Unlocking the secrets of successful spawning: laboratory techniques for breeding ornamental zebrafish *Danio rerio* (Hamilton, 1822)

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Abstract. Zebrafish, scientifically known as *Danio rerio* (Hamilton, 1822) and belonging to the Cyprinidae family, holds a prominent position as a native ornamental model species extensively utilized in fisheries research worldwide. This study aimed to evaluate the effects of sex ratio and temperature on zebrafish spawning and to observe ovarian maturity under controlled laboratory conditions. Over the course of 12 weeks, zebrafish were kept in glass aquaria measuring 25×16×26 cm^3 and subjected to five different sex ratio treatments, T_1 (1m:1f), T_2 (1m:2f), T_3 (1m:3f), T_4 (2m:1f), and T_5 (3m:1f). Daily collection of fertilized eggs revealed that T_3 exhibited the highest mean egg count (880.67±22.03), with shortest egg laying interval of 2.40±0.12 days, significantly differing from other treatments ($p < 0.05$). Moreover, water temperature demonstrated a significant effect on egg production, with the peak (470.0±23.50) observed at 29°C and the lowest (35.0±1.75) at 21°C. Zebrafish maintained at 29°C displayed a precise spawning cycle of 1.2 days. Additionally, histological examination indicated zebrafish reached sexual maturity at the third month post-fertilization under laboratory conditions. These findings advocate for maintaining a sex ratio of one male to three females and a temperature of 29°C to optimize zebrafish spawning success in laboratory environments. Additionally, these findings provide valuable guidance for small-scale fish farmers, entrepreneurs, and hatchery owners, enabling the development of zebrafish spawning techniques with minimal investment. This research paves the way for the establishment of zebrafish hatcheries and the expansion of commercial fry production.

Key Words: breeding strategies, reproductive physiology, sex ratio manipulation, spawning cycles, temperature influence.

Introduction. Bangladesh's fisheries include freshwater aquaculture and coastal shrimp, with interest in expanding into crocodile and pearl culture (AftabUddin et al 2021). Ornamental fish keeping is popular due to its simplicity and low costs, with fish being the primary product (Hossain & Mohsin 2022). Ornamental fish culture began in Bangladesh in the 1980s, spreading beyond elites to diverse demographics (Rahman et al 2009; Khatun & Mondal 2019). Global demand for ornamental fish drives growth, with Bangladesh's position still marginal despite its resources (Hossain & Mohsin 2022). Ornamental fish, including colorful and uniquely shaped aquatic species, are popular for aquarium keeping worldwide, with significant percentages of households in countries like Australia, the UK, and the USA participating (Evers et al 2019; Atalah et al 2022). In Bangladesh, the popularity and trade volume of ornamental fish are steadily increasing (Shamsuzzaman et al 2017; Hossain & Mohsin 2022).

Bangladesh's abundant natural resources support the thriving cultivation of ornamental fish, meeting the increasing demand driven by enthusiasts seeking to adorn their homes and workplaces (Khatun & Mondal 2019; Barua & Ahmad-Al-Nahid 2020).

While native species like the Rani fish (*Botia dario*) are prized, the majority of ornamental fish are exotic varieties, with efforts to breed popular species locally (Alam et al 2016; Hossain & Mohsin 2022). Across cities like Dhaka, Rajshahi, and Khulna, the aquarium fish trade flourishes, with Katabon market in Dhaka emerging as a prominent hub for aquaria and related products (Rahman et al 2009; Hossain & Mohsin 2022). With diverse species documented across the country, Bangladesh's potential in the global ornamental fish trade is underscored by the development of artificial breeding techniques by amateur breeders (Alam et al 2016).

Breeding native ornamental fish offers various benefits, including economic opportunities, employment generation, species conservation, and the development of novel strains (Husen et al 2021; Hossain & Mohsin 2022; Hoseinifar et al 2023). By utilizing local resources, it promotes patriotism and reduces costs associated with exotic fish keeping, making aquarium hobby accessible to a wider population (Arif et al 2018; Clements et al 2019).

Zebrafish (*Danio rerio*), closely related to goldfish, holds paramount significance as a model organism in scientific research, having been the first vertebrate to undergo cloning (Norton & Bally-Cuif 2010; Nielsen et al 2019; Teame et al 2019). Found predominantly in the Ganges and Brahmaputra river basins, zebrafish thrive in shallow ponds commonly associated with rice cultivation (Spence et al 2006; Parichy 2015). Their omnivorous diet, which includes zooplankton and insects, supports their rapid growth, with larvae becoming independent feeders within three days of hatching (McClure et al 2006; Spence et al 2007; Watts et al 2012). Zebrafish typically live around 5½ years, reaching lengths of 4-5 cm, and are renowned for their hardiness and adaptability to aquarium environments (Avdesh et al 2012; Singleman & Holtzman 2014). They breed year-round, with spawning often triggered by temperature changes, particularly at the onset of the monsoon season, making them a popular choice for both scientific studies and aquarium enthusiasts alike (Spence et al 2007).

Efficient propagation and maintenance of zebrafish colonies in research laboratories rely on a comprehensive understanding of their reproductive biology and behavior, both in the wild and in controlled settings (Nasiadka & Clark 2012). Zebrafish are asynchronous batch spawners, with females capable of spawning nearly daily and releasing around 100 transparent eggs per spawning event, often preferring artificial spawning sites provided in laboratory settings (Hoo et al 2016). In contrast to many other fish species, zebrafish do not depend on seasonal changes in day length to initiate breeding. This allows for year-round spawning in laboratory conditions (Spence & Smith 2006). Successful breeding hinges on accurate recognition of male and female zebrafish, typically distinguished by differences in abdominal fullness and coloration, with the placement of marbles in breeding tanks serving as a practical method to prevent egg cannibalism and facilitate egg collection (Avdesh et al 2012).

Understanding the factors influencing zebrafish spawning, such as photoperiod, sex ratio, and temperature, is crucial for maintaining consistent egg production in research settings (Abdollahpour et al 2020; Longkumer et al 2022). While photoperiod primarily triggers spawning, factors like age, size, and overall health of the fish, along with environmental conditions, also play significant roles in egg production and spawning frequency (Al-Emran et al 2024). This knowledge is essential for optimizing breeding protocols and ensuring reliable production of large numbers of zebrafish eggs for scientific research.

Sex ratio, defined as the ratio of ready-to-mate males to females, plays a crucial role in sexual selection and mating dynamics among fish populations, with the local operational sex ratio influenced by multiple factors including adult sex ratio, spatial distribution, and reproductive rates (Aronsen et al 2013). Although zebrafish exhibit a 1:1 sex ratio in their natural habitat, the effect of sex ratio on spawning behavior in laboratory conditions remains unclear, as observed variations in egg production may depend on the desirability of different mates (Rahman et al 2021). Temperature also significantly impacts zebrafish breeding, with an optimum temperature of 28.5°C often cited for breeding, but constant exposure to this temperature may lead to continuous spawning, posing welfare concerns and requiring further research (Avdesh et al 2012).

Zebrafish usually attain sexual maturity by the third month of development. Their reproductive efficiency is influenced by factors such as fish size, gonad development, and strain-specific characteristics, underscoring the importance of meticulous management for successful breeding programs (Hoo et al 2016).

Zebrafish is not only prized for its aesthetic appeal but also serves as a vital research model in biomedical studies, particularly in the investigation of vertebrate development genetics and behavior (Teame et al 2019). Given its dual significance as both an ornamental fish and a research model, understanding zebrafish reproduction in both wild and laboratory settings is crucial for effective husbandry practices, especially considering its growing importance in biomedical research worldwide.

The objective of the research is to comprehensively investigate the breeding biology of native ornamental zebrafish in Bangladesh, addressing the current lack of understanding in this area and elucidating its ecological, commercial, and biomedical significance, thereby informing improved husbandry practices and leveraging its potential as a valuable experimental model.

Material and Method

Experimental site and zebrafish collection. The investigation was conducted from October to December 2017 in the wet laboratory of the Faculty of Fisheries at Bangladesh Agricultural University (BAU) in Mymensingh, Bangladesh (Figure 1). Wild-type adult zebrafish were sourced from the field complex pond of the Faculty of Fisheries at BAU.

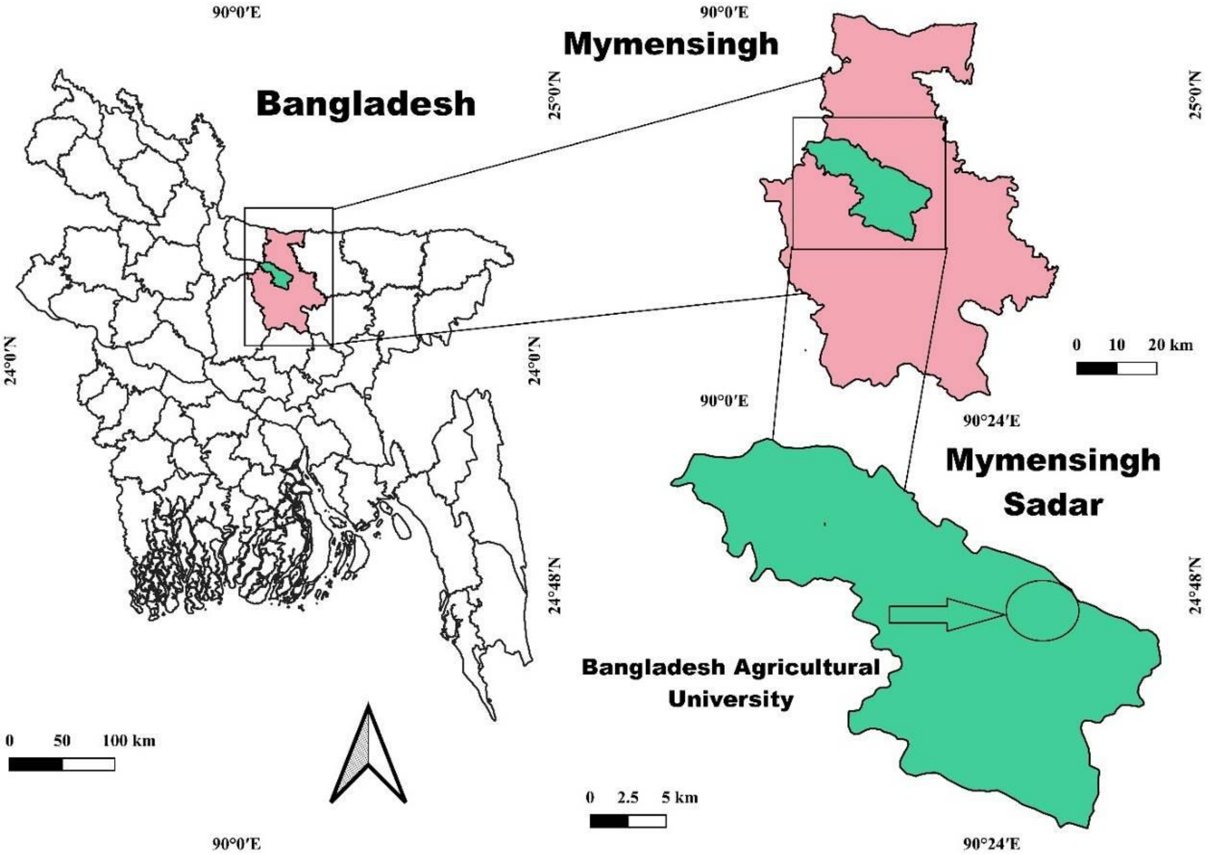

Figure 1. Map of the Bangladesh Agricultural University, where the study was conducted.

Selection of brood fish. Healthy broods were meticulously selected for spawning treatments, with males and females distinguished by specific secondary sexual characteristics. Females were identified by their noticeably swollen abdomens, while mature males were recognized by their flat abdomens, slender bodies, darker coloration, and more pronounced yellow hues in the anal fin compared to females (Figure 2).

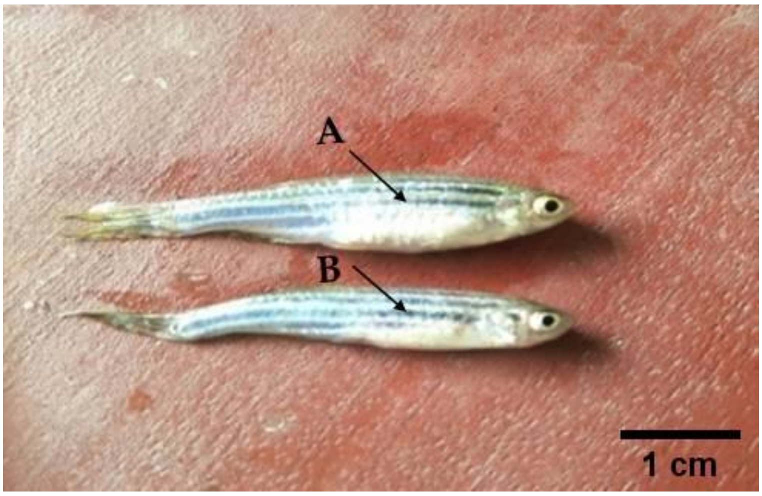

Figure 2. Mature female (A) and male (B) zebrafish.

Rearing of fish for breeding and their feeding. Ten glass aquaria, each with a capacity of 15 liters, were utilized in the experiment, housing a total of 32 male and female zebrafish across varying sex ratios. To facilitate breeding, each aquarium was equipped with two layers of marble substrate in a petri dish, along with small artificial trees. Aeration was ensured using aerators with airstones, while regular water exchange and cleaning were conducted to maintain optimal water quality, as zebrafish thrive in clean environments. Daily maintenance involved removing any uneaten food, dead eggs, fry, and detritus to prevent accumulation and maintain cleanliness within the aquariums. Zebrafish commonly consume their own eggs as a means of replenishing lost protein from egg production, emphasizing their need for a diet rich in protein. To fulfill their dietary needs, the fish were fed twice daily with a commercial floating feed (Mega Fish Feed Ltd., Bangladesh), supplemented regularly with zooplankton enriched with cyclops, *Daphnia*, and cladocerans.

Water quality parameters. The pH range for control treatments was maintained between 6.8 and 8.2, measured using a portable pH meter (HANNA, H198107, Romania). Temperature was monitored and maintained between 21 and 29°C using a normal thermometer. Dissolved oxygen (DO) levels were measured and maintained between 3.9 and 8.5 mg L^{-1} with a Portable DO meter (YSI, DO2OOA, USA).

Experimental design. A total of 16 female and 16 male zebrafish were randomly distributed into five distinct sex ratio treatment groups (T_1, T_2, T_3, T_4, T_5). Each treatment, replicated twice, involved specific combinations of males and females: T_1 (1m:1f), T_2 (1m:2f), T_3 (1m:3f), T_4 (2m:1f), and T_5 (3m:1f). Prior to spawning, zebrafish acclimated to their respective tanks for 7 days, after which 12.7-mm marbles were introduced as spawning substrate. At the end of each trial, sex ratios were confirmed by extracting gametes from the urogenital pore of each fish.

Collection and counting of eggs. Every day at 8 AM, eggs were harvested from the aquarium and carefully placed between the gaps of the marble in a Petri dish. After taking the Petri dish out of the aquarium, the eggs were retrieved from the marbles and placed in a hatching tray for counting. The eggs were then examined under a microscope with a camera (OPTICA B350, Italy) to determine their fertilization status. At the same time, the influence of temperature on zebrafish spawning was assessed during egg collection and counting. The average number of eggs was determined from two separate trials conducted at different water temperatures, which were monitored using a Celsius thermometer.

Histological study of the ovary. Specimens of fish at different developmental stages (1st, 2nd, and 3rd month post-fertilization), measuring 0.5 cm, 1.0 cm, and 1.5 cm respectively, were meticulously preserved in 10% buffered formalin using labeled vials for future analysis. To facilitate further investigation, the trunk region of older fish was excised before preservation, while younger fish were preserved intact. These samples were then transferred from the vials to cassettes and underwent a series of manual procedures, including dehydration, clearing, infiltration (using an incubator EL - 450B), embedding, sectioning (utilizing a microtome machine-KEDEE KD-3358, China), staining, and mounting. Finally, the samples were subjected to microscopic examination using an OLYMPUS microscope (Model: CX21, Japan) equipped with an OPTIKA MB3 Digital camera (3.14 Megapixel) (Siegfried & Steinfeld 2021).

Statistical analysis. The current study hypothesized that both sex ratio and water temperature would impact spawning success in laboratory conditions. Spawning success, represented as mean±SD, was assessed for each sex ratio and temperature. The impact of sex ratio on these parameters was assessed through one-way analysis of variance (ANOVA), subsequently applying Tukey's post-hoc test for further analysis. Statistical significance was determined at $p < 0.05$. The Pearson correlation matrix of dissimilarity in different spawning parameters was used to get the non-metric multidimensional scaling (nMDS) output where the ratio model and Kruskal's stress (1) were used to determine the dimensions. Data analysis was conducted using MS Excel, SPSS version 20.0 and XLSTAT 2019.2.2. software (XLSTAT 2021). The map was constructed using QGIS (QGIS Development Team 2019), and graphical presentations were generated using Origin Pro software.

Results

Influence of sex ratio on zebrafish spawning success. In this investigation, we explored the effects of various sex ratio treatments on zebrafish spawning over a span of 1-12 weeks (Table 1). Spawning success exhibited a notable decline over time, with the highest average number of eggs (181.67±2.52) observed during the initial week in T_3, and the lowest (11.33±1.53) recorded in the 11th week in T_5. Significantly distinct egg counts were evident between the first week and subsequent weeks. While consistent egg collection occurred in T_2, T_3, and T_4 weekly, interruptions were noted in T_1 and T_5. Furthermore, T_1 and T_5 displayed considerable fluctuations in spawning, with a marked decrease in egg production observed in later weeks, ultimately resulting in no eggs being produced during these periods. The average egg count differed across various sex ratio treatments, with the highest mean number of eggs (880.67±22.03) observed in T_3, while the lowest (207±20.66) was recorded in T_1 (Figure 3). Significant variations ($p < 0.05$) were observed among spawning treatment groups regarding the number of eggs spawned, with the mean number of eggs in T_3 significantly differing ($p < 0.05$) from other treatments.

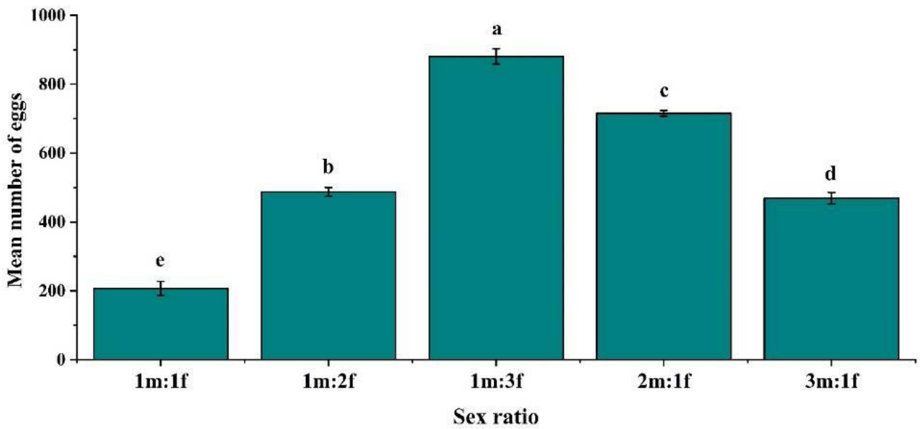

Figure 3. Variation in mean number of zebrafish eggs across different treatment configurations. Significant differences among treatments indicated by different alphabet superscripts ($p < 0.05$) (m = male; f = female).

Table 1

Variations in zebrafish spawning across different sex ratios weekly

T	*Sex ratio*	*Weeks*											
		1st	*2nd*	*3rd*	*4th*	*5th*	*6th*	*7th*	*8th*	*9th*	*10th*	*11th*	*12th*
		Number of eggs											
T_1	1:1	19.33±3.21[a]	26.67±5.69[a]	27.33±3.21[a]	29.0±5.29[a]	32.67±2.08[b]	22.67±2.31[a]	23.33±2.08[b]	26.0±1.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.0±0.0[a]
T_2	1:2	42.33±1.15[b]	60.67±1.53[b]	30.33±3.21[a]	28.33±2.52[a]	29.67±1.53[a]	68.33±2.52[c]	34.67±1.53[c]	64.33±0.58[c]	32.33±3.51[c]	25.67±2.08[e]	38.33±2.08[e]	32.33±0.58[d]
T_3	1:3	181.67±2.52[d]	129.33±0.58[d]	72.33±1.53[d]	85.67±3.06[d]	60.33±2.52[c]	82.33±3.06[d]	41.67±3.06[d]	69.33±2.52[d]	74.33±4.16[e]	18.0±4.36[c]	30.67±3.79[d]	35.0±3.61[e]
T_4	2:1	134.67±3.51[c]	121.67±3.06[d]	56.33±3.51[c]	73.33±1.53[c]	53.33±3.51[c]	51.33±3.21[b]	36.67±2.08[c]	69.33±4.04[d]	46.33±2.52[d]	22.33±3.06[d]	22.67±2.52[c]	27.67±2.52[c]
T_5	3:1	111.33±2.52[c]	83.67±3.79[c]	37.33±2.08[b]	41.33±3.06[b]	57.33±4.51[c]	20.67±1.53[a]	13.33±1.53[a]	44.33±3.51[b]	22.33±3.06[b]	12.67±2.08[b]	11.33±1.53[b]	13.33±3.21[b]

*Different superscripts within the same column indicate significant differences ($p < 0.05$).

Impact of sex ratio on zebrafish egg deposition frequency. Variations in egg deposition frequency were evident across different sex ratios. Spawning frequency, measured as the average daily egg production over a week, varied notably among treatments. The highest egg deposition occurred in T_3 (1m:3f), while the lowest was observed in T_1 (1m:1f) (Table 2). Specifically, T_3 exhibited the highest mean number of eggs per day (9.77±0.80), whereas T_1 had the lowest (1.94±0.38). Moreover, egg deposition in T3 significantly differed ($p < 0.05$) from other treatment groups.

Table 2

Impact of sex ratio treatments on daily egg deposition in zebrafish

Treatment	*Sex ratio*	*Number of eggs per day*
T_1	1m:1f	1.94 ± 0.38^{a}
T_2	1m:2f	5.25 ± 0.59^{b}
T_3	1m:3f	9.77 ± 0.80^{c}
T_4	2m:1f	8.00 ± 0.47^{bc}
T_5	3m:1f	4.89 ± 0.56^{b}

Values are presented as mean±SD. Different alphabetical superscripts signify significant differences among treatments ($p < 0.05$); (m = male; f = female).

The egg deposition frequency of zebrafish exhibited variations depending on sex ratio and days (Figure 4). The highest mean number of eggs (53.33±18.61) was recorded in T_3 (1m:3f) on the 3rd day, while the lowest (2.67±1.53) was observed in T_1 (1m:1f) on the 6th day (Figure 4). Eggs collected from T_3 (1m:3f) exhibited significant differences ($p < 0.05$) compared to other treatments on a daily basis. While eggs were consistently collected from both T_3 and T_4, interruptions in egg collection were noted in T_1, T_2, and T_5. Significant fluctuations in spawning were also observed in T_3 and T_4. Conversely, the mean number of eggs significantly decreased in T_1, T_2, and T_5 on alternate days.

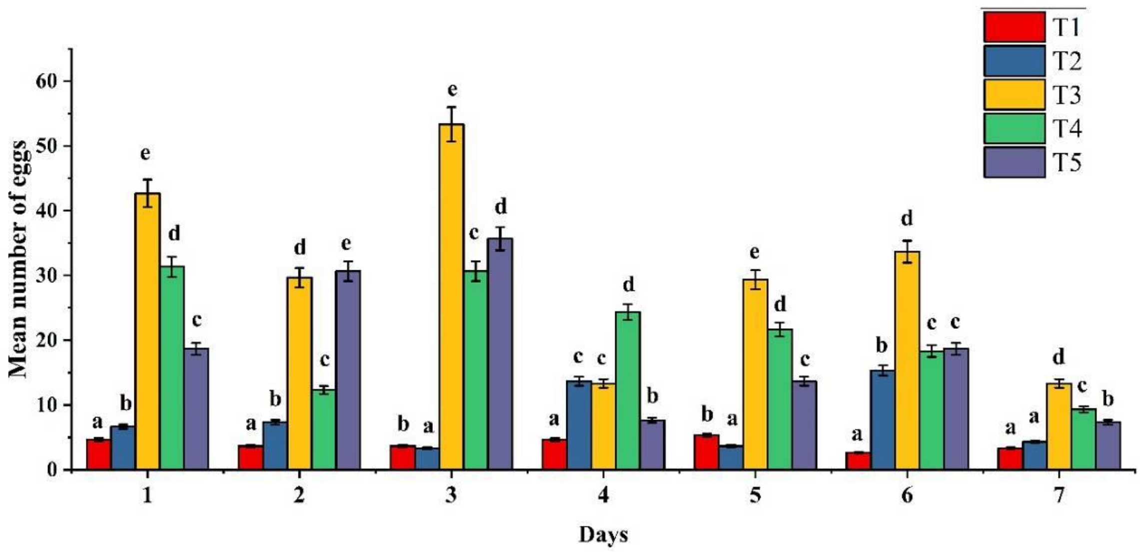

Figure 4. Egg deposition frequency varied across treatments on different days, with distinct alphabetical superscripts indicating significant differences among treatments ($p < 0.05$).

Egg laying interval of zebrafish per week. The egg laying day interval of zebrafish was monitored over a period of 12 weeks. The interval was observed to be highest (5.3±0.27) during the 12th week and lowest (1.9±0.10) during the 1st week (Figure 5). Notably, the egg laying day interval during the 1st week was significantly different ($p < 0.05$) from that of all other weeks.

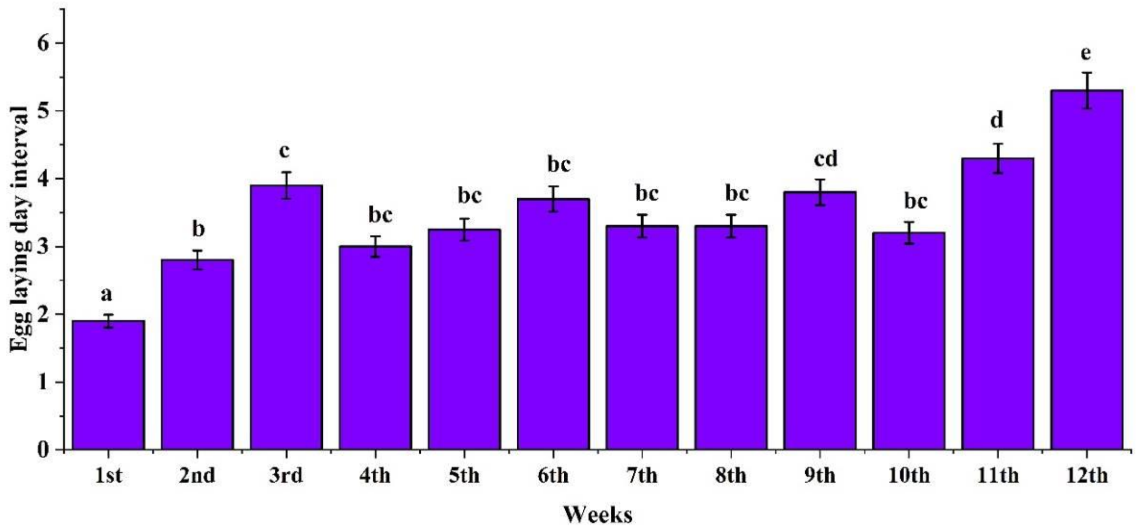

Figure 5. Egg laying day interval frequency of zebrafish per week. Different alphabet superscripts indicated significant differences among weeks ($p < 0.05$).

Effects of sex ratio on egg laying day interval. The interval between egg laying days varied among different sex ratio treatments. The longest duration (4.80±0.24) occurred in T_1 (1m:1f), whereas the shortest interval (1.91±0.12) was observed in T_3 (1m:3f) (Figure 6). It is important to highlight that the interval in T_1 significantly differed ($p < 0.05$) from the other treatments.

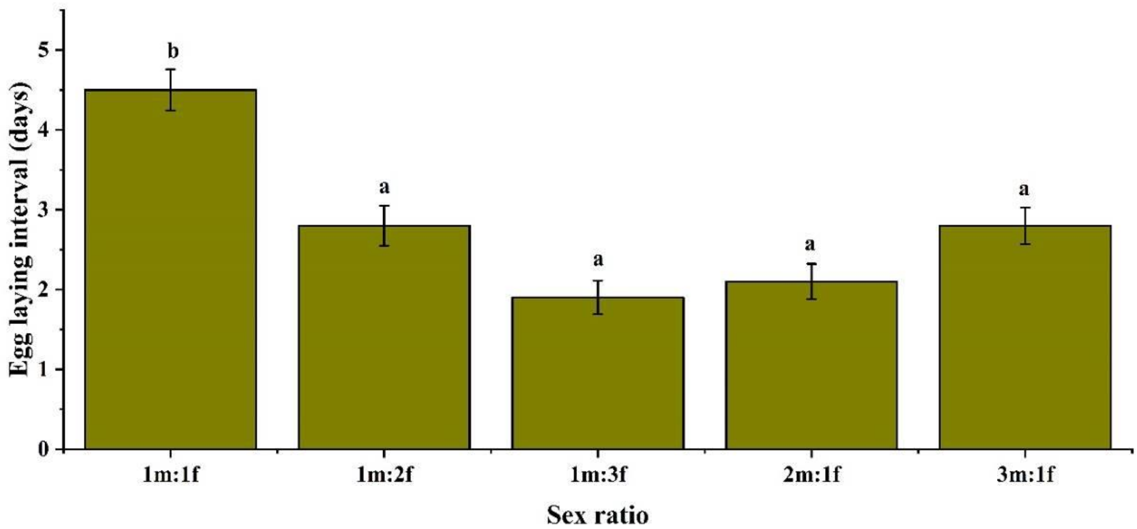

Figure 6. Variations in the frequency of egg lay day intervals were observed across different treatments for zebrafish. Statistical analysis revealed significant differences among treatments ($p < 0.05$), as indicated by different alphabet superscripts (m = male; f = female).

Correlation among spawning parameters of zebrafish breeding. With a Kruskal's stress (1) value of 0.009, the multidimensional scaling (MDS) analysis of different mean values of eggs and egg laying intervals of zebrafish spawning revealed that mean number of eggs (dimension = 0.226, 0.301), mean number of eggs per day (dimension = 0.228, 0.302) and mean number of eggs per seven days (dimension = 0.182, 0.244) were strongly correlated with each other and formed a group. While a weak correlation between egg laying intervals (dimension = -0.636, -0.847) and other mean number of eggs in different frequencies were not correlated and make an outlier position (Figure 7).

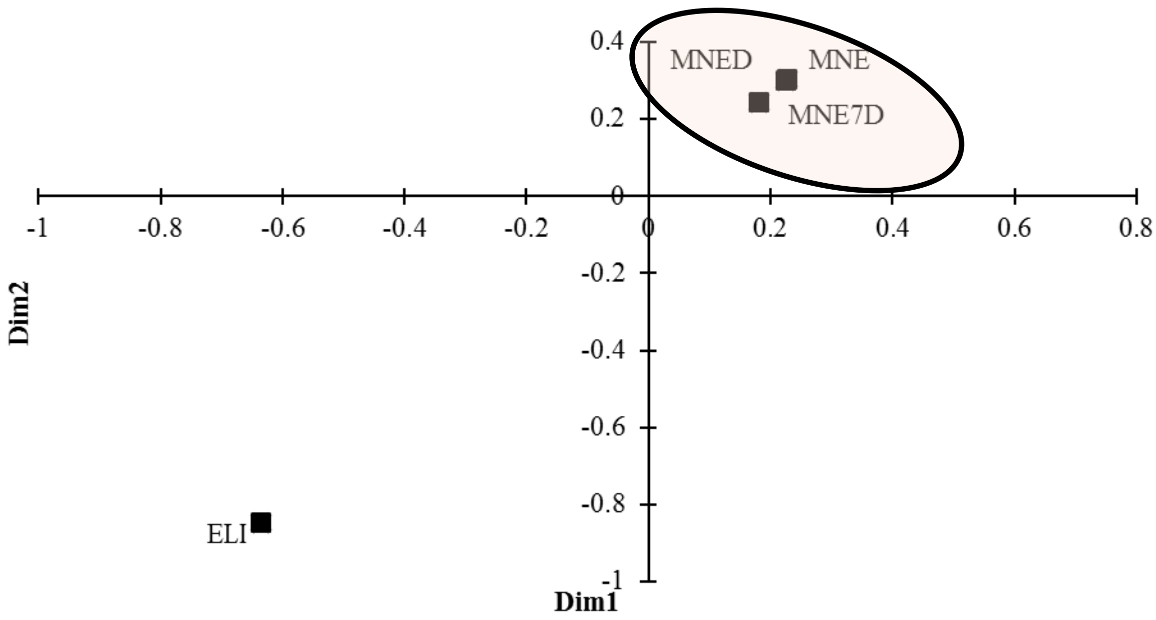

Figure 7. Non-metric multidimensional scaling (nMDS) analysis of spawning parameters of zebrafish breeding (MNE = mean number of eggs; MNED = mean number of eggs per day; MNE7D = mean number of eggs per seven days; ELI = egg laying intervals).

The Shepard diagram indicated that the MDS results of eggs and egg-laying intervals in zebrafish spawning were reliable (Figure 8).

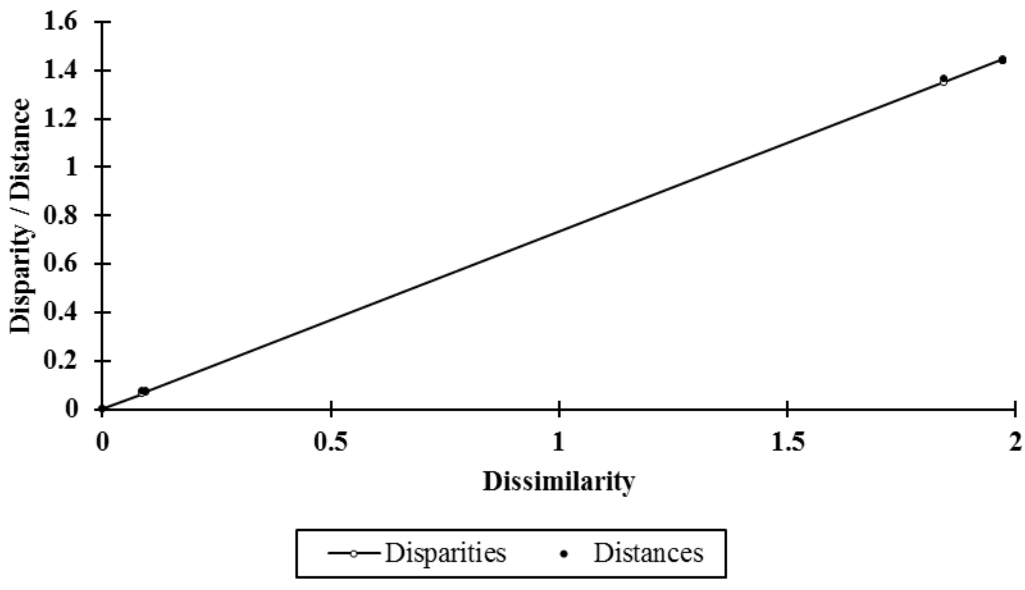

Figure 8. Shepard diagram of non-metric multidimensional scaling (nMDS) based on spawning parameters of experimental zebrafish.

Influence of sex ratio on zebrafish spawning success under varied water temperatures. Spawning observations were carried out across different sex ratio treatments at varying water temperatures. The spawning behavior of ornamental zebrafish exposed to different sex ratios showed variation based on water temperature. Notably, the highest mean number of eggs (178.31±2.57) was observed in T_3 at an average water temperature of 29°C, whereas the lowest (4.09±1.00) was recorded in T_1 at 21°C. It is noteworthy that the mean number of eggs at 29°C significantly exceeded ($p < 0.05$) those at other temperatures (Table 3).

Table 3

Variations in zebrafish spawning responses to different sex ratios across water temperatures

T	Sex ratio	Average water temperature (°C)								
		21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*
T_1	1:1	4.09±1.00[a]	9.0±1.35[a]	14.50±2.34[a]	21.87±2.99[a]	23.25±1.86[a]	19.07±3.36[a]	24.66±3.24[a]	23.45±3.18[a]	35.78±2.87[a]
T_2	1:2	21.23±1.86[c]	23.05±1.35[b]	30.92±2.84[b]	41.57±1.93[b]	26.05±2.31[a]	21.87±1.89[a]	24.02±3.43[a]	41.47±3.50[b]	51.87±2.54[b]
T_3	1:3	25.60±2.93[d]	63.08±3.45[d]	43.03±1.86[c]	83.45±1.72[d]	64.67±1.96[c]	87.58±2.22[d]	68.07±2.19[d]	138.98±3.34[e]	178.31±2.57[e]
T_4	2:1	21.03±1.95[c]	56.09±3.08[d]	29.35±1.32[b]	51.37±2.58[c]	53.21±3.21[b]	75.02±2.43[c]	55.03±2.98[c]	121.28±2.97[d]	132.54±3.45[d]
T_5	3:1	9.45±3.05[b]	33.02±2.36[c]	15.00±1.45[a]	19.21±2.13[a]	54.22±3.11[b]	44.32±2.52[b]	36.59±1.85[b]	81.64±2.85[c]	102.64±3.50[c]

*T represents treatment, where sex ratio is indicated as male:female. Values are expressed as mean±SD. Significance was determined by different alphabet superscripts indicating variations among treatments across different temperatures ($p < 0.05$).

The influence of temperature on zebrafish spawning success was evaluated through the collection and enumeration of eggs from the aquaria. Results indicated a peak in the mean number of eggs at higher temperatures, with a subsequent decline as temperatures decreased. Notably, the highest mean count (470.0±23.50) was observed at 29°C, while the lowest count (35.0±1.75) was registered at 21°C. Additionally, significant disparity ($p < 0.05$) was noted in the mean number of eggs at 29°C compared to other temperature settings (Figure 9).

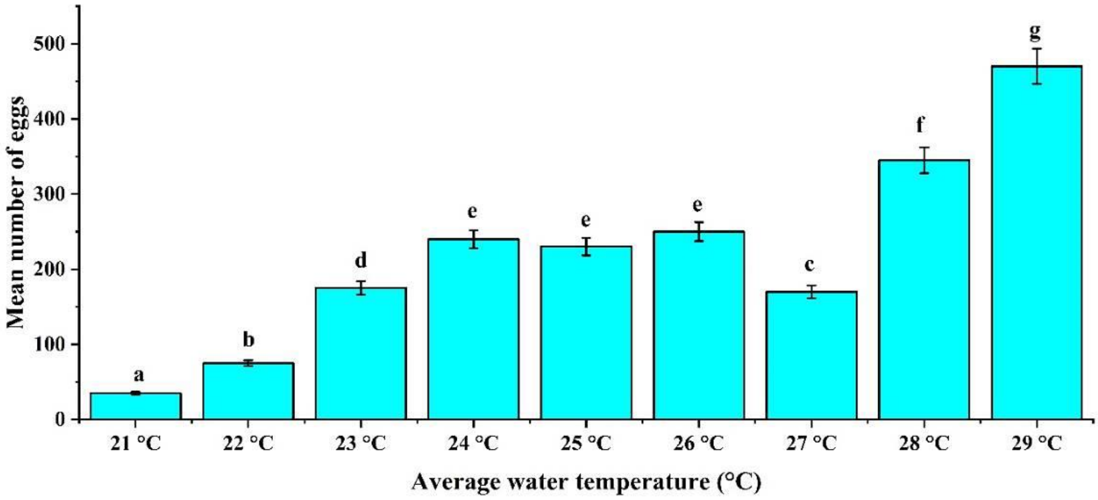

Figure 9. Variation in mean egg spawned across different temperatures. Significant differences among temperatures indicated by distinct alphabet superscripts ($p < 0.05$).

Variation in egg laying day interval across different temperatures. The egg lay day interval of zebrafish was monitored over a span of 12 weeks under varying temperatures. It was observed that the spawning interval of zebrafish exhibited variability across different water temperatures (Figure 10). The average interval between spawnings ranged from 1.2 days to 5.2 days, with the longest interval recorded at 21°C and the shortest at 29°C. Notably, the average spawning interval at 29°C demonstrated a significant difference ($p < 0.05$) compared to other temperature conditions.

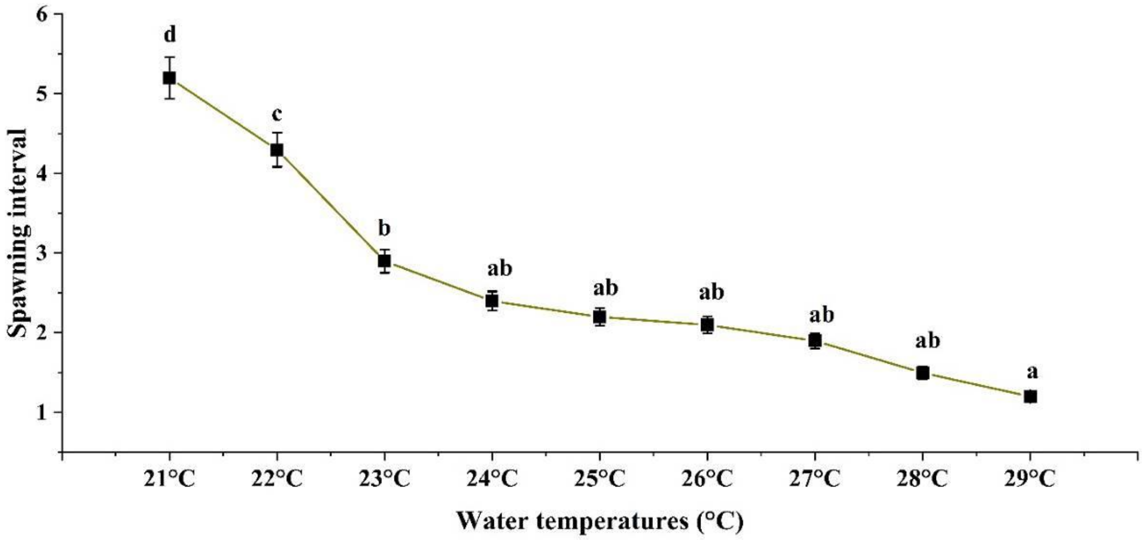

Figure 10. Variation in spawning interval of zebrafish maintained at different water temperatures. Distinct letter superscripts indicate significant variations among temperatures ($p < 0.05$).

Histological investigation of ovarian maturity. The ovarian maturity of zebrafish was examined at 1, 2, and 3 month's post-fertilization (mpf) (Figure 9). Histological analysis revealed distinct stages of ovarian development as follows: at 1 mpf, an immature stage characterized by oogonia was observed (Figure 11A); at 2 mpf, maturing stages such as chromatin nuclear, early and late perinucleolar stages were evident, with oocytes displaying cytoplasm and nucleoli staining intensely with hematoxylin (Figure 11B & C); and by 3 mpf, mature stages were identified by the presence of late yolk vesicle and early yolk granule stages, where oocytes enlarged and exhibited reduced basophilic staining while still retaining hematoxylin staining, with white yolk vesicles surrounding the nucleus (Figure 11D). Additionally, small spherical yolk globules began emerging among yolk vesicles in the peripheral cytoplasm (Figure 11E).

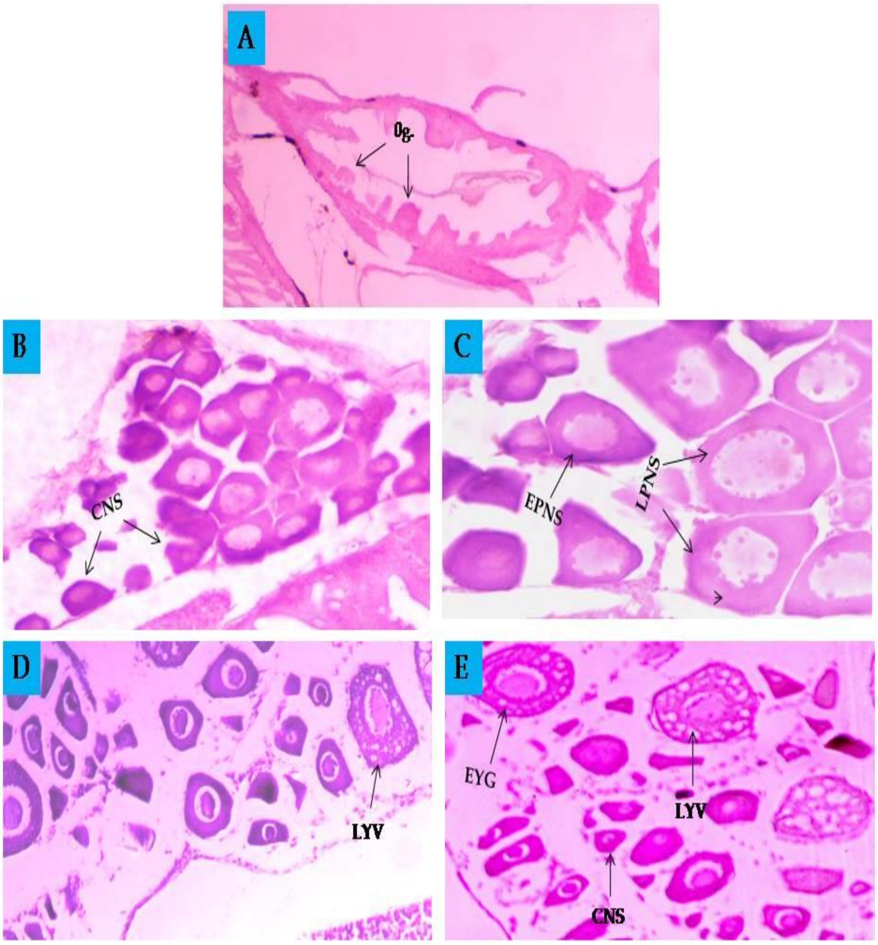

Figure 11. Histological observation of ovarian developmental stages (H and E stained, X40) in zebrafish: (A) Immature stage (1mpf); (B) and (C) Maturing stage (2mpf); (D) and (E) Mature stage (3mpf). Arrows are indicating oogonia (Og), early perinuclear stage (EPNS), late perinuclear stage (LPNS), chromatin nuclear stage (CNS), late yolk vesicle (LYV) and early yolk granule (EYG).

Discussion. The present study investigated the successful spawning of zebrafish across all treatments in sex ratio trials, aiming to identify the optimal sex ratio for maximizing zebrafish spawning in laboratory conditions. The study demonstrated that sex ratio significantly influences zebrafish spawning in laboratory conditions, with the highest success observed at T3 and the lowest at T_1, indicating increased egg production under female-biased ratios compared to equal ratios.

Previous research indicates that a sex ratio bias of 1:2 females to males had no discernible impact on zebrafish reproduction (Ruhl et al 2009). Changes in the sex ratio significantly impacted reproductive success in sand gobies (*Pomatoschistus minutus*), with males showing decreased competitiveness for mates in a female-biased environment compared to a male-biased environment (de Jong et al 2009). Male guppies (*Poecilia reticulata*) exhibited heightened responses to mating tactics, such as sigmoid displays, sneak attempts, and interference from females, when exposed to a sex ratio of 2 females to 4 males, as opposed to a ratio of 1 female to 5 males (Godin 1995). In woundfin (*Plagopterus argentissimus*), varying sex ratios comprising 1 female to 1 male, 1 female to 3 males, and 1 female to 5 males, did not yield a significant impact on fertilization success (Maskill et al 2017). The breeding performance of zebrafish in breeding tanks showed no variance among groups with one of three sex ratios (1 male:1 female; 3 males:1 female; 1 male:3 females). However, instances of aggression, evaluated by the presence of shed scales, were more common in the male-dominated treatment group (Ruhl et al 2009). The observed discrepancies in results emphasize the unique behavioral traits displayed by different fish species, indicating several notable differences compared to previous studies. These variations could arise from differences in the fish population itself (wild versus domesticated), environmental factors (such as tank size, population density, and habitat complexity), or a combination of both. Similar to gobies, alterations in sex ratio also impacted the spawning success of zebrafish.

The current findings revealed fluctuations in the daily egg deposition frequency of zebrafish, with significant variations observed based on sex ratio. Eggs were consistently collected from both female and male dominant treatments daily, while interruptions in spawning were noted in equal sex ratio conditions (1m:1f), resulting in zero spawning. Similar trends were observed in zebrafish egg production concerning pH levels (Stevens et al 2024).

Another noteworthy discovery in this study was the impact of sex ratio on the interval between egg-laying days, which ranged from 1.9 to 5.3 days on average. Notably, at T_3, the shortest interval of 1.9 days between spawning was observed. Previous research has reported inter-spawning intervals ranging from 1 to 6 days, with an average of 1.5 days, resulting in clutches ranging from 1 to over 700 eggs, with an average of 185 eggs (Spence & Smith 2006). Zebrafish possess the ability to breed continuously throughout the year, with females spawning every one to two or three days, and releasing all mature eggs within a single hour (Spence et al 2008). Under suboptimal environmental conditions (such as water quality, diet, social interactions, etc.) or when fish are frequently utilized for breeding purposes, the spawning interval is anticipated to increase.

Similar to previous findings regarding zebrafish, the sex ratio significantly influenced the egg lay day interval per week. Initially, zebrafish exhibited a shorter interval of 2.40±0.12 days between spawns in the earlier weeks, but this interval extended to 4.80±0.24 days after twelve weeks. Previous research noted that zebrafish pairs aged 12 months spawned at intervals of 1.9 days when continuously housed together, with this interval increasing to 2.7 days after three months, indicating the influence of age on the spawning cycle (Abu Rayhan et al 2023; Jenkins et al 2023). The consistent findings indicate that the interval between egg-laying days in zebrafish varies significantly depending on both sex ratio and age.

The nMDS analysis revealed that the mean number of eggs, mean number of eggs per day, and mean number of eggs per seven days were strongly correlated with each other, forming a cohesive group in the MDS plot. This suggests that these variables share similar patterns and variations across different conditions. In contrast, the egg laying intervals showed a weak correlation with the other mean number of eggs, indicating a divergent pattern from the egg production variables. This resulted in the egg laying intervals occupying an outlier position in the MDS plot. The Shepard diagram, which evaluates the goodness-of-fit of the MDS model, supported the reliability of the results obtained from the analysis. Previous studies have explored correlations between egg volume and content (Robertson & Collin 2015), as well as between egg and body size of fishes (Abu Rayhan et al 2023). However, the relationship between spawning parameters such as the mean number of eggs per day and weekly egg laying has not been thoroughly investigated until now. This study is the first to identify a strong correlation among these three parameters.

Under controlled laboratory conditions, zebrafish displayed the highest spawning success in treatment T_3 with an average water temperature of 29°C. Previous studies have indicated that the temperature during the early developmental stages, from spawning to metamorphosis, significantly influences the sex ratio of zebrafish. Male-biased populations are typically observed at lower temperatures (22°C, 87.1% males), whereas female-biased populations are more common at higher temperatures (31°C, 82.4% females) (Dimitriadi et al 2018). These findings suggest that both sex ratio and temperature may influence spawning success.

Furthermore, the experiment revealed a direct correlation between water temperature and zebrafish spawning in laboratory conditions, with temperatures ranging from 21 to 29°C. Considerable differences in spawning were noted across various water temperatures, with the greatest average number of eggs recorded at 29°C and the lowest at 21°C. Remarkably, the average egg count was notably higher at 29°C compared to other temperature settings, whereas it declined with cooler water temperatures. Prior studies have indicated an optimal temperature of approximately 28.5°C for both zebrafish breeding and embryo development (Avdesh et al 2012; Pype et al 2015). Maintaining an optimal temperature range of 27-28.5°C is essential for ensuring ideal breeding conditions for zebrafish. Temperatures below 25°C or above 30°C have been demonstrated to compromise the breeding capacity of the fish, leading to a decrease in the number of embryos produced (Miranda et al 2023). The impact of water temperature on egg production in monosex Nile tilapia (*Oreochromis niloticus*) was found to be statistically significant ($p < 0.01$). Egg production exhibited a decreasing trend with rising water temperature, reaching its peak at 25°C and declining to its lowest point at 33°C (Alam et al 2021). These findings offer evidence of the substantial impact of water temperature on fish spawning.

The study revealed fluctuations in the egg-laying interval of zebrafish across different water temperatures, ranging from 1.2 to 5.2 days. In particular, a reduced interval of 1.2 days was noted at 29°C, whereas it extended to 4.3 days at 22°C. Earlier studies have suggested a consistent ovarian cycle lasting 5 days at 26°C, during which females engage in mating and egg laying every 5 days. However, alterations in temperature could affect this cycle, with a temperature increase to 29°C shortening the cycle to 2 days, and a decrease to 22.5°C inhibiting egg-laying altogether (Nasiadka & Clark 2012; Hoo et al 2016). The consistency in these findings suggests that zebrafish are likely to spawn more frequently when kept at higher temperatures.

In this study, the early yolk granule stage was observed in zebrafish spawned in the laboratory at the third month post-fertilization. This corresponds with earlier research indicating that zebrafish raised in laboratory conditions generally achieve sexual maturity by the third month of development, with initial spawns observed in fish as young as 2.5 months old (Spence et al 2007; Singleman & Holtzman 2014).

Fish exposed to conditions promoting slower growth rates may require more than three months to attain sexual maturity, as suggested by research findings. Studies have highlighted that larger females exhibit a higher frequency of spawning and produce larger clutch sizes relative to their smaller counterparts (Spence & Smith 2006; Uusi-Heikkilä et al 2010).

The study reveals that zebrafish spawning in lab settings is influenced by sex ratio, offering a potential method to enhance wild type zebrafish production by adjusting ratios. Optimal spawning occurred at T_3, with sex ratio also impacting daily egg deposition frequency and interval. Temperature was found to affect zebrafish spawning, with highest egg numbers at 29°C. Overall, sex ratio and temperature were found to significantly influence zebrafish spawning, egg deposition frequency, and egg lay day interval in laboratory conditions.

Conclusions. The ornamental fish industry presents lucrative opportunities globally and in Bangladesh, but lacks comprehensive knowledge on native species and breeding technologies. Establishing ornamental fish hatcheries and focusing on native species like the zebrafish could yield significant economic benefits. Our study demonstrated that sex ratio significantly influences zebrafish spawning success, with the 1m:3f ratio (T_3)

proving most effective. Furthermore, temperature strongly impacts spawning success, with 29°C being the optimal temperature observed. These findings underscore the importance of carefully managing sex ratios and temperature to optimize zebrafish breeding protocols in laboratory settings, providing valuable insights for the ornamental fish industry.

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Conflict of interest. The authors declare that there is no conflict of interest.

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