Research Article

Effect of Lab Prepared and Commercially Available Activation Media on the Milt Quality and Fertilization Rate of *Clarias gariepinus* (Bruchell, 1822) African Catfish

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Abstract | African Catfish (*Clarias gariepinus*) is an improved source of nutrition because of its high biological value, high protein deposition compared to the fat content and low cholesterol level. African catfish is cultured widely all over the world through artificial propagation, but male gametes cannot be collected through simple hand stripping method. Therefore, the males require to be sacrificed in order to get the milt from the testes. As millions of sperms are present in per ml of milt so, in order to utilize milt more efficiently, various researches are based on use of different types of activation or dilution media. Therefore, the lab prepared activation media A and B, commercial activation media C and fresh water were used in this experiment to compare their effects. Experimental layout was CRD (Complete Randomized Design) with four treatments: control, media A, media B and media C (Actifish) with three replications for each. The study was conducted at National Agriculture Research Center (NARC), Islamabad, Pakistan. Seeds of African catfish were brought from Thailand which underwent acclimatization under the local culture condition. After attaining sexual maturity, six males and five females were selected for the study. Milt was collected and diluted using the above-mentioned activation media by using dilution ratio of 1:29. Sperm quality assessment of diluted milt was carried out for different traits such as sperm motility, sperm motility duration and sperm viability. Fertilization rate was determined by using same diluted milt aliquots. The results indicated that sperm quality indices and fertilization rate of milt treated with activation media show better results in comparison to control. Best results with respect to all parameters were obtained from experimental group treated with Commercial activation media (C) while lowest values were obtained for control group. The commercially available, Activation media could be useful for increasing the seed production in commercial level.

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Introduction

Different species of the genus *Clarias* and their crossbreds are produced because of their fast growth rate, resistance to diseases and environmental impacts, and favorable to high density culture since they are air-breathing fish. Among the catfish, African catfish is considered as one of the most

promising species for culture. The absence of reliable reproduction techniques is the reason for low rate of production (Haylor, 1993). Due to possession of these pre-eminent cultural qualities, African catfish was incorporated in the system of Pakistan aquaculture by importing it from Thailand. It was successfully acclimatized and bred under local environmental conditions. The process of artificial breeding has been practiced ensuring the availability of good quality seed for production at larger scale. However, the male gametes of this fish are difficult to collect through stripping and male is sacrificed to remove testes for gathering milt. Therefore, in order to strengthen the innovative techniques for the production and development of local seed production of this exotic breed, specifically male gametes were manipulated for their fertilizing ability through use of activation solutions.

In testis and seminal plasma, the spermatozoa are immotile in both freshwater and marine fish. The spermatozoa become motile after release in water when it reproduces naturally or in a diluent during artificial reproduction (Galindo *et al.*, 1999; Alavi and Cosson, 2006; Cosson at al., 2008). Sperm motility is a parameter that determines the quality of fish semen and its capacity to fertilize (Alavi *et al.*, 2004; Abascal *et al.*, 2007). This sperm motility is affected by different factors, such as temperature (Alavi and Cosson, 2005), pH (Ingermann *et al.*, 2002; Alavi and Cosson, 2005; Zuccarelli *et al.*, 2007), cations (Cosson, 2004; Alavi and Cosson, 2006; Alavi *et al.*, 2007), osmolality (Alavi and Cosson, 2006; Cosson, 2004; Linhart *et al.*, 2006; Alavi *et al.*, 2007), dilution ratio (Alavi *et al.*, 2004; Abascal *et al.*, 2007). The effects of these factors develop the methods of artificial reproduction and therefore, is important for the aquaculture industry.

In aquaculture, for *in vitro* fertilization, number of different kinds of activation media are used and it has been proved that these activation media are species specific. There is a positive effect on the spermatozoa motility and the motility duration by the activation media (Sarosiek *et al.*, 2012). Determination of factors that are responsible to influence the motility of sperm could be helpful in developing and improving the artificial reproduction in fish farms (Alavi *et al.*, 2008). There are several natural diluents (coconut water, egg yolk) that are used as fertilization media as well as synthetic diluents and are now a days in use for example Tris, glycine and $CaCl_2$ (Daniel *et al.*, 2001). Investigations also show that the type of activating solution influences the duration of egg fertilization (Zarski *et al.*, 2012). These activating solutions are widely utilized in *in-vitro* fertilization in aquaculture. The impact for the above solution on *in-vitro* fertilization has restricted evidence. For instance, the Billard solution that was originally formulated for salmonids that can also be used on percid fish like the Eurasian perch, as demonstrated by Zarski *et al.* (2012). Different form of the Woynarovich solution which was developed for cyprinids, was found to enhance fertilization rate in the same percid species (Zarski *et al.*, 2012). Whereas in cyprinid species such as common tench, *Tinca tinca* (L.), this solution reduces the chances of fertilization (Geldhauser, 1992). Therefore, it is important to develop species-specific activating solution for *in-vitro* fertilization. However, information on stimulating reproduction by artificial means using activating solutions are scarce. Due to the limited literature on the specified parameter, this work sought to compare the impact of different activation media on the motility, duration of motility, viable and fertilized spermatozoa in the African catfish.

Materials and Methods

This study was done at, AFP (NARC), Islamabad Pakistan. This research was design to assess sperm mobility activating media for determination of the quality of sperm in African catfish as well as the fertilization rate. The average standard weight of both the male and female African catfish was taken with the help of weighing balance and the range are mentioned in Table 1.

Table 1: *Weight of mixed sexes of African catfish.*

Weight of male (g)	Weight of female (g)
957.1-1009.0	1044.9-1420

Media preparation

To be precise, the activation media A was prepared with 45mM of NaCl, 5mM of KCl and 30mM of Tris dissolved in 10ml distilled water and similarly, the Media B was prepared with 50mM of NaCl, 30mM of KCl and 30mM of Tris dissolved in 12ml distilled water respectively. The fresh water served as control. Fourth treatment (media C) was commercially available activation media named as Actifish.

Milt collection

For the purpose of invitro analysis of milt, Male African Catfish was dissected to remove testes. After removal, testes were placed in 9% saline solution to prevent activation of spermatozoa. After cleaning and drying completely, the testes were lacerated with small surgical blade and placed in fine net cotton cloth and squeezed to extract the milt in a clean, dry petri dish that has been autoclaved to prevent sperm from coming into touch with room-temperature water. By utilizing a plastic syringe, the volume of milt that was collected was measured. The collected milt was divided into four aliquots and diluted using media A, B, C, and fresh water as the control in a ratio of 1:29. Next, characteristics of sperm quality were evaluated by analyzing the diluted milt.

Sperm quality parameters

Sperm motility and motility duration: Using a dropper, a cover slip was applied to one of the four aliquots of milt, and the slide was examined using a 40X magnification microscope (Optika, Italy). Sample was examined every 20 seconds to measure the percentage of motility until all spermatozoa were discovered to be dead, at which point the length of motility was measured.

Sperm concentration

Concentration was determined from the four aliquots using a conventional Neubauer hemocytometer procedure. Using a micropipette, a drop of diluted semen sample was put onto the hemocytometer. The pipette tip was inserted into the hemocytometer V-shaped groove during loading. To stop the spermatozoa from moving, the sample was put in a lab setting. By counting the spermatozoa in the central square and four corner squares at a 40X magnification, the concentration was determined. The cell calculator was used to determine the total sperm concentration in a 30 µl volume.

Sperm viability

Trypan blue (0.4%), which was made by dissolving 4 mg of the dye in 1 ml of distilled water, was used to assess the viability of the sperm. Each time the stain was used, it was filtered beforehand. 1µl of diluted milt and 1µl of thoroughly mixed stain were added to the glass slide to create a smear, which was then left to dry for 2-3 minutes. The smear was examined under a microscope using an oil emulsion and 100X magnification after 2-3 minutes. Living spermatozoa with intact membranes remained mildly stained when mixed with stain, but dead sperms with their membranes broken were strongly stained. A digital counter was used to count about 100 sperm at random, and the ratio of viable sperm to total sperm was used to determine the viability of the sperm.

Fertilization rate

To determine the rate of fertilization, the eggs were collected from female African catfish injected with ovaprim @ 0.5ml/kg. After 5-7 hours, eggs were collected by hand stripping method. The weight of the fish egg was determined and divided into four batches. Each batch weigh about 1g contained around 600-700 number of eggs. Volume of diluted milt was adjusted to equalize concentration of spermatozoa. Eggs were combined with this diluted milt. To improve fertilization, new water was added and left to stand for 10-15 minutes after the five minutes of mixing. After one to two hours, the existence of eyed eggs (brown spots) indicated fertilization. A stereomicroscope was used to calculate the percentage fertilization rate. Dead or unfertilized eggs seem opaque and yellow, whereas fertilized eggs are translucent with brown spots.

Percentage fertilization was determined by using following formula (Khara *et al.*, 2014).

$$\text{Rate of fertilization} = \frac{\text{No of fertilized eggs}}{\text{Total No. of eggs}} \times 100$$

Statistical analysis

Data were statistically analyzed through one-way ANOVA by using SPSS. DMRT was used to check the significance of means. Analyzed data was presented using suitable graphics via Microsoft Excel.

Results and Discussion

Effect of various activation medias on different sperm quality parameters i.e., sperm motility, sperm motility, duration, sperm viability and rate of fertilization in *Clarias gariepinus* (African catfish) presented in Table 2.

A significant difference ($p<0.05$) was found in sperm motility (%) among four treatments, the highest value of sperm motility was found in media C (67.65±1.58), followed by media B (63.62±1.08) and

Table 2: *Effect of various activation medias on different sperm quality parameters in Clarias gariepinus (African catfish).*

Parameters	Treatments			
	Freshwater	Media A	Media B	Media C
Sperm motility (%)	53.10±1.02^{c}	56.24±1.90^{c}	63.62±1.08^{b}	67.65±1.58^{a}
Motility duration (min)	7.8±0.81^{c}	10.16±0.65^{b}	10.54±0.75^{b}	11.81±0.67^{a}
Sperm viability (%)	42.16±0.98^{b}	50.21±0.64^{a}	53.02±0.59^{a}	54.48±0.39^{a}
Fertilization rate (%)	69.60±1.05^{c}	72.01±1.81^{c}	78.70± 1.02^{b}	90.04±1.70^{a}

media A (56.24±1.90) while it was lowest in control (53.10±1.02) (Figure 1). The percentage of sperm motility was non-significant between control (freshwater) and media A. Significantly highest value ($p<0.05$) of sperm motility duration (min) was observed by milt treated with media C (11.81±0.67) followed media B (10.54±0.75) and media A (10.16±0.65) while minimum value was for control (freshwater) (7.8±0.81), respectively (Table 2, Figure 2). Motility duration was for media A and B was non-significant from each other ($p>0.05$). The effects different activation media on sperm viability are shown in (Figure 3). The sperm viability ($p>0.05$) was better by diluted milt treated with activation media as compared to control. The values sperm viability for media A (50.21±0.98), media B (50.21±0.64) media C (54.48±0.39) were non-significant with each other as compared with control group (42.16±0.98).

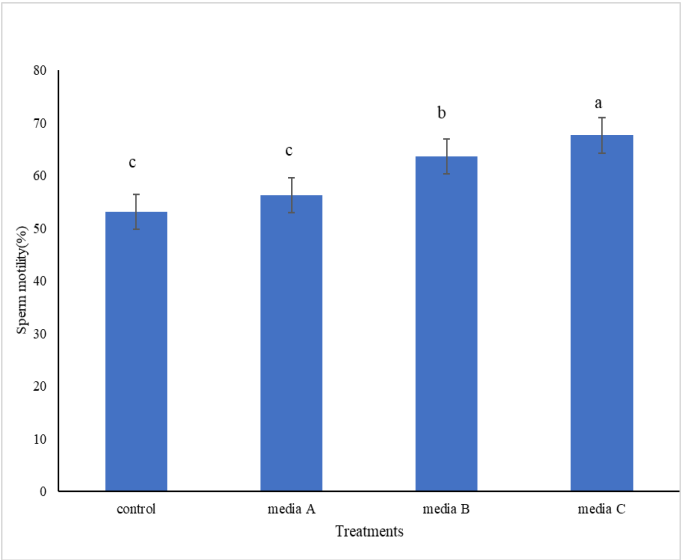

Figure 1: *Effect of different activating media on spermatozoa motility (means±standard deviation) of African catfish. Different letters above the bars show significant difference P<0.05 between treatments.*

The comparison of rate of fertilization by diluted milt treated with media A, media B, media C, and control group shown in Figure 4. Best results in term of fertilization rate were obtained by milt treated with media C (91.03±2.1) followed by media B (79.01±0.9) and media A (71.09±1.61), However, media A was equally effective in increasing fertilization rate as fresh water (control) group ($p>0.05$).

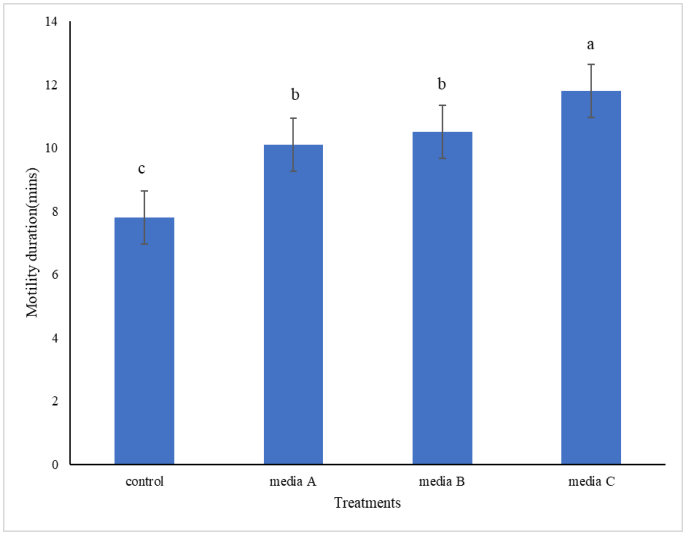

Figure 2: *Effect of different activating media on spermatozoa motility duration (means± standard deviation) of African catfish. Different letters above the bars show significant difference P<0.05 between treatments.*

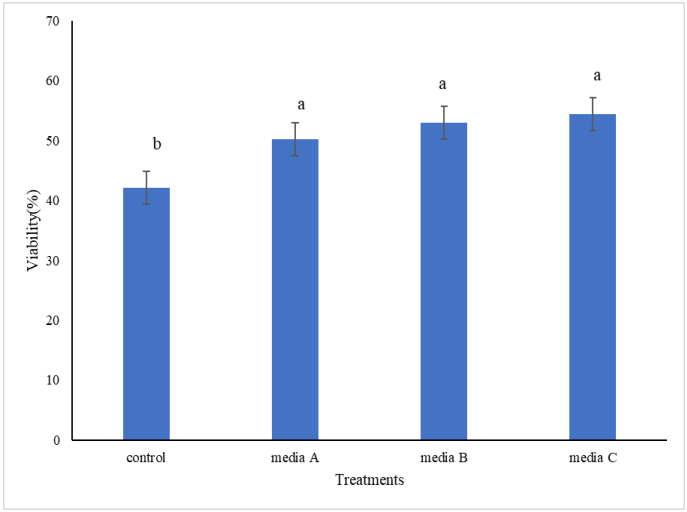

Figure 3: *Effect of different activating media on spermatozoa viability (means± standard deviation) of African catfish. Different letters above the bars show significant difference P<0.05 between treatments.*

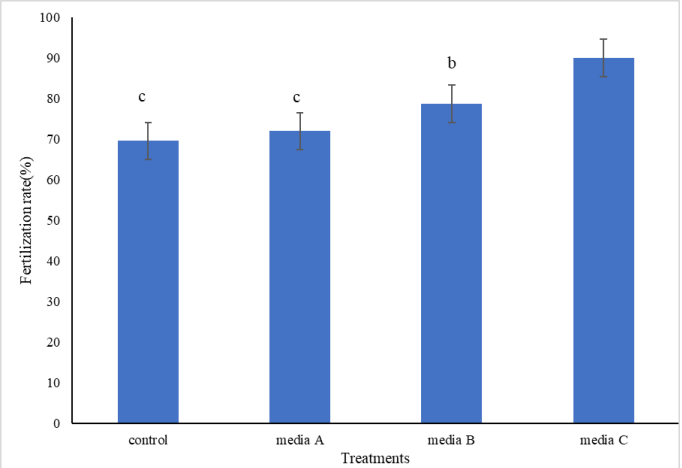

Figure 4: *Effect of different activating media on fertilization rate (means± standard deviation) of African catfish. Different letters above the bars show significant difference P<0.05 between treatments.*

Best results with respect to all parameters were obtained from experimental group treated with media C while lowest values were obtained for control group. Values obtained from media B were intermediate between media A, C and control. Our results indicate that all media show better results in comparison to control. Whereas, media C serves as best activating media for African catfish with respect to all parameters studied during our study.

The results obtained in the present study showed that use of activation media resulted in satisfactory effects on sperm quality parameters. After using these activation media, the sperm quality parameters that include sperm motility, sperm motility duration, sperm viability in male African catfish and fertilization rate in female African catfish increases as compared to control (fresh water). This study has clearly shown that the sperm of *Clarias gariepinus* gain better quality when treated with activation media as compared to control (fresh water). Highest motility of sperm in this study was observed in media C (Actifish) followed by media B and A (lab- prepared) and least value was found in control (fresh water) which showed higher percentage of motile sperm of stimulated males in activation media than the control group. In this study, both media A (45mM) and media B (50mM) contains NaCl in a moderate concentration which agrees with Alavi *et al.* (2010) who showed higher %motility of sperms in an activation media containing NaCl as compared to distilled water. This is also in agreement with the work of Wilson *et al.* (2009) who examined how different osmolality ranges affected the motility of *D. rerio* sperm and found that the maximum sperm motility rate was maintained at a moderate concentration of NaCl (80 mM), but not at low or high concentrations (0 or 120 mM). Sperm motility duration is very brief in freshwater species. Motility duration was recorded in minutes which differed significantly ($p<0.05$) among all treatments. All treatments contain moderate concentration of NaCl and KCl. When compared to the control (fresh water), media C produced the maximum value for the length of sperm motility.

However, the duration of media A and B was did not differ significantly from each other ($p>0.05$). This is in line with the findings of (Khara *et al.*, 2014), who reported the highest time span for sperm motility was observed when semen was incubated in solutions containing 88 mM KCl and 72 mM NaCl in *Cyprinus carpio* (Krasznai *et al.*, 1995), tilapia (Ahmed *et al.*, 2024; Morita *et al.*, 2003), common barbel, *Barbus barbus* (Alavi *et al.*, 2009), and bunnei, *Barbus sharpeyi* (Alavi *et al.*, 2010). Additionally, Cejko *et al.* (2013) found that the group that utilised billard solution as an activation solution had the greatest motility duration, indicating a significant effect.

All three medias have significant but equal effect on sperm viability ($p>0.05$) as compared to control (fresh water). In contrast to this study of Cejko *et al.* (2013) reported that placing non-motile sperm into a diluent (activation media) makes no difference in the percentage of viable cells. There were differences in sodium and chloride concentrations between motile and non-motile sperm of Atlantic salmon ($p< 0.05$) therefore; placing them into diluent did not revive them.

Success in fertilization is solely dependent on the gametes quality (Bobe and Abbe, 2010), the ratio of sperm to egg (Linhart *et al.* 2006), the duration of time that active sperm and active eggs come into contact (Liley *et al.*, 2002), and the activating solution that is employed (Zarski *et al.*, 2012; Kucharczy *et al.*, 2010). The media C treatment yielded the highest rate of fertilization across all treatments, with media B, A, and control following suit.

However, media A was equally effective in increasing fertilization rate as fresh water (control) group ($p>0.05$). Similar results were reported by Cejko *et al.* (2013) researchers who after applying the activation solution BS (Billard solution), a high fertilization rate

was observed. Additionally, Khara *et al*. (2014) found that when semen was diluted in saline solutions rather than freshwater, the sperm's high fertilizing ability could be increased. This means that the fish spermatozoa can fertilize more effectively when appropriate activating solutions are used to prolong their motility (Alavi *et al*., 2009; Ndimele and Owodeinde, 2012).

In this study, percentage fertilization was found to be higher in the treated groups and was significantly different from the control. This is consistent with research by Ndimele and Owodeinde (2012), which found that the induced group of *C. gariepinus* significantly increased percentage fertilization, hatching rate, and survival. Progeny of smelt hatched with milt from Ovaprim induced male had higher survival than progeny hatched naturally. Additionally, Nwokoye *et al*. (2007) results, which showed that clariid catfish *Heterobranchus bidorsalis* injected with synthetic hormone (Ovaprim) had the highest mean number of fertilized eggs, hatchability, and survival, further corroborate the effectiveness of synthetic hormones.

Novelty Statement

For the breeding of African catfish (*Clarias gariepinus*) in captivity male had to be sacrificed. In order to utilize the milt from one male for multiple females' different type of activation medias were tested to enhance the *in vitro* fertilization rates. This study will help researchers to overcome low availability of the fish seed by enhancing fertilization rate, quantity of fish seeds and fry in fish hatcheries by using activation media at local level.

Author's Contribution

Zarifshan Malik: This paper is a part of MPhil study of 1st author; she performed this research study.
Muhammad Ramzan Ali and Sabina Noor: Supervised research, help in experimental setup and data analysis and manuscript writing.
Aziz Ahmed: Helped in experimental setup and data collection.
Hasina Basharat: Helped lab analysis, reviewed and edited the manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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