Life history pathways in false clown *Amphiprion ocellaris* Cuvier, 1830: A journey from egg to adult under captive condition

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Original Article

Abstract

There is a vast literature on distribution, rearing conditions and behavioural studies of clownfishes. However, descriptive information on reproductive behaviour, early developments and life history pathways of *Amphiprion ocellaris* are scarce. In this study, 7 pairs of *A. ocellaris* having size 89 to 100 mm (presumptive females) and 40 to 60 mm (presumptive males) were developed through pair formation to generate information on breeding and spawning behaviour, egg morphology, and embryonic, larval, juvenile and adult developments. The spawning was found to be year-round with reproductive cycle of 12 to 15 days intervals and laid 300 to 1000 capsule shaped and adhesive type eggs. The eggs were 1.5-3.0 mm in length and 0.8-1.84 mm in width containing multiple oil globules of varying size and were pale yellow or white for initial two days, turned to black on 3rd to 5th day and silvery on 6th to 7th day of incubation. Hatching took place 168 h after fertilization at a water temperature of $28.5 \pm 0.5°C$. The ontogeny which began with activation of ovum was classified into embryonic, larval, juvenile, sub-adult, adult and senescent period. The embryonic developments were divided into 29 stages based on the morphological characteristics. The present study is the first scientific report in India on the life history pathways of *A. ocellaris* commencing from the time of embryonic development to senescent.

Keywords: False clown anemonefish, Amphiprion ocellaris, egg morphology, embryonic development, juvenile, adult, senescent.

Introduction

False clown anemonefish *Amphiprion ocellaris* is widely distributed in the coral reef ecosystems of tropical and subtropical region ranging from Indo-West Pacific: Indo-Australian Archipelago including India, Burma, Thailand, Malaysia, Indonesia, Philippines, New Guinea, New Britain, Solomon Islands, Vanuatu and Australia (Fautin and Allen, 1997). The anemonefishes belonging to the family Pomacentridae comprises of 27 species under the genus *Amphiprion* and one species in the genus *Premnas*, and are the most popular species in marine aquarium trade due to their attractive colouration, hardiness and interesting display behaviour, and are also model organisms for reproductive biology research. Moreover, fishes have a basal position in the vertebrate lineage and their embryos share general chordate characters with other vertebrates and most fish embryos develop externally from transparent eggs. These characteristics made them a special model for the study of vertebrate embryogenesis (Langeland and Kimmel, 1997; Falk-Petersen, 2005). Studies on embryonic and larval development of fish have been important to the basic knowledge of biology of species, primarily with relation to growth, feeding and behavioral aspects, confirm the spawning place of the species, and it also allows comparison between normal and altered development patterns (Meijide and Guerreiro, 2000;

Morrinson *et al.*, 2001). Studying the embryonic development is also important as it helps in determining the moment of yolk sac absorption and mouth opening, which varies among species. Although the embryonic development of *A. chrysogaster* (Allen, 1972) and *A. clarkii* (Wilkerson,2001) from the wild are described in general, information on the early embryonic developmental sequence from egg to new born of many marine fishes including clownfishes under captive conditions are still lacking. Observation on the morphological characters during early stages of development is the most reliable and practical indicator of quality or viability of eggs in teleost (Vallin and Nissling, 1998). Moreover interpretation of experimental studies requires the structural and temporal frame work provided by morphology and duration of developmental stages (Long and Ballard, 2001) as the embryonic development and hatching time varies with species and environmental conditions. Hence it is felt very essential to study the early ontogeny of *A. ocellaris* to enhance the existing knowledge about the developmental features and also to establish a model for comparison when the normal patterns of embryonic developments are altered. Moreover, larval fishes often display characteristic patterns of pigmentation which can be used to identify species at an early stage in their lives. Thus in the present study an attempt was made to document the behavior of courtship, spawning, parental care, morphological and embryonic, larval, juvenile and adult developments in the life history pathways of *A.ocellaris* under captive conditions to establish ontogenetic intervals to cater the knowledge for substantiating the captive breeding programme in India.

Material and methods

Seven adult pairs of *A.ocellaris* having size 89 to 100 mm (presumptive females) and 40 to 60 mm (presumptive males) were brought from Andaman and Nicobar islands during 2005, and acclimatized to the laboratory condition for two weeks and reared for 3 to 4 months in one ton FRP tanks for pair formation in the hatchery at CMFRI, Kochi. Each developed pairs were then transferred to separate 500 l perspex aquaria along with host anemone *Heteractis magnifica* to mimic the natural environment. Each tank was also provided with earthen pots, rough surfaced white ceramic tiles, PVC pipes, shells of edible oyster and coral rocks to observe their spawning behavior, egg deposition and embryonic developments. All the tanks were fitted with biological filter for water recirculation, and environmental parameters such as temperature, salinity, dissolved oxygen, pH, nitrite, nitrate, ammonia and photoperiod (12L:12D) were maintained and monitored once in 24 hrs, and once in a week 25% of water was exchanged. The brood fishes and anemones were fed two times per day with wet feeds such as meat of shrimp, green mussel and clam at the rate of 15% of their body weight and

also provided live feeds like *Brachionus plicatilis* and *Artemia* nauplii.

Behavioural observations

Observations on reproductive behaviour were made three times a day (8-9 am, 12-1 pm, 3-4 pm, and nocturnal behaviour during 8 to 10 pm) during the first 3 months prior to spawning. Parental care exhibited by the breeding pairs during incubation period and behaviour of larvae, juvenile and adults were also observed and documented.

Sampling of embryos and larvae

The samples of eggs obtained from the pairs were observed from 0 to 168 hours of post fertilization (hpf) to record the embryonic developments, and the major time intervals of each stage under water temperature 28.5 ± 0.5°C in hatchery conditions. In order to confirm and compare each embryonic developmental stage between the 7 pairs, five to seven samples per pair were collected from each spawning. Major embryonic developmental stages and the external morphology, colour changes and size of eggs, larvae, juveniles, sub-adults and adults were documented and photographed with Trinocular microscope (Zeiss) and Stereo Zoom microscope (Model:Leica S8 APO). After observation, the specimens were also fixed in 5% neutral buffered formalin.

Results

Courtship and spawning behaviour

Few days prior to spawning, an increased social interaction was observed in each breeding pair, and are expressed by chasing, fin erection, rapid up and down swimming, waggle movement and nibbling his mate with fully extended dorsal, anal and pelvic fins by the male. At this time, the pairs also select a suitable site or nest after clearing algae and debris on the tiles or pot (near to sea anemone) for laying eggs. As the time of egg laying neared, both parents actively cleaned the site with their mouth while standing in a head down and tail up position. Imminent spawning was also preceded by an obvious swelling of female's abdomen and the last indication of spawning is the protrusion of genital tubes in both sexes. A tiny white conical urogenital papilla (4-5 mm in length) appeared from the urogenital sinus as an extended part of ovipositor in females. The male also showed a small white urogenital duct, 2 mm in length, extending from the cloaca. The pairs swam side by side. At this time, it can be ascertained that the spawning will occur within an hour. Eggs were extruded through urogenital papilla on each spawning pass, when female swam slowly in a zig-zag path with her belly just brushing the nest surface and quivering side by side over the nest site, and the eggs stick on to the egg-laying substratum. Under captive conditions, all the spawning took

place between 0500 hrs to 1600 hrs during day time. Male closely followed the female and fertilized the eggs as soon as they are laid. Fresh batches of eggs were laid immediately after fertilization of the previous lot of eggs. Like this 5 to 6 times a female deposited eggs during a single spawning session giving a small gap of 2 to 3 minutes in between, and a single spawning completed within 1.00 to 1.30 h duration. The number of eggs per spawning varied between 300 to 1000 eggs/pair at every 12 to 15 days interval giving an average of two spawning/ month/ pair.

Parental care

During incubation, both the parents devoured unfertilized or dead and weekened eggs, and carefully looked after the fertilized eggs till hatching during day time by fanning and mouthing. Fanning was facilitated by fluttering the pectoral fins, and dust particles were removed by mouthing, however no nocturnal parental care was exhibited. Male assumed nearly all responsibilities of caring for the eggs and spent a higher percentage of time at the nest than the females, which increased gradually up to 70% of time as the day of hatching approached whereas the female was allowed

Fig. 1 Successive intervals of ontogeny in *A. ocellaris*

to continue feeding and thereby developing more eggs for future spawning. The environmental parameters such as temperature (28.5 ± 0.5°C), salinity (32 to 35 ppt), dissolved oxygen (4.6 to 6.2 ml/l), pH (8.0 to 8.5), NO_2 <0.01 μg-at / liter, NO_3 <0.05 μg-at / liter, NH_3 <0.02 μg-at/liter and photoperiod 12L:12D were maintained in pair formation, breeding, juvenile and adult rearing experimental tanks, whereas in larval rearing, 24h light was provided up to 20 days during the period of study.

Ontogeny

Based on the observation, the entire life history pathways of *A. ocellaris*, commencing from the time of activation of an ovum to death consisted of six periods: embryonic, larval, juvenile, sub-adult, adult and senescent (Fig. 1).

The newly spawned unfertilized egg was semitransparent and the yolk containing multiple oil globules occupied the entire space of eggs. One end of the egg, identified as animal pole was attached to the substrata (earthen pot and tiles) with a tuft of filamentous stalk or cilia with glutinous substance (Fig. 2a & Table 1). Eggs had yolk in white or pale yellow for initial two days, and as the embryo developed, it turned to black on 3rd to 5th day and silvery on 6th to 7th day of incubation.

1. Embryonic period

This period began with fertilization. The fertilized capsule shaped eggs were covered with smooth and transparent chorion and had narrow perivitelline space. The eggs were adhesive and its size ranged 1.5 - 3.0 mm in length and 0.8 to 1.84 mm in width. The rate of fertilization obtained was 95 to 98%. The embryonic period was characterized by the utilization of exclusively endogenous nutrition from the yolk. The developmental processes reflected were rapid, however, in each successive period, the developmental speed decreases until a complete standstill marks the end of senescent period or death. The embryonic period was further divided into three phases: cleavage, embryonic and eleutheroembryonic as a convenient means of morphophysiological identification of developmental levels.

1a Cleavage phase

The cleavage and embryonic development phases of *A. ocellaris* were classified into 29 stages. The cleavage phase completed within 23 h after insemination (I - XVI stages), and remaining XVII –XXIX stages were included in embryonic phase. Well defined morphogenetic movements were noticed at the lower surface of the blastodisc on the advancement of cleavage.

1a (i) Stages

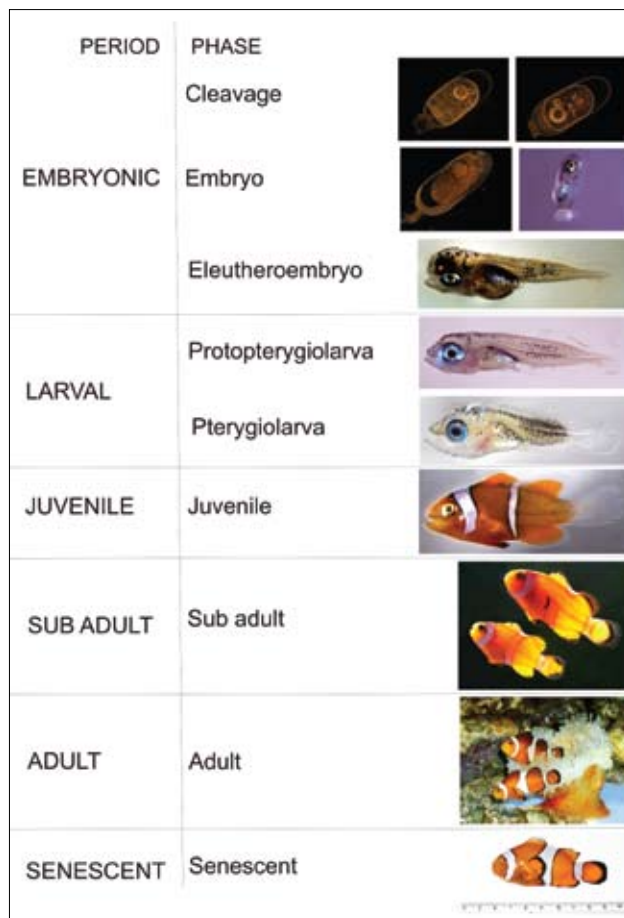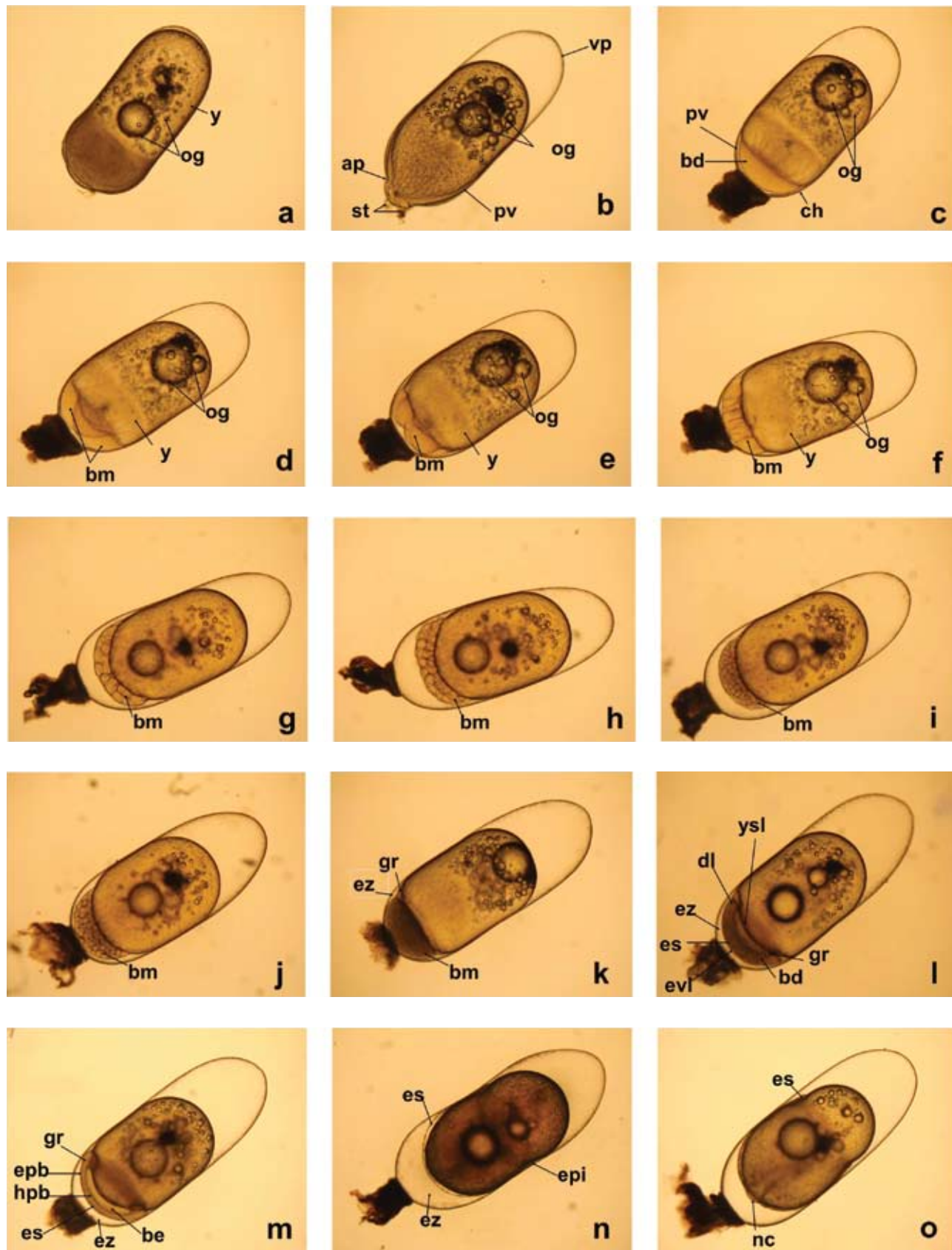**Stage I:** The fertilized eggs were transparent with multiple oil
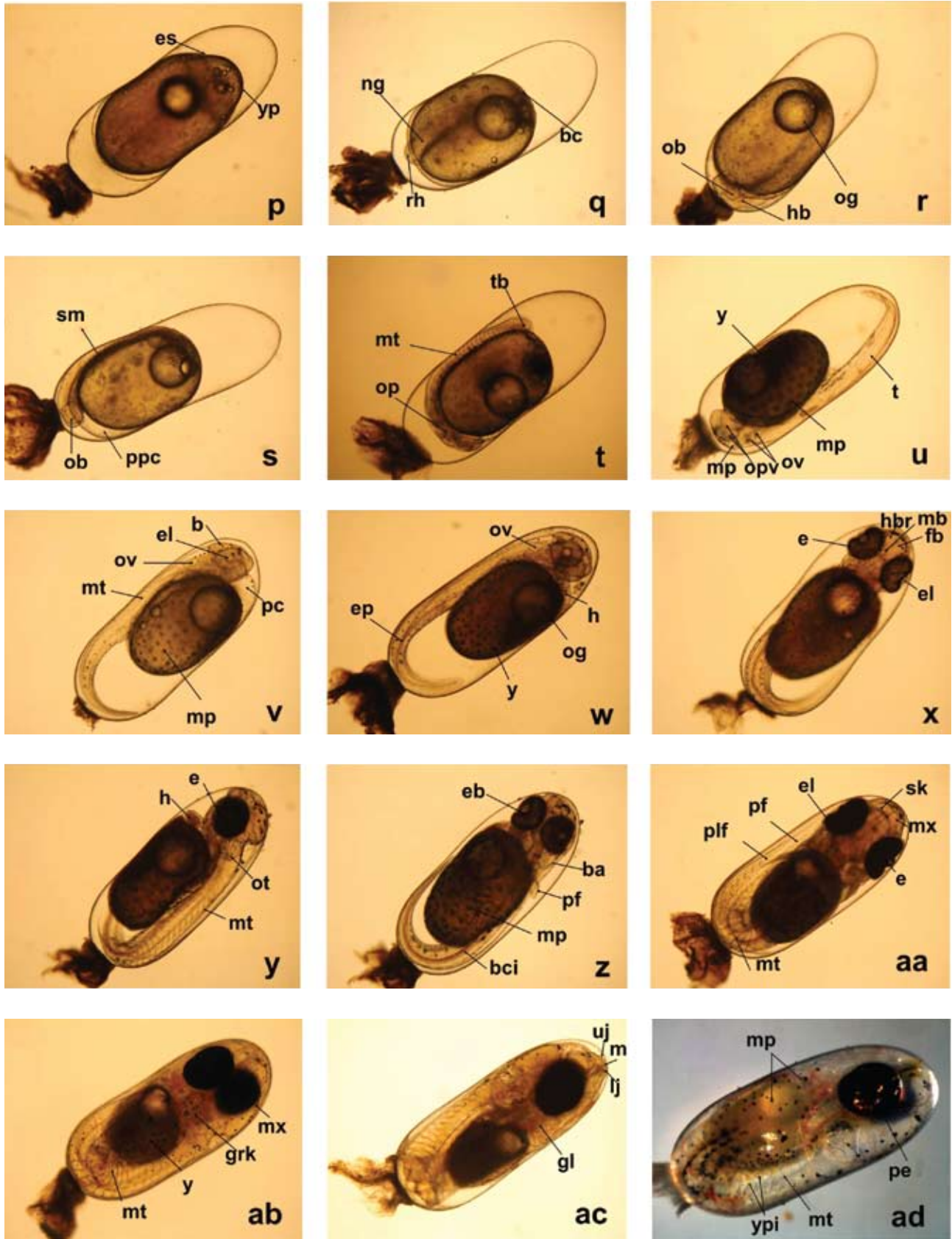
Fig. 2. Embryonic developments of *A. ocellaris* at 28.5±0.5ºC

(a) unfertilized egg soon after deposition (00:00 h); (b) Fertilized egg (00:10 h); (c) Blastodisc formation stage (00:30 h); (d) 2-blastomere stage (01:30 h); (e) 4-blastomere stage (01:50 h); (f) 8-blastomere stage (02:30 h); (g) 16-balstomeres (03:30 h); (h) 32-balstomeres (03:50 h); (i) 64-balstomeres (04:15 h); (j) Early blastula (05:58 h); (k) Late blastula/morula (06:30 h); (l) Early gastrula (12:18 h); (m) Late gastrula (18:15 h); (n) Epiboly stage (20:30 h); (o) nerve cord initiation (21:30 h); (p) Yolk plug stage (22.00h); (q) Blastopore closing (23.00h); (r) Initiation of organogenesis (23.15); (s) Notochord formation (23:40 h); (t) Somatogenesis (28.15h); (u)Development of optic and otic vesicle (40:00 h); (v) Inversion of head (48.00h); (w) heart formation and beat (72.00h); (x) Differentiation of forebrain, midbrain and hindbrain (88:00 h); (y) Appearance of myotomes and muscles (96.00h); (z) pectoral fin bud and branchial arches(106.00h); (aa) Advancement of pectoral fin ossification of maxilla and skull (120.00); (ab) Prominent maxilla and gill rackers (144.00h); (ac) visible upper and lower jaw; (158.00h); (ad) Embryo just before hatching (168:00 h);

ap- animal pole; b-brain; ba-branchial arches; bc- blastopore closing; bci-blood circulation; bd-blastodisc; be-blastocoel; bm- blastomeres; ch- chorion; dl-deep layer; e- eye; eb- eye ball; el-eye lense; ep-erythrophores; epi- epiboly; epb-epiblast; es- embryonic-shield; evl-enveloping layer; ez-evacuation zone; fb-forebrain; gl- gills; gr- germ ring; grk-gill rakers; h- heart; hb- head bud; hbr- hindbrain; hpb- hypoblast; lj-lower jaw; m-mouth; mx-maxilla; mb- midbrain; mp-melanophore; mt- myotomes; nc- nerve cord; ng-neural groove; ob-optic bud; og- oil globule; ot-otolith; op- optic bud; opv-optic vesicle; ov- otic vesicle; pc- pericardial cavity; pe-pigmented eye; ppc- presumptive pericardial cavity; pf- pectoral fin bud; plf-pelvic fin bud; pv- perivitelline space; rh-rudimentary head; sk-skull; sm- somites; st –stalk; t- tail; tb- tail-bud; uj-upper jaw; vp- vegetal pole; y- yolk; yp- yolk plug; ypi- yellow pigment; ysl-yolk syncytial layer.

Table 1.Embryonic development of *A.ocellaris* under hatchery conditions at 28.5 ± 0.5°C

Stage	Duration (h:min)	Fig. No.	Developmental Stages	Key morphological characteristics
Unfertilized egg	00.00	a	Newly spawned egg	Semi transparent with multiple oil globules. Yolk occupies entire space of capsule.
Cleavage phase				
I	00.10	b	Fertilized egg	Transparent, thick chorion, multiple oil globules of varying size, initiation of perivitelline space formation.
II	00.30	c	One blastomere	1 cell formation
III	01.30	d	2 blastomers	2cell division
IV	01.50	e	4 Blastomeres	4cell division
V	02.30	f	8 Blastomeres	8 cell division
VI	03. 30	g	16 Blastomeres	16 cell division
VII	03. 50	h	32 Blastomeres	32cell division
VIII	04. 15	i	64 Blastomeres	64cell division
IX	05.58	j	Early blastula	128 cell division
X	06.30	k	Late blastula/ Morula	Cap of cells overlying yolk. Morula, initiation of germ ring and evacuation zone formation.
XI	12.18	l	Early gastrula	Blastodisc with several cell layers, and completely separated from marginal zone of the external cell membrane by a marked perivitelline space at the animal pole.
XII	18 .15	m	Late gastrula	Flattening of blastodisc, formation of embryonic shield as a thickening of germ ring. Blastocoel with thickened small cells looked opaque, different morphogenetic movements to occur epiboly, and begins to migrate over the yolk. The number of oil droplets decreased.
XIII	20.30	n	Epiboly	Epiboly began in the animal pole forming a cap-like structure, and blastomeres moved downward and covered one-quarter of the egg.
XIV	21.30	o	Nerve cord formation	A thickened part of the blastomeres became an embryonic shield. Neural groove appeared at animal pole to form notochord .
XV	22.00	p	Yolk plug stage	Yolk is nearly covered by a thin blastoderm leaving a small area around the vegetal pole to form yolk plug.
XVI	23.00	q	Blastopore closing	Blastoderm cells spread over the yolk mass, and reaches blastophore closing stage. Neural keel and rudimentary head started to appear. Slight reduction in yolk.
Embryo phase				
XVII	23.15	r	Initiation of organogenesis	Initiation of formation of body of embryo. Neural keel extend along the embryonic axis. Optic buds noticed

XVIII	23.40	s	Notochord formation	Notochord appeared with the head and eye directed towards the animal pole, tail-bud appeared at vegetal pole and initiation of somites and presumptive pericardial cavity.
XIX	28.15	t	Embryo (Somatogenesis)	Formation of otic primodium, optic vesicles. Tail bud slightly extended beyond yolk. Myotomes recognizable
XX	40.00	u	Embryo	Stellate melanophores on yolk and cephalic region, prominent optic and otic vesicles, reduction in yolk, tail movement noticeable and extended beyond yolk.
XXI	48.00	v	Embryo	Inversion of head to vegetal pole noticed, embryo increased in size, otic vesicle prominent, eye lens formation, presumptive pericardial cavity became recognizable and yolk showed a reduction. Forebrain, midbrain, hindbrain started to appear, myotomes recognizable.
XXII	72.00	w	Embryo	Heart formation and heart beat slightly visible, yolk decreased and its absorption visible, erythropores appeared on myomeres. Prominent otic vesicle
XXIII	88.00	x	Embryo	Differentiation of forebrain, midbrain, hindbrain became prominent. Eye ball, lens and pigmentation noticed. Optic vesicles protruded laterally on head. Initiation of maxilla formation.
XXIV	96.00	y	Embryo	Myotomes, muscles, eye and heart become prominent, heart beat and tail movement clearly visible. Otolith of inner year appeared.
XXV	106.00	z	Embryo	Pectoral fin bud formation, eye become prominent, blood circulation and yolk absorption clearly visible, slight appearance of branchial arches .
XXVI	120.00	aa	Embryo	Pelvic fin bud formation, Pectoral fin advanced, ossification of maxilla and skull initiated, recogonizable upper and lower jaw, myotomes prominent
XXVII	144.00	ab	Embryo	Maxilla, gill rackers and muscles developed, reduced yolk, prominent eye with pigmentation. Size of embryo increased.
XXVIII	158.00	ac	Embryo	Upper jaw, lower jaw and mouth visible, gill rackers developed, unconstricted dorsal and ventral fin fold developed.
XXIX	168.00	ad	Embryo	Pigmented eyes and crystalline lens prominent. Embryo occupied entire space of egg capsule, well developed myotomes, wriggling movement noticed, yellow and black pigments appeared throughout the body. Embryo emerged from the egg capsule (Hatching), tail emerging first.

globules in the yolk and have thick chorion. The cortical granules release their content and thereby the eggs get attached to the substrata, and subsequently development of perivitelline space was initiated at 10 minutes of post fertilization (mpf). The yolk was homogeneous and unsegmented (Fig. 2b).

Stage II: The perivitelline space of egg was clearly differentiated between chorion and surface of the zygote with a distance of about 48 to 50μ between the chorion and deutoplasm. The animal pole was characterized by its half-circle shape which attached to the substrata with stalk while the vegetal pole contained yolk and different sized oil globules dispersed as in the case of polylecithal eggs. Accumulation of cytoplasm as a blastodisc which could be easily distinguishable from the yolk mass was noticed at the animal pole and termed as one uncleaved cell blastomere stage (one-cell) at 30 mpf (Fig. 2c).

Stage III: The egg indicated a typical meroblastic and discoidal cleavage, creating a cellular region at the posterior of the yolk mass. First sign of two cell (first cleavage) was initiated after 15 mpf at the animal pole, and the meridional

synchronous division advanced as furrow led to the formation of two blastomeres after 1.30 hours of post fertilization (hpf). Cytoplasm was clear and the oil globules moved towards vegetal pole (Fig. 2d).

Stage IV: The second cleavage, in a single meridional plane at a right angle to the first, resulting in 4 blastomeres stage clearly visible after 1.50 hpf. Each blastomere was only half size of blastomeres in the previous stage. Oil globules of varying size were also observed in the yolk (Fig. 2e).

Stage V: The egg with 8 blastomers resulting from the horizontal mitotic division were obtained after 2.30 hpf. The blastomeres were smaller than previous stage and equal in size (Fig. 2f).

Stage VI: The cleavage giving 16 blastomers happened at 3.30 hpf by another horizontal mitotic division and the blastodisc becomes oblong shape (Fig. 2g).

Stage VII: The 32 blastomere stage was reached at 3.50 hpf and the small blastomeres became overlapping due to the

limited confined space in the capsule (Fig. 2h).

Stage VIII: The sixth cleavage resulting in 64 blastomere stage was obtained at 4.15 hpf and the small blastomeres extended more laterally and arranged themselves as a flat layer at the animal pole (Fig. 2i).

Stage IX: The blastomers reached early blastula stage at 5.58 hpf and the equal sized small blastomeres were arranged in several cell layers in depth and the blastodisc became circular (Fig. 2j). At 128 cell stage, the blastomeres were not arranged on the same horizontal plane and some of the cells were cut by a horizontal plane.

Stage X: At 6.30 hpf, the blastomeres were very small and were nearing to late blastula stage. The blastodisc is seen with several cell layers, and they gradually increased in thickness to form a true cap of cells overlying the yolk and are separated from the marginal zone of external cell membrane by a marked perivitelline space at the animal pole. The blastoderm elevates like a projecting button on the surface of the yolk and the embryos were called morula having mulberry-like structures as the blastomeres were very small (Fig. 2k). During the late blastula stage, the blastoderm flattened and began migrating over the yolk. Initiations of germ ring and evacuation zone were noticed.

Stage XI: During early gastrulation, the blastoderm flattens and begins to expand (beginning of gastrulation) and the enveloping layer (superficial cells) undergoes epiboly, crawling over the surface of the yolk. In the initial phase, the deep blastoderm cells move outwardly to intercalate with the more superficial cells. On migration of blastoderm cells about half way around yolk, the deep cells undergo involution and are then divided into a superficial enveloping layer (EVL) of epiblast to form ectoderm, and a deep layer of hypoblast to form mesoderm and endoderm. Between the EVL and the yolk syncytial layer (YSL) are the deep cells that give rise to the embryo. The blastomeres started moving inward to form three germ layers called gastrulation. The rim of involuting cells known as germ ring is formed at 12.18hpf (Fig. 2l).

Stage XII: Several transitional stages were recognized when the blastoderm becomes hollow and flat. At first, the blastoderm expands along and over the yolk mass while a segmentation cavity or blastocoel was formed in its centre. A fluid filled space called blastocoel was also formed and it clearly separated from the yolk. Invagination of the marginal cells began and the very much thickened edges of segmentation disc tend to rise, all the cells became smaller, gradually disappeared and looked opaque. The late gastrula stage was marked by the germ ring and then it migrated

equidistantly around the yolk (Fig. 2m). A thickened part of the blastomeres became an embryonic shield at 18.15 hpf. Number of oil droplets showed a decrease.

Stage XIII: Gastrulation process is characterized by different morphogenetic movements. The cap-like structure of germ ring moved downwards from the animal pole to cover part of the yolk called epiboly at 20.30 hpf (Fig. 2n).

Stage XIV: The blastomeres moved further downward and covered half portion of the yolk at 21.20 hrs. The neural groove started appearing as a small projection and with a slight extension over the yolk at 21.30 hpf (Fig. 2o).

Stage XV: Yolk-plug stage was noticeable at 22 hpf and was marked by the germ ring covering three quarters of the yolk (Fig. 2 p).

Stage XVI: The blastomers covered the entire yolk as a thin layer and reached the blastopore closing stage at 22.45 hpf. The longitudinal neural groove was formed and attached to the yolk at 23.00 hpf. The number of oil globules showed a reduction (Fig 2q).

1b Embryonic phase

Stage XVII: Embryonic body formation was observed when the blastomeres covered the whole yolk by blastopore closing (end of epiboly). Neural groove extend along the embryonic axis and the notochord appeared with the head and eye buds directed towards the animal pole and tail-bud appeared at the posterior of the yolk mass. This transformation called organogenesis occurred at 23.15 hpf (Fig.2 r).

Stage XVIII: At this stage, evaginations were noted in the upper portion of embryo to form the optic placods which then successively formed the optic buds. The embryo reached 10 somite stages and the presumptive pericardial cavity became recognizable and yolk showed a reduction (Fig. 2 s). At 23.40 hpf the posterior end of embryos was characterized by the presence of a few small vacuoles (Kupffer's vesicles) and it appeared at the underside of the caudal (posterior) end of the body, which is in contact with blastopore.

Stage XIX: At 28.15 hpf, shape of the embryo became increasingly visible. The embryo began to form head, and otic primodium became recognizable. The optic buds become clearer and tail bud could be differentiated. The small oil globules started to disappear at this stage and the perivitelline space increased progressively. Myotomes and the axis of vertebral column were clear (Fig. 2 t).The first appearance of melanophores was noticed on the yolk at 30 hpf.

Stage XX: The embryo had numerous dendritic/star shaped melanophores concentrated on the yolk at 40 hpf and dotted pigmentation (melanophores) started to appear on the cephalic region and body. The tail bud extended beyond yolk. The otic vesicles become recognizable (Fig. 2u).

Stage XXI: The embryo began to move itself in the egg capsule and inverted its head position to the vegetal pole or the free end of the egg capsule at 44hpf onwards. It was also found that reorientation was essential requisite for healthy development and hatching. A slight increase in the body size was noticed, however the body was still attached to the yolk. The eye lens, otic vesicles and presumed pericardial cavity became more prominent. Dotted melanophores were appeared in the head and on the surface of yolk at 48 hpf (Fig.2v). Differentiation of brain to forebrain, midbrain and hindbrain noticed.

Stage XXII: Otic vesicles and erythrophores became more clear. The absorption of yolk and blood circulation becomes visible. The oil globules have coalesced to form a single large oil globule. Eye started to develop pigment and heart became visible at 72 hpf (Fig.2 w).

Stage XXIII: The optic vesicles became increasingly evident and protruded laterally on the head, and maxilla formation was recognizable. The body was transparent and considerable reduction in yolk noticed. Primary differentiation of forebrain, midbrain and hindbrain were prominent and the length of embryo distinctly increased at 88 hpf (Fig.2 x).

Stage XXIV: At 96 hpf, the embryo showed further differentiation of the eye with more pigmentation. The cup-shaped, red pink heart began to beat (180 to 200 beats per minute) and pumping of blood and its circulation were clearly visible. The skeletal muscles were found along the body length. Development of myotomes was evident. The small otolith of the inner ear was also appeared (Fig.2 y).

Stage XXV: Pectoral fin bud and eye ball developed. Tail further extended, blood circulation to tail can be seen clearly, and active absorption of yolk was noticed. The melanophores were increased in the head region. Slight development of branchial arches was noticed at 106 hpf (Fig.2 z).

Stage XXVI: The pectoral fin primodials and pelvic fin buds were further developed at 120 hpf. Initiation on the oscification of maxilla and differniation of upper and lower jaw were noticed. Active movement of the head and tail were noticed (Fig.2 aa).

Stage XXVII: Fin bud was progressively elongated and became parallel to the body. The large eyes contained brown pigments and their lens were prominent. The axis of vertebral column was not clear and the muscles were developed and red pigmentation of erythrophores was noticeable at 144 hpf. The yolk showed further reduction. Development of opercle, maxilla and gill rackers was noticed. Red blood cells running in the vessels could be observed indicating the functioning of circulatory system. The embryo increased in size and occupied the egg capsule leaving a very little space (Fig. 2 ab).

Stage XXVIII: The pectoral fins appeared clearly and moved frequently, and the dorsal and ventral of fin folds were well defined at 158 hpf. At this stage the anlagen of crystalline lens, the ocular pigmentation, and maxilla and gill arches with its flap were recognizable. The yolk showed considerable reduction while the embryo was growing. Increased body length was observed especially in the caudal area (Fig.2 ac).

Stage XXIX: At 168 hpf, the embryo was further enlarged and occupied entire space in the capsule. Wriggling movements were noticed. The yolk sac became quite small. The yellow and black melanophores were distributed throughout the body. The head occupied one-third of the capsule space. Retina was well pigmented and a silvery colour was evident. At this stage the glowing eyes of the developing larvae inside the egg capsule were clearly visible and these typical metallic eyes indicated that the eggs were ready to hatch (Fig.2 ad).

Hatching: The embryo began to hatch by vigorous wriggling movement to break the egg capsule, and during this the caudal part and body of embryo moved rhythmically with no exact periodicity. At this time, tail had wrapped completely around the egg, reaching its distal end. The breaking point at the caudal half of the capsule was near to the stalk and the hatchling emerged tail first (Fig. 3a). The hatching took place usually after sunset and peak hatching took place between 1900 to 2200 hours under complete darkness.

1c Eleutheroembryonic phase

Eleutheroembryo phase (eleutheros = free) commences from soon after hatching and lasts until all yolk reserve is digested or until feeding the ovarian secretion is terminated by parturition. The newly hatched larvae measured 3 to 4mm in length and each had a transparent body, large eyes, visible mouth, small yolk sac and remained at the bottom of the tank for a few seconds and soon after became free swimming. The mouth gape of newly hatched larvae measured 170 to 210μ (Fig. 3b&c). The larval fin fold (dorsal, caudal and anal fin) was continuous and perfectly transparent. The alimentary canal was short, straight, and slender and anus was placed at middle of the total length. In the post-anal region, 13 to 15 numbers of myotomes were clear but in the pre-anal region,
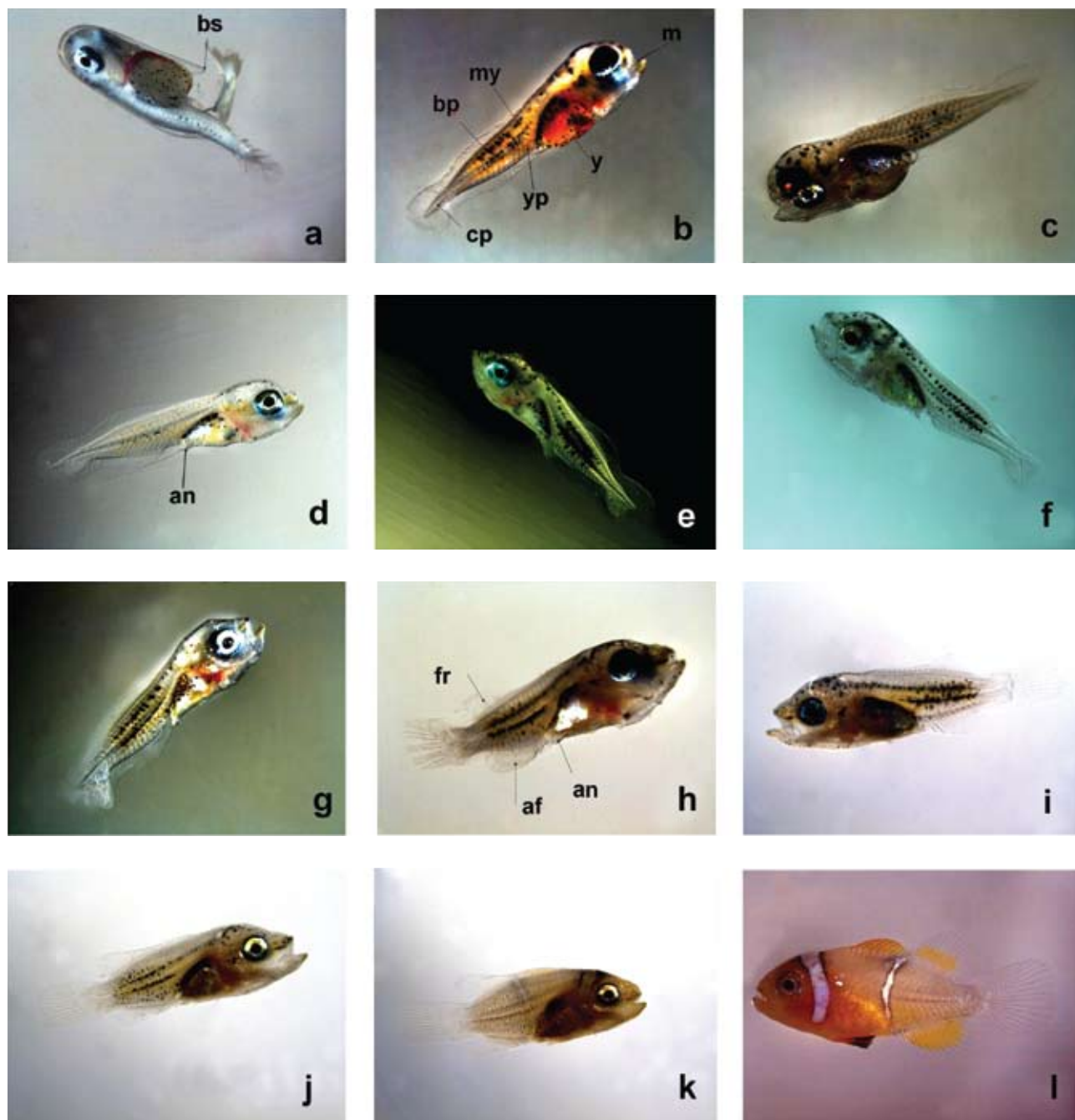
Fig.3. Hatching and larval metamorphosis of *A. ocellaris* under captivity.

(a) Hatchling emerging by breaking the capsule (168:10 h); (b) Eleutheroembryo (newly hatched larva) (00:00 hph); (c) Eleutheroembryo (2.30 hph); (d) Eleutheroembryo (5.00hph); (e) Protopterygiolarva larva (18.00hph); (f) Protopterygiolarva larva (48.00hph); (g) Pterygiolarva (3dph);(h) Pterygiolarva (5dph); (i) Pterygiolarva (6dph); (j) Pterygiolarva (8dph); (k) Pterygiolarva (10dph); (l) Juvenile (15dph).

an-anus; af-anal fin; bs- breaking shell; bp- body pigment; cp- caudal peduncle; fr-fin rays; ; m- mouth; my- myomeres; y- yolk; yp- yolk pigments.

it was discernible only up to half distance and countable only 10 to 12 numbers. Black dotted chromatophores and yellow pigment were developed on the head, middle and posterior region of the body whereas fins were transparent. The newly hatched eleutheroembryo exhausted their yolk material within 6 to 8 hours of post hatch (hph) and shift to larval period (Fig. 3d).

2. Larval period

It commences when the transition to exogenous nutrition takes place and lasted until the formation or ossification of the axial

Table 2 . Average total length variation during larval metamorphosis

Age of larvae	Average Total Length (micron)
0hph/ newly hatch	3717.5
2.5hph	3800.4
6.0 hph	4467.5
18.0 hph	5576.2
48.0 hph	5996.5
3dph	6131.6
5 dph	6897.3
6 dph	7020.5
8 dph	7234.3
10 dph	7416.4
15 dph	10898.5
20 dph	12155.2

skeleton or differentiation of median fin fold. The continuous fin fold constricted near the anus. It is also characterized by the persistence of some embryonic organs which are later replaced by different definitive organs of the same function, or disappear with the loss of their functional need. The larval period is divided into two phases i.e protopterygiolarval and pterygiolarval.

2a Protopterygiolarval phase

It encompasses the interval between transition to exogenous feeding and the commencement of differentiation of embryonic median fin fold - the first appearance of mesenchymatic buds of lepidotrichia and dorsal and anal fin outlines within the fin fold. The size of the larvae at 48 hph ranged between 5.6 to 5.9 mm in total length (Fig. 3e&f).

2b Pterygiolarval phase

This phase commence from the beginning of differentiation of the unpaired fins until the embryonic median fin fold is entirely differentiated. The crown of the head had risen in a crest and the jaws were well developed into functional structures. The lower jaw protruded slightly beyond the upper jaw giving the head a little pointed appearance. Pigmentation was also

Table 3. Development of banding pattern in *A. ocellaris* under captivity

Days of post hatch (dph)	Developments of white bands
10 – 12	feeble opercular/ head band and middle band
15 – 17	Prominent opercular/ head band and feeble middle band
30 – 40	Prominent opercular and middle band
60- 70	Feeble caudal band
80 – 90	Prominent caudal band

noticeable in the pre and post anal region and a group of stellate chromatophores on the stomach, middle region of the body and on the surface of shrunken yolk. The body gave an appearance of real solidity and feebly crossed muscle fibers (Fig 3g-k). Though larvae showed slight increase in growth rate during initial period, differential growth was noticed on 10 days of post hatch (dph) onwards (Table 2).

3. Juvenile period

It begins when the fins are fully differentiated and most of the temporary organs are replaced by definitive organs. Transition from larva to young fish involved extensive changes and some organs developed only later in the juvenile period (scales, intromittent organs), however, some special organs persist from the larval period. The larvae metamorphosed to juveniles between 15 to 17 dph and settled in the sea anemones on 17th to 20th dph (Fig. 3l). All the juveniles began to shift from partially pelagic to epibenthic, and started eating minced shrimp, fish flesh, mussel meat, clam meat and formulated diets. The juveniles reached 40 to 60mm size after rearing for three to six months under hatchery condition. If the juveniles are reared in different tank through culling, in each tank one pair grew ahead of others and become the pair. The development of banding pattern and its duration observed in the present study are given in Table 3. The juvenile period lasted until the beginning of the first maturation of gametes.

4. Sub adult period

It commences with the first maturation of gametes and is usually characterized by rapid growth. During this period the juveniles showed aggression towards subordinate juveniles for territory and space for future spawning. The study also evinced that hatchery produced sub adults attained reproductive maturity at the age of 18 months after hatching.

5. Adult period

It is characterized by the advancement in maturation of gametes resulting to male and female differentiation. In a colony, for pair formation, the largest specimen was considered as female and the second largest as the active male due to protandric hermaphroditism. These pairs were characterized by aggression, spawning behaviour, site selection for future spawning, courtship behaviour, accelerated growth and year round spawning. The first spawning occurred at 18 months of growth after hatching and the size at first maturity in males varied between 58 and 65 mm and that of females were between 70 and 80 mm depending upon the social structure. The newly formed pairs produced 300-350 eggs in the initial spawning and then after showed a gradual increase in number. Subsequently more experienced pairs started producing more eggs (Table 4).

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Table 4. Average number of eggs laid and number of spawning (in parenthesis) in 7 pairs of *A. ocellaris* under captivity

Year	Pairs						
	1	2	3	4	5	6	7
2005 (Sept - Dec)	350(8)	330(5)	380 (6)	300 (8)	315 (4)	360 (3)	355 (6)
2006	580 (22)	430 (22)	500(23)	420(19)	460 (22)	500 (22)	470(24)
2007	750 (23)	720(24)	690(24)	560(24)	590(24)	900(24)	870(24)
2008	860 (24)	830(24)	780(24)	670(24)	650(23)	925(24)	900(22)
2009	980(23)	950(24)	860(24)	780(23)	880(24)	930(24)	920(24)
2010	1000 (22)	980(20)	950(21)	960(20)	1000(22)	950(23)	990(21)
2011 (Jan - May)	870 (6)	870(6)	1000 (8)	970(6)	800(5)	780(5)	885(5)

6. Senescent period

The production of eggs, frequency of spawning and rate of growth drop substantially when the pair becomes aged. The growth and spawning were completely arrested after 6 to 7 years after first spawning and finally entered in to the senescent stage. The maximum length obtained for adult mated female is 110 mm. However, the male partner of the senescent female can change its sex to female, if a juvenile or sub adult is introduced to the colony and thereby forms a new pair under captivity.

Discussion

Yolk plays a vital role in determining the egg types, and based on this, eggs of *A. ocellaris* are classified as polylecithal egg (macrolecithal) with multiple oil globules. In the present study, size of yolk reduced gradually when the cleavage proceeds as the same basic pattern in other teleost fish, and a perivitelline space became visible after fertilization. The time and stages of embryonic development and larvae of *A. ocellaris* showed variation as per the environmental conditions. The developmental rate of fertilized egg and its hatchability of clownfishes varied with temperature and dissolved oxygen content of water (Allen,1972; Fautin and Allen,1997) and also in other teleost (Delsman,1930). In the present study, 95-100% hatchability was obtained at 168 hours.

The ontogeny begins with activation of an ovum and the entire ontogeny in *A. ocellais* is classified into embryonic, larval, juvenile, sub adult, adult and senescent period. The embryonic period was divided into three phases viz. cleavage, embryo and eleutheroembryo phases as reported in other teleost (Balon, 1984; Moyle and Cech, 2004). The cleavage pattern of *A. ocellaris* is meroblastic and equal, and slight variation in timing of defined morphogenetic movements was noticed in comparison with other marine teleost (Allen, 1972). The formation of uncleaved blastodisc stage in *A. ocellaris* was noticed at 30 mpf and the blastomeres formed through meroblastic discoidal cleavage were situated at the animal pole. First cleavage occurred at 1.30 hpf and the blastodisc was divided into two blastomeres, and it took 3.30 h to reach 16-blastomere stage and further cleavage divisions follow one another rapidly and embryo entered blastula stage (5. 58 to 6. 30 h) in *A. ocellaris* whereas Allen (1972) recorded that *A. chrysopterus* took 3 h to reach 8-cell stage, 5 h to reach 32-cell stage and 10 h to enter the blastula stage. At the blastuation stage, the cleavage process of *A. ocellaris* was recorded earlier than that of *A. chrysopterus* which may be due to the variation in temperature during incubation. Falk-Petersen (2005) also mentioned that the blastula forms after 24 hours depending upon species or incubation temperature. The early cleavage also varies depending on egg quality and fishes (Kjorsvik *et al.*, 2003). Blastulation extended from the 128-blastomere stage to the onset of gastrulation in the present study. The epiboly began 20.00 h after insemination, when the blastomeres covered one-quarter of the egg and it was evident at the animal pole forming a cap-like structure, and a thickened part of the blastomeres became an embryonic shield whereas the embryo of *A. chrysopterus* was undergoing gastrulation on the second day after spawning (Allen, 1972). During migration, one side of the blastoderm becomes noticeably thicker to form germ ring which is composed of a superficial layer (epiblast) and an inner layer (hypoblast) as reported in *Cynolebias viarius* (Arezo *et al.*, 2005). Cell-labeling experiments in zebrafish also indicated a thickening of blastoderm which marks the site for future dorsal surface of the embryo (Schmidt and Campos-Ortega, 1994). In *A. ocellaris*, appearance of evacuation-zone indicates late gastrulation, and yolk -plug stage was noticeable at 22:00hpf. The formation of embryonic body was observed when the blastomeres covered the whole yolk which is characterized by blastopore closing at 22.45 hpf, and formation of neural groove at 23:00 hpf and subsequently noticed the commencement of organogenesis as also reported in *A. clarkii* (Wilkerson,2001).

In the present study the heart beat varied between 180 to 200 beats per minute and blood circulation was clearly visible on 3rd day onwards as reported in other clownfishes (Wilkerson,2001) but in *A. chrysopterus*, a heart beat of 200 per minute with major blood circulation was visible only after 4th day (Allen,1972). The blood elements flowed from dorsal aorta formed the posterior of the yolk-sac as caudal artery to caudal vein, which ran anterior and split into posterior cardinal vein and subintestinal vitelline vein as in *Zingel streber* (Kovac, 2005). Pectoral fin bud was noticed 96hpf and it became functional on 106 hpf as also reported in *A. chrysopterus* embryo (Allen, 1972). The yolk of *A. ocellaris* was brightly pigmented as reported in *A. clarkii*, *A.perideraion* and *Premnas biaculeatus* (Wilkerson, 2001), which are also influenced by parental nutrition as well as present feeding (Allen,1972;Wilkerson,2001; Arezo *et al.*,2005; Kovac, 2005). Embryos of the same species leave the egg membranes at different times (Dziekonska, 1956; Alderdice and Forrester, 1974) and in some species, hatching occurs at different levels of development. In the present study, embryo was actively wriggling inside the capsule at 168hpf and peak hatching took place between 1900 and 2200 hours under complete darkness (Madhu *et al.*, 2006).

In eleutheroembryo phase, as long as a embryo use mainly the placental analogues or endogenous yolk supply, irrespective of it being inside of egg envelope or hatched, it was considered as free embryo or eleutheroembryo as suggested by Balon (1984). The beginning of larval period is signified by the ability to capture food items (Chen, 2005) as well as persistence of embryonic organs (Kryzhanovsky *et al.*, 1953) and by the development of special larval organs (e.g. respiratory blood vessels in fin folds, pectorals, gill covers, spines, flaps, and filamentous appendices), which are later replaced by different definitive organs of the same function, or disappear with the loss of their functional need. Alimentary canal had differentiated into esophagus, rudimentary stomach, intestine and rectum which allowed the newly hatched larva to accept exogenous feed within 6 to 8 hours after hatching. The hatched larva of *A. ocellaris* is free swimming at the more advanced stage, which enable it to capture live preys. This suggests that *A. ocellaris* larvae need to be fed soon after hatching as also observed for other clownfish species (Allen, 1972; Wilkerson, 2001; Gordon and Hecht, 2002). The hatchery produced larvae metamorphosed and settled on 17th to 20th dph, reached juvenile stage (40 to 60mm TL) after three to six months. Though the first spawning occurred at 18 months after hatching, the size at maturity in male and female varied in different pairs as it is a protandric hermaphrodite and sex change was regulated by the social structure as also reported in *A. percula* (Madhu and Rema,2006) and in *A. ocellaris* (Rema *et al.*, 2010). The size at first maturity in males of *A. ocellaris* varied between 58 to 65 mm and that of females were between 70 to 80 mm depending upon the social structure (Rema *et al.*, 2010). However the growth and spawning were completely arrested at 6 to 7 years after first spawning and finally entered into the senescent stage and its duration varies among different species of clownfishes and a longevity of 14 years for *A. clarkii* (Moyer, 1986);18 years for *A. frenatus* and *A. perideraion* (Fautin and Allen, 1997), 5 years for *A. chrysopterus* (Allen, 1972), 7 years for *A. melanopus* (Ross,1978) were reported. Balon (1984) also reported that in sturgeons, the senescent stage lasted for many years. The generated knowledge during different period of life history pathways in the present study is important as there is a significant link between morphological developments and their feeding performance which is also important in the design of feeding management strategies for its mass scale production, grow out culture and mariculture operations of other high value species of marine ornamental fishes.

Acknowledgements

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