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Broodstock development, breeding, embryonic development and larviculture of spine-cheek anemonefish, *Premnas biaculeatus* (Bloch, 1790)

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ABSTRACT

The broodstock development, breeding, embryonic development and larviculture of *Premnas biaculeatus* under different feeding and envrionmental conditions are reported for the first time. The influence of enriched rotifer (*Brachionus rotundiformis, Brachionus plicatilis*) and non-enriched newly hatched *Artemia* nauplii along with microalgae (*Chlorella marina, Nannochloropsis oculata* and *Isochrysis galbana*) on larval rearing and survival was elucidated. Fishes in the length range of 55 to 70 mm (presumptive male) and 120 to 150 mm (presumptive female) were selected for pair formation experiments along with single host sea anemone *Heteractis magnifica*. All the 10 pairs started spawning with in four months after they were shifted to the breeding tanks. Eggs were laid in round patches or clutches at intervals of 15 to 20 days with an average of 2.09 ± 0.3 spawnings per month per pair giving an annual number of 1752 -11832 eggs per pair. Upon incubation for six days in complete darkness, 95-98% of the eggs hatched with the peak hatching between 1830 and 1930 hrs. On 20th day post-hatch (d.p.h.), most of the larvae metamorphosed to juveniles and began to shift from partially pelagic to epibenthic and the juveniles reached marketable size from 60th d.p.h. onwards.

Keywords: Anemone fishes, Breeding, Larval rearing, *Premnas biaculeatus*, Spawning

Introduction

The maroon clown *Premnas biaculeatus,* the sole member in genus *Premnas* is commonly known as spine-cheek anemone fish. Adult fishes have golden yellow bands and all young ones possess white bands. Males are smaller and brighter red than females with brilliant white stripes. This reef fish is in high demand in the marine ornamental fish trade due to their most striking colouration, and proclivity to live in association with tentacle anemone *Heteractis magnifica* and bulb tipped or purple base sea anemone *Entacmaea quadricolor* (Allen, 1972, 1980). In India, this species is available only in the coral reef ecosystem of Andaman and Nicobar islands (Talwar, 1990; Madhu and Madhu, 2000). In the international trade, market for marine ornamental fish has shown a steady increase over the past few years with an estimated wholesale trade of nearly US $ 1 billion and retail trade of about US $ 3 billion (Olivotto *et al*., 2005). Many reports have focused on the prospects of reef fish breeding for aquarium trade (Holt, 2003; Olivotto *et al*., 2003). Hatchery produced seeds are more hardy, less susceptible to diseases and survive better than the wild caught counterparts. Considering these facts, the present study was undertaken on broodstock development, breeding, larviculture and juvenile production of *P. biaculeatus*.

Materials and methods

Collection of animals

Forty fishes of 55 to 150 mm total length and 18 sea anemones (*H. magnifica*) collected from different social groups from the coral reef ecosystem of Andaman and Nicobar Islands were transported to the Marine Hatchery of Central Marine Fisheries Research Institute, Kochi. During transportation, the fishes and anemones were packed in separate plastic bags as the sea anemones produce a toxin during transportation, which is harmful to fishes.

Experimental set up for pair formation and broodstock development

Ten fishes were reared together in 500 l rectangular FRP tanks (120 X 60 X 60 cm) fitted with biological filter and each tank was provided with sea anemone (*H. magnifica*) as the host to reduce the aggression. Likewise three replicates were maintained. Under gravel (UG) filter systems were used in each tank to ensure the water recirculation and water quality. One-third water was exchanged once in two days and fed thrice a day with wet feeds *viz*., mussel meat, prawn and fish egg mass at the rate of 15% of their body weight for a period of 90-120 days to develop pairs. Pairs formed were then transferred to separate 500 l perspex aquaria to develop broodstock

and were fed with wet feed such as meat of shrimp, green mussel, clam and fish egg mass at the rate of 10% of their body weight in four split doses per day along with live feed like L type rotifer (5-8 nos. ml^{-1}) and *Artemia* nauplii (3-5 nos. ml^{-1}) enriched with vitamins, minerals and fatty acids (cod liver oil). Each broodstock tank was provided with a rough surfaced substrate near the sea anemone for egg deposition and each pair was assigned code number (Pb l to Pb10).

Breeding behaviour and egg laying

Observations were made three times a day (0800–0900 hrs, 1300–1400 hrs and 1700–1800 hrs) to study courtship behaviour, breeding, spawning and parental care. Various substrata such as ceramic tiles (30 x 24 cm), asbestos sheet, coral stones, oyster shells, earthen pots and PVC pipes were provided in each tank to find out substrate preference for egg deposition. Three replicates were maintained for each substratum.

Sampling of eggs and larvae

Samples of the embryos, larvae and juveniles from the ten pairs were observed soon after fertilization to 144 h post-fertilization (h.p.f.) (6 days of incubation), soon after hatching to 20th day of post hatch (d.p.h.) and 21st to 60th d.p.h to study development. The embryonic developments were categorized in to three phases *viz*., cleavage, embryonic and eleutheroembryonic (Balon, 1975). Fifty eggs were examined for each stage from the time of laying to hatching and the developmental stages were photographed under trinocular microscope (Carl Zeiss) attached with a digital camera (Cannon Power shot-G2, Pixel 5.0). Soon after completion of each stage, samples were fixed in neutral buffered formalin. Length of the egg capsules (excluding the length of the stalk) were measured. Twenty five larvae randomly collected from each spawning of 10 breeding pairs were used to describe each larval stage and photographed using trinocular stereo zoom microscope (Leica S8-APO). Alizarin stain was used wherever the developmental stages were not recognizable.

Live feed culture

The stock culture of microalgae such as *Chlorella marina, Nannochloropsis oculata* and *Isochrysis galbana* were maintained in 3 l flasks as inoculum for mass culture in 100 l capacity perspex tanks using Walne's media (Walney, 1974). Cell density and growth phases were determined daily.

Two species of rotifers (*Brachionus rotundiformis* and *Brachionus plicatilis*) having size range of 60-100 µ and 200-250 µ respectively were cultured at optimum salinity and temperature using *N. oculata, C. marina* and *I. galbana*. *Artemia* nauplii were obtained after decapsulation of the commercially available cysts (Microfeast® Artemia, U.S.A.) as per standard procedure (Gilbert, 1996). Zooplankton were also enriched with vitamins, minerals and fatty acids (cod liver oil) for feeding the broodstock as per standard procedure (Watanabe *et al.,* 1983) as spawning and embryo development are strictly related to broodstock nutrition and high quality breeders are essential for production of quality eggs and larvae for stable fry production.

Hatching

On the expected day of hatching (6th day), the eggs along with substratum and parents were transferred from the parental tank to hatching tanks (100 l) at a water temperature of 29 ±1 ºC. Mild aeration was provided near the egg cluster to ensure sufficient water current and oxygenation under complete darkness. The sides of the tanks were also covered with black cloth. In each spawning, the substrata were taken out after completion of hatching and unhatched embryos were accounted with digital counter-pen and the imprint of the egg patches were also photographed. The total number of eggs laid in each spawning was accounted after analyzing the photographs using Adobe Photoshop software and the hatching percentage was calculated. Soon after completion of hatching, the parents were transferred back to their respective breeding tanks and the larvae were transferred to larval rearing tanks.

Larval rearing

The larvae from 10 pairs were used to estimate the role of enriched and non-enriched live feed combinations on their survival. Larvae were fed with five different diets with an equal cell density of microalgae (*C. marina* and *N. oculata* in 1:1 proportion at 1.5 x10^6 cells ml^{-1}). (Diet I: microalgae only, Diet II: microalgae and *B. rotundiformis* (6 to 8 nos. ml^{-1}), Diet III: microalgae and enriched *B. rotundiformis* with cod liver oil (6 to 8 nos. ml^{-1}), Diet IV: microalgae and *B. plicatilis* (6 to 8 nos. ml^{-1}) and Diet V: microalgae and enriched *B. plicatilis* (6 to 8 nos. ml^{-1}) with cod liver oil). All the experimental groups were subjected to an extended photoperiod (24 L/0 D) using two 40W bulbs which was hung 30 cm above the water surface in 250 l perspex tanks up to 20th dph. Three replicates of 250 ± 5 larvae were maintained for each diet. Newly hatched *Artemia* nauplii (4 to 6 nos. ml^{-1}) were provided in all experimental tanks from 9th to 20th dph. Juveniles (10 ± 2) were also randomly collected from each pair to confirm morphological changes.

In all the experimental tanks, water quality parameters such as temperature (with thermostat and thermosensors), salinity (with salinometer, ATAGO Japan), pH (with Excell XL6o pH), light (with lux meter Metravi-1330) and

dissolved oxygen (with DO Meter EUTECH Cyber Scan) were measured. In addition, NO_2, NO_3, and NH_4 levels in water were analysed following standard methodology of seawater analysis (Strickland and Parsons, 1968). The above water quality parameters were monitored regularly and maintained at optimum levels.

The results were analyzed using ANOVA, DMRT and Post Hock Test with statistical software (SPSS Ver.13).

Results

Pair formation and broodstock development

In each tank, one pair grew ahead of others and became the monogamous pair. The total length of the female varied between 120 and 150 mm (presumptive female) and that of male varied between 55 and 70 mm (presumptive male).

Substrate preference for egg deposition

Significantly higher percentage of egg deposition was obtained in earthen pots ($p<0.01$) followed by ceramic tiles ($p<0.05$) whereas other substrata showed insignificant deposition of eggs (Table 1).

Breeding behaviour and spawning

Site cleaning and nest preparation adjacent to sea anemone were observed few days prior to spawning by the pairs. Both the parents cleaned the future spawning site with their mouth, pectoral, pelvic and tail fin to remove the algae, debris and dust particles. Imminent spawning was indicated by an obvious swelling and the visibility of tiny conical ovipositor on the female's abdomen. A number of eggs were extruded through this structure on each spawning, when the female swims slowly and deliberately in a zigzag path with her belly just brushing the nest surface, the female closely followed her mate, to fertilize the eggs as they are being laid, and the spawning lasted for one to one and half hour. In all the breeding pairs, spawning mostly occurred during morning hours. However, on rare occasions the fish spawned in the evening also (1630 hrs), but no spawning was recorded after 1700 hrs. The details of spawning from all the pairs (Pb1 to Pb10) are given in Table 2.

Table 2. Spawning records of *Premnas biaculeatus*

Pair code	Number of spawning	Number of months	Average breeding frequency per pair per month (± SD)
Pb1	73	36	2.03 ± 0.31
Pb2	74	32	2.31 ± 0.21
Pb3	75	34	2.21 ± 0.3
Pb4	74	35	2.11 ± 0.24
Pb5	71	36	1.97 ± 0.22
Pb6	72	36	2.00 ± 0.32
Pb7	73	36	2.03 ± 0.33
Pb8	74	34	2.18 ± 0.3
Pb9	75	35	2.14 ± 0.32
Pb10	72	36	2.00 ± 0.3
Total	733	350	2.09 ± 0.3

Eggs shape, size and fecundity

The eggs were capsule or elliptical shaped and adhered to the surface of earthen pots with a tuft of short filamentous stalk in round patches or clutches. The egg size ranged from 2.8 to 3.0 mm in length with a width of 1.1 - 1.3 mm. The number of eggs produced varied between 150 and 1000. per spawning per pair and spawning was achieved every 15 to 20 days, giving an average of 2.09 ± 0.3 spawning per month per pair giving 1752-11832 eggs per pair per year.

Parental care and egg morphology

Both male and female breeders exhibited parental care during incubation period through two basic activities *viz.,* fanning and mouthing. The fluttering of pectoral fins for fanning created a cooling effect to reduce the decay of eggs. The unfertilized, dead or weakened eggs and dust particles were removed by mouthing. The details of colour variations observed in the eggs during incubation are given in Table 3. Newly spawned and silvery eggs during late

Table 1. Egg deposition of *P. biaculeatus* on various substrata

Pair code	Earthen pot	Ceramic tiles	Asbestos	Coral stone	Oyster shells	PVC pipes
Pb1	16	5	0	0	2	1
Pb2	18	3	0	0	1	1
Pb3	17	5	0	0	1	1
Pb4	15	8	0	0	1	0
Pb5	15	6	0	1	1	0
Pb6	14	5	0	1	1	0
Pb7	16	6	0	0	0	0
Pb8	15	7	0	0	0	1
Pb9	18	8	0	2	1	0
Pb10	19	9	0	3	0	1

incubation are shown in the Fig.1 and 2. The survival of embryo under captive conditions in the presence and absence of parental care did not show any difference on 1st day of incubation whereas from second day onwards, variations were noticed in its survival ($p<0.05$). Highly significant variation ($p<0.01$) in survival was observed on the fifth day. The results revealed that survival of embryos under captive conditions depends on environmental parameters as well as on parental care (Table 4).

Table 3. Colour variation of eggs of *P. biaculeatus* during incubation period

Days post-hatch	Colour
1	Light orange
2	Bright orange
3	Pale black
4 -5	Dark black
6	Silvery

Fig. 1. A pair of *Premnas biaculeatus* guarding the newly spawned eggs

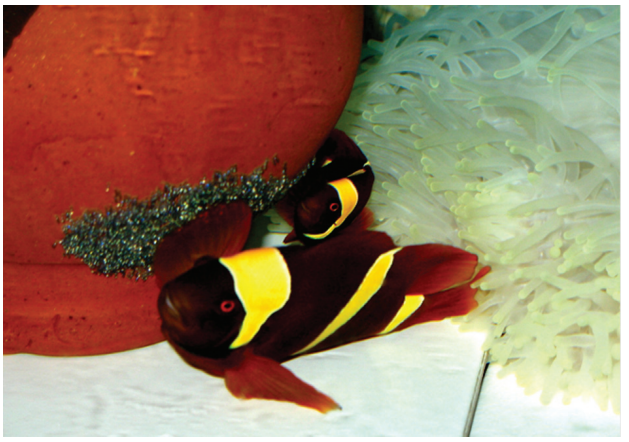


Fig. 2. Silvery eggs of *Premnas biaculeatus* on 6th day of incubation (just before hatching)

Embryonic development

The embryonic developments could be traced through three distinct phases *viz*., cleavage, embryonic and eleutheroembryonic phases.

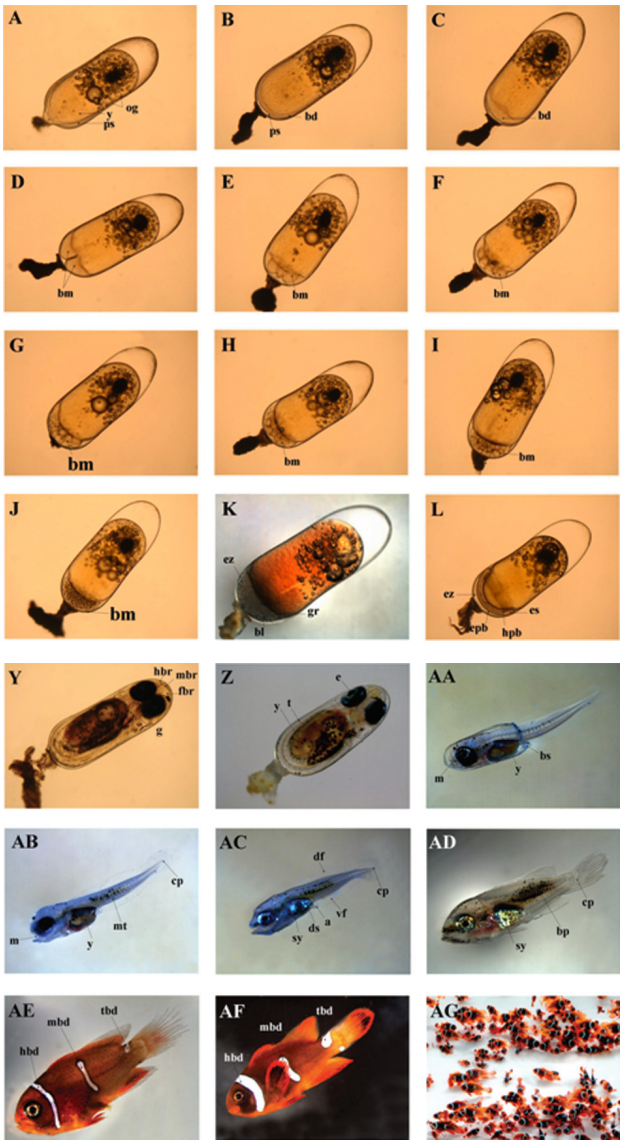
Cleavage phase

Embryonic development upto 24 h after fertilization is considered as the cleavage phase (Fig. 3; Table 5). It consists of formation of perivitelline space, bipolar differentiation and first multiplication of cells, formation of blastula, gastrula, beginning of epiboly, germ ring and embryonic shield. The newly deposited fertilized egg has a thick chorion, and contains multiple oil globules (one oil globule large in size) in the yolk. A narrow perivitelline space is formed after completion of spawning process (Fig. 3A, B). The blastodisc was noticed within 45 min after fertilization (Fig. 3C) and first meridional division (2 cells) occurred in the animal pole (posterior side) at 1:10 hours post-fertilization (h.p.f.) (Fig. 3 D).

Table 4**.** Influence of parental care on egg survival and hatchability during incubation period

Pair code	Average survival of eggs during incubation (%)						Average hatchability(%)
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
Egg clutches with parents							
Pb1	100	98.1	97.5	97	95.3	95.0	97.2
Pb2	100	100	98.5	95.2	94.7	93.4	95.1
Pb3	100	100	95.5	94.7	94.2	94.0	95.9
Pb4	100	100	98.5	98.2	98.0	95.5	96.4
Pb5	100	100	100	100	98.0	98.0	98.2
Egg clutches without parents							
Pb6	100	98.2	95.2	70.2	50.2	-	-
Pb7	100	94.2	90.1	60.2	45.2	.	-
Pb8	100	94.8	89.2	70.2	47.2	10	2
Pb9	100	90.2	81.2	74.2	30.2	-	-
Pb10	100	92.3	85.3	70.2	38.5	20	-

- No data



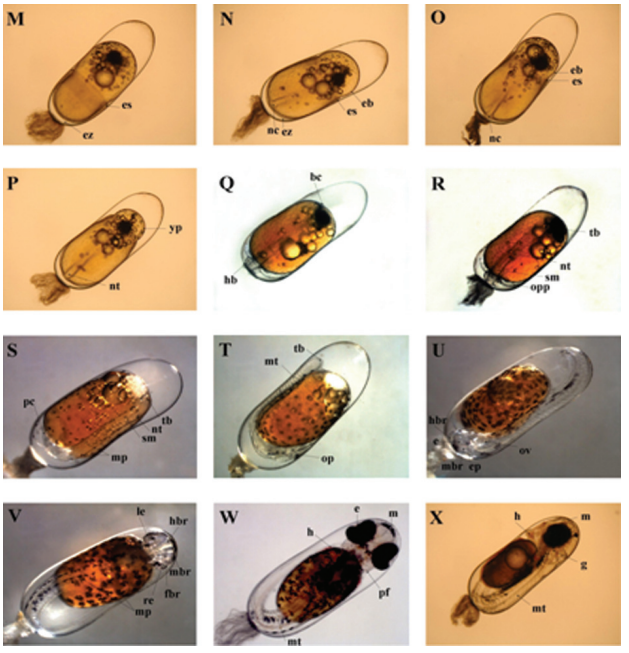


Fig. 3. Embryonic developments of *Premnas biaculeatus* (A) Soon after fertilization (00:00 h); (B) Fertilized egg (00:15 h); (C) Blastodisc formation stage (00:45 h); (D) 2-blastomere stage (01:10 h); (E) 4-blastomere stage (01:45 h); (F) 8-blastomere stage (02:30 h); (G) 16-balstomeres (03:15 h); (H) 32-balstomeres (03:30 h); (I) 64-balstomeres (03:45 h); (J) 128-balstomeres (04:10 h); (K) Late blastula (05:00 h); (L) Early gastrula (10:05 h); (M) Epiboly stage (10:10 h); (N) Late gastrula, epiboly stage and nerve cord initiation (12:30 h); (O) Extension of embryonic shield, developed nerve cord (16:40 h); (P) Notochord initiation (20:15 h); (Q) Blastopore closing (21:30 h); (R) Developed notochord and optic vesicle (24:10 h); (S) Somites stage (28:30 h); (T) Optic vesicle formation (40:00 h); (U) Prominent eye pigmentation, heart and pericardial cavity development (60:30 h); (V) Fore brain, mid brain and hind brain (72:00 h); (W) Developed eyes and maxilla formation (96:00 h); (X) Gill arches and prominent heart formation (100:00 h); (Y) Size of embryo increased with reduction of yolk (120:00 h); (Z) Embryo just before hatching (144:00 h); (AA) Hatchling (144:00 h); (AB) Newly hatched larva (144:00 h or 00:00 hph); (AC) Larva (48:00 hph); (AD) Larva (7dph); (AE) Juvenile (23dph); (AF) Juvenile (48dph); (AG) Juvenile (60dph).

a-anus; bc- blastopore closing; bd- blastodisc; bl- blastula; bm- blastomeres; bp- body pigments; bs- broken shell; cf- caudal fin; cp- caudal peduncle; df- dorsal fin; dg- digestive system; e- eye; eb- epiboly; el- eye lens; ep- eye pigments; epb- epiblast; es- embryonic-shield; ez- evacuation zone; fbr- forebrain; g- gills; gr- germ ring; h- heart; hb- head bud; hbd- head band; hbr- hindbrain; hpb- hypoblast; le- left eye; m- mouth; mbd- middle band; mbr- midbrain; ml- maxilla; mp-melanophore; mt- myotomes; my- myomeres; nc- nerve cord; nt- notochord; og- oil globule; op- otic placode; opp- otic primodium; ov- otic vesicle; pc- presumptive pericardial cavity; pf- pectoral fin; ps- perivitelline space; re- right eye; sm- somites; sy- shrunken yolk; t- tail; tb- tail-bud; tbd- tail band; vf- ventral fin; y- yolk; yp- yolk plug.

The initiation of second cleavage resulting in 4 blastomeres occured in a single meridional plane at right angles to the first at 1:30 h.p.f (Fig. 3E). The embryo attained 8, 16, 32, 64, 128 blastomere stages within 4:10 h.p.f. (Fig. 3 F-J). Blastulation was observed when the number of oil droplets decreased and it extended from the 128-blastomere stage to morula. Germ ring formation, onset of gastrulation, appearance of evacuation-zone and notochord indicates late gastrulation (Fig. 3 K-N). When the embryo is undergoing gastrulation, the individual cells are extremely small, and the blastomeres become thickened to form embryonic shield which is composed of epiblast and hypoblast layers (Fig. 3 L-N). Different morphogenetic movements were noticed at epiboly stage and the blastomeres covered three-fourth of the egg within 16:40 h.p.f. (Fig. 3 O). The yolk plug stage was noticed at 20:15 h.p.f. (Fig. 3 P) while blastopore closing occurred at 21:30 h.p.f. (Fig. 3Q).

Table 5. Embryonic development of *Premnas biaculeatus* under laboratory conditions

Phase	Time (h : min)	Stage	Characteristics
Cleavage	0. 00	Soon after fertilization	Capsule shaped egg with yolk encased in a flexible, transparent chorion, narrow perivitelline space formation, multiple oil globules situated on the dorsal side of egg.
	0.15	Fertilized egg	Prominent perivitelline space, blastodisc formation and oil globules segregated to vegetative pole.
	0.45	Blastodisc formation	1 cell formation
	1:10	2-blastomeres	2 cell division
	1:30	4-blastomeres	4 cell division initiation
	1.45	4-blastomeres	4 cell division
	2:15	8-blastomeres	8 cell division initiation
	2:30	8-blastomeres	8 cell division
	3:05	16-balstomeres	16 cell division initiation
	3.15	16-balstomeres	16 cell division
	3:30	32-balstomeres	32 cell division
	3:45	64-balstomeres	64 cell division
	4:10	Early blastula	128 cell division
	5:00	Late blastula	Morula, Germ-ring, initiation of evacuation zone
	10:05	Early gastrula	Embryonic-shield (epiblast and hypoblast), developed evacuation zone.
	10:10		Embryonic shield extended up to middle of the yolk and initiation of epiboly stage.
	12:30	Late gastrula	Epiboly stage and nerve cord initiation
	16:40		Developed nerve cord, embryonic shield extended 3/4th of the yolk.
	20.15		Yolk-plug stage.
	21.30		Blastopore closing and initiation of head bud formation.
Embryo	24:10	Embryo (somatogenesis)	Head bud prominent, otic primodium, initiation of somites, tail bud, notochord formation, melanophore appears on yolk.
	28:30		Tail bud extend up to the yolk, formation of melanophore in yolk, notochord and somites differentiation noticed, initiation of pericardium
	40:00	Embryo	Tail bud extended beyond the yolk, brain formation, stellate melanophore prominent on yolk and started to appear on cephalic region, otic placodes, myotomes.
	48.00	Embryo	Eye lenses, eye pigmentation slightly started, otic vesicles protruded laterally, tail movement noticeable, reduction in number of oil globules.
	55.00		Head inversion, midbrain, hindbrain, forebrain differentiation. presumptive pericardial cavity erythrophores formed as body pigment on myomeres, stellate pigment on yolk.
	60.30	Embryo	Heart, pericardial cavity developments, heart beat recognizable, prominent eye pigmentation, blood circulation visible, eye pigment noticeable, reduction in yolk, more stellate pigments on yolk.
	72.00	Embryo	Developed brain, pigmented eye, moving tail, size of yolk reduced.
	96.00	Embryo	Pectoral fin bud, heart developed and blood circulation visible, maxilla formation initiated.
	100.00	Embryo	Gill arches, maxilla, heart beat clearly visible, myotomes.
	120.00	Embryo	Size of embryo increased, maxilla, eye, blood circulation clearly visible, pectoral fin developed, head (hind, mid and fore brain) distinguishable, tail movement active, myotomes clearly visible.
	144.00	Embryo	Embryo occupied entire space of the capsule, wriggling movement, tail wrapped completely around the egg, eyes silvery and glittering. All embryos ready to hatch at this stage (pre-hatching stage).

Eleuthero-embryonic	00.00	Pre-larvae/ Free embryo	Newly hatched larvae 2.5 to 3.6 mm in length with a transparent body, large eyes, visible mouth, and small yolk sac. Larvae are free swimming. The mouth gape ranged from 235 to 350 µ. Caudal plecura, myomeres visible.
	48.00 hph	Larvae	Total length 5.6 to 5.9 mm, shrunken yolk, digestive system developed, presence of myomeres, pigmentation on head and abdomen. Developments of ventral fin, dorsal fin and caudal fin and caudal plecura noticeable.
	7 dph	Larvae	Total length 9.4 to 9.9 mm, shrunken yolk, dorsal, ventral, pelvic, caudal fin transparent, and fin rays developed. Blackish brown pigmentation noticed on lateral side and cephalic region.
	20 dph	Larvae	Developed caudal fin, maroon colour spread over the body, pale head bands, larvae shift from pelagic to epibenthic stage, feed on wet and artificial feed.
	23 dph	Juvenile	Larvae metamorphosed to juvenile, measured 16.3 to 17.5 mm in total length and the body deeper and rounder especially in the gut area. Metamorphosed juveniles moved from the water surface to the bottom of the tank. Three bands (developed opercular band, partially developed middle and tail band) observed. The colour of the whole body reddish maroon. Fin spines developed on the first dorsal fin.
	45 dph	Juvenile	Body more thick. Three white bands prominent and broad. Spine on the cheek developed. Fins rays developed and thick. Size 18.6 to 19.00 mm
	60 dph	Juvenile	Size of juveniles varied between 27 to 30 mm.

Embryonic phase

The initiation of embryonic body (organogenesis) formation was observed when the blastomeres covered the whole yolk. Neural keel extend along the embryonic axis and the notochord appeared with the head and eye directed towards the attached end of the egg and tail-bud appeared at the posterior part of the yolk mass (Fig. 3R). The tail-bud extend upto the end of the yolk. Embryo reached 6-somite stage with initiation of stellate melanophore (Fig. 3S). The otic placoid, brain formation and myotomes became recognizable. The melanophore appeared on yolk sac and tail is extended beyond the yolk. Throughout the initial stages described above, the yolk remains relatively large with one large oil globule and many smaller ones up to 40.00 h.p.f. (Fig. 3T) whereas the oil globules were not traceable from 40.30 h.p.f. onwards. Slight differentiation of forebrain, midbrain and hindbrain took place; pigmentation (melanophores) started to appear on the head and body; the tail was completely formed and extended beyond the yolk with frequent movement, and otic vesicles were formed on the head after 60.30 h.p.f. (Fig. 3U). The embryo inverted its position and the head was orientated to the distal end of the adhesive side, clear differentiation of midbrain, hindbrain, forebrain and presumptive pericardial cavity and melanophores were noticed at 72.00 h.p.f. (Fig. 3V).

Cup-shaped pigmented eyes and red pink heart became more visible. The heart beat and the circulation of blood cells within the whole body, pericardial body cavity and pectoral fin bud were clearly visible. A reduction in yolk mass was also observed at 96:00 h.p.f. (Fig. 3W). The pelvic fin bud and maxilla were developed at 100 h.p.f. (Fig. 3W- X). Size of the embryo increased inside the capsule, gill arches, maxilla and eye appeared, opercle, pectoral fin, head and tail became visible at 120 h.p.f. (Fig. 3 X-Y).

At 144 h.p.f., the embryo occupied the entire space of the capsule while pigmentation became more apparent during the pre-hatching stage. The wriggling movement of embryos was more visible at this stage, and tail wrapped completely around the egg. The eyes were pigmented. All the embryos were ready to hatch at this stage (Fig. 3 AA). The embryo had undergone rapid development and was clearly visible through the transparent egg membrane just prior to hatching with large eyes, silvery pupils and orange yolk sac.

On the 6th day of incubation (144 hrs), after sunset, the embryo hatched out through pliable distal end of chorion by breaking the egg capsule with its active wriggling, and the hatchlings emerged tail first. The peak hatching took place between 1830 and 1930 hrs with 95-98% hatchability.

Eleutheroembryonic phase

The eleutheroembryo (newly hatched larvae) measured 2.5 to 3.6 mm, and were actively swimming near the water surface. The mouth gape of the newly hatched larvae ranged from 235 to 350 µ.

Larval metamorphosis

First food was accepted within 12 h post-hatch, and learning process in prey capture which involves search, encounter, attack and capture was completed within 24 h post-hatch. Green water system was provided soon after hatching to enhance visibility of prey organisms as it can enhance visual contrast and light dispersion which in turn improves food detection and location, and can also reduce "nose bumping" and "head butting" syndrome. The yolk sac was almost completely reabsorbed within 24 h post-hatch. Eyes, mouth, gut and fin fold were well developed (Fig. 3 AB-AC). The larvae were in a more advanced stage of development and the fin fold had changed to dorsal, pelvic and caudal fins. Fin rays were evident and body was less transparent at 7 days post-hatch (d.p.h.) (Fig. 3AD).The larvae metamorphosed from 20th d.p.h. to juveniles and all bands (head, middle and tale bands) were observed on 23rd d.p.h. At this stage, the total length of juvenile varied between 16.3 and 17.5 mm. On 45th d.p.h, all bands were broadened. Of this, the head band was almost developed. The juveniles reached marketable size from 60th d.p.h. onwards and were kept in aquaria with other reef fishes. Most of the metamorphosed juveniles started accepting minced meat of shrimp, fish, mussel, and clam and formulated diets (Fig. 3 AE-AG).

Effect of feeding on larval survival

Survival of larvae fed on diet I showed highly significant variation ($p<0.01$) from that of larvae fed on other diets. No significant variation was obtained between diets III and V, and these two diets significantly ($p<0.05$) varied from other tested diets. The highest larval survival (78.9%) was obtained in larvae fed on microalgae, enriched rotifer (*B. rotundiformis*) and *Artemia* nauplii (diet III) whereas a survival of 70.1% was obtained in larvae fed with microalgae enriched rotifer (*B. plicatilis*) and *Artemia* nauplii (diet V). The larvae fed on non-enriched feed (diets II and IV) showed only 32.3% and 30.2% survival respectively (Table 6).

The environmental parameters such as temperature, salinity, pH, light, photoperiod, dissolved oxygen, NO_2, NO_3 and NH_3 maintained in the experimental tanks are presented in Table 7.

Discussion

Provision of optimum environmental parameters and nutritionally enriched feeds are the key factors for gonadal maturation in many marine species under captive conditions. The most significant improvements in the eighties were the conditioning and feeding of broodstock and in early nineties the use of manipulated live food organisms through quality enhancement using specified enrichment techniques which improved the quality of larvae and allowed commercial level fry production in hatcheries (Dhert *et al.,* 1997). Co-feeding of live feeds supplemented with lipid and vitamin formulations helped to increase the nutritional reserves of broodstock and hence improved the overall egg and larval quality (Dehasque *et al*., 1995). Due to small size brooders of marine ornamental clown fishes, the use of enriched live feed to enhance nutritional quality of broodstock have potential application as also reported in the freshwater ornamental fishes weighing less than 100 g (Dhert *et al.,* 1997). Spawning and embryo development are related to broodstock nutrition and high quality breeders are essential for larval rearing. Food and feeding studies of clownfishes also reported that planktonic food comprising of microzooplankton and algae is important to most clown fishes (Allen, 1974; Fautin and Allen, 1997). In the present study, apart from wet feeds, the broodstock was also fed with enriched live feed, L type

Table 6. Average survival (%) of *P. biaculeatus* larvae during 20 days post-hatch

	Days post-hatch				
Feeds	1 to 4	5 to 8	9** to 12**	13** to 16**	17** to 20**
I - Microalgae*	53.7	17.8	5.9	-	-
II - Microalgae* and *Brachionus rotundiformis* (6 to 8 nos. ml^{-1})	70.7	47.9	42.2	38.2	32.3
III- Microalgae* and enriched *B. rotundiformis* with codliver oil (6 to 8 nos. ml^{-1}).	97.4	93.1	87.7	82.9	78.9
IV- Microalgae* and *B. plicatilis* (6 to 8 nos. ml^{-1}).	67.1	45	41.6	37.5	30.2
V- Microalgae* and enriched *B. plicatilis* (6 to 8 nos. ml^{-1}) with cod liver oil	95.5	87	81.1	74.7	70.1

* Microalgal concentration: *C. marina* and *N. oculata* in 1:1 proportion at 1.5 x10^6 cells ml^{-1}.
- No data
** Newly hatched *Artemia* nauplii (4-6 numbers per ml) were provided in all treatments from 9th to 20th days post-hatch.

Table 7. Environmental parameters (water) in different rearing tanks of *P. biaculeatus*

Tanks	T (°C)	S (ppt)	D.O. (mg l^{-1})	pH	NH_3 (µg-at l^{-1})	NO_2 (µg-at l^{-1})	NO_3 (µg-at l^{-1})
Pair formation and Broodstock	29 ± 1	31 ± 1	5.2 ± 0.6	8.2± 0.2	<0.02	<0.01	<0.07
Egg hatching	29 ± 1	31± 1	6.5 ± 0.3	8.4± 0.3	<0.02	<0.01	<0.05
Larvae	30 ± 1	33 ± 1	6.2 ± 0.5	8.5± 0.4	<0.02	<0.01	<0.05
Juvenile	30 ± 2	33 ± 2	6.0± 0.5	8.5± 0.3	<0.02	<0.01	<0.07
Rotifer mass culture	30 ± 2	23± 2	5.1± 0.6	8.2 ± 0.2	<0.05	<0.02	<0.1
Artemia mass culture	32 ± 1	35± 2	6± 2	8.4 ± 0.2	<0.02	<0.01	<0.09
Enriched rotifer	30 ± 2	22 ± 2	5± 2	8.3 ± 0.2	nt	nt	nt
Enriched artemia	32 ± 1	33 ± 2	6± 2	8.5 ± 0.2	nt	nt	nt
Algae stock culture	20 ± 2	32 ± 2	7± 2	8.2± 0.3	nt	nt	nt
Algae mass culture	27 ± 2	29 ± 2	7± 2	8.4± 0.3	nt	nt	nt

T - temperature, S - salinity, D.O. - Dissolved oxygen, ppt - parts per thousand, nt - Not traceable
µg-at l^{-1}– microgram atom per litre

rotifer *B. plicatilis* and *Artemia* nauplii to improve the health of broodstock, production of quality eggs and larvae for stable fry production. Even though many marine ornamental fish and invertebrates have been induced to spawn in captivity, the larvae of only a few species have been successfully reared in captivity (Holt, 2003; Olivotto *et al*., 2003). The bottleneck is the heavy mortality encountered in the larvae during the critical period of development, mostly noticed when the larvae change over from internal yolk stores to exogenous feed.

The deposited telolecithal eggs of *P. biaculeatus* were orange-pink, elliptical/capsule-shaped, adhesive and the size of the yolk reduced gradually when cleavage proceeds as reported in *Amphiprion chrysopterus* and *Amphiprion perderaion* (Allen, 1972). The newly fertilized egg of *P. biaculeatus* contained a large oil globule in the dorsal space of the egg and had clear perivitelline space. The epidermis of embryo (egg) of marine fishes contains an outer layer of interdigitating cells and an inner layer of more uniformly shaped cells, and this epidermis layer degenerates and disappears as development proceeds (Falk-Petersen, 2005). In the present study, observation of the egg immediately after laying showed that the cortical granules release their content and thereby the eggs get attached to the substrata and then subsequent development of perivitelline space takes place, as suggested by Iwamatsu and Ohata (1976).

The duration of cleavage varies depending on species, and the eggs of *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 (Allen, 1972) whereas in the present study 8, 16, 32, 64 and 128 cell stages in *P. biaculeatus* was attained at 2.15, 3.05, 3.30, 3.45 and 4.10 h.p.f. respectively. During the blastulation stage, the cleavage process of *P. biaculeatus* was recorded earlier than that for *A. chrysopterus*. This may be due to variation in water temperature, parental care and egg quality during incubation. The early cleavage also varies depending upon egg quality and species (Kjorsvik *et al.,* 2003; Falk-Petersen, 2005). In the present study, gastrulation was characterized by the presence of a germ-ring which consisted of two cell layers called epiblast and hypoblast as reported in teleosts (Arezo *et al*., 2005). These two layers are actively involved in osmoregulation and ion transportation as in early gastrulation (Swanson, 1996). In *P. biaculeatus*, eyes started to develop pigment and heart became visible at 60:30 h when the heart started beating (200 beats per minute) and the circulation of blood cells within the whole body became clearly visible in the present study whereas Wilkerson (2001) reported that the *Amphiprion* embryo formed a heart, brain and spinal cord on day 3 after deposition, and blood circulation was clearly visible on day 4 in *A. chrysopterus* (Allen, 1974). In the present study, pectoral fin became prominent and moved frequently at 120 h.p.f. which was noticed in *A. chrysopterus* at 104 h.p.f. (Allen, 1972).

Melanophores appeared in the yolk mass region from 24.10 h.p.f., and it gradually extended to the anterior part of the head and the dorsal part of the body at 48 h.p.f. Red pigmentation of erythryophores was noticeable at 55 h.p.f. in myomeres and head of *P. biaculeatus*. In the case of *A. chrysopterus,* body pigmentation appeared in the form of 2 rows on ventral surface on 4th day of incubation and the pigment containing cells were located within the subdermal spaces and/or under the basal lamina of the ectoderma (Allen, 1972). The variation in time of appearance of pigmentation may be due to species specificity and nutrition available in yolk (Allen, 1974; Swanson, 1996; Wilkerson, 2001; Arezo *et al*., 2005; Kovac, 2005).

In nature, though marine larvae initially feed on a wide variety of marine micro-zooplankters such as protozoans (ciliates, foraminiferans), dinoflagellates, mollusc larvae, eggs and nauplii of copepods (Riley and Holt, 1993; Holt and Holt, 2000; Olivotto *et al*., 2004), they have not generally been used extensively in aquaculture, since they are difficult to culture on a continuous basis at a high density. As a result, the most widely used live feed in marine fish larval rearing are rotifers (*B. plicatilis*) and *Artemia* nauplii (Olivito *et al.*, 2005). However they are not always acceptable food for high survival of marine fish larvae without enrichment as reported in grouper (Pechmanee *et al.,* 1988). The present study agrees with their finding as maximum survival of larvae of *P. biaculeatus* was obtained in larvae fed with enriched rotifer (*B. rotundiformis*). An alternative may be to prduce high quality rotifer through bioencapsulation as they are often present in higher numbers in the water column than copepod nauplii (Kamiyama, 1994; Uye *et al.,* 1996).

Higher survival rate (78.9%) was observed in larvae fed with enriched *B. rotundiformis* (0 to 20days) followed by co-feeding with newly hatched *Artemia* nauplii from 9 to 20 days of post-hatch whereas lowest survival rate (30.2%) was observed in groups fed exclusively on the larger nonenriched rotifer *B. plicatilis* and *Artemia* nauplii. Other studies on larval rearing showed that the ω3 HUFA content of rotifers enriched with emulsified oil was higher than those fed with *Chlorella* sp. and as a result higher survival was obtained in 15 days old larvae of *Epinephelus malabaricus* (Pechmanee *et al.,*1988). The higher survival in the present study on feeding with enriched rotifer (*B. rotundiformis*) may be due to higher level of w3 HUFA which is a pre-requisite for the metamorphosis of larvae of *P. biaculeatus*. Thus the results suggest that the quality and body size of prey at first feeding may significantly affect survival rate in larval rearing as reported in other marine ornamental fishes (Holt, 2003). In all experimental groups, larval mortality was comparatively high from 36 to 48 h post-hatch probably related to onset of exogenous feeding as also suggested by Wilkerson (2001). Apart from this, the photoperiod (24 L/0 D) also influenced larval development and growth which indicate that larvae are visual feeders as reported in other marine fish larvae (Tandler and Helps, 1985; Duray and Kohno, 1988; Olivotto *et al.,* 2003). Through management of feed and environmental parameters, hatchery produced larvae metamorphosed to juveniles and shifted from partially pelagic to epibenthic way of life from 20th d.p.h. onwards. The juveniles reached marketable size within 2 months as also reported in other clown fishes (Alava and Gomes, 1989; Juhl, 1992).

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References

Allen, G. R. 1972. Behaviour. In: *The Anemonefishes : Their classification and biology*. TFH Publication, Inc., Neptune City, New Jersey, p. 199-239.

Allen, G. R. 1974. *The Anemonefishes: The classification and biology,* 2nd edn. T.F.H. Publication, Inc. USA. 352 pp.

Allen, G. R. 1980. *Anemonefishes of the World: Species, Care, and Breeding.* Aquarium Systems, Mentor, Ohio. 104 pp.

Arezo, M. J., Pereiro, L. and Berois, N. 2005. Early development in the annual fish *Cynolebias viarius*. *J. Fish Biol.,* 66: 1357-1370.

Balon, E. K.1975. Terminology of intervals in fish development. *J. Fish. Res. Board Can.*, 32:1663-1670.

Boehlert, G. W. and Morgan, J. B. 1985. Turbidity enhances feeding abilities of larval Pacific herring, *Clupea harengus* pallasi. *Hydrobiologia*, 123: 161–170.

Dehasque, M., Candreva, P., Carrascosa, M . and Lavens, P. 1995. Comparison of an artificial diet and live food as broodstock maturation diets for sea bream (*Sparus aurata*): effects on spawning and egg quality In: Lavens, P., Jaspers, E. and Roelants, I. (Eds.) *Larvi' '95* Gent, Belgium: European Aquaculture Society, Special Publication, 24: 16-21.

Dhert, P., Lim, L.C., Candreva, P.,Van Duffel, H. and Sorgeloos, P. 1997. Possible applications of modern fish larviculture technology to ornamental fish production. *Aquar. Sci. Conserv.*, 1: 119-128.

Downing, G. and Litvak, M. K. 1999. The effect of photoperiod, tank colour and light intensity on growth of larval haddock, *Aquacult. Int.,* 7: 369–382.

Duray, M. and Kohno, H. 1988. Effect of continuous lighting on growth and survival of first feeding larval rabbitfish *Siganus guttatus*. *Aquaculture,* 109: 311-312.

Falk-Petersen, I. B. 2005. Comparative organ differentiation during early life stages of marine fish. *Fish Shellfish Immunol*., 19: 397 – 412.

Gilbert, V. S. 1996. *Artemia*. In: *Manual on the production and use of live food for Aquaculture*, Lavens, P. and Sorgeloos, P. (Eds.), FAO, Fisheries Technical Paper 361, p. 79-130.

Holt, J. and Holt, S. A. 2000. Vertical distribution and role of physical process in the feeding dynamics of two larval sciaenids *S. ocellatus* and *C. nebulosus*. *Mar. Ecol. Prog. Ser.,* 193: 181-190.

Holt, G. J. 2003. Research on culturing the early life history stages of marine ornamental species. In J.C. Cato and C.L. Brown, (Eds.), *Marine ornamental species: collection, culture and conservation*. Cato, Iowa State Press. 251–254 pp.

Iwamatsu, T. and Ohta, T. 1976. Breakdown of the cortical alveoli of medaka eggs at the time of fertilization with particular reference to the possible role of spherical bodies in the alveoli. *Willhelm Roux's Archives of Developmental Biology*, 180: 297-309.

Jones, A.C. 1970. *Chlorella* for rearing of marine fish larvae, *FAO Fish Cult. Bull.,* 2 (4), p. 3.

Kamiyama, T. 1994. The impact of grazing by microzooplankton in Northern Hiroshima Bay the Seto Inland sea, Japan. *Mar. Biol.,* 119: 77-88.

Kjorsvik, E., Hoehne-Reitan, K. and Reitan, K. I. 2003. Egg and larval quality criteria as predictive measures for juvenile production in turbot (*Scophthalmus maximus* L.). *Aquaculture,* 227: 9-20.

Kovac, V. 2005. Early development of *Zingel streber*. *J. Fish Biol.*, 57: 1381-1403.

Lee, C. S. and Ostrowski, A. C. 2001. Current status of marine finfish larviculture in the United States, *Aquaculture,* 200: 89-109.

Liao, I. C., Su, H. M. and Chang, E. Y. 2001. Techniques in finfish larviculture in Taiwan, *Aquaculture,* 200: 1–31.

Madhu, R. and Madhu K. 2000. Anemone fishes in Andaman and Nicobar, *CARI, Leaflet*. p.1-6.

Madhu K., Madhu, R., Gopakumar, G., Sasidharan, C. S. and Venugopalan, K. M. 2006. Breeding, larval rearing and seed production of maroon clown *Premnas biaculeatus* under captive conditions *Mar. Fish. Infor. Serv. T&E Ser.,*190: 1-5.

Naas, K. E., Naess ,T. and Harboe, T. 1992. Enhanced first feeding of halibut larvae (*Hippoglossus hippoglossus* L.) in green water. *Aquaculture,* 105: 143–156.

Naas, K. E. Huse, I. and Iglesias, J. 1996. Illumination in first feeding tanks for marine fish larvae, *Aquacult. Eng.*, 15: 291–300.

O'Brien, W. J. 1979. The predator–prey interaction of planktivorous fish and zooplankton, *Am. Sci.*, 67: 572–581.

Olivotto, I., Cardinali, M., Barbaresi, L., Maradonna, F. and Carnevali, O. 2003. Coral reef fish breeding: the secrets of each species. *Aquaculture,* 224**:** 69– 78.

Olivotto, I., Yasumasu, S., Gioacching, G., Maradonna, F., Cionna, C. and Carnevali, O. 2004. Cloning and expression of high choriolytic enzyme, a component of the hatching enzyme system, during embryonic development of the marine ornamental teleost, *Chrysiptera parasema*, *Mar. Biol.,* 145: 1235–1241.

Olivotto, I., Zenobi, A., Rollo, A., Migliarini, B., Avella, M. and Carnevali, O. 2005. Breeding, rearing and feeding studies in the cleaner goby *Gobiosoma evelynae*. *Aquaculture,* 250: 175-182.

Pechmanee, T., Assavaarce, M., Bunliptanon, P. and Akkayanon, P. 1988. Possibility of using rotifer, *Brachionus plicatilis*, as food for early stage of grouper larvae, *Epinephelus malabaricus*. *Technical Paper No.4.* National Institute of Coastal Aquaculture, Department of Fisheries, Thailand, 10 pp.

Strickland, J. D. and Parsons, T. R. 1968. *A practical handbook of seawater analysis.* Ottawa : Fisheries Research Board of Canada, Bulletin 167, 293 pp.

Swanson, C. 1996. Early development of milkfish: effects of salinity on embryonic and larval metabolism, yolk absorption and growth. *J. Fish Biol.,* 48: 405-421.

Talwar, P. K. 1990. Fishes of the Andaman and Nicobar islands: A synoptic analysis, *J. Andaman Sci. Assoc*., 6(2): 71-102.

Tandler and Helps, S. 1985. The effect of photoperiod and water exchange rate on growth and survival of gilthead seabream (*Sparus aurata* Linnaeus, Sparidae) from hatching to metamorphosis in mass rearing system. *Aquaculture,* 48: 71-82.

Uye, S., Nagano, N. and Tamaki, H. 1996. Geographical and seasonal variations in abundance, biomass and estimated production rates of microzooplankton in the island sea of Japan. *J. Oceanogr.,* 52: 689-703.

Walney, P. R. 1974. *Culture of bivalve molluscs : 50 years experience at Conwy*. Fishing News Book Ltd. Farnham-Surrey, England, p.1-173.

Wanzenböck , J. and Schiemer, F. 1989. Prey detection in cyprinids during early development, *Can. J. Fish. Aquat. Sci.,* 46: 995–1001.

Watanabe, T., Tamiya, T., Oka, A., Hirata, M., Kitajima, C. and Fujita, S. 1983. Improvement of dietary value of live foods for fish larvae by feeding them on ω3 highly unsaturated fatty acids and fat soluble vitamins. *Bull. Jpn. Soc. Sci. Fish.*, 49: 471-479.

Wilkerson, J. D. 2001. *Clown fishes: A guide to their captive care, breeding and natural history,* 1st edn., Microsom Ltd. USA, p. 240-260.

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