) − 0.2 (SE = 0.04) × *DPI*	0.78	<0.001	0.006	0.94
	High	Y = 1.1 (SE = 0.04) − 0.2 (SE = 0.04) × *DPI*				

[1] Fixed effect of NPDE on the mean response to changes in *DPI*, hence not for *DPI* = 0 (intercept of the equation reported here) but for the mean *DPI* achieved in the experiment (*i.e.*, *DPI* = 0.85 g/d).
[2] Calculated as: DM intake/body mass gain.

Table 5
Linear effect of digestible protein intake (DPI, g/d), at each of two non-protein digestible energy intake levels (NPDE, low vs high) on the final body composition of Nile tilapia (n = 2 tanks/treatment). Fish were fed one of eight levels of DPI and one of two levels of NPDE intake, during a 42-d balance trial.

Nutrient content (*Y*, g/kg)	NPDE	Equation	R^2	*P*		
				DPI	NPDE[1]	DPI × NPDE[1]
Water	Low	$Y = 714\ (SE = 4.5) + 4\ (SE = 5.1) \times DPI$	0.63	0.028	<0.001	0.18
	High	$Y = 693\ (SE = 4.6) + 13\ (SE = 5.2) \times DPI$				
CP	Low	$Y = 153\ (SE = 2.5) + 7\ (SE = 2.8) \times DPI$	0.71	<0.001	<0.001	0.007
	High	$Y = 138\ (SE = 2.6) + 19\ (SE = 2.9) \times DPI$				
Fat	Low	$Y = 93\ (SE = 5.1) - 13\ (SE = 5.7) \times DPI$	0.76	<0.001	<0.001	0.12
	High	$Y = 121\ (SE = 5.2) - 26\ (SE = 5.8) \times DPI$				
Ash	Low	$Y = 43\ (SE = 0.7) - 2.5\ (SE = 0.81) \times DPI$	0.69	<0.001	<0.001	0.57
	High	$Y = 41\ (SE = 0.7) - 3.2\ (SE = 0.81) \times DPI$				
Calcium	Low	$Y = 12.5\ (SE = 1.24) - 2.6\ (SE = 1.40) \times DPI$	0.16	0.039	0.56	0.67
	High	$Y = 11.5\ (SE = 1.26) - 1.7\ (SE = 1.41) \times DPI$				
Magnesium	Low	$Y = 0.4\ (SE = 0.01) - 0.02\ (SE = 0.008) \times DPI$	0.63	<0.001	<0.001	0.31
	High	$Y = 0.4\ (SE = 0.01) - 0.03\ (SE = 0.008) \times DPI$				
Phosphorus	Low	$Y = 7.2\ (SE = 0.15) - 0.4\ (SE = 0.17) \times DPI$	0.65	0.003	<0.001	0.92
	High	$Y = 6.8\ (SE = 0.15) - 0.4\ (SE = 0.17) \times DPI$				
Gross energy (MJ/kg)	Low	$Y = 7.2\ (SE = 0.18) - 0.2\ (SE = 0.21) \times DPI$	0.73	0.003	<0.001	0.08
	High	$Y = 8.2\ (SE = 0.18) - 0.7\ (SE = 0.21) \times DPI$				

[1] Fixed effect of NPDE on the mean response to changes in *DPI*, hence not for *DPI* = 0 (intercept of the equation reported here) but for the mean *DPI* achieved in the experiment (*i.e.*, *DPI* = 0.85 g/d).

increasing DP intake ($P < 0.05$, Table 6). Averaged over both levels of NPDE intake, protein retention efficiency decreased by 16% per gram of additional DP intake. All energy balance parameters were affected by both DP and NPDE intake ($P < 0.01$, Table 6). Retained energy as fat increased with DP intake and was higher in fish receiving a high NPDE intake than in the ones receiving a low NPDE intake (Table 6). Averaged over all levels of DP intake, fish fed a low NPDE intake retained more energy in the form of protein than fat (9.3 vs 7.9 kJ/d), unlike those fed a high NPDE intake (9.9 vs 11.6 kJ/d). Except for gross and digestible energy intake, none of the balance parameters were influenced by the interaction effect between DP and NPDE intake (Table 6), although this effect was close to significant for the energy retention efficiency (RE/DE, $P = 0.09$, Table 6).

Linear-plateau analysis

The experiment was designed to test if growth (especially protein gain), follows distinct protein- and energy-dependent phases with increasing DP intake in Nile tilapia. To test this hypothesis, linear-plateau models were fitted to body mass gain, protein gain, lipid gain and protein retention efficiency at each of the two levels of NPDE intake. Although linear-plateau models converged for all response variables (Table 7), some did with inflection points almost equal to the lowest or highest DP intake tested in the experiment (0.44–1.23 g/d). This was the case of the two models obtained for body mass gain, with a plateau occurring at DP intakes of 1.23 and 1.20 g/d in fish fed the low and high NPDE intake, respectively (Table 7). Protein gain reached a plateau at an equal DP intake of 1.09 g/d in fish fed both a low and high NPDE intake (Table 7 and Fig. 6A), while NPDE affected the slope and the plateau in protein gain ($P < 0.05$ and $P < 0.001$, respectively, Table 7). For all variables, higher plateaux were reached in fish fed a high NPDE intake ($P < 0.05$, Table 7), compared to those fed a low NPDE intake. This difference was especially large for fat gain (Fig. 6B). Fat gain increased linearly in fish fed a low NPDE intake, while a two-phases response was observed in fish fed a high NPDE intake (Fig. 6B). In these fish, fat gain was constant at DP intake below 0.91 g/d and then increased by 0.24 g per gram of DP intake (Table 7). The lower RSS and lower AICc obtained through linear-plateau modelling for protein gain and fat gain indicated a better fit than that obtained through linear regression (Table 8). This was also the case for body mass gain, although the differences in AICc and residual sum of squares were minor, in line with the proximity in model parameters obtained through linear and linear-plateau regression. Residual sum of squares and AICc gave contradictory results for protein retention efficiency, suggesting that the relationship with DP intake was equally well described by the linear and linear-plateau models (Table 8). Fig. 6C shows the relationships obtained via linear regression.

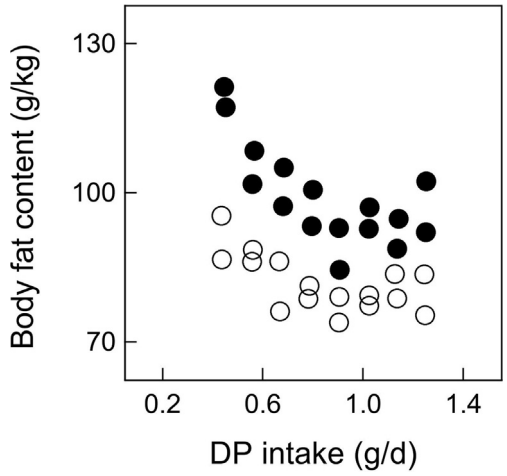

Fig. 4. Body fat content (as is) at the end of the 42-d trial decreased with digestible protein (DP) intake in Nile tilapia (n = 32 tanks) fed low (○) and high (●) levels of non-protein digestible energy intake.

Discussion

Balancing the protein and energy content of fish feeds is one of the main strategies to improve resource utilisation efficiency on fish farms. This is often achieved by formulating feeds according to a species-specific optimal dietary protein-to-energy ratio. From a physiological perspective, the existence of a single optimal dietary protein-to-energy ratio supposes that fish growth is distinctly limited by either protein or energy intake. To our knowledge, this has not been formally tested yet in Nile tilapia.

Table 6
Linear effect of digestible protein intake (DPI, g/d), at each of two non-protein digestible energy intake levels (NPDE, low vs high) on the nitrogen and energy balances of Nile tilapia (n = 2 tanks/treatment). Fish were fed one of eight levels of DPI and one of two levels of NPDE intake, during a 42-d balance trial.

Dependent variable (*Y*)	NPDE	Equation	R^2	*P*		
				DPI	NPDE[1]	DPI × NPDE[1]
Nitrogen balance (mg/d)[2]						
GN	Low	*Y* = 4 (SE = 0.4) + 162 (SE = 0.4) × *DPI*	1.00	<0.001	0.003	0.85
	High	*Y* = 5 (SE = 0.4) + 162 (SE = 0.4) × *DPI*				
BUNL	Low	*Y* = −18 (SE = 2.8) + 106 (SE = 2.7) × *DPI*	0.99	<0.001	0.001	0.11
	High	*Y* = −16 (SE = 2.8) + 100 (SE = 2.7) × *DPI*				
RN	Low	*Y* = 18 (SE = 2.8) + 53 (SE = 2.7) × *DPI*	0.97	<0.001	0.001	0.11
	High	*Y* = 16 (SE = 2.8) + 60 (SE = 2.7) × *DPI*				
RN/DN (%)	Low	*Y* = 63 (SE = 1.4) − 17 (SE = 1.3) × *DPI*	0.92	<0.001	<0.001	0.18
	High	*Y* = 63 (SE = 1.4) − 15 (SE = 1.4) × *DPI*				
Energy balance (kJ/d)						
GE	Low	*Y* = 18 (SE = 0.2) + 25.1 (SE = 0.19) × *DPI*	1.00	<0.001	<0.001	0.011
	High	*Y* = 26 (SE = 0.2) + 24.3 (SE = 0.19) × *DPI*				
DE	Low	*Y* = 16 (SE = 0.2) + 23.9 (SE = 0.22) × *DPI*	1.00	<0.001	<0.001	0.023
	High	*Y* = 23 (SE = 0.2) + 23.1 (SE = 0.22) × *DPI*				
BUEL	Low	*Y* = −0.4 (SE = 0.07) + 2.6 (SE = 0.07) × *DPI*	0.99	<0.001	0.001	0.11
	High	*Y* = −0.4 (SE = 0.07) + 2.5 (SE = 0.07) × *DPI*				
ME	Low	*Y* = 16 (SE = 0.3) + 21.2 (SE = 0.25) × *DPI*	1.00	<0.001	<0.001	0.11
	High	*Y* = 23 (SE = 0.3) + 20.6 (SE = 0.25) × *DPI*				
HP	Low	*Y* = 10 (SE = 0.7) + 8.2 (SE = 0.70) × *DPI*	0.93	<0.001	<0.001	0.76
	High	*Y* = 12 (SE = 0.7) + 8.5 (SE = 0.71) × *DPI*				
RE	Low	*Y* = 6 (SE = 0.7) + 13.1 (SE = 0.63) × *DPI*	0.97	<0.001	<0.001	0.33
	High	*Y* = 11 (SE = 0.7) + 12.2 (SE = 0.64) × *DPI*				
RE as protein	Low	*Y* = 3 (SE = 0.4) + 7.9 (SE = 0.4) × *DPI*	0.97	<0.001	0.001	0.11
	High	*Y* = 2 (SE = 0.4) + 8.8 (SE = 0.4) × *DPI*				
RE as fat	Low	*Y* = 4 (SE = 0.7) + 4.6 (SE = 0.72) × *DPI*	0.90	<0.001	<0.001	0.52
	High	*Y* = 8 (SE = 0.7) + 4.0 (SE = 0.72) × *DPI*				
RE/DE (%)	Low	*Y* = 43 (SE = 1.7) + 4.8 (SE = 1.61) × *DPI*	0.57	0.023	<0.001	0.09
	High	*Y* = 50 (SE = 1.7) + 0.7 (SE = 1.62) × *DPI*				

Abbreviations: GN = gross nitrogen intake; DN = digestible nitrogen intake; BUNL = branchial and urinary nitrogen losses; RN = retained nitrogen; GE = gross energy intake; DE = digestible energy intake; BUEL = branchial and urinary energy losses; ME = metabolisable energy intake; HP = heat production; RE = retained energy.
[1] Fixed effect of NPDE on the mean response to changes in *DPI*, hence not for *DPI* = 0 (intercept of the equation reported here) but for the mean *DPI* achieved in the experiment (*i.e.*, *DPI* = 0.85 g/d).
[2] No regression was obtained for digestible nitrogen intake because it is fully proportional to the independent variable *DPI* (*DPI* = 6.25 × digestible nitrogen intake).

Absence of distinct protein- and energy-dependent growth phases

The present experiment was designed to test the interactive effects of increasing protein intake (8 levels) and constant non-protein energy intake (two levels) on Nile tilapia growth. The purpose was to determine if Nile tilapia respond to increasing protein and non-protein energy intake by distinct protein- and non-protein energy-dependent growth phases (Fig. 1). Based on body mass gain, this hypothesis was not validated in our experiment. Instead, body mass gain was simultaneously constrained by both DP and NPDE intake, at all levels of DP intake. This is illustrated by the two parallel, non-overlapping lines represented in Fig. 3. Daily protein gain was mostly affected by DP intake, and not so much by NPDE intake (Fig. 5). Indeed, the difference in body mass gain between fish fed the low and high levels of NPDE intake mainly resulted from a difference in fat gain (Fig. 6B). Protein gain levelled-off at the two highest levels of DP intake (Fig. 6A), as indicated by the better fit obtained through linear-plateau regression analysis (Table 8). However, these results do not demonstrate the presence of distinct protein- and energy-dependent phases in protein gain in Nile tilapia, as we hypothesised. If protein gain was distinctly limited by protein and energy intake as in lambs (Black and Griffiths, 1975) or pigs (Campbell et al., 1985), the energy-limiting phase should have occurred at a lower level of DP intake in fish fed the low NPDE intake series than in those fed the high one (Fig. 1). This was not the case here since the plateau was reached at the same level of DP intake (1.09 g/d) in fish with both a low and high NPDE intake. In contrast, the fact that fish fed a high NPDE intake reached a higher plateau in protein deposition than their low NPDE-fed counterparts (0.54 vs 0.50 g/day) may indicate that NPDE intake was limiting protein deposition at the highest levels of DP intake. Within the range of DP intake tested in the present experiment, there were no distinct protein- and energy-dependent phases in protein deposition in Nile tilapia.

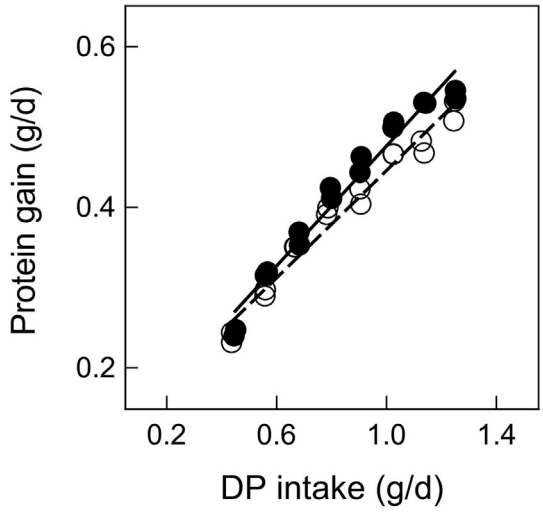

Fig. 5. Protein gain increased close to linearly with digestible protein (DP) intake in Nile tilapia (n = 32 tanks) fed low (○) and high (●) levels of non-protein digestible energy intake. Dash and solid lines represent significant linear relationships obtained for the low and high levels of non-protein digestible energy intake, respectively (Table 6).

Table 7
Linear-plateau relationships between digestible protein intake (DPI, g/d) and selected growth response variables, obtained at each of two levels of non-protein digestible energy intake (NPDE, low vs high), in Nile tilapia (n = 2 tanks/treatment). Fish were fed one of eight levels of DPI and one of two levels of NPDE intake during a 42-d balance trial.

Dependent variable (*Y*)	NPDE	DPI at inflection (g/d)	Segment equations	P_{NPDE} [1]		
				Inflection	Slope	Plateau
Body mass gain (g/d)	Low	1.23 (SE = 0.030)	*Y* (*DPI* < 1.23) = 0.6 (SE = 0.40) + 2.0 (SE = 0.08) × *DPI* *Y* (*DPI* > 1.23) = 3.1 (SE = 0.05)	0.44	0.51	0.001
	High	1.20 (SE = 0.030)	*Y* (*DPI* < 1.20) = 1.0 (SE = 0.39) + 2.0 (SE = 0.08) × *DPI* *Y* (*DPI* > 1.20) = 3.3 (SE = 0.05)			
Protein gain (g/d)	Low	1.09 (SE = 0.026)	*Y* (*DPI* < 1.09) = 0.0 (SE = 0.16) + 0.38 (SE = 0.019) × *DPI* *Y* (*DPI* > 1.09) = 0.50 (SE = 0.007)	0.82	0.038	<0.001
	High	1.09 (SE = 0.026)	*Y* (*DPI* < 1.09) = 0.1 (SE = 0.15) + 0.44 (SE = 0.019) × *DPI* *Y* (*DPI* > 1.09) = 0.54 (SE = 0.007)			
Fat gain (g/d)	Low	0.47 (SE = 0.089)	*Y* (*DPI* < 0.47) = 0.16 (SE = 0.008) *Y* (*DPI* > 0.47) = 0.10 (SE = 0.149) + 0.12 (SE = 0.042) × *DPI*	0.001	0.046	<0.001
	High	0.91 (SE = 0.089)	*Y* (*DPI* < 0.91) = 0.27 (SE = 0.008) *Y* (*DPI* > 0.91) = 0.05 (SE = 0.209) + 0.24 (SE = 0.042) × *DPI*			
Protein retention efficiency (%)	Low	0.59 (SE = 0.052)	*Y* (*DPI* < 0.59) = 53.6 (SE = 0.59) *Y* (*DPI* > 0.59) = 65 (SE = 2.6) − 20 (SE = 2.1) × *DPI*	0.99	0.27	0.034
	High	0.59 (SE = 0.052)	*Y* (*DPI* < 0.59) = 55.5 (SE = 0.59) *Y* (*DPI* > 0.59) = 65 (SE = 2.5) − 17 (SE = 1.8) × *DPI*			

[1] *P* values are given for the effect of NPDE intake level (low vs high) on the estimates of the *DPI* at inflection, the slope of the non-plateau segment and the plateau obtained through linear-plateau regression analysis.

The absence of distinct protein- and energy-dependent phases is also reflected in the negative linear relationship observed between DP intake and protein retention efficiency (*i.e.*, the ratio of protein gain to DP intake). If a distinct protein-dependent phase existed, it should translate into a constant protein retention efficiency until a drop caused by energy shortage and the transition to an energy-dependent phase. This was not the case in the present experiment, since protein retention efficiency decreased linearly over most of the DP intake range tested (Fig. 6C).

Maximal protein deposition capacity

Protein gain levelled-off at the same level of DP intake (1.09 g/d) for both levels of NPDE intake. Thus, the plateaux likely reflected a limitation common to both NPDE intake series and not the transition to energy-dependent phases. Next to energy availability, protein deposition may be limited by the genetic potential of the animal for growth. For some farm animal species, protein deposition is assumed to be limited by a maximal daily deposition capacity (**PD_{max}**) (Samadi and Liebert, 2006; Moughan et al., 2006; Soares et al., 2019). In practice, such a limitation is not seen in young animals but only occurs in certain body mass range (>20 kg in pigs) (Möhn and de Lange, 1998). Via exponential modelling, Liebert et al. (2006) estimated the maximal daily nitrogen deposition capacity of 12–150 grams all-male Nile tilapia at 388 mg/$kg^{0.67}$/d, based on a dataset in which maximal recorded N retention equalled 356 mg/$kg^{0.67}$/d. These values are not too different from those estimated through linear-plateau modelling in fish receiving low and high levels of NPDE intake in the present experiment: 329 and 342 mg/$kg^{0.67}$/d, respectively. Differences in maximal gain estimate between the two studies are attributable to the regression method used (exponential vs linear-plateau) and possibly to the fish size class, with higher estimates obtained in small fish. However, markedly higher daily nitrogen gain (439–515 mg/$kg^{0.7}$/d) was reported in 40–240 grams of Nile tilapia fed to apparent satiation (Saravanan et al., 2012). Next to differences in starting body mass (12, 40 and 60 g) in these three experiments, the main differences lie in the highest nitrogen intake achieved: 810 mg/$kg^{0.67}$/d (Liebert et al., 2006), 1 490 mg/$kg^{0.7}$/d (Saravanan et al., 2012) and 810 mg/$kg^{0.67}$/d (current experiment). The observation of a maximal protein deposition capacity, if existent, strongly depends on the feed intake capacity of the fish. The highest feed intake observed in the present experiment (16.0 g/$kg^{0.8}$/day) corresponded to that achieved at the highest DP-NPDE intake combination. This value was in line with previous observations made in 68–77 and 196–232 g Nile tilapia fed a commercial diet under optimal water temperature and oxygen conditions at our facility (Tran-Duy et al., 2012). Further studies are needed to ascertain whether there is a maximal protein deposition capacity in fish, the success of which will depend on the maximal feed intake achieved. Yet, maximal daily protein or nitrogen capacity estimates can already be a useful addition to fish growth models (Hua et al., 2010).

Absolute and marginal protein retention efficiency

The main motivation for balancing the protein and energy intake of farmed fish is to improve dietary protein utilisation for growth by exploiting the protein-sparing effect of non-protein energy substrates (Cho and Bureau, 2001; Kaushik and Seiliez, 2010). In Nile tilapia, increasing non-protein energy intake relative to protein intake (*i.e.*, reducing dietary DP/DE) increases absolute protein retention efficiency (Ali et al., 2008; Li et al., 2013; Haidar et al., 2018). In the present experiment, increasing non-protein energy intake had little effect on protein gain (Fig. 5, Table 6). The additional 6.4 kJ of NPDE consumed daily by fish fed a high NPDE intake resulted in a 0.6 kJ increase in daily protein gain while fat gain and heat production increased by 3.7 and 2.2 kJ/d, respectively. Thus, more than 50% of the additional non-protein energy intake was deposited as fat while less than 10% contributed to sparing amino acids from catabolism. The gradual increase in DP intake achieved in the present experiment caused a proportional increase in dietary DP/DE, from 17 to 28 g/MJ and 14 to 24 g/MJ in the low and high NPDE intake series, respectively. At both levels of NPDE intake, this increase in DP intake, and thus DP/DE, caused a linear decrease in protein retention efficiency, from 55 to 42%. In line with observations made in smaller Nile tilapia (7–44 g, Haidar et al., 2018), the absence of a clear plateau at the lowest levels of DP intake– and thus DP/DE – suggests that there is no limit to the increase in protein retention efficiency with decreasing DP/DE, regardless of NPDE intake. The magnitude of this effect – a 13% increase in protein retention efficiency – was similar to observations made in smaller and larger Nile tilapia subjected to large contrasts in dietary DP/DE under restricted (32–53%, Haidar et al., 2018) and apparent satiation (35–55%, Saravanan et al., 2012) feeding conditions. Similar observations were made in other

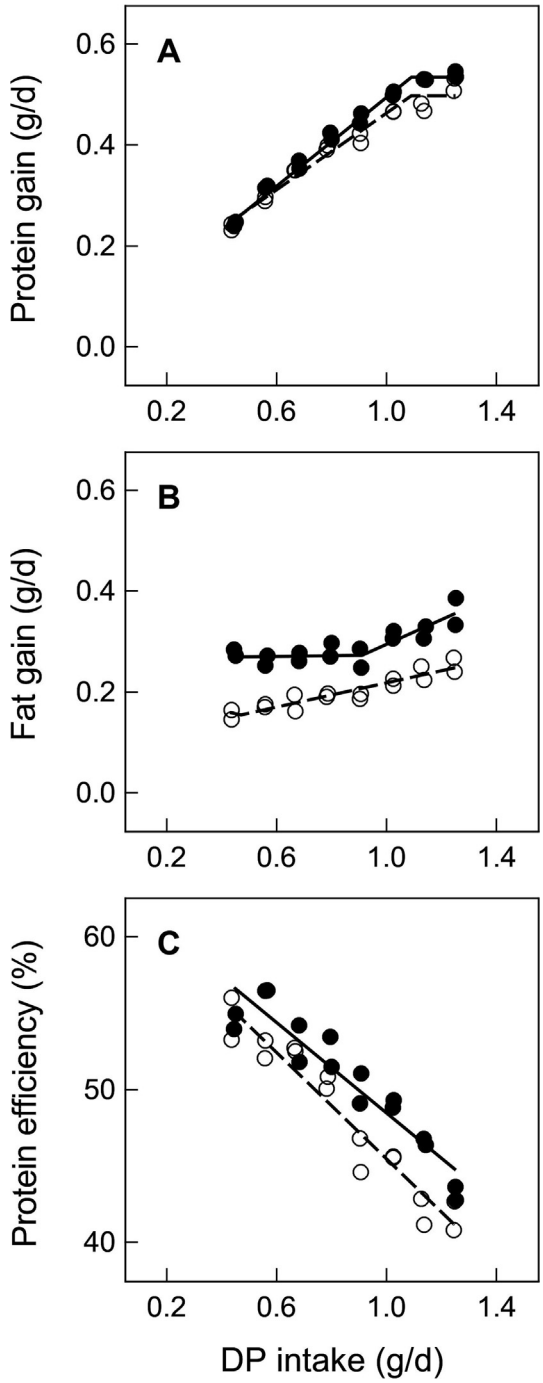

Fig. 6. Linear and linear-plateau regression analyses indicated (**A**) that protein deposition may level-off at high digestible protein (DP) intake, (**B**) that DP intake affected fat gain differently in Nile tilapia (n = 32 tanks) fed low (○) and high (●) levels of non-protein digestible energy intake, and (**C**) that protein retention efficiency decreased linearly with DP intake. Dash and solid lines represent significant linear and linear-plateau relationships obtained for the low and high levels of non-protein digestible energy intake, respectively (Table 6).

fish species like silver perch (*Bidyanus bidyanus*, 19–40%, Allan and Booth, 2004), rainbow trout (*Oncorhynchus mykiss*, 49–54%, Azevedo et al., 2004) and Atlantic salmon (*Salmo salar*, 43–53% Azevedo et al., 2004). However, compared to observations made in other terrestrial farmed animal species, this effect is moderate. For example, male broiler chickens subjected to a decrease in dietary DP-to-metabolisable energy ratio from 26 to 13 g/MJ increased their protein retention efficiency from 25 to 86% (Gous et al., 2018).

Table 8
Quality of fit of linear and linear-plateau models applied to the relationships between digestible protein intake (in g/d) and body mass gain, protein gain, lipid gain and protein retention efficiency in Nile tilapia. Corresponding linear and linear-plateau model equations are given in Tables 6 and 7, respectively.

Dependent variable	Model	RSS[1]	AICc[1]
Body mass gain (g/d)	Linear	0.15	−58
	Linear-plateau	0.14	−64
Protein gain (g/d)	Linear	0.009	−138
	Linear-plateau	0.006	−167
Fat gain (g/d)	Linear	0.010	−134
	Linear-plateau	0.007	−162
Protein retention efficiency (%)	Linear	57	107
	Linear-plateau	44	120

Abbreviations: AICc = corrected Akaike Information Criterion; RSS = residual sum of squares.
[1] Lower RSS and AICc values indicate a lower proportion of unexplained variance and a better model fit, respectively.

The utilisation efficiency of protein for growth is also reflected in the protein gain response to protein intake, which corresponds to the slopes of the lines shown in Figs. 5 and 6A. Marginal protein retention efficiency increased with the level of NPDE intake, although this effect was only significant for the estimates obtained via linear-plateau regression (38 vs 44% in fish fed low and high NPDE levels, respectively). Regardless of the regression model and NPDE intake level, marginal protein retention efficiencies ranged from 33 to 44% in the present trial. In other words, every additional gram of DP intake resulted in the retention of 0.33–0.44 g of protein. These values are in line with observations made in Nile tilapia (0.42–0.47, Castillo et al., 2017), gilthead seabream (*Sparus aurata*, 0.31–0.35, Lupatsch et al., 1998) and Australian snapper (*Pagrus auratus*, 0.29–0.38, Booth et al., 2007). However, the values obtained in the present experiment differ from those obtained in other studies in the sense that they represent "true" response to protein intake. Estimates reported in other studies often correspond to the response to changes in both protein and non-protein energy intake, as a result of feeding fish to apparent satiation with diets varying in both protein and non-protein energy contents. Again, the marginal utilisation efficiencies reported for Nile tilapia and other fish species are much lower than those reported for other farmed animal species such as pigs (0.76) and poultry (0.75) (Sandberg et al., 2005). Thus, the potential for protein sparing by increasing non-protein energy intake is much lower in fish than in some other farmed animal species. This reflects the fact that fish strongly depend on amino acid catabolism to meet their energy needs (Walton and Cowey, 1982; Kaushik and Seiliez, 2010). As a consequence, absolute protein retention efficiency of farmed fish species rarely exceeds 60% (National Research Council, 2011), as illustrated in the present experiment.

The utilisation of protein for growth not only depends on the availability of alternative energy substrates but also on dietary amino acid profile (Kaushik and Seiliez, 2010). The amino acid content of body proteins being fixed, amino acid catabolism increases when the availability of one or several amino acid(s) is limiting the use of the others for protein synthesis. In the present experiment, this risk was minimised by formulating the protein mix included in all 16 experimental diets according to recent amino acid requirement estimates for Nile tilapia (National Research Council, 2011; Diógenes et al., 2016). Protein retention efficiency reached up to 55% in the present experiment (Fig. 6C). In comparison, maximal protein retention efficiency ranged from 45 to 51% under non-limiting amino acid supply in previous amino acid requirement studies (Michelato et al., 2016, 2017; Zaminhan et al., 2017). Thus, dietary amino acid profile probably did not limit protein gain in the present experiment.

Protein and energy balance in Nile tilapia feeds

The absence of distinct protein- and energy-dependent growth phases observed in the present experiment contradicts the existence of a single optimal DP/DE for Nile tilapia feeds. In the present experiment, dietary DP/DE ranged from 16.8 to 27.5 g/MJ and 13.5 to 24.2 g/MJ in the low and high NPDE intake series, respectively. These ranges extend beyond the DP/DE range of standard commercial tilapia feeds. At both levels of non-protein energy intake, increasing DP intake, and thus dietary DP/DE, caused a linear decrease in protein retention efficiency over the whole range tested (Fig. 6C), in line with previous studies (Kaushik et al., 1995; Ali et al., 2008; Li et al., 2013; Kpundeh et al., 2015; Haidar et al., 2018). This contradicts the belief that achieving a proper balance between DP and energy (*i.e.*, optimal DP/DE) allows a shift from a situation in which protein is being "wasted" as metabolic fuel to an "optimal" situation in which it is efficiently used for body protein synthesis. In Nile tilapia, the protein-sparing effect of reducing dietary DP/DE is a linear one.

The linear change in amino acid partitioning caused by dietary DP/DE variations affects other production traits than protein retention efficiency. Previous work indicated that increasing dietary DP/DE often results in an increase in the fillet yield of Nile tilapia (Gonçalves et al., 2009; Haidar, 2017; Carneiro et al., 2020). This likely results from the fact that body fat content reduces with increasing dietary DP/DE, as shown in Fig. 4. The body fat content measured in the present experiment (70–121 g/kg) was up to twice higher than in wild (Rasoarahona et al., 2005; El-Zaeem et al., 2012) and pond-farmed (El-Sayed et al., 1996; Kabir et al., 2019, 2020; Wang et al., 2020) specimens. This is in line with other experimental observations in which Nile tilapia body fat content reached up to 166 g/kg (Saravanan et al., 2012; Haidar et al., 2018) at low DP/DE. These high levels may be caused by the combination of low DP/DE diets and low maintenance expenditures under laboratory conditions. However, high body fat accumulation (114–138 g/kg) can also occur under practical conditions, as illustrated by observations made in cage-raised Nile tilapia fed low energy-dense diets (DP = 251–260 g/kg DM and DE = 14–15 kJ/k g DM) (Coutinho et al., 2018). Body fat content is rarely mentioned as a selection criteria for the optimal dietary DP/DE. This may be due to the fact that Nile tilapia deposit fat primarily in their body cavity, alongside the viscera (Haidar, 2017), which does not affect the organoleptic properties of tilapia fillets like muscle fat storage does in other farmed fish species (Grigorakis, 2017). Another reason may be that body fat content does not only reflect the effect of DP/DE on nutrient partitioning but also varies with feeding level (Liu et al., 2018) and maintenance expenditures. As a result, predicting body fat gain (and thus body mass gain) based on dietary DP/DE is probably more hazardous than predicting protein utilisation efficiency (and thus protein gain), as illustrated in the present experiment (Fig. 6B and C). Yet, the apparent lack of body fat content regulation in Nile tilapia deserves attention. Under intensive farming conditions, the high body fat content of Nile tilapia is sometimes associated with pathological liver condition (steatosis) (Fernandes et al., 2016) and impaired lipid, glucose and amino acid metabolism (Tao et al., 2021). Body fat accumulation was also suggested to exert long-term down-regulation of voluntary feed intake in Atlantic salmon (Johansen et al., 2002), chinook salmon (*Oncorhynchus tshawytscha*, Shearer et al., 1997) and Arctic charr (*Salvelinus alpinus*, Jobling and Miglavs, 1993). Such lipostatic regulation of feed intake has not been studied in Nile tilapia but suggests that dietary DP/DE may be better evaluated in experiments longer than those conducted until now. From a human perspective, the optimal DP/DE reflects management choices with regard to outcomes such as protein retention efficiency (Haidar et al., 2018), fillet yield (Fernandes et al., 2016; Carneiro et al., 2020) and body fat content. Thus, the optimal DP/DE of a farmed Nile tilapia feed is always the result of context-dependent trade-offs (Gonçalves et al., 2009; Koch et al., 2014) rather than a physiological optimum to target.

Conclusion

The present experiment indicated that no distinct protein- and energy-dependent phases can be detected in 60–200 g Nile tilapia. Instead, body mass and protein gain were simultaneously limited by protein and non-protein energy intake over most of the range tested. Protein gain attained a plateau only at the highest two levels of protein intake, which may indicate that fish had reached a maximal protein deposition capacity. Whether this was determined by the genetic potential of the fish or by energy intake needs further study. The linear decrease in protein retention efficiency with protein intake illustrated an increase in amino acid catabolism to meet energy needs. The minor effect of non-protein energy intake on this decrease demonstrates the modest protein-sparing potential of non-protein energy substrates in Nile tilapia. Further dietary-mediated improvements in Nile tilapia protein utilisation are more likely to come from refining amino acid requirements and dietary energy evaluation than from adjusting the balance between dietary protein and energy content. Altogether, the results of the present experiment contradict the existence of a single optimal protein-to-energy ratio for Nile tilapia feeds, based on physiological and growth performance indicators.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100494.

Ethics approval

All procedures involving animals were carried out in accordance with the Dutch law on experimental animals and were approved by the Animal Experiment Committee of Wageningen University (Project number 2018.W-0033).

Data and model availability statement

None of the data were deposited in an official repository. Data can be shared by the corresponding author upon reasonable request.

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Declaration of interest

None.

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