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Evaluation of sperm mobility activation media for egg fertiliztion in locally bred American channel catfish (*Ictalurus punctatus*)

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Abstract
Fish sperm motility is now thought to be the prime indicator for the peculiarity of fish spermatozoa. Sperm motion characteristics from over 305 different fish species have been published in over 1450 scholarly articles spanning a wide variety of issues, from molecular biology to ecology. The purpose of the current study was to evaluate the impact of two activation media (A, B) created in the lab and distilled water as a control on male American channel catfish milt quality and fertilization rate (*Ictalurus punctatus*). Brooders were developed from locally bred catfish seed and selected on the basis of maturity at Aquaculture and Fisheries Program, National Agriculture Research Center (NARC), Islamabad Pakistan. The experiment used a complete randomized design (CRD) with three replicates for each of the three treatments: media A, B, and Control (distilled water). As considerable number of sperms are present in every microliter of sperm, hence several forms of activation or dilution media were utilized to utilize sperm or milt more effectively. Following milt collection, it was diluted in a ratio of 1:29 with the aforementioned matching activation media before being subjected to *in vitro* testing for a number of parameters, including sperm mobility, sperm mobility duration, and sperm viability. The same diluted milt aliquots were used to check fertilization rates, and the findings showed that the components were substantially different in the specified conducts ($p<0.05$). The sperm/egg aliquot treated with medium "B" had the highest mobility/motility and fertilization rates (81.66%±2.88 - 85.33%±2.08), followed by media "A" (73.33%±2.80 - 71.66%±2.08), and control (68.33%±2.8 - 67.33%±2.08). Sperm viability and concentration in A, B, and C, or the control, were likewise substantially different ($p < 0.05$) from one another. The media "B" had the greatest values (61.66%±2.53 -2.50±0.14), followed by "A" (57.33%±2.51 -2.37±0.76), and "Control" (48.33%±1.15 -1.93±0.45). The length of the sperm mobility duration varied considerably ($p < 0.05$) across all treatments. It was measured in seconds and was 310sec±32.94 for the control, 565sec±42.78 for media "B" alone, and 469sec±9.74 for media "A" with means + Standard Deviations.

Keywords: Activating Media (AM), *in vitro* fertilization (IVF), complete randomized design (CRD), micro liter (µL), sperm mobility, Sperm Viability

Introduction
The efficiency of reproduction depends on the quality of both gametes (eggs + sperm) Bobe, J and C. Labbe. 2010 [3]. This is the foremost aspect that donates to fruitful fertilization and development of viable descendants. (E Rurangewa, F. Olivier 2004) [9], the appropriate estimation of gamete quality is the best implement for defining the potential fertility of a sample. Using sperm from multiple males is in general in the production of many commercial species. However, as a technique for aquaculture research, measuring fish sperm motility: a historical approach, Juan F. Asturiano 2018. Male can lead to sperm competition resulting sperm variability and, consequently, Males cannot all contribute equally to the gene pool Bekkevold D, Hansen MM, and Loeschke 2002. In this intellect, the precise estimation of individual spermatozoa features, such as motility, is necessary to achieve biased info concerning separate male fecundity potential. However, there are many innovations that can provide a reliable sperm analysis, Kime DE, *et al.* Computer-assisted sperm analysis (CASA) 2001 Gallego V, *et al*., 2013.

Sperm activation is crucial for reproduction because it allows sperm to access eggs during their brief period of motility and fertilize them (Dzyuba and Cosson, 2014) [8]. Inducing spawning motility is crucial for assessing the quality of the sperm as well as for creating research methods to address sperm competition. The motility of fish sperm is activated by a variety of physicochemical factors, all of which are crucial. Most freshwater fish are given hypotonic solutions to encourage movement, while saltwater fish are given hypertonic solutions (Alavi and Cosson, 2006). For euryhaline species, such as Medaka (*Oryzias latipes*) (Yang and Tiersch, 2009a) and Tilapia (*Oreochromis mossambicus*) (Linhart *et al*., 1999) [11] hypotonic, isotonic, or hypertonic media can be used for sperm motility activation. In specific osmotic pressure ranges, electrolytic and non-electrolytic fluids can activate motility in some species (Gallego Albiach, 2013; Morisawa, 2008) [10]. Concentration level of ions are necessary to start motility in some species, either in addition to or instead of osmotic pressure. For instance, in salmonids, elevated K+ concentrations significantly decrease sperm motility prior to production, while Ca+ acts as an antagonist to this inhibitory effect (Alavi and Cosson, 2006).

The catfish are becoming more common in the culture system due to their rapid growth, consumer favor due to their high export potential and high-quality flesh with few spines (Mingkang, 2005). American channel catfish (*Ictalurus punctatus*), one of Pakistan's high-value fish species, was successfully introduced there in aquaculture (Rab *et al*., 2007).

For the purpose of producing seed stocks, channel catfish (*Ictalurus punctatus*) are bred using pond, pen, and aquarium

culture techniques (NASS, 2004) [15]. Any artificial media that improves sperm activation and shields them from extremely high osmotic conditions will result in a longer-lasting increase in sperm motility (Cosson, 2004) [6]. Since stripping channel catfish by hand is impractical, the males are slaughtered, and testicles are removed from the body to squeeze out the milt (Legendre *et al*., 1996).

Assessment and establishment of fish semen quality parameters would greatly facilitate adoption of various reproductive technologies in aquaculture, (Alavi *et al*., 2008). Therefore, this study compared the effects of two types of laboratory-prepared activating solutions with different chemical configurations, such as potassium chloride (KCl), sodium chloride (NaCl), and tris, to the control, which is distil water, on the mobility, viability, and concentration of sperm on the dimensions of fertilization in Channel catfish.

Methodology

Study area: The investigation was done between April and July 2021 at the National Agricultural Research Centre (NARC) Islamabad, National Aquaculture and Fisheries Program (AFP), Animal Science Institute (ASI), Pakistan Agricultural Research Council (PARC), Ministry of Food Security & Research Islamabad, located in the Potohar neighborhood close to Check Shahzad National Institute of Health. Additionally, there are earthen research ponds, cement raceways, hatching troughs, fiberglass circular tanks, tube wells, and hatcheries for producing channel catfish and tilapia fish seeds.

Experimental layout

In this study, In locally bred American Channel catfish, sperm mobility or motility and egg fertilization rates were assessed in response to A and B, two types of experimentally sperm motility activation solutions or media, as well as C, or pure water (control) (*Ictalurus punctatus*). The experiment's CRD (Complete Randomized Design) consisted of three groups (A, B, and control) and three replicates.

Preparation of media

At the Aquaculture & Fisheries Program (AFP), activating solutions (media) were recommended in accordance with (Bastami, 2010). Separate classifications, with various chemical (Merck) conformations in accordance, and largely by the quantity of potassium k+ ions, which includes the chemical Tris, in 10 milliliters (ml) of distillate water, while distil water served as the control otherwise.

In order to create Media "A," the following ingredients were added to 10 milli liters (ml) of distilled water: 46 milli molar (mM) NaCl correspondent to 0.0264 gm, 5 milli molar (mM) of KCl correspondent to 0.00369, and 29.76 milli molar (mM) Tris correspondent to 0.0365 gram.

In order to create Media "A," the following ingredients were added to 10 milli liters (ml) of distilled water: 45 milli molar (mM) NaCl correspondent to 0.0290 gm, 30 milli molar (mM) of KCl correspondent to 0.0320, and 30 milli molar (mM) Tris correspondent to 0.0361 gram.

Experimental Procedure

Brood Stock Preparation and Selection

At the Aquaculture & Fisheries program, NARC produced ponds, American Channel catfish brooders were created. They were fed artificial diets with a 30–35% Crude Protein (CP) content. On the basis of maturity approaches, we identified and chose six male and six female Channel Catfish brooders, with average weights of (720-950) g for females and (930-1100) g for males, respectively. During the experiment, all the fish samples were healthy, nearly disease-free, and entirely ripened at their state during the breeding season, which is May–August. The fish were taken to the lab as soon as they were caught for dissections and the collection of gametes for testing sperm motility and egg fertilization.

Sperm Mobility

Sperm mobility was examined using glass microscope slides and a diluted solution made by combining one liter of milt with 29 liters of activating medium. It was then magnified 100 times while being seen under a microscope on a slide with a cover slip. The percentage of progressively mobile spermatozoa in each stimulated field served as a proxy for sperm mobility. Only spermatozoa that are vigorously stirring were considered to be motile, while other trembling cells in the same field were rated as immotile.

Mobility Duration

Sperm mobility time is measured as the length of time it takes for all spermatozoa cells to go from fully mobilized to entirely dead. A 31 micro liter sample of diluted sperm (1.5 µL milt + 29.5 µL activating solution = 31 micro liter) was reserved from a chosen aliquot on a glass slide, and using a cover slip, the motility time was monitored under a 100 X magnification. In laboratory settings, the parameters of motility and its duration were both monitored at regular intervals of one second. Samples were tested every one to two seconds to gauge the percentage of mobilized spermatozoa until every last one was discovered to be dead.

Sperm Viability

Trypan blue (0.4 percent) solution was made by dissolving four milli gram (mg) of trypan in one milli liter (ml) of distilled water. This solution was used to assess the viability of sperm using the stain method. The stain was continuously filtered before use. A smear was made on a glass slide by mixing together µl of sperm and 1 µL of dye. The mixture was then allowed to air dry for 2–5 minutes before being examined under a microscope at a magnification of 40X. Live spermatozoa with intact membranes remained uncolored when milt was added to the stain, whereas dead sperms with damaged membranes stained blue. The following formula was used to further calculate the vitality of sperm:

$$\text{Spermatozoa Viability} = \frac{\text{No. of viable spermatozoa}}{\text{Total no. of sperm counted}} \times 100$$

Concentration/micro liter

Neuberger hemocytometer was used to calculate the concentration of spermatozoa in accordance with the recommended procedure. A micropipette was used to load a

total of 31 µL of diluted solution (1.5 µL of milt + 29.5 µL of media) onto the hemocytometer. The pipette tip was inserted into the hemocytometer's V-groove during loading. The sample was placed at lab conditions to settle the movement of spermatozoa. Using a 40 X magnification, the spermatozoa in the four corner squares and in Central Square were counted to determine the concentration. The sperm concentration per microliter was determined using the formula below:

Sperm cells that are viable per square x the dilution factor x 104.

Eggs Collection & Fertilization Rates

Specimens were taken from males and divided into three aliquots according to the measured sperm concentration/microliter. Eggs were taken from selected females and divided into three batches 01gm totaling about 220 eggs. Then a total of 30 µL of the ready solution was added. To keep the spermatozoa concentration per microliter constant across nearly all of the treatments, different amounts of milt were utilized.

In the current study, visual evaluation of fertilization rates was done by comparing the ratio of the number of eyed eggs to all eggs after fertilization. Eggs were combined with this diluted milk. For optimum fertilization, new water was added after 5 minutes of mixing, and it was left to stand for 10-15 minutes. Dead or unfertilized eggs appear impermeable, whereas water-hardened and fertilized eggs were brilliant and translucent in color, measuring three to five milli meters in diameter. The following formulas were used to calculate fertilization rates.

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

Statistical analysis

The resulting data was presented as means and standard deviations (S.D). For significant $p < 0.05$ comparisons of average means, one-way ANOVA (variances of analysis) and Duncan's Multiple Range Test (DMRT) were employed to examine the effects of the media.

Results

Examined how two lab-made sperm mobility activation media affect American channel catfish's (*Ictalurus punctatus*) sperm mobility over time and viability and fertilization rates when compared to control (distilled water).

Sperm Mobility

The current study's findings showed that within the experimental activation media within two treatments and in comparison, to control, distilled water, the highest values found significant $p<0.05$ effects within B, i-e 80.89%$\pm$ 1.96, followed by A, i-e 72.13% $\pm$1.85, and distilled water (control), was 67.33% $\pm$1.91. Presented in (Fig. 1) together with averages and standard deviation (S.D).

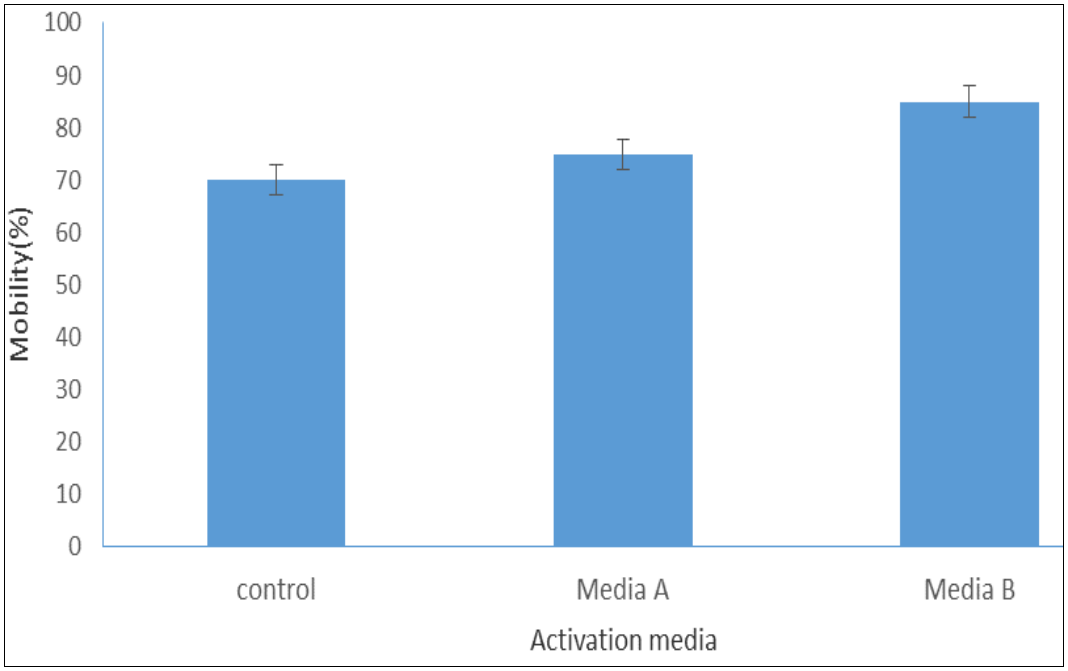

Fig 1: Effect of different activating media on percent sperm mobility (means ± S.D)

Sperm Mobility Duration

Calculated in seconds, duration recorded with A. 472 sec±9.20, B. 565sec±41.59 and control, 310sec±31.65 seconds, significantly $p<0.05$ dissimilar from each other. Presented in (Fig. 2) together with averages and standard deviation (S.D).

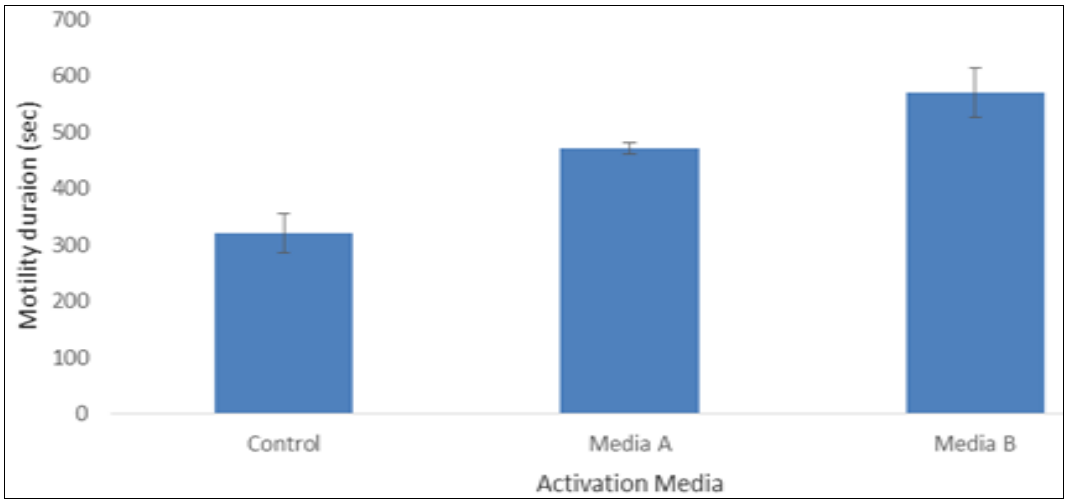

Fig 2: Effect of different activating media on sperm mobility duration (means ± S.D)

Sperm Viability

Standard Deviation (S.D.) in A.57.34%, B. 61.67%, & C. 48.34%, with highest B at 61.67% and others listed with average with means (Fig.3). Within the medium A, B, and C (control), viability varies significantly (significantly $p< 0.05$).

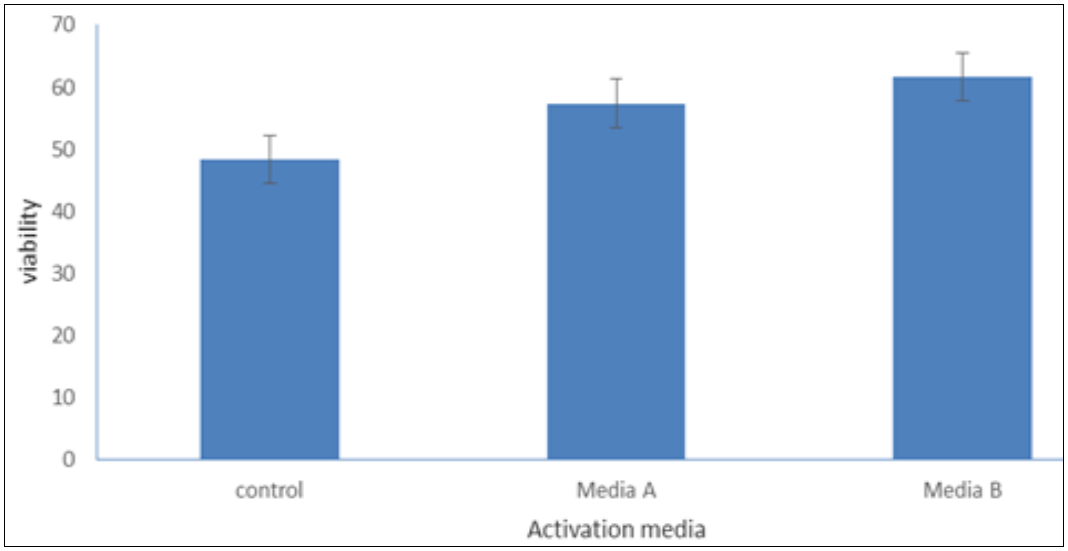

Fig 3: Effect of different activating media on percent sperm viability (means ± S.D)

Sperm Concentration/micro liter

Sperm concentration was determined to adjust the almost equal concentration of sperms in each aliquot of dilution media for determining the fertilization rate. The value of concentration was 2.37x 10 ^{4}±0.76, 2.50 x10 ^{4}±0.14 and 1.93x10 ^{4}±0.45, for media A, B and control.

Fertilization rates

Fertilization rates for media A, B, and distil water (control) individually under the almost equalized concentration of spermatozoa obtained were 72.06%±2.58, 84.93%±2.16, and 67.33%±2.08. The activation media B showed the highest percentage of fertilization rates, or 84.97%, followed by A and C (control) respectively. The fertilization rates in all of the treatments were shown to differ considerably ($p<0.05$) from one another, according to data discovered through statistical analysis. Additionally, data are shown with means and Standard Deviations (S.D.) in (F.4).

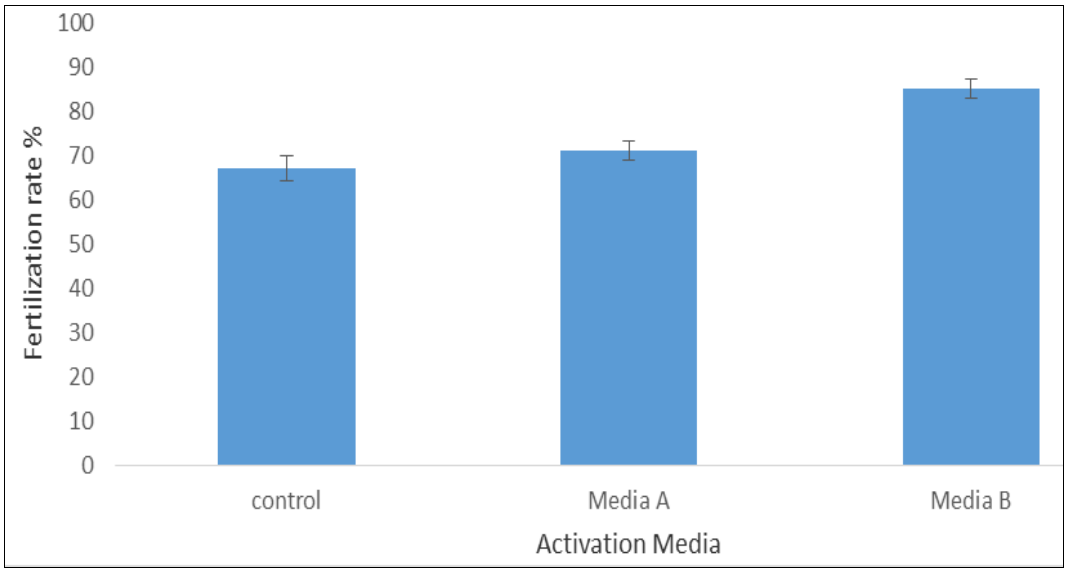

Fig 4: Effect of different activating media on percent fertilization rate (Means ± S.D)

Discussion

The results of the current research suggest that milt mobility and fertilization rate in American Channel catfish are significantly influenced by the type of activating solutions (AS) utilized. The success of fertilization is strongly correlated with the quality of gametes (Bobe and Lobbe 2010) [3], the activating solution employed (Kucharczyk *et al*. 2010; Zarski *et al*. 2012) [8], the sperm to ova ratio (Linhart *et al*. 2006), and the period of time active sperm and active eggs are in contact (Liley *et al*. 2002). Numerous studies have been done to investigate the effects of various activation media on spermatozoa's motility activation (Billard 1983). Although very little is known about how various activating solutions affect the efficacy of fertilization (Zarski *et al*. 2012a) [8].

In the presence of semen plasma or isotonic solutions, the spermatozoa of the most of freshwater fish species are immobile; therefore, they must be diluted with a peculiar medium in order to achieve optimal motility. The current study's objective was to ascertain the optimal activating solutions for Channel catfish sperm and motilities utilizing laboratory-prepared activating solutions/media to *in vitro* fertilization (Kzem Darvesh Bastami, 2010, Sperm of Feral Carp, gulfam, Activation Solution Optimization). 18(5) 771–776 (Aquaculture International, August 2010). The use of several solutions for the *in vitro* fertilization of crucian carps is described in (Daniel Zarski *et al*. Aqua. int. 22, 173–184, 2014). Sperm motility was high immediately upon dilution (0 h) (range 82.4-94.3%) in all ASP variants and even reached 98.4% in the control group using fresh sperm, but it considerably reduced with time. Beata Irena Cejko and colleagues investigated the impact of sodium, potassium, and the ideal pH on the activation of sperm motility in common carp L. during short-term storage in synthetic seminal plasma. In the current investigation, fresh sperm motility was observed to be 81.66% utilizing activation media that contained 10 milli liters (ml) of pure water, 50 milli molars (mM) of NaCl, 30 milli molars (mM) of KCl, and 30 milli molars (mM) of Tris.

The longest period of sperm motility, 570 seconds, was attained in the current investigation. Duration was reached in gulfam and zebra fish up to between 10 and 15 minutes using the same sort of activation material (Bastami *et al*., 2010). Other findings, such as 540s using sodium citrate, were also obtained (Cosson 2011). In addition to sperm viability testing, the percentage of spermatozoa that were alive and dead was determined using a trypan blue stain method (0.42 percent) milt trial, and the error was discovered using a 40-X powerful microscope. Similar research was conducted by (Rurangewa, 2002), who determined the sperm stain by calculating the number of whole (alive and injured red-stained) spermatozoa. Additionally, it was discovered by Morisawa (1980) [13] that K ions boosted sperm survival and sperm ambition speed at a lower absorption in the seminal plasma, where Na ions and none - electrolytes were less potent. According to (Sule and Adikwu, 2004), larger, more mature eggs also have a higher viability for sperm than smaller ova. on the other hand, catfish lay larger eggs.

In the current study, American Channel catfish fertilization rates were found to be around 85.11% with mean standard deviations 2.78% in media (B). Hossain (2014) [14] described the linked effects of significantly high fertilization rates, or 86.7%. With particular therapies, it can be higher than usual (More, 2010). The object may be identified by the significant changes in hormone dosages, as well as by the size of the brooder and seasonal differences (Nwokoye, 2007). The proportion of eggs to sperm and the superiority of the gonads are directly related to fertility rates (Bobe & Labe, 2010) [3]. (Linhart, 2006), the length of time that active sperm contact with active eggs (Lily, 2002), and the activation agent utilized (Zarski, 2012a) [8]. For artificial insemination of African catfish Clarias, E. Ruranwa *et al*. (2005) found that a ratio of 1500 spermatozoa to 1 egg produced fertilization and hatching rates of 80 and 68%, respectively.

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