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Breeding and Early Development of an Endemic, Endangered Freshwater Fish *Aplocheilus dayi* in Captivity

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ABSTRACT

Purpose: *Aplocheilus dayi (Ceylon Killifish) is a freshwater fish endemic to Sri Lanka, but its conservation has not received much attention as uncontrolled fishing for ornamental purposes and many other human activities have posed significant threats to its survival. Therefore, this study aimed to provide baseline information on the development of a captive breeding system and to study the embryonic and larval development of A. dayi.*

Research Method: *Matured A. dayi fish were acclimatised for two weeks prior to the experiments. A suitable breeding substrate to facilitate successful spawning was identified using two substrates; spawning mop, and sand while no substrate was used as the control. The subsequent breeding experiment (to find the best sex ratio) was performed using different sex combinations (Male: female 1:1, 1:2, 1:3, and 2:4). Subsequently, eggs were collected and analysed for fertility, hatchability, and embryonic development studies.*

Findings: *Spawning mop seemed to be the most preferred spawning substrate for A. dayi. Egg parameters, including fertilisation rate and hatchability, were not significant upon the sex ratios; however, the ratio between male: and female (1:2) had the highest significance (p<0.05) in egg production (4 eggs/day). Moreover, we found that A. dayi eggs showed a lengthy incubation period (11 - 13 days at 25±1 °C), indicating their dormancy nature to bypass the hostile conditions before being hatched.*

Research Limitations: *The breeding pattern under captivity needs to be examined over an extended period in different experimental settings, such as using other culture techniques with varying density ratios.*

Originality/Value: *A. dayi can be successfully bred in captivity, and further this study gives the readers factual information on the embryonic development of A. dayi.*

Keywords: *A. dayi, captive breeding, conservation, endemic, ornamental fish*

INTRODUCTION

Sri Lanka is blessed with diverse ecosystems with high species richness driven by various climatic and topographical variances (Gunatilleke *et al.*, 2017). Among the miscellaneous group of vertebrates inhabiting Sri Lanka, freshwater fishes are a vital group representing the highest rate of endemism (Bambaradeniya, 2006). Up to date, 139 freshwater fish species have been identified, out of which 61 are endemic to Sri Lanka (Goonatilake *et al.*, 2020). Many endemic fish species have been found in the wet zone forested areas, including marsh dwellings and riverine inhabitants, which show unique niche characteristics (Pethiyagoda, 1994; Amarasinghe *et al.*, 2006).

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The diversity of these valuable species has been impacted by various economic and developmental activities that pose a significant threat to their population decline and subsequent existence. (Amarasinghe *et al.*, 2006; Pethiyagoda, 2006). Up to date, 45 endemic fish species have been identified as threatened, including 12 critically endangered, 24 endangered, and 09 in the vulnerable stage (Goonatilake *et al.*, 2020).

Aplocheilus dayi is an endangered, endemic fish species (de Alwis Goonatilake, 2019) confined to limited locations in the western lowland wet zone of Sri Lanka. This species was previously recorded only in the Kelani River basin (Pethiyagoda, 1991); however, recent studies revealed that *A. dayi* also inhabits some other river basins, i.e., Attanagalu, Kelani, Kalu, Benthara, and Gin (IUCN, 2012; Athapaththu *et al.*, 2018). Despite the natural habitat that occupies shallow, riparian vegetation areas with a silt substrate, interestingly, it has also been recorded in mangrove swamp areas mutually inhabiting with indigenous killifish species, *Aplocheilus parvus*. *A. dayi* is a larvicidal carnivore species that primarily feed on other fish larval stages, tiny insects, and insect larvae (FishBase, 2021). Due to its carnivorous and predatory nature in the feeding habit, it was also identified as an effective mosquito controller (Wickramasinghe and Costa, 1986). Due to limited studies on its population growth and the dynamics even in the recorded locations, the available information on this species is scanty (de Alwis Goonatilake, 2019). It is assumed that the population has been declining due to various anthropogenic activities highlighted in the previous section (de Alwis Goonatilake, 2019).

Therefore, a comprehensive study on its geographical distribution and population dynamics aiming for in-situ and/or ex-situ conservation is timely and important (Pethiyagoda, 2006) to prevent this valuable species from being further declined. In this regard, developing a captive breeding technique is essential for stock enhancement through careful selection (Mijkherjee *et al.*, 2002; Pethiyagoda, 2006; Majoris *et al.*, 2018). Being a highly valued fish species in the ornamental fish market and no special restrictions have been imposed for the controlled capture of wild stocks could also lead to natural stock depletion (FishBase, 2021). According to Fernando *et al.* (2013), the total quantity of *A. dayi* exported by Sri Lanka over the past few decades was significantly reduced, suggesting the importance of captive breeding for stock enhancement. Despite the particular interest in *A. dayi*, there is no available scientific literature on reproductive biology, including breeding in captivity, embryology, and early larval development. Thus, this study aims to generate baseline information on captive breeding and embryonic development of *A. dayi*. Moreover, that could help future studies develop a comprehensive breeding strategy to conserve this valuable species and other allied species.

MATERIALS AND METHODS

A. dayi Sample Collection

Forty-five (45) specimens (in both sexes) of healthy *A. dayi* were collected from a man-made well connected to a freshwater stream located in the Namunutenna area, Ratnapura, $(60°42'14''N, 80°16'08''E)$ in Sri Lanka. The specimens were collected, adhering to environmental regulations assuring minimal stress to the existing community and natural habitat. Sexing was done using external morphology, including the body shape, the shape of the anal fin, and the body colouration. As indicated in Fig.01.A, male has a slender/slimmer body, has a pointed anal fin, and was more colourful than the female. In contrast, the female (Fig.01.B). has a bulgy abdomen area with a circular anal fin that is also prominent. In the pilot study, environmental parameters, i.e., light intensity, temperature, and water quality parameters; temperature, pH, and water depth at the collection site were examined and measured for over 02 weeks to provide favorable conditions under captivity.

Captive Breeding of A. dayi

Acclimatisation: The collected fish specimens were carefully transferred to the faculty aquaculture unit (Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka) and kept for two (02) weeks in cement tanks (Length=3', Width=2', and Height=8") for acclimatisation. Before the acclimatisation, males and females were separated based on their external morphology. During the acclimatisation and experimental periods, 12 hours-light and 12 hours-dark periods were maintained. Dechlorinated, conditioned water (temperature = 25±1°C, pH = 7±0.2, dissolved oxygen = 7±1 mg L^{-1}) was used. In addition, adequate aeration was also provided with systematic water changes to minimise the stress. Since fish refused commercial feed, they were *ad libitum* fed with mosquito larvae twice a day.

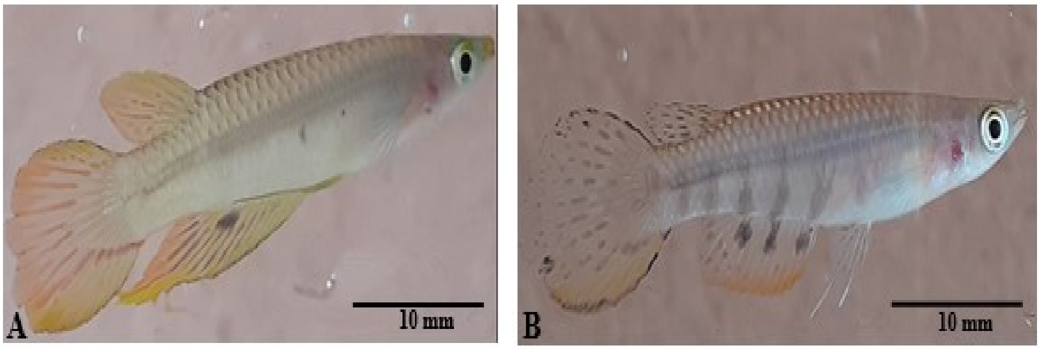

Figure 1: Sexual dimorphism of *A. dayi*; A. Male; B. Female

Substrate Effect on the Breeding Performance of A. dayi

Two weeks after accilimatization, fish (avg. size male = 5±0.1 cm, female = 4±0.1 cm) were transferred to the glass tanks (n = 09) (Length = 45 cm, Width = 25 cm and Height = 20 cm) for the breeding experiment for 30 days period. Spawning mops made of acrylic yarn and sand were used to find suitable spawning substrates under captivity. For the control, no substrate was used. The sex ratio for this experiment was kept as 1 male: 1 female. Water quality conditions were maintained similarly to the acclimatisation period, and *ad libitum* feeding was continued with mosquito larvae. The waste and the remaining feeds were removed through siphoning (twice a week) while maintaining (10 – 15%) water exchange to ensure that the fish were under minimal stress. Spawning success was evaluated in terms of egg production. Since they are serial spawners, the eggs were manually collected once every two days. The experiment was conducted in triplicates using 18 fish (9 males and 9 females).

Sex Ratio on the Breeding Performance of A. dayi

The sex ratio for breeding in fish is defined as the ratio of males and females readily available for mating purposes (Spence and Smith 2005; de Jong *et al.*, 2009). Due to insufficient information on *A. dayi*, the sex ratios used in this experiment were based on similar studies conducted for related species (Oltra and Todoli 2000; Sandkam and Fuller, 2011; Varela-Lasheras and Van Dooren, 2014; Masoudi *et al.*, 2018). Further, it was assumed that the fish used in this study attained spawning once they were provided with breeding conditions similar to their natural habitat. Based on the result of the initial experiment, the spawning mop was selected as the most appropriate substrate for this and subsequent experiments. A total of forty-five (45) individuals (Males = 15 and Females = 30) were used in different combinations/treatment groups; Male: female 1:1, 1:2, 1:3 and community (2:4) in this experiment. Fish were assigned to twelve (12) glass tanks (Length = 45 cm, Width = 25 cm, and Height = 20 cm), with each treatment in triplicates. Every tank was provided a spawning mop as the substrate and for egg collection purposes. Finally, the overall breeding performance was evaluated for egg production, fertilisation rate, and egg hatchability for 45 days. Spawned eggs were manually collected into 1 L jars and treated with 2 ppm methylene blue to prevent fungal infections while providing optimum hatching conditions. Water quality parameters, i.e., temperature, dissolved oxygen, and pH value, were maintained as 25±1°C, 7 - 9 mg/L, and 7.0, respectively. Infertile eggs were removed from the incubation jars and counted throughout the hatching process. Egg fertilisation rate and hatchability were calculated using the following equations.

$$\text{Hatchability (\%)} = \frac{\text{Total number of hatchlings}}{\text{Total number of eggs}} \times 100 \tag{2}$$

Embryonic and Larval Development of A. dayi

The fertilised eggs were collected and examined for embryonic development using the optical microscope (Carl Zeiss Primo trinocular microscope coupled with U3CMOS camera). The images were taken in 24 h intervals until the hatching was completed. Image J software (Version 1.8.0) was used to obtain egg diameter measurements.

Statistical Analysis

Statistical analysis was carried out with IBM SPSS Statistics 21 software and One Way-ANOVA, with Tukey's HSD Test used for comparison.

$$\text{Fertilization rate (\%)} = \frac{\text{Total number of eggs} - \text{Number of infertile eggs}}{\text{Total number of eggs}} \times 100 \quad (1)$$

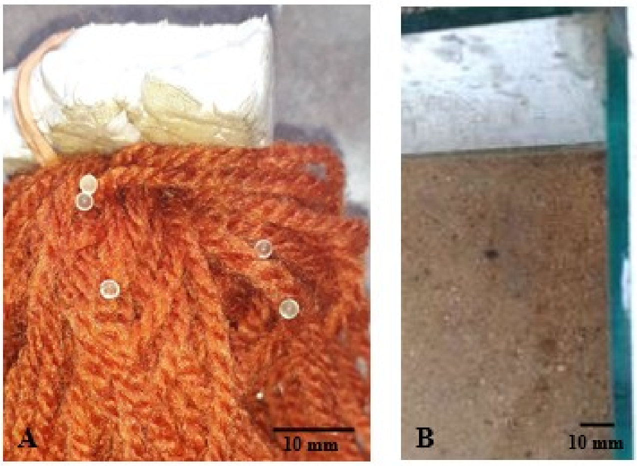

Figure 2: A. Scattered eggs observed on the spawning mops made out of yarns; B. sand

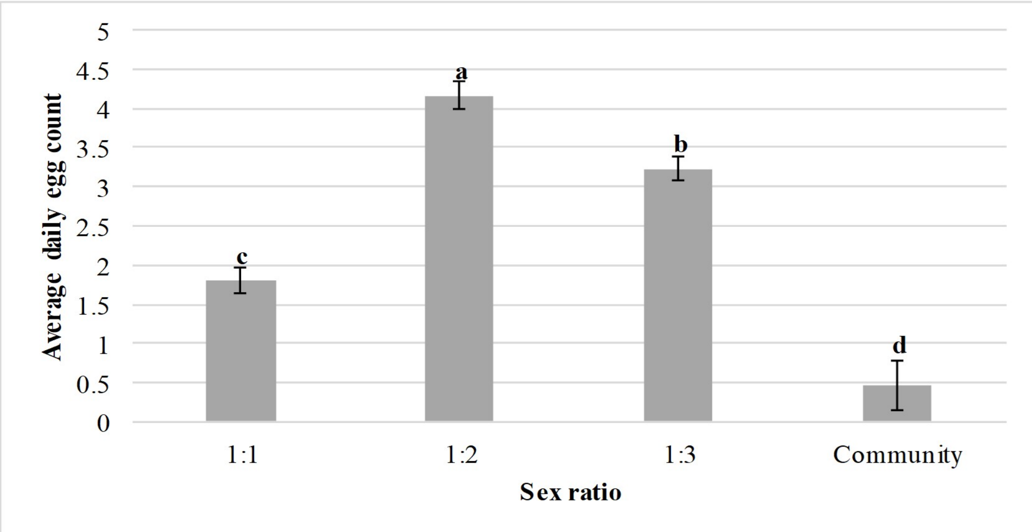

Figure 3: Mean daily egg counts (±SE) correspond to different sex ratios. Mean values (n=3) with different letters shows the results are significantly different ($p<0.05$)

RESULTS AND DISCUSSION

Habitat Assessment of A. dayi

The habitat assessment is vital for such species if we plan to breed them in captivity. The fish must be provided with optimum conditions to avoid being stressed during acclimatisation unless they may not be successful in breeding under artificial settings. During our in situ observation, we could detect slight temperature fluctuations during the daytime (24-27°C) and nighttime (23 – 25°C). The collection site usually receives 10 - 12 h of light, and the intensity fluctuates between 250-550 lux. According to our observations, *A. dayi* is sensitive; however, a social species inhabits the same niche as other related species. They usually preferred shallow waters (depth varying between 8-20 cm) with lots of live plants (ex. *Cryptocoryne wendtii*) and other detritus. We also found some *A. dayi* eggs, which were already deposited on the *C. wendtii* plant leaves and the dead plant materials. Being a larvivorous species (Wickramasinghe and Costa, 1986; Kusumawathie *et al.*, 2006), *A. dayi* prefers to consume larval stages of insects such as dipterans and other insect species. During the acclimatising period, it was evident that *A. dayi* refused to feed on commercial diets; however, the acceptance was very much apparent when provided with mosquito larvae.

Different substrates on breeding performance of A. dayi

This study was the first attempt to breed *A. dayi* in captivity. During this experiment, different substrates were tested to find the most effective one. An effective substrate creates a more natural habitat for the fish thus, giving a better performance in breeding under captive conditions (Srikrishnan *et al.*, 2017). The first successful spawning was observed 02 weeks after introduction into breeding tanks.

Males tend to carry the females towards spawning substrate (shows courtship behaviour) and force female fish to release the eggs. The results suggested that a spawning mop made out of yarns seemed the most effective substrate (average daily egg production = 1.44) compared to the other substrates, i.e., sand and zero substrates (Fig.02). However, delay in spawning in all the substrates suggests that fish require an adaptation period for given experimental conditions even after 02 weeks of acclimatisation.

Killifish species prefer to spawn on slit types of surfaces such as mops, but not in open waters (Newman, 1907). For example, *A. lineatus*, a killifish (family Aplocheilidae) preferred vegetative substrates such as aquatic plants with fine leaves as the breeding substrate (Yeo and Lim, 2010). As per the results of a pilot study, *A. dayi* favours plants like *Cryptocoryne wendtii* with fine leaves; however, they seemed less effective in captivity. This could be the reason for the highest significant ($p<0.05$) egg production in spawning mops (1.44± 0.26 eggs/day) compared to the control (0± 0 eggs per day) and sand (0.22± 0.117 eggs/day). Moreover, spawning mops and sand were effectively used as substrates in captivity for other killifish species such as *Aphyosemion gardneri*, and *Nothobranchius furzeri* (Kroll, 1984; Polačik *et al.*, 2016).

Different sex ratios on breeding performance of A. dayi

As expressed in Fig.03, the highest average daily egg production ($p<0.05$) was observed in the treatment in which the sex ratio of 1 male: 2 females was used. However, surprisingly the lowest production was recorded in community breeding tanks even if the highest number of females (04) were used in each community breeding tank.

In nature, *A. dayi* generally exhibits both solitary and schooling behaviours. According to our observations, even in a larger school of 15 - 25 fish, only 2 - 5 males were present. We observed some mortality during breeding (two males in a community tank and one male in a non-community tank) associated with the cannibalistic nature of *A. dayi*, especially when they were in a confined area under selective breeding stress. This cannibalistic behaviour has also been recorded in *A. lineatus*, another killifish species under captive breeding conditions (Yeo and Lim, 2010). In addition, the ideal sex ratio for *A. iberus*, a killifish species, was also identified as 01 male: 02 females (Oltra and Todoli, 2000). This could be allied with the territorial behaviour of the male fish, suggesting that if there were no competition for being dominant during the mating process, no cannibalistic behaviour would have been observed. When the number of females increases, it increases egg production. However, when another male is introduced, for instance, in community breeding (sex ratio = 02 males: 04 females), rather than cooperating with the breeding process, they tend to fight each other, as we have observed fish dying in some replicates. In addition, it could be an energy waste that can be used for breeding. This result and observations correspond with the breeding study of *Aphanius iberus* (Oltra and Todoli, 2000). As per the observations, *A. dayi* females also tend to devour their eggs after spawning might be another issue for less egg production when used for community breeding (sex ratio = 01 male: 03 females). This behaviour was also observed in the previous studies conducted in *A. iberus* (Polačik *et al.*, 2016; Sandkam and Fuller, 2011; Oltra and Todoli, 2000).

Different sex ratios on fertilisation rate and the egg hatchability

Table 01. showed the effect of varying sex combinations on fertilisation success and egg hatchability. According to the results, no significant difference ($p>0.05$) was observed among the treatments, indicating that even a single male fish could be enough to fertilise the whole eggs produced by the number of females.

The egg hatchability did not show a significant difference against the sex ratio, as indicated in Table 01. This is obvious that the hatching rate corresponds to the fertilisation rate. Over 80% of fertilised eggs were hatched, which is a good sign of effectiveness in captive breeding for stock enhancement. Newman (1907) described a high fertilisation rate as evident among killifish species due to their aggressive courtship behaviour. Besides, it was revealed that males release the milt directly on the eggs, simultaneously during the release of eggs by the female (Newman, 1907).

Table 1: Fertilisation and hatchability rates of *A. dayi* eggs correspond to different sex ratios

Sex ratio	Egg fertilisation rate (%)	Egg hatchability (%)
1 male: 1 female	80 ± 5.77	88.43 ± 6.42
1 male: 2 females	76.67 ± 3.33	86.9 ± 0.6
1 male: 3 females	86.67 ± 8.81	91.53 ± 4.33
2 males: 4 females (community)	87.76 ± 6.19	80 ± 11.54

Mean values (± SE) with different letters are significantly different (p<0.05)

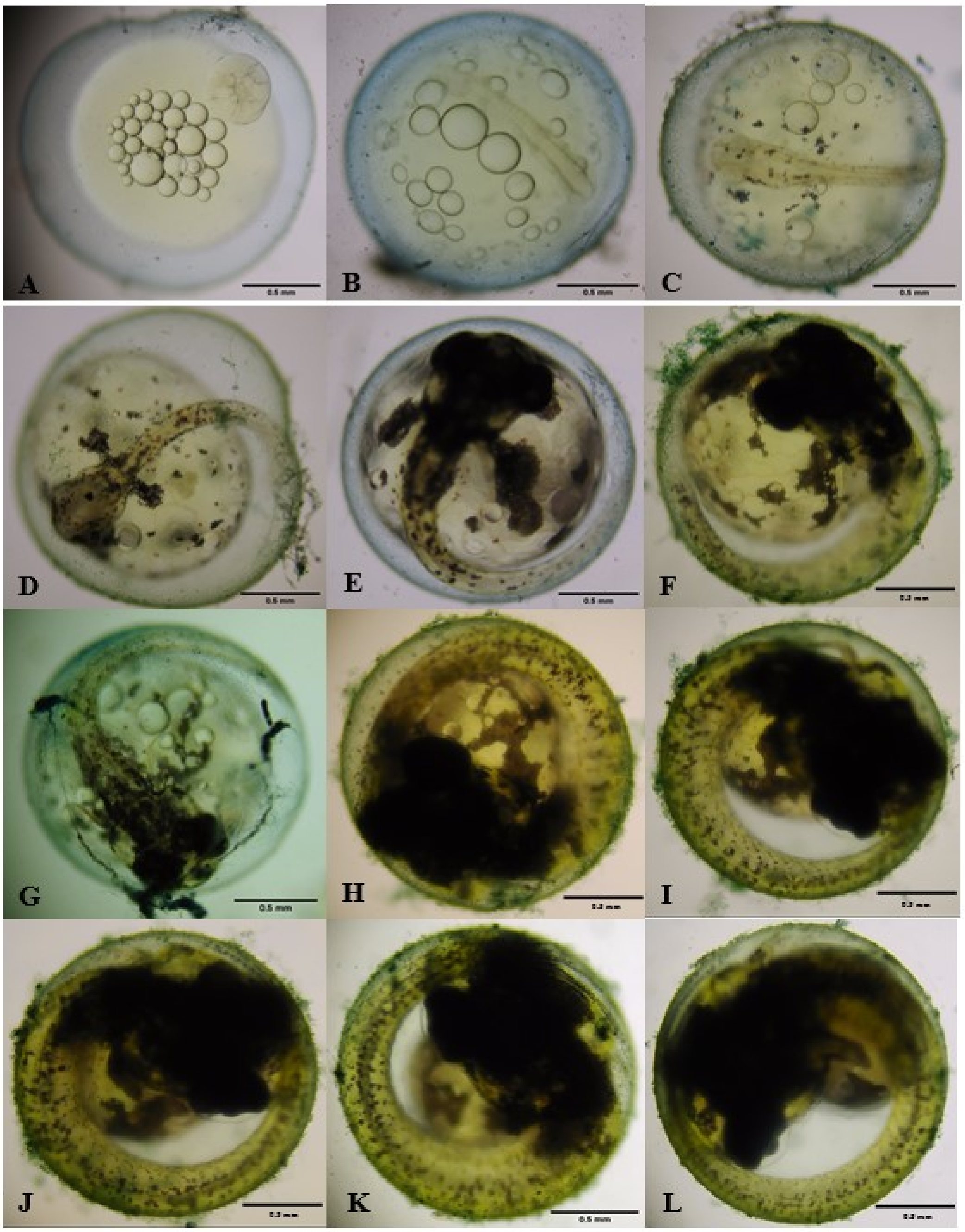

Figure 4: Embryonic development of *A. dayi*: A. Newly fertilised egg; B. 24 h old egg; C. 48 h old egg; D. 03 days old egg; E. 04 days old egg; F. 05 days old egg; G. 06 days old egg; H. 07 days old egg; I. 08 days old egg; J. 09 days old egg; K. 10 days old egg; L. 11 days old egg at 25°C

The mean fertilisation rate observed in our breeding experiments was 82.8%, a considerably higher value. A study by Polačik *et al.* (2016) reported similar results with an 85.2% fertilisation rate with *N. furzeri*. This can be concluded as the sex ratios haven't had any significant impact on fertilisation rate and the hatchability of eggs. Since there were no deviations in fertilisation rate and egg hatchability, based on the spawning success, it can be concluded that 1 Male: 2 Female sex ratio was ideal for breeding *A. dayi* in

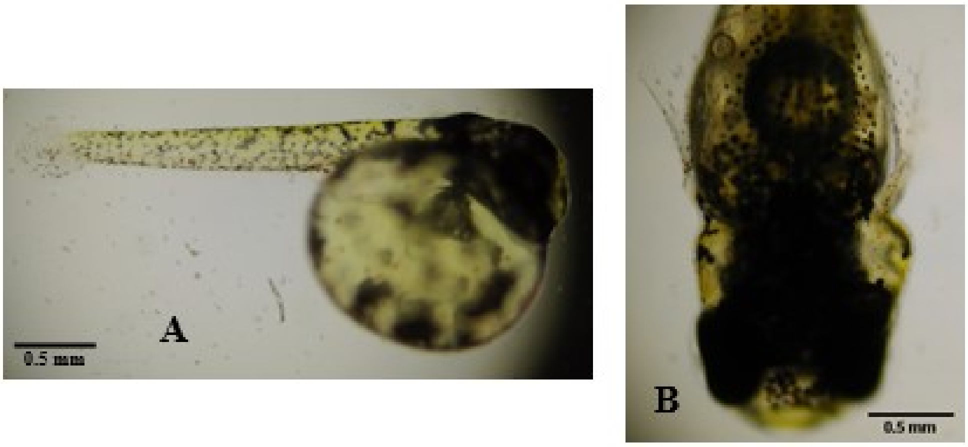

Figure 5: Early larval stages of A. dayi: A. Newly hatched *A. dayi* larvae; B. Two days old *A. dayi* larvae

captivity.

Embryonic and Larval Development of A. dayi

The eggs of killifish species are found to be varied in size range from 0.2 mm – 2 mm, and the diameter could be varied even among the same species (Job, 1940; Kroll, 1984; Kamal *et al.*, 2009; Volcan *et al.*, 2011; Motamedi *et al.*, 2019). This experiment found that the mean egg diameter in *A. dayi* was 1.5 ± 0.2 mm during early spawning and subsequently increased (1.8 ± 0.2 mm) at the late spawning period. Moreover, water temperature plays an important role in the hatchability of killifish eggs (Brown *et al.*, 2011; Furness *et al.*, 2015); thus, hatching may occur as early as the 8^{th} day or as late as the 100^{th} day from spawning could be attributed with water temperature (Kroll, 1984). In our study, hatching was observed 11 - 13 days of post fertilisation at 25 ± 1°C. Moreover, the killifish eggs have been reported to have the ability to withstand harsh conditions (Furness *et al.*, 2015) since they live in seasonal streams, and their eggs have to remain in the diapause stage, suspending growth and development during hostile environmental conditions. This diapause period can be seen among both annual and non-annual killifish species. The presence of the diapause period leads to hatching delays in killifish eggs (Podrabsky and Hand, 1999; Varela-Lasheras and Dooren, 2014). In this study also considerably extended hatching time was experienced and Fig.04, demonstrates the embryonic development stages of *A. dayi* till the hatching.

Newly fertilised egg: *A. dayi* egg is nearly spherical. The yolk is visible in a post-fertilised egg, pale in colour and translucent. As indicated in the Fig.04. A, the blastodisc is visible and positioned near the clustered oil droplets. There are 26 - 28 small oil droplets that formed a cluster and could be seen in the centre of the egg.

24 hours after fertilisation: Oil droplets dispersed throughout the yolk, and the size of an oil droplet increased; whereas the droplet counts have reduced to 20 - 22. A blastoderm with unclear margins appeared on the yolk, covering one-third of the egg. The optic vesicles emerged on both sides of the frontal area of the blastoderm. Moreover, Kupfer's vesicles appeared near the blastoderm's caudal region with unclear margins. Two elongated Otocysts were detected on both sides of the blastoderm. A neutral tube developed inside the blastoderm which runs along the blastoderm, from the frontal to the distal area.

48 hours after fertilisation: The yolk size has been reduced, and the number of oil droplets count has also reduced to 9 - 11. Optic vesicles develop into eyes which appear as large spheres in the head region. Small, irregular black-coloured spots appear on the yolk, primarily around the head area, covering nearly half the egg's circumference. Pigmentation becomes visible as pale-yellow markings and red spots are dispersed on the blastoderm.

03 days after fertilisation: The head area of the blastoderm was prominent due to its high volume. Pigmentation was more visible now, with dark red and black spots. Small, irregular black spots developed into black star blotches. Primordial structures of the pectoral fins became visible as tiny buds arose from the regions where the Otocysts appeared. Surprisingly, black blotches gathered around the aforementioned pectoral buds and the head area. Circulation was visible with the heart's rhythmic contractions at a rate of 120 cycles per minute. The yolk volume shrunk while the embryo occupied more than two-thirds of the egg's circumference.

04 days after fertilisation: As in Fig.04. G, the pectoral buds were densely surrounded by black blotches and gathered around the eye area. The black pigmentation in the head area and the yellow pigmentation in the body became denser. Only 3 - 4 oil droplets appeared on the yolk at the time. The vascular system branched, and circulation was visible in pale pink colour. The eyes protruded from the head area, becoming more visible. The gas bladder could be observed as an elongated circle in the middle. The heart beats at a rate of 160 beats per minute.

05 days after fertilisation: The pectoral fins grow in size, with no flapping. The mouth was barely visible, and pale green colour pigmentation was visible in the head area. The yolk was about two-thirds the size of the egg. Slight and random embryonic vibrations could be seen.

06 days after fertilisation: The pectorals fully develop and move at an 84-flaps-per-minute rate. The mouth was completely visible with the 02 jaws. The yolk shrunk even more, and the vitelline vessels were clearly visible in a faint red colour. There was no movement in the eyes, and they were completely dark. Random movements of the jaws were visible with vigorous vibrations of the whole body. The heartbeat was at a rate of 146 beats per minute. A line of red blotches appeared on the body in the shape of stars, running along the spinal cord. The tail grew to two-thirds the circumference of the egg.

07 days after fertilisation: 4 – 6 black-colored incomplete rays appeared on the caudal fin. Pale green colour pigmentation dispersed along the whole body. Irregular-shaped red blotches dispersed all over the lower area of the body, including the caudal fin. The tail has been lengthened to the point where the caudal fin touches the head. The yolk size was reduced to half of its original volume. The heartbeat was at about 108 beats per minute, and the vitelline fluid circulation was clearly visible.

08 days after fertilisation: Pigmentation was observed deeper throughout the whole body. The yolk accounts for roughly one-fourth of the total volume of the egg. The eye lenses were clearly visible and completely transparent. Pectoral fins grew into massive fan-like structures.

09 days after fertilisation: The tip of the caudal fin reached the neck area of the embryo. The pigmentation was much deeper, and the vitelline vessels were markedly visible due to extreme branching. Signs of optical sensitivity are reflected by the movements in the eyes to external stimuli.

10 days after fertilisation: The eyes were darker and more sensitive to stimuli. Sudden movements of the eye lenses were visible when directed to a light source. As in the Fig.04. K; Eight to ten black-coloured fin rays developed to different lengths, arising from the base of the caudal fin. The yolk has shrunk even more. A rusty golden pigmentation was visible in the head region.

Newly hatched larvae: The unabsorbed yolk was about 03 times bigger compared to the head region. Tiny oil globules and red star-shaped blotches were scattered throughout the yolk. Vitelline vessels were prominent and highly branched. The circulation of red-coloured cells and yellowish vitelline fluids were highly visible. Black and red blotches were arranged as a pattern throughout the body. The caudal fin was nearly rounded in shape, and the margin was slightly visible. 29 – 31 black colour spots appeared on the transparent caudal fin with an irregular pattern. The Operculum area and the pectoral fins were pale pink. Newly hatched larvae showed sudden movements in their body parts at irregular intervals. Due to the weight of the yolk sac, newly hatched larvae were incapable of balancing the body with their immature fins (Fig.05.A).

Two-day larvae: The total body length of a larvae was around 4.1 - 4.5 mm. Most of the yolk has been absorbed by the body, and therefore, as in Fig.05.B, gall bladder was clearly visible in the dorsal area of the abdomen as a pale-yellow-colored round vesicle and eye region appeared in dark black. The lower jaw was slightly longer than the upper jaw and opened and closed at an 84-cycle-per-minute rate—the pectorals flap at a rate of 67 flaps per minute on average. Larvae make sudden dashes in response to external stimuli by moving their caudal fins side by side. Most areas of the body are transparent and pigmented with a pale yellowish-green colour.

Larvae were tested with different feeds, and in most of the cases, formulated feeds were refused and preparably consumed live feeds, micro worms (*Panagrellus redivivus*) and brine shrimp (*Artemia salina*). Based on the results related to a pilot study, 03 weeks old *A. dayi* larvae showed a much desire to consume daphnia (*Daphnia magna*) and mosquito larvae with a desire.

CONCLUSION

Captive breeding of *A. dayi* can be successfully performed using different sex combinations and tested substrates as the spawning medium. Even though the fertilization and egg hatchability remained constant among treatments, 01 male: 02 female ratio had the highest egg production and was therefore suggested as the best sex ratio. Moreover, the egg production was highest where the mop made out of yarn was used as the spawning substrate. Moreover, *A. dayi* completed its embryonic development within 11 - 13 days. Concerning feed acceptance, brooder stages and larval stages of *A. dayi* preferred to consume live feeds with much desire.

Disclaimer

The authors understand and agree that the journal will not take any responsibility for the research process. The authors guarantee that this research was conducted adhering to ethical practices and legal boundaries.

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