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Embryonic and larval developments of brackish water catfish, *Mystus gulio* (Hamilton and Buchanan, 1822) induced with human chorionic gonadotropin and consequent larval rearing

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Abstract

Mystus gulio, the long whiskers catfish, is a popular food fish and potential candidate species for aquaculture in Sundarban area of India and Bangladesh. Recently, catch of this species has declined due to overfishing and various ecological changes. In the present study, mature fish was induced to spawn in captivity through intramuscular injection of human chorionic gonadotropin at the doses of 10 IU/g to female and 5 IU/g to male. Photomicrographs of all developmental stages of live embryo and larvae were documented with the aid of a light microscope. Results demonstrated that morula, blastula, gastrula and neurula and organogenesis ended at 1:30-, 3:00-, 5:30-, 7:30- and 17:15-hour post-spawning (hps) respectively. Heartbeat and muscular contraction of embryo commenced at 8:30 and 11.15 hps respectively. Hatching of embryo started after an incubation period of 17:30 hour at an ambient temperature of 29 ± 1°C. The newly hatched larvae measured 2.17 ± 0.29 mm in total length with a yolk volume of 0.165 ± 0.03 mm^3 started feeding 36 hr after hatching. The present study, on induced breeding and chronological development of *M. gulio* embryo, will have significant implications on conservation and seed production for aquaculture.

KEYWORDS
aquaculture, conservation, embryonic development, gonadotropin, induced spawning, *Mystus gulio*

1 | INTRODUCTION

Brackish water catfish, *Mystus gulio* (Ham.) belonging to the family of Bagridae, is also known as long whiskers catfish. This fish is distributed around India to the Malay Archipelago, especially in the estuarine and tidal waters (Jhingran, 1997). In the world's biggest mangrove ecosystem (Sundarban: Ganges–Brahmaputra estuary), catch of this species has reduced by 33.6% between 1960 and 2000 (Patra, Acharjee, & Chakraborty, 2005). Furthermore, it is reported that this species is in a state of vulnerable (IUCN, 2000; Mukherjee, Aloke, & Shamik, 2002), near threatened (Patra et al., 2005) and least concern (Ng, 2010). Although the species is not endangered, the natural population is decreasing due to over-exploitation and various ecological changes in its natural habitats (Hossain et al., 2015). As a small indigenous fish species (SIS) containing a high amount of nutrients, it fetches high market price and increasing demand, and these attributes make it a desirable candidate species for aquaculture in the Southeast Asia (Ross, Islam, & Thilsted, 2003). This species is euryhaline, therefore, well suited for both fresh and brackish water aquaculture (Siddiky, Saha, Mondal, Ali, & Washim, 2015). It is commonly cocultured with other euryhaline species, such as *Oreochromis niloticus*, *Rhinomugil corsula*, *Liza prsia* and *Penaeus monodon* (Abraham, 2014; Hossain et al., 2015). However, expansion of its aquaculture is hindered due to constraints in getting seeds either from natural

waters or hatchery. There are few published reports on size at maturity, fecundity, gonadosomatic index (GSI) and spawning of *M. gulio*. A minimum size at maturity and fecundity of this species ranged from 5.4 to 8.2 cm (Gupta, 2014) and 5,950 to 141,210 (Lal, Jaya, & Sherly, 2016) respectively. Gonadosomatic index values of both male and female *M. gulio* increase from March onwards reaching a peak in July followed by a gradual decrease up to December (Islam, Begum, Pal, & Alam, 2008; Lal et al., 2016). It is a single-spawned fish, of which spawning season ranged from March to November (Islam et al., 2008). Mukherjee et al. (2002) examined the spawning behaviour of *M. gulio* and reported that it dwells and breeds in estuaries during the monsoon months. Regardless of fragmented literature available on *M. gulio*-induced breeding, embryonic and larval developments, information is incomplete and inconclusive (Alam, Begum, Islam, & Pal, 2006; Begum, Pal, Islam, & Alam, 2009; Mukherjee et al., 2002). Moreover, earlier workers used a commercial gonadotropin-releasing hormone, 'Ovaprim' as the inducing agent and conducted breeding in a net cage, locally called *hapa*. Therefore, captive breeding and embryological studies of this species are essential for improvement of its breeding, aquaculture development and conservation programme.

The present study aimed to investigate and describe induced breeding and morphological and chronological developmental stages in the ontogeny of *M. gulio*. Furthermore, an appropriate larval-rearing technique was devised after examining stocking density, feeding frequency and live feed density in rearing tanks.

2 | MATERIALS AND METHODS

2.1 | Broodstock management

Two months before the onset of peak spawning season (May–August), 60 brood fish (body weight ranged from 50 to 300 g with a total length from 15.20 to 27.0 cm) were collected from estuarine water. Thereafter, they were stocked at a density of 10 number/m^2 in net cage (2 × 1 × 1 m) fixed in a brackish water pond of Kakdwip Research Centre of ICAR-Central Institute of Brackishwater Aquaculture (Lat. 21°51′15.01″–21°51′30.77″N, Long. 88°10′58.44″–88°11′12.09″E). Physico-chemical parameters of the pond water, such as salinity, temperature, pH, alkalinity, dissolved oxygen and total ammonia, were 18 ± 2 ppt, 29 ± 1°C, 7.80 ± 0.5, 144 ± 0.05 ppm, 4.8 ± 0.5 ppm and 0.05 ± 0.02 ppm respectively. Broodstock fish were fed with fresh chopped chicken liver to satiation once daily.

2.2 | Selection of mature broodstock

After 2 months of rearing period in the net cage, 20 matured male (mean weight: 53.70 ± 12.40 g) and 10 matured female fish (mean weight: 116.02 ± 78.49 g) in a sex ratio of 2:1 were selected during the first week of May. Male and female sex were distinguished based on the presence of pointed reddish-pink genital papilla and soft swollen abdomen with reddish vent respectively (Olaniyi & Omitogun, 2014). Maturity and oocyte diameter of the females was staged by obtaining in vivo biopsy of ovary using a polyethylene cannula (2 mm diameter) (Shehadeh, Kuo, & Milisen, 1973). Germinal vesicle position and polarization index (PI) of 10 egg samples from each female were measured. Egg samples were cleaned with cleaning solution (ethanol, formalin and acetic acid in a ratio of 6:3:1) and observed under a microscope fitted with a micrometer eyepiece. PI of the cleaned eggs was calculated as PI = $a/A \times 100$, in which 'a' is the distance between GV and cell membrane, and 'A' is the diameter of oocyte along the animal–vegetal axis (Dettlaff, Ginsburg, & Schmalhausen, 1993). Selected breeders were treated with a 50-ppm formalin bath for 3 min to remove unidentified external parasites and then transferred to an outdoor recirculatory aquaculture system (RAS), exposed to natural photoperiod.

2.3 | Induced breeding

After selection, 20 males and 10 females in the sex ratio of 2:1 were acclimatized in RAS tank (water volume: 400 L) for 24 hr, no feed was given during acclimatization. After acclimatization, simultaneously 10 separate breeding trials (Table 1) were conducted in RAS. In RAS, round the clock aeration and water flow of 8 L/hr were maintained. For induction of spawning, human chorionic gonadotropin (HCG), (IBSA Institut Biochimique SA, Switzerland) was dissolved in 0.9% physiological saline, following manufacturer instructions. HCG was injected into the dorsal musculature of fish adjacent to the first dorsal spine at a dose of 10 IU/g body weight to female and half the dose to both males. The used dose was ascertained based on a series of preliminary experiments carried out to standardize the optimal dose. Egg collector, made up of a bunch of nylon fibres (each bunch consists of 500 strips of 15 cm long nylon fibre), was kept submerged in the breeding tanks. After hormonal injection, reproductive behaviour was monitored.

2.4 | Egg incubation and embryonic development

After spawning, RAS was switched off, and the bunch of nylon fibres containing the sticky eggs was transferred to incubation tank. In order to examine the embryonic developments, 30 ± 5 eggs were taken from the incubator at every 30–60 min until hatching. Whenever there was an apparent difference during the embryonic development, photographs were captured under a trinocular microscope (Radical RXLr-5, India) at 4× or 10× magnifications. The measurement of developing embryo was carried out through image-analysing software (ProgRes CapturePro 2.7). Somatic movements, embryo twitching and heart beats per minute were recorded. Fertilization rate was determined at 2-hour post-spawning (hps).

2.5 | Breeding performance

Breeding performance of individual female was evaluated using the following criteria: latency period, total eggs spawned, fertilization rate, hatching rate and percentage of deformed larvae. The latency period is the time gap between hormone injection and the first appearance of spawned eggs. The number of eggs spawned was

TABLE 1 Total length, body weight before spawning, body weight after spawning, number of eggs spawned, oocyte diameter, polarization index, deformed larvae, fertilization rate and hatching rate of brackish water catfish, *Mystus gulio* during induced spawning through HCG at the dose of 10 IU/g body weight

Number of breeding trial	Total length of female (cm)	Weight of female before spawning (g)	Weight of female after spawning (g)	Oocyte diameter (μm)	Oocytes polarization index (%)	Number of eggs spawned (g^{-1} of fish)	Deformed larvae (%)	Fertilization rate (%)	Hatching rate (%)
1	1.52	190	167	850	3.06	96.84	10	50	55
2	2.70	300	275	1,000	2.80	58.33	20	70	72
3	2.26	132.5	112	950	2.53	154.72	15	65	75
4	1.70	61	45	1,100	2.98	183.61	21	72	65
5	1.75	65	52	900	2.78	151.20	32	65	78
6	1.80	74	62	938	2.56	105.73	24	56	65
7	1.85	85.7	72.5	750	2.40	80.25	18	74	68
8	2.20	138	125	750	2.93	49.27	23	65	65
9	1.65	50	40	870	2.87	97.80	21	69	62
10	1.78	64	50	993	1.51	150.72	14	50	70
Mean ± SD (Range)	192.1 ± 35.76 (1.52–2.70)	116.02 ± 78.49 (50–300)	100.05 ± 74.17 (50–275)	910.1 ± 110.54 (750–1,100)	2.64 ± 0.45 (1.51–3.06)	112.84 ± 45.06 (49.27–83.61)	19.80 ± 6.10 (10–32)	63.60 ± 8.70 (50–74)	67.50 ± 6.54 (55–75)

calculated by weighing 1 g of spawned eggs from each female in triplicate. The number of eggs in one gram egg sample was counted, and the average of three counts was considered. Total weight of eggs spawned by each female was ascertained by the weight difference between pre- and post-spawn female, and this was multiplied by the average number of eggs/g to calculate absolute fecundity (Ataguba, Solomon, & Onwuka, 2012). Relative fecundity was calculated as the total number of eggs/g weight of female fish.

Fertilization and hatching rates were determined for each female (three replicates of 100 eggs from each female) using the following formulae:

Fertilization rate (%) = 100 × number of fertilized eggs/total number of eggs counted.

Hatching rate (%) = 100 × number of eggs that hatched/total number of fertilized eggs.

A sample of 50 newly hatched larvae in triplicates was taken from each spawning and examined under the microscope for estimation of percentage of deformed larvae. Deformed larvae (-%) = 100 × number of deformed larvae/the total number of newly hatched larvae observed.

2.6 | Larval development and rearing

Immediately after hatching, 30 larvae from pooled larval rearing tanks were randomly measured and documented under the trinocular microscope, and further larval ontogeny was examined to evaluate the yolk sac utilization, first feeding and development. Total larval length (TL), yolk sac area and mouth size were measured with image analyzer software. The yolk sac volume (*V*) was calculated with the formula: $V = \pi/6 * Lh^2$ (where, '*h*' is yolk sac height and '*L*' is yolk sac length) (Korzelecka-Orkisz et al., 2010). After 24-hour post-hatching (hph), larval rearing tanks were inoculated with *Chlorella* spp. at a density of 1–5 × 10^3 cells/ml. To find out the commencement of oral feeding, digestive system of larvae was observed hourly under the microscope at different magnifications. After 3-day post-hatching (dph), larvae were fed with freshly hatched *Artemia* spp. nauplii measuring 150–175 μm in width and 500–580 μm in total length. Two separate experiments were carried out to standardize the optimum larval stocking density, feeding frequency and *Artemia* spp. nauplii feeding density.

2.7 | Optimization of larval stocking density and feeding frequency

Three-day-old larvae (mean length: 4.50 ± 0.54 mm and weight: 0.0028 ± 0.01 g) were stocked in 50 L tanks and fed with *Artemia* spp. nauplii at a density of 3,000 individuals/L for 15 days at natural photoperiod (12:12). A total of six experimental groups, 25 × 2, 25 × 4, 25 × 6, 50 × 2, 50 × 4 and 50 × 6, were assigned following 2 × 3 factorial experimental design with two levels of stocking density (25 and 50 number/L) and three levels of feeding frequency (two, four and six times feeding a day). Each treatment had three replicate tanks that were assigned randomly (two stocking density treatments × three levels of feeding frequency × three

replicates = 18 tanks). The San Francisco Bay strain *Artemia* spp. cysts were incubated in 25 ppt saline water at 30°C for 24 hr. The newly hatched *Artemia* spp. nauplii were kept in aerated saline water and used within 12 hr. At the end of experiment, percentage survival and weight gain of larvae were calculated and compared between the treatments.

Weight gain (%) = 100 × (average final weight − average initial weight/average initial weight).

Survival rate (%) = (Final number of fish/initial number of fish) × 100.

2.8 | Optimization of Artemia spp. nauplii feeding

Three-day-old larvae were stocked at the density of 25 individuals/L in a 50 L tank and fed with *Artemia* spp. nauplii at four different densities such as 500, 1,000, 2,000 and 3,000 individuals/L in triplicate for four times a day (7.00 a.m., 11.00 a.m., 15.00 a.m. and 19.00 am) for 15 days at natural photoperiod (12:12). At the end of the experiment, percentage survival and weight gain were calculated and compared between the treatments.

2.9 | Data analysis

Mean and standard deviation for all the data related to induced breeding, embryonic and larval developments were calculated using MS-Excel. For larval-rearing experiment, comparison of all the variables between treatments was made using two-way and one-way analysis of variance (ANOVA). The analysis was performed using SPSS for windows (version 20.0).

3 | RESULTS

3.1 | Induced spawning

The number of breeding trials, size of females, oocyte diameter, PI value, spawned eggs, deformed larvae, fertilization and hatching rates is presented in Table 1. In matured females, oocyte size ranged from 0.750 to 0.110 mm with a mean value of 0.910 ± 0.11 mm and PI of 2.64 ± 0.45% (Table 1). Successful spawning of mature male and female was achieved through single intramuscular injection of HCG at a dose of 10 IU/g body weight to female and half the dose to male. All the broodstock responded well to induce breeding and had 100% survival. The number of spawned eggs ranged between 58 and 183 eggs/g body weight with a mean value of 112.84 ± 45.06. The rates of the fertilization and hatching ranged between 50%–74% and 55%–75% with mean values of 63.6 ± 8.70 and 67.5 ± 6.54 respectively. The percentage of deformed larvae varied from 10% to 32% with a mean value of 19.8 ± 6.10.

3.2 | Courtship behaviour and spawning

The reproductive behaviour observed at a regular interval, and it was found that the male initiated courtship activity that starts 2–3-hour post-injection (hpi). The male started exhibiting active movements around the female, with occasionally synchronized swimming along with the female at 3–4 hpi. At 5–6 hpi, frequently coordinated movements were exhibited by the fish. Subsequently, an intensity of movement increased, and the male repeatedly contacted the female's abdominal area close to the urogenital aperture with the snout. At 6–8 hpi, the pair frequently moved to the surface of water. The frequency of nipping or butting activity by male increased during spawning. In all the breeding trials, spawning achieved within the latency period of 10.09 ± 2.60 hr at an ambient temperature of 29 ± 1°C.

3.3 | Embryonic development

Mystus gulio-fertilized eggs covered by a gelatinous material that gives adhesive properties to eggs. Fertilized eggs were transparent, adhesive, demersal and spherical with a mean diameter of 1.24 ± 0.13 mm and perivitelline space of 0.151 ± 0.024 mm in length. The *M. gulio* egg yolk was yellow and devoid of structural lipids visible as droplets/oil globules. As a result, the embryonic disc moved to the sides as much as the perivitelline slit size allowed. Different stages of embryonic and larval development with its specific characters are summarized in Table 2. During incubation, the multicellular morula stage visualized at 1.30 hps (Figure 1a). The blastula stage was characterized by the formation of multilayered blastodisc with numerous blastomere cells over yolk sac (Figure 1b). A cellular structure, yolk syncytial layer (YSL) formed at the fusion of blastomere marginal layer to the yolk sac and germ ring appeared (Figure 1c). A blastocoel was formed inside the blastodisc at 3:00 hps (Figure 1d). Gastrula stage started at 3:30 hps, the blastoderm proliferated and expanded as a sheet of cells towards the vegetal pole, marking the onset of epiboly. Epiboly is the slow-peristaltic cellular movement. The commencement of epiboly showed the transitional stage from late blastula to the start of gastrula. The epiboly of 30%, 50%, 70%, 80% and 90% is shown in Figure 1e–i respectively. An embryonic shield was noticed as thicken chorion (Figure 1j). Germ ring formation completed; blastopore gets closed by 5:30 hps, which indicates the end of epiboly or gastrulation, during this stage, embryo measured 1.04 ± 0.75 mm in diameter (Figure 1j). Neurula stage was characterized by formation of the neural keel, polster and tail bud (Figure 1k).

3.4 | Organogenesis

Somatogenesis, formation and development of somite block started at the cephalic parts of the embryo and progressed caudally, and during this stage, embryo measured 1.02 ± 0.8 mm (Figure 1l). At 3–4 somites stage, a neurocoele cell or brain vesicle formed (Figure 1m). At 8–9 somites stage, optic vesicles, notochord and first heart beat (80 min^{-1}) were noticed (Figure 1n,o). During 11–12 somite stages, optic vesicles differentiated to form optic cups and lens begun to develop (Figure 1p). At 15–18 somite stages, Kupffer vesicles appeared at the beneath of caudal region and muscular

TABLE 2 Embryonic and larval development of brackish water catfish, *Mystus gulio* under normal environmental condition (salinity: 18 ± 2 ppt; Temperature 29 ± 1°C)

Developmental stage	Mean development time (hr: min.)	Descriptions
Morula	1:30	Blastodisc consists of many blastomeres
Blastula		
Early blastula	2:00	Non-synchronous cell division, difficult to counts the number of cells
Mid blastula	2:30	Blastoderm flattened and spreading gradually lowers
Late blastula	3:00	Germ ring appeared and blastocoel formed
Gastrula		
30% epiboly	3:50	Blastoderm becomes flattened down onto the yolk sac and germ ring epiboled 30% of yolk sac
50% epiboly	4:30	Germ ring epiboled 50% of yolk sac, embryonic shield/body and neural plate was formed
70% epiboly	5:00	Germ ring epiboled 70% of yolk sac
80% epiboly	5:10	Germ ring epiboled 80% of yolk sac
90% epiboly	5:20	Germ ring epiboled 90% of yolk sac
100% epiboly	5.30	Yolk invasion completed
Neurula	7:30	Head and tail was differentiated
Organogenesis		
3–4 somites	8:15	Neurocoele of cells were seen in head region
8–9 somites	8:30	Brain, optic vesicle and notochord were seen
11–12 somites	10:15	Brain and neural fold is seen; Kupffer cell vesicles were seen at the beneath of caudal region
15–18 somites	11:15	Embryo movement started
12:15 hour old embryo	12:15	Blastopore closed
16:15-hour-old embryo	16:15	Rudimentary barbels formed
17:15-hour-old embryo	17:15	Two chamber heart seen
Hatching	17:30	Hatching completed by 18:00 hour
Newly hatched larvae (Pro larvae)	0 hr	Unpigmented, round to oval yolk sac
6-hr-old larvae	6 hr	Chromatophores appeared on ventral side of body
12-hr-old larvae	12 hr	Pigmentation of eye was noticed
18-hr-old larvae	18 hr	Blood circulation started
36-hr-old larvae	36 hr	Yolk sac was very little; mouth slit was seen
Post larvae	48 hr	Yolk sac absorbed; anal pore and mouth opened

contraction began at the rate of 8 min^{-1} (Figure 1q). After 12:15 hps, different parts of brain such as forebrain, midbrain and hindbrain were distinguishable, musculature formed and detachment of caudal end from yolk began (Figure 1r). During this stage, muscular contraction of the embryo was in a range of 10–15 min^{-1}. Organogenesis of the embryo was clearly distinguishable at 16:15 hps and characterized by the presence of otic capsules or auditory vesicle with two tiny otoliths, rudimentary barbells and two chambered heart (Figure 1s). After 17:15 hps, nutrient flow was from yolk to hepatocytes, an extension of tail was over the head and around 40% of the tail portions get detached from the yolk and egg chorion. At this stage, perivitelline space was nil and size of the embryo measured 1.02 ± 0.24 mm (Figure 1t). Before hatching, there were vigorous muscular contractions that consist of radial and rotational movements, especially at the caudal part of the embryo with caudal movement and heart rates of 30 and 180 min^{-1} respectively. Embryo started hatching after an incubation period of 17:30 hour and hatching completed by 18:00. During hatching, larvae broke the egg membranes/chorion and came out with tail first. Newly hatched larvae measured 2.17 ± 0.29 mm in total length.

3.5 | Larval development

Newly hatched larvae were translucent and unpigmented, which is characterized by the well-differentiated brain, otic capsule at the base end of the hindbrain (rhombencephalon) and primordial chin

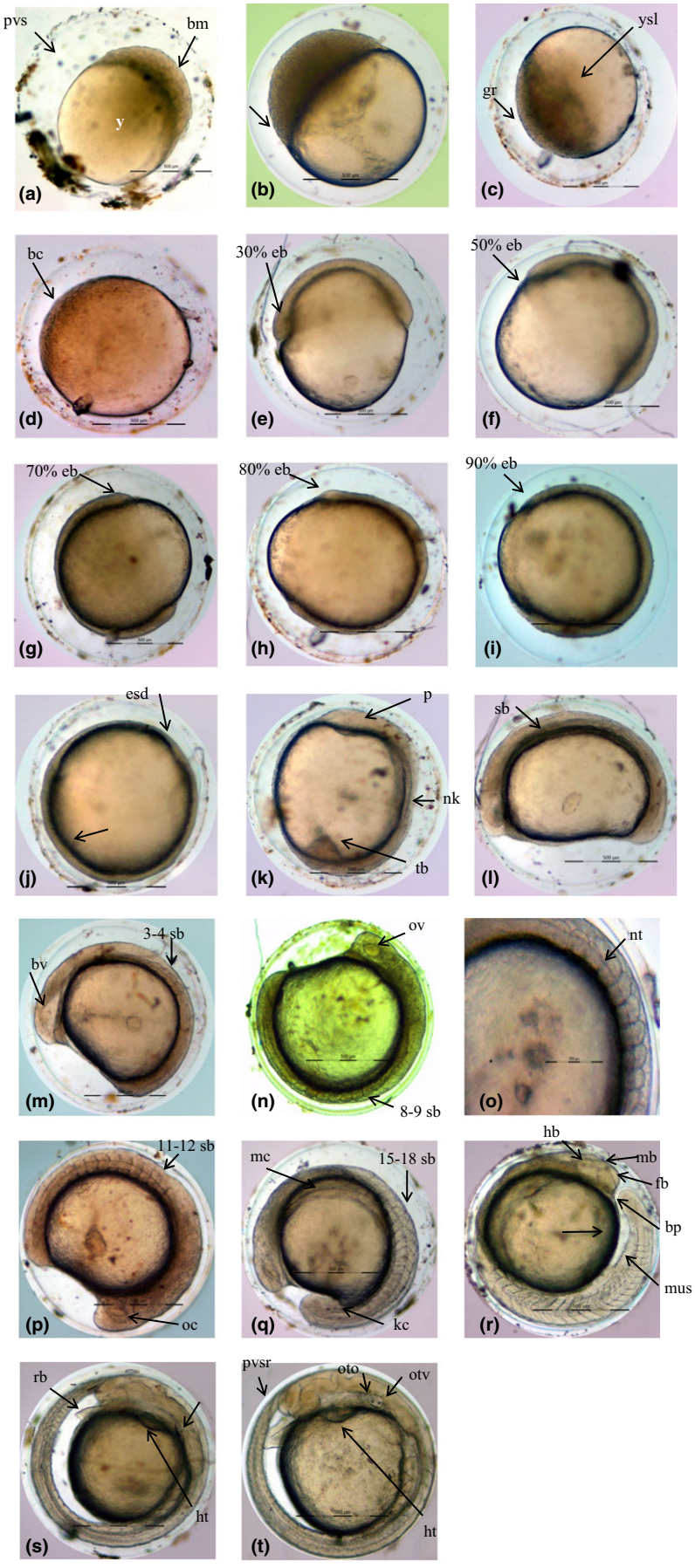
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FIGURE 1 Photomicrograph showing embryonic development of brackish water catfish, *Mystus gulio* (4× and 10×). (a) Multicellular morula stage, (b) Early blastula stage, arrow indicates spreading of blastomere cells, (c) Mid-blastula stage, (d) Late blastula stage, (e) 30% epiboly, (f) 50% epiboly, (g) 70% epiboly, (h) 80% epiboly, (i) 90% epiboly, (j) Yolk invasion completed (germ ring shown by arrow) (k) Neurula stage, (l) Start of somatogenesis, (m) 3–4 somites stage, (n) 8–9 somites stage, (o) Formation of notochord during 8–9 somites stages, (p) 11–12 somites stage, (q) 15–18 somites stage, (r) 12:15-hour-old embryo showing blastopore closing (detachment of caudal end from yolk shown by arrow) (s) 16:15-hour-old embryo showing the appearance of auditory/otic vesicles and primordial barbels, (t) 17:15-hour-old embryo with two chamber heart (bc—blastocoel, bm—blastomere, bp—blastopore, bv—brain vesicle, eb—epiboly, esd—embryonic shield, fb—fore brain, gr—germinal ring, hb—hind brain, kc—kupffer cell, mb—mid-brain, mc—muscular contraction, mus—musculature, nk—neural keel, nt—notochord, oc—optic cup, ov—optic vesicle, p—polster, p—polster, pvs—perivitelline space, rb—rudimentary barbells, sb—somite block, t—tail bud, y—yolk sac, yp—yolk plug, ysl—yolk syncytial layer)

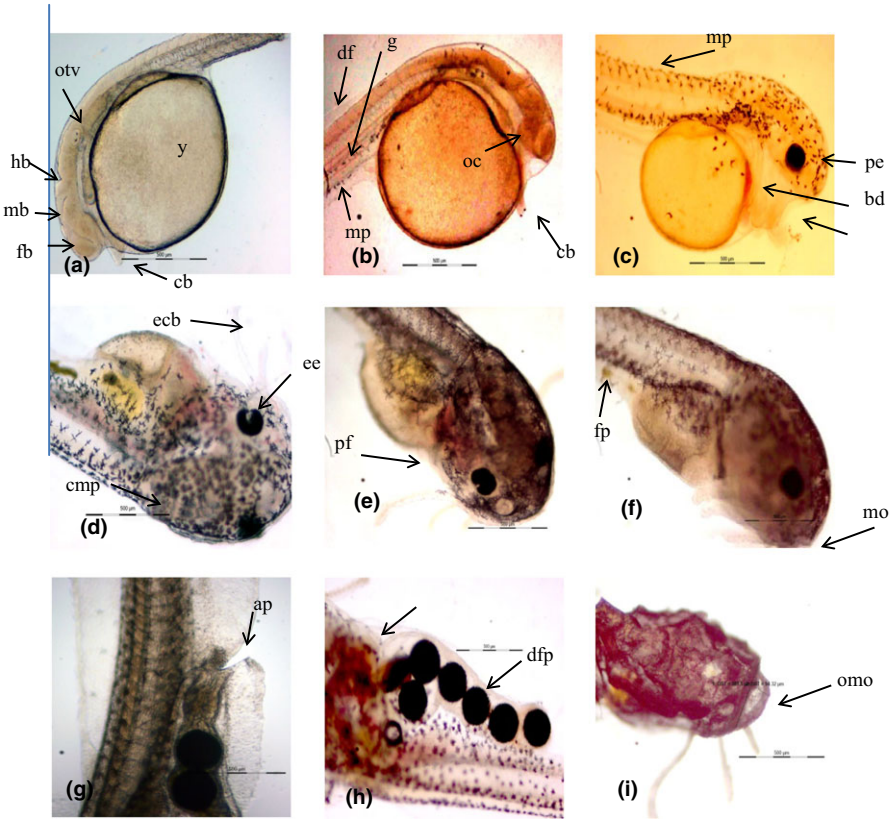

FIGURE 2 Photomicrograph showing larval development of brackish water catfish, *Mystus gulio* (4X). (a) Newly hatched larva with oval-shaped yolk sac, (b) 6-hr-old larva showing the development of chromatophores, (c) 12-hr-old larva with pigmented eye (separation of head from yolk sac shown by arrow), (d) 18-hr-old larva with epicanthus fold of eye, (e) 24-hr-old larva with pectoral fin rudiment, (f) 36-hr-old larva with open mouth and food particle in gut, (g) 48-hr-old larva with opened anal pore, (h) 48-hr-old larva with developed stomach and intestine, (i) 48-hr-old larva having wide-opened mouth (ap—anal pore, bd—blood cells, cb—chin barbels, cmp—concentrated melanophores, df—dorsal fin, dfp—digested food particles, ecb—elongated chin barbells, ee—epicanthus of eye, fb—fore brain, fp—food particles, g—gut, hb—hind brain, ht—heart, mb—mid brain, mo—mouth, mp—melanophores oc—optic cup, omo—open mouth, oto—otolith, otv—otic capsules or auditory vesicle, pe—pigmented eye, pf—pectoral fin bud, pvsr—perivitelline space reduced, sto—stomach, y—yolk sac)

barbels (Figure 2a). The newly hatched larvae were inactive and remained attached to the side and bottom of tank. The larvae had round- to oval-shaped brown yolk sac, which had a volume of 0.165 ± 0.03 mm^3 (Figure 2a). After 6-hour post-hatching, hph (total length: 2.62 ± 0.23 mm; yolk sac volume: 0.150 ± 0.02 mm^3), first melanophores appeared on the ventral side of the body and on yolk sac (Figure 1b). A thin membranous fin-fold noticed around the caudal region extending up to the yolk sac on dorsal and ventral side of the body. Twelve-hour-old larvae (total length: 2.64 ± 0.23 mm; yolk sac volume: 0.110 ± 0.08 mm^3) were characterized by pigmented eye, melanophores on the entire body, separation of head from yolk sac and onset of blood circulation (Figure 2c). Eighteen-hour-old larvae (total length: 2.72 ± 0.23 mm; yolk sac volume: 0.100 ± 0.04 mm^3) had well-spread melanophore on the entire body and heavily concentrated on the cranial region, uncovered epicanthus fold of eye and elongated barbells (Figure 2d). Twenty-four-hour-old larvae (total length: 3.26 ± 0.12 mm; yolk sac volume: 0.098 ± 0.01 mm^3) had distinct upper and lower jaws, pectoral fin buds, alimentary canal and closed anal pore (Figure 2e). Thirty-six-hour-old larvae (total length: 3.76 ± 0.40 mm; yolk sac volume:

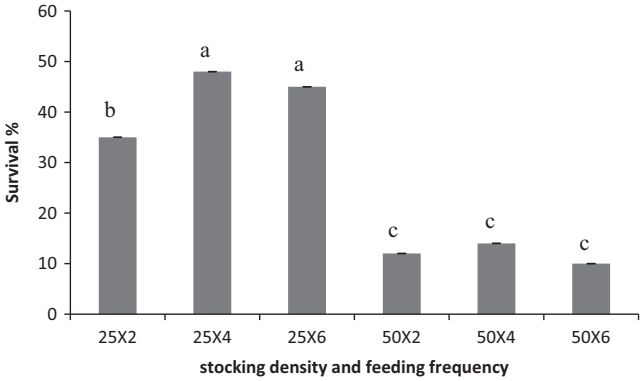

FIGURE 3 Effect of different stocking densities and feeding frequency on survival percentage of *Mystus gulio* larvae. Data bar represents mean ± *SE*. Different alphabetical superscript denotes significant differences among treatments ($p < .05$)

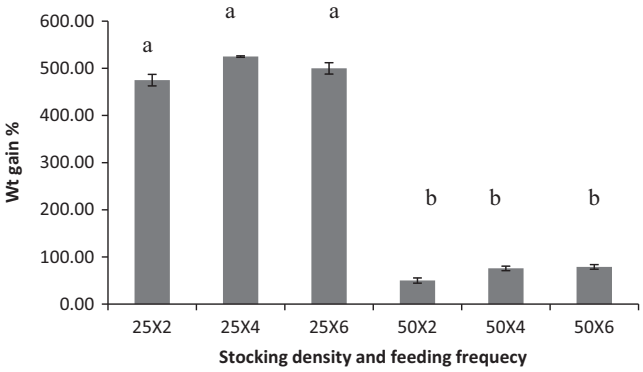

FIGURE 4 Effect of different stocking densities and feeding frequency on weight gain percentage of *Mystus gulio* larvae. Data bar represents mean ± *SE*. Different alphabetical superscript denotes significant differences among treatments ($p < .05$)

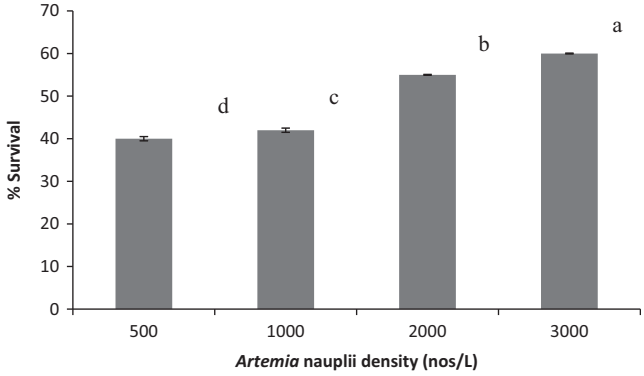

FIGURE 5 Effect of *Artemia* nauplii feeding densities on percentage survival of *Mystus gulio* larvae. Data bar represents mean ± *SE*. Different alphabetical superscript denotes significant differences among treatments ($p < .05$)

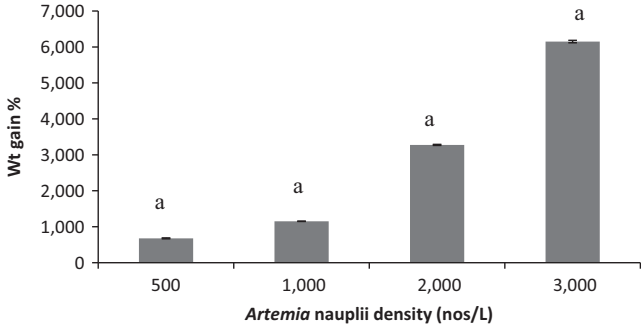

FIGURE 6 Effect of *Artemia* nauplii feeding densities on weight gain percentage of *Mystus gulio* larvae. Data bar represents mean ± *SE*. Different alphabetical superscript denotes significant differences among treatments ($p < .05$)

0.036 ± 0.02 mm^3) were characterized by little yolk sac and appearance of food particle in gut (Figure 2f). Two-day-old larvae (total length: 3.86 ± 0.18 mm) were characterized by the absence of yolk sac, elongation of barbells, an opening of anal aperture, formation of a pouch-like stomach and intestine (Figure 2 g,h). In 36-hr-old larvae, the mouth size was 32 ± 1.8 μm which gradually increased to 76 ± 0.35 μm in 48-hr-old larvae (Figure 2i).

3.6 | Larval rearing

Significantly ($p < .05$) higher survival (48 ± 0.01%) (Figure 3) and weight gain (525 ± 1.5%) (Figure 4) were observed in 25 × 4 group compared to that of other groups. Moreover, a significantly higher survival (52 ± 2.5%) was noticed in the group fed with 3,000 *Artemia* spp. nauplii/L (Figures 5 and 6).

4 | DISCUSSION

The use of HCG combined with pituitary gland extracts has been used in fish breeding (Leonardo, Romagosa, Borella, & Batlouni, 2004; Zohar & Mylonas, 2001) and has proven to be a very successful and reliable approach for catfish species (Caneppele, Honji, Hilsdorf, & Moreira, 2009; Thalathiah, Ahmad, & Zaini, 1988). Commercial GnRHa, ovaprim, was used for induced breeding of *M. gulio* (Alam et al., 2006; Begum et al., 2009 and Mukherjee et al., 2002). However, for the first time, we investigated that HCG is viable for induced spawning of *M. gulio*. In induced spawning of fish, HCG elicits shorter latency period than LHRH because of the difference in mode of action; HCG acts directly at the level of the gonad, whereas LHRH acts on the brain (Zohar & Mylonas, 2001). In this study, latency period of 10.09 ± 0.90 hr at ambient water temperature of 29 ± 1°C was similar to the report of Mukherjee et al. (2002) at 28°C, however, longer than the finding of Alam et al. (2006) and Begum et al. (2009), who documented the latency period of 6–8 hr at ambient temperature. In the present study, longer latency period might be due to the use of female at early maturity than that of female at late maturity stage. However, there is inadequate literature available on this aspect, and further studies are required to validate these results. Broodstock with 100% survival after spawning showed that the species has good potential for captive breeding. The rate of fertilization depends on egg size and its

quality (Olaniyi & Omitogun, 2013). Hatchability depends on water quality parameters, especially water temperature; higher the water temperature, faster the embryos are hatched and better the survival (Olaniyi & Omitogun, 2013).

To understand the basic biology of a species, knowledge on embryonic and larval development and organogenesis is very much essential (Borcato, Bazzoli, & Sato, 2004; Koumoundouros, Divanach, & Kentouri, 2001). Embryonic development is a complex process in which cellular differentiation and proliferation occur concurrently, though their rate is different (Hall, 1922). Fertilized eggs of *M. gulio* are transparent, spherical, demersal and adhesive in nature, which is similar to those of other catfish species such as *Clarias batrachus*, *Mystus montanus* and *Pangasius sutchi* (Islam, 2005); *Clarias gariepinus* (Olaniyi & Omitogun, 2013); and *Heterobranchus bidorsalis* (Olaniyi & Omitogun, 2014). The gelatinous coat that encapsulated the egg is a characteristic of Siluriformes and provides a form of protection for adhesive or non-adhesive eggs (Da Rocha, Sato, Rizzo, & Bazzoli, 2009). The size of fertilized eggs of *M. gulio* (1.24 ± 0.13 mm) is smaller than that of *Heteropneustes fossilis*, 1.3–1.5 mm (Puvaneswari, Kasi, Ramasamy, & Mohammed, 2009), but closer to *M. montanus*, 1.2–1.3 mm (Arockiaraj, Haniffa, Seetharaman, & Sing, 2003). Perivitelline space filled with fluid protects the embryo from mechanical injury and helps in osmoregulation (Buzollo et al., 2011). *Mystus gulio*-fertilized egg perivitelline space (0.151 ± 0.024 mm) is similar to that of *M. montanus*-fertilized eggs (0.1–0.2 mm) (Arockiaraj et al., 2003). In morula stage, due to irregular cleavage, numerous blastomeres are produced, which is referred as mulberry, half-berry or ball-like shape (Honji, Tolussi, Mello, Caneppele, & Moreira, 2012; Olaniyi & Omitogun, 2013), some buried or deep cells, piled up to give blastodisc (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Shape and size of blastomeres of *M. gulio* were uniform, which are same as in another teleost (Hall, 2008). In teleost, blastula occurred at eight zygotic cycles or 128-cell stage before the onset of gastrulation (Kimmel et al., 1995). The start of cellular movement marked the emergence of epiboly, which indicated transitional phase from blastula to the beginning of gastrula (Buzollo et al., 2011; Kimmel et al., 1995; Leme dos Santos, & Azoubel, 1996). The completion of gastrulation is marked by the closure of blastopore (Buzollo et al., 2011; Kimmel et al., 1995). In the current study, blastopore was closed by 5.30 hps, which is shorter than that in other catfish, namely *Clarias punctatus* (Munshi & Hughes, 1991) and *H. fossilis* (Puvaneswari et al., 2009). Neurulation in *M. gulio* is characterized by the formation of neural keel, polster and tail bud. Olaniyi and Omitogun (2013) reported polster and tail bud as markers for the identification of cranial and caudal parts, respectively, during neurulation. This study revealed distinct neurulation and segmentation period in *M. gulio*, which is similar to the observation of Olaniyi and Omitogun (2014) in *H. bidorsalis*, but in contrary to the report of Kimmel et al. (1995) in *Denio rerio*. During organogenesis, early segmentation stage was characterized with the formation of somite block, cephalic region, polster, auditory parts and Kupffer vesicles. The later segmentation was characterized by well-aligned somite blocks leading to the formation of myotome muscle block, commencement of heartbeat, free tail that leads to muscular contractions or caudal movement (Buzollo et al., 2011; Honji et al., 2012; Kimmel et al., 1995; Olaniyi & Omitogun, 2013). In the current study, the formation of brain vesicle, optic vesicles, Kupffer vesicles and muscular contraction was seen during early somatogenesis. In later stage, differentiation of brain, formation of musculature, detachment of tail end from yolk, auditory vesicle with two otoliths, rudimentary barbells and two chambered heart were noticed. The organogenesis of *M. gulio* is similar to other catfish such as *M. montanus* (Arockiaraj et al., 2003), *C. gariepinus* (Osman, Wiertz, Mekkawy, Verreth, & Kirschbaum, 2008), *H. fossilis* (Puvaneswari et al., 2009) and *H. bidorsalis* (Olaniyi & Omitogun, 2014). The Kupffer vesicle is an essential feature of teleost embryo (Cardoso, Alves, Ferreira, & Godinho, 1995) and indicates the allantoic rudiment (Kimmel et al., 1995). Kupffer vesicle has a role in the development of brain, heart and gut in zebrafish embryo (Essner, Amack, Nyholm, Harris, & Yost, 2005). In this study, the incubation period of *M. gulio*-fertilized eggs was 17.30 ± 1.28 hr at an ambient water temperature of 29 ± 1°C, which is similar to the finding of Begum et al. (2009) in same species. However, longer incubation period of 22 hr was observed at slightly lower temperature of 28°C in same species (Mukherjee et al., 2002).

The hatching in fish is facilitated by muscular contraction or twitching at caudal part (Honji et al., 2012; Olaniyi & Omitogun, 2013). In this study, vigorous movement of caudal part broke down perivitelline membrane at tail region and hatchling emerged out as tail first, which is similar to the observations made in *M. montanus* (Arockiaraj et al., 2003), *H. fossilis* (Puvaneswari et al., 2009) and *H. bidorsalis* (Olaniyi & Omitogun, 2014). However, Langeland and Kimmel (1997) opined that during hatching, head comes out first and tail comes out last from the embryo in most of the teleosts. Hatching period depends on fertilization period within oocytes; hence, faster the fertilization shorter the hatching period and survival of the embryos (Olaniyi & Omitogun, 2014). In this study, hatching period for *M. gulio* was 30 min.

The newly hatched larvae were inactive, with closed mouth, therefore, needed no exogenous feeding due to the availability of large yolk sac. Fish larvae contain two kinds of energy reserves, such as yolk and oil globule (Bjelland & Skiftesvik, 2006), but *M. gulio* had only yolk sac, which is similar to *M. montanus* (Arockiaraj et al., 2003) and *H. fossilis* (Puvaneswari et al., 2009). Concurrent development of barbell, removal of epicanthus from the eye, gradual widening of mouth and depletion of yolk make larvae ready for exogenous feeding. Yolk sac of *M. gulio* larvae got depleted 48 hr after hatching when larvae measured 3.86 ± 0.18 mm in total length. However, in other catfish, such as *Clarias fuscus* (Panjionghua & Zhengwenbiac, 1982), *Clarias lazera* (Panjionghua & Zhengwenbiac, 1987), *Mystus macropterus* (Wang, Zhang, & Luo, 1992), *C. batrachus* (Landge, 1995), *M. montanus* (Arockiaraj et al., 2003) and *H. fossilis* (Puvaneswari et al., 2009), and in same species (Begum et al., 2009), yolk sac gets resorbed 72 hr after hatching. Exogenous feeding of larvae of *M. macropterus* (Wang et al., 1992), *C. batrachus* (Landge, 1995) and *H. fossilis* (Korzelecka-Orkisz et al., 2010; Puvaneswari et al., 2009)

started after the complete exhaustion of yolk sac. In this study, we observed that larvae commenced exogenous feeding even before the completion of yolk absorption, which is similar to the report of Arockiaraj et al. (2003) and Begum et al. (2009) in *M. montanus* and *M. gulio* respectively. The body pigmentation due to melanophore firstly started on the ventral side of body and later concentrated on cranial region and covered entire body. The mode of spreading of melanophores was differed from other catfish such as *H. bidorsalis* (Olaniyi & Omitogun, 2014) and *Pimelodus maculatus* (Buzollo et al., 2011), where it first appeared around the eye and later spread to head and body.

Growth, survival and water quality could be affected by the feeding frequency (Aderolu, Seriki, Apatira, & Ajaegbo, 2010) and the optimum feeding frequency could make the culture economic (Davies, Inko-Tariah, & Amachree, 2006). Stocking density is one of the most significant factors in larval rearing, affecting social interactions such as aggressiveness, hierarchical phenomena and cannibalism, resulting in variation in size, survival and growth performance. (Rahman et al., 2012). Three times feeding to *C. gariepinus* larvae (Aderolu et al., 2010) and stocking density of *C. batrachus* larvae as 18 number/L (Rahman et al., 2012) were ideal. In the present study, stocking density of 25 number/L and four times feeding with *Artemia* spp. nauplii at the density of 3,000 number/L was found to be optimum for larval rearing of *M. gulio*.

5 | CONCLUSION

Induced breeding of *M. gulio* through the administration of HCG is a novel and viable protocol. This study investigated and described the morphological and chronological developmental stages in the ontogeny and organogenesis of *M. gulio*. The present knowledge also recommends larval stocking density of 25 number/L and four times feeding with *Artemia* spp. nauplii at the density of 3,000 number/L for *M. gulio* larval rearing. The data from this study will contribute immensely to the understanding of breeding and developmental biology of *M. gulio* for aquaculture and conservational researches.

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CONFLICT OF INTEREST

There is no conflict of interest between authors.

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