Spawning habits and embryonic development of the lampeye killifish *Aplocheilichthys spilauchen* in *ex situ* fresh-and-brackish water environments

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Abstract

Aside from ornamental uses of killifishes, there is growing interest in using killies for a multiplicity of purposes including baitfish and mosquito biocontrol. This experiment explored the spawning habits and embryonic development of the banded lampeye, *Aplocheilichthys spilauchen* in *ex situ* freshwater (0.04 ‰) and brackish water (5.01 ‰) to ascertaining the captive breeding prospects for mosquito control in areas where they occur. Significantly higher number of eggs were laid in the brackish water than the freshwater ($\chi^2 = 1613.0$, $P < 0.05$), and black mop was the most preferred spawning substrate, followed by green, blue and white mops. Microscopic monitoring of embryos revealed that cleavage occurred within the first 30 minutes after fertilisation, organogenesis commenced in averagely the 25th hour, and hatching in approximately 230 hours. Although certain embryonic developmental stages occurred faster in the freshwater than brackish water and freshwater eggs were relatively bigger than brackish water eggs, these differences were overall not significant and had no effects on the development and hatching. The observed outcome that *A. spilauchen* can be optimally propagated with black mops in brackish water offers a significant step in its use for the mosquito biocontrol programme, as well as other potential uses not yet explored.

Introduction

The need to explore biological control options in managing mosquitoes as disease vectors due to reported negative effects of chemical control approaches has been iteratively advocated (Dabire et al. 2008; Munhenga et al. 2008). Earlier global interest in exploring the prospects of using fish for mosquito biocontrol, and importantly the introduction of mosquitofish *Gambusia* spp. into many countries to curb mosquito populations which failed in the 1990s due to its intrinsic aggression on native fish species (Leyse et al., 2004), has reshaped the choices of fish species for mosquito control programmes (see Matias & Adrias, 2010; Okyere et al., 2019). Recent efforts have refocused on exploring indigenous killifishes (a group of small, non-aggressive cyprinodontiform fishes) due to a number of desirable traits exhibited by these fishes especially their larvivorous predatory habits and non-aggressive nature which make them suitable biocontrol candidates (Ghosh et al., 2004; Matias & Adrias, 2010). The goal is to use native killifishes for mosquito control in localities where they occur naturally rather than introducing non-native species.

One of such killifish species with wider geographical distribution along the West Coast of Africa (Wildekamp, 1995) that is being explored in Ghana for its mosquito biocontrol prospects is the banded lampeye, *Aplocheilichthys spilauchen* (Duméril 1861), the first described killifish from Africa (Wildekamp et al., 1986). A previous study by Okyere (2012) on the food habits of *A. spilauchen* population from coastal wetland pools in Ghana indicated that the fish could be a potential candidate for mosquito control due to its predatory habits on insects such as chironomid (midge) larvae. A follow up laboratory experimental study on the food selectivity and prey preference of the fish by Okyere et al. (2019) further

confirmed the killifish as a promising biocontrol tool, as it selected mosquito larvae and chironomid larvae in equal proportions depending on abundance.

Aside feeding habits, another critical determinant of the feasibility and success of utilizing the killifish for mosquito control is its reproductive habits especially the ability to spawn in captivity. Given that the species inhabits both freshwater and brackish water environments including coastal swamps, river mouths, lagoons and mangrove swamps (Wildekamp, 1995; Lanés et al., 2012), an understanding of its reproductive habits in fresh-and-brackish water environments is imperatively required to inform targeted breeding and up scaling for the biocontrol programme.

To this end, the current work explored the spawning habits and embryonic development of *A. spilauchen* in captivity under freshwater and brackish water conditions as a continuity research in ascertaining the prospects of breeding and using the species for mosquito control in areas where they occur. Premised on previous findings that the species is a continuous substrate spawner with low fecundity (Okyere, 2012), this experiment (1) assessed the spawning potential, (2) investigated whether the killifish has preference for the colour of its spawning substrate, (3) monitored the stages of embryonic development, and (4) monitored the sizes of embryos and duration of embryonic development, in *ex situ* fresh-and-brackish water environments. With the growing interests in using killifish for a multiplicity of purposes including breeding for baitfish (Chesser et al., 2019) and as tool for embryology education (Genade, 2016), the usefulness this study may possibly traverse biocontrol to other facets killifish utility.

Materials And Methods

The experiment was commenced in September 2018 and ended in April 2019. Specimens of *A. spilauchen* used were collected from the mouth of the Kakum River near Cape Coast in the Central Region of Ghana (5° 6' N; 1° 18' W; see Okyere, 2012) and transported into six round plastic experimental tanks at the Department of Fisheries and Aquatic Sciences, University of Cape Coast. Three of the tanks contained replicate brackish water of salinity 5.01 ‰ obtained through dilution of seawater from the Gulf of Guinea where the Kakum River empties into the ocean, while the other triplicate tanks contained freshwater of 0.04 ‰ from the Kakum River. Each tank was 1 m high, had a diameter of 1.3 m, contained 1m³ of water, and stocked with 7 males and 21 females of the fish (i.e. a ratio of 1:3) since the species have low fecundity and a few males are enough to fertilize the eggs produced by many females as noted by Okyere (2012). The tanks were monitored daily for water temperature, dissolved oxygen (DO) and hydrogen ion concentration (pH).

Since killifishes are substrate spawners (Joseph et al., 2018), acrylic mops commonly used by aquarists (Byrne, 1978) were provided as spawning substrates, each with one hundred strands (a strand was approximately 2 mm in diameter and 30 cm long). To investigate whether the killifish has preference for the colour of its spawning substrate, four conceivably contrasting colours of the mops (blue, green, white and black) were provided in each tank, where each colour was triplicated. The fish were fed with formulated feed commonly used in tilapia aquaculture as also used in a previous experiment by Okyere

et. al. (2019), and the mops were monitored daily for eggs. Eggs laid on each mop were removed, counted and recorded, after which the freshly spawned ova were harvested into petri dish and immediately sent to the USAID Fisheries and Coastal Research Laboratory of the Department of Fisheries and Aquatic Sciences for further studies on embryonic development.

The embryos, immersed in water in the petri dish were mounted under a Motic digital compound microscope connected to a computer. With the aid of Motic software, the stages of embryonic development were monitored on computer screen using the $\times 10$ objective, and images of key developmental stages were captured. The duration for transition from formation of blastula, cleavage and epiboly to hatching as well as the circumference of the embryos at each of these stages were recorded using the utility tools of the Motic plus software.

Chi-squared test (Zar, 1999) was used to determine the statistical significance of differences between the number of eggs laid under freshwater and brackish water conditions as well as on the different colours of mops. The significance of the differences between the time taken to reach key embryonic stages and circumference of the embryos in the fresh and brackish water was ascertained using Student t-test (Zar, 1999).

Ethical Statement

Aplocheilichthys spilarchus is not listed as threatened species on the IUCN Red List, and the use of the species for experiments do not require ethical clearance in Ghana.

Results

Summary of physico-chemical conditions in the tanks

Physico-chemical conditions were similar in both freshwater and brackish water tanks (Table 1), with a mean dissolved oxygen of 3.1 mg/l, temperature of averagely 28 °C and neutral (7.4 in freshwater) to near alkaline (7.9 in brackish water) pH recorded during the experiment.

Size distribution of fish specimens

A total of 168 *A. spilarchus* specimens were used for the study, the smallest being 4.1 cm TL and the largest measuring 6.1 cm long. The fish sample showed a normal distribution with individuals within the 5.0-5.1 cm class constituting the mode (Fig. 1). A Kolmogorov-Smirnov Normality Test (D) confirmed that the length distribution of the specimens was normal ($D = 0.070$; $P > 0.05$).

Table 1: Physicochemical conditions in the experimental tanks (standard error in parenthesis)

Parameter	Range		Mean $\pm$ (SE)
	Min	Max	
<i>Freshwater</i>			
DO (mg/L)	2.9	3.4	3.1 $\pm$ 0.2)
Temperature ($^{\circ}$ C)	27.5	28.6	28.0 ($\pm$ 0.4)
pH	7.2	7.5	7.4 ($\pm$ 0.1)
<i>Brackish water</i>			
DO (mg/L)	2.8	3.4	3.1 ($\pm$ 0.2)
Temperature ($^{\circ}$ C)	27.6	28.8	28.2 ($\pm$ 0.3)
pH	7.5	8.3	7.9 ($\pm$ 0.2)

Figure 1

Spawning potential and substrate colour preference

A total of 30,263 eggs were collected over the period of study, of which 12,978 were spawned by the fish in the freshwater set-up, and 17,285 from specimens in the brackish water tanks (Table 2). Significantly higher number of eggs were laid in the brackish water than the freshwater ($\chi^2 = 1613.0$, $P < 0.05$). Of the four mop colours, black mops appeared as the most preferred spawning substrate, with the number of eggs laid on the black being about twice higher than the other colours in both waters ($\chi^2 = 1669.8$, $P < 0.05$ for freshwater; and $\chi^2 = 2735.0$, $P < 0.05$ for brackish water). The white mop recorded the lowest egg counts in both water systems, suggesting white as the least preferred spawning substrate by the fish. Generally, the observed substrate colour preference by the killifish in a decreasing order was black, green, blue and white.

Embryonic developmental stages, sizes and duration in fresh-and –brackish water set-ups

The embryonic developmental stages observed and monitored were the cleavage stage characterised by cell divisions (2, 4, 8, 16, 32, 64), blastulation stage observed from 128 cell divisions and beyond, gastrulation stage (occurring from 15% epiboly, 50% epiboly, 75% epiboly and 100% epiboly), the early organogenesis characterised by appearance of eye buds, brain and the heart, pigmentation stage with appearance of dark pigments, late organogenesis and finally hatching. Key among these stages where clear images were captured are shown in Figure 2.

Table 2: Number of eggs collected from the freshwater and brackish water set-ups, and for the different colours of mops

Tank Replicate	Mop colour and number of eggs collected				Ratio	χ^2	P _(0.05)
	Blue (B)	Green (G)	White (W)	Black (Bk)			
Freshwater (FW)							
1	1144	1100	1365	2384			
2	736	889	667	1779			
3	700	689	432	1093			
Sub-Total	2580	2678	2464	5256	12978	1: 1.1: 1: 2.1	1669.8 S
Brackish water (BW)							
1	1359	1356	948	2483			
2	1064	990	1028	2260			
3	1024	1396	874	2503			
Sub-Total	3447	3742	2850	7246	17285	1.2: 1.3: 1: 2.5	2735.0 S
Overall					FW:BW		
Total FW & BW					30263	1: 1.3	1 613.0 S
S indicates significant at the 5% significance level (α = 0.05)							

Figure 2

As presented in Table 3, the cleavage stage was rapid, occurring within the first 30 minutes after fertilisation and completing in roughly 24 hours (in Day 1 of development). Organogenesis commenced in averagely the 25th hour while pigmentation occurred between the 43rd and 44th hour (i.e. both commenced in Day 2), and both progressed through to 230 hours (between Day 9 and 10) when the embryos hatched. Development of embryos to reach most of the stages was comparatively faster in the freshwater than the brackish water, however, there was no statistical difference in the overall duration taken to hatch in the two waters. Similarly, results of the measurement of egg circumference showed that the freshwater eggs were comparatively bigger than the brackish water eggs at any development stage although the differences were not statistically significant after the early cleavage stages.

Table 3: Duration and circumference of *A. spilauchen* embryos in freshwater and brackish water tanks at the different stages of development; Mean $\pm$ (Standard Error)

EMBRYONIC STAGES	CIRCUMFERENCE OF EMBRYO (µm)			DURATION OF DEVELOPMENT		
	Freshwater	Brackish water	P-values	Freshwater	Brackish water	P-values
<i>Cleavage</i>						
2 Cells	249.1 (±18.4)	236.0 (±12.5)	0.01 (S)	27 min (± 2 min)	32 min (± 9 min)	0.08 (NS)
4 Cells	249.6 (±19.7)	235.2 (±9.32)	0.01 (S)	1 h, 11 min (± 2 min)	1 h, 19 min (± 11 min)	0.01 (S)
8 Cells	250.3 (±17.9)	238.2 (±11.3)	0.01 (S)	1 h, 32 min (± 8 min)	2 h, 17 min (± 25 min)	0.00 (S)
16 Cells	248.7 (±19.3)	237.1 (±11.2)	0.03 (S)	2 h, 14 min (± 5 min)	2 h, 45 min (± 32 min)	0.00 (S)
32 Cells	248.7 (±18.7)	236.4 (±12.2)	0.02 (S)	3 h, 5 min (± 20 min)	3 h, 35 min (± 35 min)	0.00 (S)
64 - Beyond	245.2 (±20.7)	239.2 (±15.0)	0.30 (NS)	3 h, 20 min (± 17 min)	4 h, 13 min (± 35 min)	0.00 (S)
15% Epiboly	244.6 (±17.6)	237.5 (±10.3)	0.13 (NS)	11 h, 23 min (± 19 min)	12 h, 38 min (± 40 min)	0.00 (S)
<i>Gastrulation</i>						
50% Epiboly	244.2 (±16.6)	238.6 (± 9.4)	0.20 (NS)	13 h, 55 min (± 30 min)	14 h, 17 min (± 51 min)	0.05 (NS)
75% Epiboly	246.0 (±18.3)	237.4 (±12.6)	0.09 (NS)	17 h, 1 min (± 56 min)	17 h, 23 min (± 62 min)	0.19 (NS)
100% Epiboly	245.6 (±15.9)	236.1 (±11.4)	0.04 (S)	21 h, 17 min (± 17 min)	22 h, 5min (± 1 h)	0.00 (S)
<i>Organogenesis</i>	239.8 (±14.5)	237.5 (±12.8)	0.18 (NS)	25 h, 22 mins (± 41 min)	25 h, 55 min (± 58 min)	0.02 (S)
<i>Pigmentation</i>	243.1 (±7.0)	239.6 (±10.0)	0.20 (NS)	43 h, 1 min (± 2 h)	44 h, 22 min (± 2 h)	0.04 (S)
<i>Hatching</i>	-	-	-	232 h, 3 min (± 12 h)	233 h, 30 min (± 8 h)	0.56 (NS)
S indicates significant at $P_{(0.05)}$ significance level; NS indicates Not Significant						

Discussion

Killifishes have evolved to occupy a wide range of habitats within freshwater, brackish water and saline ecosystems (Wildekamp, 1995). Even different species within a genus (e.g. *Fundulus*) are reported to show varied levels of salt tolerance and are adapted to surviving at different salinities (Whitehead, 2010). In Eurasia, the Mediterranean coast of Africa and North America, more species live in brackish water while in South America and sub-Saharan Africa, a majority are freshwater inhabitants (Ghedotti and Davis, 2013; Van der Zee et al., 2007; Wildekamp et al., 1986; Wildekamp and Van der Zee, 2003;). Of the few species in sub-Saharan Africa that inhabit brackish water environments, *Aplocheilichthys spilauchen*, *Poropanchax scheeli*, *Pantanodon madagascariensis* and *P. stuhlmanni* are the well-known members (Wildekamp et al., 1986) although these species are also tolerant of freshwater. The stronger association with brackish water possibly explains why *A. spilauchen* spawned significant number of eggs at the salinity of 5.01 ‰ than the 0.04 ‰ during the present experiment. This spawning habit is inconsistent with observations on other killifishes such as the Iberian killifish *Aphanius iberus* where Oltra and Todolí (2000) found no significant differences between the number of spawns at different salinities.

A considerable number of studies have been carried out on colour preference of fin-and-shellfishes widely covering effects of colour preference on fish learning (Roy et al., 2019), fish feeding (Cole and Endler, 2015), prawn shelter (Kawamura et al., 2017), shrimp growth (Freire et al., 2012), fish mating behaviour (Seehausen et al., 1998), among others. In the bluefin killifish *Lucania goodei*, pecking behaviour has been found to be associated with blue and red colours (Johnson et al., 2013), but beyond this report, studies on colour preference of killifishes for other biological and behavioural purposes remain dearth despite the growing interests in varied utilisation of these fishes. Traditionally, killifish breeders have chosen spawning mops that are darker to enable easy spotting of the eggs laid since the whitish transparent eggs are easily visible on a dark background. The selection of spawning substrate by breeders has not been based on empirical studies on substrate colour preference by the fish. In fact, some killifish breeding sources even suggest that the fish will not care about the spawning substrate colour (Aquatic Community, 2004). Results of the current study, however, shows that the banded lampeye killifish has preference for the colour of its spawning substrate, with the most preferred colour being black, followed by green and blue, and white being the least preferred. Although the black mop preference observed in this study may appear to corroborate the practice of using dark mops by breeders, it possible that different species may have preference for different spawning substrate colours and spawning could be optimised if the preferences are indentified.

In the field of killifish embryology, the early work of Wourms (1972) on *Austrofundulus myersi* as well as recent works of Mourabit et al. (2011) together with Genade (2016) on *Kryptolebias marmoratus*, Podrabsky et al. (2017) on *Austrofundulus limnaeus* and Masoudi et al. (2018) on *Aphanius sophiae*, among others, have well documented the developmental stages from cleavage to hatching including pictorial appearance of the stages. In the present study, the development of *A. spilauchen* embryos followed same stages as reported for the other killifishes. No differences were observed between the physical appearance of the embryos in the freshwater and brackish water tanks. Although some minor disparities were observed for the duration of certain stages (faster in the freshwater than brackish water) and embryo size (freshwater eggs were bigger than the brackish water), these differences were overall not

significant and had no effects on the overall duration of 230 hours (i.e. 9 to 10 days) taken to hatch. Osmotic effects could likely account for these imperceptible differences as freshwater eggs are susceptible to the influx of water while eggs in saline waters may tend to lose water and shrink in size due to the differences in osmotic gradient (Holliday, 1969).

In conclusion, this experiment has demonstrated that *A. spilauchen* can be propagated through captive breeding. Using brackish water of 5 ‰ salinity yielded more seed than freshwater, and providing black mops as spawning substrate also yielded significantly higher spawns than green, blue and white mops. Inferably, for optimal spawning of the lampeye, breeding should be carried out under brackish water condition using black mops. The outcome of this study offers a significant step for the mosquito biocontrol programme since the predator, *A. spilauchen*, can be optimally propagated and disseminated in a few weeks. It is important to emphasise that for future breeding programs and introduction of *A. spilauchen*, local populations should be used since reports indicate there are large genetic distances between different populations. Aside from the biocontrol, the work also opens a new discussion on the prospects of breeding the lampeye as baitfish for the Tuna Fishing Industry in Ghana given that the declined small pelagic stocks in country has rendered anchovies (*Engraulis encrasicolus* - the traditional baitfish for the industry) unavailable and alternatives are currently being sought. Finally, our research complements the works of others such as Bragança and Costa (2019) in promoting research on African lampeyes.

Declarations

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Contribution of authors: Isaac Okyere was responsible for overall coordination of the project including data collection and analysis, and drafting of part of the manuscript. Juliet Afrah Obeng and Samuel Ayitey carried out the in the experimentation, including data collection and analyses, while Jouke Rients Van der Zee and Herman Meeus designed the experiment, provided the spawning mops, identified embryonic stages, and carried out final revision of the manuscript. All authors have approved the final article.

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Figures

Figure 1

Length-frequency distribution of *Aplocheilichthys spilarchus* used for the experiment

Figure 2

Stages of development of *Aplocheilichthys spilauchen* embryo showing (a) cleavage (2-cell stage), (b) cleavage (4-cell stage), (c) blastulation stage, (d) gastrulation stage, (e) early organogenesis, (f) pigmentation, (g) late organogenesis, and (h) hatchling (fry)