

Larval development and growth of Red Saddleback Anemonefish, *Amphiprion ephippium*, (Bloch, 1790) under captive conditions

Rohini Krishna, M. V^{1*}, Anil M. K² & Neethu Raj. P³

¹ Department of Zoology, TKM College of Arts and Science, Kollam - 691 005, Kerala, India

^{2,3} Vizhinjam Research Centre of ICAR-Central Marine Fisheries Research Institute,
Vizhinjam, Thiruvananthapuram-695521, Kerala, India

* [E.mail: rohini.krishna09@gmail.com]

Received 01 May 2017; revised 24 July 2017

On the 1st day of hatching, the body of the larva was transparent and all the fins were fused together to form a single fin fold. Hatchlings measured 4.96 mm in total length. On the 10th day, all the fins were visible and body colouration had begun to develop, the larvae then measured 7.08 mm in total length. The banding began to appear from the 10th day and on the 15th day, the head and middle band were clearly visible. From the 25th day onwards, the larva measured 9.66 mm in total length. On the 30th day, adult pigmentation had begun to appear in the larva. After the 45th day, the bands started to disappear. By the 160th day, the middle band had completely disappeared. On the 310th day all the bands had disappeared and now the juvenile has transformed into an adult fish.

[Keywords: *Amphiprion ephippium*, Chromatophores, Pigmentation, Clownfish]

Introduction

The marine ornamental trade industry has been flourishing in the recent years even though there was a slight decline in the reef fish trade late 1990¹. Many of the marine ornamental fish species habitats are on the brim of endangerment due to human activities^{2,3}. In 2012 a petition was submitted by the Centre for Biological Diversity before the US Department of Commerce to list 8 species of Pomacentrids including *Amphiprion ephippium* and seven other damselfishes as threatened or endangered under the US endangered species act. So breeding and thereby conservation of Pomacentrids is of prime importance as far as a conservational biologist is concerned. Ornamental fish breeding programmes tasted success in India when the breeding of the seahorse *Hippocampus kuda* was carried out successfully⁴. In recent years a plethora of breeding programmes has been launched for breeding many Pomacentrid species. Breeding of *Premnas biaculeatus* and *A. nigripes* have also been carried out.^{5,6} Although some of these programmes have tasted success the detailed information on the embryonic development and larval growth of many species are very meagre. This paper focuses on the larval growth and embryonic development of *Amphiprion ephippium* popularly known as red saddleback anemonefish. The

fish is widely distributed throughout Eastern Indian Ocean and has been known to associate with 3 different sea anemones. They usually associate with *Entacmaea quadricolor*, *Heteractis crisper* and *H. magnifica*⁷. This is indeed advantageous since it has high demand in international trade. *A. ephippium* is the only clownfish which is unstriped as an adult. In the juvenile stage, they resemble *A. frenatus* with 2 white bands on their head and middle region and sometimes the third band may also be present in the tail region. This retention of the 2 barred colour pattern may be due to the fact that they arose from a clarkii-like ancestor⁸. These bands disappear as the larvae grow older. The disappearance starts from the headband and slowly progresses to the middle band.

Materials and Methods

In April 2015, 2 sub adult fishes were brought from Mandapam. The fishes were stocked in rectangular FRP tanks of 500 L capacity. Tanks were fitted with a biological filter for maintaining the water quality. Uneaten food was syphoned out twice daily, the syphoned out water was then replaced with fresh seawater, in addition to this sea water was exchanged at the rate of 25 % once in a week. During the rearing period, water quality parameters such as water temperature, pH, salinity, NH₃, nitrate and phosphate

were measured using standard methods. Temperature, salinity and pH in the breeding tanks were maintained at 27 ± 0.2 °C, 32-34 ppt and 8 to 8.2 respectively by replacement of sea water whenever required. The fishes were fed 4 times a day. The feeding schedule followed was pellet feed at 10.00 am, boiled mussel meat at 12.00 pm and 2.00 pm and *Artemia* nauplii at 4.00 pm. Fishes put in the bloodstock tanks showed pairing behaviour. The pair started laying eggs on July 2015. Fishes were provided with earthen pots for laying eggs.

Live feed such as phytoplankton, rotifers and *Artemia*, were used for the larval rearing experiments. For feeding rotifer culture and for maintaining green water in the rearing tanks, the stock culture of algae such as *Nanochloropsis oculata* and *Isochrysis galbana* were maintained in a stock culture room at 24 °C in 500- 4000 ml flasks and then the cultures were upscaled to 20-litre flasks for feeding. Rotifer, *Brachionous rotundiformis* and *B.plicatilis* were cultured by feeding a mixed culture of *N. oculata*, *I.galbana* in equal proportions. *Artemia* nauplii were produced by hatching commercially available artemia cysts (Microfeast® Artemia, U.S.A.).

Algal density was maintained between 1×10^5 – 3×10^5 cells ml^{-1} in the larval rearing tanks. Larvae were stocked at the rate of 2 nos. L^{-1} and 100 larvae were stocked in 50 l of water. For calculating larval survival and growth, three replicates were provided. Larvae were fed on rotifers from 1 to 10th day of hatch, from 1st to 5th day on *B.rotundiformis* and from 5th to 10th day on *B.plicatilis* at the rate of 8 nos. ml^{-1} . *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. The particulate feed was started from the 25th day onwards using ground pellet feed from 200 to 500 micron. They were given boiled mussel meat from the 30th day onwards. Morphological changes were noted and measurements were taken on every 5th day from the 1st to 45th day of hatching. After that measurement were taken every 10 days upto the 110th day. Each time 5 individuals were taken for making the measurements.

Results

Spawning took place in the early morning hours. Eggs were found attached to the substratum and they were capsule shaped (Fig 1).

Male parent exhibited a high degree of parental care for the eggs, such as fanning the eggs with the pectoral and caudal fins and removing the infected



Fig 1 — *A.ehippium* broodstock

eggs with its mouth. Eggs measured 1350.147 - 1541.976 μ along the vertical axis and 535.925 - 717.463 μ along the horizontal axis. Spawning frequency was observed from 07-05-2015 to 20-03-2016. Spawning frequency was found out to be 2-3 times per month and 1 per month during January, February and March. The eggs hatched on the 6th day of incubation. Hatching took place in the early evening hours at about 7.00-8.00 pm. Since the larvae are photopositive they were attracted by a torch and transferred to a small trough and then transferred to the larval rearing tanks.

Eggs were observed soon after fertilisation, they were capsule shaped and attached to the substratum. At the initial stage, the cytoplasm was not visible as the egg is macrolecithal and highly telolecithal. The yolk was yellowish-orange in colour and contained many tightly packed oil globules. Fertilisation helps in egg activation and thereby activates the cytoplasmic movements. Now the cytoplasm at the animal pole starts to cleave, after few minutes after fertilisation the micromeres occupy the top of the yolk and the micromeres at the top come to possess definite cell boundaries in contrast to the ones and the bottom which merge with the underlying yolk mass. The first divisions occur in synchrony and the cleavage is meroblastic and discoidal. (Fig 2)

The cleavage occurring in the blastodisc is described alongside. The first cleavage is meridional producing 2 equal blastomeres. Second cleavage is at right angles to the first producing 4 blastomeres. The successive cleavages produce 8,16,32 and 64 celled stages respectively after the 6th cleavage the division became asynchronous. From the 10th division onwards the mid-blastula transition begins which is characterised by the slowing down of cell division and increased cell movement.

During blastulation 3 distinct cell populations can be recognised such as yolk syncytial layer consisting of the cell which remains in contact with the underlying yolk, the enveloping layer which consists of purely blastodermic cells and an extraembryonic protective layer known as the deep cell layer which lies between the above 2 layers⁹. The blastoderm resembles a mount perched on top of the yolk¹⁰

The next phase is gastrulation which results in the formation of the three germ layers the first step is epiboly, here the blastoderm moves and expands over the underlying yolk. Blastoderm develops a thickening called the germ ring which is composed of an outer epiblast and inner hypoblast. The hypoblast functions as the presumptive endoderm and mesoderm. Epiblast and hypoblast intercalate together to form the embryonic shield which functions as an organiser. Tail bud begins to develop during this stage.

On the 2nd day the yolk showed pigmentation with the prominence of stellate melanophores and during this stage, the head bud was clearly visible. Neurulation is initiated. Organogenesis also begins during this stage and metamerism is also evident in the larval body which is manifested by the presence of paired somites. Oil globules were also apparent. Body length does not increase considerably. Rudiments of the optic cup begin to appear (Fig 3)

On the 3rd day, the larval body was visible and melanophores are also visible on the yolk mass and the larva has started actively utilising the yolk. Brain differentiates into 3 parts and optic vesicle develops as outgrowths of the prosencephalon and the optic vesicle transforms into the optic cup which then induces the overlying ectoderm to produce a lens placode which then differentiates into a lens. The lens then induces the differentiation of the cornea. Pigmentation of the eye is also clearly visible. Detection of heartbeat and blood flow can be taken as an indication of the development of heart and a definite circulatory system. Embryo also shows body reversal by positioning its head opposite to the stalk. (Fig 4)

On the 4th day, the full larval body is visible and melanophores have started to develop in the head region. Some part of the larval body shows movement (Fig 5)

On the 5th day, Larva shows full-fledged movement inside the egg and the larva has come to occupy 3/4th of the egg capsule. (Fig 6)

On the 6th day, Branchial arches and jaws have completed development and the embryo completely

encompasses the capsule with the eyes showing silver colour as an indication that they are about to hatch and before hatching the larva shows vigorous movements in the egg capsule and the body shows muscular contractions. The rigorous movement of the larva enables them to break the capsule and come out. (Fig 7)

The newly hatched larvae measured about 4.96mm in total length and 3.9mm in standard length. (Fig 8)



Fig 2 — *A.ehippium* egg 1st day



Fig 3 — *A.ehippium* egg 2nd day

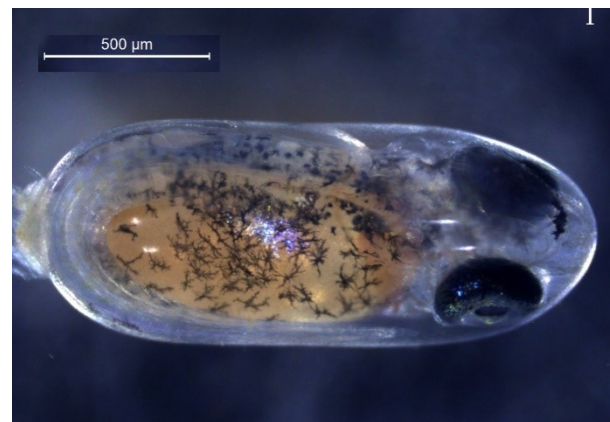
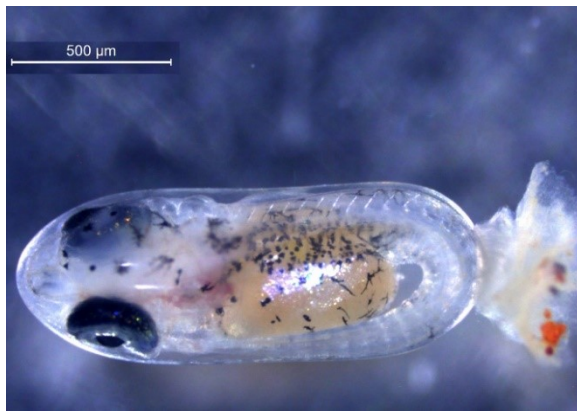


Fig 4 — *A.ehippium* egg 3rd day

Fig 5 — *A. ephippium* egg 4th dayFig 6 — *A. ephippium* egg 5th day

and Table 1. Larvae were transparent except for the presence of yellow pigmentation in the gut. Gut is slightly protruded. Scattered melanophores were also observed in the ventral midline till the 3/4th of the body. Dorsal, pelvic and anal fins were fused together to form a single fin fold which is present along the 3/4th of the body along the sagittal plane. Mouth is open and the larva has started feeding on the first day itself. On the 2nd day pigment granules in the middle of the body increased in size and the head pigmentation which had begun to manifest itself on the first day had begun to show prominence. The caudal fin had also begun to separate from the rest of the fin folds. Larval dissection revealed the presence of a well-equipped digestive system. On the 3rd day, the caudal rays were clearly visible and notochord flexion is also evident. Dorsal and anal fins also have started to develop and the fins rays were clearly visible. Pigments on the middle of the body gave way for stellate melanophores. The operculum also began to show signs of pigmentation. On the 5th day, the yellow pigmentation began to spread along the whole

body leaving out the head which was transparent with the exception of stellate melanophores (Fig 9)

The yolk sac has shrunk to a considerable size. On the 10th day, a distinct black line had begun to form in the middle of the body. Table 1 & (Fig 10).

The fins were transparent without any pigmentation. Teeth had begun to form in the upper and lower jaws. The yellow pigment had also begun to spread to the head. The headband and middle band had begun to appear on the 10- 12th day but the caudal fin showed no

Fig 7 — *A. ephippium* egg 6th dayFig 8 — *A. ephippium* newly hatched larvaeFig 9 — 5th-day larva

signs of bifurcation and the whole larval body now attained a yellowish colouration. The adult body colouration was not yet visible. On the 14th day, the larva started to display adult pattern of body colouration. The yellowish orange colour of the adult predominated in the head region and in the parts of the anal, dorsal and caudal fins which are attached to the body. The pectoral fins showed adult colouration. Bands showed increased thickness and intensity. (Fig 11)

On the 20th day, the headband showed a drastic increase in thickness and the yellowish pigmentation had started to progress from the margins proximal to the distal region but still much of the fin was transparent at the tips. On the 25th day, the thickness of the middle band increased and the larva transformed into a juvenile fish in which most of the adult characters except the banding pattern is the same. The larvae began to move from the top to the bottom layer of the tank. On the 30th day, the middle band has extended into the dorsal fins(Fig 12).

Table 1 — Summarised morphometric data for hatchery-reared *A. ephippium*. The firstline:mean ±standard deviation, secondline: range.

Days	Total length (mm)	Standard length (mm)	Head length (mm)	Body Width (mm)
1	4.96±0.21	3.9±0.1	1.3±0.19	0.89±0.11
	4.7-5.5	3.8-4	1.1-1.4	0.75-1
5	6.19±0.19	4.42±0.13	1.34±0.11	1.04±0.09
	6-6.5	4.3-4.6	1.2-1.5	1-1.2
10	7.08±0.28	5.02±0.15	1.77±0.11	1.45±0.1
	6.9-7.5	4.8-5.2	1.6-1.8	1.3-1.5
15	8.08±0.30	5.34±0.29	1.96±0.15	1.74±0.05
	7.7-8.5	4.9-5.5	1.7-2.1	1.7-1.8
20	9±0.21	6.64±0.23	2.24±0.09	2.14±0.11
	8.7-9.2	6.4-7	2.1-2.3	2-2.23
25	9.66±0.27	6.78±0.22	2.3±0.1	2.18±0.08
	9.4-10.1	6.5-7	2.2-2.4	2.1-2.4
30	10.64±0.19	7.36±0.17	2.63±0.08	2.8±0.21
	10.4-10.9	7.1-7.5	2.5-2.7	2.5-3
35	11.76±0.33	7.66±0.86	2.74±0.21	2.98±0.36
	11.4-12	6.6-8.7	2.6-2.9	2.6-3.4
40	13.4±0.26	11.74±0.92	3.9±0.28	4.7±0.42
	13-13.5	10.4-12.6	3.6-4.2	4-4.9
45	14.8±0.18	12.5±0.22	4.82±0.52	5.72±0.19
	14.6-14.9	12.2-12.8	4.3-5.5	5.5-6
50	15.58±0.18	13.72±0.13	5.2±0.76	7.3±0.45
	15.4-15.8	13.6-13.9	4.5-6	7.0-8
60	18.8±0.16	15.68±0.19	6.4±0.65	9±1.00
	18.6-19	15.4-15.9	5.5-7	8-10
75	19.48±0.13	17.44±0.18	7.1±1.24	11.1±1.24
	19.3-19.6	17.2-17.6	6-9	10-13
85	20.28±0.28	18.42±0.18	8.8±1.79	14±2.00
	20-20.6	18.2-18.6	7-11	12-16
110	22.62±0.33	18.76±0.34	12±0.11	21±0.27
	22.1-22.9	18.3-19.2	12-14	18-25



Fig 10 — 10th-day larvae



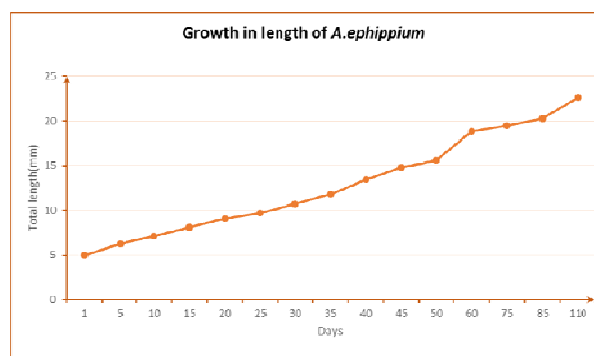
Fig 11 — 15th-day larvae



Fig. 12 — 30th-day larvae



Fig 13 — 160-day old sub-adult

Fig 14 — *A. ephippium* 300th dayFig 15 — Growth in length of hatchery produced *A. ephippium*

The banding pattern has reached its peak on the 45th day in which both the bands are visible and were surrounded by black pigments. After the 45th day, the bands start to disappear. On the 50th day, much of the middle portion of the middle band has disappeared and the surviving portion is restricted to two sides of the body. On the 55th day of the remaining 2 sides of the band, one near the dorsal fin had disappeared completely. Some larvae showed complete banding pattern. This broken middle band started regressing from the 120th day onwards and completely

disappeared in all the larvae by the 160th day (Fig 13).

Now the fish resembles an adult *frenatus*. Colour pattern is now deep orange which corresponds to the colour pattern of an adult fish. Fins are light orange in colour. The headband had begun its disappearance by the 240th day and by the 310th day all the bands have disappeared and now the sub-adult fish has transformed into adult fish. (Fig 14). The growth in length was slow at first but it reached its maximum by the 110th day (Fig 15).

Discussion

The hatching took place on the 6th day of incubation. Similar observations were made in *Premnas biaculeatus*¹¹ and in *Amphiprion nigripes*⁵. Though in *A. nigripes* prevalence of low temperature prolonged the hatching to 7 days. Organogenesis began as early as the 2nd day in which the rudiments of the optic cup were visible, this was in contrast with *Pseudochromis polycanthus* in which the lens development began as early as the 2nd day¹². Muscular development in *A. ephippium* starts from the 2nd day which is evident from the presence of myotomes but in *A. percula* the muscle development is inconspicuous during the 2nd-day¹³. But the development of the heart of *A. chrysopterus* and *A. trilineatus* precedes that of *A. ephippium* with the presence of a heart detectable on the 2nd day itself¹⁴. The brain differentiation took place on the 3rd day, a similar pattern of brain development was observed in *A. polymnus*.¹⁵ The hatching was enabled by the vigorous movements of the larva in the capsule which is a common observation among Pomacentrids. But it is uncommon in other fish species such as Cichlids, in a cichlid, *Cichlasoma dimerus*, the larva secretes eclosion enzymes to moisten the capsule and comes out. *A. ephippium* juveniles hatched at a total length of 4.96 mm with functional eyes whereas the total length of the hatchlings were 3.9 in the case of *A. nigripes*⁵ and 2.44 mm in *Neopomacentrus cyanomos*¹⁶. Larvae had a well-developed eye and mouth which differentiates clown fish development from that of damselfishes in most of the damselfishes like *Pomacentrus amboinensis* the mouth and eye development is incomplete at the time of hatching¹⁷. The larvae were transparent and the dorsal, pelvic and anal fins were fused together to form a single fin fold which was present along the 3/4th of the body. Similar observations were made in *A. nigripes*⁵, but in *A. ephippium* yellow pigmentation was present in the gut

and the middle of the body. On the 10th day, the fins were clearly visible in *A. ephippium*, fin development in *A. sebae* followed a similar pattern¹⁸. Banding began to appear in *A. ephippium* from the 10th day onwards, whereas no band formation was evident in *A. nigripes* on the 12th day. Larvae started feeding actively from the first day onwards. The food included rotifers, artemia, boiled mussel meat and pellet feed. This could also be supplemented with mysids which can be cultured in the hatchery¹⁹. On the 20th day, the black pigment granules increased in number and black pigment granules were also visible on the fins whereas in the case of *A. nigripes* scattered dark pigment granules were observed on the body. On the 20th day the headband showed a drastic increase in thickness and on the 25th day the thickness of the middle band also increased but in case *A. ocellaris* all the three body bars become opaque and fully developed from the 14th -30th day²⁰. Another notable difference between the banding pattern of the two species is that but in the case of *A. ephippium* the banding pattern has reached its peak during the 45th day and after that, the bands slowly regress. Whereas in *A. ocellaris* the 45th day is marked by the production of heightened pigmentation on the caudal fin. On the 14th day the pectoral, pelvic, dorsal and anal fins display pigmentation whereas in the case of *A. ocellaris* early stages of pigmentation are visible only on the caudal fins by the 40th day. In *A. ephippium* the 50th to 55th day is characterised by the breaking and partial disappearance of the middle band whereas in *A. ocellaris* heavy black lines develop along bars from 40- 58 days. In *A. ephippium* the middle band disappeared by the 160th day whereas in *A. ocellaris* all the larvae attained adult pigmentation by about 80 days. The fishes reach a marketable size on the 6th month.

Conclusion

The present research is a complete study on the larval development of economically important fishes. This helps us to find out different strategies to compare the growth rate of different clown fishes and find out the fastest growing one and understand which one exhibits the fastest growth when provided with optimum feed conditions.

Acknowledgment

We would like to extend our warm gratitude to Dr A. Gopalakrishnan, Director, ICAR-Central Marine Fisheries Research Institute, Kochi, for the permission granted and facilities provided.

Reference

- 1 Dam Roy, S., Raymond, S., Jani Angel and Rohith Kumar, K., National Seminar on "Fisheries and Aquaculture: Livelihood Security, Sustainability and Conservation" (2016).
- 2 Leal, M.C., Puga, J., Serodio, J., Gomes, N.C.M. and Calado, R., Trends in the discovery of new marine natural products from invertebrates over the last two decades – where and what are we bioprospecting? PLoS ONE 7, e30580. (2012)
- 3 Thornhill, D.J., Ecological impacts and practices of the coral reef wildlife trade. (2012) 179 pp.
- 4 Anil, M.K., Kakati, V.S., Ganga, U and Sherly Zacharia., Larval rearing of seahorse *Hippocampus kuda* under laboratory conditions. *Marine Fisheries Information Service*. (1999)
- 5 Anil, M. K., Santhosh B., Prasad, O and Rani Mary George., Broodstock development and breeding of black-finned anemonefish *Amphiprion nigripes* Regan, 1908 under captive conditions *Indian J. Fish.*, 59(1) (2012): 77-82
- 6 Anil, M. K., Santhosh B., Jasmine. S., Reenamole, S., Unnikrishnan, C and Anukumar, A., Techniques for the mass production of two species of clown fish: Clown anemonefish *Amphiprion ocellaris* Cuvier, 1830 and Spinecheek anemonefish *Premnas biaculeatus* (Bloch, 1790). In: Felix, S. (Ed.), The proceedings of the National Seminar on technology and trade prospects in Ornamental aquaculture, Fisheries Research and Extension Centre, Tamil Nadu Veterinary and Animal Sciences University, Chennai., (2010) 96-102.
- 7 Rema Madhu and K. Madhu., Occurrence of anemonefishes and host sea anemones in Andaman and Nicobar Islands, *J. Mar. Biol. Ass. India*, 49 (2): (2007) 118 – 126
- 8 Allen, G. R. *The anemonefishes: their classification and biology*, T.F.H. Publications, (1972) 210 pp
- 9 Scott, F. Gilbert. *Developmental biology 9th ed.* Sinauer associates (2010).
- 10 Mejjide, F.J and Guerrero, G.A. Embryonic and larval development of a substrate-brooding cichlid *Cichlasoma dimerus* (Heckel, 1840) under laboratory conditions *J. Zool.*, Lond. (2000) 252, 481-493
- 11 Madhu, K., Rema Madhu and Rethesh.T. Broodstock development, breeding, embryonic development and larviculture of spine-cheek anemonefish, *Premnas biaculeatus* (Bloch, 1790) *Indian J. Fish.*, 59(1) : (2012) 65-75
- 12 Ike Olivotto., Arianna Rollo., Roberto Sulpizio., Matteo Avella., Luca Tosti., Oliana Carnevali. Breeding and rearing the Sunrise Dottyback *Pseudochromis flavivertex*: the importance of live prey enrichment during larval development. *Aquaculture* (2006) 255: 480 – 487
- 13 Dhaneesh, K.V., Ajith Kumar, T.T. and Shunmugaraj, T. Embryonic Development of Percula Clownfish, *Amphiprion percula* (Lacepede, 1802). *Middle East Journal of Scientific Research*. (2009) 84-89.
- 14 Allen, G. R., *Damsel fishes of the World*, Mergus Publishers, Melle, Germany, (1991) 10, 11, 271 pp.
- 15 Sukjai Rattanayuvakorn., Pisut Mungkorakarn., Amara Thongpan and Kannika Chatchavalvanich Embryonic Development of Saddleback Anemonefish, *Amphiprion polymnus*, Linnaeus (1758) *Kasetsart J. (Nat. Sci.)* (2005) 39: 455 – 463
- 16 Rohini Krishna, M.V., Anil, M.K., Neethu Raj P and Santhosh, B. Seed production and growth of *Neopomacentrus cyanomos* (Bleeker, 1856) in captivity. *Indian J. Fish.*, (2016). 63(3) : 50-56.

- 17 Murphy, B. F., Leis, J. M and Kavanagh, K. D. Larval development of the Ambon damselfish *Pomacentrus amboinensis*, with a summary of Pomacentrid development. *Journal of Fish Biology* (2007) 71, 569–584
- 18 Sreeraj, G., .Studies on the reproductive biology, breeding and larval rearing of selected marine ornamental fishes belonging to the Family Pomacentridae. PhD Thesis. Central Institute of Fisheries Education, India, 2002.
- 19 Neethu Raj, P., Anil, M.K and Rohini Krishna, M.V. Seasonal availability and population structure of *Eurobowmaniella simulans*. W. Tattersall, 1915 (Crustacea: Mysida) along Vizhinjam coast, Kerala, India. *J. Mar. Biol. Ass. India*, (2016). 58 (1), Jan-June.
- 20 Bertschy, A. Essais de reproduction en aquarium d'*Amphiprion ocellaris* Cuv. (Pomacentrides). *Rev. Fr. Aquariol.*, 6 (Sept 79), (1979) 3:91-94