RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2011.37.179

ScienceAsia 37 (2011): **179–185**

Larval rearing of clownfish using *Brachionus plicatilis* rotifer as starter food

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Received 11 Feb 2011
Accepted 23 Aug 2011

ABSTRACT: Sebae anemonefish, *Amphiprion sebae*, is currently one of the most demanded marine ornamental fish species in tropical countries. The development of controlled larval rearing procedures are required for the sustainable culture of these valuable fish. In the present study, the suitability of the marine rotifer *Brachionus plicatilis* as a starter food for larviculture of *A. sebae* was investigated. After the yolk absorption, the larvae were stocked in 250-l fibreglass reinforced plastic tanks under different feeding conditions: clear water rearing conditions with rotifers *Brachionus plicatilis*, 8–10 ml^{-1} for 10 days (R), green water conditions (*Chlorella* sp., 1.1–2.6 $\times$ 10^5 cells/ml) with rotifers (8–10 ml^{-1}) offered for 10 days (C+R), green water conditions (*Chlorella* sp., 1.1–2.6 $\times$ 10^5 cells/ml) for 3 days followed by clear water in combination with rotifers (8–10 ml^{-1}) feeding for 7 days (3C+7R), and clear water conditions with *Artemia* nauplii offered for 10 days (4–6 ml^{-1}). After the 10-day feeding, all groups received *Artemia* nauplii up to 35 days post-hatching. Larval survival was counted at day 10 and at the end of the 35-day rearing experiment. At day 35, a significant survival difference was noted between the groups where rotifers were supplemented with algae versus only *Artemia*. At the end of the experiment, the highest survival rate (68.2 $\pm$ 2.3%) was obtained with larvae receiving only algae in the first 3 days of feeding. Lowest survival rate (23.9 $\pm$ 10.3%) was obtained with larvae receiving only *Artemia* for 35 days. This indicates that smaller preys are essential for clownfish larvae at first feeding. Larval length and wet weight were measured at the time of mouth opening, at days 7, 10, and 21, and at the end of the experiment (day 35). On day 35, mean length of the larvae varied significantly between the treatments. However, the final wet weight of the larvae did not vary significantly between the treatments.

KEYWORDS: first feeding, live feed, captive breeding, *Chlorella marina*, *Artemia*

INTRODUCTION

In the last decade, the increasing demand of fish by the aquarium trade has stimulated many studies on ornamental larval fish development and nutrition to improve production in captivity and thereby harnessing the aquatic biodiversity[1–4]. In many developing countries ornamental fish production through aquaculture forms an important way of income generation, but, even if the majority ($>$ 90%) of freshwater ornamental fish are captively bred, only 25 species of marine fish are commercially produced[4]. However, efforts are being made to breed and rear some of the highly valued marine ornamental species using sea and estuarine waters in India and other tropical countries[5–8].

The sebae anemonefish (*Amphiprion sebae*), a member of the family Pomacentridae, is an extremely beautiful tropical marine aquarium fish suited for aquariculture and in great demand in the international market. These fish, popularly known as "clownfish" or "anemone fish" are distributed in the tropical and subtropical seas. The popularity of clownfish among the aquarists all over the world is due to the generally small and hardy nature of the fish, their attractive colours, high adaptability to life in captivity, and the interesting display of behaviour due to their association with sea anemones[1,6]. But at present its habitat loss through cage fishing, dynamite fishing, pollution, and climatic warming may have resulted in population decline of the fish[9,10]. Potential measures such as stocking or introduction of young fish in brackish and marine environments have been suggested for the protection of these fish populations[3]. An essential prerequisite for any stocking or reintroduction programme would be the rearing of large numbers of fish in captivity[4].

The success in the hatchery production of fish fingerlings for stocking in the grow-out production system is largely dependent on the availability of suit-

able live food organisms such as marine rotifer (*Brachionus plicatilis* and *B. rotundiformis*) and *Artemia* nauplii [11,12] during their transition from endogenous to exogenous feeding. Small prey (rotifers) are needed to fulfil the demand of clownfish larvae in the early period of exogenous feeding because they cannot ingest macro zooplankters (*Moina*, *Daphnia*, and copepods; 643–728 μm) at the time of initial feeding. Rotifers are usually mass-cultured as feed for the early stages of marine fish larvae because of their size, nutritional value, and behaviour [5,13,14].

The freshwater rotifer, *Brachionus calyciflorus*, is a suitable organism for ornamental freshwater fish larvae and can serve as an adequate food source [15,16]. For the mass-rearing of marine fish larvae the rotifer *Brachionus plicatilis* has been used as an indispensable source of initial live food [16–18]. There is also no suitable live feed for feeding early fish larvae with small mouth [19]. Many freshwater ornamental fish farmers have shifted from *Moina* to the cleaner *Artemia* nauplii for feeding their young fish. As the nauplii (length of instar-1 *Artemia* $<$ 0.55 mm) are only half the size of *Moina* (length $<$ 1.20 mm), it is necessary to look for bigger organisms, both to fill in the size gap, and as a substitute of *Tubifex* for feeding larger fish such as brooders [11]. Furthermore, the high price of *Artemia* cysts has increased the fish production cost, and cheaper alternative diets with comparable nutritional quality are needed to maintain the cost competitiveness of ornamental fish in the global market [11,20].

One important aspect of larval nutrition is providing adequate levels of lipids, proteins, carbohydrates, vitamins, and minerals through the diet [21,22]. Highly unsaturated fatty acids (HUFAs) including eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) are also essential, since deficiencies in these lipids result in poor growth, low feed efficiency, anaemia, and high mortality [2,23,24]. It has been demonstrated that in the wild, marine fish larvae mainly feed on copepod nauplii and copepodites which are naturally rich in HUFAs [25,26]. Thus it is vital to enrich the most widely used live prey such as rotifers and *Artemia* nauplii with HUFAs before offering them to the fish larvae in captive fish production [1,27].

Clownfish larvae are fed usually with the rotifer *Brachionus rotundiformis*, from the same day of hatching, even though complete yolk sac exhaustion occurs after two days [7]. Earlier reports on the Mauritian anemonefish (*Amphiprion chrysogaster*) larvae reared in captivity fed with the rotifer *Brachionus rotundiformis* (average lorica length 150 μm) showed a larval survival during the critical period (from the day of hatching to the fifth day) that ranged from 50 to 60% [6]. Using *B. plicatilis* as starter feed from the 2nd day onwards to rear the larva of *A. sebae* in captivity gave about 55% larval survival [10].

In a field study, the burbot (*Lota lota*) larvae started to feed on zooplankton 5 days after hatching; of 25 burbot larvae, only two larvae contained zooplankton prey in their stomach at the first feeding [28]. These authors indicated that the first food items taken by burbot larvae were rotifers. Also in a laboratory study, the larvae first ate phytoplankton and did not switch to copepod nauplii until the third day of exogenous feeding [18].

To rear larvae of marine ornamental fish species successfully, it is important to investigate food size preference of larvae, which can be a basis to establish an optimal feeding regime. Influence of green water systems in the larval rearing of clownfish is well established as a water conditioner [5,7]. So, the aim of the present research was to develop a suitable method for larval rearing of *A. sebae* under controlled conditions using *B. plicatilis* as a starter food.

MATERIALS AND METHODS

The study was conducted at the marine ornamental fish breeding centre at the Aquaculture Laboratory, Centre of Advanced Study in Marine Biology, Annamalai University. Cultures of the microalgae *(Chlorella* sp.) were started by agar plating techniques, upscaled to test tubes, and then to Erlenmeyer flasks of 500 ml. The content of the flasks was used to inoculate 5-l Hoffkin flasks, which, in turn, were used to seed 20-l carboys and then to 100 l capacity FRP tanks in a mass culture level. Dechlorinated and filtered (0.45 μm) estuarine water was used for algal cultures. Microalgae were fertilized with previously reported Walne [29] and agricultural level fertilizers (10:2:2, ammonium sulphate, super phosphate, and urea) media. Temperature was maintained at 25 °C.

Rotifer *B. plicatilis* (lorica length 70 to 239 μm) was cultured using adaptation of previously reported techniques [30]. Rotifer resting eggs were incubated in centrifuge tubes of 50 ml containing prefiltered and dechlorinated estuarine water (26 ± 2 ppt). The tubes were exposed to 1000 lux artificial light for hatching of the rotifers. Upon hatching, the rotifers were fed micro algae *(Chlorella* sp.). Thereafter, the cultures were upscaled to 500 ml Erlenmeyer flasks, then to 15 l bottles, and after 1 week the 15 l bottles were used for the inoculation of rotifers on a larger scale. Total ammonia levels in the rotifer cultures were generally kept below 5 mg/l by water exchange.

Rotifers were added to the fish tanks each morning and their concentration adjusted to the desired density (8–10 rotifer/ml) on the following morning.

Hatching of *Artemia* cysts was performed according to standard methods developed in the Laboratory of Aquaculture and Artemia Reference Centre[30]. Newly hatched *Artemia* nauplii were used for feeding the larvae.

Newly hatched clownfish larvae reared in the clownfish hatchery at the Centre of Advanced Study in Marine biology were acclimatized to experimental conditions for 3 days in the holding FRP tanks (250 l), using UV filtered estuarine water. Water temperature was constant during rearing (28 ± 1 °C). Dissolved oxygen ranged between 4.5 and 6.8 mg/l. pH varied from 8.1–8.6. NH_4/NH_3 and NO_2 values ranged from 0 mg/l and NO_3 levels were > 0.2 mg/l. An ambient photoperiod of 12L:12D was maintained at a light intensity of 800 lux during the experiment.

After yolk absorption, the larvae were stocked at random in the FRP tanks each containing 250 l of estuarine water using a recirculated system. There were four treatments arranged in triplicates ($n = 3 \times 4$). The water flow through each tank was similar and constant (0.5 l/min) with 50% water exchange per day. Water was gently aerated with a single air stone. Stocking density in the larval rearing tank was 3 larvae/l of water. Initial larval total length (mean $\pm$ SD) and average wet body weight were 2.9 ± 0.3 mm and 0.8 ± 0.2 mg, respectively. Each day, just before feeding, bottom debris was siphoned from every tank.

The experiment was performed under four different feeding conditions in a way of three times per day (8.00, 12.00, and 16.00 h). Clear water rearing conditions with rotifers (*B. calyciflorus*) fed at a density of 8–10 rotifer/ml for 10 days (R), green water conditions (*Chlorella* sp., $1.1–2.6 \times 10^5$) with rotifers (8–10 ml^{-1}) offered for 10 days (C+R), green water (*C* sp., 1.1–2.6×10^5) conditions for 3 days followed by clear water in combination with rotifers (8–10 ml^{-1}) for 7 days (3C+7R), and *Artemia* nauplii (4–6 ml^{-1}) offered for 10 days (*Art*). Artemia and rotifers remain to be available in culture tanks about 2 h after each administration of feeding. After 10 days of feeding with rotifers, all groups were given solely *Artemia* nauplii up to 35 days post-hatching (Table 1). There were three replicates per group. The number of fish was obtained by direct counting. Growth parameters (length and wet weight) were measured on days 0, 7, 10, 21, and 35 post-hatching. Fish length was measured to the nearest 0.1 mm with a binocular microscope equipped with an ocular micrometer. For length measurement, 20 larvae were collected randomly from each replicate. Survival of the larvae was recorded by counting the fish in the tank on the 10th day and at the end of the experiment.

Table 1 Feeding regimes of larvae raised in different experimental groups.

Treatment group	Feeding regimes (3 times per day; 8.00, 12.00, and 16.00 h)		
	Algae[a]	Rotifers[b]	Artemia[c]
R (clear water + rotifer)		D1–D10	D11–D35
C + R (green water + rotifer)	D1–D10	D1–D10	D11–D35
3C + 7R (3 days green water + rotifer)	D1–D3	D4–D10	D11–D35
Art (clear water + *Artemia)*			D1–D35

[a] $1.1–2.6 \times 10^5$ cells/ml.
[b] 8–10 ml^{-1}.
[c] 4–6 ml^{-1}.

Table 2 Survival rate of larvae counted on day 10 and at the end of the experiment (Mean $\pm$ SD).

Treatment Group	Day 10	Day 35
R (clear water + rotifer)	$98.6^a \pm 0.6$	$38^b \pm 7$
C+R (green water + rotifer)	$97.8^a \pm 0.6$	$60^a \pm 13$
3C+7R (3 days green water + rotifer)	$99.0^a \pm 0.3$	$68^a \pm 2$
Art (clear water + *Artemia)*	$98.5^a \pm 0.6$	$24^b \pm 10$

Different superscript letters within a column indicate significant difference ($P < 0.05$).

Only data collected on day 10 (switching of food items) and day 35 (the end of experiment) were subjected to ANOVA to determine any significant difference among treatments. Significant differences between treatments were determined by Tukey's multiple range test ($P < 0.05$).

RESULTS

Larval survival was counted at day 10 and at the end of the 35th day rearing experiment. No significant difference ($P > 0.05$) was observed in larval survival at the 10th day of post hatching (dph), among the different groups (Table 2). However, a significant difference ($P < 0.05$) in survival rate was noticed among the groups at the end of the experiment. The survival rate in group *Art* (clear water + *Artemia)* was significantly lower than C+R (green water + rotifer) and 3C+7R (green water for 3 days) after 35 days. Other groups were not significantly different from each other. At the end of the experiment, the highest survival rate ($68.2 \pm 2.3\%$) was obtained with the larvae receiving only algae (3C+7R) in the first 3 days of feeding. Average survival rate of the larvae cultured in green water (C+R) condition for 10th day was $60.2 \pm 13.2\%$.

Table 3 Length (mm) of larvae measured on days 7, 10, 21, and 35 of the experimental course (mean ± SD).

Treatment group	Day 7	Day 10	Day 21	Day 35
R (clear water + rotifer)	4.02 ± 0.22	$4.13^{b} \pm 0.54$	6.03 ± 0.92	$7.33^{b} \pm 0.49$
C+R (green water+ rotifer)	3.98 ± 0.26	$4.55^{a} \pm 0.46$	6.51 ± 0.70	$8.45^{a} \pm 0.50$
3C+7R (3 days green water + rotifer)	3.70 ± 0.20	$3.84^{b} \pm 0.46$	5.74 ± 0.59	$7.49^{b} \pm 0.19$
Art (clear water + *Artemia)*	3.45 ± 0.24	$3.96^{b} \pm 0.44$	6.05 ± 0.84	$7.87^{b} \pm 0.09$

Different superscript letters within a column indicate significant difference ($P < 0.05$).

The survival rate of the larvae receiving rotifers in clear water (R) condition was lower ($38.2 \pm 6.6\%$) compared with the other two groups receiving rotifers. Lowest survival rate ($23.9 \pm 10.3\%$) was obtained with the larvae receiving only *Artemia* (*Art*) during 35 days.

On the 10th dph, mean size of the larvae receiving rotifers in green water condition (treatment C+R) was significantly ($P < 0.05$) higher than in the *Artemia* (*Art*) fed fish group. A similar observation was detected on the 35th dph ($P < 0.05$). The larvae cultured in green water conditions for 10 days had the largest size (8.45 mm total length) after 35 days (dph) of culture (Table 3).

On day 10, the mean weight of the rotifer fed larvae for 10 days (R and C+R) was not significantly different ($P > 0.05$). On the 10th dph, average wet weight of larvae receiving rotifers supplemented with algae was significantly ($P < 0.05$) higher than in the group fed on *Artemia* (*Art*) (Table 4). However, final wet weight of the larvae did not vary significantly among the groups at the end of the experiment ($P > 0.05$). The larvae started metamorphosing from 15–17th day of hatching and all the larvae metamorphosed by 20th day in all the four treatments.

DISCUSSION

Although all larvae in different groups were fed *Artemia* after 10 days, survival of the larvae receiving rotifers in green water condition was significantly better than the group fed solely on *Artemia* during the experiment. The artemia nauplii are much bigger and faster than rotifers. The high survival rate achieved in the groups of larvae fed on rotifers could have been influenced by the quality of the starter food and also size of the prey offered. These findings may suggest that quality of the starter food is crucial to the later developmental stages of clownfish larvae. This is consistent with previous reports[31,32] on burbot larvae and a low survival of burbot larvae fed on *Artemia* was described in the study. *B. plicatilis* rotifers can be mass cultured with many of the techniques as previously reported[10,33]. These culture practices of rotifers marked the first regular successes in the mass larval rearing of several marine species of economic value such as red sea bream (*Pargus major*)[34], grey mullet[35], and milkfish[36]. As the mass production of unicellular algae is labour-intensive and expensive, optimum methods must be developed for the mass culture of *B. plicatilis* rotifer when using artificial commercial preparations (Selco, Algamac-2000, Sander's Rich, or Microfeast) as diet and these may found to be more cost-effective[37,38].

Several studies that have demonstrated the positive effect of enriched live food on the growth performance of various marine aquaculture species[39]. It is obviously evident from this study that while spawning in the clownfish *A. sebae*, was fairly straight forward enrichment of food offered was found to be crucial in the early larval rearing stage.

Previous reports on the influence of green water systems in the larval rearing of clownfish clearly shown to be in agreement with the present study[5,7]. A significantly higher mean length and wet weight of the larvae cultured in green water condition in comparison with larvae fed on *Artemia* was detected on the 10th dph, indicating that feeding rotifers (8–10 ml^{-1}) along with algae ($1.1–2.6 \times 10^5$ cells/ml) accelerated fish larval growth (97.8%, Table 2). Pomacentrid larvae are very sensitive to light and in the presence of bright light reflection, they exhibit "head butting syndrome" and consequent mass mortality[40]. It is found from the present study that "green water techniques" of reducing light might have stopped the same disorder of the fish and improved the water quality. This seen to be increased the contrast for feeding and acted as food for rotifers[40].

In our present experimental work, improvement of the first feeding of larvae also included the addition of microalgae together with rotifers to the rearing tanks[41–43]. In this respect, nutritional fortification of rotifer with microalgae for larviculture of the clownfish *Amphiprion ocellaris* and larval survival from 0 to 15 dph in captive condition was reported[44]. Feeding the larvae of clownfish with rotifer (100–150 ml^{-1}) enriched with *Chlorella salina* ($60–70 \times 10^6$) and

Table 4 Wet weight (mg) of Clownfish larvae measured on days 7, 10, 21, and 35 of the experimental course (mean ± SD).

Treatment group	Day 7	Day 10	Day 21	Day 35
R (clear water + rotifer)	12.53 ± 0.04	$22.54^{a} \pm 0.04$	32.86 ± 0.40	$53.46^{a} \pm 0.22$
C+R (green water+ rotifer)	12.52 ± 0.03	$22.77^{a} \pm 0.10$	40.20 ± 0.30	$54.86^{a} \pm 0.63$
3C+7R (3 days green water + rotifer)	12.45 ± 0.01	$25.58^{b} \pm 0.05$	34.62 ± 0.16	$54.08^{a} \pm 0.12$
Art (clear water + *Artemia)*	12.42 ± 0.02	$20.56^{b} \pm 0.06$	30.87 ± 0.20	$53.16^{a} \pm 0.14$

Different superscript letters within a column indicate significant difference ($P < 0.05$).

Nannochloropsis oculata ($60–70 \times 10^6$ cells/ml) in 1:1 proportion showed 80 to 85% larval survival. However, larvae fed with oil enriched rotifer along with green algae *N. oculata* ($60–70 \times 10^6$ cells/ml) and *C. salina* ($60–70 \times 10^6$ cells/ml) showed only 35–40% survival. The same authors investigated the influence of green water system on larval rearing of the clownfish *A. percula* using *Nannochloropsis* (60×10^6 cells/ml) and *C. salina* (60×10^6 cells/ml) in 1:1 proportion, immediately after hatching to 3rd dph gave 80 to 90% larval survival.

In the present study a "green mass" was observed in the gut of clownfish larvae offered *Chlorella* sp. in the first 3 days. This is in line with the earlier findings[28,31] on the burbot larvae that in the first days of feeding they preferred only phytoplankton. Filter feeding on algae by drinking activity and using the visceral arches as a trap was also reported for cod larvae[45,46]. Positive effects of adding microalgae (green water technique) to the larval tanks are well documented[47,48]. Highest larval survival (65%) of *A. sebae* using enriched rotifers with various livefeeds (*N. salina*, *C. marina*, *I. galbana*) as "green water technique" was reported[7]. Feeding on algae during early developmental stages may provide the larvae with essential nutrients, may act as an initiator for the digestive system, or may have an effect on the microflora of the larvae[49]. This requires an increased knowledge about mechanisms of algal-larval interaction at the first feeding stages. The fish larvae showed better growth, survival and viability through rearing them by feeding rotifers with size of higher selectivity[50]. Thus it is evident from this study that the rotifer, *B. plicatilis*, size chosen is within the mouth gap size of the targeted fish. It should be noted that the feeding selectivity of larvae is not only dependent on mouth size of larvae, but also on species specific characteristics[51,52]. In this regard, the importance of the transfer of fatty acids and other nutrients through the algae-rotifers-larvae food chain was also reported[11,17] and these nutritional factors can maintain an appropriate HUFA content in the live prey before they are eventually ingested by the fish larvae[20].

In conclusion, the results indicated that clownfish larvae in the first feeding period need food of small size and that algae may play an important role during this period. However, even if rotifers are an adequate starter diet for the first larval stage of the clownfish larvae, they must be replaced after 7–8 days by larger crustaceans such as *Artemia*. The application of the rotifers would enable intensive larviculture of marine ornamental fish species with small larvae, which would eventually lead to exponential increase in the yield of the fry, as demonstrated in this study. As the two marine live feeds rotifers and *Artemia* naturally lack n-3 HUFAs, being rich in linolenic acid, they must be supplemented with n-3 HUFAs to ensure successful growth and metamorphosis of the larvae. Also the availability of other small live food organisms would also facilitate breeding of new fish species with small larvae that could not be raised previously using the existing macro zooplankton culture method. This would eventually enhance the number of fish species for captive breeding.

Acknowledgements: The authors are thankful to the anonymous reviewers for their critical suggestions on the manuscript, and to the higher authorities of Annamalai University for the facilities provided during the study period.

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