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Enrichment of Adult *Artemia* Biomass and Squid Mantle Muscle, *Dosidicus gigas*, with Different Ascorbic Acid (L-Ascorbyl-2-Monophosphate-Na/Ca) Concentrations

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Abstract

L-ascorbyl-2-monophosphate-Na/Ca (AMP-Na/Ca) was used as a vitamin C source to investigate its ascorbic acid (L-AA) enrichment and retention in boosted *Artemia* biomass (AB) and squid mantle muscle (SM). Different doses of AMP-Na/Ca (500, 1000, and 1500 AMP-Na/Ca mg/kg) were gradually dissolved into the culture tanks at time 0 (T_0) and at each hour until Hour 6 (T_6). Samples of AB and SM were taken for AMP-Na/Ca and L-AA analysis at T_0, T_1, T_2, T_3, T_4, T_5, T_6, T_{12}, and T_{24}. There were no significant differences ($P > 0.05$) among the AB groups at T_1. The T_6 enrichment analysis for AB resulted in significant differences ($P < 0.05$) in the AMP-Na/Ca content for the 1500 mg/kg treatment, in which the initial concentration (0.001 ± 0.002 mg/kg) increased by more than 16-fold. For all AB enrichment treatments, the AMP-Na/Ca content demonstrated a decrease (32–11%) for the T_6, T_{12} and T_{24} analysis. The T_1 analysis for SM at the higher AMP-Na/Ca enrichment concentration registered 30 mg/kg of L-AA and decreased (27.6%) at T_6. This study demonstrated that AB and SM can be boosted with AMP-Na/Ca.

The importance of vitamin C has been demonstrated for the development and reproductive processes of aquatic animals. It has been suggested that vitamin C is an essential nutrient for the reproductive physiology of crustacean species (Nguyen et al. 2012). For example, ascorbic acid (L-AA)-supplemented diets improve survival, body weight gain, feed

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efficiency ratios, gonadal maturation, and stress resistance in penaeid shrimp (Lee and Shiau 2002).

Established techniques have demonstrated the ability to boost the endogenous L-AA levels in *Artemia* nauplii and juveniles (Merchie et al. 1995a; Smith et al. 2004a; Monroig et al. 2007). However, the transfer of this methodology to larger juveniles or adults has not resulted in similar levels of enrichment (Lim et al. 2002). Thus, it is of particular interest to researchers involved in culturing species that utilize adult *Artemia* (Lim et al. 2002; Smith et al. 2002; Ritar et al. 2003) as a food source to look for other vitamin C boosting strategies.

In Latin America, vitamin C is mainly used in both live food and/or fresh diets for breeding white shrimp (*Litopenaeus vannamei*). The rate of incorporation and loss of vitamin C in both *Artemia* adults and squid mantle muscle during the enrichment process could be understood and thus combined to develop targeted feeding regimes for crustacean broodstock.

The use of enriched squid as a dietary component in shrimp maturation has rarely been documented. It has been reported that the postmortem stage, which is associated with the destruction of muscle tissue structure, occurs earlier in squid meat than it does in other common fish (Kugino et al. 1997). The disintegration of the squid muscle (SM) tissue structure at the cellular level was studied during storage under refrigeration, clarifying that squid flesh autolysis advanced rapidly after the beginning of cell necrosis. The muscle tissue structure disintegrated because of the decomposition of muscle proteins, and muscle transparency was lost because the entire muscle developed a mixed coarse-minute structure (Kugino et al. 2009); however, the adhesion of a vitamin to the mantle muscle tissue (mixed coarse-minute structure) has not been studied. The aim of this study was to compare the efficacy of different concentrations of vitamin C, in the L-ascorbyl-2-monophosphate-Na/Ca (AMP-Na/Ca) form, to enrich *Artemia* biomass (AB) and squid mantle muscle for use as a possible feeding strategy for fish and crustacean broodstock.

Materials and Methods

Artemia Production

Decapsulated *Artemia* cysts (ARC No: 1320, INVE Aquaculture, Baasrode, Belgium) from the Great Salt Lake (UT, USA) were hatched in 50-L white fiberglass cones. The cysts (4 g/L) were disinfected with a hypochlorite solution of 200 µg/L for 20 min before hatching. The hatching and culture of *Artemia* were conducted in aerated 1 mL filtered seawater maintained at 34 ± 1 ppt and 28 ± 1 C (mean ± SE). After a 24-h incubation period, newly hatched *Artemia* nauplii were removed from the hatching cones, rinsed in freshwater for 2 min, and cultured at a density of 5 org/mL in 800-L conical tanks containing seawater. *Artemia* were grown to 1.0 mm (instar II/III, 2 d), 1.5 mm (early juvenile, 5 d), 2.5 mm (late juvenile, 8 d), and 7.0 mm (adult, 12 d) on a blended brine shrimp food containing primarily rice pollard, soybean, and wheat flour. The *Artemia* diet was added to the culture water three times daily at a rate to maintain a Secchi disk depth of 25–30 cm.

Enrichment of AB and SM Tissue

The enrichment product, AMP-Na/Ca, is primarily L-AA in the form of L-AMP-Na/Ca and contains a minimum of 35% L-AA activity, with a minimum of 33% in the monophosphate form (ROVIMIX STAY-C® 35; DSM Nutritional Products, Inc., Parsippany, NJ, USA). The content of AMP-Na/Ca in the AB and SM was studied during a 24-h enrichment period. The retention of AMP-Na/Ca following enrichment was also monitored. Water temperature and salinity during the enrichment process were 28 C and 34 ppt, respectively. AB and SM were enriched with AMP-Na/Ca dissolved in the culture water in a 50-L triplicate tank, containing 100 g of AB (adults of 7.0 mm) and 100 g fillets of fresh squid. Triplicate samples of 7.0-mm *Artemia* adults and 10 g of fresh squid fillets were collected prior to enrichment at different times and stored for L-AA analysis, as described below (Figs. 1 and 2).

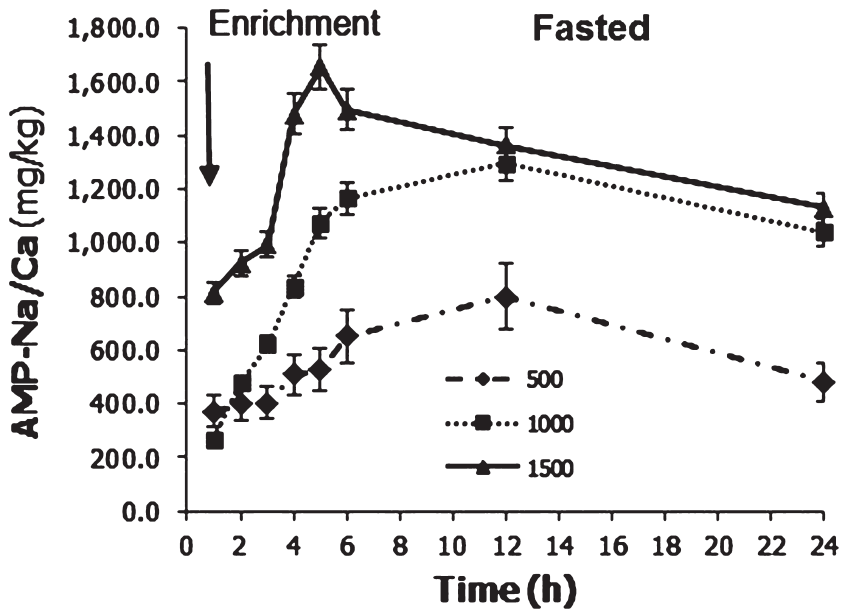

FIGURE 1. *Concentration of the L-ascorbyl-2-monophosphate-Na/Ca (mg/kg) in different levels on* Artemia *biomass. Error bars signify SEMs. Data are means of three replicates.*

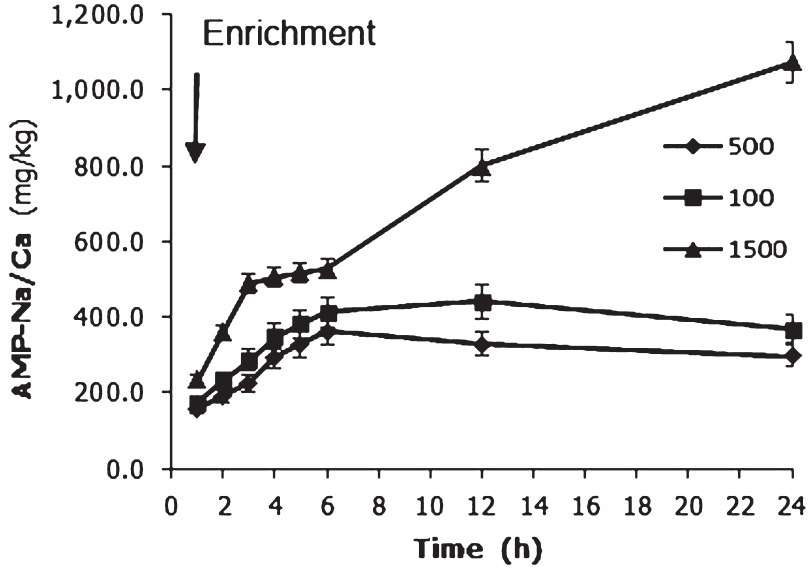

FIGURE 2. *Concentration of the L-ascorbyl-2-monophosphate-Na/Ca (mg/kg) in different levels on squid muscle tissue. Error bars signify SEMs. Data are means of three replicates.*

Experimental Design

Different doses of AMP-Na/Ca were used to evaluate the effects of the feeding concentration. Three concentrations (500, 1000, and 1500 AMP-Na/Ca mg/kg) were tested. The AMP-Na/Ca concentration was applied at enrichment time 0 (T_0) and at each hour until Hour 6 (T_6). Samples of AB and SM were taken for AMP-Na/Ca and L-AA analysis at T_0, T_1, T_2, T_3, T_4, T_5, T_6, T_{12}, and T_{24}.

L-AA Analysis

AB and SM samples were freeze-dried for 24 h (Thermo Scientific Revco ExF -86 Ultra Low Freezer, 13 cu ft – 208-230V, Waltham, MA, USA) and lyophilized (FreeZone 2.5 Liter Benchtop Freeze Dry System, Kansas City, MO, USA) for storage in glycine bags until AMP-Na/Ca determination. The AB and fresh squid were evaluated based on AMP-Na/Ca incorporation at the end of the experiment. Vitamin C in the form of L-AA and AMP-Na/Ca was determined by high-performance liquid chromatography (HPLC) analysis using the isocratic ion pair method and a Hewlett Packard HPLC (1050) system (Bristol, WI, USA), with a Zorbax-SB C18 column (150 mm width); the vitamin C forms were detected at 254 nm.

Changes in Gut Contents after AMP-Na/Ca Enrichment in Adult Artemia

Adult *Artemia* in 50-L batch cultures were sampled during enrichment at 0, 30, 60, 90, 120, 150, 180, 360, 540, and 720 min to determine the gut evacuation (%) and body pigmentation (% whole body).

Statistical Analysis

Data represent the means of triplicate analyses. Data were analyzed using Student's *t*-test and a one-way ANOVA, followed by Tukey's test ($P < 0.05$) (Sokal and Rohlf 1981).

Results

AMP-Na/Ca and L-AA content in AB and SM

Boosted AB and SM tissue resulted in a significant ($P < 0.05$) increase in the levels of AMP-Na/Ca compared with the non-enrichment treatments. When the higher AMP-Na/Ca level was used, the L-AA in the AB was seven times greater than it was in the non-enrichment group (Table 1); meanwhile, the AMP-Na/Ca concentration for SM was over 14 times higher than that obtained for the non-boosted SM. The maximum L-AA enrichment for both diets was attained using AMP-Na/Ca at a dose of 1500 mg/kg. L-AA was detected in very low concentrations in both non-enriched and enriched *Artemia*. Enriched AB resulted in a significant ($P < 0.05$) increase in the levels of L-AA compared with non-enriched *Artemia*, except for the 500 mg/kg AMP-Na/Ca concentration. The concentration of L-AA in AMP-Na/Ca at a dose of 1500 mg/kg increased 10-fold in response to short-term enrichment (1 h). The levels of L-AA were significantly (30.6-fold) higher compared with the AMP-Na/Ca enrichment (Fig. 3).

AB and SM Enrichment Experiments

The T_6 enrichment analysis for AB resulted in significant differences ($P < 0.05$) in the AMP-Na/Ca content for the 1500 mg/kg treatment, with which the initial concentration (0.001 ± 0.002 mg/kg) increased by more than 16-fold. For all AB enrichment treatments, the AMP-Na/Ca content demonstrated a tendency to decrease (11 to 32%) in the T_6, T_{12}, and T_{24} analyses. The T_1 analysis for SM at the higher AMP-Na/Ca enrichment concentration registered 30 mg/kg of L-AA, decreasing (27.6%) at T_6. At T_{24}, there were differences ($P < 0.05$) in the L-AA concentration across the SM treatments. For the AMP-Ca/Na content in SM, the initial concentration (14.9 ± 6.1 mg/kg) increased from 159.9 ± 19.7 mg/kg for the 500 mg/kg enrichment treatment to 235.8 ± 23.6 mg/kg for the 1500 mg/kg group at T_1. There was a tendency of the AMP-Ca/Na concentration to increase until T_6 for all groups. At T_{24}, the AMP-Na/Ca concentration for the 500 and 1000 mg/kg groups was below 400 mg/kg; meanwhile, the 1500 mg/kg treatment had an AMP-Na/Ca content over 1000 mg/kg.

Gut Evacuation and Pigmentation of Artemia

Artemia adults showed the appearance of a full gut cavity (Fig. 4) before the enrichment experiments. Animals began gut evacuation during the first few hours of the experiment; 3 h later, the gut cavity was empty and reddish pigmentation increased all over the body and gut cavity. Complete gut evacuation was determined for the 1500, 1000, and 500 mg/kg AMP-Na/Ca treatments at 2, 2.5, and 3 h after enrichment, respectively (Table 2). Complete reddish body

TABLE 1. *Body composition of* Artemia *and squid muscle tissues L-ascorbic acid (L-AA) and L-ascorbyl-2-monophosphate-Na/Ca (AMP-Na/Ca) content (mg/kg).*[1]

		L-AA		AMP-Na/Ca	
	Treatment (mg/kg)/Time (h)	0	1	0	1
Artemia	500	0.001 ± 0.002^{a}	0.003 ± 0.001^{a}	102.2 ± 18.9^{b}	268.52 ± 22.4^{a}
	1000	0.001 ± 0.002^{b}	0.007 ± 0.002^{a}	102.2 ± 18.9^{b}	373.33 ± 25.9^{a}
	1500	0.001 ± 0.002^{b}	0.010 ± 0.003^{a}	102.2 ± 18.9^{b}	814.58 ± 42.1^{a}
Squid muscle	500	2.2 ± 1.5^{a}	2.6 ± 1.1^{a}	9.7 ± 2.8^{b}	159.92 ± 19.7^{a}
	1000	4.1 ± 1.8^{b}	16.1 ± 3.7^{a}	14.9 ± 6.1^{b}	176.41 ± 18.2^{a}
	1500	3.6 ± 2.1^{b}	30.6 ± 7.1^{a}	16.6 ± 5.4^{b}	235.85 ± 23.6^{a}

[1] Means within the same row with the same letter are not significantly different ($P > 0.05$).

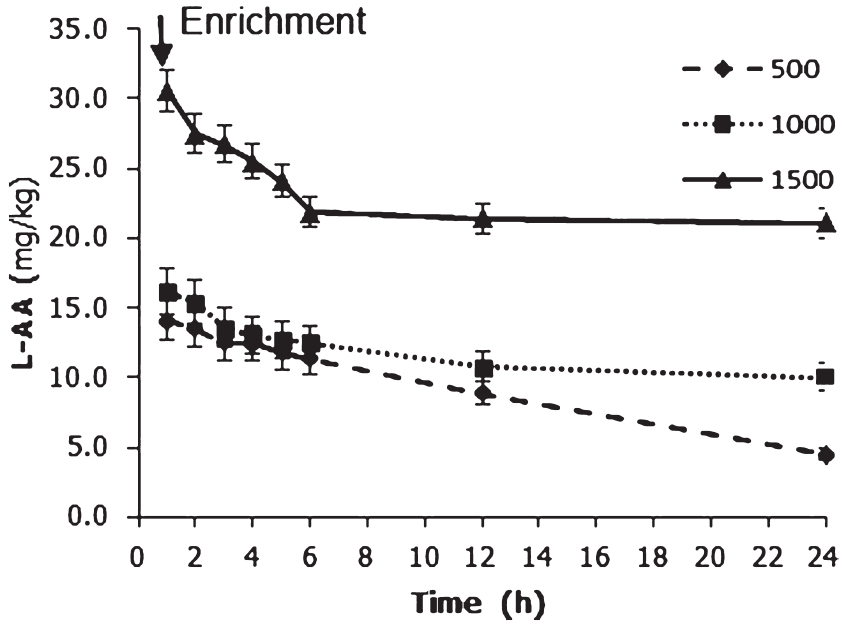

FIGURE 3. *Concentration of the L-ascorbic acid (mg/kg) in different levels on squid muscle tissue. Error bars signify SEMs. Data are means of three replicates.*

pigmentation was determined for the 1500 and 1000 mg/kg treatments, at 2.5 and 3 h after enrichment, respectively, and between 3 and 6 h after the 500 mg/kg enrichment treatment.

Discussion

In recent years, the rearing of new aquaculture species with specific life stage requirements has required diversifying the use of *Artemia* by including live juvenile and adults as well as frozen or freeze-dried AB (Lim et al. 2002; Smith et al. 2002, 2004a). The ability of *Artemia* to metabolize and store specific biochemical substances (Sorgeloos et al. 1998; Narciso et al. 1999; Smith et al. 2002) while suffering no obvious detrimental physiological effects enables their use as a vehicle for the delivery of chemotherapeutics, including L-AA.

The basal L-AA requirement for many commercially important aquaculture shrimp species is generally <100 mg/kg dry weight and may be as low as 20 mg/kg during juvenile and adult stages (He and Lawrence 1993; Shiau and Hsu 1994; Giri et al. 1995). In general, adult *Artemia* enriched with the commercial AMP-Na/Ca contained very low L-AA levels, which supports the results of other studies (Kolkovski et al. 2000;

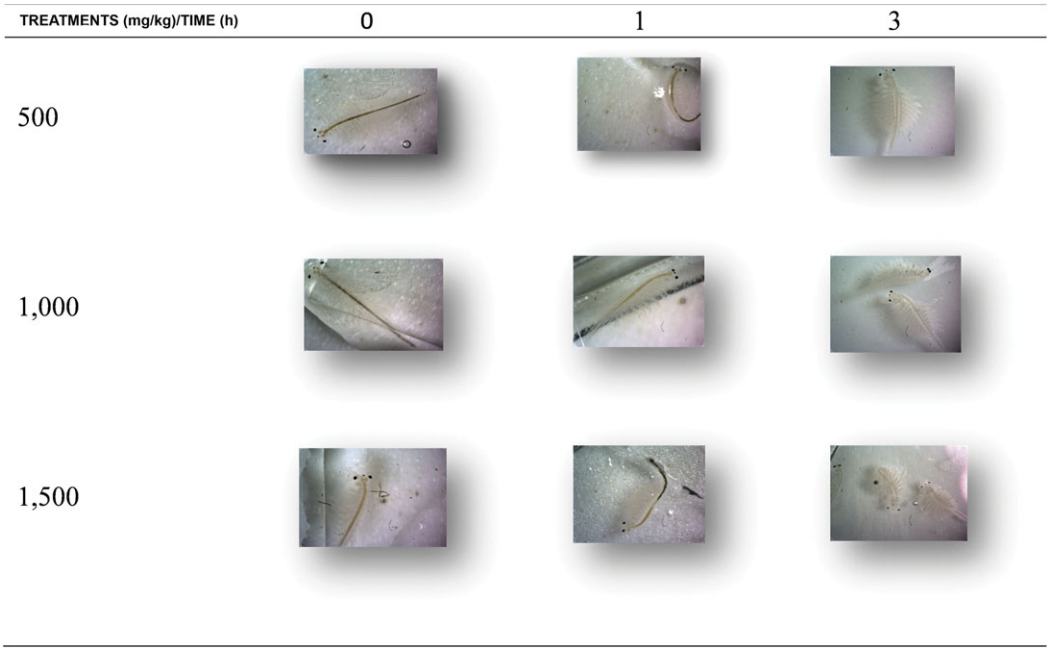

FIGURE 4. *Changes in gut content and body pigmentation of adult* Artemia *after 0, 1, and 3 h of L-ascorbyl-2-monophosphate-Na/Ca enrichment in all treatments.*

TABLE 2. *Changes in gut evacuation (GE; %) and body reddish pigmentation (BRP; %) of adults* Artemia *after L-ascorbyl-2-monophosphate-Na/Ca enrichment.*

	500 (mg/kg)		1000 (mg/kg)		1500 (mg/kg)	
Time (min)	GE	BRP	GE	BRP	GE	BRP
0	0	0	0	0	0	0
30	10	0	10	10	10	10
60	20	0	40	30	50	15
90	40	15	80	40	90	40
120	60	40	95	70	100	60
150	80	50	100	80	100	100
180	90	90	100	100	100	100
360	100	100	100	100	100	100
540	100	100	100	100	100	100
720	100	100	100	100	100	100

Tonheim et al. 2000; Smith et al. 2004b; Monroig et al. 2007). However, the low L-AA concentration obtained for AB was similar to the concentrations reported by Smith et al. (2004b). The authors revealed that the enhancement of the L-AA content in *Artemia* nauplii was more efficient when L-AA was encapsulated in liposomes or simply dissolved in the water compared with the oil emulsion formulated with ascorbyl palmitate. *Artemia* enriched with methionine demonstrated that enriched liposomes did not improve the cost efficiency of the enrichment because of the high expense of the liposome lipid itself; thus, the liposome technique is likely not recommended in the enrichment of *Artemia* with free methionine for nutritional purposes (Tonheim et al. 2000).

AB quickly assimilated AMP-Na/Ca and gathered greater L-AA concentrations within 1 and 6 h of enrichment compared to the results reported by Merchie et al. (1995b) and Smith et al. (2004b), who enriched *Artemia* with AMP-Na/Ca for 6 and 24 h, respectively. In this study, AMP-Na/Ca levels higher than 500–1500 mg/kg were obtained in AB after a 6-h enrichment period. However, Dobbeleir et al. (1980) reported that juvenile and adult *Artemia* have a poor ability to ingest soluble products compared with nauplii, which effectively remove soluble and small particulate matter up to 30 µ in size. Juvenile and adult *Artemia* are predisposed to removing particulate matter up to 50 µ and effectively starve when

only soluble products are used (Dobbeleir et al. 1980). Such an enrichment strategy for adult *Artemia* is more effectively delivered via particulate presentation (Smith et al. 2004b), such as the AMP-Na/Ca in this study. However, even for this size class of *Artemia*, AMP-Na/Ca enrichment may be beneficial by allowing the targeted delivery of L-AA mega doses (Dobbeleir et al. 1980). It was found that losses during fasting are synonymous with the losses obtained when feeding *Artemia* to predators in aquaculture systems. If continuous mega doses of L-AA must be maintained, appropriate feeding protocols should be investigated. Such protocols may include frequently feeding *Artemia* to predators in small doses (initial, 3, 6, 12, and 24 h) during a 24-h period, rather than once daily, and minimizing the L-AA losses in the surplus feed by storing L-AA enriched *Artemia* at low temperatures (Merchie et al. 1995b).

For *Artemia*, its gut contents were evacuated during the first 3 h at the highest concentrations (1000 and 1500 mg/kg AMP-Na/Ca) and over a period of 3–6 h at the lowest concentration (500 mg/kg), which supports the findings of Smith et al. (2002, 2004a, 2004b) in juvenile *Artemia* 6 h after oil enrichment and fasting. The understanding of the rate of incorporation and loss of L-AA and AMP-Na/Ca in *Artemia* nauplii and metanauplii during both enrichment and the subsequent fasting has assisted in the development of feeding regimes that target crustaceans and fish (Evjemo et al. 1997; Smith et al. 2002). While some research has conducted on the enrichment of juvenile *Artemia* (Dhont et al. 1991; Smith et al. 2004b, 2008), there has been little emphasis on adult *Artemia* and its comparison with fresh food for shrimp broodstock nutrition.

Although *Artemia* juveniles and adults represent one of the most efficient ways to deliver nutrients to target species, squid has been shown to improve the percentage of normal sperm and the rate of ovarian maturation (Wouters et al. 2001; Meunpol et al. 2005; Coman et al. 2007) and good reproductive performance in shrimp (Naessens et al. 1997). Most shrimp broodstock nutrition studies are based on trial-and-error experiences or experimental research using fresh and fresh-frozen chopped squid prior to feeding (Harrison 1997; Coman et al. 2007).

It is known that squid mantle texture is related to its particular structure (Otwell and Giddings (1980). The muscle fibers show both radial and circular arrangements and are supported by connective tissue with longitudinal, radial, and circular orientations (Melendo et al. 1996). In this experiment, the samples were cut into pieces (Melendo et al. 1996) and the stable collagen (Ando et al. 2001) and pores between the muscle cells in the softened raw samples (Ando et al. 1999) favored the vitamin C boosting as vitamin C was retained in the squid mantle muscle, which kept its particular texture profile unchanged and achieved tenderization because of the water's pH (7.3) and temperature (27 C). This could explain the fact that the treatment with the highest concentration of vitamin C (1500 mg/kg) reached the maximum AMP-Na/Ca concentration.

In general, SM enriched with the commercial AMP-Na/Ca contained moderate L-AA levels, which supports the results of other studies (Kolkovski et al. 2000; Tonheim et al. 2000; Smith et al. 2004b; Monroig et al. 2007). Enrichment with vitamin C dissolved in water for the SM proved to be a quick and inexpensive technique. In this study, 100% of the determined concentration was fixed at a maximum of approximately 6 h, which coincides with the results obtained by Monroig et al. (2007).

In conclusion, the enrichment of AMP-Na/Ca in adult *Artemia* and squid mantle muscle increased by more than 16- and 15-fold, respectively, for the 1500 mg/kg group. The concentrations were fixed at a maximum of approximately 6 h. The enrichment with vitamin C dissolved in water for the SM proved to be a quick and inexpensive technique. The use of enriched AB and SM with AMP-Na/Ca might provide another feeding strategy for using essential nutrients, such as L-AA, in aquatic diets for fish and shrimp broodstock.

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