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Optimization of dietary vitamin C in fish and crustacean larvae: a review

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Abstract

HPLC techniques were adapted and standardized for quantification of ascorbic acid (AA) and its derivatives in both diets and target organisms. To assess the dietary needs for AA at start of exogenous feeding, the AA content in the various live diets currently used in aquaculture (algae, rotifers, *Artemia*) was analyzed. Application of techniques for boosting vitamin C using ascorbyl palmitate as the source enabled the transfer of elevated levels (up to 2500 μg AA/g DW) of bioactive vitamin C. Larvae of fish (*Clarias gariepinus*, *Dicentrarchus labrax*, *Scophthalmus maximus*), white shrimp (*Penaeus vannamei*) and prawn (*Macrobrachium rosenbergii*) were enriched via the live food chain. This vitamin C enrichment procedure has proven to be a valuable technique for the evaluation of the effects of high levels of dietary vitamin C on stress resistance. However, in most of the species examined, the initial level of AA in *Brachionus* and *Artemia* impaired the determination of the AA requirements for optimal growth and survival. Formulated diets containing variable levels of stable AA-phosphate esters were used for the determination of minimal requirements for AA in the early post-weaning stage of marine fish species (*D. labrax*, *S. maximus*) and the postlarval stage of penaeid shrimp (*Penaeus monodon*, *P. vannamei*). For both fish species, results indicated that, within the concentration range tested, 20 mg AA/kg diet is sufficient for normal growth and survival. For production of postlarval shrimp, this level amounted to a minimum 20 and 130 mg AA/kg diet for *P. monodon* and *P. vannamei*, respectively, while a level of 2000 mg AA/kg diet was needed to enhance the resistance of shrimp postlarvae to stress conditions and bacterial infections. © 1997 Elsevier Science B.V.

Keywords: Ascorbic acid; Enrichment; Fish; Larviculture; Live food; Shrimp; Vitamin C

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1. Introduction

In aquaculture, awareness of the nutritional importance of vitamin C and more specifically ascorbic acid (AA), has grown steadily over the past 25 years. Whereas most animals can synthesize AA from glucuronic acid, fish and crustaceans lack the enzyme gulonolactone oxidase necessary for the last step in this biosynthesis (Chatterjee, 1973; Dabrowski, 1990). Consequently, they are dependent on constant supplies of adequate quantities of vitamin C through the feed. The National Research Council (1993) recommends 25–50 mg AA/kg diet as a requirement to secure an optimal performance of juvenile fish, while levels of about 100 mg AA/kg diet are suggested for shrimp (D'Abramo and Conklin, 1995). Lower dietary AA levels cause typical deficiency symptoms, e.g. the broken back syndrome in fish and the black death disease in shrimp. Dabrowski (1991a) suggested that the metabolic rate is the primary factor regulating the AA requirements. Therefore, larval fish, displaying a relatively faster growth and metabolism than juveniles and adults, might need higher dietary AA levels to sustain optimal growth and physiological condition (Dabrowski et al., 1988; Dabrowski, 1990). Moreover, high AA concentrations are detected in fish eggs, e.g. 267–316 μg/g WW for rainbow trout (Dabrowski and Blom, 1994), which might be an indication of the importance of this micronutrient during early development. Little research, however, has been devoted to the larval stages, mainly because of the complexity of the hatchery-rearing process resulting in variable and relatively low production figures, the need of large numbers of animals for evaluation, and the dependency on a live food diet during start-feeding stages.

This article covers data on the nutritional requirements for AA and effects on stress and disease resistance during the hatchery and early-nursery phase of various fish and crustacean species. Culture tests using standardized larval culture systems and well-defined diets, and a thorough analytical control via standardized analyses of the different vitamin C compounds were adopted.

2. Standardized analytical techniques for ascorbic acid and its derivatives

Any thorough nutritional study implies the availability of accurate analytical methods for detection of the nutrient studied both in the diet (during preparation, storage and feeding) and in the animal tissues. Up to present, fish nutrition studies relied on indirect colorimetric methods involving derivatization (Roe, 1967; Roy et al., 1976; Dabrowski and Hinterleitner, 1989), of which specificity is often questionable. By contrast, few HPLC methods have been used for the determination of vitamin C compounds in aquatic organisms (Carr and Neff, 1980; Felton and Halver, 1987; Hapette and Poulet, 1990; Wang and Seib, 1990).

Reversed-phase HPLC techniques were developed for the various AA esters [AA, AA-monophosphate (AmP), AA-polyphosphate (ApP) and AA-sulphate (AAS)]. Ion-pair chromatography coupled with electrochemical detection was selected for the routine determination of AA. The methods were verified for algae, rotifers, *Artemia*, fish and shrimp tissue. This combination allowed a superior chromatographic selectivity com-

pared to ion-suppression procedures and/or UV detection for which interfering peaks were observed in extracts of complete fish or shrimp. Dehydroascorbic acid (DHAA) was quantified after its reduction to AA with homocysteine. Different extraction solutions were compared with regard to recovery and stability of AA and compatibility with the chromatographic system. The use of strong acids for extraction, e.g. metaphosphoric acid (MPA), afforded adequate recovery (> 80%) of AA, but resulted in a progressively negative drift in its retention time after repeated injections. Non-acidic extraction solutions such as homocysteine-EDTA did not interfere with the chromatography, but yielded lower recovery and partly reduced DHAA to AA, despite the low pH of 4. Using weak acids, particularly 1% acetic acid (HAc), the recovery was also slightly lower (76.4%), but the addition of a small amount of MPA (0.1%) to 1% HAc increased this figure to 86.2%. Retention times were subject to considerably less variability than in MPA. A typical chromatogram is presented in Fig. 1. Using the various extraction solutions described, AA and occasionally DHAA were determined on a routine basis in both live diets and in the test species.

The extraction for determination of AA phosphates in the formulated diets was carried out in distilled water instead of 1% HAc–0.1% MPA-1 mM EDTA. For the phosphatase digestion, 0.5 ml acetate buffer was added to the same volume of the final extract. Oxidation of the AA formed was prevented using homocysteine 3% and EDTA

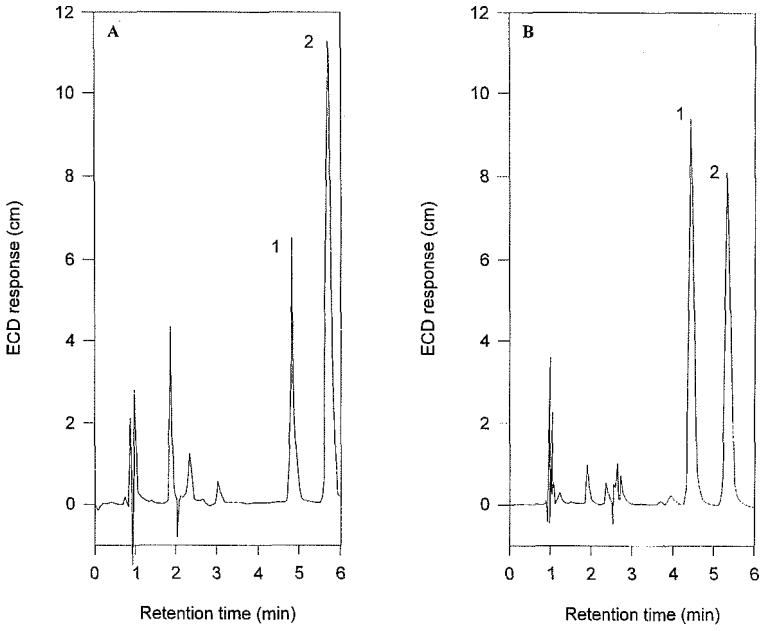

Fig. 1. Representative chromatograms of extracts of *A. nauplii* in 5% MPA–0.54 mM EDTA (A) and in 1% HAc–0.1% MPA–1 mM EDTA (B). Peak identification: 1, AA; 2, IAA (internal standard). Chromatographic conditions: column, 5 μm Hypersil ODS, 15×0.46 cm; eluent, 0.04 M sodium acetate containing 0.54 mM Na_2EDTA and 1.5 mM dodecyltriethylammonium phosphate, pH 4.75; flow rate 1 ml/min; detection: amperometry, +0.72 V.

10 mM. Acid phosphatase (2 mg/ml acetate buffer) was added and the mixture incubated for 30 min at 37°C before injection on the column.

Existing spectrophotometric and liquid chromatographic methods for the determination of AAS in animal tissue and feeds suffered from poor selectivity end sensitivity, mainly because of insufficient sample pretreatment (Nelis et al., 1994). More recently, Felton et al. (1994) reported a direct method allowing a better separation from conflicting metabolites and constituents of extraction mixtures. A direct analytical procedure was developed in which AAS in *Artemia* cysts was initially determined based on reversed-phase ion pair extraction on an octadecylsilica cartridge using tetrabutylammonium as the counter-ion, followed by elution with methanol. The relatively low recovery (63.0%) and reproducibility (CV of 6.3–18.7%) of this procedure led to the development of a second method using solid-phase extraction of AAS. In the latter method, yielding a better recovery (88.7%) and reproducibility (CV of 3.6–7.7%), AAS was retained on a DEAE silica cartridge and eluted with sodium salicylate. Both approaches were applied as extraction techniques part of a reversed-phase HPLC method with detection at 254 nm (Nelis et al., 1994).

3. Vitamin C content and its manipulation in various live food organisms

The variability of the nutritional value of the main live food organisms currently used in aquaculture (i.e. microalgae, rotifers and brine shrimp) is well documented, especially with respect to fatty acids (Léger et al., 1986; Volkman et al., 1989; Lavens et al., 1995b). In order to better assess the transfer of AA in aquatic food chain, AA levels in the different live feeds were determined (Merchie et al., 1995a). Three laboratory-cultured microalgae (*Isochrysis* sp., *Chlorella* sp. and *Tetraselmis suecica*) and *Isochrysis* and *Nannochloropsis* sampled from routine cultures in a commercial marine fish hatchery were analysed for AA concentrations. Laboratory-cultured *Isochrysis* and *Chlorella* contained, respectively, 3806 and 3740 μg AA/g DW, i.e. 3 to 4 times the levels found in *Tetraselmis* (1090 μg AA/g DW), during the late logarithmic phase, the preferred stage of harvest in aquaculture facilities. Brown and Miller (1992) detected levels of the same order of magnitude in *Tetraselmis* and *Isochrysis*, i.e. 1700–2600 and 3800–4400 μg AA/g DW, respectively, while inappropriate storage and/or extraction in the study of De Roeck-Holtzhauer et al. (1991) probably led to the underestimation of vitamin C levels, e.g. only 498 and 772 μg AA/g DW were retrieved in the same species, respectively. *Isochrysis* samples from a commercial hatchery contained only one third of the AA concentration detected in our lab cultures. Differences in culture conditions probably contributed to this variation in AA content. Despite the wide range in the AA composition of the various microalgae, the four species examined provide a rich source of AA for hatchery-reared shrimp and fish larvae, be it directly or indirectly through the feeding of algae-grown rotifers.

Routine cultures of *Brachionus plicatilis* on instant baker's yeast and *Chlorella* contained up to 150 and 2300 μg AA/g DW, respectively. Using the vitamin C-boosted rotifer diet Culture Selco® (CS, INVE Aquaculture N.V., Belgium) AA levels of 565 μg/g DW were obtained after a 7-day culture period. After a subsequent boosting with

Isochrysis during 6 h, the AA levels of yeast-cultured rotifers could be enhanced up to 10-fold (1599 μg AA/g DW). Analysis of samples from commercial hatcheries demonstrated the use of a wide range of products for the culture and enrichment of rotifers consequently affecting the AA levels (506–1559 μg AA/g DW).

AA levels in freshly-hatched *Artemia nauplii* varied considerably from batch to batch, and among strains (310–524 μg AA/g DW) and reflected the concentrations of AAS, the stored form of vitamin C, found in the respective cyst sources (296–517 μg/g DW, expressed as AA). The latter values are slightly higher than those reported by Dabrowski (1991b). This variation in AAS concentration may be due to differences in adult nutrition during egg production as was demonstrated for the fatty acid content by Lavens et al. (1989) and may consequently affect the nutritional value of newly-hatched *nauplii* for larval fish and shrimp.

In order to obtain better tools for studying the nutritional requirements and the role of vitamin C during early larval phases, a methodology was developed for optimal enrichment of *A. nauplii* and rotifers for application both in the laboratory and on a commercial scale (Merchie et al., 1995a). For this purpose, the bioencapsulation technique used for the enrichment with HUFA (Léger et al., 1987) was adopted, using the lipophilic ascorbyl palmitate (AP) as a stable and bio-available source of AA in emulsions and particulate boosters for the live food prior to their administration to predator larvae. Applying experimental self-emulsifying concentrates supplemented with 10% and 20% AP (w/w), high levels of AA could be obtained in 24-h enriched brine shrimp *nauplii*: whereas a 10%-AP inclusion resulted in a doubling of the levels found in freshly-hatched *nauplii* (550 μg AA/g DW), 20% AP additions increased the AA content 4-fold. Moreover, these concentrations did not drop when the 24-h enriched *nauplii* were cold stored for another 24 h. In fact, after 12 h a slight increase in AA was observed, which could be attributed to a continued assimilation of AP still present in the gut at the time of harvest (200–300 mg/g DW, expressed as AA). The addition of 20% AP in the diet of *Brachionus* enhanced their AA content 10-fold over 3 days of culture. An increase in AA from 565 μg AA/g DW after culture up to 1670 μg AA/g DW was demonstrated using vitamin C-boosted Protein Selco® (PS, INVE Aquaculture N.V., Belgium) during a 24-h enrichment period (Merchie et al., 1995a). Important is the fact that apparently AP is immediately assimilated by the live food under the most active form of vitamin C, i.e. AA, and thus, readily available to the predator larvae (Merchie et al., 1995a).

4. Vitamin C during hatchery rearing

The AA requirement of poikilotherms appears to be directly related to their metabolic rate (Matusiewicz et al., 1994). Therefore, a higher supply of vitamin C during the hatchery period may be needed, due to a higher rate of larval growth and metabolism, i.e. a higher ascorbate supplementation is required to gain the same tissue concentration. Moreover, the high AA concentrations detected in fish eggs further support the idea that this micronutrient is of high importance during early larval development. In this respect, culture tests were performed to evaluate a possible beneficial effect of additional dietary vitamin C on larvae of both freshwater and marine crustaceans and fish.

Three different enrichment levels (0%, 10% and 20% AP) in the live food were compared for the African catfish (*Clarias gariepinus*; Merchie et al., 1995b, 1997b), European sea bass (*Dicentrarchus labrax*; Merchie et al., 1995b), turbot (*Scophthalmus maximus*; Merchie et al., 1996a) and the giant freshwater prawn (*Macrobrachium rosenbergii*; Merchie et al., 1995c).

In all experiments reported, boosting of the live food with vitamin C via bioencapsulation resulted in levels up to an average of 1400 and 2800 μg AA/g DW for the 10%- and 20%-AP groups, respectively (Table 1), whereas non-enriched *A. nauplii* contained about 550 μg AA/g DW. Considerable variability in enrichment levels occurred within each experiment (CV of 10–25%) and among the different trials (CV of 14–22%). Also for enrichment with essential fatty acids, a high variability was demonstrated in laboratory tests: e.g., total $n-3$ HUFA varied from 15 to 28% of total fatty acids in *Artemia* enriched using a standard protocol (Lavens et al., 1995a).

The AA content in the predator larvae reflected the dietary AA levels demonstrating the applicability of the AP-boosting technique (Table 2). However, the increases were not further increased following feeding with a 20%-AP enriched diet compared to the one boosted with 10% AP. This suggests a saturation of the body AA pool when levels of approximately 1400 μg AA/g DW diet were fed. This might be the optimal dietary AA concentration since Dabrowski (1990) suggested that the latter is equivalent to the one allowing the maintenance of a constant tissue concentration of the vitamin in larval fish. Furthermore, under standard culture conditions the AA levels naturally occurring in *A. nauplii* ($\pm$550 μg AA/g DW) appeared to be sufficient to prevent a depletion of the initial larval AA levels (Table 2). On the contrary, a culture test with startfeeding turbot

Table 1
Ascorbic acid concentration (μg AA/g DW) in *A. nauplii* boosted with 0%, 10% and 20% ascorbyl palmitate (AP) in different larviculture tests; data compiled from Merchie et al. (1995b,c, 1996a, 1997b)

Experimental species	*Artemia* enrichment level		
	0% AP	10% AP	20% AP
Crustaceans: freshwater prawn			
Experiment 1	530 [1]	–	2920
Experiment 2	660	1310	2760
Fish: African catfish			
Experiment 1	560	1380	2260
Experiment 2	530	1180	1640
Experiment 3	530	1720	2280
Experiment 4	480	–	2620
Mean $\pm$ sd [2]	530 $\pm$ 30 [a]	1430 $\pm$ 270 [b]	2200 $\pm$ 410 [c]
European sea bass	580	1430	3580
Turbot	500	1580	2600
Mean $\pm$ sd [2]	560 $\pm$ 60 [a]	1440 $\pm$ 110 [b]	2810 $\pm$ 510 [c]

[1] Figures are an average of various samples taken during the actual rearing period; dry weight (%) of the *Artemia* samples varied between 9 and 13%.

[2] Values were statistically evaluated using one way analysis of variance. Tukey's multiple range test was applied to determine significant differences between means ($p < 0.05$) using log transformation of values to obtain homoscedasticity of variances; values sharing common superscript are not significantly different.

Table 2
Ascorbic acid concentration (μg AA/g DW) in the larvae of different species before first feeding and after feeding live diets enriched with three ascorbyl palmitate (AP) levels during approximately one month (except for the African catfish: 20 and 10 days for experiments 1 and 2 to 4, respectively); data compiled from Merchie et al. (1995b,c, 1996a, 1997b)

Experimental species	Before first feeding	Live food enrichment levels (μg AA/g DW)		
		600	1400	2800
Crustaceans: freshwater prawn				
Experiment 1 larvae	154	365 [a]	–	552 [b]
Postlarvae		288 [a]	–	325 [a]
Experiment 2 larvae	293	352 [a]	448 [b]	507 [b]
Postlarvae		255 [a]	389 [b]	432 [b]
Fish: African catfish				
Experiment 1	–	469 [a]	543 [b]	547 [b]
Experiment 2	285	408 [a]	490 [ab]	552 [b]
Experiment 3	235	433 [a]	619 [b]	751 [b]
Experiment 4	462	509 [a]	–	815 [b]
European sea bass	–	756 [a]	1603 [b]	1624 [b]
Turbot				
Experiment 1	498	863 [a]	1215 [b]	1204 [b]
Experiment 2	467	889 [a]	1118 [b]	1232 [b]

Values were statistically evaluated using one way analysis of variance. Tukey's multiple range test was applied to determine significant differences between means ($p < 0.05$); numbers in the same row with common superscript are not significantly different. Average dry weights (%) of the samples of the various species were, before first feeding and at the end of the trial, respectively: freshwater prawn: 15 and 20%, African catfish: 9 and 16%, European sea bass: not analysed and 15%, turbot: 9 and 17%.

larvae showed that feeding rotifers containing only 363 μg AA/g DW did not allow the initial AA concentration to be maintained (Fig. 2). The latter, i.e. 1940 μg AA/g DW, however, was four times higher than AA levels (490 μg AA/g DW) detected by Merchie et al. (1996a). This high variation of AA levels in turbot eggs and newly-hatched larvae was probably due to different conditions of raising broodstock as previously reported by Lavens and Sorgeloos (1991). Nevertheless, the rapid decrease in AA during the first days of development suggests a high requirement for this micronutrient during the yolk sac stage and thus, may explain the tendency of the female to store high amounts of AA during gametogenesis.

Remarkable was the significant decrease in AA in post-larvae of the freshwater prawns as compared to the levels detected in the larvae (Table 2), suggesting a higher requirement for this vitamin during metamorphosis than during the larval stage. This is first of all a stressful and physiologically sensitive period since the animals undergo major morphological and biochemical changes and, moreover, growth in crustaceans during moulting is associated with a fast collagen formation and thus, extra needs for AA as a co-factor (Hunter et al., 1979).

For larvae of the African catfish (*C. gariepinus*), supplemental dietary ascorbate resulted in a positive effect on growth (significant in three out of four experiments;

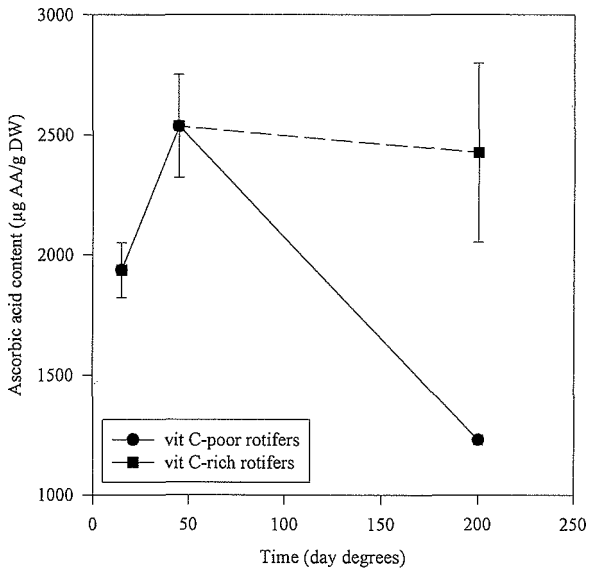

Fig. 2. Ascorbic acid concentration (μg AA/g DW) in turbot larvae fed rotifers containing 363 (vitamin C-poor rotifers) and 1070 μg AA/g DW (vitamin C-rich rotifers), respectively (modified from Plañas et al., 1995).

Merchie et al., 1995b, 1997b): e.g., in the first experiment, the 20%-AP group weighed 30% more than the control (0% AP). No significant differences occurred between the 10%-AP and 20%-AP groups (average dietary AA levels of 1400 and 2200 μg/g, respectively), confirming the earlier hypothesis that in the range of concentrations tested, a dietary level of approximately 1400 μg AA/g DW was sufficient to combine an optimal AA tissue concentration and larval performance. Furthermore, co-feeding tests using various supplementation levels (100–2600 μg AA/g diet) demonstrated a clear relationship between larval growth and dietary AA (Fig. 3). It was verified that the growth effect of the boosted *Artemia* diet was the result of the extra AA incorporation and not of the concomittant palmitic acid, which is set free after hydrolysis of AP, and which could possibly have been used as a supplemental energy source. The subsequent observation supported a causal relationship between dietary AA and growth in larval African catfish. Moreover, growth results were supported with data from the ultrastructural evaluation of the hepatocytes, i.e. a more organised cell compartmentation, better-structured cell organelles in the 20%-AP group compared to the control are indicative of a more active metabolism. These results indicate that in the case of *C. gariepinus*, vitamin C requirements appear to be much higher during the hatchery phase than the values reported for juveniles and ongrowing fish, e.g. a need for 10–25 mg AA/kg diet has been shown to be sufficient for normal growth in channel catfish (*Ictalurus punctatus*: Mustin and Lovell, 1992), Asian sea bass (*Lates calcarifer*: Boonyaratpalin et al., 1994), red sea bream (*Pagrus major*: Kosutarak et al., 1994) and Atlantic salmon (*Salmo salar*: Sandnes et al., 1992). This higher requirement may

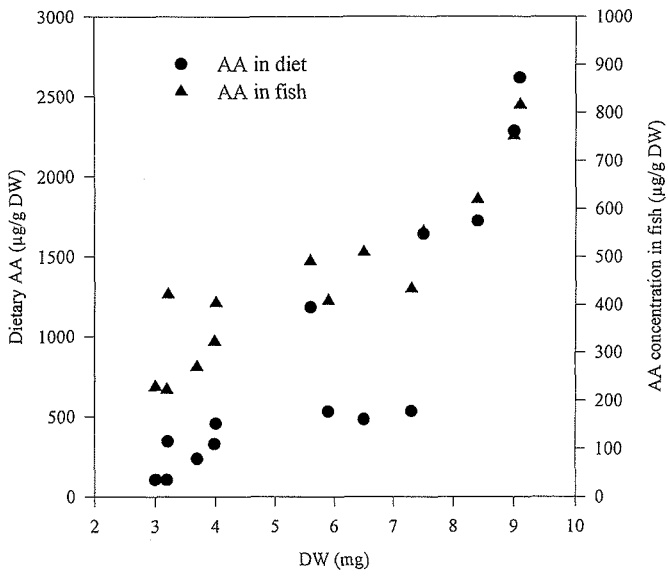

Fig. 3. Relationship between the larval dry weight and either the dietary AA (μg/g DW) or the AA incorporated (μg/g DW) in larvae of the African catfish, 8 days old (calculated over three experiments, modified from Merchie et al., 1997b).

indeed be due to a higher rate of growth and metabolism in the larvae compared to juveniles. In this respect, Matusiewicz et al. (1994) proposed that the AA requirements of poikilotherms were linked directly to the metabolic rate. This might explain the difference with the marine fish species examined, i.e. no differences in production output could be observed between the different dietary treatments for larvae of European sea bass or turbot, indicating that the nutritional requirement of these species was below 500 μg AA/g DW.

In studies with European sea bass using diets supplemented with high AA concentrations, positive effects on stress resistance have been observed in salinity stress tests (Merchie et al., 1995b). Also for turbot, cumulative mortalities after challenge with *Vibrio anguillarum* increased up to 50% for the control, while only 40% mortality occurred in the groups fed vitamin C-enriched diets, providing evidence for an immunostimulatory effect of high AA doses. This lower stress sensitivity was confirmed also for African catfish (Merchie et al., 1995b, 1997b), freshwater prawn (Merchie et al., 1995c) and tiger shrimp (unpublished data) receiving high dietary vitamin C supplementation.

These effects of high levels of AA supplementation on stress and disease resistance might be of importance under some sub-optimal rearing conditions in commercial operations. Mortalities of fish reared under intensive conditions might be manifested only when these animals are subjected to various types of stressors, such as handling, transportation, crowding, poor water quality or disease outbreaks. The results summarized here support the hypothesis of Dabrowski (1992) that stress creates increased

ascorbate requirements for larval fish, and that in this respect, body vitamin C concentrations may reflect the potential for fish survival more accurately than variations in growth rate. Characterization of general physiological indicators of fish health status (e.g., AA content) should, in this respect, allow us to predict more accurately their chances of survival after stocking (Dabrowski and Ciereszko, 1993).

An additional effect of vitamin C was verified through the study of the occurrence of opercular deformities in gilthead sea bream (*Sparus aurata*) in a French commercial fish hatchery (Merchie, unpublished data). Abnormal gill opercula were indeed a possible sign of vitamin C deficiency since AA is involved in cartilage and fibrous tissue formation (Halver et al., 1969). A positive effect of dietary vitamin C supplementation on survival and frequency of shortened opercula was noticed, i.e. the percentage of shortened opercula decreased from 7.4% to 2.2% when vitamin C-boosted live food (20% AP) was fed. For milkfish (*Chanos chanos*), the difference was even more pronounced: in two consecutive experiments run in the Philippines, the occurrence of deformed opercula dropped by half (from 36 and 38% to 17 and 20%, respectively) when feeding AA-enriched rotifers and *Artemia* (Gapasin et al., unpublished). Since Mediterranean sea bream hatcheries currently face problems with opercular deformities of the fry, this could be related to the changes in live food production management which reduced the levels of vitamin C. It was noticed that boosting of rotifers with microalgae at commercial scale tended to score lower with respect to AA content than under laboratory conditions (Merchie et al., 1995a). However, further research is necessary to confirm these preliminary observations, since more factors are probably involved in operculum development, e.g. vitamin K, calcium and zinc.

It can be concluded that in general the AA levels in rotifers and brine shrimp *nauplii* are sufficient to support a normal growth and survival of most fish and crustacean larvae; on the other hand, a rise in the dietary AA content to 1500–2500 μg/g DW not only enhances the larval AA content but also its physiological condition, pointing to an important role of AA in dealing with stress.

5. Vitamin C during early nursery stages

The relatively high AA levels present in the live feeds *Brachionus* and *Artemia*, impair the determination of the minimal requirements for vitamin C in most larval fish and crustaceans, since these needs are met by the initial AA content of the live prey even without enrichment. Exact AA requirements can, therefore, only be verified for those fish/shrimp stages which readily accept formulated diets, thus, allowing the evaluation of a control feed containing no AA. Artificial diets made up with semi-purified ingredients and containing variable levels of AA phosphates (0–2000 mg AA/kg diet) were used to determine minimal needs for AA in the postlarval stages of penaeid shrimp (*Penaeus vannamei* and *P. monodon*: Kontara et al., 1997; Merchie et al., 1997a) and the early post-weaning stage of two marine fish species (*D. labrax* and *S. maximus*: Merchie et al., 1996b).

Retained levels of AmP and ApP, included in the fish and shrimp diets, after extrusion-coating (Coutteau et al., 1995) or micro-binding (Camara et al., 1995),

Table 3
Ascorbic acid phosphate levels (mg AA/kg diet) included in formulated feeds for fish (Merchie et al., 1995c) and shrimp (Kontara et al., 1997; Merchie et al., 1997a)

Fish diets (extruded)			Shrimp diets (micro-bound)		
ApP formulated	ApP retained	AmP formulated	AmP retained	ApP formulated	ApP retained
0	– [a]	0	– [a]	0	– [a]
20	23	10	7	20	10– 29
200	246	100	99	40	20– 44
2000	1751	1000	990	100	68– 107
		2000	1907	2000	1491–2251

[a] Below detection limit (< 5 mg AA/kg).
[b] Variation of values in three consecutive tests.
ApP: ascorbic acid polyphosphate; AmP: ascorbic acid monophosphate.

respectively, are summarized in Table 3. In the extruded fish diets, ApP retention exceeded 88%, confirming the finding of Gadient and Fenster (1994) that ApP is highly stable in aquaculture diets. In the pelleted shrimp diets, ApP inclusion varied from 50 to 100% of the supplemented levels, while for AmP, a 70–99% retention was obtained. In the pelleted feeds, the lower supplementation levels (10–40 mg AA/kg) were subject to losses of the AA-phosphate esters during processing. After the shrimp feed had been in contact with seawater for 10 min, another 50% of the vitamin C was lost. This rapid leaching of the hydrophilic AA phosphates into the water probably explains the low AA incorporation levels in the slow-feeding white shrimp (*P. vannamei*) postlarvae, especially at the lower dietary concentrations (10–100 mg AA/kg; Table 4) and demonstrates the need to monitor the feed not only during processing but also for losses, from leaching after feeding.

Leaching of the extruded diets on the other hand was limited to a 26–34% decrease in soluble dry matter after a 1-h immersion. The significant fatty acid incorporation into the sea bass larvae from the coated lipid fraction of the feed provides evidence for a satisfactory ingestion of the complete particle before the coating was detached. A frequent feed distribution (every hour) promoted a fast and efficient intake. AA incorporation levels in the fish reflected the ApP supplemented in the diet, confirming that the phosphate derivative is bio-available and can be readily assimilated by the fry (Table 4). The efficacy of ApP as feed additive has previously been demonstrated for salmonids (Roem and Oines, 1993; Völker and Fenster, 1994), channel catfish (Wilson et al., 1989) and penaeid shrimp (He and Lawrence, 1993). Feeding AP-supplemented dry diets, however, did result in a significantly lower AA concentration in post-weaning sea bass larvae (Merchie et al., 1996b), indicating that AP is not fully assimilated by these young fish stages. This is in accordance with Albreksten et al. (1988) who suggested that the presence of AP in the gut requires a specific enzyme system which is absent at the beginning of start-feeding. Dabrowski, on the other hand, states that available esterases should be sufficient for the release of AA from AP (personal communication).

Significant differences in survival and growth for tiger shrimp (*P. monodon*) and

Table 4
Ascorbic acid concentration (μg AA/g DW) in postlarval white shrimp and marine fish before weaning and after feeding formulated diets, containing various AA phosphate levels, during 3–5 weeks; data compiled from Kontara et al. (1997) and Merchie et al. (1996b, 1997a)

Species	White shrimp			Sea bass	Turbot
	Experiment 1	Experiment 2	Experiment 3		
Before weaning	594	372	301	569	552
After feeding	AmP	ApP	ApP	ApP	ApP
0	99 ^{bc}	79 ^{b}	65 ^{a}	68 ^{a}	89 ^{a}
10	82 ^{bc}	–	–	–	–
20	–	65 ^{ab}	67 ^{a}	112 ^{a}	94 ^{a}
40	–	53 ^{a}	76 ^{ab}	–	–
100	23 ^{a}	73 ^{b}	95 ^{b}	–	–
200	–	–	–	189 ^{b}	229 ^{a}
1000	62 ^{b}	–	–	–	–
2000	126 ^{c}	193 ^{c}	215 ^{c}	273 ^{c}	497 ^{b}
mg AA/kg diet					

Values were statistically evaluated using one way analysis of variance. Tukey's multiple range test was applied to determine significant differences between means ($p < 0.05$); numbers in the same column with common superscript are not significantly different; average dry weights (%) of the samples of the various species were, before weaning and at the end of the trial, respectively: white shrimp: 19 and 20%, European sea bass: 17 and 23%, turbot: 17 and 14%; ApP: AA-polyphosphate Na salt (Stay-C®, Roche, Switzerland); AmP: AA-monophosphate Mg salt (Phospitan® C, Showa Denko, Japan).

white shrimp (*P. vannamei*), respectively, were observed after 10–14 days of feeding, indicating a differential sensitivity to ascorbate deficiency of early post-larval shrimp. Results show that 20, respectively, 130 mg AA/kg diet is sufficient to meet the postlarval needs for an optimal production output (Merchie et al., 1997a). After 25 days of feeding with 2000 mg AA/kg, white shrimp postlarvae weighed 10-fold of the control. Repeating this experiment, however, could not confirm this effect on growth (Kontara et al., 1997). He and Lawrence (1993) estimated an AA requirement for normal survival of juvenile *P. vannamei* (0.1 and 0.5 g initial WW) of 120 and 41 mg AA/kg diet, respectively, indicating size-dependent needs decreasing with age. For tiger shrimp (0.1–0.5 g) dietary AA needs for production (survival and growth) were found to be in the range of 50 to 200 mg AA/kg diet (Catacutan and Lavilla-Pitogo, 1994; Chen and Chang, 1994), decreasing to 20 mg AA/kg for 1-g juveniles (Shiau and Hsu, 1994).

The control diet, containing no vitamin C, did not result in a slower growth rate of European sea bass during the first 3 weeks of culture, whereas after 5 weeks of culture, a strongly reduced growth as well as survival was found (Merchie et al., 1996b). Apparently a 3-week culture period seems to be too short to deplete sufficiently the AA reserves still present from the previous *Artemia* feeding period, i.e. a body AA content of 569 μg/g DW (Table 4). Also turbot, containing 552 μg AA/g DW before weaning, did not yield significant differences in production characteristics after 4 weeks of culture (Merchie et al., 1996b). Results of a broken line regression between dietary AA and sea bass biomass denote that a level of 20.7 mg supplementary AA/kg diet was sufficient for a normal production output. A similar need (12.6 mg AA/kg diet) has

been proven to be sufficient for normal growth in Asian sea bass (*L. calcarifer*) fry of 1.9 g (Boonyaratpalin et al., 1994). Although for turbot no significant differences were demonstrated, survival and growth were poorest for the fish fed the AA-free diet. This might indicate that here again 20 mg AA/kg diet is sufficient for normal development at this stage. The production results for both sea bass and turbot are confirmed by the ultrastructural findings of the hepatocytes, indicating a poorly developed cellular compartmentation when the AA-deficient diet was administered, while the organization of organelles improved in parallel with the increase in dietary AA content (Merchie et al., 1996b). This phenomenon was also observed for larval African catfish (Merchie et al., 1997b). The improved ultrastructure of sea bass hepatocytes is indicative of a higher nutritional quality of the AA-supplemented diets compared to the control. This resulted in a considerable storage of glycogen as well, demonstrating that the metabolism increased when 20 mg AA/kg or more was administered to the early nursery stages of sea bass and turbot.

The method of measuring resistance to salinity shocks has proven to be a good tool for quality evaluation of penaeid post-larvae and can easily be applied in hatcheries (Tackaert et al., 1992; Rees et al., 1994). Using this technique, a significantly improved physiological condition of *P. monodon* was shown when feeding a minimum of 200 mg AA/kg diet in comparison to the control (Merchie et al., 1997a). Whereas in a preliminary test no effect of dietary AA on the osmotic stress resistance of *P. vannamei* post-larvae was observed, a significantly better survival (averaging 70–90%) was noticed in a second trial when feeding 40 mg AA/kg diet or more, compared to the 40–60% survival of low-AA groups after a 1-h incubation in freshwater (Merchie, unpublished). In another experiment, shrimps were shown to have a significantly enhanced disease resistance to a *Vibrio harveyi* infection after 18 days of feeding: shrimp fed 0–40 mg AA/kg showed a cumulative mortality over 1 week which ranged from 63% to 73%, whereas the shrimp receiving the highest ascorbate level (1500 mg AA/kg diet) exhibited no mortality (Kontara et al., 1997). Kanazawa (1996) also reported that vitamin C was effective in increasing resistance of penaeid shrimp to bacterial infections: one week after infection of *P. japonicus* juveniles with a *Vibrio* sp. isolated from diseased shrimp, only 14% of the animals fed an AA-deficient diet survived, while 80% survival was obtained when incorporating 50 mg AA/kg diet.

Also for juvenile fish, inclusion of high levels of vitamin C (1000–5000 μg AA/g DW) was demonstrated to enhance tolerance to environmental stressors (e.g., aldrin toxicity: Agrawal et al., 1978; intermittent hypoxic stress: Ishibashi et al., 1992) and to increase immunoresistance (Li and Lovell, 1985; Navarre and Halver, 1989; Hardie et al., 1991; Obach and Laurencin, 1992). In our studies, no significant differences in resistance to a salinity shock were observed, however, fish displaying the highest body-AA content performed best (Merchie et al., 1996b). During acclimation of the sea bass before the challenge test, only 20% of the fish fed the AA-deficient diet survived, adding evidence to their reduced viability when subjected to stress conditions. Results of both challenge tests were inconclusive and do not provide evidence for an immunostimulatory effect of vitamin C during the post-weaning phase of sea bass (112 days old) or turbot (71 days old). Probably the mortality criterion used after the challenge test was not sensitive enough to properly evaluate the effects of dietary AA levels at this stage of

fish development. A clear positive effect of extra dietary vitamin C was noticed when larval turbot were challenged (35 days old). The use of other parameters to evaluate disease resistance (for the non-specific immune system: phagocytosis, serum complement activity) during the nursery period might be more appropriate.

It can be concluded that relatively low AA levels (20–130 mg AA/kg diet) supplied as a stable and bio-available source allow a normal growth and survival during the nursery phase of both fish and shrimp; on the other hand, a rise in the dietary AA content up to 1500 mg/kg enhances the resistance to stress and diseases, especially in shrimp.

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The effect of supplemental ascorbic acid in enriched live food for *Clarias gariepinus* larvae at startfeeding

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Abstract

The effect of three dietary ascorbic acid (AA) concentrations, each applied via two feed types, on production characteristics and physiological condition of African catfish (*Clarias gariepinus*) larvae has been assessed in two 10-day culture trials. Three treatments received only *Artemia* nauplii enriched with an experimental emulsion containing 0, 10, or 20% ascorbyl palmitate (AP) and yielding 530, 1200 and 1600 μg AA g^{-1} DW *Artemia*, respectively; the other three treatments were fed the same *Artemia* diets which were partially substituted by an artificial diet containing no vitamin C (ratio 20:80). No differences in survival could be observed; however, from day 6 onwards the 20%-AP group showed significantly better growth compared to the 0%- and 10%-AP treatments. For the cofeeding series, the same positive, but not significant, influence of vitamin C on dry weight was found. Moreover, the animals receiving the highest vitamin C supplementation displayed a considerably lower stress sensitivity than those of the 0%- and the

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