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EMBRYONIC DEVELOPMENT OF THE INDIAN NATIVE KILLIFISH APLOCHEILUS LINEATUS FROM THE RIVER TAMBRAPARANEI

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ABSTRACT
The characteristics and stages of early development of *A. lineatus*, a cyprinodont fish endemic to India, were obtained from *in vivo* observations of embryos reared under laboratory conditions. Gravid females produced 25 - 30 eggs in a batch. Eggs measured 1 - 1.2mm in diameter, were phytophilic, and possessed a transparent chorion. Eggs were attached to the aquatic plants using sticky filamentous projections of the chorion. The incubation period of *A. lineatus* was found to be 9 - 12 days. The index of hatching was 96%, and that of fertility was 96.3%. Early development was divided into 11 stages, similar to the development in other killifishes. The hatchlings were transparent and measured 4 - 6 mm in TL. The yolk sac got fully absorbed on the 9th day. The fingerlings appeared more like adults.

KEYWORDS: Killifish, Embryonic development, Diapause, *Aplocheilus lineatus.*

INTRODUCTION
The primary objective of any fish hatchery is to produce the maximum number of quality seeds from the available brood stock. According to Blaxter (1974) studies on fish eggs and larvae were of value in estimating the size of the fish stocks. The embryonic development of killifish, a group of small, freshwater fish in the family Cyprinodontidae, is a fascinating process that offers insights into developmental biology, ecology and evolutionary adaptation. Killifish are particularly well known for their ability to produce desiccation-resistant eggs, allowing them to thrive in extreme and variable environments like ephemeral pools and seasonal wetlands.

The studies on the larval development of cyprinodontiformes *viz.*, Kapakos *et al*., 2024 in killifishes; Brown *et al*., 2011 in *Fundulus grandis*; Chaibi *et al* 2015 in *Aphanius fasciatus*; Kamal *et al*., 2009 in *Aphanius sophiae*, Korwin (2012); Podrabsky & Culpepper (2012) in *Austrofundulus limnaeus* and Podrabsky (2017) in *Austrofundulus limnaeus* have been reported.

The pattern of development and the timing of the ontogenetic events were a reflection of both internal and external conditions (Arezo *et al*., 2005). During the embryonic growth of *Fundulus heteroclitus* the yolk material provides substitute energy (Huang *et al*., 2019). Arenzon *et al.* (2002) reported the early developmental description of *Cynopoecilus melanotaenia.*

Shun *et al.*, 2014 described 19 embryonic stages in *Oryzias latipes*, 36 stages in *Adinia xenica* (Seegers, 2000), and 13 stages in *Cynolebias viarius* (Arezo *et al.*, 2005). Among killifishes' embryonic development was described in *Cynolebias viarius* (Arezo *et al.*, 2005), *Rivulus marmoratus* (Kim *et al.*, 2003), *Fundulus heteroclitus* (Huang *et al*., 2019), *Cynopoecilus melanotaenia* (Arenzon *et al*., 2002), *Austrofundulus limnaeus* (Podrabsky (2017), (Kapakos *et al*., 2024) killifishes, (Brown *et al*., 2011) *Fundulus grandis*, (Chaibi *et al*., 2015) *Aphanius fasciatus*, (Kamal *et al*., 2009) *Aphanius sophiae*, Korwin (2012) and Podrabsky & Culpepper (2012); Podrabsky (2017) *Austrofundulus limnaeus.* A thorough survey of the literature showed that no such work had been reported earlier in the killifish *A. lineatus.*

MATERIALS AND METHODS
Captive Breeding and Incubation of fertilized eggs
Mature males were transferred to separate breeding tanks (30 × 20 × 30cm deep) and isolated from females for a week. All aquaria in this study were maintained at 25 ± 2° under a 12h: 12h L/D photoperiod. Twice daily, the adults were fed *ad libitum* with mosquito, *chironomus* larvae and *tubifex*. Brood females were then exposed to the isolated males. Each of the pairs was allowed to have physical contact. The courtship and mating behaviours of

A. lineatus were continuously monitored and the stages were photographed. The number of eggs laid and infected in each tank was recorded daily. Fertilized eggs of *A. lineatus* were collected from the submerged plants (phytophilic) and transferred to pert dishes divided into compartments. Eggs were kept in a medium adjusted to the quality of river water (pH - 7.35, hardness - 20mg L^{-1}). The antibiotic Penicillin G was added to the medium (40mg L^{-1}). Water quality parameters were carefully monitored.

Embryonic development

The eggs were manually separated every day from the substrate and observed under a stereoscopic microscope. Triplicates were maintained for different batches. The hatching rate was observed after substrate hydration. The hatching process started a few hours after substrate hydration. After completion of development the eggs were transferred from the substrate to glass beakers containing adjusted water medium. The eggs were kept under the same water quality conditions as mentioned above.

Post embryonic development

The hatched larvae were transferred to glass tanks (30 × 15 × 15cm) containing *Hydrila* plants. The newly hatched larvae were fed with plankton soup containing *Daphnia* and *Moina* for 10 days. Water was changed daily without disturbing the hatchlings for 30 days. The larvae were then transferred to small cement tanks and fed with mosquito larvae and *Chironomus ad libtum*. The survival rate, length and weight of the fry were noted during development.

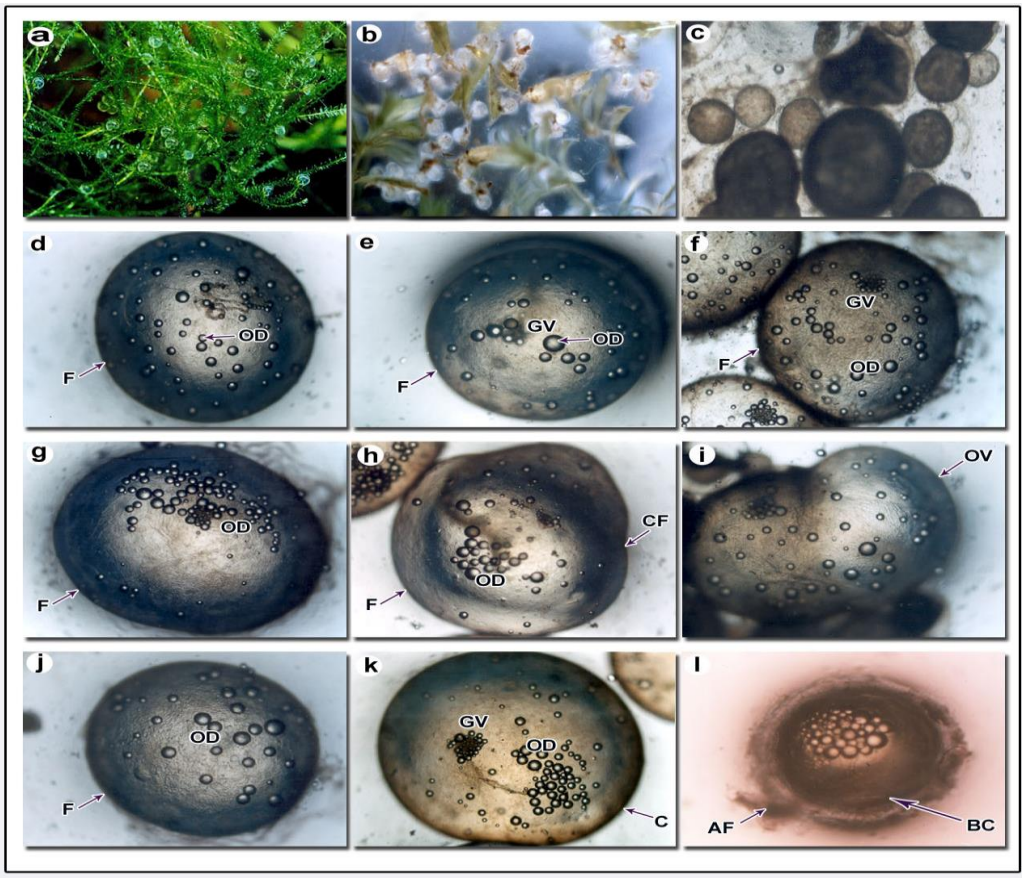

Plate 2. Embryonic development of Indian killifish *Aplocheilus lineatus*.

a. Phytophilic egg (plant spawning behaviour.) **b.** Just laid eggs (sticky filaments

c. Ovarian fragment showing follicles of all stages. **d.** Matured follicle (F) about to ovulate.

e. Hydrated oocyte with germinal vesicle (GV) near the upper surface. **f.** Hydrated oocyte having oil drops (OD) peripherally attached.

g. Hydrated oocyte where oil drops no longer peripherally attached. **h.** Egg ovulating out of its follicle : Note halo of (CF) chorionic fibres (arrow).

i. Ovulating egg. **j.** Unfertilized egg.

k. Just fertilized egg with perivitelline space (pv) **l.** Balstodisc stage.

OD - Oil droplet, F - Follicle, GV - Germinal vesicle, CF - Chorionic fibre, OV - Ovulating follicle, BC - Blastodermal cap, AF - Adhesive fillament, C - Chorion.

Data collection

The numbers of eggs laid by each pair were noted. The number of fertilized eggs and spoiled eggs was recorded. The fertilization rate and hatching rate were calculated by the following formulae (Amornsakun *et al.*, 2004):

$$\text{Fertilization rate (\%)} = \frac{\text{Number of fertilized eggs}}{\text{Total number of eggs}} \times 100$$

$$\text{Hatching rate (\%)} = \frac{\text{Number of the newly- hatched}}{\text{Total number of eggs}} \times 100$$

RESULTS

The embryos of *Aplocheilus lineatus* were successfully reared in captivity. The fertilized eggs of *A. lineatus* showed oil drops and perivitelline space between the cytoplasm and the egg cover. The freshly laid eggs were approximately 1mm in diameter. Female *A. lineatus* did not release all the eggs at a time, but rather spawned clutches of eggs in a series of mating events. They released an average of 5 - 6 eggs per clutch. It was also observed that *A. lineatus* laid 12 - 15 eggs/batch. Each batch consisted of 2 - 3 clutches. They laid eggs on alternate days, but in some cases daily. They laid their eggs both day and night. When they spawn, they never feed. They lay 25 - 30 eggs in a spawn, but in a fraction of 5 - 6/clutch. The same pair started to spawn within the next 15 days (*pers. observ)*. However, the fertilization rate and the hatching success were different within the same batch of eggs. The spawn sex ratio of 1:1 was found to be perfect in the case of killifishes. *A. lineatus* can be bred easily in captivity without the administration of any hormones.

Characteristics of unfertilized eggs

The appearance of egg follicles undergoing maturation and ovulation is shown in Plate 2d to i. As the egg enlarged, a clearly visible germinal vesicle migrated to the periphery and disappeared. At the same time, the oocyte became more translucent and oil droplets in the periphery gradually coalesced with one another (Plate 2d, e, f). Eventually, the oil droplets were able to move freely and congregated at the upper surface (Plate 2g) and dislocated to the opposite pole if the oocyte was inverted. After this stage, the oocytes were ovulated into the ovarian lumen (Plate 2i). Germinal vesicle breakdown (GVBD) *in vivo* generally occurred when the follicle reached a diameter of 465 - 623µm. The diameter of the ovulated egg was about 697 - 925µm.

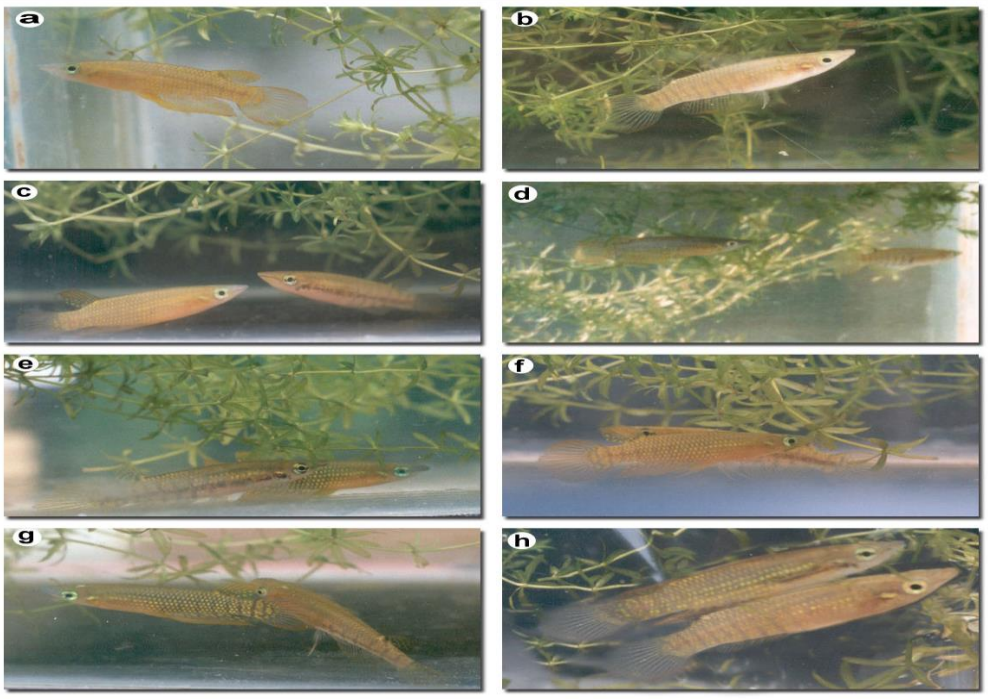

Plate 1. Courtship in *Aplocheilus lineatus*

a. Mature gravid male. **b.** Mature ripe female.
c. Male approaching female. **d.** Male chasing female.
e. Male making dangling movements inducing female. **f.** Receptor female responding to male.
g. Female following male to the spawning area. **h.** Nuptial mating.

Courtship behaviour under captivity

1. Isolated mature males and females were left in the ratio of 1:1 (Plate 1c).
2. The male approached the female and displayed its bright colour and showed s-shaped (undulatory) movements in front of the female to attract her (Plate 1c).
3. The males were motionless, caudal fin became red in colour, the rest of the fins became orange with white spot between the rays (Plate 1a).
4. The male approached the female and both of them spread their fins fully like fighters. They stood in opposite directions. The female responded by wagging its tail (S- movement). For 5 to 10 minutes, they chased one another nibbling each other (Plate 1c).
5. The male located a bushy area and came back to the female indicating the breeding area. The receptive female followed the male and returned to the original place. They swam parallel for 2 - 3 minutes (Plate 1d).

Spawning behaviour under captivity

1. After sometime the male induced the female by pressing on the dorsal region with its jaws (Plate 1g).
2. The male always stood beside the female and made undulatory movements till she responded to his call. Suddenly the male and the female moved to the breeding area and the male clasped the female and the eggs were released. At the same time the male also released his milt and fertilized the eggs. Spawning took place only in the dawn but the courtship activity was exhibited during the day time between 0900 to 1500hrs. The eggs were laid on the leaves/branches of aquatic plants (Plate 1h; Plate 2a, b).
3. After 12 hours the female again laid the next clutch of 5 - 10 eggs. The size of the eggs varied from 1.0mm to 2.5mm. A female laid 25 to 30 eggs in a fortnight. The spawn sex ratio was tested with 1(male): 2 (females), 1(male): 3 (females) and 1(male): 1 (female). The latter was observed to be very effective. Territorial behaviour was observed between males and pairs.
4. The hatching rate was 96% and the fertilization rate was 98.9 % (Table 1). Fertilization was external in *A. lineatus*. Eggs were fertilized by the male as and when the female laid the eggs in the plants. The eggs got attached to the plants with the help of sticky thread like projections. The unfertilized or dead eggs were identified by the change in colour from transparent to translucent and /or milky white.

Table 1: *Aplocheilus lineatus* spawning performance.

Performance / Spawner*	Mean ± S.E.
No.of eggs laid first spawn	35±5
No.of eggs laid second spawn	28±3
Percentage of fertilized eggs	96.3±2.6
Percentage of hatchlings	96.0±2.5
Length of hatchling (mm)	5.0±1.0
Weight of hatchling (g)	0.002±0.0005

Stages in the development of *A. lineatus*

The transparency of the egg membrane (chorion) rendered it possible to follow the stages in the embryonic development of *A. lineatus* (Table 2). The rate of development of the killifish was neither too rapid nor too slow. The incubation period was about 10 - 15 days when kept at 27°C. In the egg cluster interior eggs developed more slowly than the outer ones.

Table 2: *Aplocheilus lineatus*: Summary of development.

Stage	Characterization	Time (hours from fertilization)
1	Blastodisc stage	6
2	Blastula	12
3	Early gastrula	24
4	Late gastrula	48
5	Closure of blastopore	72
6	Pulsation of heart	96
7	Vitellocaudal vein formation	120
8	Circulation in vitellocaudal vein	144
9	Embryo encircles 1/2 of yolk sac	168
10	Embryo encircles 2/3 of yolk sac	192
11	Embryo encircles 3/4 of yolk sac	216
12	Embryo encircles 7/8 of yolk sac	222
13	Embryo encircles entire yolk sac	228
14	Tail tip reaches beyond eyes	234

15	Hatched fry	240
16	Yolk sac absorbed completely	246
17	Newly hatched larva	264

1. **The unfertilized egg:** The unfertilized egg was a round spheroid (Plate 2d) measuring about 1.0 - 2.5mm in diameter. The egg proper was closely surrounded by a chorion. The micropyle was situated on the animal pole of the ovum. The chorion had a number of evenly distributed short villi. Long sticky filaments were tufted on the vegetal pole of the egg (Plate 13l), by which eggs were attached to any water plants or solid substrates. There was a minute, funnel-shaped perforation in the chorion on the animal pole of the unfertilized ovum. This aperture was a micropyle for the entrance of the sperm.

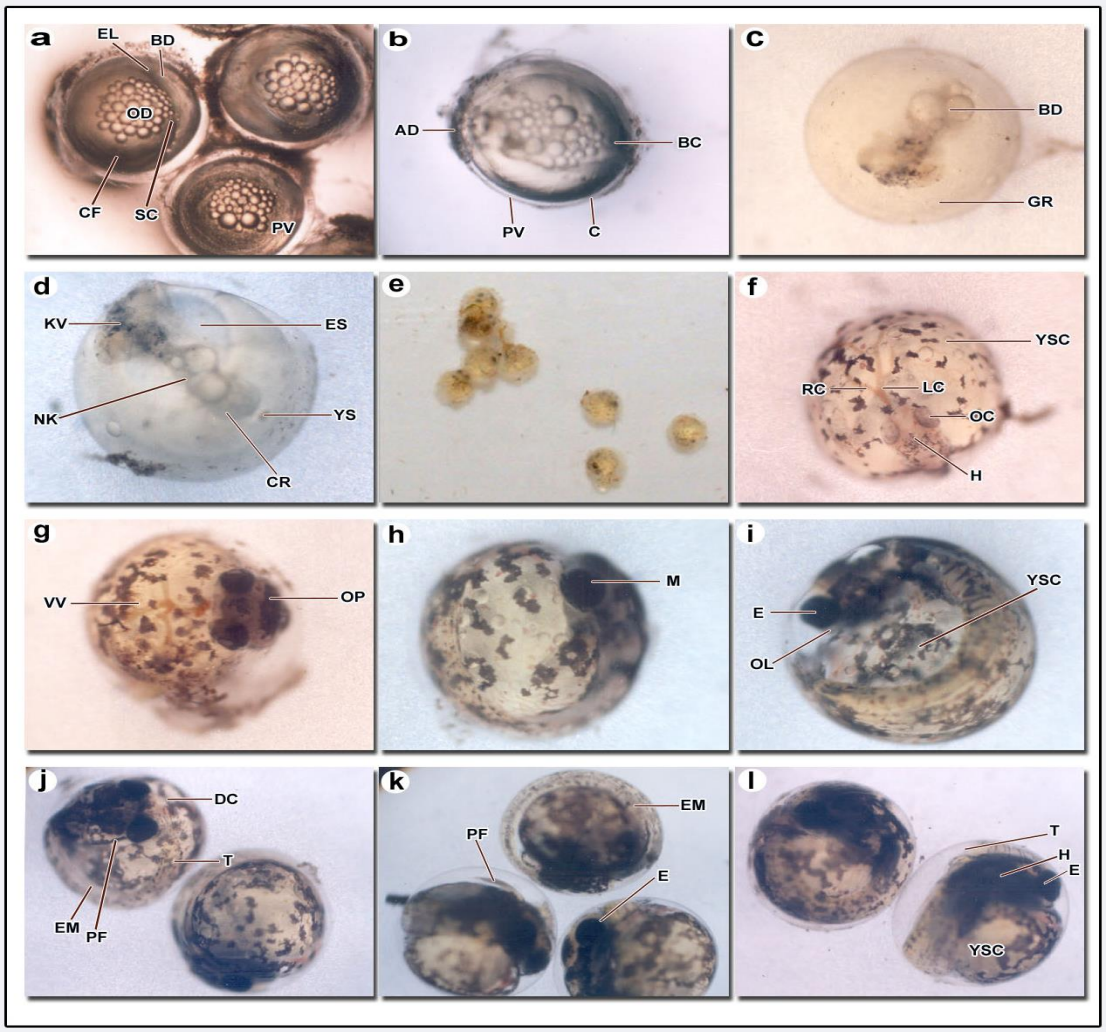

Plate 3. Embryonic development of Indian killifish *Aplocheilus lineatus*.

a. Blastula stage **b.** Early gastrula.
c. Late gastrula.. **d.** Closure of blastopore.
e. Fertilized and advanced stage. **f.** Pulsation of heart beat.
g. Formation of Vitellocaudal veins. **h.** Circulation in three vitello veins.
i. Embryo encircles 1/2th of yolk sac. **j.** Embryo encircles 2/3rd of yolk sac.
k. Embryo encircles 3/4th of yolk sac. **l.** Embryo encircles 7/8th of yolk sac.

EL - Enveloping layer, BD - Blastoderm, SC - Segment cavity, PV - Perivitelline space, CF - Chorionic fillament, GR - Germ Ring, KV - Kupffer vesicle ES - Embrtonic shield, T - Tail, NK - Neural Keel, YS - Yolk sphere, CR- Cephalic Region, YSC- Yolk Sac, RC - Right Cuvier duct, LC - Left Cuvier duct, OC - Optic Cup, H - Head, VV - Vitello caudal Vein, Op - Optic Plexus, M - Melanin, OL - Olfactory lobe, E - Eye, DC - Duct of Cuvier, EM - Embryo, PF - Pectoral fin.

2. **Fertilized egg**: After fertilization, the cortical alveoli embedded in the protoplasmic layer began to break down, starting from the animal pole and proceeding towards the vegetal pole (Plate 2e). After that the chorion was separated from the plasma membrane forming the perivitelline space.

3. **Blastula:** The blastoderm cap consisted of cells smaller than that of the 2 celled stage. The number of the marginal cells increased (Plate 2l; Plate 3a, b). Nuclei from marginal cells wandered out of the cells and entered the central periblast. The blastoderm expanded over the surface of the yolk sphere.

4. **Gastrula:** The blastoderm covered half of the yolk sphere. Gastrulation began at one portion of the germ ring where the thickening was greater than other parts, forming the embryonic shield (Plate 2j; Plate 3b, c, d, e). The germ ring reached two thirds of the yolk sphere. The embryonic shield became narrow and the neural keel was more clearly visible. The embryonic shield developed to form the embryo. The entire embryonic area at this stage rose slightly above the yolk sac. It was made of the neural keel. Small vesicles (two or three) appeared as small vacuoles underneath the posterior end of the embryo. These vesicles later became a single Kupffer's vesicle.

5. **Formation of heart:** The lens was formed at this stage. The division of the brain into fore, mid and hind brain became well defined. A tubular heart was formed underneath the mid brain (Plate 3f). The heart began to pulsate beneath the head region. The left and right ducts of Cuvier were formed as semi-circular tubules on the yolk sac between the head and the middle part of the embryo. Somites were 13 or 14 in number. Brick-red colored leucophores (larval form) appeared beneath the mid-brain region.

6. **Vitello-caudal vein:** The vitello-caudal vein was formed, which circulated around the median part of the yolk sac and reached to the heart. At this stage all the three vitelline veins, *i.e.*, left and right ducts of Cuvier and the vitello-caudal vein were formed (Plate 3g). A pair of the nasal sacs were formed between the fore brain and eyes. Two otoliths were discernible in the otic capsule. Circulation in the three vitelline veins was established. Melanin was found deposited in the eye. Somites were 22 - 23 in number.

7. **Yolk sac stage:** The tail tip became free of the yolk sac. The third brain ventricle enlarged. A pair of olfactory lobes appeared in front of the eyes. The optic capsules enlarged. The pectoral fins began to move (Plate 3i, j, k, l, m; Plate 4a, b, c). Circulation was observed in pectoral fins. Embryonic melanophores extended to the tail. The lower jaw was discernible both from top and side. The air bladder was visible beneath the embryo. Opaque hatching glands were seen in the buccal cavity. The yolk significantly reduced one day before hatching.

8. **Fry stage**: In the larva just hatched, the yolk was greatly decreased in amount while the oil globule was not significantly decreased in size. The peritoneum on the dorsal part of the swim bladder was pigmented (Plate 3c, d, e; Plate 4c, d, e).

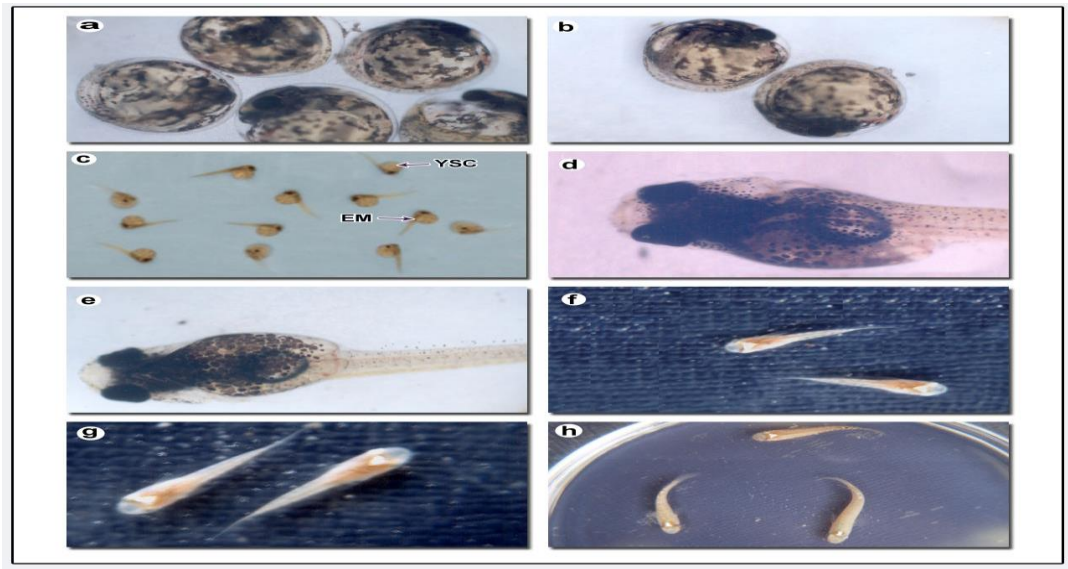

Plate 4. Larval development of *Aplocheilus lineatus*.

a. Embryo encircles the entire yolk sac. b. Tail tip reaches beyond eyes.
c. Just hatched larva wriggles. d. Hatched fry.
e. Yolk completely absorbed. f. Newly hatched larva. (TL 4.5mm).
g. 7 day old hatchling. h. 30days old fry.

The mean total length (TL) of newly hatched fry of *A. lineatus* was about 5.0 - 5.5mm (Plate 4e). The fry was found active immediately after hatching. The reduced yolk sac extended from beneath the pectoral fins to the anus (rectum) which was situated slightly anterior to the mid-point of the body. The transparent median fin fold was still continuous. The dorsal fin fold formed at slightly posterior to the mid-point of the body and extended along the whole dorsal and ventral rim of the tail. The ventral fin fold extended from the anus to the caudal end. It was broadest at the prospective anal fin region. The caudal fin area was broader than the peduncular area and rudiments of the caudal fin-rays could be seen at the ventro-posterior part. The pectoral fins were transparent fan-like structures. The pelvic fins were not formed and differentiated during larval stage. Chromatophores of just hatched fry appeared dark brown in transmitted light and light brown in falling light.

DISCUSSION

The eggs of *A. lineatus* exhibited oil drops and perivitelline space between the cytoplasm and the egg cover. *A. lineatus* eggs had 25 - 30 oil droplets where as *Lucania goodie* exhibited an average of 22.8 oil droplets/egg. Huang *et al*., (2019) reported that *Fundulus heteroclitus* eggs had chorionic filaments with a diameter of 150 - 220µ and a mean oil droplet number of 109 - 172/egg. The eggs of *A. lineatus* had a sticky adhesive tuft of filaments by which the eggs were attached to the plants. Adhesive eggs were reported by Kapakos *et al.* (2024) in killifishes; Brown *et al*., (2011) in *Fundulus grandis*; Chaibi *et al* (2015) in *Aphanius fasciatus*; Kamal *et al*., (2009) in *Aphanius sophiae*, Korwin (2012); Podrabsky & Culpepper (2012) in *Austrofundulus limnaeus* and Podrabsky (2017) in *Austrofundulus limnaeus*. The freshly laid eggs of *A. lineatus* measured about 1mm in diameter. *A. lineatus* eggs had adhesive threads at one end and they were found hanging like a bunch of grapes on the branches of aquatic plants. Similar features were reported in *Fundulus heteroclitus*(Huang *et al.,* (2019).

The pattern of courtship behaviour of *A. lineatus* was similar to those described by Crawford and Balon (1994) in *Lucania goodei*, Kapakos *et al.,* (2024) in killifishes; Brown *et al.,* (2011) in *Fundulus grandis*; Chaibi *et al* (2015) in *Aphanius fasciatus*; Kamal *et al*., (2009) in *Aphanius sophiae*, Korwin (2012); Podrabsky & Culpepper (2012) in *Austrofundulus limnaeus* and Podrabsky (2017) in *Austrofundulus limnaeus*. During courtship, *A. lineatus* exhibited more spawning colours than usual. Huang *et ai.,* (2019)) reported that mature males were identified in *Fundulus heteroclitus* by the characteristic spawning colours (Huang *et al* ., 2019) and females by their swollen anal sheath (Huang *et al* ., 2019) and their robust abdomen. *A. lineatus* females showed a breeding territory that enabled them to guard the eggs from the predators. Females were aggressive with the males even after spawning.

In the present study, 11 stages of larval development were observed in *A. lineatus.* Seegers (2000) reported 36 stages in *Adinia xenica,* Arezo *et al*. (2005) 13 stages in *Cynolebias viarius* and Arenzon *et al.* (2002) 13 stages in *Cynopoecilus melanotaenia.*

The incubation period of *A. lineatus* was found to be 9 - 12 days. Hung *et al.,* (2014) reported 9 - 11 days of incubation period in *Oryzias latgipes*, and Huang *et al.,* (2019) reported 17 days in *Fundulis heteroclitus*, Arezo *et al*. (2005) 40 days in *Cynolebias viarius*, Greeley and Mac Gregor (1983) 14 - 15 days in *Fundulus grandis* and 4 - 5 days Podrabsky (2017) in *Austrofundulus limnaeus*. It was also observed in *A. lineatus* that incubation time varied (10 - 18 days) even in a single batch of eggs. Arenzon *et al.* (2002) reported that developing embryos of *Cynopoecilus melanotaenia* underwent diapause to survive the long dry season Kapakos *et al*., (2024) in killifishes; Brown *et al*., (2011) in *Fundulus grandis*; Chaibi *et al* (2015) in *Aphanius fasciatus*; Kamal *et al*., (2009) in *Aphanius sophiae*, Korwin (2012); Podrabsky & Culpepper (2012) in *Austrofundulus limnaeus. Fundulus heteroclitus* (Huang *et al.,* 2019), had delayed hatching within siblings. The distinction of the embryo of *A. lineatus* was that the appearance of the optic vesicle did not take place until the germ ring closure at the end of epiboly. A similar observation was reported in other fishes such as *Oryzias latipes* (Shun *et al., 2014*), and *Fundulus heteroclitus*. (Huang *et ai.,* 2019).

The formation of the vitelline circulatory network in *A. lineatus* reflected the hypoxic environment in which these fish survived and reproduced. In *A. lineatus*, the vitelline network formed at 144 hours after fertilization. Shun *et al.,* (2014) reported that the formation of the vitelline circulatory system in *Oryzias latipes* occurred 51 hours post-fertilization. Huang *et al.,* (2019) reported that it took 92 hours in *Fundulus heteroclitus*.

The yolk-sac larvae of *A. lineatus* measured 5 - 6mm in total length, whereas the *Fundulus heteroclitus* were 4.6 - 6.8mm (Huang *et al.,* (2019). Chondrification of some skeletal elements in *A. lineatus* began before hatching of the embryo, but calcification of most structures was not observed until late in larval development. This observation was reported by Crawford and Balon (1994) in *Lucania goodie* and in *Fundulus heteroclitus* by Huang *et al.,* (2019. Like *Oryzias latipes* (Hung *et al.,* 2014), the free embryos of *A. lineatus* had not exhausted their yolk supply by the time they hatched from their egg envelopes. The newly hatched fry of *A. lineatus* weighed 0.002gm. The index of hatching was 96 %, and that of fertility was 96.3%.

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