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EFFECTS OF DILUTION RATIO ON THE POTENCY AND VIABILITY OF THE SPERMATOZOA OF AFRICAN CATFISH (*Clarias gariepinus* Burchell, 1822) USING NORMAL SALINE SOLUTION

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ABSTRACT

Normal saline solution is one of the most common diluents in artificial breeding and to dilute the milt of *C. gariepinus*, however the most desirable quantity or volume to be used is still a problem leading to several failures in artificial propagation of fish. The study determined the effects of dilution ratio on the potency of the spermatozoa, fertilization and hatchability rate of the eggs and survival rate of the hatchlings. The dilution ratio used included milt to saline solution ratio of: 1:10 (T2), 1:50 (T3), 1:100 (T4), and zero saline inclusion (T1), as the control. The experiment was laid out in a complete randomized design (CRD) with 3 replications each for the treatments. The result showed that there was significant difference ($P<0.05$) in percentage motility of the milt, egg fertilization and hatchability rate, and survival rate of the larvae across the treatments. The highest percentage motility (87.67%), fertilization (97.55%), hatchability (87.78%) and survival rate (I83.53%) occurred at T2 (1:10), while the least percentage motility (49%), fertilization (75.89%), hatchability (40.73%) and survival rate (45.43%) occurred at T1 (zero saline inclusion). It can be concluded that normal saline improves artificial propagation of *C. gariepinus* and with further increase of normal saline solution there is a negative effect on the potency of the milt, fertilization and hatchability of the eggs and survival rate of the hatchlings. A dilution ratio of 1:10 is therefore recommended to fertilize eggs of *C. gariepinus* for improving artificial propagation of fish.

Keywords: *Clarias gariepinus;* dilution ratio; normal saline; spermatozoa

INTRODUCTION

The high increase in the demand for fingerlings in the phenomenal growing aquaculture industry has stimulated the demand for artificial reproduction of cultured fishes (Nwokoye *et al*., 2007). Artificial propagation or reproduction methods constitutes the major practicable means of providing enough quality seed for rearing in confined fish enclosures such as fish ponds, reservoirs and lakes (Charo and Oirere, 2000). Artificial propagation of fish is a most promising and reliable way of ensuring availability of good quality fish seeds all year round and sustainability of the Aquaculture industry. It involves the use of natural

(hypophysation) or synthetic hormones to induce ovulation and spawning in farmed fishes (Olumuji *et al*., 2012). It is a method used for fishes that do not normally breed in captivity.

The fish farming industry has been more focused towards the quality of eggs and larvae rather than that of spermatozoa, even though the spermatozoa quality of male brood stock also affects the production of healthy larvae (Rurangwa *et al*., 2004). Spermatozoa quality or potency is defined as a measure of the ability of sperm to successfully fertilize an egg. The ability of spermatozoa to successfully fertilize an egg mostly depends on qualitative parameters of milt composition of seminal fluid, milt volume, sperm density and sperm motility (Rurangwa *et al*., 2004). The viability of spermatozoa is defined as the ability or the capacity for the spermatozoa to move and fertilize an egg (Rurangwa *et al*., 2002). Hajirezaee *et al*. (2010), grouped the factors that affect the qualitative parameters of spermatozoa of fish into biological characteristics, rearing conditions of brooders, artificial induction of spawning, spawning season and post stripping factors such as chemical properties of diluents and short and long term storage of milt. In most farms, physiological or normal saline solution is used as a diluent of spermatozoa obtained from the male broodstock. Normal saline has been used for different method of fertilization, the most common being the dry method where few drops of physiological or normal saline can be added to facilitate mixing (Ankakali *et al*., 2011). Semen can easily be collected from males injected with *Clarias* pituitary homogenate unlike in the untreated fish (van der Waal, 1985). In case of insufficient sperm availability, males are sacrificed and the milt is extracted from the testis cut into small pieces extended in a saline solution of intra testicular sperm is inactivated in Normal saline solution (dilution 1:10 or 1:100) (Hogendoon and Vismans, 1980). Anne (2016) defined a normal saline solution as a sterile mixture of salt and water. Normal saline which is the commonly used form of saline solution is prepared by dissolution of 9 g of NaCl in 1 litre of water (Madu 1989). Saline solution is used not only as a carrier of homogenate but also as a preservative for the milt. Orji *et al*. (1997) reported that the success of induced spawning is as a result of the application of saline solution. Saline solution has been used for storing and preserving animal cells. Different investigations of fish reproductive materials have shown that the use of physiological or normal saline solutions cause the protection of flagella structure and increases the time of motility, thus researchers used these solutions instead of tap water for activating spermatozoa (Kalbassi and Lorestani, 2007). The quantity or rate of application differs among researchers (Adebayo and Popoola, 2008).

The quantity of saline mixed with milt before it is used to fertilized stripped eggs is suspected to have an effect on the fertilization rate, hatchability rate and survival rate of the eggs (Okunsebor *et al*., 2014). Failure in fertilization and hatching rate may be due to environmental and physiological factors such as salinity (Orji *et al*., 1997). Many fish hatchery in Nigeria, the quantity of normal saline used as diluent for milt often constitute dilution ratios different from the 0-6-07% recommended by Delince *et al*. (1987) either to increase or reduce the concentration of milt during induced breeding of *Clarias gariepinus*. Too little or too much saline has a lethal effect on the cells (Orji *et al*., 1997 and Okunsebor *et al*., 2014). Hence there is a need to evaluate the potency and viability of different spermatozoa dilution ratio in achieving high performance in fish yield. There is paucity of knowledge on the effect of dilution on the potency and viability of spermatozoa. Okunsebor *et al*. (2014) studied the effect of different Dilutions of saline on some sperm parameters and their influence on fertilization, hatchability and survival rate of hatchlings. While Ukwuani *et al*. (2014) conducted a study on the Induced breeding of catfish (*C. gariepinus*) as influenced by milt dilution through different levels of normal saline inclusion. They

considered the fertilization, hatchability and survival rate of the hatchlings but did not study the effect on the motility of the spermatozoa as well as not establishing the best milt: saline water dilution ration for the species. This research attempts to study the effect of dilution ratio on the motility of the spermatozoa, with a view to establishing utmost dilution ratio so as to avoid over dilution, since diluting the milt with normal saline increases its volume with tendency to increase the volume of eggs it can be used to fertilize. Farmers may be able to fertilize large amounts of eggs using a single gene pool when the milt is appropriately diluted.

MATERIALS AND METHODS

Study Area

The study was carried out at the indoor Fish Hatchery unit of Department of Aquaculture and Fisheries Management, University of Benin, Benin city, Edo State. The hatchery has several plastic and concrete tanks with flow through facilities.

Experimental Procedure

A total of 3 males (of 2kg each, same age and nutritional history) and 6 females (1.8kg average weight same age and nutritional history) broodstocks of *C. gariepinus* were obtained from a reputable fish farm in Benin city, Edo state and transported to the fish farm in well aerated plastic drum to avoid stress. The Broodstocks were selected based on the recommendations of Olaniyi and Omitogun (2014). Ovaprim (0.5ml per kg of fish) was administered to the female broodstock following the method in Egwenomhe and Obi (2012).

A male broodstocks was sacrificed per trial by dissecting the abdomen to obtain the gonads. Blood clots and other tissues were rinsed away from the gonads which were lacerated using a sterilized surgical blade and milt was collected into a clean cup. Milt was diluted with measured quantity of normal saline solution (0.9% NaCl) into clean cups. Ovulated eggs flowed out easily in a thick jet from the genital vent of the females by gently pressing the abdomen with a thumb from the pectoral fin towards the genital papilla. The first free running eggs obtained were collected for the study (Omoniyi *et al*., 2014). From the ovulated eggs samples were taken for each Treatment. Thus, eggs from the same female were used in each trial.

Experimental Design

The experiment was laid out in a completely randomized design (CRD) with 3 treatments and a control with 3 replications each. The milt for the different dilution ratio was measured. One ml of milt was used to 10ml, 50ml, and 100ml of normal saline solution. This was to produce two solutions and one solution with milt concentrations less and more respectively, than that recommended by Delince *et al,* (1987). A total of 36 spawning bowls were used for the experiment (3 bowls/ treatments for the 3 trials).

Lab Experiment

Twelve grams of egg was collected for each treatment including the control. The 12g eggs were separated into 4 different bowls. The already diluted milt was then used to fertilize

the eggs in 3 of the bowls. For the control the dry method of fertilization was used, 1ml of fresh milt obtained with a syringe was directly used to fertilize the egg without the addition of normal saline. The eggs were thoroughly mixed with plastic spoon to ensure proper fertilization takes place after which they were placed on spawning mat for incubation.

Data Collection

Water flow through was done consistently to prevent pollution of the water. Also, water quality parameters were closely monitored. Temperature and DO which were measured *in situ* with WTM, Oxical-SL portable electronic probe, pH was monitored using Hanna Hep pH meter and adequate oxygen level (above 5mg/l) was maintained with RESUN LP- 100 low noise air-pump. Ammonia and Nitrate were measured with the aid of a visible spectrophotometer after it had been treated with Nessler's reagent. Total Alkalinity was measured by titration method.

In collecting the data, part of the sperm sac/lobe was taken to Splendid Stan laboratory, Isiohor Benin City, just to determine that each of the males used per trial were viable. The sperm sac was thereafter lacerated and the milt was collected into a test tube. The milt was measured and diluted with the different volume of normal saline solution. The manual motility estimation method was used. Each sample was estimated using the light microscope at magnification of 400X immediately after addition of normal saline solution. The fraction of the population that was motile was estimated. Only forward moving sperm were judged motile, those simply vibrating or turning on their axis were considered immotile (Oguntuase and Adebayo, 2014). During spermatozoa activation immotile sperm cell (ISC) were counted and when activation stopped, whole sperm cells were counted (Canyurt and Akhan 2008). The motile sperm cells (MC) were calculated as:

$$\mathrm{WSC} - \mathrm{ISC} = \mathrm{MC}$$

$$MC\% = \frac{ISC}{WSC}100$$

The stripped eggs were weighed using a Dura scale D2^{TM} 300g x 0.01g capacity pocket scale (Precision 0.01g). The weight of 12g of eggs was taken and then counted. Fertilization rate was determined 3 hours after fertilization (late morula to early gastrular stage) from sub samples kept (Egwenomhe and Obi, 2012). The fertilized eggs and unfertilized eggs were counted physically for each treatment. Fertilization rate was calculated as;

$fertilization\ rate\ (\%) = \frac{no.of\ fertilized\ eggs}{total\ no.of\ eggs}100$ (Egwenomhe and Obi, 2012).

Actual fertilized eggs (translucent ones with embryonic eyes) were counted by the third hour of incubation. Translucent eggs without embryonic eyes were regarded as unfertilized eggs. The hatching rate in each trial was evaluated 24 – 48 hours, depending on temperature, after fertilization. The number of eggs that hatched from each of the 3 kakabans (with 12g of eggs each) per Treatment was also recorded and means values used to calculate mean percentage hatching rate.

$Hatching\ rate\ (\%) = \frac{no.of\ hatched\ eggs}{total\ no.of\ fertilized\ eggs}100$ (Egwenomhe and Obi, 2012).

The percentage survival rate was determined 3 days after hatching. The percentage survival was calculated following the method of Omitogun *et al.* (2012).

$$\text{Survival } rate\ (\%) = \frac{Ni - Nf}{total\ no.of\ fertilized\ eggs} 100$$

Where;
N_f = number of larvae remaining by day 4
N_i = initial number of hatched eggs

Data Analysis

Data collected were subjected to Analysis of variance (ANOVA) using Genstat (version 2005) software. Where significant differences ($p<0.05$) exist, means were separated using Duncan's Multiple Range Test.

RESULTS

Summary of the Three Trials

Results in Table 1 showed similar trend in all studied parameters where significant differences ($p<0.05$) was observed among treatments. T2 recorded significantly ($p<0.05$) higher values in terms of percentage motility (87.67 ± 2.5166), fertilization (97.55 ± 0. 3208), hatchability (84.78 ± 5.6467), and survival (83.53 ± 1.8086), while T1(control), had the least values of percentage motility (49.00 ± 5.2915), fertilization (75.89 ± 10.6384), hatchability (40.73 ± 7.2705), and survival (45.43 ±5.0552). The percentage values of the studied parameters increased with addition of normal saline up to T2 and decreased with further addition of normal saline solution.

Table 1: Mean value (summary of the three trials) showing percentage motility, fertilization rate, hatchability rate and survival rate of larvae of the studied fish

Dilution ratio	Motility (%)	Fertilization rate (%)	Hatchability rate (%)	Survival rate (%)
T1 100% (1ml M+0ml NS) control	49.00 ± 5.2915^{d}	75.89 ± 10.6384^{c}	40.73 ± 7.2705^{c}	45.43 ±5.0552^{d}
T2 10% (1ml M+10ml NS)	87.67 ± 2.5166^{a}	97.55 ± 0. 3208^{a}	84.78 ± 5.6467^{a}	83.53 ± 1.8086^{a}
T3 0.25% (1ml M+50ml NS)	73.00 ± 1.7321^{b}	93.67 ± 1. 2503^{ab}	65.53 ± 3.5501^{b}	74.49 ± 4.2533^{b}
T4 0.01% (1mlM+100mlNS)	58.00 ± 3.6056^{c}	86.29 ± 3.9923^{bc}	49.23± 7.8897^{c}	62.08 ± 4.7440^{c}

NS = Normal Saline; M = Milt; Mean values in rows with same superscripts are not significantly different (P > 0.05)

Water Quality Parameters

The water quality parameters measured where all within the desirable range as shown in Table 2. The same water quality was maintained for all treatments in each trial.

Table 2: Range of water quality variables recorded during experimental period

Variable	Minimum	Maximum
Dissolved oxygen (mg/l)	5.0	6.7
pH	6.8	7.0
Ammonia (mg/l)	0.19	0.28
Temperature (ºC)	28	29
Total alkalinity(mg/l)	120	127

DISCUSSION

There was significant difference ($P<0.05$) among the treatments in all the parameters measured. The highest percentage motility occurred in T2 and the lowest occurred in T1. The lowest percentage motility obtained in T1 showed that addition of normal saline solution has an effect on the sperm cells as it activates the sperm cells in agreement with the result obtained by Atse *et al*. (2002) who reported that salinity can be a prime factor ensuring male reproduction success in fishes. Okunsebor *et al*. (2016) suggested that when sperm cells of fish are not protected by the fish skin, an isotonic medium must be provided to extend the cell's lifespan; this explains the low percentage motility obtained in T1 with no saline inclusion. The highest motility obtained in T2 shows optimal addition of the solution as further addition meant possible plasmolysis of sperm cells as more volume of saline solution yielded lower results. This is in line with the findings of Orji *et al*. (1997) and Okunsebor *et al*. (2014) which stated that too much saline solution has a lethal effect on the sperm cells.

The highest fertilization rate in T2 can be attributed to the higher percentage of motility of spermatozoan in the Treatment. A direct relationship between percentage motility and fertilization capacity of spermatozoa has been established in many fishes (Billard and Cosson, 1992). When dense milt (containing more count of sperm cells) is used for fertilization, the chance of collision of a sperm with an egg is higher than a milt sample containing lower density of spermatozoa. The high value of measured parameters obtained in moderate dilution at T2 and T3 happened probably because of tolerable salinity and wider spermatozoa distribution Ukwuani *et al*. (2016). The low fertilization rate at T4 was probably due to an exceeded tolerable range in salinity beyond the 0-2.5 ppt recommended for larvae of *C. gariepinus* by Chervinski (2006). Non normal saline inclusion probably provided little volume when compared to the quantity of eggs available for fertilization and as a result the spermatozoa probably did not distribute widely over the surface area of the eggs to ensure high rate of egg fertilization resulting in many unfertilized eggs (Ukwuani *et al*., 2016), this explains the result obtained in T1. For the fertilization to be more effective, addition of more milt or dilution of milt using Normal saline will be required.

The highest percentage hatchability occurred in T2 (84.78 ± 5.6467) and the lowest in T1 (40.73 ± 7.2705). The low percentage hatchability in T4 might have been as a result of excess salinity of the milt solution which may have led to some denatured fertilized eggs due to an exceeded tolerable range (Chervinski, 2006) in salinity for a fresh water species, this is in agreement with the result obtained by Okunsebor *et al*., (2014) and Ukwuani *et al*., (2016). The high percentage hatchability in T2 and T3 might be due to improved incubation condition which occurred at moderate range of salinity.

There were significant differences ($P<0.05$) in the measured parameters across the treatments. The percentage survival rate decreases with the increase of normal saline solution in T4 and increase with the decrease of normal saline solution in T2 and T3 i.e., as the salinity tended towards that of fresh water with less quantity of saline water per ml of milt. This may be attributed to the adaptation to fresh water habitat of this species. The result obtained was in line with that obtained by Ukwuani *et al*. (2016), that an inversely proportional relationship exists between increase in salinity and survival rate of the hatchlings. The result obtained in T4 is in agreement with that obtained by Ogunseye and Sogbesan (2004) which stated that survival is optimum in low levels of salinity considering the fact that *C. gariepinus* fry is a freshwater fish at a developing stage of growth, higher concentration of salinity can lead to stress and death at this stage due to osmotic effect. Orji *et al*. (1997) in a study on

Heterobranchus bidorsalis reported that it was possible that lower salinity might have given a better result. They opined that the lower salinity tolerance might be based on the fact that *H. bidorsalis* was a freshwater fish and higher salinity would probably result in the shrinkage of the cells and result to death. *C. gariepinus* just like *H. bidorsalis* is a freshwater fish and its survival could be affected by high salinity.

CONCLUSION

Although normal saline solution plays a vital role in the successful artificial propagation of *C. gariepinus*, the quantity used can have an effect on the potency of the spermatozoa, fertilization and hatchability of the eggs and survival rate of the hatchlings. Better performance was achieved when the milt was diluted with normal saline solution with optimum values obtained at 10% inclusion level. A dilution ratio of 1:10 (milt to saline water) is thus recommended as the optimal dilution rate in the artificial propagation of *C. gariepinus.*

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