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Evaluation of vitamin C-enriched *Artemia* nauplii for larvae of the giant freshwater prawn

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The effect of high levels of ascorbic acid (AA) delivered through enriched live food has been verified through the successful culture of larval giant freshwater prawn, *Macrobrachium rosenbergii*. Two successive feeding trials were set up using a control (550 μg AA g^{-1} DW) and two different AA-enrichment levels in *Artemia* (1300 and 2750 μg AA g^{-1} DW). Under standard culture conditions, no differences in growth nor survival could be observed demonstrating that the nutritional requirements are below 550 μg AA g^{-1} DW, which is the normal level occurring in freshly-hatched *Artemia*. However, a significantly positive effect could be demonstrated on the physiological condition of the postlarvae, measured by means of a salinity stress test, when vitamin C-boosted live food was administered. Since the AA levels in the predator larvae are linked with the enrichment levels in the live prey, it may be assumed that a positive influence on stress resistance was caused by feeding vitamin C-enriched *Artemia*. It is expected that under suboptimal conditions, supplementation of high vitamin C levels might also enhance production characteristics.

KEYWORDS: Freshwater prawn (*Macrobrachium rosenbergii*), Ascorbic acid, Vitamin C, Larviculture, *Artemia*, Enrichment

INTRODUCTION

Vitamin C, more specifically ascorbic acid (AA), is generally considered to be an essential dietary component for the various stages of aquaculture species (Magarelli *et al.*, 1979; Sandnes, 1991). Values of 10–30 μg AA g^{-1} DW are recommended for normal growth and collagen formation (in this way preventing skeletal deformities) in juvenile and adult fish e.g. channel catfish (*Ictalurus punctatus*) (El Naggar and Lovell, 1991; Mustin and Lovell, 1992), Asian sea bass (*Lates calcarifer*) (Boonyaratpalin *et al.*, 1995), red sea bream (*Pagrus major*) (Kosutarak *et al.*, 1995), Japanese flounder (*Paralichthys olivaceus*) (Teshima *et al.*, 1993) and Atlantic salmon (*Salmo salar*) (Sandnes *et al.*, 1992). For shrimp postlarvae e.g. white-legged shrimp (*Penaeus vannamei*) (He and Lawrence, 1993) and tiger shrimp (*P. monodon*) (Giri *et al.*, 1995), 40–100 μg AA g^{-1} DW (size-dependent and decreasing with age) is sufficient to prevent vitamin C deficiency-related mortality and to maintain normal growth. Inclusion of high levels of vitamin C (1000–5000 μg AA g^{-1} DW) has been demonstrated to enhance tolerance to environmental

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stressors in fish (e.g. aldrin toxicity: Agrawal *et al.*, 1978; intermittent hypoxic stress: Ishibashi *et al.*, 1992) and to increase immunoresistance (Li and Lovell, 1985; Liu *et al.*, 1989; Navarre and Halver, 1989; Hardie *et al.*, 1991).

In larval stages of aquaculture species, several biological functions (e.g. skeletal development, growth, survival) as well as physiological functions (e.g. resistance to toxicants and stress, immunoactivity) might be enhanced by supplemental dietary ascorbate (Dabrowski, 1992). Recently we were able to demonstrate that for larvae of the African catfish (*Clarias gariepinus*) high dietary levels of AA (up to 2500 μg AA g^{-1} DW) significantly increased the growth rate and stress resistance (Merchie *et al.*, 1995c), whereas for European sea bass (*Dicentrarchus labrax*) larvae only stress resistance was affected (Merchie *et al.*, 1995b). To our knowledge there is no other published evidence of vitamin C effects on larval stages and only a paucity of information exists about the requirements during hatchery production. This paper reports on the effects of vitamin C-enriched *Artemia* for larvae of the giant freshwater prawn, *Macrobrachium rosenbergii*.

MATERIALS AND METHODS

For each experiment freshly-hatched prawn larvae from a single female from two different experimental brood stocks were used (A. Bastin, Leuven, Belgium). They were acclimated for 2 days in a stock tank and fed instar I *Artemia* nauplii (Great Salt Lake, Utah-USA origin; GSL). At day 3 the larvae were transferred into 10 and 15 respectively, cylindro-conical tanks with a culture volume of 20 l, each connected to an individual recirculation system consisting of a gravel bed biofilter with a volume of 12 l (adapted from Léger *et al.*, 1989). An upwelling system with a flow rate of 1.2 l min^{-1} was selected to avoid patchiness of live food while creating minimal disturbance. No extra aeration of the culture medium was required. Larvae were reared at a density of 50 larvae l^{-1} at 28 (1)°C in 12‰ natural seawater diluted with deionized water. Water quality parameters (temperature, salinity, dissolved oxygen (DO > 4 mg l^{-1}) and nitrite (<0.1 mg NO_2–N l^{-1}) concentrations) were monitored daily. Twice a day a diet of instar III *Artemia* nauplii (GSL) was administered to install a food density of 3 nauplii ml^{-1} at day 3 increasing to 12 nauplii ml^{-1} at the end of the experiment (day 28). Before each food delivery, the remaining *Artemia* nauplii were removed by means of a small filter installed in the biofilter at the water inlet from the culture tank (Léger *et al.*, 1989). *Artemia* enrichment with vitamin C followed the same procedure as for fatty acids (Léger *et al.*, 1987).

Two experiments were designed. In the first, *Artemia* nauplii enriched with an emulsion containing 50% highly unsaturated fatty acids (HUFA) and 20% ascorbyl palmitate (AP) were compared with a control (50% HUFA, 0% AP). For the second trial a control (50% HUFA, 0% AP) and two levels of vitamin C enrichment (50% HUFA, 10% and 20% AP) were tested. Preliminary testing demonstrated that these AP levels were immediately metabolized into the active AA form in the *Artemia* (respectively 1200 and 2500 μg AA g^{-1} DW). In both experiments, all treatments were run with 5 replicates each. The treatments were assigned at random to the 10 and 15 aquaria for the first and second experiment, respectively.

The diets were evaluated based on production characteristics (survival, dry weight, length from the tip of the rostrum to the end of the telson, and rate of metamorphosis) and physiological condition (measured by means of a salinity stress test) at day 28. The latter vitality test was carried out by transferring 30 post-larvae into 65‰ artificial seawater for a period of 1 h. Subsequently they were allowed to recover during 1 h in

their regular environment of 12‰ and assessed by counting the number of animals showing active movement of the pleopods (Romdhane *et al.*, 1995). This type of activity test for quality evaluation of hatchery-produced fry has also been applied to marine shrimp (Tackaert *et al.*, 1992) and fish (Dhert *et al.*, 1992). It offers the possibility of rapid larval quality control by exposing the test organisms to a short but extreme stress condition (salinity shock) in which the physiological condition of the animals determines their ability for osmoregulation and consequently their chances to survive.

A paired-ion, reversed phase, high-performance liquid chromatography (HPLC) procedure coupled with electrochemical detection and internal standard quantitation based on iso-ascorbic acid (IAA) was used for the determination of AA in *Artemia* and prawn larvae. The HPLC apparatus consisted of a Varian 8500 pump (Varian Assoc., Palo Alto, CA), an N60 valve injector fitted with a 20µl loop (Valco, Houston, TX), and a Coulochem 5100A electrochemical detector (ESA, Inc., Bedford, MA) equipped with a model 5010 or 5011 analytical cell. The HPLC column (15 × 0.46 cm), packed with 5 µm Hypersil ODS (Shandon, Runcorn, UK) and equipped with a 5 × 0.3 cm Chromguard RP guard column (Chrompack, Middelburg, The Netherlands) was eluted with an aqueous buffer, (40 mM sodium acetate, 0.54 mM Na_2EDTA, 1.5 mM dodecyltriethylammonium phosphate adjusted to pH 4.75 with glacial acetic acid). The flow rate was 0.8 ml min^{-1}. Settings of the electrochemical detector were as follows: detector 1, +0.05 V; detector 2, +0.5 V; guard cell, +0.95 V. Extraction was done following a modification of the method of Kutnink *et al.* (1987).

For AP detection a PU4025 UV detector (Pye Unicam, Cambridge, UK) was set at 265 nm. The eluent consisted of a 75:25 mixture of acetonitrile and phosphoric acid (pH 1.6). Other parts of the HPLC apparatus and chromatographic conditions were identical to the ones mentioned above for the AA analysis. Samples were prepared applying solid phase extraction (Merchie *et al.*, 1995a).

Values in the tables represent means (SD). Parameters were statistically evaluated using one way analysis of variance (ANOVA). Tukey's multiple range test was applied to determine significant differences ($p < 0.05$) between means.

RESULTS

At the day of stocking, the prawn larvae contained 154 and 293 µg AA g^{-1} DW in the first and second experiment, respectively. AA incorporation into the live diet is summarized in Table 1. Boosting of the *Artemia* diet with vitamin C resulted in high levels of free AA, while after the 24 h enrichment period only a small fraction of non-assimilated AP (< 120 µg g^{-1} DW, expressed as AA) remained. No significant differences in enrichment levels were observed between both experiments. Dietary AA levels were significantly reflected in the prawn postlarval body tissue and were not significantly different between the two culture trials (Table 2). The AA content in the postlarvae was significantly lower as compared with the larvae. An absolute drop of ± 75 µg AA g^{-1} DW occurred in both experiments, except for the 20%-AP group in the first trial in which the decline reached 227 µg AA g^{-1} DW.

Production results of the two experiments are summarized in Table 2. In general the larvae performed better during the second trial which is demonstrated by the higher growth and metamorphosis rate. No significant differences in hatchery production among treatments for each experiment were detected. Only a significantly positive effect

TABLE 1. Mean (SD) ascorbic acid and ascorbyl palmitate (AP) content (μg AA g^{-1} DW) in *Artemia* nauplii boosted with AP; n = number of samples taken during the 28 day experiments

	0%AP	10% AP	20% AP
Experiment 1			
AA (n=20)	529 (102)	–	2920 (571)
AP (n=2)	nd[a]	–	130 (17)
Experiment 2			
AA (n=10)	656 (59)	1305 (129)	2759 (458)

[a]nd: not detectable

on the physiological condition of the postlarvae, measured by means of a salinity stress test, was demonstrated when vitamin C-boosted live food was administered (Table 2). In the first trial a four-fold increase of survival after a 1 h incubation in a 65‰ artificial seawater was obtained when the larvae were fed the 20% AP-enriched *Artemia*. Also in the second experiment the survival of the stressed organisms corresponded well to the AA content of the live food.

DISCUSSION

The technique of bio-encapsulation of live food or enrichment using self-emulsifying concentrates (Léger *et al.*, 1987) is widely applied at marine and crustacean hatcheries all over the world for enhancing the nutritional value of *Artemia* with respect to essential fatty acids. More recent developments involve a further improvement of the nutritional composition of *Artemia* through enrichment with specific components (docosahexaenoic acid 22:6ω-3, phospholipids, vitamin C) in order to fulfil the nutritional requirements of the predator. In this way tests have been conducted to incorporate this latter nutrient in live feeds (Merchie *et al.*, 1995a). In the experiments reported here non-enriched *Artemia* nauplii already contained more than 500 μg AA g^{-1} DW (control treatment, 0% AP), while boosting of the nauplii with vitamin C via bio-encapsulation resulted in very high levels of AA: i.e. more than 1300 and 2700 μg AA g^{-1} DW was incorporated after 24 h enrichment for the 10%-AP and 20%-AP treatment, respectively (Table 1). AP was quickly assimilated and converted by *Artemia* into the active component AA. These observations are in agreement with previously reported data of vitamin C-enrichment in live food organisms (Merchie *et al.*, 1995a).

The lower growth and metamorphosis numbers obtained during the first experiment (Table 2) might be explained by a lower larval quality as the brood stock providing eggs for this trial was kept on a low HUFA diet. Consequently, the larvae at first feeding may have had a less optimal biochemical composition as demonstrated in our laboratory (De Caluwe *et al.*, 1995), eventually resulting in a lower larval quality (Kjørsvik *et al.*, 1990). The eggs used for the second trial and yielding a higher larval production, were obtained from a female fed a HUFA-rich diet. In this respect Romdhane *et al.* (1995) demonstrated clearly that HUFA were essential for first feeding larvae of the giant freshwater prawn, resulting in improved growth rate and more synchronous metamorphosis. In fact, this

TABLE 2. Production characteristics (survival, dry weight, length and metamorphosis), physiological condition (stress resistance) and AA incorporation of giant freshwater prawn larvae cultured until day 28 and fed an *Artemia* diet containing different levels of vitamin C (mean (SD) values)

	First experiment		Second experiment		
	Control	20% AP	Control	10% AP	20% AP
Survival (%)	72.1 (13.0) [a*]	48.4 (10.6)[a]	57.5 (2.7)[a]	57.8 (9.2)[a]	57.1 (5.0)[a]
Length (mm)	9.31 (0.36)[a]	9.34 (0.48)[a]	9.67 (0.69)[a]	9.73 (1.24)[a]	9.58 (0.65)[a]
Dry weight (μg)	831 (106)[a]	888 (91)[a]	1130 (150)[a]	1200 (320)[a]	1310 (250)[a]
Metamorphosis rate (%)	12.9 (7.0)[a]	16.2 (5.2)[a]	40.6 (11.4)[a]	53.3 (20.7)[a]	49.1 (6.9)[a]
Stress resistance (% survival)	8.7 (8.0)[a]	32.7 (6.7)[b]	40.0 (14.1)[a]	62.0 (4.5)[ab]	74.0 (16.7)[b]
AA in larvae (μg g^{-1} DW)	365 (18)[a/x]	552 (10)[b/x]	352 (28)[a/x]	448 (40)[b/x]	507 (47)[b/x]
AA in postlarvae (μg g^{-1} DW)	288 (0)[a/y]	325 (36)[a/y]	255 (8)[a/y]	389 (73)[b/x]	432 (68)[b/x]

*means with the same superscript were not significantly different ($p<0.05$); (a, b, c): comparison among treatments; (x, y): comparison before and after metamorphosis.

less optimal biochemical composition is also demonstrated here with respect to the vitamin C content: the initial AA content in the larvae of the first trial was only 50% of the value detected in the second experiment. It has been demonstrated that a lower AA deposition in the eggs due to lower maternal intake may lead to deficiency signs (both biochemical and morphological) and/or decreased survival in newly hatched tilapia (*Oreochromis mossambicus*) (Soliman *et al.*, 1986).

During the second trial production results were still slightly inferior to those obtained under similar culture conditions by Romdhane *et al.* (1995) for survival (55 vs. 72%), length (9.8 vs. 11.6 mm), dry weight (1.3 vs. 1.8 mg) and metamorphosis data (60 vs. 94%).

The *Artemia* diets used were analysed for AA and HUFA (not presented) and differed only in AA content. Since the extra dietary vitamin C also resulted in increased AA levels in the predator larvae (>500 μg AA g^{-1} DW for the 20%-AP diet), and the only biological response measured was an increased stress resistance, it may be assumed that the latter positive effect was caused by feeding vitamin C-enriched live food (Table 2). These results support the hypothesis that stress creates increased ascorbate requirements for larval fish and crustaceans, and that in this respect body vitamin C concentration may reflect the survival potential more acurately than variation in growth rate (Dabrowski, 1992). Moreover, at day 28 a significant drop in AA concentration was detected in the postlarvae as compared with the levels found in the larvae. This may reflect an extra need for vitamin C during metamorphosis, i.e. a stressful period as the larvae undergo major morphological and physiological changes. In addition, growth in crustaceans only occurs during moulting by an uptake of water via osmosis. In this respect vitamin C has been linked to collagen formation (Hunter *et al.*, 1979) and to ion-exchange regulation in aquatic organisms (Thomas, 1984).

No significant differences could be identified for any production characteristic examined, illustrating that dietary vitamin C requirements of prawn larvae are met by non-enriched *Artemia* nauplii containing already 500 μg AA g^{-1} DW. This is not surprising since a need of <100 mg AA kg^{-1} has been proven to be sufficient for normal production in juvenile Natantia, e.g. prawn (D'Abramo *et al.*, 1994) and penaeid shrimp (He and Lawrence 1993; Giri *et al.*, 1995), respectively. Nevertheless, when comparing these results with those reported for the hatchery production of two fish species, European sea bass and African catfish (Merchie *et al.*, 1995b), it convinces us that the requirements for vitamin C are probably species dependent. Indeed, whereas for larvae of the European sea bass, no significant effects on production, nor on physiological condition were shown (Merchie *et al.*, 1995b), for larval African catfish a clearly positive effect on growth was demonstrated as well as a significantly higher stress resistance when supplementing extra vitamin C in the *Artemia* diet (Merchie *et al.*, 1995c). In this respect Dhert *et al.* (1990) demonstrated that feeding HUFA-enriched *Artemia* for 2–5 days before metamorphosis of Asian sea bass (*Lates calcarifer*) was required to accelerate metamorphosis rate and to prevent subsequent mortalities.

CONCLUSIONS

1. The extra cost for supplementing vitamin C to the diet in optimal routine production of the freshwater prawn may be seen as a luxury, since it did not result in a better production yield.

2. It is important to realize, however, that small differences in management or in physical parameters are unavoidable and result in a stress exposure.
3. In this respect, the extra investment in supplementation of high vitamin C levels might assure a stable production output during stressful periods, e.g. metamorphosis, transportation and restocking.
4. Eventually, an adopted feeding strategy in which vitamin C-enriched live prey is administered during a short period before such stressful situations, might be sufficient to maintain an optimal physiological condition.

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