

## Effect of artificial diets on growth, survival and condition of adult cuttlefish, *Sepia officinalis* Linnaeus, 1758

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**Abstract.** The effects of artificial diets on growth and body condition of adult cuttlefish, *Sepia officinalis* were tested in two experiments. Supplemented prepared diets (fish myofibrillar protein concentrate) were fed during a 30-day and a 21-day experiments. Growth, feeding rate and food conversion of group-reared cuttlefish were analyzed. The first of these experiments tested four artificial diets, made with increasing levels of lysine, on adult cuttlefish. According to the chemical analysis, diets 1–3 had limiting concentrations of lysine and other essential amino acids (compared to mantle composition of the cuttlefish), while diet 4 was the only one where almost all essential amino acids were present in concentrations similar or higher than the ones present in cuttlefish mantle. A second experiment was conducted by isolating 16 adult cuttlefish individually, and feeding them the same four artificial diets, in order to obtain individual data. During Experiment 1, only the diet with the best chemical score (diet 4) produced growth ( $p < 0.05$ ), with a mean instantaneous growth rate (MIGR) of 0.30% wet body weight (BW)  $d^{-1}$ . Similarly, individually reared cuttlefish fed diet 4 produced the highest IGR's (0.26, 0.38 and 0.48% BW  $d^{-1}$ ) and grew larger ( $p < 0.01$ ). Comparison of cuttlefish fed the artificial diets vs. thawed shrimp and unfed cuttlefish indicated that cuttlefish fed the artificial diets were in an intermediate state. Growth rates obtained with the artificial diets ( $< 0.4\%$  BW  $d^{-1}$ ) were considerably lower compared to natural prey, live or frozen, reported by other authors.

### Introduction

The potential for the mariculture of cephalopod mollusks has been recognized for many years (Hanlon et al. 1991; Lee et al. 1998) because of their rapid growth rates, short life cycles and high food conversion efficiencies (Forsythe and Van Heukelem 1987; Domingues et al. 2001a, 2003b). There are several natural foods on which cephalopods can be maintained or grown Boletzky and Hanlon 1983; Toll and Strain 1988; DeRusha et al. 1989; DiMarco et al. 1993; Domingues et al. 2002, 2003a, b, 2004). In cephalopod aquaculture, over 50% of the labor required involves collecting, holding and feeding live prey (Lee 1994). Collecting and immediately freezing the prey reduces labor costs by one-third. In Contrast, the labor required to prepare an artificial food can be

significantly lower, and if a prepared diet is substituted for live food, an additional cost reduction of at least 20% can be estimated. Greater cost reductions might be obtained by using commercial production methods to produce larger quantities of diet (Lee 1994).

Prepared diets for cephalopods have been tested over the past few years to lower the costs of mariculture (Lee et al. 1991; Domingues 1999). Until now, feeding, growth and survival of cephalopods are comparable to fish larvae being weaned from natural to prepared diets (Dabrowski et al. 1978; Lindberg and Doroshov 1986). This inability to grow cephalopods on an inexpensive and stable prepared diet has inhibited cephalopod mariculture on a commercial basis (O'Dor and Wells 1987; Lee et al. 1991). Therefore, the formulation of such a diet is one of the primary and achievable goals needed for the successful large-scale culture of cephalopods (Lee 1994).

Since cephalopods grow at rates between 3 and 15% BW d<sup>-1</sup>, the amino acid requirement for protein synthesis is high (Lee 1994). Cephalopod bodies are composed of 75–85% protein on a dry weight basis. In contrast to fishes, cephalopods contain 20% more protein, 80% less ash, 50–90% less lipid and 50–90% less carbohydrate (Lee 1994). Since protein is used almost exclusively for energy, cephalopods are extremely efficient in assimilating protein, with apparent protein digestibility greater than 85–90%. Cephalopod water content is approximately 80% with protein (16.6%) being the next most abundant component (Iwasaki and Harada 1985). Carbohydrates are approximately 1% (Vlieg 1984) and lipids are usually less than 2%; neither are candidates for energy production (Hochachka et al. 1975).

The cuttlefish, *Sepia officinalis*, is one of the most easily cultured cephalopods, having been maintained, reared and cultured in aquaria in both Europe and North America (Richard 1971; Pascual 1978; Boletzky 1983; Boletzky and Hanlon 1983; Forsythe et al. 1994; Lee et al. 1998; Domingues et al. 2001a, b, 2002, 2003a). This species is highly adaptable to life in captivity since it has (1) large eggs, (2) voracious hatchlings, (3) high hatchling survival, (4) sedentary behavior, (5) tolerance to crowding, handling and shipping, (6) acceptance of nonliving foods and (7) most importantly, been shown to easily reproduce in captivity (Forsythe et al. 1994). In the last few years, feeding experiments have been conducted with either moist or dry pellets (Lee et al. 1991; Castro et al. 1993) or surimi (fish myofibrillar protein concentrate; Castro et al. 1993; Castro and Lee 1994; Domingues 1999), demonstrating that cuttlefish readily accept prepared diets. These characteristics underscore this species as a promising candidate for successful commercial mariculture.

The rapid growth rate of cephalopods requires highly efficient ingestion, digestion and assimilation of protein. This reliance on amino acid metabolism makes it essential for prepared diets to contain balanced levels of amino acids in order to promote growth. In contrast to the protein-to-energy (P/E) ratio that is utilized as an indicator of the nutritional value of terrestrial vertebrate diets, amino acid ratios are the best measure of the nutritional value of cephalopod diets (Lee 1994). Therefore, the addition of known levels of amino

acids, particularly the essential amino acids, is of paramount importance in the determination of well balanced diets for cephalopods. The artificial diets were initially designed in order to obtain increasing concentrations of lysine, one of the most limiting of the essential amino acids in marine organisms (Zarate and Lovell 1997). *Post hoc* diet composition revealed that not only did lysine concentrations increase from diets 1 to 4, but also that there was high variability in overall amino acid composition in the diets, which made it impossible to determine lysine effects. Nevertheless, protein content and the chemical score of diet 1 was considerably lower compared to the remaining diets, and the chemical score of diet 4 was higher than for diets 2 and 3. Therefore, experiments were conducted to determine the effects of four artificial diets to a natural diet on growth of adult cuttlefish, and to try and determine cuttlefish condition by analyzing amino acid composition and compare it with cuttlefish fed natural diets and starved animals.

#### **Materials and methods**

During previous experiments at the National Resource Center for Cephalopods (NRCC) in Galveston, Texas, USA, cuttlefish had been tagged, in order to obtain individual data. Nevertheless, mortality was very high, due to stress in handling and infections probably due to the tagging procedure, which required mantle perforation, therefore opening doors for possible infections. For these two reasons, cuttlefish were not tagged during Experiment 1, and all values were determined as population means of the cuttlefish in each diet treatment. Individual data was obtained for cuttlefish reared individually in Experiment 2. Experiments were conducted at the NRCC during 1998.

For Experiment 1, a total of 96 cuttlefish ( $321.8 \pm 57.9$  g) were distributed equally into 12 circular tanks (each tank was 500 l in volume, 1.6 m diameter) connected to a common filtration system. This tank system was similar to others used for the culture of cephalopods (Yang et al. 1989; Lee 1994; Lee et al. 1998). Experiment 1 lasted 30 days. Each cuttlefish was weighed on days 1, 15 and 30.

For Experiment 2, 16 cuttlefish ( $451.5 \pm 103.7$  g) were distributed into individual chambers ( $35 \times 25$  cm, water depth of 10 cm) and one of each of the four lysine prepared diets was fed to four randomly chosen cuttlefish. Experiment 2 lasted 21 days and cuttlefish were weighed on days 1, 14 and 21.

Before each experiment, the cuttlefish were weighed and distributed randomly so that there were no differences ( $p > 0.05$ ) among the means or variances in wet body weight of the cuttlefish in each tank.

Environmental conditions were standardized for the two experiments. Salinity was maintained at  $35 \pm 2$  ppt and temperature was stable at  $23 \pm 1.5$  °C. Since nitrate-nitrogen concentrations above  $80 \text{ mg l}^{-1}$  cause cuttlefish to become highly agitated and easily startled (Forsythe et al. 1994), ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were kept below 0.1,

0.1 and 50 mg l<sup>-1</sup>, respectively, by making water changes when necessary. The pH was maintained between 7.8 and 8.1 by addition of sodium bicarbonate. Low light intensities measured at surface water level of the tanks (2.8 lux), were maintained 24 h d<sup>-1</sup>.

Experiment 1 was initially set to test four diets that were made with different levels of lysine (7.2, 23.1, 65.4 and 106.9 mg g<sup>-1</sup> ww). These levels were chosen in order to obtain increasing levels of lysine from diet 1 to 4, the first three being deficient (according to the chemical score) when compared to lysine concentrations in adult cuttlefish mantle (106.2 ± 31.4 mg g<sup>-1</sup>). Also, diet 1 had a lower protein content (achieved by adding corn starch and cellulose) compared to the remaining three diets, since we wanted to determine the effect of a lower protein diet (about 60% protein content in diet 1, compared to > 90% protein content for diets 2, 3 and 4). Finally, casein was also added to diet 4. This allowed us to test a lower protein diet, where many essential amino acids were present in low concentrations, two high protein diets but deficient in some essential amino acids, and a supplemented diet that was similar to the amino acid composition of cuttlefish mantle, but with higher concentrations of some amino acids. Artificial diets were formulated using surimi as a base (Castro et al. 1993). The content of each nutrient of the diets in percentage is shown in Table 1. Chemical score of the amino acids was determined using the formula proposed by Tacon (1987) to determine the most limiting amino acids in each diet, compared to the amino acid concentrations in adult cuttlefish mantle. Chemical score (CS) was calculated using the equation  $CS = ((\text{mg per gram of aa in diet}) / (\text{mg per gram of aa in cuttlefish mantle})) * 100$ .

During the week prior to the start of each experiment, cuttlefish were slowly weaned from thawed frozen shrimp to thawed catfish fillets. This procedure was done to ensure better acceptance of the prepared diets by the cuttlefish, as small pieces of catfish resembled our prepared diets in shape. For Experiment 1, each of the four diets was fed to cuttlefish in three randomly chosen tanks.

Initial ration levels in both experiments were set at 5% BW d<sup>-1</sup> allowing for a slight excess of food. Later, these rations were adjusted based on ingestion and mortality rates. The daily amount of food given to each tank was presented in two rations: 50% at 9:00 h and 50% at 16:00 h. The food was presented for an hour and then uneaten food was removed, weighed and multiplied by a correction factor. This factor was determined by weighing pieces of surimi, then soaking them in water for 1 h and then weighed again, to determine bleaching and soaking. The correlation factor was 1.12, meaning that a piece of surimi that weighed 1 g before being introduced in the tanks, weighed 1.12 g after 1 h in the tanks.

Data were used to calculate: (1) Mean Instantaneous Growth Rate (MIGR) (% Body Weight d<sup>-1</sup>) =  $((\ln W_2 - \ln W_1) / t) * 100$ , where  $W_2$  and  $W_1$  are the final and initial wet weights of the cuttlefish,  $\ln$  the natural logarithm and  $t$  the number of days of the time period; (2) Feeding Rate (FR) (% BW d<sup>-1</sup>) =  $(FI / \text{Average } W(t)) * 100$ , where FI is the food ingested in a period of time ( $t$ ),  $W(t)$  is the average wet weight of the cuttlefish during the period of time ( $t$ ) in

Table 1. Ingredients used to prepare the artificial diets fed to cuttlefish, expressed as a percent of the final weight of the diet.

LYSINE diets	Diet 1 (%)	Diet 2 (%)	Diet 3 (%)	Diet 4 (%)
<i>Ingredients</i>				
Catfish	51.5	85.52	85.07	80.49
Casein <sup>a</sup>	0	0	0	6
NaCl	2	2	2	2
Corn starch	16.04	0	0	0
Cellulose	16.04	0	0	0
<i>Amino acids</i>				
THR	0.63	0.48	0.48	0.41
GLU	1.98	1.40	1.40	1.01
PRO	2.38	2.15	2.15	1.80
VAL	0.47	0.35	0.35	0.22
MET	0.41	0.34	0.34	0.0
ISO	0.60	0.48	0.48	0.36
LEU	1.01	0.68	0.68	0.54
TYR	0.46	0.37	0.37	0.25
LYS	0	0	0.45	0.67
ARG	1.50	1.23	1.23	1.27
TRP	0.5	0.5	0.5	0.5
Vitamins <sup>b</sup>	2	2	2	2
Minerals <sup>c</sup>	2	2	2	2
Chromic oxide	0.5	0.5	0.5	0.5

<sup>a</sup> Vitamin free casein, ICN Biomedicals Inc. Cat. # 904798.

<sup>b</sup> AIN Vitamin mixture '76', ICN Biomedicals Inc. Cat. # 905454.

<sup>c</sup> AIN Mineral mixture '76', USB Corp., Catalog # 10664.

days; and (3) Food Conversion (FC) (%) =  $FI/(W_2 - W_1)$ . Mean weight gained was determined by subtracting the mean weight at the start from the mean weight at the end of each experiment. Non-parametric Kruskal–Wallis ANOVA tests (Zar 1999) determined significant differences in feeding rates, food conversions, weight and IGR's of cuttlefish fed the different diets, between the start and the end of the experiment, for each diet.

Samples of each artificial diet were taken, and amino acid composition of each diet was performed. At the end of experiment 1, two cuttlefish were sacrificed per tank (6 animals per diet). Samples were taken from the mantle, digestive gland and blood of each cuttlefish to determine and compare the amino acid composition of these tissues. All samples were frozen before being analyzed by high-performance liquid chromatography (Lehninger et al. 1993). The total amino acid composition of the artificial diets, cuttlefish mantle and digestive gland tissue was determined along with the free amino acid composition of the blood. Additionally, the amino acid composition of these tissues was compared with the amino acid composition from mantle, digestive gland and blood of cuttlefish fed shrimp and unfed cuttlefish. Non-parametric Kruskal–Wallis ANOVA tests (Zar 1999) determined significant differences

between the amino acid composition of cuttlefish fed the prepared diets and both cuttlefish fed shrimp and unfed cuttlefish.

## Results

Results from the three experiments showed that only diet 4 (with less limiting essential amino acids, according to the chemical score) produced growth ( $p < 0.05$ ) and higher food conversions. The remaining three diets with limiting levels of several essential amino acids (being lysine the one in lower concentration in all 3 diets) did not promote growth, and diet 1, with the lowest protein levels, gave negative growth.

### Experiment 1

#### (a) Feeding, food conversion, growth and mortality

Table 2 shows feeding rates, food conversions, growth rates and average individual weight gained for Experiment 1. Feeding rates for cuttlefish fed the four diets were not different ( $p > 0.05$ ), during the experiment (Table 2).

Diet 2 was the only one that showed a decrease in food conversion ( $p > 0.05$ ) from the first to the second half of the experiment. Cuttlefish fed diet 4 produced the best overall food conversion (Table 2), which was significantly higher ( $p > 0.05$ ) compared to the other three diets. A food conversion for of 10.7 for diet 4 means that for a cuttlefish to grow 1 kg, it has to be fed 10.7 kg of this diet; when feeding diet 3, it would mean that we would have to feed it 80.9 kg for it to grow 1 kg. As for negative food conversions, they only indicate negative growth (loss of weight).

The MIGR's for diets 2, 3 and 4 were higher in the first half of the experiment. Cuttlefish fed diet 1 had negative growth during the first half of the experiment ( $-0.64 \pm 0.14\% \text{ BW d}^{-1}$ ), but had positive growth during the second half ( $0.08 \pm 0.06\% \text{ BW d}^{-1}$ ). For the entire experiment, diet 4, with higher amino acid concentrations, promoted higher ( $p > 0.05$ ) growth (Table 2). Cuttlefish fed diet 1 lost weight (Table 2). There were no differences in weights ( $p > 0.05$ ) for cuttlefish fed diets 1, 2 and 3, compared to weights at

Table 2. Feeding rates, food conversions, growth rates and average weight gained during 30 days by cuttlefish fed four artificial diets, compared to weight at the start of for Experiment 1.

	Diet 1	Diet 2	Diet 3	Diet 4
Feeding rate (% BW d <sup>-1</sup> )	2.69	2.67	2.77	2.79
Food conversions	-40.8	26.7	80.9	10.7*
Growth rate (% BW d <sup>-1</sup> )	$-0.02 \pm 0.12$	$0.16 \pm 0.09$	$0.14 \pm 0.11$	$0.30 \pm 0.09^*$
Weight gained (g)	$-2.4 \pm 12.3$	$17.7 \pm 10.9$	$16.5 \pm 12.1$	$29.8 \pm 11.8^*$

The \*\* indicates that values were significantly different ( $p < 0.05$ ).

the start of the experiment. In contrast, cuttlefish fed diet 4 weighed more ( $p > 0.05$ ) at the end of the experiment (Table 2).

Mortality was 18.75%, being of 12.5% for cuttlefish fed diet 1 and 20.8% for the remaining diets.

*(b) Comparison between the amino acid composition of the diets*

The amino acid composition of the four diets was analyzed and compared. Overall, the amino acid composition of diet 1 was lower ( $p > 0.05$ ) from the composition of diet 4 (due to the lower protein content in diet 1, and addition of casein in diet 4); the overall composition of diets 2 and 3 was not different ( $p > 0.05$ ).

*(c) Composition of cuttlefish tissues*

For the cuttlefish fed the four artificial diets, Table 3 shows the total amino acid composition of mantle tissue ( $\text{mg kg}^{-1}$ ), digestive gland tissue ( $\text{mg kg}^{-1}$ ), and the free amino acid composition of the blood ( $\mu\text{g kg}^{-1}$ ). This table also shows composition of mantle, digestive gland and blood from cuttlefish fed thawed shrimp. This was used to feed cuttlefish in our laboratory throughout the majority of the life cycle, and they were considered by us to be in normal condition. A total of 6 cuttlefish were sacrificed and analyzed for each diet (2 from each tank), making a total of 24 cuttlefish used in the determination of amino acid composition. Weight of all cuttlefish sacrificed varied between  $316.8 \pm 73.9$  g.

*(d) Chemical score of the diets*

The chemical score of the four diets is shown in Table 4. Lysine was the most limiting essential amino acid in diets 1, 2 and 3, while the most limiting in diet 4 was phenylalanine.

## *Experiment 2*

Feeding rates, IGR's and food conversion for surviving cuttlefish from the individually reared experiment are presented in Table 5. Feeding rates varied between 1.0 and 3.9%  $\text{BW d}^{-1}$ ; there were no differences in feeding rates ( $p > 0.05$ ) between any of the diets. Food conversion for cuttlefish fed diet 1 was lower ( $p > 0.01$ ) than diet 4. There were no differences ( $p > 0.05$ ) in food conversions between the remaining diets. Cuttlefish fed diet 4, had the highest ( $p < 0.01$ ) IGR's at the end of the experiment (0.48, 0.36 and 0.26%  $\text{BW d}^{-1}$  for the three surviving cuttlefish). Cuttlefish fed diet 1 (lowest protein level and more limiting amino acid concentrations) had negative IGR's ( $-0.37$ ,  $-0.35$  and  $-0.18\%$   $\text{BW d}^{-1}$ ). The highest food conversions were from cuttlefish fed diet 4 (between 6.14 and 6.74), and only cuttlefish fed diet 4 had significant growth ( $p > 0.01$ ).

Table 3. The adult cuttlefish amino acid composition of the tissue in wet weight ( $n = 6$ ) fed 4 artificial diets, and cuttlefish fed thawed shrimp.

	MET	CYS	ARG	LYS	ALA	PRO	ASP	GLU	GLY	SER	THR	VAL	LEU	ISO	TYR	PHE	HIS
<i>Diet 1</i>																	
Mantle	41.2	27.0	104.3	113.3	77.7	84.7	139.4	181.2	65.4	19.0	60.0	58.7	107.6	60.7	38.8	52.8	28.9
	14.5	11.5	43.6	41.1	26.8	18.1	49.1	96.8	19.9	10.2	21.2	19.8	29.9	20.5	14.4	19.2	9.9
Digestive gland	19.3	20.9	39.3	60.7	36.8	75.5	76.7	89.9	36.4	9.4	36.5	37.2	57.4	37.1	25.9	36.3	39.3
	3.1	3.9	7.4	10.1	6.6	17.7	14.0	17.3	7.9	1.8	6.1	7.7	9.4	6.0	3.9	6.2	7.4
Free blood	9.3	11.4	7.5	13.2	23.5	30.5	4.0	9.8	14.5	1.0	12.0	17.4	23.9	15.2	16.2	7.8	7.5
	4.2	5.9	5.1	6.9	13.5	6.6	2.9	8.1	4.0	0.9	4.1	0.7	7.2	4.4	6.1	4.0	5.1
<i>Diet 2</i>																	
Mantle	36.8	22.9	89.5	99.3	68.7	107.0	122.7	177.3	62.8	50.1	52.3	53.8	95.6	54.7	35.7	47.7	25.8
	20.5	11.0	53.1	54.2	36.1	65.1	66.5	89.4	34.6	26.9	28.0	26.9	46.7	27.1	17.9	24.6	12.9
Digestive gland	33.9	32.2	68.6	104.3	62.3	60.7	124.0	146.9	62.1	56.4	40.0	65.6	95.5	62.9	43.0	54.9	33.4
	9.1	11.8	18.4	30.4	15.1	12.3	33.8	28.5	20.9	16.7	24.5	20.8	24.1	19.7	13.6	20.0	11.6
Free blood	10.2	8.6	2.9	10.6	18.7	30.5	1.4	2.2	13.7	9.7	8.7	17.8	24.6	18.4	11.3	4.4	6.6
<i>Diet 3</i>																	
Mantle	48.7	30.4	131.2	128.6	98.1	195.8	164.5	229.8	78.0	64.1	70.8	70.4	123.9	71.0	46.5	64.1	34.3
	21.3	15.1	53.5	60.2	34.3	100.6	71.2	78.0	27.2	33.2	31.2	32.2	30.7	28.4	22.7	27.0	15.3
Digestive gland	27.9	29.5	58.0	88.2	54.3	45.8	113.1	130.8	50.7	48.6	52.4	57.0	81.3	52.9	33.7	52.3	29.3
	4.2	16.1	28.9	44.7	23.9	22.9	49.0	54.6	23.9	22.0	24.7	29.6	33.9	26.4	18.3	26.1	14.8
Free blood	9.7	42.6	2.8	14.2	19.5	34.3	2.0	4.4	13.4	14.5	11.9	20.8	30.3	17.3	19.6	6.2	11.6
	4.6	41.2	1.7	5.5	5.1	8.2	1.7	3.9	4.4	3.6	1.6	7.6	8.5	4.5	5.5	2.5	3.5
<i>Diet 4</i>																	
Mantle	44.2	20.5	117.8	121.8	91.4	173.4	137.6	199.7	69.6	52.2	58.7	58.7	116.1	60.6	42.9	55.3	29.4
	12.7	11.0	45.9	38.1	36.1	92.5	45.6	60.7	17.2	23.9	19.2	18.8	28.1	19.1	12.7	16.6	8.7
Digestive gland	22.1	22.3	42.7	71.9	40.4	47.0	85.9	110.9	39.1	42.0	32.2	47.3	66.7	44.7	27.6	39.0	24.0
	6.0	6.4	13.2	16.3	12.4	10.2	22.4	22.4	15.7	9.7	16.5	10.5	16.3	10.4	9.0	11.6	5.1
Free blood	6.6	36.4	4.6	14.4	16.7	42.3	2.0	5.1	12.1	11.7	11.7	27.8	39.8	23.1	12.0	4.3	7.9
	3.7	46.2	2.5	8.9	4.3	18.5	1.6	4.6	4.2	4.9	5.7	16.8	24.0	12.7	2.4	1.9	3.0
Fed SHRIMP																	
Mantle	41.7	24.8	84.1	106.1	88.4	164.3	130.7	190.0	64.3	56.6	57.0	52.3	98.6	51.3	39.2	46.6	21.5
	13.4	9.8	30.7	31.3	12.8	18.9	40.3	46.5	19.7	17.8	17.1	13.2	22.4	12.6	12.5	13.4	5.6
Digestive gland	46.3	81.9	91.0	162.0	95.0	65.9	224.7	78.1	73.7	22.6	86.4	114.3	56.6	79.1	37.0	91.0	46.2
	21.4	36.5	79.7	73.0	43.9	34.3	86.1	33.9	31.4	28.1	39.0	36.0	24.5	37.3	16.8	79.7	34.3
Free blood	11.9	45.6	1.2	17.5	26.4	52.9	0.7	1.4	12.7	18.3	21.8	28.8	16.7	36.9	11.9	11.6	15.8
	0.9	7.9	0.3	4.7	9.4	23.2	0.2	0.6	0.6	5.7	6.8	10.0	3.3	3.2	3.2	3.3	0.7

Total amino acid from the mantle and digestive gland is expressed in  $\text{mg Kg}^{-1}$  and free amino acid composition of the blood as  $\mu\text{g Kg}^{-1}$  (means, in bold; standard deviations below).



Table 4. Chemical score of the four artificial diets used to culture cuttlefish during Experiments 1 and 2.

	<b>MET</b>	<b>CYS</b>	<b>ARG</b>	<b>LYS</b>	<b>ALA</b>	<b>PRO</b>	<b>ASP</b>	<b>GLU</b>	<b>GLY</b>	<b>SER</b>	<b>THR</b>	<b>VAL</b>	<b>LEU</b>	<b>ISO</b>	<b>TYR</b>	<b>PHE</b>	<b>HIS</b>
Diet 1	68.1	63.7	186.3	6.8 *	42.8	187.7	23.3	110.3	41.5	39.0	74.4	142.1	78.4	69.0	46.4	40.3**	57.7
Diet 2	88.0	80.2	216.9	21.8*	67.8	182.4	34.7	118.4	63.1	53.0	71.8	118.4	104.7	85.0	75.0	48.9**	73.5
Diet 3	88.5	81.9	215.1	61.6*	87.3	141.0	81.3	114.1	79.6	67.8	103.2	101.3	101.6	110.3	71.4	62.4**	67.9
Diet 4	101.2	98.8	222.1	100.8	95.2	124.6	108.3	140.6	95.2	69.8	117.0	92.4	128.6	117.3	101.5	82.8*	83.3**

The essential amino acids are represented in bold. The \*\* indicates the most limiting essential amino acid and the \*\*\* indicates the second most limiting essential amino acid in each diet.

Table 5. Feeding rates, food conversions and Instantaneous Growth Rates for cuttlefish kept in isolated chambers and fed 4 different artificial diets.

Diets	I	I	I	II	II	II	III	III	IV	IV	IV
Feeding rate (%BW d <sup>-1</sup> )	1.3	1.1	1.0	2.2	3.9	2.1	1.6	1.9	1.7	3.0	2.2
Food Conversion	-3.5	-2.8	-5.4	13.7	-34.3	16.5	28.1	16.0	6.5	6.1	6.2
IGR (%BW d <sup>-1</sup> )	-0.37	-0.39	-0.18	0.16	-0.11	0.13	0.06	0.12	0.26	0.48	0.36

#### *Comparison between artificial-fed, shrimp-fed and unfed cuttlefish*

Table 6 compares amino acid concentrations from tissues of cuttlefish fed the prepared diets with both cuttlefish fed shrimp and unfed cuttlefish, respectively. When compared with cuttlefish fed shrimp, no amino acid concentration was higher ( $p > 0.05$ ) in the cuttlefish fed any of the artificial diets. For both tables, the '-' or '+' indicates that the concentration of the amino acid in that tissue was significantly lower or higher ( $p > 0.05$ ) when compared to shrimp fed or unfed cuttlefish.

When compared with cuttlefish fed shrimp, cuttlefish fed the diets with lowest (diet 1) and the highest (diet 4) lysine levels had lower ( $p > 0.05$ ) overall amino acid concentrations, especially in the digestive gland (Table 6). The aromatic amino acids (tyrosine, phenylalanine and histidine) were also lower ( $p > 0.05$ ) in the blood of cuttlefish fed diets 2 and 4, and in the digestive gland of cuttlefish fed diet 2. Cystine was lower ( $p > 0.05$ ) in the digestive gland of cuttlefish fed all artificial diets, and serine was lower ( $p > 0.05$ ) in all three tissues of cuttlefish fed diet 1. Cuttlefish fed the intermediate diets (diets 2 and 3) had a similar overall amino acid composition ( $p > 0.05$ ) when compared with cuttlefish fed shrimp.

When comparing cuttlefish fed the prepared diets with unfed cuttlefish (Table 6), proline was higher in the blood of cuttlefish fed all four diets, in the digestive gland of cuttlefish fed all but diet 3 and in the mantle of cuttlefish fed diet 1 (all at  $p > 0.05$ ). The basic amino acids (arginine and lysine) were also higher ( $p > 0.05$ ) in the digestive gland of cuttlefish fed diet 2. The branch-chained amino acids (valine, leucine and isoleucine) were higher ( $p > 0.05$ ) in the blood of cuttlefish fed diets 1, 3 and 4 and in the digestive gland of cuttlefish fed diet 2. In contrast, serine was lower in all three tissues of cuttlefish fed diet 1. Cystine was lower in digestive gland of cuttlefish fed diets 1 and 4, and lower in the blood of cuttlefish fed diets 1 and 2. Histidine was also lower in the blood of cuttlefish fed diet 1 (all  $p > 0.05$ ).

#### **Discussion**

A diet low in energy may be sufficient for meeting metabolic needs, but no growth or subnormal growth can result. The artificial diets tested in the present

Table 6. Comparison of cuttlefish fed the four artificial prepared diets with shrimp fed and unfed cuttlefish.

	MET	CYS	ARG	LYS	ALA	PRO	ASP	GLU	GLY	SER	THR	VAL	LEU	ISO	TYR	PHE	HIS
<i>Prepared diet vs. shrimp</i>																	
<i>Diet 1</i>																	
Mantle						-				-							
Digestive gland	-	-	-	-	-		-	-	-						-	-	-
Free blood	-	-	-	-	-					-					-	-	-
<i>Diet 2</i>																	
Mantle																	
Digestive gland	-	-	-	-	-	-				-					-	-	-
Free blood	-	-	-	-	-					-					-	-	-
<i>Diet 3</i>																	
Mantle																	
Digestive gland	-	-	-	-	-	-									-	-	-
Free blood	-	-	-	-	-										-	-	-
<i>Diet 4</i>																	
Mantle																	
Digestive gland	-	-	-	-	-	-	-	-	-						-	-	-
Free blood	-	-	-	-	-										-	-	-
<i>Surimi vs. unfed</i>																	
<i>Diet 1</i>																	
Mantle						+				-							
Digestive gland	-	-	-	-	-	+				-				+			-
Free blood	-	-	-	-	-	+				-			+	+			-
<i>Diet 2</i>																	
Mantle																	
Digestive gland	-	-	-	-	-	+								+			+
Free blood	-	-	-	-	-	+								+			+
<i>Diet 3</i>																	
Mantle																	
Digestive gland	-	-	-	-	-	+				+				+			+
Free blood	-	-	-	-	-	+								+			+
<i>Diet 4</i>																	
Mantle																	
Digestive gland	-	-	-	-	-	+				+				+			+
Free blood	-	-	-	-	-	+								+			+

The '-' or '+' indicates that the concentration of the amino acid in that tissue was significantly lower or higher ( $p < 0.05$ ) when compared to shrimp fed or unfed cuttlefish.

experiments produced either modest or negative growth. During early trials cuttlefish did not accept commercial surimi (Lee et al. 1991), but more recently, Castro et al. (1993) reported that cuttlefish more readily accepted prepared surimi diets (fish based) than pelleted diets (shrimp based). No growth was obtained during those experiments due to the low nutritional value of the diets. It was postulated that the lack of growth was due to the low nutritional value of the diet, and that this could be improved with supplementation. Castro and Lee (1994) tested growth on plain surimi diets and surimi diets with casein supplementation. Only the supplemented diet promoted significant growth. In the present experiments, only the diet with the highest amino acid concentrations (according to the chemical score from Table 4) promoted growth ( $p > 0.05$ ). Even the remaining high protein diets (diets 2 and 3) but with limiting levels of several essential amino acids, (lysine being the most limiting (Table 4)) did not promote growth ( $p > 0.05$ ). The diet with cellulose and corn starch, with lower protein content (diet 1) even gave negative growth. A positive association was found between growth and the level of the essential amino acids in the diets. Food conversions were also higher for the diet 4. These facts show that *S. officinalis* has an amino acid based metabolism, as all cephalopods do (Lee 1994). Thus, ideal prepared diets for cephalopods will have to take into account the type and amount of amino acids used for supplementation.

Nevertheless, even in the diets with higher (and non-limiting) levels of this and other essential amino acids, acceptable growth was not obtained. One possible cause for the low growth promoted by these diets could be poor diet composition. In fact, it is likely that free amino acids as used to make our diets, in their pure states, may be assimilated less efficiently by cuttlefish, compared to proteins and amino acids present in natural prey. According to Cowey and Sargent (1979), Cowey and Walton (1988), absorption of protein bound amino acids was higher in fish, compared to free amino acids. Accordingly, Zarate and Lovell (1997) reported a 175% higher absorption of protein bound lysine, compared to free lysine in catfish. De la Higuera et al. (1998) reports a significant increase in protein synthesis by catfish with a supplement of coated lysine, compared to free lysine. Sierra (1995) indicates that encapsulation of nutrients in the feeds to be beneficial when feeding aquatic animals. Since amino acids in the present diets were added in their free form, a proportion of them could have been liberated into the water and lost. Another factor that could explain the lower growth compared to our expectations was the cooking of the diet, necessary to deliver consistency to the diets. This could have contributed to protein breakdown, and a consequent loss in quality of the diet.

Lysine supplementation in diets for catfish did not promote improved growth (Li and Robinson 1998). However, we do not believe that the lower concentration of lysine in the first three diets was the major cause, or even one of the major causes for the small growth obtained. This is supported by the fact that the diet with high lysine content (and overall high amino acid content) did not promote acceptable growth, compared to natural feeds. As previously

mentioned, we believe that diet preparation was most likely the major factor contributing to these low growth rates. The analysis of the chemical score (Table 4) shows that there were many essential amino acids that were limiting in diet 1, with the lysine concentration being very limiting (6.8). Also, with the exception of arginine and valine, all essential amino acids were limiting in this diet. This could have contributed to the very poor (negative) growth for this diet. Also, the very high content in corn starch and cellulose (fibers) should have resulted in a lower assimilation of this diet by the carnivorous cuttlefish. Their presence in the diet not only reduced protein content in the diet, but also fibers accelerate the pathway of the food through the digestive tract, leaving the nutrients less time to be assimilated. Also, for diet 2, with the exception of valine, arginine and leucine, all essential amino acids in the diet were limiting, which concurs with the poor growth obtained here.

Besides visual stimuli from the food, the potential effects of chemical stimuli are of great importance (DiMarco et al. 1993), since a good diet can high in energy and digestible, but if it is not detected it will not stimulate consumption. Some nucleotides such as ATP and AMP, as well as amino acids (proline) have proved to be chemo attractants for cephalopods (Lee 1992). It was noted that feeding rates on prepared diets during the present experiments were considerably lower than feeding rates on a normal laboratory maintenance diet of thawed shrimp, (of 6–8% BW d<sup>-1</sup>), determined in previous experiments (Castro et al. 1993), and also considerably lower than rates during transition periods when cuttlefish were fed thawed catfish fillets. During these transition periods, feeding rate varied between 3.5% and 10% BW d<sup>-1</sup>. Rates obtained here were also considerably lower than the ones reported by Domingues et al. (2002, 2003a, b, 2004), which were always higher than 10% BW d<sup>-1</sup>. This is (low palatability and diet consumption), in our opinion, the main reason for the low growth obtained. Furthermore, the texture of the artificial diet was such that pieces of surimi were broken down into small portions while being eaten, and they were not consumed as they fell to the bottom. Although this contributed to low feeding rates, it does not explain by itself the much lower feeding rates compared to natural diets, which we attribute to the low palatability and attractiveness of our artificial diets.

Despite the acceptance of the prepared diets, the highest growth rate in the present experiments (0.48% BW d<sup>-1</sup>) for a cuttlefish fed the diet with highest amino acid levels in the individual trial was approximately seven times lower than growth rates recorded during normal laboratory maintenance of this species (3.5% BW d<sup>-1</sup>) by Forsythe et al. (1994). Growth rates higher than 5% BW d<sup>-1</sup> for similar size cuttlefish were obtained by Domingues et al. (2002) when using live shrimp as food. Even higher growth rates (> 20% BW d<sup>-1</sup>) were even reported by Domingues et al. (2001a) for juveniles. We must stress the fact that studies with artificial diets for cephalopods are still in their early stages, and that results obtained here are similar to the ones obtained when first substituting natural prey by prepared feeds for fish.

In the individually reared experiment, feeding rates on the low protein diet were lower ( $p > 0.05$ ) than for the remaining diets, which was probably due to negative post-ingestive feedback as well as low nutritional value of the diets. This could be due to the high content in fiber of the diet, which lowers absorption by the cuttlefish. Also, since cephalopods have high-energy costs for feeding (O'Dor and Wells 1987), a low energetic diet may inhibit feeding. Besides, from the first half to the second half of Experiment 1 there was also a decrease ( $p > 0.05$ ) in feeding rates in all diets tested, indicating that cuttlefish possibly learned that the diets were not nutritionally satisfactory.

The overall composition of the mantle of cuttlefish fed the prepared diets showed little variation (Table 6), except for the amino acids proline and serine. Along with alanine, proline is utilized for energy (Ballantyne et al. 1981). These two amino acids are some of the most common in cephalopod muscle; in fact, proline and alanine, together with glycine and arginine account for about 60% of the free amino acids in the muscle of several cephalopods (Ruíz-Capillas et al. 2002). This might indicate that proline was being used by cuttlefish fed the artificial diets, since it is the amino acid that is most easily oxidized into glutamate (Lehninger et al. 1993). This in turn is used to produce energy either by deamination (into  $\alpha$ -ketoglutarate) or transamination by aspartate or alanine aminotransferases (Ballantyne et al. 1981). Therefore, proline is the first amino acid to be naturally depleted. The lower ( $p > 0.05$ ) concentration of proline in the blood of animals fed three of the four prepared diets (Table 6) is also explained by the fact that proline is naturally broken down into carbohydrates (sugars) and used as energy. Alanine is also a very important for cephalopods, and is abundant in the brain of *S. officinalis* (D'Aniello et al. 1995). The fact that this amino acid is used for energy could explain the significant reduction in digestive gland concentration from cuttlefish fed diets 2 and 4, compared to cuttlefish fed natural diets. For Experiment 1, amino acid concentrations in the tissues of cuttlefish fed the prepared diets were never higher than concentrations in cuttlefish fed shrimp.

Higher ( $p > 0.05$ ) concentrations of proline were found in tissues of cuttlefish fed the prepared diets, compared to unfed cuttlefish, especially in the blood (Table 6). This indicates that cuttlefish fed the prepared diets were in better condition than unfed cuttlefish. The probable explanation is that breakdown of proteins and amino acids was still occurring, especially in the blood. Higher ( $p > 0.05$ ) concentrations of branch-chained amino acids such as valine, leucine and isoleucine were also found in the blood of cuttlefish fed the high protein content prepared diets, compared to unfed cuttlefish. Higher concentrations of these three amino acids were also found in the digestive gland for diet 2 (Table 6). These amino acids are converted into succinyl-CoA, an intermediate of the Krebs cycle, and used as energy, especially in the mantle (Lehninger et al. 1993).

Cephalopods mobilize amino acids from the mantle and digestive gland tissues during starvation (O'Dor and Wells 1987; Castro et al. 1992). Amino acid concentrations in cuttlefish fed the artificial diets were similar or lower

when compared with cuttlefish fed shrimp. Nevertheless, compared with unfed cuttlefish, higher ( $p > 0.05$ ) concentrations of the amino acids used for energy (proline and the branch-chained amino acids) were generally present in the digestive gland and/or blood (Table 6). Another indicator that the diets were not sufficiently energetic to promote acceptable growth is that, compared with cuttlefish fed shrimp, there was a lower ( $p > 0.05$ ) concentration of histidine in the blood of cuttlefish fed the prepared diets, particularly the one with low protein content (Table 6). Histidine is also a precursor to glutamate, but has a more complex pathway than proline to transform into glutamate (Lehninger et al. 1993), and is depleted at a slower rate. This is based on the fact that its concentration was not lower ( $p > 0.05$ ) in the mantle, the most stable of the tissues analyzed, but was lower ( $p > 0.05$ ) in the blood, where breakdown was taking place. Since alanine is produced when proline is catabolized by transamination to glutamate (Storey and Storey 1978), an increase in alanine is expected if cuttlefish eat a high-energy diet. This was not observed in Experiment 1, even when comparing with unfed cuttlefish, indicating that the cuttlefish fed the artificial diets were not in good condition.

The digestive gland is the most metabolically active organ in cuttlefish, and showed a greater change in amino acid concentration than the other tissues analyzed. In the digestive gland, concentrations of several amino acids, particularly essential amino acids, were lower ( $p > 0.05$ ) when compared with cuttlefish fed shrimp (Table 6). This may have been caused by a decrease of substrate due to poor nutritional quality of the diets. Since less protein or amino acids arrive in the digestive gland, fewer enzymes are needed for catabolic enzyme production. Also, after using their small lipid reserves, mainly concentrated in the digestive gland, cuttlefish begin to rapidly use protein and amino acids as their main fuel reserve (Boucher-Rodoni et al. 1987). Being that the digestive gland is an extremely well irrigated organ, the breakdown of amino acids occurs here faster than in other tissues like the mantle.

By analyzing Table 6, we consider the unfed cuttlefish to be in a state of nutritional stress with extremely low blood levels of the energetically important amino acids. We conclude that our prepared diets, including the one with higher (not limiting) lysine level, were not sufficiently energetic to promote acceptable growth. Nevertheless, they appear to be energetic enough to maintain cuttlefish with minimum growth rates, particularly with the higher lysine level tested.

Another important aspect that was not taken in consideration during the elaboration of the diets was lipid composition. Until recent years, it was believed that lipids were not important to cephalopod metabolism, since they have a protein metabolism, using proteins for energy and growth. Nevertheless, recent research by Navarro and Villanueva (2000), Villanueva and Navarro (2003), Villanueva et al. (2002, 2004), and Domingues et al. (2003a, 2004) indicate that lipids, particular polar and structural lipids are of vital importance, especially during early stages of cephalopod life cycles. The diets used here had no lipids added, and the only ones present were from catfish fillets.

Centrifuging should have eliminated significant portions of lipids in the catfish fillet, particularly neutral lipids, but part of the structural lipids from cell walls, and internal lipids were still probably present in the diets, and available to the cuttlefish. Nevertheless, polar lipid composition of the diets should be considered in the formulation of diets for cephalopods, although in small concentrations.

Research in the future should focus on the preparation of the diets. One possible way to prevent the pure amino acids from being lost into the water is to encapsulate them (with gelatin), since coated nutrients have been used in aquatic feeds with success. This might prevent their loss and allow their assimilation by the cuttlefish. The mechanisms of assimilation of these purified forms of amino acids or proteins by the cuttlefish should be better understood. Also, digestibility and attractiveness of prepared diets, which were substantially lower compared to natural feeds, should be studied and improved. The use of semi-dried pelleted diets, with the addition of squid meat could improve attractiveness. Also, the mechanism of epidermal absorption, already observed in cephalopods, although in a lesser scale in adult cuttlefish (de Eguileor et al. 2000) should be studied, to better understand requirements, assimilation, and other mechanisms that could help the development of artificial feeds for cephalopods, which remain the last bottleneck preventing large-scale culture.

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