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Broodstock development and breeding of black-finned anemone fish *Amphiprion nigripes* Regan, 1908 under captive conditions

M. K. ANIL, B. SANTHOSH, B. O. PRASAD AND RANI MARY GEORGE
Vizhinjam Research Centre of Central Marine Fisheries Research Institute, Vizhinjam
Thiruvananthapuram - 695 521, Kerala, India
e-mail: mkanil65@gmail.com

ABSTRACT

Due to recent advances in saltwater fish-keeping, there is great demand for marine ornamental fishes, especially for those from the tropical coral reef habitats. Exploitation of wild stock has depleted their availability, thus making it difficult to meet the market demand. This paper describes the development of brood stock and a viable technology for commercial production of black-finned anemone fish *Amphiprion nigripes***.** Fecundity of this species ranged from 350-450 per spawning and continuous spawning could be achieved at 12 to 16 days interval. The incubation period was 6 to 7 days. Larvae were successfully fed with rotifer *Brachionus plicatilis*, *Artemia* nauplii and particulate feed at appropriate stages of development using green-water system of larval rearing. Juveniles with average size of 15 mm were produced in 40 days with an average survival rate of 72% and marketable size of 25 mm or more was achieved in less than four months time.

Keywords: *Amphiprion nigripes*, Breeding, Broodstock, Clownfish

Introduction

Saltwater fish keeping is a multi-million dollar industry with more than 1,400 species of marine fishes being collected from coral reefs providing livelihood for thousands of fishers in developing countries. This industry began in the 1930s in Sri Lanka as small export units of wild caught fishes (Wood, 2001). More than 98% of the marine ornamental fish marketed currently are collected from coral reef ecosystems of tropical countries. Marine aquarium keeping as a hobby has recorded rapid and steady growth in recent years, mainly due to the scientific advancements of marine aquarium and reef tank technologies, resulting in the proportionate expansion in global marine ornamental fish trade.

Even though more than 100 species of marine fishes have been bred in captivity, relatively few have been bred to commercial quantities (Dawes, 1999). Many species of anemone fishes were bred and reared from different parts of the world (Alava and Gomes, 1989; Allen, 1991; Hoff, 1996; Wilkerson, 1998; Gopakumar *et al*., 1999; Ignatius *et al*., 2001; Anil *et al*., 2010). *Amphiprion nigripes* is popularly known as black-finned anemone fish or Maldives anemonefish, distributed in Maldives, Central Indian Ocean and Sri Lanka. This fish is commonly associated with the anemone *Heteractis magnifica* (Fautin and Allen, 1992). Patzner (2008) has carried out a detailed ecological study of *A. nigripes* in Maldivian coral reefs and confirmed that this fish is associated only with *H. magnifica*. This species is characterized by an orange coloured large head and body, a pinkish snout, a single white bar behind the eye, red-orange colored dorsal fin and pectorals; yellow-orange caudal fin and black pelvics and anal fin (Allen, 1971). They are of high demand in international trade due to their beautiful colouration and hardy nature. This clownfish has been bred and mass produced under captive conditions for the first time in India and the results are presented in this paper.

Materials and methods

Eighteen numbers of black-finned anemone fishes in the size range of 7 to 10 cm were brought from Lakshadweep islands in oxygenated polythene bags at the rate of one specimen per polythene bag containing 500 ml of seawater. They were acclimatized to filtered and aerated seawater of 30-32 ppt salinity in six FRP tanks of 500 l capacity, provided with stable biological filters. Each tank was stocked with three adult specimens of 70 - 100 mm size. Anemones were not provided in the brood stock tanks. About 10% of rearing water along with faecal matter and excess feed was siphoned off daily and was replaced with fresh seawater. They were fed thrice daily; first feeding at 0900 hrs with pelleted feed, second at 1400 hrs with boiled mussel and the third feeding in the evening with *Artemia* nauplii/juveniles or mysids or copepods as per availability. The feeding was regulated to avoid feeding in excess of 8% of their body weight. Leftover food, if any, was siphoned out in the afternoon. All the tanks were

provided with clay pots as substratum for egg laying. The broodstock tanks were observed regularly to remove aggressive fishes and to study pair formation and spawning. Spawned egg clutch was observed daily and developmental changes of the embryo were recorded and photographed using stereozoom as well as trinocular compound microscopes. Continuous aeration was given in all the brood stock tanks and mild aeration in larval rearing tanks.

Live feed culture

Microalgal stock culture of *Chlorella marina, Nannochloropsis oculata* and *Isochrysis galbana* were maintained in three litre capacity Haufkins flasks. They were subcultured at regular intervals to maintain purity. Walne's enrichment medium (Walney, 1974) was used for the culture of microalgae. Cell density was monitored using haemocytometer at regular intervals. Rotifer, *Brachionus plicatilis* was cultured by feeding mixed culture of *N. oculata, C. marina* and *I. galbana* in equal proportions. *Artemia* nauplii were produced by hatching commercially available artemia cysts (Microfeast® Artemia, U.S.A.).

Hatching and larval rearing

Date of hatching was calculated based on the number of days after spawning, external appearance of egg clutch and microscopic observations of the egg. On the day of hatching, an hour before sunset, the clay pot with the egg clutch and parents were transferred to 100 l capacity larval rearing tank having the same physico-chemical characteristics as the breeding tanks or the eggs were allowed to hatch in the brood stock tank itself. All the lights were switched off in the evening or the hatching tank was covered with dark cloth. Mild aeration was provided in the tank in both the cases.

In the case of hatching in spawning tank, the clay pot and the parents were taken out after completion of hatching. In the case of hatching inside the broodstock tank, an electric torch was used to congregate the larvae as the newly hatched larvae are phototropic in nature. The total number of eggs laid in each spawning were counted using the digital images of the clutch and the percentage hatching was calculated based on the number of unhatched eggs remaining after the hatching period. Usually hatching would be completed by 2000 hrs.

Green water used for larval rearing was prepared using a combination of *N. oculata* and *I. galbana* at equal proportions and the algal density was kept between $1x10^5 – 3x10^5$ cells ml^{-1}. Larvae were stocked at the rate of 2 nos. l^{-1} and 100 larvae were stoked in 50 l of water. Three replicates were provided to calculate the survival and growth. The larvae were fed with rotifer at the rate of 10 nos. ml^{-1} from 0 dph (days post hatch) to 15 dph. *Artemia* nauplii were given at the rate of 2 nos. ml^{-1} from 7 dph and slowly increased to 4 and 6 nos. ml^{-1} on 15 and 20 dph respectively. From 15th dph onwards, they were given particulate feed of 200-300 micron made by grinding and sieving Varna feed (Cadalmin brand, CMFRI) and increased to 300-400 micron from 25 dph. Larvae were randomly taken from the tank and observed for morphological changes and measurements were taken on every 5th day from 0 dph to 30 dph and every tenth day from 30 dph to 60 dph and final measurements were taken on 100 dph. Mouth size measurements were taken every 5th day till day 20. Ten individuals were observed for recording morphological changes.

Hydrographic parameters such as salinity, pH, dissolved oxygen, NO_2, NO_3, and NH_4 were measured twice weekly from all the experimental tanks. Salinity was measured with a salinometer (ATAGO, Japan), pH with Eutech pH meter. Dissolved oxygen, NO_2, NO_3, and NH_4 were estimated using standard procedures of seawater analysis (Strickland and Parsons, 1968). These results were analysed and water quality was maintained at optimum level by taking corrective measures like water exchange and aeration. Nitrate level of less than 30 ppm, nitrite and ammonia levels of less than 0.01 ppm were always maintained in the tanks. Temperature, salinity and pH in the breeding tanks were maintained at 27±02 °C, 30-32 ppt and 8 to 8.2 respectively.

Results

Of the total six broodstock tanks setup, pairing was noticed in three tanks in 4 to 7 days time. Two fishes succumbed during this period. From the tanks with fishes showing strong pairing behaviour and aggression towards the third specimen was seen, the unpaired specimens were removed to separate tanks. Altogether 4 pairs were formed in one month. First spawning was recorded on 74th day of pair formation. In six months time, two spawning pairs were obtained. During the spawning and incubation period, the non-functional male (NFM) was seen disturbing the parents and were being chased out by the breeding pairs. They were later removed from the tanks.

Two days prior to spawning, pairs started clearing a site for the nest and the process intensified one day prior to spawning. Spawning usually takes place from 09 00 to 15 00 hrs. Female fish sticks eggs inside the pot on the cleared area. Each spurt of egg attachment is followed by release of sperms by male at the site (Fig. 1). The whole process of egg laying and fertilization takes around 20 to 40 min. The eggs were pale pink in colour at the time of spawning (Fig. 2). They were capsule shaped with a small stalk for attachment to the substratum. The egg size ranged

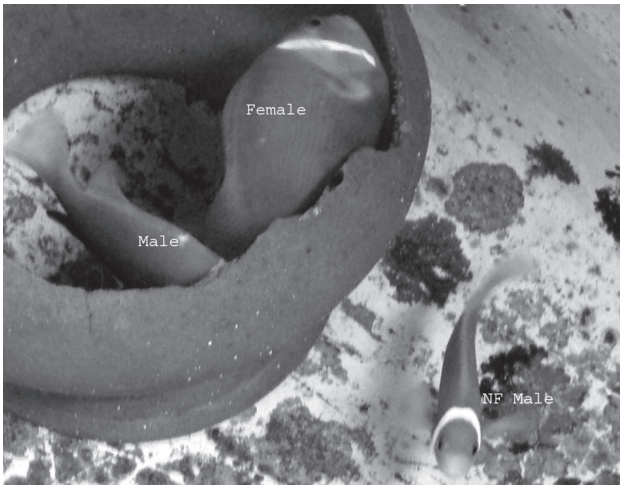

Fig. 1. Spawnng of *Amphiprion nigripes*

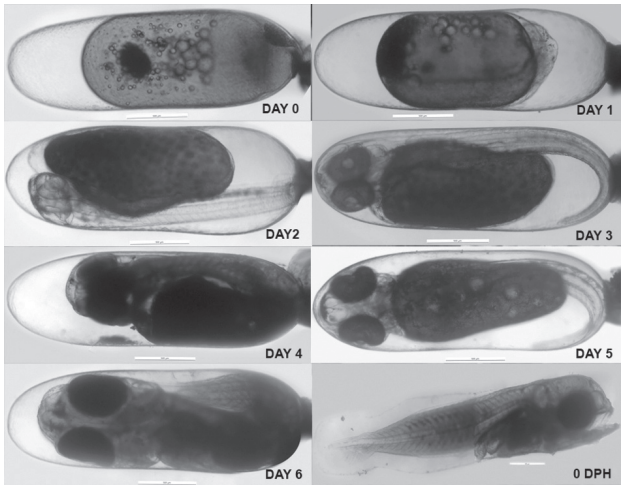

Fig. 3. Embryonic development in *A. nigripes*

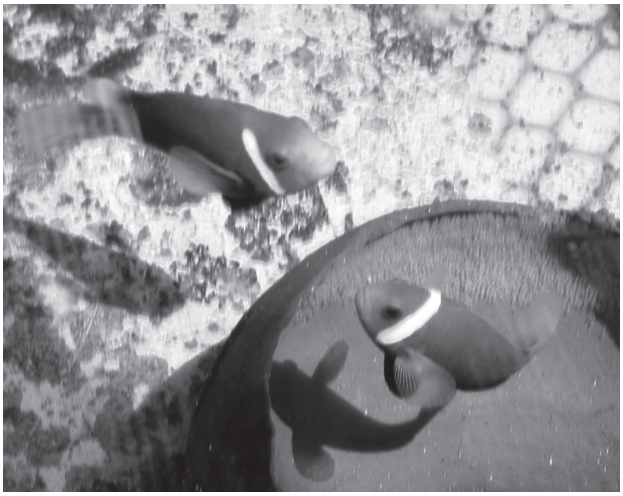

Fig. 2. *A. nigripes* guarding the eggs

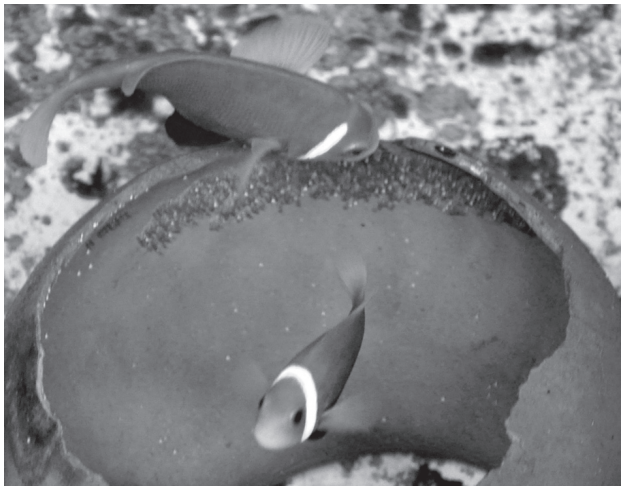

Fig. 4. Silvery eggs of *A. nigripes* on the day of hatching

between 2.4 to 2.6 mm in length and between 0.9 to 1.0 mm in width. During incubation, parents took care of the eggs by fanning and mouthing. With the movement of fins, they aerated the eggs. The frequency of fanning increased as the incubation advanced. Colour variations were observed in the eggs during incubation with progressive development of the embryo (Fig. 3). The eggs turned black and then silvery (Fig. 4) by the end of the incubation period indicating organogenesis and development of eyes. Parents removed the unfertilized, dead or weakened eggs and dust particles by mouthing. The number of eggs produced varied from 350 to 450 per spawning from a single pair and spawning was achieved at every 12 to 16 days interval. Incubation period was 6 days and rarely 7 days when the temperature was low. Hatching percentage varied between 94 to 95%.

Table 1. Growth and survival of *Amphiprion nigripes* Regan

Day	0	5	10	15	20	25	30	40	50	60	100
Avg. survival (%) ± SD	100	96.4 ± 1.2	89.7 ± 1.5	83.5 ± 1.8	78.4 ± 2.2	75.9 ± 2.2	73.6 ± 3.4	71.8 ± 3.5	68.2 ± 4.9	65.9 ± 5.2	63.4 ± 6.1
Avg. TL (mm) ± SD	3.9± 0.05	4.5 ± 0.06	6.5 ± 0.10	7.3 ± 0.13	8.5 ± 0.43	9.5 ± 0.37	13.5 ± 1.43	15 ± 1.39	15.5 ± 1.52	17.2 ± 1.93	27.3 ± 2.16
Avg. SL (mm) ±SD	3.1± 0.05	3.5 ± 0.05	5.1 ± 0.09	5.8 ± 0.11	6.5 ± 0.31	7.3 ± 0.29	10.0 ± 0.59	12.1 ± 1.1	13.5 ± 1.2	14.2 ± 1.5	17.4 ± 1.8
Avg. mouth size (µm) ± SD	180± 5.3	626 ± 12.5	635 ± 15.4	780 ± 15.5	870 ± 20.2	_	_	_	_	_	_

Data on average survival, total length (TL), standard length (SL) and mouth size of the larvae are presented in Table 1. On the day of hatching, the larvae measured 3.1 mm in standard length (SL) and 3.9 mm in total length (TL). Larvae looked transparent and the dorsal, caudal and ventral fins were merged as a fin-fold bordering three fourth of the body. Mouth size of the freshly hatched larvae was 180 µm. On the 10th day, dark chromatophores were seen on the body with a definite horizontal pattern. Fins were without any pigment spots. Minute denticles were observed on the upper and lower jaws. Light yellow colour was observed between the dark chromatophores. No distinct band formation was noticed. On 15th day, the larvae were bright yellow with some scattered dark pigments along the posterior part of the body. Anterior region or the belly region was silvery. Fins were clear, without any pigmentation. Minute denticles could be seen along the anterior margin of upper and lower jaw. At 20th day, scattered dark pigments were observed on the body. Ventral side remained silvery in colour. Denticles were found arranged in one row at the anterior region of upper and lower jaw. On 25th day, small dark spotted chromatophores were seen scattered all over the body including the caudal region. Ventral region (belly) was silvery and fins were developed. The base of the fins was also found scattered with dark chromatophores. Minute denticles were observed on the margins of upper and lower jaw. Minute orange red pigments were seen scattered on the body in between the dark chromatophores. One white narrow band was seen along the outer region of the posterior part of operculum..

On 30th day, all fins were well developed and dark chromatophores were found scattered along the base of fins. On the anal fin, more than half portion was seen coloured with thick dark chromatophores. Only one white band was seen in the anterior region with thin black outer lining. In the dorsal region, the band was very thin, and the band did not encircle the whole body. However, at the opercular region the band was a bit thicker, The whole body was covered with light brown coloured chromatophores. There was no pigmentation in the mouth region. On the 40th day, the body was entirely pigmented with light brown colour including fins. Ventral region along the caudal part showed more dark chromatophores scattered all over the region. Only one band was seen, which was irregular in shape, broader at the centre of operculum, with a slight curvature and tapering towards the ventral region. At the dorsal region, the band was very thin, still not encircling the whole body and with thin black linings. On 50th day, body pigmentation was light brown including the fins (Fig. 5). The white band was slightly broader at the dorsal region, but still not encircling the whole body and the thin black lining along the margin of the white band was thicker. The body was light brown in colour including all fins on the 60th day with the pelvic fin slightly dusted with dark pigmentation. On day 100, body was light brown including dorsal, pelvic and caudal fins. The pectoral fin was purely blackish and anal fin almost black. Minute dark chromatophores were seen scattered along the body. Only one anterior band was clearly visible, which was broader at the dorsal region and tapering towards the lower opercular region. Bands were without any dark margins. Caudal, dorsal and pelvic fins were slightly yellowish.

Fig. 5. Seeds of *A. nigripes*

Discussion

An advantage with pairing clownfish is their ability to change sex naturally. During the present study, medium sized specimens of 7-10 cm were successfully used to develop the broodstock. According to Hoff (1996), the best and easiest approach in pairing clownfish is to keep three or four fish of equal size in a tank. Sex reversal is common in clownfish and they themselves choose which shall become the male/ female. Clown fishes are protandric, and the males can change to female but not *vice versa* (Hoff, 1996). When we keep 3-4 specimens, the probability that all of them may be females is rather low. Eventually, two fishes will be moving jointly chasing others. Sometimes a pair will permit a few smaller individuals to remain as a reserve.

During the present study with *A. nigripes,* fishes which remained in the broodstock tank other than the pair were seen disturbing spawning and parental care and so they were later removed. Though host anemone was not stocked in the broodstock tank, the fishes formed pairs and spawned successfully. Hoff (1996) was also of the opinion that brooder fish can be stocked in a tank with or without a host anemone. Conditioning of the pairs depends on manipulation of a combination of environmental factors to induce gonadal maturation and spawning. The factors may include light intensity, light duration, temperature, water

quality, nitrogen, phosphate, ammonia, p^H, type of food, tank size, tank shape, aeration and habitat.

During the present study, salinity was maintained between 30-32 ppt. The salinity of around 28-32 ppt was better while conditioning the fish. Lower salinity helps to reduce disease problems associated with parasites that demand higher salinities to survive. It also allows a large variance in salinity due to evaporation of tank water in the hatchery. A nitrate level less than 30 ppm, nitrite and ammonia levels at less than 0.1 ppm and pH around 8 - 8.3 were found ideal in conditioning tanks. Broodstock diet is one of the main factors for gonadal maturation and successful spawning. Eggs contain considerable amount of lipids which are energy resources needed for the development of the embryos within the eggs. Hence, nutritious diet in adequate quantities must be fed to the broodstock fish. If the broodstock fish were not duly fed, the results are directly reflected in the number of eggs laid, fertilization rate, hatch rate and the quality of hatched larvae. Poor quality eggs develop slowly, hatch late and often result in significant early larval mortalities. Present food regime of compounded feed in the morning, a second feeding at noon with boiled mussel and the third feeding in the evening with a variety of live feed resulted in good fecundity, continuous spawning good hatching rate, good survival and growth of the larvae.

The eggs were laid always on clean and stable substratum during morning hours in contrast to evening hours as reported by others in the case of *Amphiprion clarkii (*Alava and Gomes, 1989) and *A. percula* (Malpass, 1996). Spawning of clown fishes in the morning hours was reported in *A. chrysogaster* (Gopakumar *et al*., 1999), *A. sebae* (Ignatius *et al*., 2001) and in *A. ocellaris* and *Premnas biaculeatus* (Anil *et al*., 2010). Rotifer (*B. plicatilis*) and *Artemia* nauplii are widely used in rearing marine ornamental fishes (Gopakumar *et al*., 1999; Olivotto *et al*., 2003; 2005), however, they do not always ensure good survival and growth of marine fish larvae without enrichment as reported in grouper (Pechmanee *et al.,* 1988).

During the present study, good survival was achieved at stages fed with rotifer and *Artemia* in green water with mixed algal culture, which ensured that rotifers were always in well fed condition ingesting sufficient quantity of microalgae. Larval mortality was noticed on day 2 post-hatch when the yolk sac almost disappeared, during day 6-10 of metamorphosis and around day12-14. The larvae reached juvenile stage of around 13-15 mm size at 30-40 days post-hatch (Fig. 5), and were then transferred to growout tanks. It took a total of four months to rear them to a marketable size of around 25-30 mm. Juvenile growth and development are strongly influenced by water and food quality and the amount of food intake. The survival and growth achieved during the present study convincingly proved that *A. nigripes* rearing can be taken up on a commercial scale. It is during juvenile to grow-out phase that filtration capabilities become vital. It was observed that providing anemones in the juvenile rearing tanks (Fig. 6) could keep them healthy without any diseases especially those due to parasites.

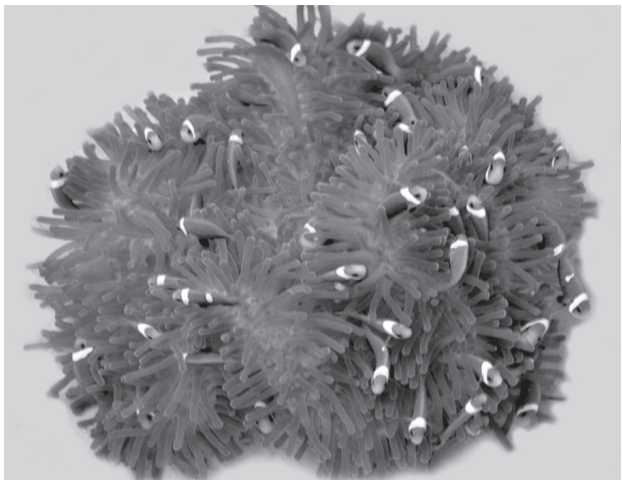

Fig. 6. Juveniles of *A. nigripes* in the sea anemane *Hectractis magnifica*

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