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J. Environ. Biol.
33, 623-628 (2012)
ISSN: 0254-8704
CODEN: JEBIDP

Hatchery production of the clownfish *Amphiprion nigripes* at Agatti island, Lakshadweep, India

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Publication Data

Paper received:
11 November 2010

Revised received:
05 May 2011

Accepted:
26 May 2011

Abstract

Healthy individuals of matured clownfish, *Amphiprion nigripes* and sea anemone, *Heteractis magnifica* were collected from the Agatti island lagoon by snorkeling. During 'conditioning' for 3 months, pair formation occurred and the same were transferred to rectangular fiber glass spawning tanks of 1000 l capacity. Suitable water quality parameters were maintained. The fishes were fed with tuna eggs, boiled clam meat, squid, octopus and trash fish thrice in a day. Reproductive behaviour and embryonic development were documented. Spawning took place in between 0900 - 1100 hr and hatched-out occurs, after sunset following an incubation period of 8-9 days. Size of the newly laid egg was 2.0-2.2 mm in length and 1.0-1.2 mm in width. The larval rearing tanks were maintained with UV-treated water and followed the optimal physico-chemical parameters as in the parent tanks. The different light regimes and diets were used for the successful larval rearing. The maximum larval survival (61%) was achieved at the photoperiod of 24 L/0D. Within 15-17 days, the larvae metamorphosed and took up parent colouration and comparatively high growth rate was observed when fed on algae enriched rotifer than those with poly unsaturated fatty acid (PUFA).

Key words

Hatchery production, *Amphiprion nigripes*, Embryonic development, Larval rearing, Photoperiod

Introduction

Tropical and subtropical countries are among the world's largest exporters of marine ornamental species for the aquarium trade (Olivotto *et al*., 2003). Due to the increasing demand, many studies on ornamental fishes, particularly, larval rearing and nutrition have been performed (Avella *et al*., 2007). In the year 2007, the global trade on 'marine and fresh water fishes and invertebrates' were valued at US$ 327 million. The global demand for aquarium fish has been estimated to worth US$ 200-330 million yr^{-1} (Wabnitz *et al*., 2003), of this, the annual global trade ranged between 11 and 12 million coral fishes, clearly indicating the need of hatchery production of coral fishes like clownfishes.

In India, Lakshadweep has rich marine ornamental fish diversity. The complex interaction of mega, meio and micro-fauna makes Lakshadweep as an example of coral reef ecosystem (Murty *et al*., 2002). Agatti island is part of the Chagos-Maldives-Lakshadweep archipelago, which is the largest atoll system in the world (Jones, 1986). As many as 29 species of clownfishes and 10 species of symbiotic sea anemones reported worldwide (Allen *et al*., 2008), among them, the Maldives clownfish, *Amphiprion nigripes* is restricted to Maldive and Lakshadweep islands.

Protandrous sex change is a peculiar feature of the clownfishes. They are born as male and dominant member of the group changes sex to female and the second largest sub-adult then becomes the functioning male (Wood and Aw, 2002). At present, natural population of clownfishes is declining due to overexploitation to meet the burgeoning demand in aquarium trade. Incidentally, *A. nigripes* survives longer in aquarium, has more attractive body coloration and fetches a higher price in the global market. Several attempts have been made to replenish the clownfish population by mass-producing them (Ajith Kumar and Balasubramanian, 2009). In the present study, breeding, embryonic development, mass scale larval rearing and experiments on influence of photoperiod and diet on larval growth and survival were carried out from the Lakshadweep, the coral paradise of the Arabian sea.

Materials and Methods

Fish collection: Fourteen numbers of clownfish, *A. nigripes* (total length 6.5 ± 5 cm) and seven sea anemone, *Heteractics magnifica* were collected using snorkeling at a depth of 1 to 2 m at different locations of Agatti lagoon (Lat. 10º 51'N, Long. 72º 11'E) during April, 2009. They were accommodated in 100 l capacity cylindrical fibre glass tank, fitted with battery aerator and transferred to the hatchery at Agatti island. Subsequently, the fishes and anemones were introduced into a 3000 l tank for quarantine.

After one month period, ten healthy fish and five sea anemones were shifted to the conditioning tank. Pair formation occurred during three month period of conditioning and the paired fish were transferred to separate 1000 l capacity fibre glass tank. The bio-filtered water was used for the brooder tanks. Water quality parameters such as temperature (26±2ºC) was measured by mercury centigrade thermometer with 0.5ºC accuracy, salinity (34-35 psu) by salinity meter (Ecoscan, Singapore), pH (8.0-8.3) by pH pen (Eutech, Singapore), dissolved oxygen (5-6.5 mgl^{-1}) by D.O probe (Ecoscan, Singapore), ammonia (less than 0.01 ppm) by ammonium test kit (Merck, Germany). The photoperiod was maintained at 13 hr light: 11 hr dark by 25 W fluorescent bulbs. Ceramic tiles and live rocks were provided as substratum for egg laying. The fish were fed with tuna egg mass, boiled clam meat, squid and octopus, thrice a day (0800, 1200 and 1600 hr) at 4% of the body weight, and the sea anemones were fed with fresh shrimp. Uneaten food particles and excreta were removed by siphoning 30 min after feeding. Approximately 50% of water was exchanged weekly once.

Behavioural observations: Courtship behaviour and spawning activities were monitored daily (0900-1000, 1300-1400 and 1700-1800 hrs). The fishes used to lay eggs at morning hours. The fecundity varied from 300 to 700 eggs and the incubation period was 8 to 9 days. The eggs were tended and guarded by the male. The area (A) of the nest was calculated by a constant factor 3.14 with minimum and maximum radius of nest as proposed by Robertson *et al.* (1988) modified by Hoff (1996). The total number of eggs was estimated by counting the number of eggs per cm^2 and multiplying with deposition area.

Preparation of algal enriched rotifer diet: The algal stock culture (*Nannochloropsis* spp. and *Chlorella* spp) was maintained using F2 medium and outdoor mass culture by commercial fertilizers. Rotifer, *Brachionus plicatilis* was mixed with algae and mild aeration was provided for enrichment and after one hour they were filtered using plankton net and used as larvae feed.

Embryonic development: A total number of 5 eggs were sampled randomly at each sampling to study the embryonic development. The sampling was started immediately after completing the spawning until hatch out. The eggs were transferred into sterile plate with UV-filtered seawater. Time was recorded when 50% of the collected embryos attained the stages such as cleavage, blastulation, gastrulation, organogenesis, embryo, pre-hatching and larval stage clearly. The main morphological and functional features of each developmental stage were recorded by light microscope (Motic, B1-series Biological Microscope) and digital camera (Canon, China).

Larvae rearing: The eggs hatched out after sunset and occasionally it extended to night (1900 to 2300 hrs). After completion of hatching, the floating larvae were gently collected by glass bowl and transferred to previously set larval rearing tank. Cylindrical white coloured fibre glass tanks (100 l capacity) were used for larval rearing. Prior to larvae transfer, the tanks were filled with 20 l treated seawater and 5 l algae to "green up" the system. A 15 W white fluorescent bulb was fixed on top of the tank for illumination. After stocking the larvae (3 nos. l^{-1}), approximately 10% water exchange was made every day. The newly hatched larvae were divided into groups to study the effect of photoperiod and suitability of diets on survival and growth. Three numbers of larvae were randomly collected on 1st, 5th, 10th and 15th day after hatch to measure the total length and mouth gap in nearest millimeter by ocular micrometer (Erma, Tokyo) and light microscope.

The larvae from same parents were stocked in different experimental tanks with three modes of photoperiod as well as three different feeds. The larvae were divided into five groups (A-control, B1, B2, C1 and C2) and stocked them as 100 larvae per tank. The feeding schedule was commenced after 7 hrs of hatched out and fed 4 times in a day.

The experimental tank B1 was maintained with algal enriched rotifer and 16L/8D photoperiod, and B2 retained with poly unsaturated fatty acid (PUFA) enriched rotifer and 16L/8D photoperiod. While, the tank C1 was maintained with algal enriched rotifer and 24L/0D photoperiod, and C2 was maintained with PUFA enriched rotifer and 24L/0D photoperiod. The control tank (A) was maintained with 13L/11D photoperiod and rotifer, without enrichment. All the experiments were done in duplicate. The larvae were fed S-type rotifer *B. plicatilis* with a concentration of 7-10 ml^{-1} and on 11th day onwards newly hatched *Artemia* nauplii (15-20 ml^{-1}) were gradually introduced.

The rotifer was enriched by PUFA 6 hr before feeding and the emulsion used at a concentration of 0.25 ml l^{-1} of seawater. The mixture was aerated for 10 min to ensure good integration before feeding. Prey density was approximately counted for every 6 hr until metamorphosis. The physico-chemical parameters excluding light were maintained as such in the parent tank. Survival (%) and growth increment (mm) of larvae and juveniles in each set up was documented daily. Further, the mortality, either due to starvation or indigestion was recorded visually.

Statistical analyse : Statistical analyses were done with the software STATISTICA. The results were analyzed using analysis of variance (ANOVA) with different variables namely survival and growth. The probability level of 0.05 was utilized to account the statistical difference between the means.

Results and Discussion

Behavioural changes: In all the pairs, courtship began just two days before spawning with the initiative taken by the male. One day before spawning, the male showed morphological and behavioural changes such as fin erection, chasing, nest preparation and biting the sea anemone. During this period, the conical ovipositor of the female becomes visible. Female started to lay capsule shaped eggs on the cleaned substratum in mostly oval patch and the male subsequently fertilized the eggs by scattering their sperm over the eggs which took about 30 to 45 min. The fecundity was 300-700 eggs and the clutch was orange in colour on the first two days. Male mostly contributed for fanning the eggs by fluttering the pectoral fins, which created cooling effect to the clutch and the unfertilized eggs and dust particles were removed by mouthing. Eggs were turned to

Table - 1: Embryonic developmental characteristics of *A. nigripes* during 178 hr

Time (hr)	Stages	Developmental characteristics
0:00	Fertilized egg (1st stage)	Yolk in the capsule, one cell stage
1:20	2-blastomeres (2nd stage)	2 cell division
1:45	4-blastomeres (3rd stage)	4 cell division
2:25	8-blastomeres (4th stage)	8 cell division
3:05	16-blastomeres (5th stage)	16 cell division
3:35	32-blastomeres (6th stage)	32 cell division
4:00	64-blastomeres (7th stage)	64 cell division
4:45	Early blastula (8th stage)	128 cell division
5:35	Late blastula (9-12th stage)	Morula
12:10	Early gastrula (13th stage)	Germ ring stage, embryonic-shield
15:05	Late gastrula (14-15th stage)	Epiboly stage, nerve cord formation yolk-plug stage
22:43	Organogenesis	Blastopore closing, notochord
	(16-17th stage)	Formation
24:10	Embryo	Presumptive pericardial cavity, otic
	Somitogenesis	Primodium, midbrain, hindbrain,
	(18-19th stage)	Forebrain, otic placode becomes
		Visible, melanophore appears on
		Yolk-sac
41:30	Embryo (20-21st stage)	Tail, eye, otic vesicle, forebrain, hindbrain, midbrain formation,
		Melanophore started to appear on myomeres
78:30	Embryo (22nd stage)	Heart, eye pigment noticeable, pectoral fin completely developed,
		Blood circulates the whole body early
84:30	Embryo (23rd stage)	Erythrophores formed as body pigment on myomeres
96:30	Embryo (24th stage)	Size of embryo increases and develops around egg capsule,
		Maxilla and eye appear clearly
144:30	Embryo (25th stage)	Head, pectoral fin and tail move frequently
178:15	Free larva (26th stage)	Newly hatched larva

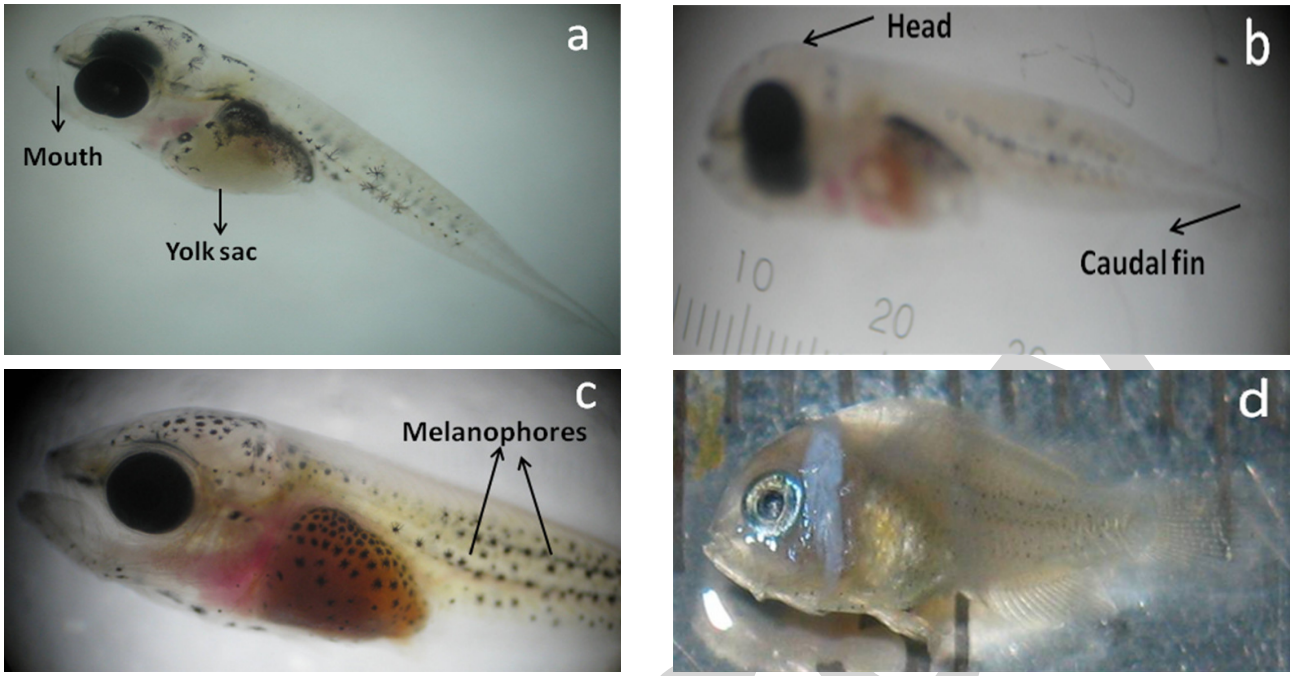

Fig. 1: Larval developmental characteristics on days, a = 1, b = 5, c = 10 and d = 15

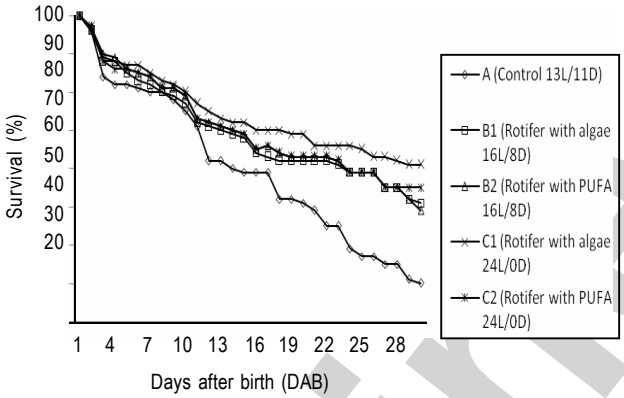

Fig. 2: Survival percentage of *A. nigripes* larvae (0-30 DAB) in different photoperiods and diets

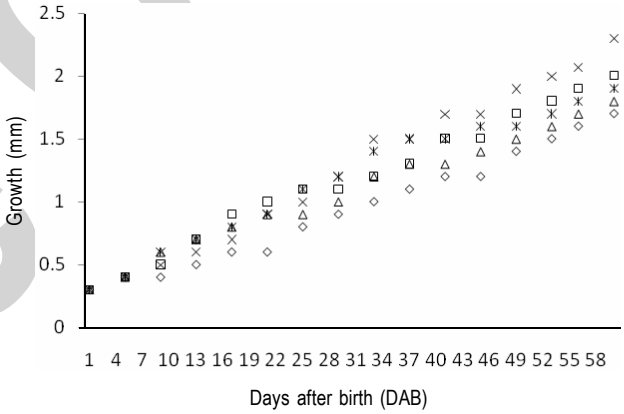

Fig. 3: Growth (mm) of *A. nigripes* larvae in different photoperiods and diets

black colour on third day onwards and the tip of eggs appeared as silver, 5 hrs before hatching. Clutch had an average length of 5-7 cm and width of 3-4 cm. A total of five clutches from the same tank at different spawning at regular interval were analyzed for embryonic development study. The average number of eggs 1 cm^{-2} ranged from 20 to 25 eggs.

Embryonic and larval development: The animal pole of the egg was attached with the substratum by a mass of adhesive threads. The egg size was about 2.0-2.2 mm in length and 1.0-1.2 mm in width. After fertilization, the cytoplasm was clear and the vegetal pole has yolk with different sized fat globules. The first cleavage started by dividing the blastodisc into blastomeres. Later, the blastomeres cleaved in every stage at particular time duration and fat globules moved slowly and reached the top of the vegetal pole. At the gastrula stage, the blastomeres were extended towards the vegetal pole and the number of fat globules was decreased, which occurred after 12 hrs. Organogenesis process occurred after 22 hrs, when the blastopores closed and notochord started to form. At 24 hrs, the appearance of forebrain, midbrain, hindbrain and melanophores was noticed. The other organs were developed at 41 hrs later and melanophore started to appear. The heart commenced beating and blood circulation was observed at 78 hrs. The head, pectoral fin and tail moved frequently at 144 hrs and embryo reached the pre-hatching stage at 178 hrs (Table 1).

Hatching took place 8-9 days post-fecundation during the first 2 hr of darkness. The dorsal, caudal and anal fins were continuous in a longitudinal line. The distal end of the chorion becames soft and pliable and the movements of the embryo became

more frequent and violent until the weakened chorion gives way. The hatching percentage was recorded an average of 95% in all batches. The larvae that emerged from capsule still possessed a small yolk sac, well developed mouth and eye ball.

During larval rearing, important changes were observed (Fig. 1 a-d). The newly hatched larva (day 1) had an average length ranged from 3.8 to 4.1 mm. The mouth opening ranged from 300 to 400 µm (Fig. 1a). The caudal ray appeared and there were marked changes in the head length and depth with respect to body depth (day 5). The pigmentation also increased. The average length was 5.0 mm and the mouth opening was 420 µm (Fig. 1b). The notochord and caudal fin were fully developed (day 10). Total length was 7 mm and the mouth opening was 800 to 860 µm. The fins were developed but not attained adult colouration (Fig. 1c). Fins were developed completely and adult colour patterns were noted (day 15). Total length was about 9 mm and the mouth opening ranged from 900-940 µm (Fig. 1d).

Effect of photoperiod and diet on larval survival: Based on the experiment, it was noticed that enriched initial feed is essential for larval survival. The control tank (13L/11D photoperiod) showed 30% larvae survival and the 16L/8D photoperiod tanks (B1 & B2) showed 51% and 49%, respectively (Fig. 2). The good survival (61 and 55%) and growth (2.3 and 1.9 mm) was observed in C1 & C2 under 24L/0D photoperiod, respectively. The larvae fed with rotifer which was enriched by algae under 24L/0D photoperiod (C1) had shown better survival and growth than rotifer enriched by PUFA (B2 and C2) (Fig. 3). The differences between different experiment groups were statistically significant ($F_{4, 145}$=0.004, P<0.05).

The embryonic development could be divided into 26 stages based on morphological characteristics which revealed the study made in other clownfishes as *A. polymnus, A. ocellaris* and *A. percula* (Rattanayuvakorn *et al.*, 2005; Liew *et al*., 2006; Dhaneesh *et al.*, 2009). The newly hatched larvae were small in size and the yolk sac was sufficient to sustain them for during the first 24 hrs. But in the present study, the initial feed was supplied on 7-9 hrs after post hatch and it gave healthier larvae and provided good survival. In the case of sea bass larvae, which can survive even up to 96 hrs without feed (Kailasam *et al*., 2007) the larvae could not find out and consume the prey actively in preliminary level, and a sufficient amount of feed to be required to the larvae.

The active obligation was observed in parental care made by male and the same was reported in other clown species also (Gopakumar *et al*., 1999; Ignatius *et al*., 2001). The days spent for metamorphosis by *A. sebae* varied between 12 and 15 days (Ignatius *et al*., 2001), *A. ocellaris,* 9-10 days (Madhu *et al*., 2006a; Ajith Kumar and Balasubramanian, 2009), *Premnas biaculeatus,* 11-12 days (Madhu *et al*., 2006b) and *A. chrysogaster,* 12-15 days (Gopakumar *et al.*, 1999) but in the present study the completion of metamorphosis occurred between 15-17 days.

In the present study, *A. nigripes* larval developments were observed more rapidly by an extended photoperiod of 24 hrs light. Under this circumstance, the fish could take feed for longer period and thus able to attain higher growth and development to achieve metamorphosis. After completion of metamorphosis, the photoperiod was reduced to 13 hrs light and 11 hrs dark which help to practice nature condition. According to previous reports, algae enriched rotifer can enhance the development of larval growth compared to rotifer enriched by PUFA (Kraul *et al*., 1989; Immanuel *et al*., 2007).

Agh and Sorgeloos (2005) reported that the algae was used for boosting the fatty acids like eicosapentaenoic (EPA 20:5n-3) and docosahexaenoic (DHA 22:6n-3) in rotifer, *B. plicatilis.* A common practice is to enrich the rotifer overnight and feed the fish larvae on the following morning. The implications of these results show that general health and well-being of the fish larvae have been significantly improved by feeding of algae enriched rotifer. The most interesting result of this work is that *A. nigripes* can indeed be successfully reared in captivity, as long as the physico-chemical parameters, photoperiod and food chain are optimized. In this study, it is revealed that a diet suitably rich algae and 24 hrs light and 0 hrs dark lighting cycle are indispensable for successful rearing of *A. nigripes in* captivity.

Acknowledgments

The authors are grateful to authorities of Annamalai University for providing facilities and the Centre for Marine Living Resources and Ecology (CMLRE), Ministry of Earth Sciences, Government of India for financial support.

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