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Performance of formulated diets with different level of lipids and glutamate supplementation in *Octopus vulgaris*

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Abstract

Growth, feed efficiency and proximate and lipid class composition of subadults *Octopus vulgaris* (788 ± 133 g; 18.5°C) fed formulated diets of low lipid (LL: 8 g kg^{-1}) and high lipid levels (HL: 84 g kg^{-1}) and each one of these with three different levels of glutamate supplementation (0, 5 and 20 g kg^{-1}) were compared. All the animals accepted the diets with a survival of 100%. The addition of glutamate did not stimulate feeding rates in any of the assays (2.48–2.64 and 1.86–2.01%Body weight day^{-1} for LL and HL, respectively; $P > 0.05$). The best growth, feed efficiency and protein productive value were observed in the groups fed 5 or 20 g kg^{-1} glutamate supplementation at both lipid levels, with significant differences for LL diet ($P < 0.05$). A better feed efficiency was achieved with the HL diet (14.6–27.5% vs. 2.5–19.2% for LL diet). There were no significant differences in the proximate composition of carcass (animal excluding the digestive gland). However, a substantial amount of lipids accumulated in the digestive gland, mainly triglycerides, was detected as a consequence of higher lipid ingestion or glutamate supplementation. It was notable the lower percentages of phosphatidylethanolamine and phosphatidylcholine in the digestive gland of animals with best growth.

Keywords: formulated diet, glutamate, lipids, nutrition, *Octopus vulgaris*

Introduction

The successful commercial ongrowing of any species needs a formulated diet, given the advantages of such compared with natural diets, and this is the case with seabream, seabass and turbot (Cho & Bureau 2001; Davies, Gouveia, Laporte, Woodgate & Nates 2009). In the case of cephalopods, our knowledge is still in the early stages of finding feeds of suitable nutritional composition and satisfactory performance, and this is one of the barriers to full scale commercial production (Lee 1994; Domingues, López, Muñoz, Maldonado, Gaxiola & Rosas 2007).

Among different cephalopod species, common octopus (*Octopus vulgaris*) has awakened much interest in recent years due to its biological characteristics and good commercial value (Vaz-Pires, Seixas & Barbosa 2004). In this species, the lack of formulated diets, combined with the impossibility of obtaining benthic juveniles on a commercial scale in captivity (Navarro & Villanueva 2000, 2003; Iglesias, Sánchez, Bersano, Carrasco, Dhont, Fuentes, Linares, Muñoz, Okumura, Roo, Meeren, Vidal & Villanueva 2007), implies the capture of subadults at sea and their ongrowing based on feeding with different species of fish and crustaceans of a low commercial value (Rodríguez, Carrasco, Arronte & Rodríguez 2006; García García, Cerezo Valverde, Aguado-Giménez & García 2009). This explains why such an activity is still not an attractive commercial proposition (García García, Rodríguez González & García 2004).

Although much research effort has gone into preparing formulas for cephalopods, including cuttlefish and octopus, in most studies published until 2008, growth and feeding rates have not been satisfactory and differ much from those obtained using natural diets (Lee, Forsythe,

Dimarco, DeRusha & Hanlon 1991; Castro & Lee 1994; Domingues, Dimarco, Andrade & Lee 2005; Rosas, Cuzon, Pascual, Gaxiola, Chay, López, Maldonado & Domingues 2007). This has sometimes been attributed to the unpalatability of the diets and, if ingested at all, to their deficient nutritional composition. Nevertheless, some research groups have recently obtained formulas that were acceptable to octopus and produced significant growth (Cerezo Valverde, Hernández, Aguado-Giménez & García García 2008; Quintana, Domingues & García 2008; Rosas, Tut, Baeza, Sánchez, Sosa, Pascual, Arena, Domingues & Cuzon 2008). All these diets have in common that they use a paste of fish or crustaceans mixed with different binders, such as alginate or gelatine, which provide a moist feed (>70% water), but with final texture that is suitable for manipulation and ingestion. In this sense, formulated feeds with a known nutritional composition could be developed to improve our knowledge on the nutritional requirements of cephalopods. According to previous studies, both the quantity and type of lipids (Navarro & Villanueva 2000, 2003; García García & Aguado Giménez 2002; Petza, Katsanevakis & Verriopoulos 2006; García-Garrido, Hachero-Cruzado, Garrido, Rosas & Domingues 2010; Estefanell, Roo, Guirao, Afonso, Fernández-Palacios, H., Izquierdo, M. & Socorro 2011; Cerezo Valverde, Hernández, García-Garrido, Rodríguez, Estefanell, Gairín, Rodríguez, Tomás & García García 2012) and balance of amino acids (Lee 1994; Villanueva, Riba, Ruíz-Capillas, González & Baeta 2004; Domingues *et al.* 2005) should be borne in mind when designing a formulated feed.

This study compares the growth, feed efficiency and proximate and lipid class composition in common octopus after feeding with low or high lipid diets, each of these diets supplemented with three different levels of glutamate, the most abundant amino acid in cephalopods and the natural diets they usually consume (Zdzislaw, Kolakowska & Sun Pan 1994; Villanueva *et al.* 2004).

Material and methods

Experimental animals and maintenance

The octopuses (*O. vulgaris*) were caught at Mediterranean Sea (Murcia, S.E. Spain) and kept in 2000 L tanks in the laboratory. The experiments began when the animals had acclimatized (2 weeks) and were seen to be feeding on the amount of feed calculated according to Aguado Giménez and García García (2002). Bogue (*Boops boops*) and crab (*Carcinus mediterranus*) were supplied on alternate days before the experiment started. Subsequently, the animals were transferred to individual 216 L circular tanks -both to avoid cannibalistic behaviour and for an exact determination of food intake-, containing PVC tubes as shelters and connected to a recirculation seawater system with the controlled temperature, UV lamps and mechanical and biological filtration systems. The water temperature varied between 17 and 20°C, photoperiod was 12L:12D, salinity 37 g L^{-1}, pH 7.7–8.1, dissolved oxygen was maintained above 90% saturation, so that this factor was not limiting (Cerezo Valverde & García García 2005), and total ammonia nitrogen was below 0.2 mg L^{-1}. All animals used were male at the same maturity stage to avoid the influence of reproductive processes.

Experimental design

Two experiments lasting 29 days were carried out, using a low lipid diet (LL, Dec 07) and a high lipid diet (HL, Feb 08). Each of these diets was supplemented with three different degrees of glutamate in crystalline form (0, 5 and 20 g kg^{-1}; Sodium L-Glutamate 1-Hydrate, PANREAC, Barcelona, Spain). In each experiment, 16 animals were isolated and distributed into three experimental groups. In the LL trial: low lipid and 0 g kg^{-1} glutamate supplementation (LL-0; $N = 4$), low lipid and 5 g kg^{-1} glutamate supplementation (LL-5; $N = 6$) and low lipid and 20 g kg^{-1} glutamate supplementation (LL-20; $N = 6$). In the HL trial: high lipid and 0 g kg^{-1} glutamate supplementation (HL-0; $N = 4$), high lipid and 5 g kg^{-1} glutamate supplementation (HL-5; $N = 6$) and high lipid and 20 g kg^{-1} glutamate supplementation (HL-20; $N = 6$). The initial weights and temperatures throughout the experiments were 871 ±125 g (mean ± standard deviation) and 18.5 ± 0.4°C for LL diet, and 703 ± 78 g and 18.5 ± 0.4°C for HL diet. All the diets were administered daily to satiety 6 days a week at 9 a.m., whereas the uneaten food was collected 4–5 h later (13–14 p.m.) to be dried with absorbent paper and weighed to calculate daily ingestion rates.

Manufacture and water stability of the formulated diets

Six formulated diets were elaborated using sodium alginate as binder (see Table 1 for composition) according to Cerezo Valverde *et al.* (2008). To prepare the formulated feeds, a purée of sodium alginate was prepared (65 g of the commercial product POKEL MERL® in 1 L of distilled water; Productos Sur S.A., Murcia, Spain), mixed with a paste of fish (*Boops boops*) and prawn (*Hymenopenaeus muelleri*) previously boned and shelled, and the quantity of glutamate according to the degree of supplementation required, using a household blender. In the LL assay, the *Boops boops* used (obtained using traditional fishing methods) had a low lipid level (2.2%), whereas in HL, the same species (by-catch from marine fish farm) had a high lipid level (17.6%). Changes in lipid content and their classes in *Boops boops* from different origin have been detailed in previous studies (Estefanell *et al.* 2011; Cerezo Valverde *et al.* 2012). Subsequently, a solution of calcium sulphate (10 g POKEL CALS® for every 100 mL distilled water; Productos Sur S.A.) was added at a rate of 100 g kg^{-1} to harden the mixture. After homogenization, the preparations were placed on plastic trays and kept at 4°C for 24 h. Finally, the diets were cut into 3-cm cubes and kept in the freezer until use.

The water stability of the feeds was determined by weighing five samples of each and then soaking in water for 4 and 24 h. With the data obtained, the mean values of the following indices were calculated:

VW (%) = (Wf−Wi)/Wi × 100, which expresses the variation in weight of the feed after soaking in water, where Wi and Wf are the initial and final wet weights respectively (Table 2).

F = Wi/Wf, which represents a correction factor (Table 2). The weight of uneaten food was multiplied by this correction factor to account for water absorption or disintegration to estimate the change in weight.

Analytical method

The proximate composition (moisture, protein, fat and ash) was analysed in three octopuses of similar weight (initial control) to those used in the experiment and in all the octopuses at the end of the experiment. To do this, the carcass (animal

Table 2 Results of the water stability tests for formulated diets

	4 h in water		24 h in water	
Diet	**VW (%)***	**F†**	**VW (%)***	**F†**
Low lipid (LL)				
LL-0	−3.33	1.03	−17.20	1.21
LL-5	−4.10	1.04	−23.97	1.32
LL-20	−9.19	1.10	−24.45	1.32
High lipid (HL)				
HL-0	3.17	0.97	−0.38	1.00
HL-5	3.36	0.97	1.15	0.99
HL-20	2.59	0.97	1.06	0.99

*Mean variation in weight of feed (% alter after immersion in water).

†Correction factor for calculating real intake.

Table 1 Composition (g kg^{-1}) of the experimental formulated diets

Diet	Distilled water*	Sodium alginate†	Calcium sulfate‡	Sodium glutamate§	Fish paste¶	Prawn paste**
Low lipid (LL)						
LL-0	373	18	9	0	500[a]	100
LL-5	368	18	9	5	500[a]	100
LL-20	354	17	9	20	500[a]	100
High lipid (HL)						
HL-0	373	18	9	0	500[b]	100
HL-5	368	18	9	5	500[b]	100
HL-20	354	17	9	20	500[b]	100

*Used in elaboration of purée of alginate and calcium solution.

†Used in elaboration of purée of alginate [65 g of Pokel Merl (Productos Sur. S.A.) in 1 L of distilled water].

‡Used in elaboration of calcium solution [1 g of Pokel Cals (Productos Sur. S.A.) in 10 mL of distilled water].

§Sodium L-Glutamate 1-Hydrate (PANREAC, Barcelona, Spain).

¶*Boops boops*. edible portion. [a]From traditional method fisheries (low lipid level); [b]From by-catch of fish farms (high lipid level).

***Hymenopenaeus muelleri*. edible portion.

excluding the digestive gland) and the digestive gland (DG) were ground and mixed separately until a homogenous sample was obtained. Subsequently, carcass and DG analyses were carried out. The nutritional composition of the formulated feeds was also analysed (Table 3).

A 1 g sample (in the case of moisture, protein and ash) or 2 g sample (in the case of lipids) was used for the analyses, which were carried out in triplicate. Protein content was determined using the Kjeldhal method using a conversion factor of 6.25 (AOAC 1997; Method nº. 954.01). The lipid content was obtained using ether extraction in a SOXTEC AVANTI 2058 (AOAC 1997; Method nº 920.39). Moisture was obtained by drying (105 ± 1°C, 24 h) to constant weight in a drying chamber KOWELL D2 NOVA (AOAC 1997; Method nº. 930.15), and ash by incineration (loss in weight: at 450 ± 1°C, 24 h) in an oven MUFLA HOBERSAL, HD-230 (AOAC 1997; Method nº 942.05). Nitrogen-free extract (NFE) was calculated by difference. Gross energy and the protein energy ratio (P/E in g MJ^{-1}) were estimated using the Miglavs and Jobling (1989) energy coefficients: protein 23.6 kJ g^{-1}, lipid 38.9 kJ g^{-1} and carbohydrate 16.7 kJ g^{-1}.

The lipid classes were obtained in formulated diets, digestive gland and carcass of animals for each experiment. For this, the quantity of sample necessary to extract 10 mg of lipids was processed according to Folch, Lees and Sloane-Stanley (1957); in duplicate. The lipids were kept dissolved in chloroform:methanol (2:1) containing butylated hydroxytoluene (0.01%) as antioxidant at −80°C (Christie 1982), adjusting the concentration to 10 μg lipids μl^{-1} before analysis. The lipid classes were separated using HPTLC according to Olsen and Henderson (1989). Lipids (15 μg) were applied to 20 × 10 cm silica gel plates (MERCK, Darmstadt, Germany) using an automatic injector (Linomat 5; CAMAG, Muttenz, Switzerland). The plates were developed using methyl acetate, isopropanol, chloroform, methanol and 0.25% (w/v) KCl (10:10:10:4:3.6 by vol.) as the polar solvent system and hexane, diethyl ether and glacial acetic acid (32:8:0.8 by vol.) as the neutral solvent system. The lipid classes were visualized by charring at 160°C for 15 min after spraying with 3% (w/v) cupric acetate in 8% phosphoric acid. Final quantification was made using densitometry in a TLC 3 scanner (CAMAG) at a wavelength of 254 nm. To identify the lipid classes, their order of appearance and reference position was determined by applying each one separately and using solutions made up with pure standards (Larodan Fine Chemicals, Malmo, Sweden). Polar and neutral lipids were included: lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylethanolamine (PE), monoacylglycerols (MG; monoolein), diacylglycerols (DG; diolein), cholesterol (CHO), free fatty acids (FFA; oleic acid), triacylglycerols (TG; triolein) and steryl esters (SE; Cholesteryl oleate).

Parameters calculated and data analysis

All the samples were weighed at the outset and at the end of the experiment (Wi = initial weight in g; Wf = final weight in g). The following indices were calculated as follows:

Weight Increment: WI = Wf−Wi; Absolute Feeding Rate: AFR = IF/t; Absolute Protein Feed-

Table 3 Nutritional composition (g kg^{-1} wet substance) of formulated diets

Diet	Moisture (g kg^{-1})	Protein (g kg^{-1})	Lipids (g kg^{-1})	Ash (g kg^{-1})	NFE (g kg^{-1})	Gross energy (kJ kg^{-1})	P/E (g MJ^{-1})
Low lipid							
LL-0	820.4	132.8	9.1	28.0	9.7	3650.1	36.38
LL-5	816.6	141.7	7.8	30.7	3.2	3701.0	38.29
LL-20	792.1	146.3	8.1	31.3	22.2	4138.5	35.35
High lipid							
HL-0	744.0	120.2	84.0	22.5	29.3	6593.6	18.23
HL-5	723.9	125.5	83.8	22.8	44.0	6956.4	18.04
HL-20	721.8	143.3	83.4	24.2	27.3	7082.1	20.23

NFE, Nitrogen-free extract; P/E, Protein/energy ratio. Protein 23.6 kJ g^{-1}, lipids 38.9 kJ g^{-1} and carbohydrates 16.7 kJ g^{-1} according to Miglavs and Jobling (1989).

ing Rate: APFR = IP/t; Absolute Lipid Feeding Rate: ALFR = IL/t; Specific Feeding Rate: SFR = AFR100/Wa; Absolute Growth Rate: AGR = (Wf−Wi)/t; Specific Growth Rate: SGR = (LnWf−LnWi) 100/t; Feed Efficiency: FE = (Wf−Wi)100/IF; Protein Productive Value: PPV = 100(Retained protein/IP); Lipid Productive Value: LPV = 100 (Retained lipid/IL); Digestive gland index: DGI = (DGW/Wf) × 100; where Wa = average weight between sampling in g; t = time in days; IF ingested food in g (food supplied-food uneaten × F, where F values were obtained as correction factor from Table 2); IP ingested protein in g; IL ingested lipid in g; DGW = digestive gland weight.

The distribution of the different lipid classes was obtained as a percentage with respect to the total of the areas detected in the densitometer. The effect of glutamate supplementation on the above indices was analysed using one-way ANOVA at both lipid levels. The significance of the differences in mean values was analysed using Duncan's test, establishing a significance level of $P < 0.05$. A Neperian logarithmic transformation of the indices and percentages was made before the ANOVA.

Results

Water stability, nutritional and lipid class composition of the formulated diets

All the formulated diets had a firm texture before being put into water. However, after soaking in water, the three LL diets demonstrated lower stability than the three HL diets. Table 2 shows how the LL feeds lost between 17% and 25% of their weight after immersion for 24 h, whereas the HL feeds showed hardly any change in weight. The lipid content of the HL feeds was between 83 and 84 g kg^{-1}, Whereas it did not exceed 10 g kg^{-1} in LL (Table 3). As a consequence, the HL feeds contained double the energy content of LL, and had lower humidity and protein/energy ratio. In the feeds as a whole, the protein content varied around 120–147 g kg^{-1}, increasing as glutamate supplementation increased. The LL diet lipid class composition was 10.8% in polar lipids and 89.2% in neutral lipids, predominating free fatty acids (31.6%), triacylglycerols (28.0%) and cholesterol (11.9%; Table 4). Polar lipids were not detected in HL diet, triacylglycerols being particularly well represented (66.7%; Table 4).

Table 4 Lipid class composition of formulated diets (% ± SD total lipids detected)

	Diet	
Lipid classes	**Low lipid**	**High lipid**
Polar lipids		
LPC	4.01 ± 2.88	n.d.
SM	3.31 ± 0.12	n.d.
PC	3.52 ± 1.54	n.d.
LPE	n.d.	n.d.
PS/PI	n.d.	n.d.
PA	n.d.	n.d.
PE	n.d.	n.d.
Neutral lipids		
MG	11.37 ± 1.65	8.54 ± 0.64
DG	2.42 ± 0.34	6.15 ± 0.68
CHO	11.94 ± 0.22	4.82 ± 0.20
FFA	31.63 ± 0.77	13.14 ± 1.45
TG	28.02 ± 1.41	66.68 ± 0.91
SE	3.80 ± 0.36	n.d.

LPC, Lysophosphatidylcholine; SM, Sphingomyelin; PC, Phosphatidylcholine; LPE, Lysophosphatidylethanolamine; PS/PI, Phosphatidylserine and Phosphatidylinositol; PA, Phosphatidic acid – including phosphatidylglycerol and cardiolipin; PE, Phosphatidylethanolamine; MG, Monoacylglycerols – including pigments; DG, Diacylglycerols; CHO, Cholesterol; FFA, Free fatty acids; TG, Triacylglycerols; SE, Steryl esters – including waxes; n.d. = not detected.

Growth and feed efficiency parameters

All the animals accepted the diets with a survival of 100% in both assays. The specific feeding rates were ranged between 2.49 and 2.69%Body weight day^{-1} (%BW day^{-1}) for LL diet, without significant differences for glutamate supplementation degree (Table 5). There were no significant differences for APFR or ALFR ($P > 0.05$). The WI, AGR, SGR, FE and PPV values were increased for the 20 g kg^{-1} glutamate supplementation with respect to those without glutamate supplementation, with significant differences ($P < 0.05$; Table 5). The degree of glutamate supplementation did not affect LPV or DGI ($P > 0.05$). With reference to HL diet, the specific feeding rates were ranged between 1.86 and 2.05%BW day^{-1}, without significant differences for glutamate supplementation degree (Table 5). There were no significant differences for APFR or ALFR either ($P > 0.05$). The best WI, AGR, SGR, FE and PPV were observed in the groups fed 5 or 20 g kg^{-1} glutamate diets, although the high degree of variability observed for these did not allow significant differences to be detected (Table 5). There were no significant differences for DGI or LPV ($P > 0.05$).

Table 5 Mean values and standard deviations for every index in *Octopus vulgaris* fed with diet of low lipid level (8 g kg^{-1}) or high lipid level (84 g kg^{-1}) and different levels of glutamate supplementation

		Glutamate supplementation (g kg^{-1})			
Low lipid diet		**0**	**5**	**20**	**ANOVA**
N		4	6	6	
Wi	g	842 ± 66	902 ± 163	859 ± 126	ns
Wf	g	857 ± 85	959 ± 173	1002 ± 151	ns
WI	g	15 ± 45^{a}	57 ± 86^{ab}	143 ± 69^{b}	*
AFR	g day^{-1}	22.23 ± 2.35	23.42 ± 5.06	24.98 ± 4.72	ns
APFR	g day^{-1}	2.82 ± 0.31	3.31 ± 0.72	3.65 ± 0.69	ns
ALFR	g day^{-1}	0.19 ± 0.02	0.21 ± 0.04	0.22 ± 0.04	ns
SFR	%BW day^{-1}	2.49 ± 0.13	2.52 ± 0.37	2.69 ± 0.39	ns
AGR	g day^{-1}	0.53 ± 1.57^{a}	1.96 ± 3.06^{ab}	4.93 ± 2.39^{b}	*
SGR	%BW day^{-1}	0.06 ± 0.17^{a}	0.21 ± 0.33^{ab}	0.53 ± 0.24^{b}	*
FE	%	2.51 ± 7.11^{a}	7.08 ± 12.05^{ab}	19.18 ± 8.34^{b}	*
PPV	%	9.63 ± 5.52^{a}	13.93 ± 17.57^{ab}	28.82 ± 12.54^{b}	*
LPV	%	12.08 ± 9.95	9.60 ± 7.89	22.85 ± 19.58	ns
DGI	%	3.41 ± 0.41	2.96 ± 0.35	3.44 ± 0.47	ns
High lipid diet					
N		4	6	6	
Wi	g	700 ± 73	685 ± 90	722 ± 79	ns
Wf	g	758 ± 87	786 ± 127	838 ± 121	ns
WI	g	58 ± 43	101 ± 88	116 ± 46	ns
AFR	g day^{-1}	13.51 ± 1.71	15.34 ± 4.68	15.71 ± 5.89	ns
APFR	g day^{-1}	1.85 ± 0.23	2.00 ± 0.61	2.25 ± 0.84	ns
ALFR	g day^{-1}	1.18 ± 0.15	1.28 ± 0.39	1.43 ± 0.54	ns
SFR	%BW day^{-1}	1.86 ± 0.18	2.05 ± 0.37	1.97 ± 0.57	ns
AGR	g day^{-1}	2.08 ± 1.56	3.61 ± 3.17	4.14 ± 1.67	ns
SGR	%BW day^{-1}	0.28 ± 0.20	0.33 ± 0.48	0.52 ± 0.15	ns
FE	%	14.65 ± 10.81	21.52 ± 19.16	27.53 ± 7.84	ns
PPV	%	14.99 ± 9.25	34.65 ± 23.49	31.31 ± 11.87	ns
LPV	%	9.78 ± 9.74	5.35 ± 2.07	2.76 ± 2.56	ns
DGI	%	2.98 ± 0.41	3.03 ± 1.04	3.24 ± 0.41	ns

Values on the same line and different superscripts are significantly different.

Wi, Initial weight; Wf, Final weight; WI, Weight increment; AFR, Absolute Feeding Rate; APFR, Absolute Protein Feeding Rate; ALFR, Absolute Lipid Feeding Rate; SFR, Specific Feeding Rate; AGR, Absolute Growth Rate; SGR, Specific Growth Rate; FE, Feed Efficiency; PPV, Protein Productive Value; LPV, Lipid Productive Value; DGI, Digestive Gland Index.

ANOVA: ns, not significant ($P > 0.05$); *$P < 0.05$.

Proximate composition

There were no significant differences in the proximate composition of carcass in animals fed LL diet ($P > 0.05$; Table 6). However, these animals showed higher percentage of protein in the digestive gland (64.0–66.2%) compared with the initial values (60.5%; $P < 0.05$). Lipid percentages of digestive gland were between 7.8% for animals fed 5 g kg^{-1} glutamate supplementation and 13.9% for animals fed 20 g kg^{-1} glutamate supplementation, with significant differences ($P < 0.05$; Table 6). There were no significant differences in moisture, protein or ash composition of carcass in the HL assay, although a lower lipid percentage was detected for animals fed 20 g kg^{-1} glutamate supplementation (0.6% lipids; $P < 0.05$; Table 6). Furthermore, the high percentage of lipids measured in the digestive gland in all the HL groups (25.1–33.2%) compared with the initial values (7.7%; $P < 0.05$) or LL groups (7.8–13.9%) was of particular note. There were lower protein and NFE percentages in the digestive gland for the HL groups (46.1–55.4%, 10.5–15.4%, respectively), compared with initial values (62.6% and 22.5%, respectively; $P < 0.05$; Table 6).

Lipid class composition

Phosphatidylcholine (17.0–19.4%), phosphatidylserine/inositol (13.4–15.9%), phosphatidylethanolamine (16.7–18.6%), cholesterol (24.2–25.3%)

Table 6 Proximate composition (% dry substance; mean ± standard deviation) of *Octopus vulgaris* fed with diet of low lipid level (8 g kg^{-1}) or high lipid level (84 g kg^{-1}) and different levels of glutamate supplementation

		Glutamate supplementation (g kg^{-1})			
	Initial	0	5	20	ANOVA
Low lipid diet					
N	3	4	6	6	
Carcass					
Moisture	80.7 ± 0.6	80.3 ± 0.4	79.8 ± 1.0	79.6 ± 0.6	ns
Protein	80.0 ± 1.9	80.6 ± 3.6	79.1 ± 3.2	80.1 ± 3.3	ns
Lipids	1.4 ± 0.4	1.6 ± 0.4	1.5 ± 0.4	1.4 ± 0.2	ns
Ash	11.2 ± 0.2	11.5 ± 0.4	11.5 ± 0.8	11.3 ± 0.5	ns
NFE[1]	7.3 ± 2.3	6.2 ± 3.7	7.9 ± 3.7	7.1 ± 3.4	ns
Digestive gland					
Moisture	71.6 ± 0.7[a]	69.1 ± 0.9[ab]	69.6 ± 2.1[ab]	67.4 ± 2.6[b]	*
Protein	60.5 ± 2.6[a]	65.9 ± 1.3[b]	66.2 ± 2.3[b]	64.0 ± 5.1[ab]	*
Lipids	11.2 ± 2.4[ab]	9.3 ± 2.9[ab]	7.8 ± 3.2[a]	13.9 ± 5.5[b]	*
Ash	8.4 ± 1.1	7.0 ± 0.3	7.5 ± 1.2	6.6 ± 2.0	ns
NFE[1]	19.9 ± 1.2	17.8 ± 2.6	18.5 ± 2.9	15.5 ± 3.3	ns
High lipid diet					
N	3	4	6	6	
Carcass					
Moisture	81.7 ± 1.0	80.5 ± 0.5	80.8 ± 0.3	80.1 ± 1.0	ns
Protein	80.4 ± 2.6	79.0 ± 3.0	81.2 ± 3.2	79.1 ± 3.1	ns
Lipids	1.4 ± 0.3[a]	1.0 ± 0.3[b]	1.0 ± 0.2[b]	0.6 ± 0.2[c]	*
Ash	10.2 ± 1.9	10.9 ± 0.8	11.3 ± 0.7	10.8 ± 0.8	ns
NFE	8.0 ± 2.6	8.2 ± 3.8	6.5 ± 2.7	9.4 ± 3.3	ns
Digestive gland					
Moisture	68.6 ± 2.0[a]	62.9 ± 1.5[b]	68.4 ± 3.4[a]	65.3 ± 1.0[ab]	*
Protein	62.6 ± 3.0[a]	46.1 ± 4.9[b]	55.4 ± 9.8[ab]	51.5 ± 3.7[b]	*
Lipids	7.7 ± 2.9[a]	33.2 ± 2.1[b]	25.1 ± 9.0[b]	30.5 ± 5.1[b]	*
Ash	7.2 ± 0.7	5.3 ± 1.2	6.2 ± 1.3	7.4 ± 2.3	ns
NFE[1]	22.5 ± 1.0[a]	15.4 ± 6.6[b]	13.3 ± 2.3[b]	10.5 ± 3.9[b]	*

Values on the same line and different superscripts are significantly different.
NFE, Nitrogen-free extract.
ANOVA: ns, not significant ($P > 0.05$); *$P < 0.05$.

and free fatty acids (9.5–11.9%) were the main lipid classes detected in the carcass of animals fed LL diet, without significant differences for glutamate supplementation degree (Table 7). However, a higher triglycerides percentage was detected for animals fed 20 g kg^{-1} glutamate supplementation (Table 7). Diacylglycerols and triacylglycerols reached higher percentages in the digestive gland of animals fed 20 g kg^{-1} glutamate supplementation ($P < 0.05$; Table 8). On the contrary, phosphatidylcholine and phosphatidylethanolamine showed lower percentages in these animals ($P < 0.05$). Furthermore, free fatty acids showed lower percentages in animals with 5 or 20 g kg^{-1} glutamate supplementation ($P < 0.05$; Table 8).

There were no significant differences in the lipid class percentages of carcass in animals fed HL diet (Table 7). The main lipid classes detected in the animal carcass fed HL diets were phosphatidylcholine, phosphatidylserine/inositol, phosphatidylethanolamine, cholesterol and free fatty acids (as in animals fed LL diets). Triacylglycerols (39.1–46.1%) and free fatty acids (16.3–23.3%) were the main lipid classes detected in the digestive gland of these animals, although there were no differences with respect to the glutamate supplementation. Lowest phosphatidylethanolamine and steryl esters percentages were detected for 20 g kg^1 glutamate supplementation group ($P < 0.05$; Table 8).

Discussion

The addition of glutamate did not stimulate ingestion in any of the assays (Table 5). However, the same amino acid attracts this species and stimulates ventilation frequency (Chase & Wells 1986).

Table 7 Lipid class composition (% ± SD total lipids) in the carcass of *Octopus vulgaris* fed with diet of low lipid level (8 g kg^{-1}) or high lipid level (84 g kg^{-1}) and different levels of glutamate supplementation

	Low lipid diet				High lipid diet			
	Glutamate supplementation (g kg^{-1})				Glutamate supplementation (g kg^{-1})			
Lipid classes	**0**	**5**	**20**	**ANOVA**	**0**	**5**	**20**	**ANOVA**
Polar lipids								
LPC	0.48 ± 0.46	0.57 ± 0.45	0.16 ± 0.31	ns	n.d.	n.d.	n.d.	ns
SM	1.10 ± 0.49	1.31 ± 0.17	1.02 ± 0.65	ns	0.60 ± 0.39	0.33 ± 0.38	0.67 ± 0.40	ns
PC	19.36 ± 1.45	17.02 ± 1.20	18.13 ± 1.89	ns	19.28 ± 0.46	17.66 ± 1.33	20.17 ± 1.76	ns
LPE	0.75 ± 0.16	0.90 ± 0.25	0.53 ± 0.26	ns	0.30 ± 0.60	n.d.	n.d.	ns
PS/PI	15.96 ± 1.79	13.49 ± 1.93	13.44 ± 3.14	ns	13.71 ± 1.31	10.99 ± 0.91	10.94 ± 2.15	ns
PA	3.19 ± 0.46	2.67 ± 0.83	2.43 ± 1.18	ns	1.30 ± 0.69	1.15 ± 0.97	0.83 ± 1.66	ns
PE	18.00 ± 0.91	16.72 ± 0.75	18.56 ± 2.11	ns	19.02 ± 1.86	19.23 ± 0.68	20.85 ± 2.09	ns
Neutral lipids								
MG	1.61 ± 0.14	2.56 ± 0.88	2.68 ± 1.26	ns	1.37 ± 0.51	1.50 ± 0.72	1.68 ± 0.43	ns
DG	n.d.	n.d.	n.d.	ns	n.d.	n.d.	n.d.	ns
CHO	25.11 ± 1.79	24.24 ± 2.33	25.30 ± 1.58	ns	27.60 ± 3.55	28.96 ± 3.46	27.16 ± 2.83	ns
FFA	10.74 ± 2.54	11.93 ± 2.67	9.49 ± 0.81	ns	5.69 ± 2.02	9.82 ± 3.58	6.42 ± 1.65	ns
TG	1.11 ± 0.32^{a}	1.62 ± 0.39^{ab}	2.79 ± 1.23^{b}	*	2.93 ± 0.43	1.58 ± 2.10	2.33 ± 0.79	ns
SE	2.88 ± 0.76	6.99 ± 7.00	5.91 ± 5.47	ns	8.81 ± 3.47	10.85 ± 7.18	8.11 ± 2.45	ns

Values on the same line and different superscripts are significantly different.
LPC, Lysophosphatidylcholine; SM, Sphingomyelin; PC, Phosphatidylcholine; LPE, Lysophosphatidylethanolamine; PS/PI, Phosphatidylserine and Phosphatidylinositol; PA, Phosphatidic acid – including phosphatidylglycerol and cardiolipin; PE, Phosphatidylethanolamine; MG, Monoacylglycerols – including pigments-; DG, Diacylglycerols; CHO, Cholesterol; FFA, Free fatty acids; TG, Triacylglycerols; SE, Steryl esters – including waxes; n.d., not detected.
ANOVA: ns, not significant ($P > 0.05$); *$P < 0.05$.

In this respect, it is necessary to differentiate between substances that favour the capture of prey and those that favour its ingestion (Lee *et al.* 1991). Neither did Domingues *et al.* (2005) observe differences in the SFR of *S. officinalis* fed diets containing different levels of lysine supplementation.

The results for both LL and HL diets as regards acceptability were poorer (1.8–2.7%BW day^{-1}) than those obtained with natural diets based on crustaceans (5.9–7.0%BW day^{-1}; Aguado Giménez & García García 2002; García García & Cerezo Valverde 2006), or squid (4.6–5.8%BW day^{-1}; Quintana *et al.* 2008; Domingues, García, Hachero-Cruzado, Lopez & Rosas 2009), but similar to those obtained with a fish-based diet (2.3–2.6% BW day^{-1}; Aguado Giménez & García García 2002; García García & Aguado Giménez 2002) or with diets that contained alginate as binder (3.1% BW day^{-1}; Cerezo Valverde *et al.* 2008). According to other studies, the use of gelatine as binder increases the acceptability of diets in *O. maya* (Rosas *et al.* 2008) and *O. vulgaris* (Quintana *et al.* 2008), for which similar SFR values were obtained as with natural diets of crustaceans or squid respectively. However, the SFR obtained in this last study (8.6%BW day^{-1}) may have been overestimated because the feed leaching rates were not taken into account. The same authors also used smaller samples (468–506 g) than are used in this study, once again increasing the SFR values obtained. The use of alginates would provide a more stable texture, although the acceptability and digestibility of the diet may be compromised (Rosas *et al.* 2008; Seiça Neves *et al.* 2010).

According to this study, the best growth rates and feed efficiency were obtained with a 5 or 20 g kg^{-1} glutamate supplementation rates in both assays, although none of the diets formulated have managed to reproduce the high growth rates reached when crustacean were supplied. These results suggest that octopus benefit from supplementary glutamate and underline the idea that the protein and amino acid needs of cephalopods should be high on the list of priorities when formulating diets. Cephalopods are exclusively carnivorous species, and amino acids represent their main energetic source (O'Dor, Mangold, Boucher-Rodoni, Wells & Wells 1984; Lee 1994), arginine, lysine and leucine predominating the essential

Table 8 Lipid class composition (% ± SD total lipids) in the digestive gland of *Octopus vulgaris* fed with diet of low lipid level (8 g kg^{-1}) or high lipid level (84 g kg^{-1}) and different levels of glutamate supplementation

	Low lipid diet				High lipid diet			
	Glutamate supplementation (g kg^{-1})				Glutamate supplementation (g kg^{-1})			
Lipid classes	**0**	**5**	**20**	**ANOVA**	**0**	**5**	**20**	**ANOVA**
Polar lipids								
LPC	1.64 ± 0.91	1.78 ± 1.43	1.80 ± 0.84	ns	n.d.	n.d.	0.21 ± 0.33	ns
SM	n.d.	n.d.	n.d.	ns	n.d.	0.09 ± 0.22	0.19 ± 0.29	ns
PC	4.03 ± 1.22^{ab}	5.20 ± 1.78^{a}	1.76 ± 0.70^{b}	*	6.01 ± 0.59	3.68 ± 1.07	5.77 ± 1.89	ns
LPE	1.53 ± 0.50	1.43 ± 0.27	1.23 ± 0.57	ns	n.d.	n.d.	n.d.	ns
PS/PI	7.20 ± 1.99	6.57 ± 3.02	4.42 ± 1.56	ns	n.d.	n.d.	2.66 ± 4.15	ns
PA	n.d.	n.d.	0.23 ± 0.36	ns	n.d.	n.d.	n.d.	ns
PE	2.66 ± 0.45^{a}	2.89 ± 0.91^{a}	1.05 ± .051^{b}	*	10.44 ± 1.75^{a}	8.23 ± 1.95^{ab}	6.50 ± 1.03^{b}	*
Neutral lipids								
MG	16.76 ± 3.21	20.90 ± 4.60	18.87 ± 1.69	ns	5.21 ± 0.82	5.63 ± 0.52	5.58 ± 0.69	ns
DG	0.64 ± 0.23^{a}	0.66 ± 0.51^{a}	2.29 ± 0.81^{b}	*	5.20 ± 0.68	4.58 ± 1.57	6.45 ± 1.24	ns
CHO	6.95 ± 2.45	6.74 ± 2.42	5.80 ± 2.19	ns	5.66 ± 1.48	6.43 ± 1.58	7.68 ± 1.91	ns
FFA	41.75 ± 3.60^{a}	33.70 ± 4.80^{b}	35.90 ± 3.15^{b}	*	16.31 ± 3.00	23.32 ± 3.24	20.69 ± 6.90	ns
TG	3.01 ± 2.81^{a}	4.45 ± 6.95^{a}	18.56 ± 9.29^{b}	*	46.15 ± 6.45	39.14 ± 9.73	43.11 ± 6.36	ns
SE	12.37 ± 2.61	9.59 ± 3.44	8.29 ± 1.94	ns	4.80 ± 0.25^{a}	4.73 ± 3.19^{a}	0.41 ± 0.67^{b}	*

Values on the same line and different superscripts are significantly different.
LPC, Lysophosphatidylcholine; SM, Sphingomyelin; PC, Phosphatidylcholine; LPE, Lysophosphatidylethanolamine; PS/PI, Phosphatidylserine and Phosphatidylinositol; PA, Phosphatidic acid – including phosphatidylglycerol and cardiolipin; PE, Phosphatidylethanolamine; MG, Monoacylglycerols – including pigments; DG, Diacylglycerols; CHO, Cholesterol; FFA, Free fatty acids; TG, Triacylglycerols; SE, Steryl esters – including waxes; n.d., not detected.
ANOVA: ns, not significant ($P > 0.05$); *$P < 0.05$.

amino acids, and aspartate and glutamate the non-essential (Rosa, Costa & Nunes 2004; Villanueva *et al.* 2004). Furthermore, glutamate is the most abundant amino acid in the natural diets they usually consume (Zdzislaw *et al.* 1994; Goodman-Lowe, Carpenter, Atkinson & Ako 1999). Glutamate also has a specific enzyme system for its oxidation both in *O. vulgaris* and in other cephalopods (Rocca & Ghiretti 1958; Storey, Fields & Hochachka 1978; Hoeger, Mommsen, O'Dor & Webber 1987). Using this amino acid as energy source would save others included in the diet – thus explaining the better performance and PPV obtained with the supplemented diet-. Domingues *et al.* (2005) also obtained best results with a diet representing the highest degree of supplementation with lysine (6.7 g kg^{-1}) in *Sepia officinalis*. On the other hand, the addition of hydrolysed proteins to the diet of *Octopus maya* (Aguila, Cuzon, Pascual, Domingues, Gaxiola, Sánchez, Maldonado & Rosas 2007) or amino acids in crystalline form to the diet of *S. officinalis* (Castro & Lee 1994) resulted in negative or low growth, which was partly attributed to the low acceptability of the diets in question.

With reference to lipids, better feed efficiency was achieved with the HL diet (14.6–27.5% vs. 2.5–19.2% for LL diet). In other studies, the FE exceeded 40% when bogue (59 g kg^{-1} lipids; García García & Aguado Giménez 2002), hake or squid (13 g kg^{-1} lipids; Domingues *et al.* 2009) were supplied, and was between 20% and 35% for crustaceans (<10 g kg^{-1} lipids). Similarly, García García and Cerezo Valverde (2006) observed a poorer degree of FE when the diet was composed exclusively of crustaceans, suggesting an ideal lipid level of 20–30 g kg^{-1} when a mixed diet of bogue and crab is supplied (39.3% and 33.1% for FE and PPV respectively). Furthermore, Estefanell *et al.* (2011) have recently obtained excellent results in *O. vulgaris* ongrowing using *Boops boops* from by-catch of fish farms, which contain high levels of neutral lipids and suggesting an efficient utilization of dietary lipids. Cephalopods also show high levels of phospholipids, cholesterol and polyunsaturated fatty acids (PUFA), especially series n-3 HUFA (Sinanoglou & Miniadis-Meimaroglou 1998; Navarro & Villanueva 2000, 2003; Almansa, Domingues, Sykes, Tejera, Lorenzo & Andrade 2006). The lack of some such lipid classes may be a limiting factor

for the adequate development of octopus and for the energy efficiency of the diet.

Numerous studies have discussed the utility of DGI as nutritional indicator of cephalopods (Castro & Lee 1994; Moltschaniwskyj & Johnston 2006; Cerezo Valverde *et al.* 2008). There is a consensus that low values of DGI are related to malnourished or fasting animals (Castro, Garrido & Sotelo 1992; García-Garrido *et al.* 2010; Morillo-Velarde, Cerezo Valverde, Serra Llinares & García García 2011). However, in our study, there were animals with poor growth, but maintained a good DGI, regardless of dietary lipid level. These results rethink the usefulness of this index by itself and reinforce the need to accompany with digestive gland analysis. Similarly, Domingues, Ferreira, Marquez, Andrade, López and Rosas (2008) observed a higher DGI for cuttlefish fed sardine, which did not promote significant growth during the experiment, but feeding rates were acceptable.

With reference to nutritional composition of the animals, the most relevant changes were observed in proximate composition for the digestive gland, without notable differences detected for the carcass. According to Almansa *et al.* (2006) and Sieiro, Aubourg and Rocha (2006), the distribution of lipid classes in cephalopods fed natural diets or analysed in different seasons is more uniform in the mantle, where polar lipids predominate. On the contrary, a greater variability is detected in the digestive gland. In this study, a substantial amount of lipids accumulated in the digestive gland (25.1–33.2%), mainly triacylglycerols, as a consequence of higher lipid ingestion (see Fig. 1). In the case of a low lipid diet was supplied, the ability of *O. vulgaris* to store triacylglycerols could be improved with a 20 g kg^{-1} glutamate supplementation. In fact, a low lipid diet without glutamate supplementation is characterized by higher percentages of free fatty acids and lower diacylglycerols and triacylglycerols. These results suggest that amino acids supplementation could improve the biosynthesis or storage of lipids from diet, and a possible relationship between lipid and protein metabolism. According to our findings and those of Sieiro *et al.* (2006), triglycerides are the predominant lipid class in the digestive gland of *O. vulgaris*; Castro *et al.* (1992) and García-Garrido *et al.* (2010) demonstrated that stored lipids can be mobilized in starvation conditions. However, both the lipid content of the digestive gland and their distribution into various classes vary among species and normally reflect the respective feeding strategies (Rosa, Pereira & Nunes 2005). For example, Moltschaniwskyj and Johnston (2006) observed in the squid *Eupryma tasmanica* that most of the lipids present in the digestive gland were structural components of the membrane (polar lipids) or digestion-derived products, such as sterols or free fatty acids, with low levels of storage lipids. Also in our study, high percentages of FFA and TG in the digestive gland of animals fed low or high lipid diets, respectively, could be attributed to the predominance of these lipid classes in their diets.

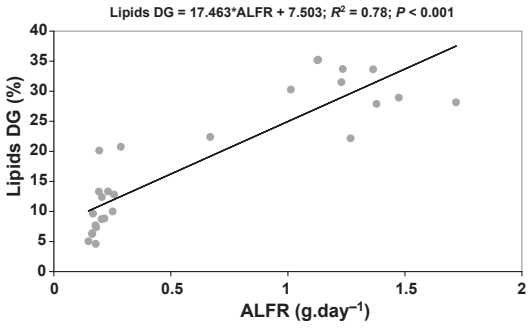

Figure 1 Percentage of lipids (% dry substance) in the digestive gland (Lipids DG) as a function of absolute lipid feeding rate (ALFR) in *Octopus vulgaris*.

Furthermore, the results of this study suggest several interesting hypotheses, which should be checked by lipid metabolism studies. It was notable the lower percentages of phosphatidylethanolamine and phosphatidylcholine in the digestive gland resulting from LL diet, and lower percentage of phosphatidylethanolamine resulting from HL diet, both when a 20 g kg^{-1} glutamate supplementation is provided and best growth is registered. Together with cholesterol, these are the main lipid classes in mantle of cephalopods (Navarro & Villanueva 2000; Almansa *et al.* 2006) and have been recently proposed as key nutrients for cephalopods growth (Cerezo Valverde *et al.* 2012), it being suggested that these lipids may be mobilized from the digestive gland when a protein intake is enough to stimulate growth. In this sense, Heras and Pollero (1990, 1992) described the presence of three lipoproteins of different densities and lipid composition (LP-I, LP-II and LP-III) that might act as transports of lipids among organs in *Octopus*. Most of the cholesterol and phospholipids are transported by LP-I and LP-II,

whereas free fatty acids, steryl esters and triglycerides are transported by LP-III. Therefore, the high percentages of steryl esters (8–11%) and cholesterol (27–29%) in the carcass of animals fed HL diet could be as a result of an active lipid transport to the muscle tissue. Whatever the case, the use of formulated diets supplemented with different classes of lipids may be an effective tool for throwing light on their role in the nutrition of cephalopods.

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