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ORIGINAL ARTICLE

Temperature-optimized, hormone-induced spawning of Asian striped dwarf catfish, *Mystus vittatus* in early-stage F1 generation

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Abstract
In the present study a trial for induced breeding of a minor bagrid *Mystus vittatus* was made by stripping methods using various doses (0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ml/kg body weight) of a synthetic hormone, Ovasis at 26, 28 and 30ºC to evaluate their breeding performance. During spawning their latency period ranged between 12-17h. Highest number of absolute fecundity (3785 nos.) and relative fecundity (209.04 nos./g) were recorded at the dose of 0.5 ml/kg body weight of female at 28ºC. Similarly highest breeding success was observed at this dose and temperature when female was bred with male counterpart treated with a dose of 0.2 ml/kg body weight. In this condition the rate of fertilization and hatching of eggs were 84.15% and 75.48% respectively. Lowest breeding performance was recorded at the dose of 0.7 ml/kg body weight of female at 26ºC. Further, the developmental stages of fish (fertilized egg to 28th day old fish) were also characterized chronologically along with their behavioral tendency in this study. Morphological deformities of 6.3% was recorded at 26ºC in the hatchlings developed from the eggs under the administration of dose of 0.7 ml/kg body weight. Recommended dose of ovasis is 0.5ml/kg of *Mystus vittatus* at 28ºC.

Keywords: *Mystus vittatus*, Morphological development, Behaviour, Larval deformities.

INTRODUCTION

The strong decline of freshwater fish species globally emphasizes the need escalate efforts towards ex-situ conservation in the near future (Manubens et al. 2020). As a result, captive breeding programs are increasingly being implemented as a management technique to prevent endangered species and/or endemic populations from becoming extinct (Nath et al. 2021). For the goal of breeding, more commercially important fish species are being placed into captivity for long or short periods of time. Many times, the offspring are kept in captivity and raised for food or other purposes. However, it is very common to release the stock into the wild at various phases of growth in order to supplement or strengthen the natural population (Fraser 2008). In addition, captive breeding can serve the purpose of generating foreign exchange by introducing indigenous species into the global market (Biondo & Burki 2020). However, in addition to having sufficient knowledge about the biology of the species of interest i.e., breeding behavior, fecundity, successful techniques for breeding, fertilization, hatching and rearing in captivity is also critical (Moorhead & Zeng 2010; Mylonas et al. 2010).

Induced spawning is a technique applied to species attain maturity in captivity but are unable to spawn, as well as for species which can spawn when specific environmental conditions are provided (Migaud et al. 2013; Borah et al. 2020). For the latter, since spontaneous spawning is largely by chance, the resort to induced spawning techniques has become necessary to allow fish farmers to optimize brood-stock management by increasing the quantity of produced fry (Comizzoli & Holt 2019). Hormonally induced spawning has been applied to an increasingly diverse number of species using the knowledge of reproductive endocrinology to improve the outcome of the techniques (Mylonas et al. 2013; Ogidan et al. 2018). Aspects of successful outcome of these induced reproduction in fish have also entailed the manipulation of environmental conditions (temperature, photoperiod, water quality, social factors) (Lee et al. 2020). Manipulating water temperature can often improve the reliability of spawning in fish by influencing the embryonic maturation of fish and their subsequent development (Borah et al. 2020). Thus its application as an adjustable extrinsic factor in improving the spawning outcome of fish species on commercial scale has been

documented (Thépot & Jerry 2015).

Aspects of successful spawning outcome and determinants of production quality and quantity of induced finfish includes paying attention to parameters such as the embryologic development, hatching rate, survival rate, fertilization rate and larval quality, and deformation rates during larval and juvenile developmental stages from eggs (Żarski et al. 2015). Although the occurrence of malformations are mostly associated with skeletal and swim bladder morphology (Castro et al. 2008), the frequency of malformations may be species-specific issue. Aside nutritional imbalances including tryptophan, essential fatty acids, genetic factors or their interaction (Cahu et al. 2003; Sfakianakis et al. 2006), malformation rates are also influenced by water temperature, eggs density, metal contamination or hydrodynamics during development (Sfakianakis et al., 2004; Kranenbarg et al., 2005; Sfakianakis et al. 2015). Deformation rate in the eggs and larval quality has also been linked to hormone treatments for induced reproduction (Tan et al. 2015), but compared to other causes of larval deformation, hormonal treatment as a factor and co-factor, has been sparsely reported. The Asian striped dwarf catfish, *Mystus vittatus* (Bloch, 1794), is a freshwater fish species belonging to the Bagridae family (order Siluriformes). It has high culture potential in freshwater due to its delicious taste, tender flesh, few spines in the body and high nutritive value (Mou et al. 2018), and has already gained a promising status in the international ornamental fish market with moderate export value (Gupta & Banerjee 2014). During last few decades its steady decline in the wild, largely due to habitat disruption, and unfavorable agro-climatic conditions has earned it a place among threatened fish species (Borah et al., 2020). Thus, trials for induced breeding and larval development of *M. vittatus* in captivity to sustain the entrepreneurial supply chain and facilitate their restoration and conservation in wild has become imperative. Although successful breeding using synthetic inducing agents, Ovatide and Ovaprim have been documented (Mijkherjee et al. 2002), morphological abnormalities in the induced-bred larvae is an issue (Teji & Thomas 2006). So far, the significant variation in nature and dose of the stimulant necessitates efforts towards repeated standardization of the process (Priyadarshi et al. 2017). This study induced breeding trials for *M. vittatus* using various doses of synthetic gonadotropin releasing hormone, Ovasis at different temperatures (26º, 28º and 30ºC). These trials were targeted towards attaining a higher success rate with respect to fecundity, latency period, incubation period, fertilization and hatching. The morphological characters from fertilized egg to 28thday chronologically at regular interval, frequency of morphometric abnormalities in the hatchlings along with behavioural characteristics of their progeny was observed.

MATERIALS AND METHODS

Test animal: For the experimental trials, healthy and gravid male (where mean total length 10.68±0.61cm and average weight 10.33±1.16g) and female (average total length 11.94±0.36cm and average weight 16.53±1.33 gm) *Mystus vittatus* were used for induced breeding (supplementary material, SM 1). The gravid fish were chosen based on exterior morphological characteristics. The appearance of a soft and enlarged abdomen with a short, round, button-shaped genital papilla characterized gravid female fish (SM 2). Eggs released by light pressure on the swollen belly were observed for size uniformity of egg as a measure of the female's prime maturity. The slim and streamlined bodies of fully gravid males were distinguished by an extended, conical, and creamy genital papilla with a pointed tip (SM 3). They were collected from local unpolluted pond in the month of July and were acclimatized in the laboratory condition for 48h prior to breeding operation in the glass aquaria (120cm x 45cm x 45cm) filled with un-chlorinated and iron free tap water (pH 7.3±0.25, Dissolve Oxygen 5.33±0.47mg/l, CO_2 15.12±2.7mg/l, total alkalinity 170±7.5mg/l as $CaCO_3$; total hardness 112±7.4mg/l as $CaCO_3$and total ammonia nitrogen 0.05±0.01mg/l). The aquarium was partly covered with fresh and

floating aquatic weeds (*Pistia stratiotes*) provided with aeration facilities. During acclimatization, the male and female fish were kept separately and no feed were supplied for a day prior to breeding operation. Both the fish and aquatic weeds were dipped into 1mg/l $KMnO_4$ solution for 2-3 minutes to avoid any pathogenic contamination before their use in the experiment (Dangi et al. 2021).

Inducing agent: In the present study, synthetic inducing agent, Ovasis^{TM}, a combination of 20 μg salmon gonadotropin releasing hormone analogue (sGnRHa) and 10mg domperidone (as a dopamine receptor antagonist) dissolved in calibrated quantities of non-toxic organic solvent, propylene glycol (manufactured by USV Private Ltd., Mumbai, India and marketed by Apisa Bitech Extn. Private Ltd., Hyderabad, India) was administered in *Mystus vittatus*.

Breeding operation: The breeding operations were conducted at three different temperatures (26º, 28º and 30ºC) following Dhara & Saha (2013). During the stripping method of artificial propagation in fish, different doses of Ovasis (0.2, 0.3, 0.4, 0.5, 0.6, and 0.7ml/kg body weight) were employed for females. Males, on the other hand, received only a single dose of the inducing chemical (0.2ml/kg body weight). After a series of rough range-finding tests, the doses of inducing chemicals were chosen (data not shown). Inducing agents were given to two distinct groups of brood fish (total 96 fish, male 64 and female 32), two males and one female, in a 2:1 ratio (SM 1).

The needed dosages of inducing agent were delivered intramuscularly by hypodermic syringe with a tiny size needle (Beckton Dickinson needle No.26) at a 45^0 angle in the evening at the caudal peduncle area above the lateral line sense organ. After stimulant administration, both the males and females were kept separately in the water filled glass aquaria (60cm x30cm x 30cm) having 20 l of unchlorinated iron free tap water. All the aquaria were provided with submerged weeds (*Hydrilla verticillata*) and aeration facilities. For female, different aquaria were also maintained according to their different doses of stimulant. The fish were undergone breeding operation after desired latency period of 12-17 hours their full maturity determined by the trial-and-error method. Under this operation, the testes of males were cut open. The testes were carefully retrieved in intact condition after opening and cleaned with distilled water. It was quickly cut into small pieces with a scissor and thoroughly squeezed before being mixed with a small amount of 0.9 percent sodium chloride (NaCl) solution. In this state of suspension, the sperm stays inactive. The sperm suspension was used to fertilise eggs right away. Simultaneously, female fish were stripped after a period comparable to the latency period in order to gather eggs into a previously washed, dried, and clean enamel dish. As soon as blood came out with the eggs, the stripping stopped. However, a dropper was used to gently drip freshly prepared milt suspension into the stripped-out eggs during and immediately after stripping, preferably within 2-3 minutes. With the help of a dry, clean, and sterilised bird's feather, these were thoroughly combined. To activate the sperm, a small amount of fresh water was introduced. The tray was then gently jerked for 2-3 minutes to ensure that the eggs and milt were properly mixed, allowing for even fertilisation. Unfertilized eggs, blood clotting, muscular dirt, and foam, if any, were rapidly rinsed and cleaned many times with fresh tap water to eliminate water hardening as well as unfertilized eggs, blood clotting, muscular dirt, and foam, if any. After that, the fertilised eggs were moved to a flow-through system to be incubated.

To determine the total number of released eggs, a small amount of egg mass was randomly taken in a transparent petri dish from each group. Weighing 1g of produced eggs from each female in triplicate yielded the total number of eggs spawned in each batch. Each egg sample was counted, and the average of each set was considered. The weight difference between pre- and post-spawned females was then used to calculate the total weight of eggs spawned by each brood fish. To calculate absolute fecundity, multiply this by the average number of eggs per gramme

(Ataguba et al. 2012). The total number of eggs was divided by the total weight of the female fish to calculate relative fecundity (g). For each set, a little part of the egg mass was randomly collected in a transparent petridish filled with unchlorinated and iron-free tap water to assess the fertilisation percentage. A magnifying glass was used to count the total number of fertilised eggs in the collected egg mass. The figure was then multiplied by one hundred and divided by the total number of eggs collected.

A small-scale pool was constructed in the shade by installing a set of high-density polyvinyl chloride (PVC) trays (57cm x 37cm x 10.5cm) on a platform in a water flow through system with an aerator for hatching. Each tray had two exits at various depths, each of which was connected to a common drainage pipe installed six inches below the tray. A white enamel tray (46cm x 30cm x 7.5cm) was also placed inside each PVC tray. In the enamel trays, the fertilized eggs were evenly distributed in a single layer. Water was delivered to the trays via separate taps in the showers, which were fed by a 2cm diameter galvanized iron (GI) pipe. The above tank was connected to the common GI pipe, which was mounted one foot above the trays. As required, water was released from the overhead shower at a rate of 1-1.5 l/min. Aeration was also maintained in each tray throughout the experiment to keep dissolved oxygen levels over 5mg/l in the water after the fertilized eggs were added. To prevent eggs and hatchlings from escaping, the outlets of all the trays were closed with No. 60 bolting silk cloth. During hatching operations, the water level was kept at a safe level by opening and closing the outlets.

In addition, dead eggs were gently siphoned out from the tray time to time to avoid any biological pollution. Water flow was stopped as soon as hatching starts, but aeration facilities were continued for better oxygenation. Dead hatchlings and debris were also sucked out gently time to time from tray. The hatchlings were counted in total by eye estimation after hatching, and the hatching rate was calculated by multiplying hundred by the total number of hatchlings obtained. The total number of eggs retrieved was then divided by the outcome. A sample of 50 freshly hatched larvae was obtained in triplicates from each set and inspected under the microscope to determine the rate of deformity in hatchlings (percent) by multiplying hundred by the average number of deformed larvae detected.

Rearing Operations: The hatching unit served as a raising unit as well. Hatchlings were grown in the same flow-through system at a rate of 200-2000 hatchlings per tray. Each dish was aerated to keep the dissolved oxygen level above 5mg/l. The outlets were also opened and closed to maintain a sufficient water level. After complete absorption of yolk materials, 5^{th} days old young fish were shifted to indoor glass aquaria (60cm x 30cm x 30cm) with aeration facilities and reared for 23 days (5^{th} to 28^{th} day old) under 12L: 12D photoperiod (6:00-18:00) condition. The stocking density was maintained at the rate of 500 developing fish per rearing container. Few submerged aquatic weeds (*Hydrilla verticillata*) were kept in the rearing systems to provide hiding place. Aeration was continued in each aquarium to maintain sufficient dissolved oxygen level throughout the rearing operations. A frequent water replacement (30-50 percent) was performed in the morning to remove fecal matter, unused food ingredients, and any dead fish. To avoid weariness of the fish during their vertical journey, the water depth in the rearing medium was gradually increased from 9 to 18 cm, based on the size of the developing fish.

During rearing no feed was supplied to the developing fish up to 3^{rd} day due to nutritive contribution of yolk material. Daily collected fresh and finely sieved live freshwater zooplankton was given as natural feed on and from 4^{th} to 13^{th} day of rearing at 4h interval to ease ingestion. During 14^{th} to 28^{th} day of rearing, finely chopped tubificid worms were supplied as feed at the rate of 3% of their body weight at 8h interval. Prior to usage, the worms were treated with oxytetracycline at a dosage of 250 mg/l of water for 15 minutes to ensure that they were pathogen-free.

Morpho-sequential study: For chronological developmental study of the fish on the basis of their morphological characteristics, 10 developing eggs were monitored at every 10min. interval until the morula stage was attained; afterward, it was performed on an hourly basis until hatching and then 10 developing fish were sampled once a day up to 28th day of development (Dhara & Saha, 2013; Okomoda et al. 2017). To assess the diameter of an egg along the animal-vegetal axis, collected egg samples were cleaned with a solution (a 6:3:1 mixture of ethanol, formalin, and acetic acid) and examined under a microscope using a micrometer eyepiece (Gisbert & Nam 2018). Measuring the total length (mm) of the developing fish (from the tip of snout to the end of caudal fin) and weight (mg) were made using conventional method sat each sampling time (Crawford 1986).

Behavioural study: During the rearing period of developing fish, their common behavioural tendency was also recorded systematically by naked eye observation three times per day.

Statistical analyses: All experiments were replicated to overcome methodical errors. All values are expressed as the mean (SD) of three replicates and statistically examined using a Two-way ANOVA followed by a DMRT (Duncan's Multiple Range Test) to find significant differences between the means (Gomez & Gomez 1984).

Calculation of IBR: Integrated biomarker responses (IBRs) using various parameters (latency, egg size, absolute fecundity, relative fecundity, fertilization, hatching and larval deformity) exposed to various doses of Ovasis was estimated and multi-biomarker response in *Mystus vittatus* depicted as star plots. The responses of multi-biomarkers were examined using the methods given by Beliaeff & Burgeot (2002) with revisions by Guerlet et al. (2010). In brief, the overall mean (m) and standard deviation (s) of a given biomarker were computed for each parameter (containing data from all temperatures), and then standardized to generate Y, i.e., $Y= (X – m) / s$, where X is the mean biomarker value of a specific dose. Thereafter Z was calculated as Z= -Y or Z= +Y according to the expected biological effect, with "-" representing an inhibition of a biological effect and "+" representing an induction (such evaluation was based on the average baseline biomarker values). Then, biomarker scores (S) were calculated as $S= Z+|Min|$, where $Z\geq 0$ and |Min| is the absolute value of all Y calculated for a given biomarker (including all measurements). Star plots were used to represent the scores (S) of all biomarkers measured in a specific therapy and tissue. The IBRs were computed using the following formulas:

$$Ai= S_i/2 \sin\beta (S_i \cos\beta+S_{i+1} \sin\beta)$$

Where $\beta= \text{Arc tan} (S_{i+1} \sin\alpha/ S_i- S_{i+1} \cos\alpha)$ and $\alpha= 2\pi/ n$, $S_{n+1} = S_1$.

Where Ai is the area between the two scores (S), Si and Si+1 are two successive clockwise scores (radius coordinates) of a specific star plot, and n is the number of biomarkers utilized in the calculations. The IBR index for each biochemical parameter was then standardized in order to establish the mean value of each biomarker.

RESULTS

Breeding performance: The female brooders were found to release eggs through the genital opening by giving gentle pressure on the abdomen after latency period of 12-17h based on the rough range finding test for the doses (0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ml/ kg body weight) administered at all temperatures (26º, 28º and 30ºC) (Fig. 1a). Minimum latency period of 12.0h was recorded at the dose of 0.7ml Ovasis/kg body weight at 28ºC and at the dose of 0.5ml/kg and above at 30ºC, while maximum period of 17.5h was found at 26ºC in the female brood induced with 0.2 ml/kg. Comparing among the observed latency periods at different treatments, a range between 15.5-17.5h of latency period was recorded at 26ºC and it was 12.0-15.0h and 12.0-13.5h at 28º and 30ºC respectively (Fig. 1a). The smooth release of eggs was recorded in all the doses irrespective of temperatures except 0.2ml/kg of the stimulant at all temperature. Female fish treated with inducing agent at the rate of 0.3 ml/kg body weight and above at both 28º and 30ºCrespondedwell in

respect of their stripping property and released smoothly the cluster less eggs but the female administered with 0.4ml/kg of the Ovasis at 26ºC responded partially. The female induced with 0.2ml/kg body weight of the stimulant did not show smooth stripping and release of eggs. On the other hand, a little effort caused smooth release of eggs from the female treated with 0.7ml/kg body weight of the stimulant but the released eggs were found to lose their normal shape and consistency. The size of released eggs was between 0.39±0.014 and 0.59±0.021mm with a mean value of 0.48±0.05mm (Fig. 1b). Depending upon the dose of inducing agent applied to the fish at different temperature total number of eggs produced or absolute fecundity ranged from 2304 ± 56.78 to 3785±55.15 nos. and relative fecundity were between 129.00±3.18 and 219.04±3.19nos./g (Fig. 1c-d). In male, their testes were fully developed and swollen treated with Ovasis at the rate of 0.2ml/kg body weight. The fertilized eggs were very small in size (0.67±0.02mm), round in shape, transparent, demersal, adhesive and yellowish in color. They were devoid of structural lipids visible as droplets/oil globules and remaining attached to the bottom of the hatching tray. Unfertilized eggs were opaque and whitish in color. Time required for hatching after fertilization was 21.5±1.5h, 18.4±0.5h and 17.2±1.0h at 26º, 28º and 30ºC, respectively irrespective of doses of the stimulant used. Frequent agitation or twitching movement of the embryo at the rate of 12-18 times per minute with an uneven pace in between movement was recorded inside the egg capsule before hatching and hatchling emerged out as tail first. Immediately after hatching the hatchlings came to the water surface with a sudden jerk and then they settled down to the bottom slowly. Hatchlings usually preferred to take rest at the corner of the container but the hatchlings were also found to move frequently in isolation from the 2ndday after greatly absorption of yolk material. The study revealed that the breeding performance in *M. vittatus* was significantly high (P<0.05) at 28ºC over the results found at 26º and 30ºC irrespective of doses (Fig. 1e-f). On the other hand, the rate of fertilization and hatching were significantly higher (P<0.05) at dose of 0.5ml/kg body weight of the stimulant over all other doses at all temperatures (Fig. 1e-f). At the dose of 0.4ml/kg body weight the results were moderate. Regardless of temperature, the quantity of eggs, rate of fertilization, and hatching were substantially higher (P<0.05) at this dose than at 0.2, 0.3, and 0.6ml/kg body weight of Ovasis.

Frequency of morphological deformities: Frequency of morphological deformities in *M. vittatus* larvae was ranged between 2.1 and 6.3% (**Figure 1g**). Every breeding set wherein female brooder administered with different doses of Ovasis showed deformities which were significantly different from each other. Comparatively higher deformed larvae were observed at 26ºC with highest frequency of 6.3% at the dose of 0.7ml/kg body weight. Again, more deformity in larvae at lower (0.2ml/kg) or higher doses (0.6 and 0.7ml/kg) was recorded irrespective of temperatures. Rudimentary barbels, enlarged yolk sac, trunk region with 'coma' shaped, underdeveloped dorsal and pelvic fin formation, curved tail or feeble tail lashing were most common in the abnormal hatchlings with one or more characters. The hatchlings generated from the brooder female treated with the lowest (0.5ml kg/body weight) at 28^{o}C had the least deformation (P<0.05).

Morpho-sequential development and behavioral characteristics in the developing progeny: The developmental stages of *M. vittatus* from fertilized egg to 28thd recorded in the present experiment were characterized chronologically. Noticeable behavioral characteristics of the progeny were also recorded at their respective stage.

Fertilized egg: **0 h**: Water hardened eggs were round, non-filamentous, demersal, adhesive, transparent, yellowish in colour with 0.67±0.02mm in diameter. They were devoid of oil globule.

5h: Embryo formation within egg capsule was almost completed.

7h: Head and tail ends of the embryo were differentiated.

10h: Tail end was considerably elongated.

15h: Embryo started twitching movement within egg capsule.

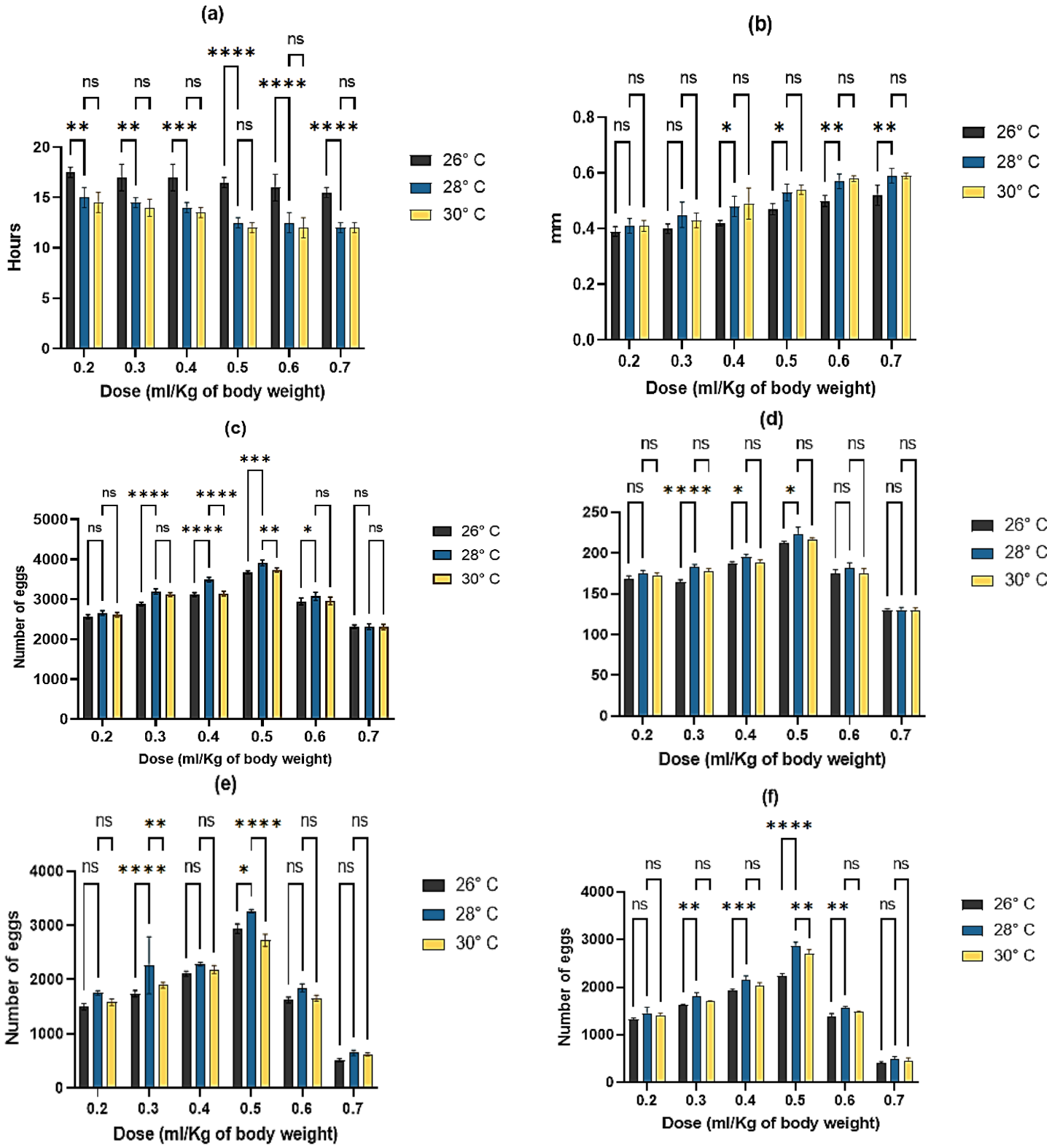

Fig.1. Effects of various doses of Ovasis on (a) latency, (b) egg size, (c) absolute fecundity, (d) relative fecundity, (e) fertilization and (f) hatching of *Mystus vittatus* at different temperatures (26º, 28º and 30ºC).

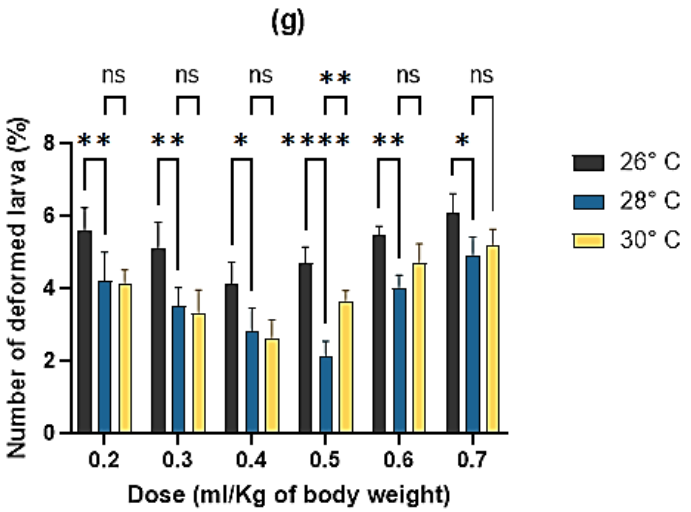

Fig.1. Continued; (g) larval deformity of *Mystus vittatus* at different temperatures (26º, 28º and 30ºC).

20h: Tail part was further elongated. Twitching movement was more frequent.

21-22h: Vigorous twitching movement was observed.

23-26h: Spawn hatched out rupturing the egg membrane.

Newly hatched fish (0h old fish): The mean length was 2.10±0.30mm. The yolk sac was large and ovoid with a mean length 0.40±0.05mm and mean height 0.28±0.03mm. The body was dull whitish to yellowish in color, translucent and without pigmentation. Slender and laterally compressed body with prominent oval shaped yolk sac. The protruding head part of fish bended downwards and with large unpigmented eyes. The fish rested their body at any one side on the bottom. The body was devoid of distinct mouth and fins.

6h old fish: The fish were achieved mean length of 2.19±0.25mm. Pigmentation appeared on the ventral part of the body but large eyes were still unpigmented. A conspicuous depression developed at the location of mouth to form silt. A thin membranous fin fold appeared at caudal region.

12h old fish: The fish were characterized by the pigmentation on their cephalic region and separation of head part from the yolk sac. They were enhanced by the mean length of 2.28±0.31mm. Barbels were not developed till the stage.

18h old fish: The mean length of fish was 2.57±0.13mm. Pronounced pigmentation was noticed on the whole fish body. Mouth slit and anal pore started to appear. Appearance of barbels was observed.

1d old fish: Mean length of the fish was 2.75±0.60mm. The mean length and height of the yolk sac were 0.38±0.03mm and 0.25±0.04mm respectively. Fish was dull yellowish in color with elongated slender body. Pigmentation was noted throughout the body especially over the cephalic region. A slit like mouth appeared in the ventral side at this stage. Pectoral fin buds were noticed as a small protuberance.

The fish remained confined to the bottom and started body movement lashing the tail. They became very sensitive to light and usually preferred to keep themselves at the corner of the tray but schooling tendency was not so dominant. A good number of hatchlings became active and moved separately keeping one side on the bottom.

2d old fish: The mean length of fish was 3.35±0.70mm. The yolk sac started to reduce (mean length 0.32±0.05mm and mean height 0.22±0.02mm). The head of developing fish with minute developing maxillary barbels was observed. Both upper and lower jaws were formed. The body was more pigmented on the edge part especially on the cephalic region. Pectoral fin started to appear. Anal aperture became distinct.

The tiny fish were also found to move frequently in isolation after gradual absorption of yolk material.

3d old fish: The mean length of fish was

4.00±0.50mm. The yolk sac was greatly reduced in size to a small tube-like rudimentary structure with mean length 0.25±0.04mm and mean height 0.15±0.02mm. Eyes were prominent and pigmented. Barbels were elongated.

The fish started swimming freely but most of the time they continued to remain in resting condition.

4d old fish: The mean length of fish was 4.85±0.60mm. The yolk sac was completely absent.

5d old fish: 5.55±0.30mm was the average length of developing fish. The body was lengthened, and the eyes were fully pigmented. All across the body, distinct coloring emerged. The maxillary barbels on the head were distinct. Mandibular barbels of varying sizes occurred. The fish preferred to hide in the tray's corner.

7d old fish: Fish was tiny in size with mean length of 7.18±0.50mm. Yolk sac was fully absent. Fish was creamy white in color with elongated slender body. Snout of the fish became angular. Well-developed head part with prominent black eyes and prominent barbels was observed. Pronounced pigmentation was noticed on the cephalic region. Dorsal fin appeared. Vertical movement was recorded for aerial respiration. Active swimming and foraging behavior were also noticed in fish.

10d old fish: The body was yellowish in color and more pigmented. Body contour was adult like. The mean length of body was 10.25±1.00mm. Head was fairly broad. The dorsal fin was distinct and pectoral one started to appear. Bifurcation was found in the caudal fin region.

14d old fish: The mean length of body was 12.10±1.00mm. Pigmentation was more concentrated over the broad head part and along the length of the body. A golden ring encircling the black prominent eyes was found. The ventral mouth cleft was distinct. Dorsal fin with fin rays was well developed. Pectoral fins were prominent and caudal fin was bifurcated.

18d old fish: The mean length of body was 16.50±1.50mm. The body was similar to the adults with dorso-ventrally compressed head. Four pairs of barbels were prominent. Pinkish red patch at gill region was noticed.

21d old fish: The mean length of the body was 18.00±0.75mm. Two pigmented lines appeared along both sides of the body. A golden ring encircling the black prominent eyes was found. The pinkish red patch at the gill region was recorded and a transparent hollow mark was found behind the gill. The dorsal, pectoral and caudal fins with fin rays were prominent and a ventral fin bud appeared. The 1stfin ray of the dorsal fin was comparatively hard. A distinct 1stfin ray was found in the pectoral fin.

25d old fish: The mean length of the body was 22.20±0.84mm. Body colour was golden yellowish. The transparent hollow mark was still present along both sides of body behind the gill region. Barbels were prominent and further elongated. Hard fin rays in the dorsal and pectoral fin were noticed.

28d old fish: The mean length of body was 27.50±1.00mm. Band like pigmentation was present all over the body. The base of dorsal fin showed the presence of prominent pigmentation. The transparent hollow mark behind the gill region became pale. The adipose fin was fully formed and lost its connection with caudal fin. The1stfin ray of the pectoral fin was serrated.

Integrated Biomarkers Response (IBR): The IBR index (Table 1) was used to compare the effects of biomarkers on different *Mystus vittatus* parameters. For the 0.2 Biomarker group, the IBR mean values had relative higher values for all parameters in the 0.2 Biomarker‘s column in Table 1 except for absolute fecundity and egg size. Similarly, for the 0.3 and 0.4 Biomarker group the IBR mean values had relative higher for all parameters in the 0.3 and 0.4 Biomarker‘s column in Table 1 except for deformed larva and egg size. The IBR index values were more significant in the 0.5 Biomarker group for all parameters except deformed larva and latency, with the most significant IBR index values in parameters like absolute Fertilization, hatching, and relative fecundity (Fig. 2). The 0.6 Biomarker group had higher IBR mean values for egg size and malformed larva, intermediate values for absolute fecundity,

Table 1. Summary of IBR mean values of different biomarkers.

Summary of IBR mean values							
Category	Absolute fecundity	Fertilization	Deformed larva	Hatching	Relative fecundity	Egg size	Latency
T0_0.2. Biomarker	0.48	2.03	4.21	2.17	2.91	0.00	7.84
T1._0.3.Biomarker	3.02	3.67	1.55	3.89	3.32	0.13	4.67
T2._0.4.Biomarker	3.82	5.03	0.03	6.17	5.92	0.94	3.36
T2._0.5.Biomarker	10.97	12.10	0.00	11.57	12.13	3.44	0.22
T2._0.6.Biomarker	2.53	2.44	5.28	2.57	3.71	6.15	0.11
T2._0.7.Biomarker	0.00	0.00	8.96	0.00	0.00	7.72	0.00

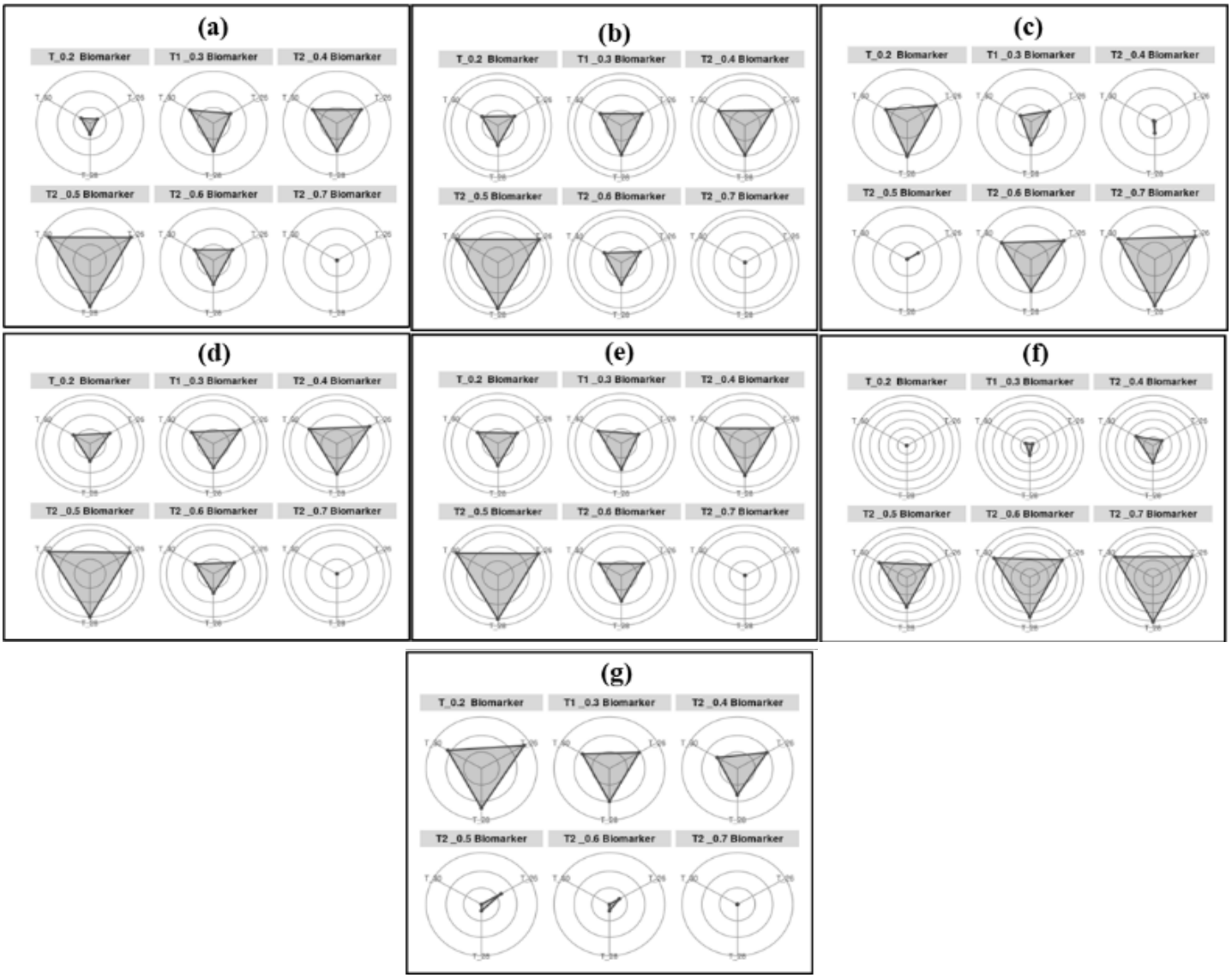

Fig.2. IBR star plots for evaluating (a) latency, (b) egg size, (c) absolute fecundity, (d) relative fecundity, (e) fertilization, (f) hatching, (g) larval deformity of *Mystus vittatus* at different temperatures (26º, 28º and 30ºC).

fertilization, hatching, and relative fecundity, and the lowest for latency. The IBR mean was found higher in the 0.7 Biomarker group for the deformed larva and egg size parameters but found lower IBR mean values for absolute fecundity, fertilization, hatching, relative fecundity, and latency.

DISCUSSION

In this study, the fish administered with Ovasis at a rate of 0.5 ml/kg body weight for females produced the best results in terms of egg release, fertilization, and hatching of eggs, regardless of temperature. This is most likely due to the appropriate activation of gonadotropin hormone-II (GTH-II) in the treated fish's pars distalis, which aids in the proper release of GTH-II. This released GTH-II interacts to a specific receptor in the granulosa cells of the ovary, stimulating steroid hormone synthesis in these cells and resulting in improved ovulation (Nuraini et al. 2017).

The result indicated that 0.2 ml/kg body weight for female was not suitable for complete ovulation for which stripping was not easy. This is consistent with reports for *Clarias batrachus* where the stripping response was low at lower dose of inducing agent (Zonneveld et al. 1988; Dhara & Saha 2013). The poor responses at lower doses of Ovasis (0.2ml/kg body weight for female) may be due to insufficient secretion of gonadotropin which leads to ovulation failure. Similar findings in finfish species have also been documented (Crawford 1986; Sahoo et al. 2005; Sahoo et al. 2008; Nargesi et al. 2022; Nargesi et al. 2023). Another reason for such low response in respect of fertilization might be due to asynchrony between maturation and ovulation leading to low hatching success (Rottmann et al. 1991).

At a dose of 0.6ml/kg body weight, the released eggs were not normal in their shape whereas translucency increased in the eggs at the dose of 0.7ml/kg body weight and the released eggs even lost their consistency. Such type of deterioration in egg quality at these higher doses was also reported and was implicated in the significantly lower fertilization and hatching rates compared to the other doses (Sahoo et al. 2008; Dhara & Saha 2013). Early ovulation in the higher doses in fish and the ovulated eggs remain in the ovarian lumen in a hypoxic condition for a long time leading to over-ripeness of eggs. The cytoplasm in these eggs accumulated at the animal pole region which results in lower fertilization and hatching success (Lam 1994).

Lower responses at the higher doses of stimulant may also be due to ovulation failure or blocking of ovipore by disintegrated ovarian tissue and egg masses (Sahoo et al. 2005). On the other hand, the result of intermediate success in fertilization and hatching rates at the dose of 0.3 and 0.4ml stimulant/kg body weight of female revealed as suboptimal in which ovulation was probably not fully completed at different level (Sahoo et al. 2005). Islam et al. (2011) observed 57-80% fertilization rate and 32-56% hatching rate in *M. vittatus* stimulated with different doses of inducing agent. Ray (2005) recorded 90% fertilization rate that yielded hatchings about 80% of fertilized eggs in *M. gulio* while Alam et al. (2006) found fertilization rate of 81-85% and hatching success of 71-73% with different doses of ovaprim in the same species. Bailung & Biswas (2014) recorded fertilization and hatching rates between 34.83-77.54% and 20.61-74.32% respectively in *M. dibrugarensis*. Such variation in the effects of doses of stimulant may be attributed to different level of dopamine activity in different fish species (Billard et al. 1984). Higher age and puberty age of fish, lower fertilization and hatching rates is often the case in smaller sized fish species compared to the larger (Nurullah et al. 2003). But the findings of the present experiment revealed that proper treatment combination may cause better reproductive performance in terms of fertilization and hatching.

The study indicated that a latency period of 12-17h depending upon the dose of inducing agent and water temperature probably due to release of gonadotropin at different degree. Islam et al. (2011) recorded similar time (16-18h) of latency period in the same species induced by different doses of pituitary gland extracts. Similarly, dose dependent latency periods in other bagrids were observed elsewhere (Alam et al. 2006; Bailung & Biswas 2014). Further, highest rate of fertilization and hatching was recorded at 28ºC in all the treatments as temperature plays a vital role in the control of all reproductive processes in fish from gamete development and maturation, ovulation,

spawning, embryogenesis and hatching to larval and juvenile development and survival (Pankhurst & Munday 2011; Anpe et al. 2017). In brood fish, it is generally considered as a secondary cue in synchronizing the final stages of reproductive maturity (Pankhurst & Porter 2003). An increased in temperature within an optimal range lead to faster embryonic development and thereby makes shorter hatching time (Small & Bates 2001). Olaniyi & Omitogun (2014) advocated that hatchability of the fertilized fish egg depends on various water quality parameters especially temperature resulting in faster hatchability and better survival at higher water temperature. Okunsebor et al. (2015) and Anpe et al. (2017) reported higher value of fertilization above 80% in *Heterobranchus bidorsalis* and *Clarias gariepinus* eggs at the temperatures of 28ºC and 30ºC, respectively.

In the present study on *M. vittatus*, time required for hatching after fertilization was 21.5±1.5 h, 18.4±0.5h and 17.2±1.0 h at 26º, 28º and 30ºC respectively irrespective of doses of the stimulant used. Almost similar incubation period was reported in other bagrids (Alam et al. 2006; Begum et al. 2009; Kumar et al. 2018). Ramanathan et al. (1985) recorded the incubation period of 18-24h in *M. punctatus* at a temperature of 28.5±1.8ºC. Graaf & Janssen (1996) advocated that the development and incubation periods of embryo in most fishes are fully temperature dependent and species specific. Other reports have demonstrated that an increased in temperature within an optimal range lead to faster development and shorter hatching time, also opined that fertilized eggs develop properly if water quality parameters especially temperature is within tolerable limit (Small & Bates 2001; El-Gamal 2009). However, in this study, *M. vittatus* showed comparatively lower reproductive performances at 26ºC. It is probable that this temperature was suboptimal for sexual maturation and spawning. Furthermore at 30 ºC, fish showed significantly higher reproductive performances than those at 26ºC but comparatively lower than the parameters at 28ºC. Such temperature specific changes in results may be due to conformational alteration in proteins in the reproductive cascade (Pankhurst & Munday 2011). The varied effects of temperature in reproductive performance of *M. vittatus* may also be attributed to the effect of temperature on HPG axis in fish at multiple sites through its reaction-rate-determining effects on hormone synthesis and action, and its effects on hormone structure at different degree (Van Der Kraak et al. 1998; Pankhurst & Munday 2011). Further developing fish also showed lower survival and growth at 26ºC as temperature specificity plays the key role to optimize all the physiological activities of fish (Buckley et al. 2000).

In the present study, absolute and relative fecundity for *M. vittatus* ranging from 2304 to 3785 nos. and 129.0 to 219.04nos./g. These values were within the range observed by some earlier findings on the same species with some variations (Basu et al. 2015; Rahman et al. 2016). However, the present observation with the mean of 33386nos. per female of *M. vittatus* is a greater variation from the fecundity estimated by similar studies (Islam et al. 2011). Such differences may be due to environmental conditions, location and also size group, age and genetic potential of the species (Mohammad & Pathak 2010). Egg quality emphasizes the egg's ability to generate viable progeny, and high-quality eggs are distinguished by a higher rate of fertilization, hatching, and larvae survival (Bromage, 1995; Aristizabal et al., 2009). Egg quality is crucial for creating high-quality progeny; if it is impaired, a large number of malformed larvae and mortality will ensue (Migaud et al. 2013; Reading et al. 2018). The rate of fertilization and subsequent hatching is determined by the size and quality of the egg (Olaniyi & Omitogun 2014).

Egg quality is profoundly influenced by different environmental factors (Bobe & Labbé 2010; Migaud et al. 2013). In this study, egg diameter and larval length were correlated with the dose of stimulant and temperature. In turn, reproductive parameters such as fertilization and hatching rates also showed a relationship with the egg size. Deterioration in egg

quality due to an increased dose of gonadotropin has previously been reported in some earlier studies on catfish (Haraldsson et al. 1993; Sahoo et al., 2008). On the other hand, lower rate of fertilization in lower dose (0.2 ml/kg body weight of female) followed by poor response during stripping might be attributed to the blood on the stripped egg and protein from ruptured eggs which coagulate and clog the micropile leading to poor breeding performance (Piper et al. 1982). The higher doses of stimulant might have influenced early ovulation of eggs, and the ovulated eggs were exposed to a long stay in ovocoel, possibly resulting in over-ripening of ovulated eggs (Ohta et al. 1996). Alternatively, hypoxic conditions in the ovarian lumen after ovulation, could have reduced the viability of the eggs.

In the present study, all the eggs appeared to be healthy and well developed in all temperatures at the beginning. But after 2-4h of mixing with milt suspension, some of the eggs became white or opaque and had turbid contents. They remained as unfertilized may be either due to injuries sustained during the stripping process or due to improper maturation or improper synchronization with sperm during mixing with milt. They died either during the morula stage or before the closing of the blastopore (Anpe et al. 2017). Such unfertilized eggs were not distinguishable from the fertilized ones at the beginning as they swelled in the same way. The fertilized eggs of *M. vittatus* were transparent, spherical and adhesive in nature. Their contents were cleared. The similar characters of fertilized eggs were also recorded in other catfish under other genus (Puvaneswari et al. 2009; Islam et al. 2011; Dhara & Saha 2013) and within the same genus (Bailung & Biswas 2014).

The adhesive nature of the fertilized eggs, which is a characteristic of Order Siluriformes, provides an adaptation to prevent from the flowing of eggs in the water currents and helps to get optimal oxygen supply from the flowing water (Puvaneswari et al. 2009). The mean size of mature egg (0.50 ± 0.05 mm) and fully swollen fertilized egg (0.67±0.02mm) of *M. vittatus* recorded in the present study was comparatively smaller than the other *Mystus* species (Kumar et al. 2018). The yellowish colour of the fertilized eggs of *M. vittatus* without oil globule found in the present study almost corresponds with the colour of the eggs of members of the same genus (Arockiaraj et al. 2003; Kumar et al. 2018). The hatching in fish is generally facilitated by twitching at their tail part (Olaniyi & Omitogun 2014). Prior to hatching out from the egg of *M. vittatus* in the present study, vigorous twitching movement in the tail part and hatchling emerged out from the tail region first corroborates to the observations made in various catfish species such as *M. montanus* (Arockiaraj et al. 2003), *M. gulio* (Kumar et al. 2018), *H. fossilis* (Puvaneswari et al. 2009), *Heterobranchus bidorsalis* (Olaniyi & Omitogun 2014) and *C. batrachus* (Dhara & Saha 2013). After hatching the fish was inactive, this is like *M. gulio* as observed in the study of Kumar et al. (2018).

Hatchlings usually preferred to take rest at the corner of the container probably due to load of yolk sac but they were also found to move frequently in isolation from the 2nd day onwards after extensive absorption of yolk material as observed in the present study. The mean length (2.10±0.30mm) of the newly hatched fish in the study was comparatively smaller than the other *Mystus* species (Arockiaraj et al. 2003; Kumar et al. 2018). The smaller size of the hatchlings was probably due to the smaller size of the fertilized eggs. The positive correlation between the egg diameter and the size of hatchlings was also recorded by Bagarinao & Chua (1986). In the present study, the yolk in the hatchlings of *M. vittatus* was almost exhausted on the 3rd day of development and became completely absent on the 4th day but the yolk was found absent on the 2nd day of developing *M. gulio* (Kumar et al. 2018) and on the 3rd day of *M. macropterus* and M*. montanus* (Wang et al. 1992; Arockiaraj et al. 2003). The body pigmentation due to melanophore firstly started on the head part then covered entire body. Such type of spreading of melanophores was differed from other bagrid, *M. gulio* (Kumar et al. 2018) but corroborates with

some other catfish such as *Pimelodus maculatus* (Buzollo et al. 2011) *C. batrachus* (Dhara & Saha 2013) and *H. bidorsalis* (Olaniyi & Omitogun 2014). The vertical movement of the young *M. vittatus* on the 7thday of development was found probably due to the formation of aerial respiratory organ. Similar findings were also recorded in the development of *M. montanus* (Arockiaraj et al. 2003). All morphological characters of the adult *M. vittatus* were distinctly observed in the young developing fish in the present study on 18th day corresponding to that of *M. seenghala* (Saigal & Motwani 1961) but 20th day in *M. montanus* (Arockiaraj et al. 2003). Such type of difference may be due to species specificity or due to availability of proper and adequate food with optimum physico-chemical properties of the rearing medium.

Time required for hatching after fertilization was 17.2-21.5h irrespective of doses of the stimulant. The present time requirement for hatching corresponds with the time taken by *Mystus seenghala* (Chondar 1999), but it was slightly higher than the time required by *Mystus montanus* (Arockiaraj et al. 2003). In the present study, the time required for incubation of eggs for hatching in all the treatments was largely influenced by temperature. The incubation period of the eggs varies from species to species (Graaf & Janssen 1996).

Morphological deformity in hatchlings is not uncommon in induced bred freshwater catfish (Fagbuaro et al. 2006), with incidences ranging from 11% up to 50% in some other species (Linhart & Billard 1995; Sahoo et al. 2007). Morphological abnormalities like underdeveloped head and barbel, deformed trunk, enlarged yolk sac, curved tail and vertebral deformities in induced bred larvae of *M. vittatus* (Teji & Thomas 2006) and other species (Sahoo et al. 2007; Dhara & Das 2018). In the present study, observation of more deformity in larvae at lower (0.2ml/kg) or higher doses (0.6 and 0.7ml/kg) irrespective of temperature may be due to the fertilization of unripe and over-ripe eggs (Sharma et al. 2010). Release of unripe eggs during the stripping of incomplete ovulated female brooder administered with lower dose of inducing agent led to occurrence of moderately higher frequency of deformed larvae (4.1-5.6%) probably due to the fertilization of unripe ova in the present study corroborated with some earlier studies on catfish (Dhara & Das 2018). Similarly, eggs discharged from brooder fish triggered by larger doses were viable and fertile, but likely resulted in altered embryogenesis, resulting in malformed hatchlings (Rath et al. 1995; Sahoo et al. 2004). Furthermore, Lam (1994) claimed that when over-ripe eggs were placed in fresh water, they did not form a perivitelline gap, implying that the chorion's permeability was reduced. The reduced permeability of the chorion to water may have a detrimental influence on the usage of yolk components, resulting in slowed or aberrant embryogenesis in over-ripe eggs.

Figure 2 shows the converted data for all of the biomarkers evaluated as a star plot for each site. The star plots, which demonstrate a progressive decrease in the size of forms acquired for each experimental group, essentially reflect the effect of various doses of ovasis at varying temperatures. For the parameters-latency, egg size, relative fecundity, and fertilization, the T2 0.5 biomarker group with the greatest shape star plot and grey area was the least influenced, followed by T2 0.4, T2 0.6, T1 0.3, T 0.2, and T2 0.7. In the case of absolute fecundity and hatching, when substantial grey areas for some biomarkers imply unfavorable influence, the opposite patterns are found.

CONCLUSION

To expand culture of catfish, adequate knowledge of their breeding as well as their early development is imperative. In the present study most successful results in respect of fecundity, egg size and embryonic development, rate of fertilization and hatching were recorded in *Mystus vittatus* injected with Ovasis at the dose of 0.5 ml/kg body weight of female fish at 28ºC. Further, larval deformities were also least at that condition. The present findings will provide some valuable information in breeding technology for the development of commercial hatcheries of *M. vittatus*. From the study, it is suggested that the proper dose and

temperature combination may be ideal for getting better result in respect of yielding viable progeny of *M. vittatus.* Furthermore, the chronological characterization of different developmental phases of *M. vittatus* (fertilized egg to 28th day old fish) described in the current study will open up new avenues in management methods, particularly during rearing of their most vulnerable earlier stages.

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مقاله کامل

تخم‌ریزی القایی با هورمون و بهینه‌سازی شده با دما در گربه‌ماهی کوتوله راه‌راه آسیایی، *Mystus vittatus* در مراحل اولیه نسل F1

کیشور دارا[1]، اودی نارارایان داس[2]، پراسیناجیت پال[3]، نیمای چاندرا ساها[4]، شوبهاجیت ساها[*4]

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چکیده: در مطالعه حاضر، آزمایش تکثیر القایی گربه ماهی کوچک؛ *Mystus vittatus* با روش‌های تخم‌گیری دستی با استفاده از دوزهای مختلف (۰/۲، ۰/۳، ۰/۴، ۰/۵، ۰/۶ و ۰/۷ میلی‌لیتر بر کیلوگرم وزن بدن) از یک هورمون مصنوعی، Ovasis در دمای ۲۶، ۲۸ و ۳۰ درجه سانتی‌گراد برای ارزیابی عملکرد اصلاحی آنها انجام شد. در طول تخم ریزی، دوره نهفتگی آن‌ها بین ۱۷-۱۲ ساعت بود. بیشترین تعداد هم‌آوری مطلق (۳۷۸۵ عدد) و هم‌آوری نسبی (۲۰۹/۰۴ عدد در گرم) در دوز ۰/۵ میلی‌لیتر بر کیلوگرم وزن بدن ماده در دمای ۲۸ درجه سانتی‌گراد مشاهده شد. به‌طور مشابه، بالاترین موفقیت تکثیر در این دوز و دما مشاهده شد که ماده با همتای نر تحت تیمار هورمونی با دوز ۰/۲ میلی‌لیتر بر کیلوگرم وزن بدن تکثیر داده شد. در این شرایط میزان لقاح و تفریخ تخم ها به ترتیب ۸۴/۱۵ درصد و ۷۵/۴۸ درصد بود. کمترین عملکرد تکثیر در دوز ۰/۷ میلی‌لیتر بر کیلوگرم وزن بدن ماده در دمای ۲۶ درجه سانتی‌گراد مشاهده شد. علاوه بر این، مراحل رشد ماهی (تخم بارور شده تا ماهی ۲۸ روزه) نیز به‌صورت زمانی همراه با تمایل رفتاری آن‌ها در این مطالعه مشخص شد. ناهنجاری‌های ریختی، ۶/۳ درصد در دمای ۲۶ درجه سانتی‌گراد در لاروهای تفریخ‌شده از تخم‌ها با دوز ۰/۷ میلی‌لیتر بر کیلوگرم وزن بدن ثبت شد. دوز توصیه شده Ovasis، ۰/۵ میلی‌لیتر بر کیلوگرم *Mystus vittatus* در دمای ۲۸ درجه سانتی‌گراد است.

کلمات‌کلیدی: *Mystus vittatus*، توسعه مورفولوژیکی، رفتار، تغییر شکل لارو.