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The cuttlefish *Sepia officinalis*
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INFLUENCE OF DIET PEPTIDE CONTENT ON SURVIVAL, GROWTH AND DIGESTIVE ENZYMES ACTIVITIES OF JUVENILE CUTTLEFISH *SEPIA OFFICINALIS*

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CUTTLEFISH
DIET
ENRICHMENT
ENZYMES
GROWTH
REARING

ABSTRACT. – Juvenile cuttlefish hatched in the laboratory were reared for 40 days with different enriched diets (LBBMA4 or LBBMA25, which are viscera silages). Survival was 100% in every group. Cuttlefish fed with enriched diet had better growth and conversion rate. These results indicate that autolysed proteins from enrichment can be used in juvenile cephalopod nutrition. Differences observed in juvenile cuttlefish fed with non-enriched food and cuttlefish receiving enriched food are due for a large part to the protein and peptides contained in the enrichments. In fact, LBBMA4 contains less peptides than LBBMA25, whereas the content in proteins (> 20kDa) was similar. Low molecular weight peptides present in enriched shrimps are also responsible for the remaining specific activity of proteolytic enzymes in the juvenile cuttlefish fed with enriched shrimp. Moreover, high concentration of carbohydrates in enriched shrimps improved a decrease of amylase specific activity. Specific activity of lipases was not modified by diet.

INTRODUCTION

Marine animals are mainly commercialized in eviscerated frozen form, which improves the development of transformation factories. In consequence, many scraps are produced, principally viscera. Traditionally, viscera have been considered as waste and utilized only to a minor extent (Gildberg & Almas 1986). Therefore, given their biochemical composition, viscera could be the basis of marine autolysates to be used in aquaculture diet supplementation. Silage is described as a liquid product made only from animals. Liquefaction is caused by the action of enzymes already present in the animal, and is accelerated by the acid, which in addition to creating the right conditions for the enzymes to work, helps to break down bone and limits the growth of spoilage bacteria (Tatterson 1976). Temperature at which silage is processed may affect the nutritional value of the resulting meal, mainly due to differences in protein digestibility (e.g. Pike *et al*. 1990). In fact, the higher the temperature of fabrication of silage the higher the quantity of peptides compared with protein composition of silage.

Since there is no formulated diet suitable for marine larvae and juvenile, they are fed live prey. Therefore, rearing marine larvae and juveniles represents high cost that limits the development of marine aquaculture. In fact, cuttlefish eat mainly live prey such as crustaceans and fish only subadults and adults can be reared easily with inert food (Castro *et al*., 1993). During post-hatch development, they exclusively hunt small crustaceans such as young shrimp (Koueta *et al*. 2002). This is the main problem to succeed in rearing juvenile cuttlefish for aquaculture. Many attempts have been made to rear juvenile cuttlefish with alternative diets, but young animals in these trials were fragile and their growth rate was low (Castro 1991). Nevertheless, Perrin *et al*. (2004) obtained encouraging results in rearing juvenile cuttlefish with enriched frozen shrimps from 10-days-old. Over the last two decades, several studies have been conducted to determine the nutritional requirements of marine larvae and juveniles (Zambonino-Infante *et al*. 1997, Perrin *et al*. 2004). As demonstrated by Zambonino-Infante *et al*. (1997) in sea bass larvae, the molecular size of the dietary protein fraction could play a major role in larval and juvenile development. Protein hydrolysates are potential ingredients that are used in aquaculture mainly as protein supplements, attractants and palatability enhancers (Hardy 1991). The high concentration of free amino acids in silage permitted to use it as a food additive in aquaculture (Viana *et al*. 1993).

MATERIAL AND METHODS

Enrichment characteristic and prey enrichment: In this paper, our viscera silages made at two different temperatures were investigated in enrichment of juvenile cuttlefish diet. Table I top describes our two silages.

The *Crangon crangon* shrimp were captured at Luc-sur-Mer (France) and placed in a tank (90 l). Shrimp were fed with mashed shrimp. In this way, fresh shrimp surimi was soaked for 24 h at 4°C for control diet and for 24 h at 4°C in silage (500 mg powder / 2 g surimi) for test diet. Surimi was then distributed to young *C. crangon* at a ratio of 5 g of surimi for 10 g of live prey (Koueta 2002). After feeding, shrimp were weighted and frozen.

Rearing: The rearing took place at the Marine Station in Luc-sur-Mer (France) in the facility described by Koueta & Boucaud-Camou (1999). At hatching, juvenile cuttlefish were placed individually in small tanks, which were separated in 4 compartments by a transparent surface. A total of 60 juvenile cuttlefish, hatched the same day, were divided in 3 groups of 20 animals that were housed and fed separately and reared in the same conditions. Each tank contained 4 animals separated by a thick partition; the animals were housed and fed separately with the same quantity of food for each treatment and reared in the same conditions.

The 60 cuttlefish were fed *ad libitum* with simple live shrimps (*Crangon crangon*) during the first 5 days. After 5 days, the first group received simple frozen shrimps, the second group received frozen shrimps enriched with LBBMA4 and the third group received frozen shrimps enriched with LBBMA25 (*ad libitum*). At 40-days-old, 8 h after receiving the diet, cuttlefish were frozen in liquid nitrogen and stored at –80°C until enzymatic analysis.

Growth parameters: The amount of food ingested by the animals in each container was measured by weighting the food remaining in the individual tanks each day. Weight (mg) was estimated each 5 days. Food conversion efficiency (%) was calculated as (growth weight/ weight of food ingested)*100.

Assays:

Extraction: The tissues were disrupted in a potter in 2.5 vol. of the extraction buffer which corresponded to 1% KCl containing 1 mM of EDTA for acid proteases assays (Le Bihan *et al*. 2004). The homogenate was centrifuged for 60 minutes at 10000 g at 4°C. The supernatant liquid was used for the assays. The tissues were disrupted in a potter in extraction buffer (1 ml of buffer per 60 mg of sample) containing: 0.09 M TRIS-base, 0.08 M boric acid, 3 mM EDTA, 0,5 mM mercapto-ethanol, glycerol 10%, pH 8,3 (Koueta 1983) for the alkaline proteases and amylase assays. Then, the crude extract was centrifuged 30 min at 10000 g at 4°C. The supernatant liquid was used for assays.

Enzymatic assays: Total acid and alkaline proteases were measured according to Charney & Tomarelli (1947). Substrate was caseine Yellow 0,005% in a phosphate buffer (0,096 M, KH2P04, 0,004 M, NaH2PO4), pH 2 for the acid proteases and pH 10 for alkaline proteases. 0.5 ml was used for each 0.1 ml of supernatant. The incubation was carried out for 1 hour at 37°C and the intensity of the yellow coloration was estimated at 442 nm. Enzyme activity was expressed as specific activity ($U.mg^{-1}$ protein) where one unit is the variation of one unit of O.D. (optic density).

The technique used for the amylase assay was described by SIGMA-ALDRICH using the starch as substrate. The substrate buffer contains starch at 1% in a monobasic phosphate buffer at 20 mM and 6.7 mM of sodium chloride, pH 6.9. We add 100 µl of substrate buffer to 100 µl of extract. The incubation was 3 min at 20°C. After that, 100 µl of revelator was added and incubated 15min in boiling water. The revelator corresponds to tartrate sodium potassium 0.94 M, NaOH 0.4 M and 3-5-dinitrosalicilic acid 48 mM. The absorbance was recorded at 640 nm. Enzyme activity was expressed as specific activity ($U.mg^{-1}$ protein).

Lipase activity was assayed according to Mckellar & Cholette (1986). Substrate was β-naphtyl caprylate 200 mM in DMSO. Sodium taurocholate 200mM (50µl), BES 50mM pH7.2 (450µl), substrate (5µl) and supernatant (100µl) were mixed and incubated 30 min at 40°C. After the incubation, 5µl of Fast BB salt 100mM in DMSO was added. The incubation was carried out during 5 min at 40°C. Finally, 50µl of TCA 0.72N and 675µl of a solution ethanol 95%/ ethylacetate (v/v) was added. The intensity of coloration was estimated at 540 nm. Enzyme activity was expressed as specific activity ($U.mg^{-1}$ protein) where one unit is the variation of one unit of O.D.

Molecular weight of proteins and peptides: Molecular weight of proteins and peptides contained by shrimps or silages was determined using gel filtration. Dry tissue was added to Tris buffer containing 10 mM Tris-HCl and 150 mM NaCl, pH 8 (0.1 g to 10 ml). The mixture was stored at 4°C for 1 h, then centrifuged for 10 min at 4°C at 10 000g. The supernatant contains the Tris soluble proteins. The protein content was assayed according to the Lowry method (1951) using BSA as standard. The protein concentration in the supernatant was then adjusted to 10 mg/ml. The molecular weight of the extract obtained was estimated using a Sephadex column (Pharmacia PD 10 Sephadex), which had been calibrated with the following molecular weight standards: β-amylase (200,000), alcohol deshydrogenase (150,000), albumine (66,000), carbonic anhydrase (29,000), vitamin B12 (1,350). The amount of protein in the different fractions (500 µl) was assessed by the absorbance at 280 nm. Results were used to calculate the proportion of peptides contained in each assay. Proportion of peptides (%) was calculated as (area of peak 2 / (area of peak 1 + area of peak 2))*100.

Protein contents: The protein content in each extract was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as the standard.

Prey composition: Frozen shrimps from the 3 groups (100 g) were freeze-dried and transformed to powder using a ball grinder. Total proteins contained in freeze-dried shrimps was determined using the Lowry method (1951). Ten ml of NaOH 1M were added to the powder of freeze-dried shrimps (10 mg). After vortexing, the solution was stocked for 12 h at room temperature. A solution of Na_2CO_3 2% in NaOH 0.1M (100 ml) was added to solutions of $CuSO_4$ 0.5% (1ml) and tartrate sodium potassium 1% (1 ml). Later on, 0.5 ml of sample was added to 0.5 ml of H_2SO4 0.5M and 5 ml of Na_2CO_3 solution. The incubation took place at room temperature during 10 min. After that, 0.5 ml of Folin-Cioacalteu solution 0.5M was added to the solution. After vortexing, the incubation took place at room temperature during 1h30. The intensity of coloration was estimated at 750 nm.

Total carbohydrates contained in freeze-dried shrimps were extracted using the Staats *et al*. method (1999) and assayed according to Dubois *et al*. (1956). Dry tissue (500 mg) was added to distillated water (5 ml). Assays were placed at 35°C during 1 h, after that, assays were

centrifuged 10 min at 3000 g. Two ml of supernatant were taken and added to 8 ml of absolute ethanol. Assays were placed during 16 h at –20°C and centrifuged 30 min at 4000 g and 10°C. The supernatant contains low molecular weight carbohydrates and pellets contain high molecular weight carbohydrates. Ethanol rests on pellets and supernatant were evaporated at 60°C. We added 1 ml of distilled water on dry pellets thus obtained. Assays of carbohydrates were made adding 1 ml of phenol solution at 5% and 5 ml of sulfuric acid 96%. The incubation took place at room temperature during 30 min using glucose as standard. Intensity of coloration was estimated at 485 nm.

Total lipids contained in freeze-dried shrimps were extracted using the Bligh & Dyer method (1959) and assayed according to Marsh & Weinstein (1959). Dry tissue (10 mg) was added to 1 ml of chloroform and 2 ml of methanol. Assays were centrifuged 10 min at 4000 g. Supernatant was taken and 1 ml of chloroform and 2 ml of methanol were added to pellets. Assays were centrifuged 10 min at 4000 g. All the supernatant was taken and 4 ml of distillated water were added. Assays were centrifuged 10 min at 4000 g. Lipids were contained in the lower phase. Chloroform remains were evaporated at 60°C. Then we added 10 ml of H_2SO_4 on dry pellets thus obtained. The incubation took place at 200°C during 20 min using tripalmitate as standard. Intensity of coloration was estimated at 360 nm.

Statistical analysis: Results are given as mean ± standard deviation (n=10). Data were compared with an ANOVA followed by a Tukey's test when significant differences ($p<0.05$) were found (Sokal & Rohlf 1981).

RESULTS

Molecular weight of proteins in silage

The peak, which contains proteins of a molecular weight higher than 20 kDa represents 19 or 11%, respectively in the LBBMA4 and LBBMA25, whereas the second peak (proteins >6.5 KDa) represents 81 or 89%, respectively (Fig. 1).

Growth parameter

The survival was 100% in all three groups during the experimental rearing. The cuttlefish weight significantly increased ($p<0.05$) from 0 to 40 days in all the groups (Fig. 2). The cuttlefish which received enriched food have significantly higher weights ($p<0.05$) at 10, 35, 40-days of age. The conversion rate was significantly higher ($p<0.05$) in the groups fed with enriched diet at 10 and 35-days-old than in the control (Fig. 2).

Enzymatic activity

Specific total proteolytic acid activity increased significantly ($p<0.05$) in cuttlefish fed with shrimp enriched with LBBMA25 (Table I top). Specific total proteolytic alkaline activity increased significantly ($p<0.05$) in cuttlefish fed with shrimp enriched with LBBMA25. Specific lipase activity increased significantly ($p<0.05$) in cuttlefish fed with shrimp enriched with LBBMA4 (Table I middle). Specific amylase activity increased significantly ($p<0.05$) in cuttlefish fed with enriched shrimp (Table I top).

Prey composition

The amount of total proteins in g/ 100g of dry weight was significantly ($p<0.05$) lower in enriched shrimp in comparison to non enriched shrimp (Table I bottom). The amount of total carbohydrates in g/ 100g of dry weight was significantly ($p<0.05$) higher in enriched shrimp in comparison to non enriched shrimp (Table I bottom).

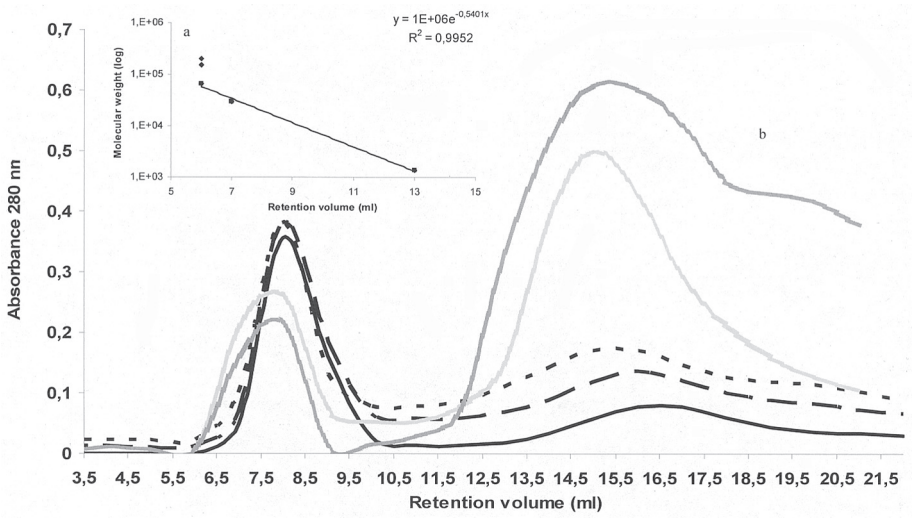

Fig. 1. – a, Calibration graph of molecular weight for Pharmacia G25M PD 10 Sephadex. b, Shrimp proteins molecular weight distribution using gel filtration. –: Simple frozen shrimps, — —: Frozen shrimps enriched with LBBMA4, - -: Frozen shrimps enriched with silage LBBMA25, "clear grey bar": LBBMA4, "dark grey bar": LBBMA25.

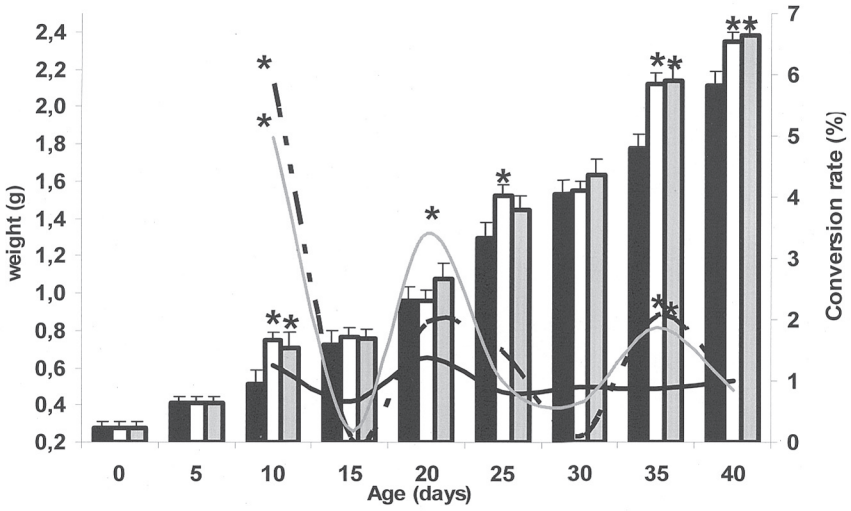

Fig. 2. – Weight (g) and conversion rate (%) of cuttlefish according to their diets. Weight of cuttlefish fed with simple feed: "white bar", with feed enriched with LBBMA4: "clear grey bar", with feed enriched with LBBMA25: "dark grey bar". –: Ration of cuttlefish fed with simple feed, — —: with feed enriched with LBBMA4, - -: with feed enriched with LBBMA25. *: Significantly different from control ($p < 0.05$).

HMW (high molecular weight) carbohydrates in µg/g of dry weight were significantly ($p<0.05$) higher in shrimp enriched with LBBMA4 in comparison to non enriched shrimp. Moreover, LMW (low molecular weight) carbohydrates in µg/g of dry weight was significantly ($p<0.05$) lower in shrimp enriched with LBBMA4 and higher in shrimp enriched with LBBMA25 in comparison to non enriched shrimp. The amount of total lipids in g/100g of dry weight was significantly ($p<0.05$) lower in enriched shrimp in comparison to non enriched shrimp (Table I bottom). The quantity of proteins, which have a molecular weight higher than 20 kDa contained in shrimps was not significantly influenced by enrichment. On the contrary, the content of peptides (> 6.5 kDa) in shrimps was directly influenced by the amounts of peptides of enrichment. In this way, shrimps received the LBBMA25 contains significantly ($p<0.05$) more peptides than shrimps receiving the LBBMA4, which contain significantly ($p<0.05$) more peptides than control shrimps.

DISCUSSION

From a metabolic point of view, one of the most apparent differences found in comparison to cephalopods and other marine organisms is a high-protein content (75-85% of dry weight) due to the predominance of their amino acid metabolism (Villanueva *et al*. 2002). Artificial diets based on less expensive protein sources are becoming increasingly important as an alternative to live feeds in the aquaculture industry (Coutteau & Sorgeloss 1992). In fact, silage has proved to be a good protein source for sea-farmed fish (Raa & Gildberg 1982). In our silages, there are more than 81% of proteins in LBBMA4, which have a molecular weight lower than 6.5 KDa, whereas there are 89% in LBBMA25. Silages obtained contain mainly peptides, which can possess active properties and amino acids, which can be ingested rapidly by juvenile animals in rearing.

Nobody has reared juvenile cuttlefish with alternative diet before this experiment, from 5-days-old with such good survival (100%). The weight highly increased to 40-days old. In this way, cuttlefish, which received simple frozen prey, have a weight of 2.11g, cuttlefish receiving frozen enriched shrimp with LBBMA have a weight of 2.35g (+11.37% compared to control cuttlefish) and cuttlefish which received frozen enriched shrimp with LBBMA25 have a weight of 2.38g (+ 12.8% compared to control cuttlefish) after 40 days of rearing. Moreover, enrichments permit juvenile cuttlefish to have better growth and higher weight. The conversion rate was better for the cuttlefish fed with enriched frozen prey at 10, 20, 25 and 35-days-old. This higher food conversion rate shows that the methodology of enrichment of the prey with silages was effective. The animal fed with enriched diet refused excess food as previously observed by Richard (1975) and Koueta *et al*. 2002 in experimental rearing. Other studies showed the same phenomena in *Haliotis fulgens* where the addition of viscera silage to the diet permitted to induce higher growth rates compared with natural food (Viana *et al*. 1993). Moreover, Oliva-Telez *et al*. (1999) showed that the effect of silage processing temperature on fish performance is more or less pronounced, depending on the species considered. In Atlantic salmon *Salmo salar* (Pike *et al*. 1990)

Table I. – Top, Short presentation of the silages used in this study. Middle, Specific activity (Unity/mg of proteins) of digestive enzymes of cuttlefish according to their diets. Bottom, Shrimps composition on HMW carbohydrates (µg/g of dry weight), LMW carbohydrates (µg/g of dry weight), total carbohydrates (g/100g of dry weight) proteins (g/100g of dry weight) and lipids (mg/g of dry weight).

Commercial name	Raw material	Manufacturer	Presentation (aspect)	Temperature of fabrication	Peptides content (<6.5 kDa)
LBBMA4	Marine animal viscera	LBBM	Powder	4°C	81%
LBBMA25	Marine animal viscera	LBBM	Powder	25°C	89%

	Cuttlefish fed with simple shrimp:	Cuttlefish fed shrimps enriched with LBBMA4:	Cuttlefish fed shrimps enriched with LBBMA25:
Specific activity of total acid proteolytic enzymes:	$4.58.10^{-5} \pm 2.63.10^{-6}$	$3.97.10^{-5} \pm 2.1.10^{-6}$	$5.5.10^{-5} \pm 2.67.10^{-6}$ +
Specific activity of total alkaline proteolytic enzymes:	$3.42.10^{-5} \pm 2.24.10^{-6}$	$3.8.10^{-5} \pm 2.27.10^{-6}$	$4.17.10^{-5} \pm 2.2.10^{-6}$ +
Specific activity of lipases:	$2.10^{-4} \pm 8.81.10^{-6}$	$2.17.10^{-4} \pm 7.10^{-6}$	$1.9.10^{-4} \pm 8.01.10^{-6}$
Specific activity of amylase:	$7.99.10^{-6} \pm 2.9.10^{-6}$	$7.14.10^{-5} \pm 2.8.10^{-6}$ -	$5.53.10^{-5} \pm 2.49.10^{-6}$ -

+: Significant increase compared to cuttlefish fed with simple shrimp ($p < 0.05$).

-: Significant decrease compared to cuttlefish fed with simple shrimp ($p < 0.05$).

	Total proteins (g/100g of dry weight):	HMW carbohydrates (µg/g of dry weight):	LMW carbohydrates (µg/g of dry weight):	Total carbohydrates (g/100g of dry weight):	Total lipids (g/100g of dry weight):	Peptides (>6.5 kDa):
Frozen simple shrimps:	28.80 ± 0.123	61328.6 ± 311.72	79624.7 ± 108	14.095 ± 0.325	9.588 ± 1.2	27,8 %
Frozen shrimps enriched with LBBMA4:	18.31 ± 0.13 -	82552.1 ± 121.29 +	69671.6 ± 6.9 -	15.222 ± 0.127 +	6.8498 ± 0.8 -	35,2 % +
Frozen shrimps enriched with LBBMA25:	25.54 ± 0.0986 -	64548.7 ± 356.33	84601.3 ± 131 +	14.915 ± 0.23 +	8.3184 ± 0.5 -	41,5 % +

+: Significant increase compared to from control shrimps ($p < 0.05$).

-: Significant decrease compared to control shrimps ($p < 0.05$).

diets including silage processed at low temperatures supported higher growth rates, while in rainbow trout *Oncorhynchus mykis* (Pike *et al.* 1990) and in cuttlefish differences were not as pronounced as in salmon. Nevertheless, given the high difficulty to feed juvenile cuttlefish with artificial diet, we used enriched frozen shrimps as alternative diet. Therefore, the impact of silages supplementation on juvenile cuttlefish was made by using indirect enrichment. Prey composition shows that enrichment implied an increase of total carbohydrates and peptides. In contrast, total proteins and lipids decreased with the enrichment of shrimps. Our results show, despite the decrease of total proteins content on enriched shrimps, that the quantity of peptides increased according to the peptide content of silages. Even in indirect enrichment, we have success in obtaining alternative diet enriched on peptide and free amino acids. Nevertheless, shrimp composition was not adjustable indefinitely. Thus, since shrimp metabolism is based on carbohydrates, protein enrichment induces an increase of carbohydrate content after metabolization. We had only success to obtain alternative diet enriched on peptides and free amino acids. This limitation can explain the fact that no differences are observed in comparison to cuttlefish fed with shrimp enriched with LBBMA4 or LBBMA25. We can imagine that the utilization of indirect enrichment limits the effect of silage. In future experiments, we have to test the impact of silages using direct enrichment.

At 40-days of age, the digestive system of cuttlefish was similar to the adult system (Boucaud-Camou 1973, Perrin *et al.* 2004). Therefore, all differences observed in specific digestive enzyme activities are correlated to cuttlefish diet. Our results show that total acid proteolytic activity was stimu-

lated by the contents of shrimps fed with LBBMA25. Moreover, total alkaline proteolytic activity stimulation by the contents of shrimps fed with enrichment was correlated with shrimp quantity of peptides. So, higher peptide composition of enriched shrimps improved higher proteolytic enzyme stimulation. Furthermore, the contents of shrimp carbohydrate quantity inhibited amylase activity.

The utilization of silage as enrichment of juvenile cuttlefish shows very interesting results. Thus, it permits to reduce food ration, to increase weight and to increase the food conversion rate. Several factors can be at the origin of this phenomenon. Enrichment improves higher quantity of peptides and carbohydrates in shrimps as observed by Perrin (2004). Thus, autolysate from viscera can be used as enrichment in cuttlefish rearing.

CONCLUSION

The use of dried fish protein hydrolysate in diets has been shown to improve growth and food utilization of salmonids such as *Salmo salar* (Berge & Storebakken 1996) and carp larvae *Cyprinus carpio* (Carvalho *et al*. 1997). This positive effect can be attributed to increased digestibility of the diet due to the enzymatic treatment; free amino acids released in the process might also act as attractants, increasing food intake and growth (Berge & Storebakken 1996, Oliva-Telez *et al*. 1999). In this way, incorporation of a protein hydrolysate in the diet has a beneficial effect on larval development as in *Carassius auratus* or *Dicentrarchus labrax* (Szlaminska *et al*. 1991, Carvalho *et al*. 1997, Zambinino-Infante *et al*. 1997). The digestive system of juvenile cuttlefish only becomes mature 30 days after hatching (Boucaud-Camou 1973, Perrin 2004). During this maturation period, important changes occur. Therefore supplementing the diet contributed to healthy and rapid growth and higher survival rate.

Autolysate is used as a food for farmed fish (Haaland & Njaa 1989). The nutritional quality of the silage may depend on the degree of autolysis and the products formed. Haaland & Njaa (1989) showed that the temperature has an influence both on the degree of autolysis reached after storage and on the degree of hydrolysis of the amide groups. Moreover, the undissolved fraction, which always remains in an autolysate, was smaller in the autolysate stored at high temperatures than in the autolysate stored at 2°C (Haaland & Njaa 1989). The liquefaction of silages is markedly favoured at acid pH values and above room temperature (Raa & Gildberg 1976). The process of ensilage is very simple and the capital cost of equipment can be low. The basic equipment could consist of a grinder, a means of adding the acid, an acid-resistant storage tank, a means of stirring the silage and containers to distribute the product (Tatterson & Windsor 1974). Thus, our experimentally silage fabrication can be easily applied industrially to produce a protein concentrate from viscera.

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