Research Article

Captive breeding and larval rearing of the endemic ornamental fish Moustached Danio, Danio dangila (Hamilton, 1822) – First report

Dr. Mangesh Bhosale[1,2], **Rohini R Mugale**[3]

1. Tamil Nadu Dr. J Jayalalitha Fisheries University, Nagapattinam, India; 2. Centurion University of Technology and Management, Khurda, India; 3. Chhattisgarh Kamdhenu University, Raipur, India

Moustached danio, *Danio dangila,* is a potential ornamental fish of India but the stock of this endemic Cyprinid is declining in the wild due to environmental variation and anthropogenic activities. Hence, the present work on induced breeding and larval rearing was undertaken to conserve this species. During the investigation, live fish were brought from Assam (North-East Indian region) to ARTP, Chennai, Tamil Nadu. In experiment 1, trials to breed the species using the synthetic hormone WOVA-FH, since the species did not respond to breed in captivity, revealed the highest spawning (1002.50±52.6), fertilization (84.0±2.72%) and hatching rate (86.5±2.9%) after intraperitonial injection at 0.5 ml/kg for females and 0.3 ml/kg for males. In experiment 2, larval rearing in nursery raceways and FRP tanks at different stocking densities for 45 days showed that the RAS-based nursery raceways produced the highest survival (91.79±1.67%) and specific growth rate (0.13±0.001%/day) at 30 numbers/L stocking density. The present investigation standardized the breeding and larval rearing protocol in advanced aquaculture systems and can give significant implications on the conservation and seed production of the species.

Corresponding author: Dr. Mangesh Bhosale, mangesh.bhosale@cutm.ac.in

1. Introduction

Worldwide, around 4000 fish species are traded by 145 countries of which tropical species contribute 85% share (Sneddon *et al.,* 2016; King, 2019). Northeast India (21^{o}57'N to 29^{o}30'N Longitude and 89^{o}46'E to 97^{o}30'E Latitude) is endowed with a vast expanse of freshwater habitats mainly flood plain (1.59 lakh ha), rivers (20,875km), reservoirs (0.33 lakh ha), tanks and ponds (3.71 lakh ha) including the mighty Brahmaputra and the adjoining wetlands. Sandilyan (2016) reported that India is endowed with rich ornamental fish diversity having around 600 species of which North-East India is blessed with 296 (49.33%) potential ornamental fish species contributing 11.6% share to the world freshwater ornamental fish species trade (Dhar and Ghosh, 2015). Diversification of fish species and the development of a fish seed production system are essential to meet the trading demand (Bhendarkar *et al.,* 2020).

Cyprinids, a popular group among the ornamental fish trade, include 2,420 species having bright colours, large scales, schooling behavior, and easy to maintain in captivity (Bera *et al.,* 2022). *Danio dangila* (Hamilton, 1822), commonly known as moustached danio, is an endemic fish inhabiting mountain streams of the Ganga-Brahmaputra drainage. The species have brilliant coloration with brownish to olive on the back and silver yellowish on the belly and flanks. Three to four silvery bluish lines on the flanks and the bands of the body break up anteriorly forming a mottled pattern, which makes the species more attractive and unique among the Cyprinids.

However, aquatic habitats have witnessed rapid changes in their environment and caused greater habitat degradation as a consequence of natural and ever-increasing human interference. This led to unavailability of the optimum environmental conditions and hence, loss of natural breeding grounds (Geist and Hawkins, 2016 and Pandi *et al.,* 2021). Although North-East India is one of the 34th mega biodiversity hotspots identified by Conservation International, the Himalayas and Indo-Burma (Bijoya *et al.,* 2014), reports on breeding and larval rearing of endemic fishes especially away from their local grounds are fairly available. Mahapatra (2010) has succeeded in inducing breeding and rearing of indigenous ornamental fish *P. shalynius* using Ovaprim in the local ground. Prakash *et al.* (2014) have developed a captive breeding and larval rearing protocol for *Danio aequipinnatus*, while Motilan *et al.* (2014) succeeded in induced breeding of the state fish of Manipur, *Puntius manipurensis*, using the synthetic hormone WOVA-FH. Likewise, Udit *et al.* (2014) used Ovatide at higher concentrations for successful breeding of *Puntius sarana*. By reviewing numerous reports, Stevens *et al.* (2017) reported that the population of *Danio spp.* in the wild is continuously declining in the extent of occurrence due to over-exploitation, especially in aquarium trade.

Although the majority of exotic freshwater fishes involved in the aquarium trade are from captive-bred sources, significant trials on captive breeding of indigenous and especially endemic fish species are still in vogue (Rahdari *et al.,* 2014). Therefore, standardization of captive breeding and larval rearing techniques of the species could play a major role in increasing the population of this incredible species' biodiversity. Hence, to conserve and meet the demand of the aquarium trade, the breeding and larval rearing protocol for the endemic fish, *Danio dangila,* has been standardized in captivity.

2. Materials and Methods

2.1. Experimental design and setup

Live fishes were procured from Brahmaputra stretch near Rajabari Grant in Assam (Lat. 26°28'24.44" N, Long. 92°32'02.3" E), packed in oxygenated polythene bags and brought to the hatchery situated at ICAR-Research Complex for North-East Hilly region, Manipur for acclimatization. Thereafter, the fishes were re-packed and brought to Aquatic Rainbow Technology Park (ARTP), Dr. MGR Fisheries College and Research Institute, TNJFU Madhavaram campus, Chennai (Lat. 13° 9'42.81"N, Lon. 80°15'1.86"E) through the air following the methods of live fish transportation given by Rosten (2009). The fish were stocked at 100 numbers/tank in circular fiberglass reinforced plastic (FRP) tanks (10000 L) attached to a recirculatory aquaculture system facility (RAS). The fishes were fed with live food organisms and an artificial diet (35% protein) at 4-5% of their body weight. Fishes were observed periodically for morphological indicators of maturity and health issues. Reverse Osmosis (R.O.) and U.V. filtered water were used for domestication, breeding, and larval rearing of the species since the groundwater hardness was above 800 mg/L. In the experimental tanks, water quality parameters were maintained at pH - 6.9-7.2, total dissolved solids - 30-50 mg/L, dissolved oxygen - 5.5-6.5 mg/L, free CO_2 - <1.0 mg/L, ammonia - <0.05 mg/L, alkalinity - 45-50 mg/L and were determined periodically by standard methods (APHA, 2012). Water temperature was maintained at 20-25°C using regulated water heaters (Thermostat).

2.2 *Selection of brood pair*

Velon screen (10mm mesh) made hapas (30cm×30cm×30cm) were fixed in 20 aquarium tanks (60cm×30cm×45cm; L×B×H) and filled with water up to 15 cm. Male brooders with ventrally straight belly, oval anal opening, and slightly rough anal fin with red linings were selected, while female brooders with bulged and ventrally curved belly, round anal opening, and whitish yellow lining on anal fins were segregated (Anon, 2012). For confirmation of maturity, oocyte diameter was staged by obtaining an *in-vivo* biopsy using a polyethylene cannula and observed under a trinocular microscope (NLCD-120E, Lawrence and Mayo).

2.3. Hormonal inducement

A synthetic hormone, WOVA-FH, was obtained from Biostadt India Ltd., Worli, Mumbai, India. The hormone was injected twice into the females while males were injected once during the resolving dose of the female (6 h) intra-peritoneally using an insulated syringe (1.0mL, Terumo U40) after dilution (Table 1). While injecting care was taken to reduce the chance of cardiac puncture by needle. The present experiment followed a completely randomized design (CRD) using four treatments in triplicate with control for each hormone.

Treatments	Hormonal dosage for female	Hormonal dosage for male
T_0	No inducement	No inducement
T_1	0.3	0.1
T_2	0.4	0.2
T_3	0.5	0.3
T_4	0.6	0.4

Table 1. Treatments based on the hormonal dose applied (ml/kg)

2.4. Breeding performance and embryonic developments

The breeding performance of an individual female was evaluated based on breeding behavior, latency period (h), number of spawned eggs, fertilization rate (%), and hatching rate (%). The latency period was the time gap between hormonal inducement to both the parents and the first appearance of the spawned eggs (Kumar *et al.,* 2018). The number of spawned eggs from each female was calculated by collecting the spawned eggs in a graduated measuring cylinder and counted in unit volume (Behera *et al.,* 2007). The fertilization rate was determined by counting eggs with an intact nucleus from the total number of eggs following Das *et al.* (2016). The hatching rate was calculated by dividing the number of hatchlings by the total number of fertilized eggs multiplied by a hundred (Mangesh *et al.,* 2017). Three days old hatchlings were transferred in 200 L capacity circular FRP tanks connected with RAS and 0.2 m/sec water flow facilities arranged by fixing ducklets-like structures.

After spawning, brooders from the aquarium tanks (hapa) were transferred to FRP tanks. The eggs were collected using a dropper for embryonic development observations under a trinocular microscope while post-embryonic developments were observed under a stereozoom microscope attached to a camera (EM 33 MEIJI).

2.5. Experimental setup for larval rearing

The experiment was conducted following a 2×4 factorial design for 45 days at 10, 30, 50, and 70 numbers/L stocking densities in triplicate (Table 2). Spawns (4 DPH) were stocked in two rearing systems *viz.* nursery raceways (10000 L) (6×2×1.15 m) and FRP tanks (1000 L) attached to RAS and 0.4-0.5 m/sec water flow. Spawns were fed to satiation initially with rotifers, *Brachionus calyciflorus*, followed by moina (*M. micrura*), cyclops (*T. hyalinus*) and artificial diet (52% protein) at 5-8% of body weight. The results were obtained by analyzing final weight gain, average daily gain (ADG), specific growth rate (SGR), and survival rate (%).

Rearing systems		Treatments	T_1	T_2	T_3	T_4
1.	Nursery Raceways	Stocking density (numbers/L)	10	30	50	70
2.	FRP tanks					

Table 2. Experimental setup based on rearing system and stocking densities

2.6. Statistical analysis

Initially, the experimental data were tested for normality of distribution by Shapiro-Wilk's W-statistic (PAST, Version 16.0). For experiment 1, one-way ANOVA was performed to compare significant differences in hormonal treatments following Duncan's multiple range test ($p<0.05$). For experiment 2, the nursery rearing data was analyzed following two-way ANOVA in SPSS (version 20.0, SPSS Inc., Michigan Avenue, Chicago, Illinois, USA). The results are presented as mean ± standard error (SE).

3. Results and Discussion

The breeding performance of *D. dangila* induced with cumulative doses of hormone and nursery rearing in two systems with different stocking densities showed significant variation ($p<0.5$) and are described in Tables 3 and 4.

3.1. Breeding behavior and latency period

In almost all induced sets, brooders showed mating behavior after 2.0-2.5 h except in the control and low dosage sets. Mating was preceded by an elaborated courtship behavior where the male started chasing the female and sprayed milt over the eggs released by the female. In the case of T_0 and T_1, no breeding behavior was seen in all groups, while in the case of high dosage (T_4), although the males were active, the females were stressed. In captive conditions, most of the fish lose the natural breeding behavior due to the non-availability of an optimum environment for breeding and the breeding behavior can be stimulated through hormone administration (Motilan *et al.,* 2014). Captive breeding by using a suitable inducing agent is the best tool for the breeding of fish and it ensures the hatchery-bred seed round the year (Pandi *et al.,* 2021). Similarly, Behera *et al.* (2007) and Karim *et al.* (2016) reported no inducement in the low dosage sets while the higher dosage of synthetic hormones caused stress to *Labeo bata* and *Mugil cephalus*, respectively. It is expected that excess dosage might have caused negative stimulation (Bondarenko *et al.,* 2015). Almost all brood fishes were

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spawned within 07-08 h of inducement. Differences in latency time might be due to the varied level of dopamine activity and target organ of the hormone as the luteinizing hormone-releasing factor of the anterior pituitary act on the brain to have well-matured sperms or eggs. A similar report on the variations in the latency time of tench (*Tinca tinca*) with different spawning agents was observed by Jamorz *et al.* (2008). The latency or response time is also related to the water temperature which decreases with sudden changes. In the present study, the latency period of 6-8 h at ambient water temperature (20-25^{o}C) was similar to the reports of Behera *et al.* (2007) in *Labeo bata*, Mangesh *et al.* (2017) in *Hemichromis bimaculatus*, Pandi *et al.* (2021) in *Dawkinsia rohani* at different doses of WOVA-FH.

3.2. Number of eggs spawned

A significantly higher (p<0.05) number of spawned eggs were observed in T_3 (1002±52.6) followed by T_2 (675.50±52.61). Nonetheless, fish injected with excess and low doses showed poor performance. In a similar way, Motilan *et al.* (2014) reported the maximum number of spawned eggs in *P. manipurensis* (6218.75±32.75) and *P. chola* (893.34±41.34) using WOVA-FH at 0.4 ml/kg than other synthetic agents. Pandi *et al.,* (2021) also reported the highest spawning capacity (1142.3±40.20) in *D. rohani* when induced with WOVA-FH at 0.7 ml/kg for the females and 0.3 ml/kg for the males. In contrast, Udit *et al.* (2014) reported a maximum number of spawned eggs after inducing a higher dosage of Ovatide (1.0 ml/kg) for *P. sarana* (78500±124).

3.3. Fertilization rate and Hatching rate

The fertilized eggs were spherical and translucent with a pale brownish color measuring around 0.8 mm to 1.0 mm. The twitching movement of the embryos was observed within 60:00 hours of spawning and the young ones were hatched out within 68–72 h at 25.5±1.5^{o}C. In the experiment, the administered hormone and its dosage apparently affected the rate of fertilization and hatching. Significantly highest (*p<0.05*) fertilization (84.0±2.72%) and hatching rate (86.5±2.9%) was observed in T_3 (Table 3). In a similar way, Behera *et al.* (2007), Motilan *et al.* (2014), Das *et al.* (2016), Mangesh *et al.* (2017), and Pandi *et al.,* (2021) found an increased percentage of egg production, fertilization and hatching rate in carps, *L. bata*, *P. manipurensis*, *O. belangri, H. bimaculatus* and *D. rohani* when induced with WOVA-FH at 0.4-0.7 ml/kg for the females and 0.2-0.4 ml/kg for the males.

However, the lower fertilization rate was observed with excess inducement, which might be due to its mode of action of forcing the gonad to open up even when it is underdeveloped for fertilization (Zohar and Mylonas, 2001). Over-dosing of the inducing agents caused early milting, and under-dosing caused late inducement in one of the mating partners, triggered fertilization and ultimately hatching rate (Karim *et al.,* 2016). The dose of hormone obviously affected the hatching rate, the optimum range of water quality parameters in the breeding tanks also might have led to higher fertilization and hatching rates. According to Svinger *et al.* (2013) and Bondarenko *et al.* (2015), the ideal incubation temperature (25.5±1.5^{o}C) for tropical barb and carp varieties resulted in higher fertilization and hatching rates. Similar findings were reported by Behera *et al.* (2007) and Das *et al.* (2016) in *L. bata* and *O. belangeri*, respectively. The chronological developments of the embryo have been described in Fig. 1.

Treatments	Weight (g)		Latency Period (h)	Number of eggs spawned	Fertilization Rate (%)	Hatching Rate (%)	Remarks
	Male	Female					
T_0	5.25±0.95	6.57±0.20	0^a	0^a	0^a	0^a	No spawning
T_1	4.75±0.30	6.50±0.14	0^a	0^a	0^a	0^a	No spawning
T_2	5.02±0.34	6.30±0.19	$8.82±0.56^c$	$675.5±52.61^b$	$72.0±3.39^{bc}$	$75.0±3.0^b$	Partial spawning
T_3	4.8±0.23	6.60±0.23	$7.87±0.12^b$	$1002.50±52.6^c$	$84.0±2.72^c$	$86.5±2.9^{bc}$	Complete spawning
T_4	4.9±0.31	6.6±0.23	0^a	0^a	0^a	0^a	No spawning
p-value	-	-	0.024	0.025	0.037	0.026	

Table 3. Induced reproductive performance of *Danio dangila*.

Note: *Values with different superscripts in the column differ significantly at p<0.05 (n=3)*

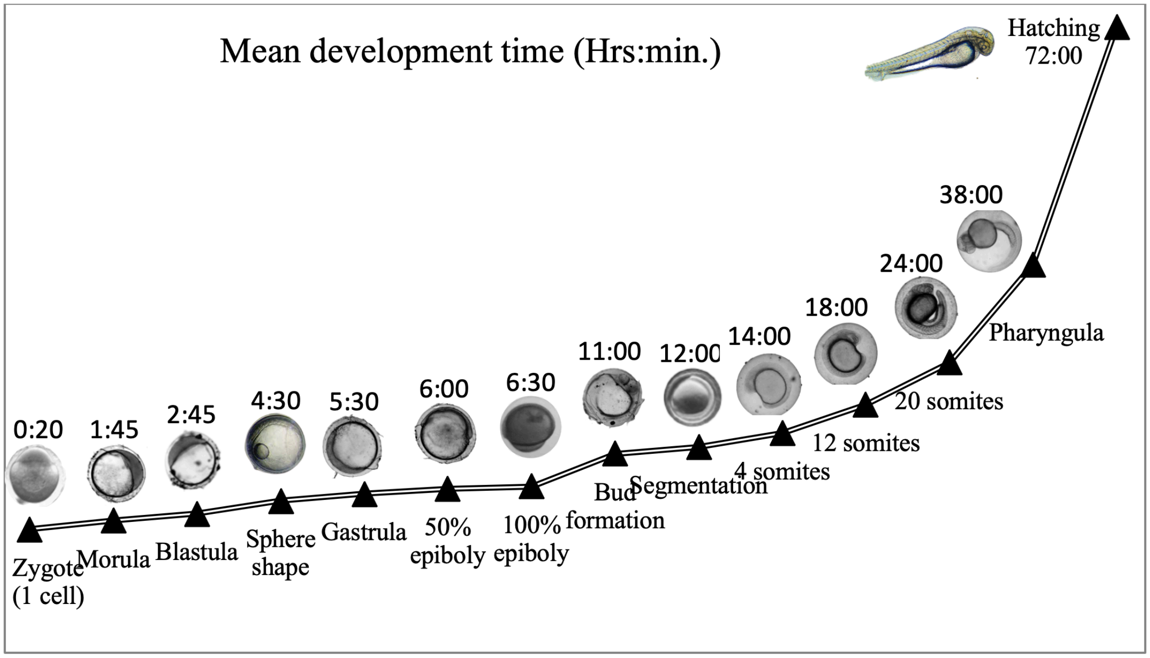

Fig. 1. Embryonic development of *Danio dangila* with mean development time

3.4. Larval rearing

The newly hatched larvae were transparent and measured around 1.8-2.1 mm in total length. The yolk sac was fully absorbed within 68-72 h. Intensive aquaculture systems are used to efficiently produce large quantities of fish or shrimp under controlled conditions (Watanabe *et al.,* 2002). One such system is RAS-based nursery raceways which constantly circulate water by infusing an adequate amount of oxygen by giving a riverine condition led to increased production rates more than the conventional culture systems (Fornshell *et al.,* 2012; Felix *et al.,* 2021). In the present experiment, the raceways and FRP tanks-based nursery rearing systems at four cumulative stocking densities showed a significant interaction effect (p = 0.041) (Table 4). The spawns (DPH 4) reared in nursery raceways significantly (p<0.05) exhibited a higher survival rate (62.86%), daily weight gain (0.039), final weight gain (1.229 g), and specific growth rate (0.132%) compared to FRP tanks. Similarly, Felix *et al.,* (2021) reported the highest survival (98%) and a 10-fold increase in weight gain of genetically improved farmed tilapia (GIFT) fry reared in nursery raceways. Furthermore, they suggested that the stocking density could be increased again up to 33% in an aerobic microbial flock-driven raceway system. As with any aquaculture species, the initial developmental stages are the bottleneck of finfish and shellfish hatchery systems and are relatively susceptible to pathogens, especially ciliate parasites and bacterial infections (Plumb and Hanson, 1999). But in the present experiment no such incidences were observed throughout the study even at higher stocking densities.

Mortality is an important indicator of the adaptation of fish to the rearing system. In several studies, high stocking densities resulted in huge mortalities (Ellis *et al.,* 2005; Ashley, 2007). In our study, the highest and almost equal survival rate was observed at 10 numbers/L (81.00%) and 30 numbers/L (80.55%) stocking densities. However, mortality was observed in higher stocking densities which might be due to over-crowding, aggressive behavior of fast grew fishes and mechanical injuries against the system components, mainly air lift pumps and water outlet systems. Similar results have been reported by Hengsawat *et al.* (1997), North *et al.* (2006), Sophie *et al.* (2009), Laizcarrion *et al.* (2012), Alhassan *et al.* (2012) and Kucharczyk *et al.* (2020) for *Clarias gariepinus*, *Oncorhynchus mykiss*, *Dicentrarchus labrax*, *Pagrus pagrus*, *Oreochromis niloticus*, and *Leuciscus idus*, respectively.

The stoking density is connected to the production parameters in the commercial farming systems even though the fish were fed to satiation (Ashley, 2007; Jha and Barat, 2014). In traditional open culture systems, fish are grown at a very low stocking ratio (0.2–0.3 fish/L) (Jahedi *et al.,* 2012). The well-studied physiological parameter in relation to the rearing environment is growth (Sloman and Armstrong, 2002). It can be measured easily and used as an indicator of stress and other related issues. In our experiment, the average daily weight gain (0.042±0.002 g) and mean weight gain (1.323±0.051 g) was found maximum at 10 and 30 numbers/L. Furthermore, the specific growth rate remained constant (0.134%) at 10 numbers/L and 30 numbers/L with significant interaction effect (p = 0.016). However, the increased stocking density of more than 30 numbers/L significantly affected survival and growth parameters. According to Tan *et al.* (2018) and Jha and Barat (2005), stocking more than the optimum rate increased stress on the fish and led to heavy mortality. Furthermore, the findings suggest that stocking densities lower than the optimum range might not alter the growth efficiency of cultured fish but the production potential. These results are consistent with the trials made by Oliver and Kaiser (1997) on guppies, Luo *et al.* (2013) on *Scortum barcoo*, and Jha and Barat (2016) on *Cyprinus carpio* vr. *Koi.*

	Survival (%)	Final weight (g)	Average daily gain (g)	Specific growth rate (%)
Rearing system				
FRP tanks	60.53^b	1.078^b	0.034^b	0.128^b
Nursery Raceways	62.86^a	1.229^a	0.039^a	0.132^a
SEM	0.123	0.031	0.001	0.001
p-value	0.02	0.003	0.003	0.016
Stocking density (numbers/L)				
10	81.00^a	1.272^b	0.040^a	0.134^a
30	80.55^a	1.323^a	0.042^a	0.134^a
50	58.05^b	1.073^b	0.034^b	0.130^a
70	28.07^c	0.945^c	0.029^b	0.123^b
SEM	0.783	0.044	0.001	0.001
p-value	0.000	0.000	0.000	0.000
Rearing system × Stocking density (numbers/L)				
p-value	0.041	0.043	0.038	0.022

Table 4. Growth performance of *D. dangila*

4. Conclusion

Danio dangila can be bred and reared successfully under captive conditions by obtaining sustainable techniques and standard management practices. The objective of the present study was fulfilled and found WOVA-FH, a gonadotropic signaling molecular analogue, inducement at 0.5 ml/kg for females and 0.3 ml/kg for males with the highest egg production, fertilization, and hatching rate. Furthermore, the raceway-based larval rearing system gave surprising results and recommend to rear the fishes at 30 numbers/L stocking rate. The subject matter in this paper is useful for fish breeders and aquarium keepers for expanding aquariculture, species restoration, and conservation.

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Author contribution

Dr. Mangesh M. Bhosale – designed, conducted, and noted the observations and data analysis of the research study. Dr. R.R. Mugale – Helped in research paper writing.

Conflict of Interest

The authors declare that they do not have any conflict of interest between authors.

Ethical Approval

Not Required.

Data Availability Statement

The data that support the research findings are available within the article.

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Declarations

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Potential competing interests: First report on Captive Breeding of Endemic ornamental fish Mass Production in Advanced Aquaculture (RAS based raceways) systems Standardisation of Larval rearing technique with minimum mortality