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# Spawning behaviour and embryonic development in the sebae anemonefish *Amphiprion sebae* (Bleeker, 1853)

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## ABSTRACT

The present paper describes the spawning behaviour and embryonic development of *Amphiprion sebae* (Bleeker, 1853) under captive conditions. Ten functional breeding pairs of *A. sebae* ranging in total length from 90 to 100 mm (female) and 60 to 80 mm (male) collected from the inshore waters of Gulf of Mannar, along with sea anemones, *Stichodactyla haddoni* were acclimatised to captive conditions in 500 l glass tanks fitted with biofilter. Fishes spawned within 45 to 60 days of rearing. Sexual dichromatism was visible in spawning pairs during the breeding season. The number of eggs in each clutch varied between 300 to 1500 and the interval between successive spawning ranged between 15 - 26 days. The eggs were adhesive, capsule shaped and bright orange in colour measuring 2.10±0.031 mm in length and 1.02±0.037 mm in width. The eggs were bright orange for initial two days, turned black on 3rd to 5th day and silvery on 6th to 7th day of incubation. The embryonic development was divided into two phases based on the morphological characteristics. The overall understanding of spawning behaviour and embryonic development of *A. sebae* could help to improve the commercial production and culture of other coral reef fishes as well.

Keywords: *Amphiprion sebae*, Anemonefish, Embryonic development, Sebae anemonefish, Spawning

## Introduction

The tropical marine anemonefishes belonging to the family Pomacentridae and subfamily Amphiprioninae are of prime importance in marine ornamental fish trade. Among the marine ornamental fishes, the family Pomacentridae, is more commonly traded which accounts for 43% of all fish traded (Wabnitz *et al.,* 2003; Gopakumar, 2008). *Amphiprion sebae* (Bleeker, 1853) is one of the popular species in the aquarium trade due to its attractive colouration, hardy nature, symbiotic association with carpet sea anemone and display of interesting behaviour. Colour pattern is the most important feature for identifying anemonefishes. Identifying characters of *A. sebae* are dark brown to blackish colour with two milky white bars, the mid-body bar slanting slightly backwards and extending onto rear part of dorsal fin, snout, breast and belly often orange or yellow orange in colour and orange or yellow coloured tail.

Compared to other marine ornamental fishes, anemonefishes have some remarkable behavioural characteristics such as their distribution in shallow waters due to their fascinating relationship with specific sea anemone species (Fautin, 1991). Most fish species are repelled by the poisonous stings of anemones, while clownfishes have immunity to this defence and is therefore able to use it for its own protection against predators. In return, clownfish keep their host anemone in a healthy state and prevent them from being attacked by predators, and produce sounds during agonistic interactions involving conspecifics or heterospecifics (Colleye *et al.,* 2009). The anemonefishes are characterised by the formation of a small social group that includes a monogamous pair of sexually functional adults and from zero to three sexually non-functional sub adults or juveniles (Moyer and Nakazono, 1978). They are protandrous hermaphrodites, starting life as male and later changing to female (Allen, 1972; Fautin, 1991). In an anemonefish population, the juveniles are ambosexual. At first, only testicular tissues mature and under certain conditions conversion of a functional male into a female take place by degeneration of testicular tissue and transformation to ovaries (Moyer and Nakazono, 1978).

Studies on anemonefish taxonomy, behavioural ecology and rearing methodology of many tropical and subtropical pomacentrid species have been well documented. However, description of spawning behaviour

and development of the embryo are available only for few pomacentrids. Hatching and chronological flow of embryonic development stages differs with species and the specific environmental conditions. The present study investigated the spawning behaviour and embryonic development of *A. sebae,* which would help to improve our understanding of the developmental biology and rearing methods of coral reef fishes in general.

## Materials and methods

*Broodstock maintenance*

Functional pairs of *Amphiprion sebae* having total length ranging from 90 to 100 mm (female) and 60 to 70 mm (male) and host anemone *Stichodactyla haddoni* were collected from the Gulf of Mannar (8°47' to 9°15'N; 78°12' to 79°14'E). The anemonefish colonies were located by diving and the individual colonies were collected with scoop net and along with sea anemones and they were transported to the Marine Finfish Hatchery at Mandapam Regional Centre of ICAR-CMFRI, Tamil Nadu, India. These functional pairs were acclimated to captive conditions in 500 l glass tanks (temperature: $28\pm5^{0}C$ and salinity: 33±2 ‰) fitted with biological filters. The fishes were fed *ad libitum* twice daily (09.00 and 16.00 hrs) with wet feeds such as squid meal, fish roe and also live feeds like *Artemia* nauplii. Fifty percent of water exchange was done weekly. Earthen pots were placed inside the tanks to provide spawning substratum.

*Behavioural observations*

Observations on spawning behaviour were made three times a day (at 08.00, 11.00 and 18.00 hrs) during the experimental period. Spawning took place after 45 to 60 days of rearing. The total number of eggs per clutch was estimated by counting all eggs in 1 $cm^2$ area and then multiplying with the total area of deposition. Even though both the parents showed parental care, male fish was more involved in caring the eggs until hatching.

*Sampling of embryos and embryological observations*

Egg samples were collected immediately after fertilisation by scraping individual eggs from each clutch using 'needle' and collected with 'ink filler' every day at a prefixed time till hatching. The eggs were observed immediately and their size was measured under light microscope. The length and width of the eggs were measured to the nearest 0.01 mm. The developmental stages were observed and sequenced based on morphological features. Photographs were taken with a digital camera for documenting major morphological and functional features of embryos. Five embryos were sampled at a time and images of embryonic development were taken at 15 min interval on first day, at 1 h interval on the second day and at 6 h interval from third day after fertilisation until the eggs hatched. The embryonic development time was expressed as hours post-fertilisation (hpf).

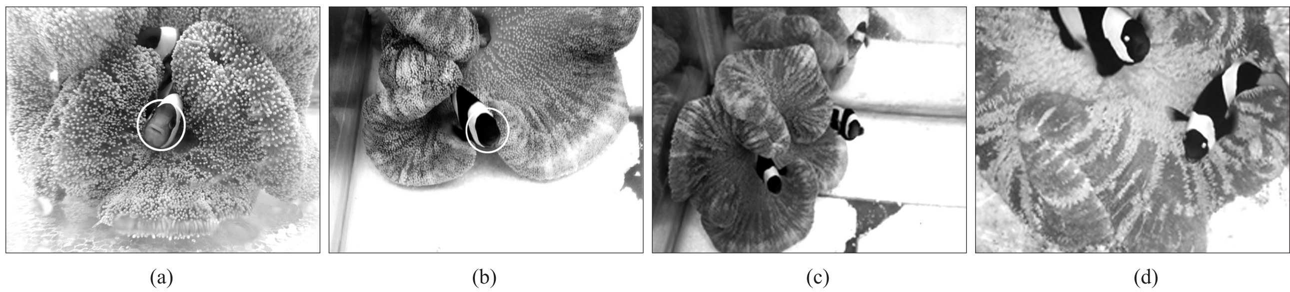
## Results and discussion

*Spawning behaviour*

*A. sebae* are found together with their host anemones in small social groups that included a monogamous pair of sexually functional adults and from zero to three sexually non-functional sub-adults or juveniles. The single female in a particular social group was larger than her mate. Sexual dichromatism was visible in spawning pairs during the breeding season (Fig. 1). The snout of the male was conspicuous with an intense yellow colour (Fig. 1a, c), whereas that of the female was dusky yellow (Fig. 1b, c). Snout colour became dusky at the end of the breeding season in both male and female (Fig. 1d). The first indication of the spawning readiness is that the male swims up and down in front of the female and this behaviour is termed as "clownfish waggle" (Gopakumar *et al*., 2001). In the present study, before spawning, an increased social interaction was observed in each breeding pair, which was expressed by chasing, fin erection, rapid up and down swimming, waggle movement and nibbling his mate by the male which was also reported in studies on other species of anemone fish (Madhu *et al*., 2012). At this time, the pair also selected a suitable site after clearing algae and debris on the pot near to sea anemone for laying eggs. Prior to egg laying, both parents actively cleaned the site with their mouth while standing in a head down and tail up position. Before spawning, the mating pair swam side by side and spawning commenced within an hour. Female laid adhesive, capsule-shaped eggs on the cleaned substratum in almost circular or oval patches and subsequently the male fertilised the eggs. The spawning activity lasted from an hour to an hour and fortyfive minutes. The eggs were deposited on the earthen pots. Spawning occurred between 11.00 to 13.00 hrs, and this is supported with the findings of Ignatius *et al*. (2001) in *A. Sebae*. This is in contrast to other species in the same family, as in the case of *Amphiprion clarkii* (Alava and Gomes, 1989) and *Amphiprion percula* (Malpass, 1996), fishes spawned during the evening hours. The number of eggs per spawning varied between 300 to 1500 at every 15 to 26 days interval.

*Parental care*

Both male and female brooders exhibited parental care during incubation period through two basic activities *viz*., fanning and mouthing. The unfertilised, dead or weakened eggs and dust particles were removed by mouthing. Colour change in the clutch was observed as development progressed. For the first two days, the eggs were pale yellow/orange in colour, later turned to dark brown



(a) (b) (c) (d)

Fig. 1. Sexual dichromatism in *Amphiprion sebae* during breeding season. (a): male with intense yellow colour in snout, (b): female with dusky yellow colour in snout, (c): functional breeding pair showing sexual dichromatism and (d): change in sexual dichromatism at end of breeding season (both male and female with dusky yellow colour in snout)

to dark black. As embryonic development progressed, the eggs turned silvery on 7th day due to the development of large eye, which is usually a good indication for the hatching of eggs within 12 h. The present observations on colour change, shape of the eggs and parental care observed were similar to the observations as reported in other anemone fish species (Ignatius *et al*., 2001; Madhu *et al*., 2006; Rema *et al*., 2012). The details of colour variation observed in the eggs during incubation are given in Table 1 and Fig. 2.

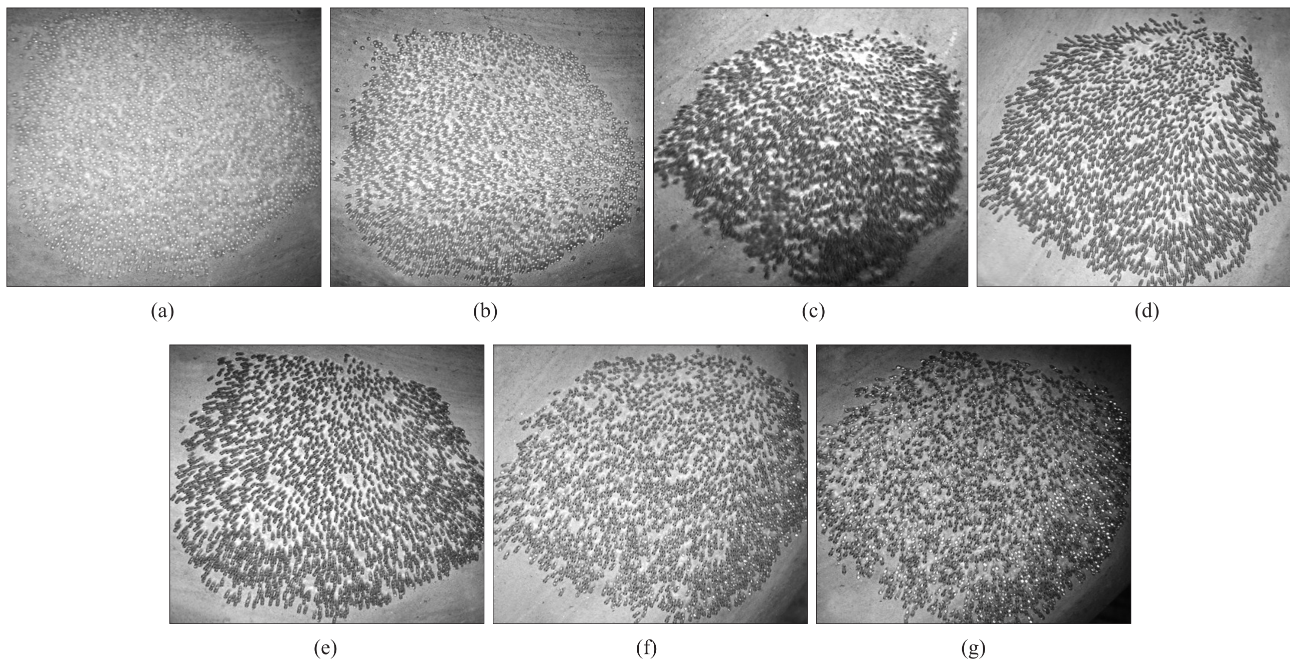
The embryonic development of *A. sebae* can be classified into two phases (Fig. 3, 4 and 5). The first phase is the egg cleavage phase; the interval between the first cell division and the appearance of recognisable precursors of the organ systems, namely, the neural plate. The second or the embryo phase begins when the embryo becomes recognisable as a vertebrate.

Table 1. Colour variation in the fertilised eggs of *A. sebae* during incubation period

| Days post-hatch | Colour |
|---|---|
| 1 | Light orange |
| 2 | Bright orange |
| 3 | Dark brown |
| 4-5 | Pale black |
| 6 | Dark black |
| 7 | Dark black with silvery ring on head |

*Oocytes*

The eggs of *A. sebae* are classified as polylecithal with multiple oil globules and the newly laid eggs were



(a) (b) (c) (d)

(e) (f) (g)

Fig. 2. Colour variation in fertilised eggs of *Amphiprion sebae* during incubation period (a): day 1, (b): day 2, (c): day 3, (d): day 4, (e): day 5, (f): day 6 and (g): day 7

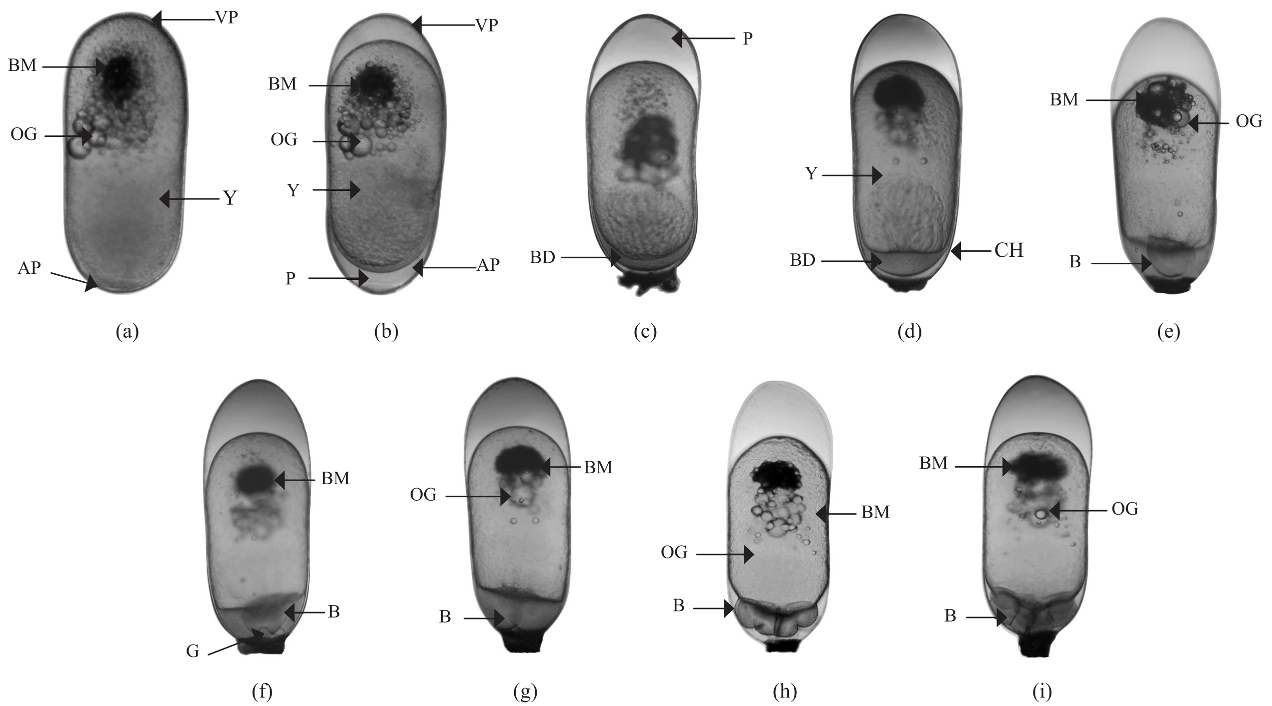



Fig. 3. Embryonic developmental stages of *A. sebae* from unfertilized egg to 8 cell stage, showing animal pole (AP), black mass (BM), blastodisc (BD), blastomeres (B), chorion (CH), groove (G), oil globule (OG), perivitelline space (P), vegetal pole (VP) and yolk mass (Y).

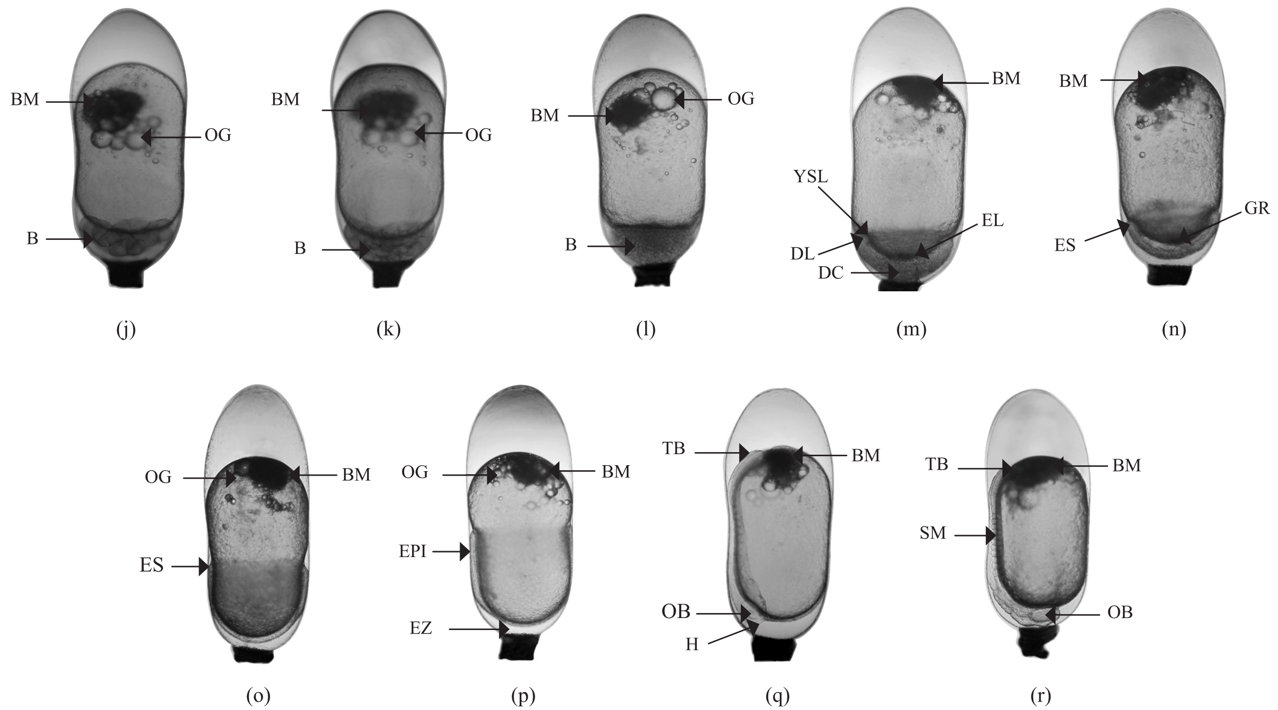



Fig. 4. Embryonic developmental stages of *A. sebae* from 16 cell stage to neurula stage, showing black mass (BM), blastomeres (B), deep cell (DC), dorsal lip (DL), embryonic shield (ES), envelope layer (EL), epiboli (EPI), evacuation zone (EZ), germ ring (GR), head (H), optic bud (OB), oil globule (OG), somites (SM), tail bud (TB) and yolk syncytial layers (YSL).

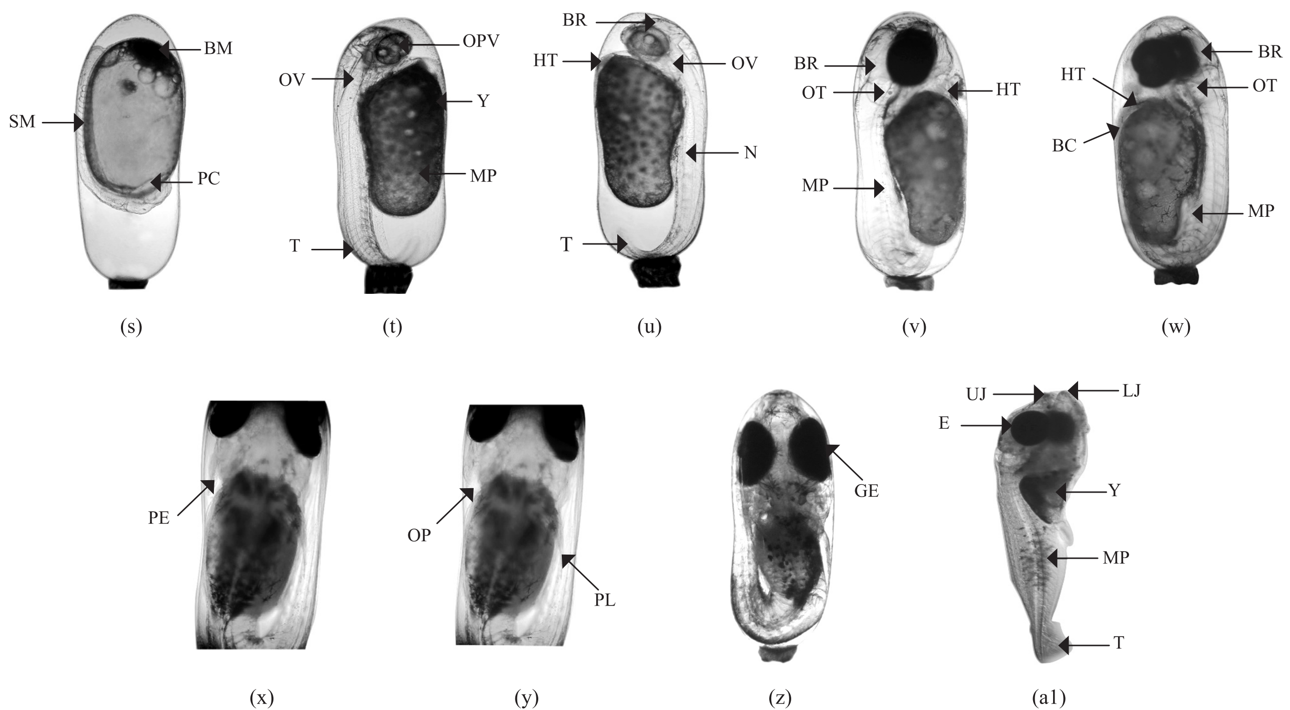



Fig. 5. Embryonic developmental stages of *A. sebae* from notochord formation to hatching, showing black mass (BM), brain (BR), blood circulation (BC), glowing eyes (GE), heart (H), lower jaw (LJ), melanophore (MP), notochord (N), optic vesicle (OPV), otic vesicle (OV), otolith (OT), opercula (OP), pericardial cavity (PC), pectoral fin (PE), pelvic fin (PL), somites (SM), tail (T), upper jaw (UJ) and yolk mass (Y)

orange in colour, capsule shaped and slightly curved around the middle part of yolk. Present study showed that the eggs of *A. sebae* were larger in size (2.1 mm length and 1.02 mm width) as compared to those of *A. ocellaris* (1.8 mm in length and 0.8 mm width) (Yasir and Qin, 2007), and similar to those of *A. percula* (2.0 to 2.3 mm length and 1.0 to 1.2 mm width) (Dhaneesh, 2011). The eggs were covered with transparent chorion with narrow perivitelline space. After attachment of eggs to the substrate, a border between the animal pole and the vegetal pole was developed by the accumulation of cytoplasm at the animal pole. The egg yolk was orange in colour with dispersed fat globules (Fig. 3a). Newly laid eggs had one large oil droplet and assorted small ones. The small oil droplets unite with the large ones during development. There was a large black mass at the vegetal pole surrounded by oil droplets. The animal pole was characterised by its half circle shape which attached to egg laying material, while the vegetal pole had yolk and fat globules of varying sizes. The fertilised eggs hatched after 6-7 days of incubation.

*Egg activation*

Few minutes after spawning (5 to 15 min), there was a flow of water into the space between the cortex and chorion, starting from the animal pole to the vegetal pole. Water entering the egg forcibly compressed the yolk line and reached the vegetal pole (Fig. 3b) to form the perivitelline space. This process took about 30 min to complete. The black mass observed in the embryo was surrounded by numerous small oil globules dispersed in the vegetal pole, as reported by Yasir and Qin (2007). Perivitelline space at the vegetal pole was larger than that at the animal pole. Immediately after formation of the perivitelline space, the cytoplasmic area in the animal pole became thicker with an obvious blastodisc (Fig. 3c). Yolk sac in fertilised eggs shrank in size after the formation of the perivitelline space but the size of the whole egg did not change. One hour after spawning, a dome shaped blastodisc was formed, signaling the completion of the activation process (Fig. 3d).

*Cleavage phase*

Stage I (one cell): Cytoplasm of fertilised egg was clear in the uncleaved cell (Fig. 3e). Animal pole was characterised by its half circle shape which attached to the egg laying material while the vegetal pole contained yolk and fat globules of different sizes dispersed in it. This stage began at fertilisation and lasted until the first cleavage furrow was readily visible, about 1 hpf (Fig. 3f).

Stage II (2 cell): Cytoplasm moved as a stream down to the blastodisc, then retracted back, leaving a groove in the middle. Following the retraction of the cytoplasm, the first cleavage started by dividing the blastodisc into two blastomeres at 2 hpf. The cytoplasmic division followed meroblastic cleavage. Fat globules were very small and moved towards the vegetative pole (Fig. 3g).

Stage III (4 cell): Second cleavage took place in a vertical plane at right angle to the first and resulted in 4 blastomeres which were clearly visible after 2.20 hpf. (Fig. 3h).

Stage IV (8 cell): Eight blastomeres appeared after 3 hpf as a result of horizontal mitotic division and they were smaller than the ones in the previous stage (Fig. 3i).

Stage V (16 cell): Fourth cleavage was vertical and parallel to the first one, giving rise to 16 blastomeres which were clearly visible after 4.40 hpf. At this stage, the animal pole consisted of solid cluster of cells. One or two cells were centrally located with the others completely surrounding them (Fig. 3j).

Stage VI (32 cell): Fifth round of cleavage was noticed at 5.30 hpf. The small blastomeres became overlapping due to the confined space in the capsule (Fig. 4k).

Stage VII (64 cell): Sixth cleavage resulted in 64 blastomere stage which was observed at 6 hpf and the small blastomeres extended more laterally and arranged themselves as a flat layer at the animal pole (Fig. 4l). At the 64 cell stage, the blastomeres rearranged into two layers, indicating the completion of the cleavage stage at 11 hpf.

Stages VIII (Blastula): Blastomeres were very small and extended more laterally due to limited space within the capsule. Blastoderm expanded along and over the yolk mass while a cavity (segmentation cavity or blastocoel) was formed in its centre. Outer blastomeres formed the enveloping cell layer which roofed the segmentation cavity. Within the cavity, the spherical blastomeres (deep cells) were loosely arranged and centrally located. At the edge of the expanding blastoderm, small nuclei were organised in rows which appeared in the yolk syncitial layer. Embryos developed from a two layer blastomere are called morula having mulberry-like structures (Fig. 4m), where the blastodisc developed into blastoderm. This stage with the presence of yolk syncytial layer (YSL) and the dorsal lip (DL) was reached at 13.20 hpf, in *A. sebae*, whereas Rema *et al*. (2012) reported that 2, 4, 8, 16, 32, 64 cell stages and blastula stage was attained at 1.30, 1.50, 2.30, 3.30, 3.50, 4.15 and 5.58 hpf respectively in *A. ocellaris*. Duration of cleavage varies depending on species and environmental factors.

Stage IX (Gastrula): Blastomeres extended towards the vegetal pole and the expansion of blastoderm increased the coverage over the yolk sac. Yolk syncytial layer continued to expand along the embryonic shield (ES) and thus formed the germ ring GR (Fig. 4n). Prior to completion of the gastrula stage, the embryonic shield (ES) increased in length and the germ ring disappeared (Fig. 4o). The periblast (P) and the envelope layer gradually migrated towards the vegetal pole and the blastomeres moved from animal pole to cover a part of yolk called epiboly and it took about 19.00 hpf. At the end of epiboly, the yolk mass was covered by the yolk syncytial and enveloped cell layers (Fig. 4p).

*Embryo phase*

Stage I (Neurula): At 24 hpf, the process of cephalisation started and a beak like structure appeared in the anterior part of the embryo. Embryo began to form head and neural ectoderm. Brain was formed at the end of the embryonic body close to animal pole and optic buds were also noticed (Fig. 4q).

Stage II (Notochord formation): At 29.30 hpf, neural keel extended along the embryonic axis and the notochord appeared with the head and eye directed towards the attached end of the egg. Tail-bud appeared at the posterior part of the yolk mass. Blastopore covered 60 to 70% of the yolk sac and began to form the somites (Fig. 4r). Subsequently Kupffer's vesicle appeared at the ventral side of the embryo. Head was clearly visible and chromatophores appeared as black dots spreading all over the blastopore (Fig. 5s).

Stage III (Turnover): Body and tip of the tail was still attached to the yolk sac. Embryonic body started to turnover. Head orientation could be identified by the eye buds. Embryo made a 180° shift in its orientation by turning its head that was facing the attached end of the egg shell to the free end (Fig. 5t). Body turnover of *A. sebae* embryos occurred at 40 hpf while it was reported to occur at 44 hpf in *A. ocellaris* (Rema *et al*., 2012). Turnover stage seems to be a critical stage for survival since failure to turn the body around would lead to mortality of embryo (Yasir and Qin, 2007). By 44.30 hpf the nasal vesicle and brain were clearly visible in the transparent head, while the notochord was visible along the posterior body.

Stage IV (Heart formation): A few melanophores appeared and migrated across the embryo. During this time heart started to beat (50 to 65 bpm), which was much slower than that of *A. ocellaris* as reported by Rema *et al*. (2012). Clear differentiation of midbrain, hindbrain, forebrain and presumptive pericardial cavity and melanophores were noticed at 50.00 hpf (Fig. 3u).

Stage V (Otolith formation): Tail became separated from the yolk and moved freely with jerking movements. A pair of otolith was observed in ear as small granules at 56.00 hpf (Fig. 5v). Melanophores increased in the entire body especially in the head region and eye. Further increase in the tail movement and number of somites (30 to 40) were also observed.

Stage VI (Blood formation): At 78.00 hpf, the body length increased and tail moved freely. Embryo attained size larger than that of egg capsule. Blood circulation was clearly visible. Number of blood cells quickly increased and got circulated within the body and in blood vessels on the surface of the yolk sac (Fig. 5w). The heart beat also increased (75 to 80 bpm).

Stage VII (Pectoral fin bud formation): At about 90.00 hpf, the head got enlarged with a prominent big eye and brown pigments and the peritoneal cavity became dark as a result of melanophore migration. Heart developed and blood circulation was visible. Pectoral fin bud and maxilla formation also initiated (Fig. 5x).

Stage VIII (Opercular movements): Embryo further enlarged and occupied most of the space in the capsule. Its movement in the capsule was continuous. Melanophores became abundant in the head region. The pectoral fin was quite large. By 128 hpf gills and the opercles were distinct with movements (Fig. 5y).

Stage IX (Pelvic fin bud formation): Pelvic fin bud formation and pectoral fin advanced. Yolk sac became quite small and covered by the abdomen of the embryo. Head occupied one third of the capsule space. At 135 hpf, the embryo further enlarged about 1.5 times of the capsule and continuous movements were observed inside the capsule (Fig. 5y). *A. sebae* formed gills and opercula on the 5$^{th}$ day and the developmental rate of eye and digestive system were similar to those reported by Rema *et al*. (2012).

Stage X (Glowing eyes): At 150.00 hpf, the capsule was fully occupied by the embryo and the size of the yolk gradually decreased. Eyes were found glowing and rotating. Embryo completely occupied the space inside egg capsule and showed vigorous movement inside (Fig. 5z).

Stage XI (Hatching): Embryo reached 1.75 times of that of the egg shell length and the digestive tract was distinct. Embryo began to hatch by moving vigorously to break the capsule. Eyes were fully developed, body movements were intensified and the tail ruptured the egg shell and the larva with dorsal, pectoral, anal and caudal fins was released. Total length of larvae ranged between 4-5 mm. Fertilised egg took about 158 hpf to hatch and release larva (Fig. 5a1), whereas *A. ocellaris* embryo hatched at 168 hpf (Rema *et al*., 2012).

Spawning behaviour and time required for each major development stages from fertilisation to hatching in *A. sebae* was documented in this study. Comparison of developmental stages between *A. sebae*, as documented in the present study with other anemonefish species highlighted the variation among closely related species in the *Amphiprion* genus. Understanding of the spawning behaviour and embryological studies will be helpful for the hatchery production of these fishes. Development of culture technology is widely accepted as an environmentally sound way of increasing the supply of marine ornamental fishes and thereby reducing pressure on wild population. The overall understanding of spawning behaviour and embryological development of *A. sebae* may help to improve the commercial production and culture of other coral reef fishes as well.

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