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Effects of dietary energy density and digestible protein:energy ratio on *de novo* lipid synthesis from dietary protein in gilthead sea bream (*Sparus aurata*) quantified with stable isotopes

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Abstract

The effects of varying dietary digestible protein (DP) and digestible energy (DE) content on performance, nutrient retention efficiency and the *de novo* lipogenesis of DP origin were examined in triplicate groups of gilthead sea bream (*Sparus aurata*), fed nine extruded experimental diets. In order to trace the metabolic fate of dietary protein, 1·8 % fishmeal was replaced with isotope-labelled whole protein (>98 % ^{13}C). The experiment was divided into a growth period lasting 89 d, growing fish from approximately 140 to 350 g, followed by a 3 d period feeding isotope-enriched diets. Isotope ratio MS was applied to quantify the ^{13}C enrichment of whole-body lipid from dietary DP. Between 18·6 and 22·4 % of the carbon derived from protein was recovered in the lipid fraction of the fish, and between 21·6 and 30·3 % of the total lipid deposited could be attributed to dietary protein. DP retention was significantly improved by reductions in dietary DP:DE ratio, while the opposite was true for apparent digestible lipid retention. Both overall DE retention and whole-body proximate composition of whole fish were largely unaffected by dietary treatments, while feed conversion ratios were significantly improved with increasing dietary energy density. The present study suggests that gilthead sea bream efficiently utilises dietary nutrients over a wide range of DP:DE ratios and energy densities. In addition, they appear to endeavour a certain body energy status rather than maximising growth, which in the present trial was apparent from inherently high *de novo* lipogenesis originating from DP.

Key words: Gilthead sea bream: Stable isotopes: Protein-to-energy ratio: Lipogenesis

One of the biggest expenses in modern aquaculture is feed cost. Thus, maximised feed utilisation per unit cost is of paramount importance in maintaining an economically viable aquaculture enterprise. In commercial fish feed production, the main concern is the quality, quantity and ratio between nutrients supplied through the raw materials used, and the cost of combining these to fit the nutritional requirements of a specific farmed species at a specific life stage. These nutrient requirements, however, are not absolute. Rather, they should be present in the correct proportion to each other as pointed out by Wilson[1] with respect to protein requirements. According to him, the protein requirement of an animal comprises a well-balanced mixture of essential and non-essential amino acids, where protein digestibility, amino acid profile and energy concentration of the diet are also considered.

Consequently, two of the most commonly used diet optimisation 'tools' in aquaculture comprise amino acid optimisation ('ideal protein concept')[2–4], and optimisation of the ratio between digestible protein (DP) and digestible energy (DE)[5–9]. The optimal DP:DE ratio refers to the minimum amount of DP required for optimising a certain production trait, such as growth, feed conversion or protein retention at a given DE density. Diets containing DP in excess of requirements will lead to excessive protein deamination, which in turn increases the discharge of nitrogenous compounds into the environment[10,11]. Additionally, protein is the most costly macronutrient in aquaculture diets. Thus, there is an economic incentive not to include this nutrient in excess of requirements.

Historically, gilthead sea bream (*Sparus aurata*) has been perceived to have a high dietary protein requirement, and

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relatively poor protein utilisation and feed conversion compared with other aquacultured species such as salmonids. This is also reflected in the reported optimal DP:DE ratios for this species[12,13], which are considerably higher than for farmed salmonids at the same life stage[14–16]. Irrespective of species, practically all DP/DE studies reported so far have focused on optimising protein retention. In practice, this is typically done by reducing the dietary DP/DE level, by substituting DE supplied from DP with DE supplied from non-protein DE sources such as fat[17–20] or carbohydrates[18,21–25]. Only a few studies have commented on the metabolic fate of non-retained (deaminated) protein in this respect[26,27].

Recent studies have indicated that a substantial part of the deaminated amino acids in blackspot seabream (*Pagellus bogaraveo*) were converted into fatty acids *de novo*[27]. This was expressed by increased hepatic lipogenic enzyme activities and the increased hepatic content of palmitic and stearic acids, which are generally recognised to be the main products of *de novo* lipogenesis[28]. Additionally, studies by Enes[29,30] have shown a positive correlation between dietary protein level and lipogenic enzyme activity both in gilthead sea bream and European seabass (*Dicentrarchus labrax*), indicating that protein may contribute to lipid biosynthesis in this species. However, since deaminated protein can precede both gluconeogenesis, lipogenesis and complete oxidation for energy purposes, it is hard to quantitatively conclude on the fate of deaminated protein.

The main purpose of the present study was to quantify the amount of dietary protein endogenously converted to body lipid *de novo* in gilthead sea bream using nine diets enriched with stable isotopes that differed in DP/DE levels and energy density. Additionally, macronutrient retention efficiencies, growth and feed performance parameters were determined. To achieve this, a study comprising two trial periods was conducted. First, an 89 d growth period was carried out feeding gilthead sea bream nine diets differing in DP and DE content. Based on this specific growth rate (SGR), feed conversion ratio (FCR), nutrient digestibility coefficients (ADC), DP retention (DPR), DE retention (DER) and apparent lipid retention (aDLR) were determined. Second, and immediately following the growth period, fish were fed their respective diets for three more days, only now diets were added trace amounts of ^{13}C-labelled protein isolate. This was done to determine the extent to which dietary protein was converted into body lipid endogenously, and to determine how much this lipid biosynthesis contributed to the overall lipid deposition in the fish. As deposited lipid could originate from both dietary and endogenous sources (*de novo* lipogenesis), digestible lipid retention efficiencies are henceforth referred to as 'apparent'.

Materials and methods

Culture conditions and fish

Gilthead sea bream with an average individual weight of approximately 120 g were obtained from a commercial fish farm (Ferme Marine de Douhet). They were subsequently stocked into a recirculated aquaculture system comprising twenty-seven fibreglass tanks with a volume of 800 litres, each at a stocking density of twenty fish/tank (BioMar research facility; The North Sea Research Centre). The tanks were fitted with a central bottom drain designed to quickly and efficiently remove faeces and uneaten feed pellets from the water by means of externally mounted swirl separators. The trial facility was supplied with filtered North Sea water with a salinity of 34 g/l, and the temperature was kept at 24°C throughout the trials. Water quality was monitored daily, maintaining O_2 saturation between 80 and 100 %, NH^{4+} below 1·0 mg/l, NO^{2-} below 1·0 mg/l and NO^{3-} below 100 mg/l. pH was adjusted to 7·0 using sodium bicarbonate when necessary. The tanks were supplied with system water at a flow rate of 1200 litres/tank per h. A 14 h light−10 h dark photoperiod was maintained throughout the trials. All fish were acclimatised to the facility for 2 weeks during which they were fed a commercial diet (BioMar EFICO YM 664; DP/DE level 21·7 g/MJ) according to a commercial feeding table value (1·5 % of the biomass per d).

Experimental diets

A total of nine experimental diets were prepared using Allix[2] feed formulation software (A-systems S.A.; Table 1). The main dietary ingredients were fishmeal, fish oil, wheat and field peas, and the diets were formulated to contain three DP levels (330, 360 or 380 g/kg) and three DE levels (20, 21 or 22 MJ/kg) in a 3 × 3 factorial design. The diets were named according to their DP and DE content (LP, MP or HP for low, medium or high DP content, respectively, and LE, ME or HE for low, medium or high DE content, respectively). For example, the diet LPLE refers to the low DP:low DE diet (expected to contain 330 g/kg DP and 20 MJ/kg DE). The following two versions of each diet were made: one where approximately 1·8 % of the dietary fishmeal was substituted with a ^{13}C-labelled (97–98 % [13]C) *Spirulina* protein isolate (Cambridge Isotope Laboratories, Inc.), and one unlabelled version. The *Spirulina* protein isolate was chosen as an isotope marker due to its close resemblance to fishmeal regarding the amino acid profile and the lack of non-protein macronutrients that could potentially adversely affect the enrichment data. The diets were supplemented with a vitamin and mineral premix, as well as monocalcium phosphate. Guar gum was added (5 g/kg) to enhance pellet stability and accurately quantify feed waste, and yttrium oxide (Y_2O_3) was added (0·3 g/kg) as an inert marker enabling indirect measurements of nutrient and stable isotope digestibility. The diets were prepared at the BioMar TechCenter using a twin-screw extruder (Clextral BC-45; Firminy) to produce 4·5 mm pellets. Following extrusion, the diets were dried in a six-level Geelen counterflow continuous dryer (Geelen Counterflow), vacuum coated with fish oil and cooled.

Experimental procedures

The study comprised two trial periods: (1) an 89 d growth period (growing fish from approximately 140 to 340 g) feeding

Table 1. Diet formulation, chemical and isotope composition of the experimental diets

(Mean values and standard deviations; percentages)

Diets...	LPLE	LPME	LPHE	MPLE	MPME	MPHE	HPLE	HPME	HPHE
Ingredients (g/kg)*									
Fishmeal†	442 (434)	448 (440)	453 (445)	498 (489)	504 (495)	509 (500)	554 (544)	559 (549)	565 (555)
Fish oil	180	218	257	163	202	240	146	185	223
Wheat	207	161	115	154	108	61	101	55	8
Field peas	147	149	151	166	168	170	185	186	188
Guar gum	5	5	5	5	5	5	5	5	5
Vitamin and mineral premix	3	3	3	3	3	3	3	3	3
Monocalcium phosphate	16	16	16	11	11	11	7	7	7
Y_2O_3	0·3	0·3	0·3	0·3	0·3	0·3	0·3	0·3	0·3
^{13}C protein isolate†	(8)	(8)	(8)	(9)	(9)	(9)	(10)	(10)	(10)
Proximate composition (g/kg)									
DM	930	921	920	915	909	922	917	918	920
Crude protein	385	372	375	409	404	404	453	443	444
Crude fat	230	274	308	219	256	304	210	254	283
Starch	193	192	126	168	152	102	133	106	84
Ash	74	72	72	73	74	73	77	76	77
Energy calculations									
Gross energy (MJ/kg)‡	22·4	23·1	23·9	22·0	22·7	24·0	22·1	23·0	23·7
DE (MJ/kg)§	19·9	21·2	21·4	19·6	20·7	21·6	19·7	20·6	21·3
DP (g/kg)‖	344	331	334	363	363	361	405	395	395
DP:DE ratio (g/MJ)	17·3	15·6	15·6	18·5	17·5	16·7	20·6	19·1	18·5
Carbon content of the diets (%)									
Non-enriched diets									
Mean	47·74	49·89	51·25	48·69	48·44	50·04	47·17	49·19	50·11
SD	1·06	1·15	1·44	0·51	0·51	1·39	0·38	1·07	0·49
^{13}C-enriched diets									
Mean	46·19	50·20	51·08	46·50	50·38	50·74	48·70	49·50	49·61
SD	1·01	1·29	1·36	0·63	1·20	1·64	0·74	1·12	0·82
$\delta\,^{13}$C values of the diets (‰)									
Non-enriched diets									
Mean	−22·48	−22·40	−22·23	−22·12	−21·44	−21·52	−21·25	−21·59	−21·81
SD	0·17	0·06	0·09	0·13	0·27	0·71	0·25	0·30	0·12
^{13}C-enriched diets									
Mean	594·54	596·93	593·91	661·68	663·74	663·23	753·28	754·48	753·25
SD	17·12	15·01	16·27	18·90	17·30	26·67	25·18	8·53	34·83
^{13}C APE of the diets (%)									
^{13}C-enriched diets	0·6738	0·6762	0·6728	0·7461	0·7476	0·7471	0·8443	0·8459	0·8448
^{13}C enrichment of protein C (%)									
^{13}C-enriched diets	1·718	1·943	1·950	1·805	1·982	1·998	1·931	2·010	2·010

LPLE, low digestible protein (DP):low digestible energy (DE) diet; LPME, low DP:medium DE diet; LPHE, low DP:high DE diet; MPLE, medium DP:low DE diet; MPME, medium DP:medium DE diet; MPHE, medium DP:high DE diet; HPLE, high DP:low DE diet; HPME, high DP:medium DE diet; HPHE, high DP:high DE diet; APE, atom per cent excess.

* Fishmeal: TripleNine Fish Protein; Fish oil: South American (Peru); Guar gum: HV200; LCH A/S; ^{13}C-labelled protein isolate: Cambridge Isotope Laboratories, Inc., algal crude protein fraction isolated from *Spirulina* algae (U-^{13}C, 97–98 %), lot no. BP-733, catalogue no. CLM-3348-0; Cambridge Isotope Laboratories, Inc.; vitamin and mineral mix is estimated to meet the requirements according to the US National Research Council recommendations[49].

† Each of the nine diets were produced in a ^{13}C-enriched version (where ^{13}C-labelled protein isolate replaced approximately 1·8 % of the con-kix fishmeal; shown in brackets) and a non-enriched version in order to determine ^{13}C APE of the experimental diets.

‡ Gross energy (MJ/kg) was calculated as the sum of the dietary content of protein, lipid and N-free extract (NFE), multiplied by their respective energetic values upon complete oxidation[50]: Gross energy = ($P_{diet} \times 23\cdot66$) + ($L_{diet} \times 39\cdot57$) + ($NFE_{diet} \times 17\cdot17$), where P_{diet}, L_{diet} and NFE_{diet} refer to the dietary protein, lipid and NFE content (%), respectively. NFE was calculated as the sum of dietary protein, lipid, ash and water deducted from 100 % (by difference).

§ The DE (MJ/kg) content was calculated as the dietary gross energy, but with the apparent nutrient digestibility coefficients (ADC) of each nutrient multiplied into their respective terms: DE = ($P_{diet} \times 23\cdot66 \times ADC_{protein}$) + ($L_{diet} \times 39\cdot57 \times ADC_{lipid}$) + ($S_{diet} \times 17\cdot17 \times ADC_{starch}$), where S_{diet} is the dietary starch content, and $ADC_{protein}$, ADC_{lipid} and ADC_{starch} are the ADC of protein, lipid and starch, respectively.

‖ DP (g/kg) = dietary crude protein content × $ADC_{protein}$.

each of the nine unlabelled diets to three replicate tanks (i.e. twenty-seven tanks in all), and concluded by a faeces stripping procedure to determine the ADC of macronutrients, and subsequently (2) a 3 d enrichment period feeding the ^{13}C-enriched versions of the experimental diets to determine the proportion of dietary protein converted into body lipid *de novo*, and to determine the ADC of the two stable carbon isotopes (^{12}C and ^{13}C). All procedures were carried out in accordance with the EC directive 86/609/EEC for animal experiments[31].

Growth period. At start-up, five randomly chosen fish were removed from each of the twenty-seven tanks and euthanised using 250 mg/l of tricaine methanesulfonate (MS-222). The 5×27 fish were subsequently pooled and stored at $-20°C$ until analysis, constituting the initial fish sample. The remaining fifteen fish in each tank were weighed, and the nine experimental diets were fed to triplicate tanks for 89 d. The fish were fed a ration recalculated from a commercial feeding table value for gilthead sea bream, allowing a restrictive iso-DE feeding regimen based on the expected DE content of the respective experimental diets. Any uneaten feed was collected daily and subtracted in the calculations of feed intake. Fish were fed continuously from 08.00 to 14.00 hours using automatic belt feeders. On day 89, the final meal was administered 18 h before faeces stripping, where the fish were anaesthetised using MS-222 (50 mg/l), and a gentle bilateral pressure was applied to the hindgut in order to provoke defecation. Faeces obtained from the fish within each tank were pooled and immediately frozen at 20°C. At 24 h after the stripping procedure, fish were bulk weighed, and seven fish from each tank were removed, euthanised using MS-222 (250 mg/l) and subsequently stored at 20°C for chemical and isotopic analysis.

^{13}C enrichment period. Following the stripping procedure of the growth trial, the eight remaining fish in each tank were fed their respective nine experimental diets for three more days, only now in the ^{13}C protein-enriched version at a feeding rate calculated as described in the 'Growth period' section. Uneaten feed was collected daily and subtracted in the calculations of feed intake. At 18 h after the final meal, fish fed the diet MPME (three tanks in all) were stripped for faecal matter according to the method described in the 'Growth period' section. This was done in order to determine the ADC of the two stable carbon isotopes (^{12}C and ^{13}C), assuming that they were representative of all the experimental diets. Fish were starved for 48 h after the final meal, and subsequently euthanised using MS-222 (250 mg/l), weighed and stored at 20°C for chemical and isotopic analysis.

Sample preparation and chemical and isotopic analysis

Feed samples. Feed samples were homogenised before analysis using a Krups Speedy Pro homogeniser. Crude protein was determined according to the ISO[32], crude fat according to Bligh & Dyer[33], and DM and ash according to Kolar[34]. Yttrium was determined according to the ISO[35] and Danish Standards[36]. Starch analyses were carried out according to the method by Bach Knudsen[37], while amino acids were determined according to the EC[38] and ISO[39]. Aliquots of the homogenised feed samples were lyophilised and finely

ground using a mortar and pestle before the determination of ^{13}C isotope enrichment and elemental carbon (see the 'Isotopic analysis' section).

Faecal samples. Faecal samples were freeze-dried before analysis using a Christ Beta 2-16 freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH). Faecal protein was determined by elemental analysis according to the method described in the 'Isotopic analysis' section, assuming that protein equals $6.25 \times N$. Faecal lipid was determined according to Bligh & Dyer[33]. Faecal starch was determined using a BioVision Starch assay kit (catalogue no. K647−100, Tecan GENios microplate reader (Austria) fitted with a 570 nm colorimetric filter), and yttrium was determined according to the ISO[35] and Danish Standards[36]. Aliquots of the faecal samples were lyophilised and finely ground using a mortar and pestle before the determination of ^{13}C isotope enrichment, and elemental C and N (see the 'Isotopic analysis' section).

Fish samples. Fish sampled initially (one pooled sample), at the end of the growth trial (twenty-seven samples), and after the ^{13}C enrichment trial (twenty-seven samples) were homogenised in a two-step procedure before chemical and/ or isotopic analysis. Frozen fish samples were homogenised for 60 s using a Tecator 1094 homogeniser (Tecator AB), and an aliquot of each sample was further homogenised for 30 s using a Büchi Mixer B-400 (BÜCHI Labortechnik AG). All sample aliquots were subjected to crude protein, crude lipid, DM and ash analyses, using the same methodology as described for feed (see the 'Feed samples' section). Samples obtained at the end of the growth trial and at the end of the isotope enrichment trial were additionally subjected to stable carbon isotope analysis of their respective lipid fractions. Lipid samples for isotopic analysis were obtained during the lipid extraction process of the Bligh & Dyer[33] procedure.

Isotopic analysis. Feed (enriched and unenriched) and isolated whole-body lipid samples were all subjected to stable isotope (δ^{13}C) and elemental carbon analysis, while faecal samples were additionally subjected to elemental N analysis. Before isotopic analysis, aliquots of all samples were packed and weighed into tin capsules (standard weight pressed tin capsules 5×3.5 mm, catalogue no. D1002; Elemental Microanalysis Limited) using an analytical microbalance (Mettler Toledo MT5; Mettler). All stable isotope enrichment, elemental C and N analyses were carried out using a Thermoquest EA1110 CHNS-O elemental analyser coupled to a Thermo Scientific Delta V advantage isotope ratio mass spectrometer via a Thermo Scientific ConFlo IV module.

Calculations

Stable ^{13}C isotope enrichment (δ^{13}C, ‰) of samples was calculated as:

$$\delta^{13}C = ((R_{sample} - R_{standard})/(R_{standard})) \times 1000,$$

where R_{sample} is the ^{13}C:^{12}C ratio of the sample, and $R_{standard}$ is the ^{13}C:^{12}C ratio of the reference standard calibrated against the international standard V-PDB (Pee Dee Belemnite). The ^{13}C atom per cent excess (APE, %) of the samples was

determined as the difference between the atom percentage (^{13}C atm%) of the enriched sample and the unenriched sample ('blank'), according to:

$$APE\,(^{13}C) = {}^{13}C\,atm\%_{sample} - {}^{13}C\,atm\%_{blank}.$$

Atomic percentages were calculated as:

$$^{13}C\,atm\% = (100 \times AR \times ((\delta^{13}C/1000) + 1))/(1 + AR \times ((\delta^{13}C/1000) + 1)),$$

where AR is the absolute ^{13}C:^{12}C ratio of V-PDB (0·0112372) as given by Craig[40].

Lipid deposition of protein origin (LDPO, %) expressed as a fraction of total lipid deposited was calculated according to:

$$LDPO = ((BM_{end} \times BL_{end} \times C_{wbl} \times {}^{13}C\,APE_{wbl})/PE_{diet})/(FI_{enr} \times DL \times ADC_{lipid} \times aDLR \times C_{wbl}),$$

where BM_{end} is the end biomass (in g); BL_{end} is the end body lipid content (in %); C_{wbl} is the end whole-body lipid carbon content (in %); $^{13}C\,APE_{wbl}$ is the ^{13}C APE in the whole-body lipid fraction of the fish (in %) at the end of the 3 d enrichment period; PE_{diet} is the ^{13}C enrichment of dietary protein (in %); FI_{enr} is the intake of ^{13}C-enriched feed (in g); DL is the dietary lipid content (in %); ADC_{lipid} is the ADC of dietary lipid (in %); aDLR is the aDLR (in %) obtained from the growth trial.

The recovery of carbon derived from dietary protein in whole-fish lipid (RPCL, %) was calculated according to:

$$RPCL = (BM_{end} \times BL_{end} \times C/_{wbl} \times {}^{13}C\,APE_{wbl})/(FI_{enr} \times C_{diet} \times {}^{13}C\,APE_{diet} \times ADC_{protein}),$$

where C_{diet} is the diet carbon content (in %); $^{13}C\,APE_{diet}$ is the ^{13}C APE of the enriched diets (in %); $ADC_{protein}$ is the ADC of protein (in %).

The ADC of nutrient X ($ADC_{(X)}$) was calculated according to:

$$ADC_{(X)} = 1 - ((I_{diet} \times X_{faeces})/(I_{faeces} \times X_{diet})),$$

where I_{diet} and I_{faeces} are yttrium concentrations recovered in the diet and faeces, respectively, and X_{faeces} and X_{diet} are the concentrations of X (protein, lipid, starch or carbon isotope) recovered in the faeces and diet, respectively[41].

Statistical analysis

Data on FCR, SGR, ADC, DPR, aDLR, DER, DE intake, LDPO, RPCL, and the proximate composition of whole fish were subjected to two-way ANOVA to test for the main effects of,

Table 2. Feed conversion ratio (FCR), specific growth rate (SGR), digestible energy (DE) intake (DEI) and apparent nutrient digestibility coefficients (ADC) of macronutrients and stable carbon isotopes

(Mean values and standard deviations, n 3)

Diets…	LPLE	LPME	LPHE	MPLE	MPME	MPHE	HPLE	HPME	HPHE	Two-way ANOVA*		
										DP	DE	DP × DE
SGR (%/d) and DEI (MJ) during the growth period												
FCR†												
Mean	1·48^x	1·32^y	1·27^y	1·42^x	1·38^{x,y}	1·29^y	1·38^x	1·29^{x,y}	1·24^y	0·052	<0·001	0·338
SD	0·03	0·06	0·05	0·03	0·02	0·09	0·07	0·03	0·05			
SGR‡												
Mean	0·96^a	1·02	1·02	1·01^{a,b}	0·99	1·01	1·03^b	1·05	1·04	0·028	0·336	0·266
SD	0·01	0·04	0·04	0·01	0·01	0·06	0·04	0·01	0·03			
DEI§												
Mean	83·5	86·8	82·5	85·1	83·7	85·7	87·0	87·7	87·0	0·027	0·584	0·218
SD	2·9	1·4	1·7	1·5	2·6	2·1	2·2	2·3	2·8			
ADC of dietary macronutrients and stable carbon isotopes (%)												
Protein												
Mean	89·2	89·0	89·1	88·7	89·8	89·3	89·4	89·0	89·1	0·957	0·959	0·849
SD	2·2	0·8	0·8	0·9	1·6	1·1	0·7	0·5	1·0			
Lipid												
Mean	95·0	94·6	94·1	95·0	95·1	94·5	95·3	94·9	94·9	0·605	0·482	0·969
SD	1·6	0·5	1·1	0·6	0·4	1·1	1·8	0·7	0·7			
Starch												
Mean	94·8	95·7	94·8	95·7	95·5	94·7	95·2	95·5	94·6	0·933	0·307	0·924
SD	1·4	1·0	0·2	0·8	0·7	0·9	1·3	1·9	1·5			
^{12}C												
Mean	NA	NA	NA	NA	84·8	NA	NA	NA	NA	0·668$^{\parallel}$		
SD					0·8							
^{13}C												
Mean	NA	NA	NA	NA	84·3	NA	NA	NA	NA			
SD					1·3							

LPLE, low digestible protein (DP):low DE diet; LPME, low DP:medium DE diet; LPHE, low DP:high DE diet; MPLE, medium DP:low DE diet; MPME, medium DP:medium DE diet; MPHE, medium DP:high DE diet; HPLE, high DP:low DE diet; HPME, high DP:medium DE diet; HPHE, high DP:high DE diet; NA, not available.
^{a,b} Mean values of DP within the DE groups were significantly different ($P<0.05$; Holm−Sidak method).
^{x,y} Mean values of DE within the DP groups were significantly different ($P<0.05$; Holm−Sidak method).
* Two-way ANOVA (df = 2, 26) on the effects of DP, DE and their interaction (DP × DE).
† FCR = feed consumed/biomass gain.
‡ SGR[51] = ln(biomass$_{(final)}$/biomass$_{(initial)}$)/(days in the trial) × 100.
§ DEI = feed intake$_{(growth\,trial)}$ × DE$_{diet}$.
$\parallel$ P value is based on a t test comparing the ADC of the two carbon isotopes.

and interactions between, dietary DE and DP, respectively. Significant differences caused by a main effect were subsequently assessed using the Holm–Sidak all-pairwise multiple comparison test. A probability of $P < 0.05$ was considered as significant in all analyses.

Results

Diets and dietary ^{13}C enrichment

The ingredient composition and chemical and isotopic analyses of the experimental diets are shown in Table 1. The nine experimental diets were designed to comprise three different DP levels combined with three different DE levels in a 3×3 factorial design. The crude protein content of the LP, MP and HP diets ranged between 372–385, 404–409 and 443–453 g/kg feed, respectively. Similarly, the DE levels ranged between 19·6–19·9, 20·6–21·2 and 21·3–21·6 MJ/kg feed for the LE, ME and HE diets, respectively. Collectively, the nine experimental diets covered a DP/DE range from 15·6 to 20·6 g/MJ. The dietary carbon content ranged between 46·2 and 51·3 %. The δ^{13}C values of the non-enriched diets ranged between −22·5 and −21·3 ‰, while the δ^{13}C values of the enriched diets ranged between 593·9 and 754·5 ‰, cor-responding to the ^{13}C APE values from 0·673 to 0·846 %. The measured ^{13}C enrichment of dietary protein carbon ranged between 1·718 and 2·010 %. The indispensable amino acid (IAA) profile of the nine experimental diets is presented in Fig. 2. Also, the IAA profile of the *Spirulina* protein isolate employed and the IAA requirements of gilthead sea beam approximated by Kaushik[3] are presented.

Digestibility of macronutrients, energy and carbon isotopes

The ADC of macronutrients and stable carbon isotopes are shown in Table 2. The ADC of protein, lipid and starch ranged from 88·7 to 89·8, 94·1 to 95·3 % and 94·6 to 95·7 %, respectively, and were not significantly affected by the dietary treatment. The ADC of the two stable carbon isotopes, ^{12}C and ^{13}C, were 84·8 and 84·3 %, respectively. No significant differences between the ADC of the two carbon isotopes were observed.

Feeding, growth, feed conversion ratio and mortality

The results on SGR, FCR and total DE intake from the 89 d feeding trial are presented in Table 2. FCR (ranging from 1·24 to 1·48) were significantly lowered by increasing DE in

Table 3. Chemical and isotopic composition of whole fish

(Mean values and standard deviations, *n* 3)*

Diets...	Initial	LPLE	LPME	LPHE	MPLE	MPME	MPHE	HPLE	HPME	HPHE	Two-way ANOVA* DP	DE	DP × DE
Proximate composition of fish (%)													
Protein													
Mean	17·0	17·1	16·9	16·9	17·2	17·1	17·1	17·3	17·2	17·4	0·015	0·176	0·826
SD		0·1	0·1	0·1	0·2	0·2	0·2	0·1	0·1	0·2			
Lipid													
Mean	16·4	21·5	22·3	22·3	21·9	21·5	21·9	21·9	21·4	20·6	0·273	0·508	0·657
SD		0·9	0·2	0·8	1·3	0·6	0·7	0·8	0·8	1·6			
Ash													
Mean	3·4	3·4	3·3	3·2	3·3	3·3	3·3	3·3	3·3	3·2	0·474	0·560	0·749
SD		0·1	0·2	0·1	0·1	0·1	0·1	0·0	0·1	0·1			
DM													
Mean	35·6	40·8	41·5	41·4	41·0	41·0	41·1	41·2	41·1	40·0	0·584	0·738	0·487
SD		0·4	0·7	0·5	0·8	0·5	0·8	0·7	0·6	1·0			
Carbon content of the whole-fish lipid fraction (%)													
Pre-enrichment													
Mean		68·30	67·42	67·43	68·03	68·11	68·08	69·08	68·50	68·33	0·175	0·497	0·907
SD		0·75	0·44	0·25	0·93	1·63	0·89	0·83	1·08	1·44			
Post-enrichment													
Mean		70·01	69·86	69·40	69·57	69·32	68·55	69·84	68·70	68·32	0·294	0·156	0·919
SD		0·14	0·29	0·21	0·11	0·06	0·34	1·04	0·61	0·25			
δ^{13}C values of the whole-fish lipid fraction (‰)													
Pre-enrichment													
Mean		−23·75	−23·50	−23·33	−23·80	−23·57	−23·34	−23·75	−23·58	−23·36			
SD		0·03	0·06	0·06	0·01	0·06	0·02	0·05	0·05	0·04			
Post-enrichment													
Mean		−15·84	−15·11	−15·05	−13·17	−13·87	−14·47	−11·88	−12·53	−12·04			
SD		0·13	0·46	0·60	0·43	0·78	0·31	0·38	0·47	0·51			
^{13}C atom per cent excess (APE) in the whole-fish lipid fraction ($\times 10^{-3}$ %)													
Post-enrichment													
Mean		8·69	9·23	9·10	11·68	10·67	9·75	13·04	12·15	12·44			
SD		0·11	0·58	0·60	0·48	0·92	0·32	0·39	0·46	0·57			

LPLE, low digestible protein (DP):low digestible energy (DE) diet; LPME, low DP:medium DE diet; LPHE, low DP:high DE diet; MPLE, medium DP:low DE diet; MPME, medium DP:medium DE diet; MPHE, medium DP:high DE diet; HPLE, high DP:low DE diet; HPME, high DP:medium DE diet; HPHE, high DP:high DE diet.
* Two-way ANOVA (df = 2, 26) on the effects of DP, DE and their interaction (DP × DE).

all the DP groups (LP, MP and HP), while no significant effects of DP were observed in FCR. SGR (ranging from 0·96 to 1·05 %/d) were slightly, but significantly, higher with increasing DP in fish fed the LE diets, while no significant effects of DE on SGR were observed for any of the dietary treatment groups. DE intake ranged between 82·5 and 87·7 MJ, and was significantly different among the DP groups. No mortality occurred throughout the trial.

Chemical composition of fish

The chemical composition of whole fish at the beginning and at the end of the 89 d growth period is presented in Table 3. A two-way ANOVA showed no significant effects of dietary treatment on whole-body lipid, ash or DM, while the whole-body protein content was significantly higher in fish fed the HP diets than in fish fed the LP and MP diets. After 3 d of feeding using diets with a ^{13}C-enriched protein content, ^{13}C APE in the lipid fraction of whole fish ranged between 8·69 and 13·04 × 10^{-3} % (Table 3).

Nutrient retention efficiencies

The results on DPR and aDLR based on the 89 d feeding trial are presented in Table 4 and Fig. 1(a) and (b). DPR ranged between 30·6 and 39·2 %, and was significantly affected by both dietary DP and DE levels, showing increased retention efficiency with increasing DE and decreasing DP (Fig. 1(a)). aDLR ranged between 70·4 and 95·1 %, and was also significantly affected by both dietary DP and DE levels, showing increased retention efficiency with increasing DP within the LE groups and/or with decreasing DE within the MP and HP groups (Fig. 1(b)). The results on DER are also presented in Table 4 ranging between 48·0 and 54·9 %. DER was not significantly affected by the dietary treatment.

Recovery of protein-derived carbon in fish lipid and contribution to the total lipid deposition of lipid synthesised from dietary protein de novo

RPCL as determined from the stable isotope analyses ranged between 18·6 and 22·4 %, and was significantly affected by DE and the interaction between DP and DE. Pairwise comparisons showed that RPCL was significantly increased by increasing DP in the LE group and by decreasing DE in the MP group (Table 4; Fig. 1(c)). The contribution to the total lipid deposition of lipid synthesised from dietary protein de novo (LDPO) ranged between 21·6 and 30·2 %, and was significantly affected by both dietary DP and DE levels, showing increased contribution with decreasing DE and/or increasing DP (Table 4; Fig. 1(d)).

Discussion

The main purpose of the present study was to determine: (1) the magnitude of de novo lipogenesis originating from dietary protein in gilthead sea bream fed nine diets with different DP (330, 360 or 380 g/kg) and DE (20, 21 or 22 MJ/kg) levels set up in a 3 × 3 factorial design, and (2) the overall contribution to lipid deposition from de novo lipogenesis originating from dietary protein in this species. Simple, high-quality raw material matrices were applied to assure the highest possible quality of dietary nutrients and to avoid possible anti-nutritional effects associated with certain plant raw materials[42]. DP/DE levels of the nine experimental diets were deliberately formulated to also cover a lower range (from 15·6 to

Table 4. Digestible macronutrient retention and recovery of protein-derived carbon in the whole-fish lipid fraction

(Mean values and standard deviations, n 3)*

Diets...	LPLE	LPME	LPHE	MPLE	MPME	MPHE	HPLE	HPME	HPHE	DP	DE	DP × DE
										Two-way ANOVA*		
DPR (%)†												
Mean	33·2[x]	37·3[a,y]	39·2[a,y]	32·1[x]	32·6[b,x]	35·4[b,y]	30·6[x]	33·0[b,x]	34·5[b,y]	<0·001	<0·001	0·237
SD	0·8	1·5	1·0	1·0	0·7	2·5	2·3	0·3	0·5			
aDLR (%)†												
Mean	79·4[a]	78·4	70·4	92·2[b,x]	80·5[y]	74·7[y]	95·1[b,x]	86·3[x,y]	76·0[y]	0·007	<0·001	0·448
SD	8·7	1·7	4·5	6·0	2·2	2·1	6·4	7·2	7·9			
DER (%)†												
Mean	48·0	51·6	52·1	52·8	51·0	53·4	53·2	54·9	53·0	0·101	0·524	0·405
SD	3·8	1·2	2·7	3·0	1·3	1·9	3·3	3·4	3·9			
RPCL (%)												
Mean	19·2[a]	19·8	19·3	22·4[b,x]	18·9[y]	18·6[y]	21·1[b]	19·5	19·8	0·243	0·001	0·005
SD	0·9	0·7	0·3	0·9	0·9	0·2	1·0	0·8	1·5			
LDPO (%)												
Mean	25·9[a,x]	22·1[a,y]	21·9[a,y]	28·8[b,x]	23·4[a,b,y]	21·6[a,z]	30·3[b,x]	25·2[b,y]	26·3[b,y]	0·002	<0·001	0·542
SD	2·4	1·2	0·9	2·3	1·9	1·1	2·5	2·2	2·5			

LPLE, low digestible protein (DP):low DE diet; LPME, low DP:medium DE diet; LPHE, low DP:high DE diet; MPLE, medium DP:low DE diet; MPME, medium DP:medium DE diet; MPHE, medium DP:high DE diet; HPLE, high DP:low DE diet; HPME, high DP:medium DE diet; HPHE, high DP:high DE diet; DPR, digestible protein retention; aDLR, apparent digestible lipid retention; DER, digestible energy retention; RPCL, recovery of protein-derived carbon in fish lipid; LDPO, lipid deposition of protein origin.
[a,b] Mean values of DP within the DE groups were significantly different (P<0·05; Holm−Sidak method).
[x,y] Mean values of DE within the DP groups were significantly different (P<0·05; Holm−Sidak method).
* Two-way ANOVA (df = 2, 26) on the effects of DP, DE and their interaction (DP × DE).
† DPR, aDLR and DER were calculated as the ratio between the amount of protein, lipid and energy retained by the fish and the amount of protein, lipid and energy digested by the fish during the growth trial, respectively.
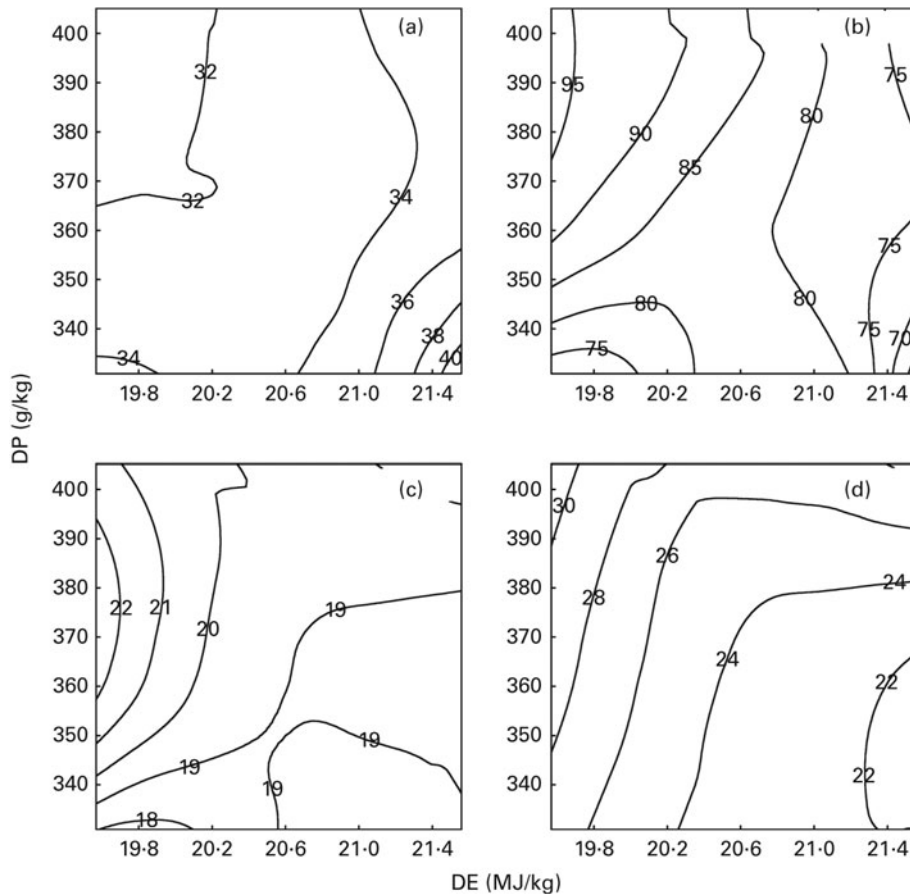
Fig. 1. Contour plots of the effects on (a) digestible protein (DP) retention (%), (b) apparent digestible lipid retention (%), (c) recovery of protein carbon in the lipid fraction of fish (%) and (d) the percentage of total lipid deposit originating from dietary protein (%) in fish fed the nine diets differing in DP content and digestible energy (DE) content for a period of 89 feeding days. The response values of changes in DE (horizontal axis) and DP (vertical axis) are given directly on the contour curves seen in the four plots. All diets were fed to triplicate tanks.

20·1 g/MJ) than previously recommended for gilthead sea bream by, for example, Lupatsch *et al*.[12] (ranging from 19·0 to 22·6 g/MJ for present fish size). This was done in order to incite the possible effects of protein deficiency on *de novo* lipogenesis, performance and nutrient retention efficiencies. Also, recommendations on dietary DE densities from the same authors were slightly more conservative (ranging between 15 and 20 MJ/kg) than dietary DE densities of the present study (ranging between 19·6 and 21·6 MJ/kg).

The dietary IAA profile of all the experimental diets satisfied the requirements put forward by Kaushik[3]. However, since these recommendations were expressed relatively to dietary N content, fish fed the low DP/DE diets might have experienced a general lack of DP.

The present study clearly demonstrated that DP, irrespective of the diet, did indeed contribute significantly to endogenous lipid biosynthesis in gilthead sea bream, as seen both from RPCL and from the contribution of lipid synthesised *de novo* to total lipid deposition (LDPO; Table 4; Fig. 1(c) and (d)). The results thereby corroborate the findings by Enes *et al*.[30] and Figueiredo-Silva *et al*.[27] who both observed a significant correlation between hepatic lipogenic enzyme activity (glucose-6-phosphate dehydrogenase) and dietary protein level in diets for gilthead sea bream

and blackspot seabream, respectively. These studies, including the present study, are thereby in contrast to the review by Tocher[43], who claims that biosynthesis of fatty acids *de novo* is not likely to occur to any significant extent in marine predatory species. Also, using ^{13}C-labelled dietary protein, Campbell[26] found that between 9·7 and 44·5 % of whole-body lipids in rainbow trout (*Oncorhynchus mykiss*) juveniles were derived from dietary protein, using diets with a protein:energy ratio ranging between 17·7 and 26·6 g/MJ, respectively. In the present study, approximately one-fifth (18·6–22·4 %) of the dietary DP supplied was converted into body lipid, irrespective of the dietary treatment. Fish fed the MPLE and HPLE diets displayed slightly higher RPCL values (22·4 and 21·1 %, respectively) than fish fed the remaining seven diets. This might have been due to these two diets having the highest DP/DE level in their respective DE groups, triggering excessive protein deamination and donation of extra carbon for lipid biosynthesis (Table 4; Fig. 1(c)). The contribution of lipid synthesised *de novo* from DP to total lipid deposition (LDPO) ranged between 21·6 and 30·2 %, confirming that *de novo* lipid synthesis from DP plays a major role in the overall lipid deposition in gilthead sea bream. LDPO was clearly elevated in the low-energy diets of each DP group, and by increasing
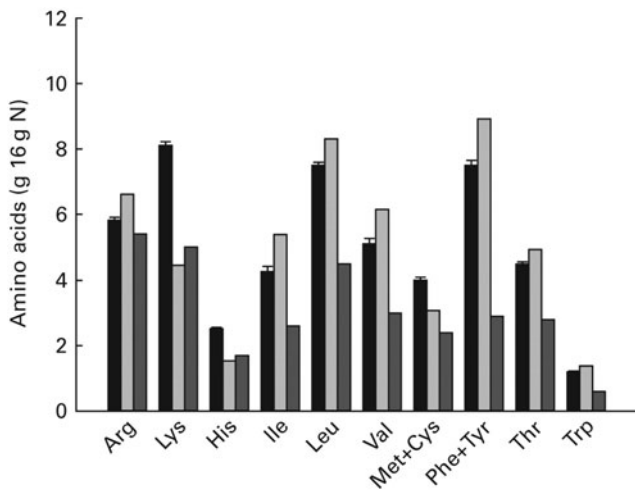
Fig. 2. Indispensable amino acid (IAA) profile including Cys and Tyr of the diets (■) and *Spirulina* protein isolate (□). The IAA requirements of gilthead sea bream (*Sparus aurata*) as approximated by Kaushik[3] are shown as ■. Values are means (*n* 9), with standard deviations represented by vertical bars.

DP generally (Table 4; Fig. 1(d)). Thus, LDPO values were directly related to dietary DP/DE levels. This was also reflected in the aDLR values that ranged between 70·4 and 95·1 % (Table 4; Fig. 1(b)). Hence, similar to the LDPO results, aDLR increased with increasing dietary DP and decreasing DE (i.e. increasing DP:DE ratio). Conversely, the DPR results showed increasing retention efficiencies with decreasing DP and/or increasing DE levels (i.e. decreasing DP:DE ratio). These results substantiate the so-called protein-sparing effect of substituting DE originating from DP with DE from non-protein sources, as already reported in a number of aquacultured species[23,44,45], including gilthead sea bream[19,24,46] (Table 4; Fig. 1(a)). Thus, the aDLR and DPR results combined suggest that while protein was spared by a decreasing dietary DP/DE level, the opposite was true for lipid, substantiating that deaminated DP was indeed converted into body lipids. The results of these opposing nutrient retention dynamics have supposedly rendered differences in the overall DER insignificant, as shown in Table 4. In addition, the proximate composition of whole fish was largely unaffected by the dietary treatment. No significant effects were observed in whole-body lipid, ash and DM, while a very small, but significant, effect of dietary DP was observed on whole-body protein content. This is in accordance with the finding of Bonaldo et al.[47], who found no difference in the body composition of gilthead sea bream fed three diets differing in dietary DP:DE ratio for 81 d. Thus, considering lipogenesis, nutrient retention and body composition results of the present study collectively, fish appeared to endeavour to rigorously maintain a certain whole-body energy status under a wide variety of dietary DP:DE ratios, even if substantial amounts of dietary protein were sacrificed to achieve this.

It was expected that the SGR values obtained from the growth period would not differ significantly among the dietary treatment groups, as fish were fed iso-DE throughout the trial. However, a small, but significant, difference was observed between the LPLE and HPLE fish. This could be partly explained by the slightly lower DE intake observed in fish fed the LPLE diet (83·5 MJ) compared with fish fed the HPLE diet (87·0 MJ), or by a possible general lack of DP experienced by LPLE fish. However, since dietary DP levels did not have any significant effect on the FCR obtained from the growth trial, the latter point probably does not apply. In contrast, FCR were clearly improved by a dietary DE increase. This clear link between dietary DE and FCR responses has been reported earlier in a number of aquacultured fish species[17,48], including gilthead sea bream[12,13] when growth was not limited by dietary protein content. The present results thereby indicate that gilthead sea bream have the ability to efficiently utilise diets with lower DP:DE ratios and higher energy densities (virtually resembling commercial diets for salmonid species) than previously recommended[12] without showing adverse effects on the proximate composition or performance of the fish.

The measured ADC of protein, lipid and starch did not differ significantly among the dietary treatments, which was also expected from diet optimisation, considering that the same raw materials were used in all diets, and only inclusion levels differed.

An inherent problem by using a tracer to investigate metabolic pathways is the potential difference in functional behaviour between the tracer and the tracee. In the present study, a uniformly ^{13}C-labelled *Spirulina* protein isolate was used to trace the fate of dietary protein, which mainly originated from con-kix fishmeal. However, possible differences in overall protein digestibility, amino acid profile and individual amino acid digestibility between the *Spirulina* protein isolate and dietary protein could potentially lead to differences in the way that the tracer and the tracee were metabolised, rendering the tracer unsuitable for the purpose. However, when comparing the IAA profile of the *Spirulina* protein isolate and experimental diets, only small differences were apparent (Fig. 2). The only clear difference was a considerably higher lysine content of the experimental diets. This difference, however, was unlikely to change the overall pattern in amino acid deamination since the remaining amino acids were basically in balance, and all experimental diets, as such, fulfilled the general IAA requirements of gilthead sea bream[3,4]. The ADC of individual amino acids were not determined in the present trial. However, the ADC of measured stable carbon isotopes displayed no significant differences between ^{12}C and ^{13}C, indicating that *Spirulina* whole protein was indeed digested similarly to the remaining dietary protein fraction. Thus, it was assumed that the *Spirulina* protein isolate could be considered a true tracer, not behaving functionally different from the tracee.

Conclusion

For the first time, orally administered ^{13}C-labelled protein was applied to quantify *de novo* lipogenesis originating from dietary protein, and to determine the importance of this in the overall body lipid deposition in gilthead sea bream. Irrespective of the dietary treatment, the fish converted substantial

amounts of carbon derived from dietary protein into body lipids, which in turn contributed significantly to total body lipid deposition. Despite dietary effects on protein and lipid retention efficiencies and *de novo* lipogenesis, the fish were able to maintain a constant retention of DE with no significant effects seen on the whole body composition. The results indicate that this species may have evolved to maximise energy storage (in the form of lipid) for seasonal, migratory or maturation purposes at the expense of increasing body size through more efficient use of protein for growth. Additionally, the improvement of FCR by increased DE combined with an improvement of DPR with decreasing DP/DE levels suggest that gilthead sea bream is able to efficiently utilise feeds within a wide range of dietary DP:DE ratios, which could be taken into consideration in the future production of commercial feeds for this species.

Acknowledgements

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