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Review Article

Cryopreservation and artificial insemination in African Catfish (*Clarias gariepinus*, Burchell 1822): A review

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Abstract: *Despite the high potential for the production of African Catfish, Clarias gariepinus, and its market demand there exists a serious lack of fish fingerlings to supply for producers and (re)stock water bodies. Unlike other species, the traditional method of obtaining C. gariepinus milt is sacrificing the male, removing its testes and macerating over the stripped and collected eggs. This is a loss for a farm as male brood stock is going to be killed every time. The C. gariepinus fish shows seasonal gonadal maturation that is usually associated with the rainy season as the hormonal level increases during this season. Recent efforts are becoming successful in multiplying C. gariepinus artificially by fertilizing the striped eggs with preserved sperm. The preservation of sperm is a means to ensure year-round availability and supply of fingerlings and overcomes the scarcity of seed. Fish sperm can be preserved in dry ice, freezing the semen and storing the frozen semen in liquid nitrogen (cryopreservation). The preservation period of fish male gamete is usually short, compared to mammals due to its biochemical structure and temperature exposure effects on the sperm cells. Hence, the paper focuses on reviewing the efforts so far made on the preservation of male C. gariepinus and the use of the preserved semen for insemination. It also addresses the methods to evaluate the quality of sperm, milt collection and preparation as well as the amount of sperm used to fertilize an egg. The aim of this review is to gather the efforts made so far on the amount of sperm required and parameters considered while evaluating the sperm, cryopreservation and artificial insemination in C. gariepinus and provide the available information for the hatcheries to have an alternative means of getting milt without sacrificing broods in the hatchery. Milt can be collected easily by dissecting the selected and matured testes with scissors, removing the two testes, cutting each testis into smaller pieces and squeezing it in a loosely woven cloth. This milt can, then, be placed in a freezer and the frozen semen can be preserved in liquid nitrogen at a temperature of -196 ^{0}C. The ratio of semen to egg in C. gariepinus fish is recommended at 6 to 24x10^3 semen to an egg. Cryopreservation of C. gariepinus semen in liquid nitrogen invariably helps to conserve the genetic resources of desirable male fish for future use any time when the females are ready.*

Keywords: Broodstock, Courtship, Fish, Milt, Spermiation
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1. Introduction

Catfish is a diverse group of ray-finned fishes representing more than 3000 species, 478 genera, and 36 families (Teugels, 1986; Ferarris and Pinna, 1999). The Clariid freshwater fishes belong to the order Siluriformes with a wide geographical distribution in Africa. Although Teugels (1984) described more than 100 different species of the Genus Clarias in Africa, a recent systematic revision based on morphological, anatomical and biographical

studies recognizes 34 species and two of these species, *Amphilius lampei* and *Chiloglanis modjensis*, are endemic to Ethiopia (Fishbase.org., 2020). The African catfish (*C. gariepinus)* is native to Africa and one of Africa's most suitable species for aquaculture. Since the 1970s, it has been considered to hold a great promise for fish farming in Africa.

The African catfish is hardy and adaptable to diverse environments even with poor water quality and lower oxygen levels due to its air-breathing ability (Hetch *et al.,* 1996), high growth potential at intensified stocking densities (Basharat *et al*., 2020) and has higher biomass compared to Nile Tilapia, *Oreochromis niloticus* (Shaw *et al*., 2022). Some other merits of *C. gariepinus* fishes are the higher growth rate reaching a market size of 1 kg in 5–6 months under intensive management conditions: high adaptability and resistance to handling and stress; can be artificially propagated by induced spawning techniques for the reliable mass supply of fingerlings; commands a very high commercial value (Olaleye, 2005). However, the procurement of reliable broodstock (of good genetic quality), fingerlings and juvenile fishes for stocking fish farms have been a major setback in the development of catfish culture in Africa (Omitogun *et al*., 2012). This is because these cultivable species are not easily obtained from the wild like the *O. niloticus* which reproduces every 25-30 days (Tahoun *et al*., 2008).

The supply of fish in Ethiopia is still very small and cannot sufficiently satisfy the increasing demand of its population that projected at 118,959,000 in 2027 (CSA, 2013). The *C. gariepinus* has a very high demand and market value at Gambella and the eastern part of Ethiopia and the diplomatic community of the African states in Addis Ababa. The preference and market value of African catfish is more than double in Metehara and Gambella towns compared to the *O. niloticus* fish (Yalew and Spliethoff, 2016). To solve the populace's high demand for fish, Ethiopians need to resort to aquaculture that faces major constraints, including a lack of quality fish seed and feed (Natea, 2019). The scarcity of good quality *C. gariepinus* broodstock requires the need to conserve the fish's genetic resources from selected parents' fishes killed indiscriminately during fishing practices. One way of solving the scarcity of selected (both in number and quality) *C. gariepinus* fingerlings for aquaculture in the country is by devising the means to preserve fish gametes and capable of hatching fry to ensure a year-round supply of fish seed through cryopreservation. The objectives of this paper is to make a review of the efforts made on the cryopreservation of semen and artificial insemination of *C. gariepinus* fish and generate relevant scientific information for the hatcheries to have an alternative option to get milt without losing the life of male parents.

2. Reproduction in African Catfish

The *C. gariepinus* can be reproduced both by artificial means and naturally through fulfilling the requirements that nature can provide (Bruton, 1979). In nature, the *C. gariepinus* reach sexual maturity after 2-3 years of age (Hogendoorn and Vismans, 1980; Bruton, 1996) and between 7-10 months of age in captivity, of which males mature earlier than females (Legendre *et al*., 1996) and shows seasonal gonadal maturation (de Graaf and Janssen, 1996).

The annual changes in water temperature and photoperiodicity influence the maturation process of *C. gariepinus* and the final triggering agent for spawning is a rise in water level due to runoff water (de Graaf *et al*., 1995) due to the changes and elevation in level on the hormone responsible for spawning during this season (Goos and Richter, 1996). As the rain commences the *C. gariepinus* fishes get stimulated and migrate pairwise for mating (de Graaf and Janssen, 1996). When rivers and streams flood to shallow vegetated areas, which is an ideal condition for the fish to get the appropriate site to spawn and put the fertilized eggs.

During this time the fish performs a courtship full of phenomenon. While in a courtship the male gets attracted, follows the female and bends his body in front of the female's head in a U-shaped posture (Figure 1) where the female enters in the amplexus, stays for 7-10 seconds and performs mating (Olshanskiy *et al*., 2009). During mating, the female bent the anterior part of its body to the right or left, depending on where the male's head was turned to, pressing the caudal part of the male's body to the bend of the female's body (de Graaf, 1994; Olshanskiy *et al*., 2009). The eggs spawned immediately as the male began to detach itself from the female by straightening the anterior part of the

body and pressing against the abdomen of the female (Olshanskiy *et al*., 2009).

Spawning in the *C. gariepinus* takes place at night and a batch of milt and eggs is released followed by a vigorous swish of the female's tail to distribute the eggs over a wider area. In *C. gariepinus*, parental care is not exhibited and hence the survival of the offspring is ensured by the selection of an appropriate site. The development of eggs and hatching of the larva is rapid. The hatchlings can swim within 48-72 hours after fertilization depending on the temperature of the culture water.

The fecundity of this fish varies between 30,000 - 90, 000 depending on its size. An adult and ripe female can spawn up to 70,000 per kg body weight, which is expected to balance the non-parental care of the hatched larvae (De Graaf and Janssen, 1996; Hogendoorn, 1977). The sooner after the spawning migration, the parents return back to the lake or river while the juveniles remain in the inundated area till they grow and attain a size of 1.5 and 2.5 cm long (Witte and van Densen, 1995).

However, the natural way of reproduction cannot supply the required number of fingerlings as there is no parental care, like *O. niloticus* (mouth brooders) fishes. Due to the increased fish demand, it is more than important to investigate the large-scale spawning of commercial fish species in order to culture them and/or restock water bodies. Most of the suitable areas of *C. gariepinus* breeding areas are being changed and threatened by anthropogenic effects (mainly crop farming, overgrazing, settlement, urbanization, and destruction of the aquatic plants). This is reducing the population of the *C. gariepinus* fishes in many water bodies in Ethiopia. The establishment of aquaculture to supplement the dwindling protein resources of the world has necessitated the development of large-scale artificial spawning techniques for a number of fish species. Aquaculture techniques enable the brood *C. gariepinus* to be transferred from nature to the hatchery, inducing them artificially using a variety of hormonal treatments to spawn (Omitogun *et al*., 2012; Tkacheva *et al*., 2020).

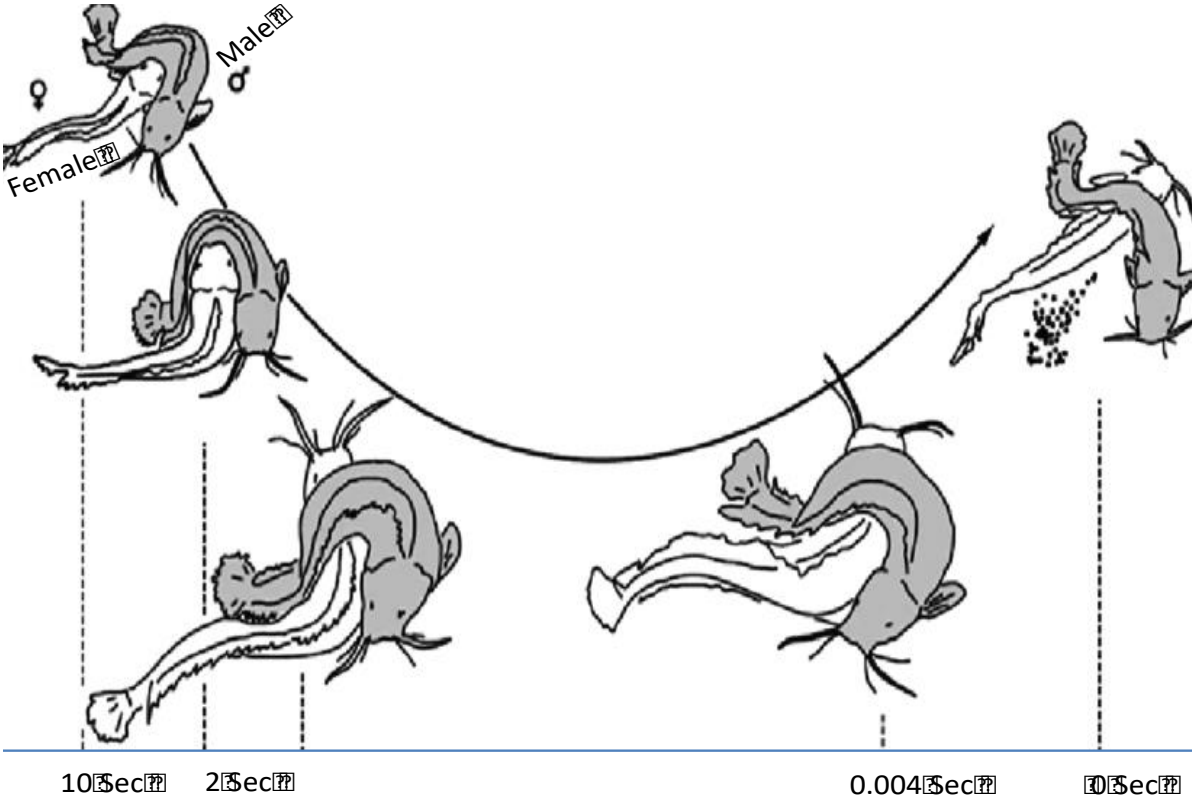

Figure 1: Courtship and mating ritual in the catfishes (modified from Olshanskiy *et al*., 2009)

3. Fish Sperm Collection and Management

Fish sperm preservation has contributed to the development and application of methods of reproductive control by favoring genetic manipulation, broodstock selection, and reduction of male stock demands since it provides gametes for future uses (Migaud *et al*., 2013). The development of methods of semen collection, preservation and the implementation of effective artificial reproduction protocols require a good knowledge of reproductive biology and seminal characteristics (sperm concentration, semen color and volume, sperm morphology) of fishes. Sperm can be preserved both naturally (*In vivo*) and artificially (*In vitro*) for future use. Naturally, sperm can be stored either in the testes or in the vas deference until being spawned. Studies showed that sperm motility as well as fertilizing ability decreased during the period of spermiation (liberation of mature spermatids) leading to the conclusion that gamete development has a series of stages to occur (Billard and Cosson, 1992). Therefore, this decrease in sperm quality in the course of spermiation shows that *in vivo* preservation is somewhat deficient and does not keep the sperm at its initial state.

In the neotropical species of interest for fish breeding, the possibility of *ex-situ* genetic preservation has been proved. The efforts made so far include; the evaluation of fresh semen of the *C. gariepinus* and the cryopreserved semen in dry ice, the studies towards the conditions for the preservation of Curimbata (*Prochilodus scrofa*) semen and also the freezing and cryopreservation of Piau (*Leporinus silvestrii*) semen, the cryopreservation of the Lake Tana endemic species, *Labeobarbus breciphalus*, the freezing of Pacu (*Piaractus mesopotamicus*) semen and the sperm evaluation, cryogenic preservation, semen fertility of *P. mesopotamicus* are noteworthy (Ninhaus *et al*., 2006; Galoa *et al*, 2017; Abdissa *et al*., 2022).

The availability of gametes throughout the year is important to ensure a constant supply of fish juveniles. In pond or tank conditions, at a temperature of 25^0C and 12 h light per day, *the process of gamete development in sexually mature C. gariepinus* is continuous (Huisman and Richter, 1978). Spermatogenesis and male reproductive behavior do not take place spontaneously (Van Oordt *et al*., 1987) like in females, even after the fish is induced with spawning hormone (Omitogun *et al*., 2012). Unlike males, females can be stripped of eggs after treatment with pituitary extracts or other hormones (Huisman and Richter, 1987; Omitogun *et al*., 2012). Accessing the sperm of the *C. gariepinus* by stripping the abdomen is impossible due to the physiology of the fish and hence it is a must to kill male brood fish or surgically remove the testes (Tkacheva, *et al*., 2020). Storing batches of spermatozoa by appropriate preservation methods significantly improved the reproductive potential of male *C. gariepinus* (Omitogun *et al*., 2012). The development of preservation procedures for sperm of *C. gariepinus* aids in the recovery of threatened and endangered species as well as in the genetic selection and maintenance of lines of selected stocks (Hatipoglu and Akcay, 2010; Omitogun *et al*., 2012).

Gamete preservation is usually short (several hours to several days) at a temperature above 0^0C. At subzero temperatures, sperm fertilizing ability may be kept for several weeks. Techniques of sperm preservation in liquid nitrogen (cryopreservation) are now established for catfish species. Fish sperm preserved under cryogenic temperature can be employed for the improvement of the genetic make of fish species through selective breeding, production of foundation stocks and hybrids, and reducing the number of broodstocks to be kept and labor involved in maintaining them (Bozkurt *et al*., 2005). For the successful preservation and qualitative control, advanced knowledge of the structure of spermatozoa is required. In fishes of internal fertilization, spermatozoa usually display an elongated head and a more elaborate and structured one and in species with external fertilization, spermatozoa commonly have a round, or oval head, and a small midpiece (Ninhaus *et al*., 2006). However, recent studies indicated that species with higher levels of sperm competition have faster sperm with longer flagella relative to the head length (Ito *et al.*, 2022).

In spite of much progress in the field of cryopreservation of fish sperm, fertilization results vary on the cryopreserved semen. The fertilization rate also varied between the cryopreserved semen and that of the fresh one. Kovacs *et al*. (2010) proved

that the highest fertilization rate with cryopreserved sperm is when eggs are fertilized with sperm activated for 20 seconds. According to Viveiros *et al*. (2000) hatching rate of cryopreserved semen is equal to fresh spermatozoa frozen at -5^{0}C/min. Kovács *et al*. (2010) confirmed that the cryopreserved sperm was proven to give significantly higher fertilization percentages than freshly extracted semen. Other studies indicated that the fertilization percentage between the cryopreserved and fresh sperm does not have a significant difference as far as the appropriate cryoprotectant and concentration is used (Viveiros *et al*., 2000; Muchlisin *et al*, 2015; Schachter-Safrai *et al*., 2017). However, most studies achieved higher fertilization percentages while using freshly extracted semen than the cryopreserved one (Galoa *et al*, 2017). Fertilization success on the cryopreserved semen is basically influenced by the extender used and dilution rate, equilibration duration, process of cryopreservation, management of the cryopreserved semen, thawing process and temperature, and activation time during fertilization (Viveiros *et al*., 2000; Kamaruding *et al*., 2014; Schachter-Safrai *et al*., 2017; Doğan *et al*., 2023). When using freshly collected sperm, water temperature, quality of the fish sperm, and timing during mixing with the egg are the key factors for fertilization (Galoa *et al*, 2017).

3.1. Sperm viability

Viability of the sperm is the crucial component of any fruitful animal production operation and the process of reproduction is successful with the availability and supply of high-quality gametes (Cruz-Casallas *et al*., 2005; Solomon and Ataguba, 2015). Sperm viability is a measure of counting the number of fertilization-capable sperm and evaluating the proportion of sperm with intact cell membranes prior to insemination (Kommisrud *et al*., 2020). Lower viability occurs when there exist changes in physicochemical characteristics of culture water, fish are exposed continuously to xenobiotics and endocrine-disrupting compounds like ethinylestradiol, higher concentrations of some metals (like Cu, Fe, Mn, Zn), and differences in nutrition (Gárriz and Miranda, 2020; Fritze *et al*., 2021). Siring success is of particular interest in sperm viability and studies confirmed that courting males have higher sperm viability than small males that sneak copulations (Smith, 2012). The effect of sperm viability on siring success also depends upon the duration of time sperm were stored. Sperm quality is highly variable and depends on various internal and external factors (Kime *et al.*, 2001; Khara *et al*., 2012). According to Kime *et al* (2001), the factors affecting sperm quality in fish include rearing photoperiod and temperature, feeding regime and the quality of the feed, water and food contamination, stress, age of broodstocks, breeding season, diseases of broodstocks, hormonal induction and spermiation. The age of parent fishes could be associated with their body weight and that plays a greater role in determining the time required for a sperm to mature.

As preserving gametes for a certain time incurs a cost, their viability should be checked before processing it to preserve. The sample sperm has to be taken and the ionic composition and osmolality have to be investigated before preservation (Alavi and Cosson, 2006). The development of appropriate activation media; preparation of immobilization solutions and cryoprotective agents; as well as fixing equilibration time, cooling rates, sperm packaging unit, semen: extender ratio, storage vessel and thawing rates (Scott and Baynes, 1980) have to be done priorly. The important precautions that help to prolong sperm viability include prevention of desiccation, lowering the storage temperature to below 0^{0}C, optimizing the gaseous atmosphere as an oxygen atmosphere can be sub-optimal, securing sterility by using sterile dilution media or antibiotics and avoiding the contamination of sperm with urine (Jenkin and Tiersch, 1977; Billard and Cosson, 1992; Cierezko and Dabrowski, 1994). Preservation parameters should be set so that sperm is judged as viable and fulfills the requirements. Parameters used to estimate the viability of gametes include fertilizing ability, motility and others (like respiration, and minera1 content of the seminal plasm) (Rurangwa *et al*., 2004).

3.1.1. Fertilizing ability

The quality of the male gamete is the most important factor for the success of reproduction in fishes and hence preservation of fish under cryogenic temperature should be taken into consideration (Khara *et al*., 2012). A standard procedure is established to estimate the fertilizing ability of sperm

in fish by taking batches of about 200 fresh eggs from a pool of several females, mixed with 10 ml of diluent and inseminated with 1/100 (10^{-2}) to 1/10,000 (10^{-4}) sperm to be tested (Billard *et al*., 1978; Billard, 1978a). The percentage of fertilization is estimated by the percentage of embryonated eggs at 100 degrees days (Hatipoglu and Ackay, 2010). The use of gamete from genetically superior male increases the productivity of a farm and the preservation of germ-plasm through employing strategies to extend the semen of such superior males is required (Adeyemo *et al*., 2007).

3.1.2. Motility

The quality of the genetic material introduced into the egg during artificial insemination an important parameter to achieve a higher fertilization rate. Sperm motility or the ability of the sperm to move towards the female gamete is very crucial for fertilization (Oyeleye and Omitogun, 2007; Mishu *et al*, 2020). Sperm motility, which is measured as the proportion of progressively motile sperm prior to insemination (Rurangwa *et al*., 2001), is a prerequisite factor determining sperm quality and fertilizing ability (Alavi *et al*., 2004; 2007). Several factors influence sperm motility, such as pH (Alavi and Cosson, 2005), cations (Cosson, 2004; Khara, 2012), osmolality (Cosson, 2004; Alavi *et al.,* 2007) and dilution ratio (Alavi *et al*., 2004) in either the aqueous environment or diluent. Rurangwa *et al*. (2001: 2004) identify sodium (Na^{+}), potassium (K^{+}), calcium (Ca^{+2}), and magnesium (Mg^{+2}) as the major ions involved in improving motility characteristics in *C. gariepinus*. In most cases, sperm quality was only evaluated in terms of motility after thawing (Kowalski and Cejko, 2019). Using excess spermatozoa for fertilization obviously masks the quality of cryopreserved spermatozoa, making a comparison of protocols difficult (Alavi *et al.,* 2004).

Fish sperm motility assessment is a useful parameter to indicate the success of the cryopreservation technique, as it provides baseline information on the fecundity of *C. gariepinus* and quality biomarker for fish spermatozoa (Horvath and Urbanyi, 2000; Kamaruding *et al*., 2012; Albiach and Nemesio, 2018; Kowalski and Cejko, 2019). Motility is induced after dilution either in water or saline (6-10% salinity) for freshwater fish (Jaspers, 1972; Billard, 1978b). Spermatozoa motility can be assessed by motility score (Hoyle *et al*., 1968; Guest *et al*., 1976) or by the duration of the initial score observed immediately after dilution. The motility test can be proved by diluting a drop of post-thawed or fresh spermatozoa either with Phosphate Buffered Saline (PBS), Ginzburg Fish Ringer (GFR), or 0.9% saline solution at a ratio of 1:100 from which one drop of the solution is put on the hemocytometer and viewed subsequently under the microscope 10X and 40X, low and high-power objectives of the microscope (Sigma, 1994). Only samples with 80% motility and above should be extended and preserved (Adeyemo *et al*., 2007). Studies assured (Embong *et al*., 2011) that *C. gariepinus* having large body weight gave the highest total motility (85%) and small-sized ones have the lowest total motility (50%). The sperm motility rate fluctuates with a combination of equilibration and vapor exposure factors due to multiple steps and their interactions (Albiach and Nemesio, 2018).

To keep the sperm cells immotile until ready for use, a good extender should be isotonic to the seminal plasma of the fish. As that of the cryopreserved sperm cells, those in the body of the fish are nonmotile and motility is initiated in freshwater fishes when the sperm is released in the water osmolality goes down (Maria *et al.,* 2006; Khara *et al*., 2012). Motility is initiated by exposure of the semen to a hypotonic solution and the motility of *C. gariepinus sperm* is completely but irreversibly suppressed in electrolytes and non-electrolytes with an osmolality of 200 mOsmol/kg (Hwang and Idler, 1969; Morisawa and Suzuki,, 1980; Mansour *et al*., 2002; Cosson, 2004). However, the study conducted by Omitogun *et al*. (2012) proved that the 200 mOsmol/kg of calcium Free Hanks' Balanced Salt Solution (Ca-F HBSS) retained motility till the twelfth day as it became closer to being isotonic to the seminal plasma of *C. gariepinus.* Though the sperm fulfills the important parameters, other factors such as the nature of the extender, rate of dilution, temperature and oxygen availability may interfere. Rurangwa *et al*. (1998) demonstrated and proved that both excess semen and low sperm concentration reduced fertilization success in *C. gariepinus*. Respiration, minera1 content of the seminal plasma,

especially Na/K ratio and enzymatic activities are some other parameters used to estimate the viability of sperm (Rurangwa *et al*. 2004).

3.2. Milt collection and preparation

The African catfish has enormous potential for the development of fish farming, however the availability of fingerlings is constrained by the inability to strip milt from males like most fishes (Huisman and Richter, 1987; Tkacheva *et al*., 2020). The traditional method of obtaining milt is by killing the male, opening its abdominal cavity, removing part or the entire gonad with developed sperm, extracting the milt, and macerating over stripped eggs (Mansour *et al*., 2002; Rurangwa *et al*., 2004; Omitogun *et al*., 2012; Tkacheva *et al*., 2020). The testes are removed and dissected with scissors, putting them in a plastic plate, cutting each testis into smaller pieces into a loosely woven cloth, squeeze it and then spermatozoa will come out and can be collected to preserve (Omitogun *et al*., 2012). The sacrifice of male *C. gariepinus* to collect sperm is hard work and loss for a farm, as many males have to be killed. Instead, methods are devised to collect and preserve male gamete from fishes slaughtered during harvest by collecting the semen and ensuring year-round artificial propagation to ease the hatchery operation.

Other than the traditional method, there are also techniques that milt from the fish can be accessed without sacrificing it. One is the physiological invasive method with the resection (ectomy) that enables to extraction of part of the gonad through making an incision in the abdominal wall and the other is a physiological invasive method with gonad puncture which can be done by biopsy with a puncture through the abdominal wall without a surgery (Tkacheva *et al*., 2020). The disadvantage of the methods of taking reproductive products without the sacrifice of male *C. gariepinus* is the requirement of special skills and practice.

In the artificial reproduction, selected male *C. gariepinus* fishes are injected with synthetic (like ovaprim) or natural hormones extracted from fish pituitary glands to induce spermiation. Before injection, fish are usually anesthetized to calm them by immersing them in an anesthetic bath containing a suitable concentration of the drug (Potongkam and Miller, 2006). Milt has mostly collected 10 hrs after injection using plastic syringes. Milt should be diluted with extenders like Hanks' Balanced Salt Solution (HBSS), Ca-F HBSS or 0.9% NaCl. Diluted sperm is activated using freshwater and then only sperm samples with motility higher than 50% are preserved (Rurangwa *et al.*, 2001). The diluted sperm is held at 4^0C until freezing (10-30 min) and mixed with different cryoprotectants like DiMethyl-Acetamide (DMA), Dimethyl Sulphoxide (DMSO), Ethyl Glycol (EG), Methyl Hydroxide (MeOH) at a rate of 1:1. The mixture is, then, loaded with a given equilibration period, sealed with a heated hemostat and preserved in liquid nitrogen (Potongkam and Miller, 2006).

4. Fish Sperm Collection and Management

Cryopreservation of fish spermatozoa is a technology that enables the long-term preservation of valuable genetic material under cryogenic temperatures without losing their biological function. It is one of the important *ex-situ* methods whereby fish germ plasma can be conserved till been used (Rahman et al, 2020; Bøe *et al*., 2021). This preservation method works best with the use of selected extenders, as the extender is its sole source of energy, protects the cells from temperature shock, and maintains a suitable environment for the survival of the sperm (Whaley *et al.*, 2021). Development of successful techniques for the cryopreservation of fish sperm must take into account considerations mainly those related to fish species such as the biochemical structure and life span of the sperm after release into the water (van der Walt *et al*., 1993).

Cryoprotectants protect sperm cells from being damaged during the process of freezing and thawing, but the extent of damage varies according to the species. Scientists successfully cryopreserved the spermatozoa of *C. gariepinus* and obtained 40% motility in 24 h after storage in liquid nitrogen (Steyn *et al.*, 1985; Oyeleye and Omitogun, 2007).). Later, glucose in combination with glycerol or DMSO has become the most widely used and effective cryoprotective solution (Urbanyi *et al*., 1999). Recently, DMSO, DMA, EG, and MeOH have commonly been used as the internal cryoprotective agent for the cryopreservation of fish sperm (Steyn *et al.*, 1985; Urbanyi *et al*., 1999; Rurangwa *et al*.,

2001; Akcay *et al*., 2004; Abdissa *et al*., 2022). Seminal plasma imitating media and simple carbohydrate-based solutions are commonly exploited extenders for the cryopreservation of fish spermatozoa. Cryoprotected sperm can be frozen using the straw or pellet methods to reduce the time required for sperm packaging and thawing and facilitate sperm handling during fertilization. Freezing rates can be rapid (*e.g*., pellet freezing on dry ice or in liquid nitrogen vapor) or slow (*e.g*., at fixed rates in programmable freezer) (Steyn, 1993). Cryoinjury occurs due to temperature shock during freezing and thawing, pH fluctuation, ice crystal formation, osmometric effect, and cryoprotectant toxicity (Urbanyi *et al*., 1999; Akcay *et al*., 2004).

The success history of cryopreservation rests on maintaining the viability of the sperm of fish spermatozoa which is dependent basically on the extender, the cryoprotectant agent, the diluent, the substances to maintain the osmolarity, the energy source, the freezing and thawing protocols, enzymes, antibiotics and the cryo-container (Rana, 1995; Holt, 2000; Horvath and Urbanyi, 2000; Linhart *et al*., 2000; Viveiros et al., 2000; Embong *et al*., 2011). A cryoprotectant keeps the cells during freezing and thawing by maintaining the size and shape of ice crystals formed during freezing (van der Walt *et al*., 1993). The effectiveness of a cryoprotectant varies with animal species due to sperm size, shape, and biochemical characteristics (Baynes *et al*., 1981; Suquet *et al*., 1993; Lin *et al*., 1996; Yang and Tiersch, 2009; Herranz-Jusdado *et al*., 2019). The development of sperm cryopreservation in *C. gariepinus* is difficult as the sperm has high lipid content (Denniston *et al*., 2000; Kamaruding *et al*., 2012). The sperm cryopreservation medium consists of a non-penetrating cryoprotectant (milk and egg yolk), a penetrating cryoprotectant (glycerol, EG, DMSO, combinations), a buffer (Tris or Test), sugars (glucose, lactose or sucrose), salts (sodium citrate or citric acid) and antibiotics (Penicillin or Streptomycin), (Urbanyi *et al*., 1999; Vishwanat and Shannon, 2000; Chao and Liao, 2001; Kwantong and Bart, 2008; Ponchunchoovong and Plime, 2010; Omitogun *et al.*, 2012). The extenders employed for the cryopreservation of sperm for *C. gariepinus* includes fructose solution with $NaHCO_3$ buffer and glucose that resulted in a capacity of fertilizing 96% of the eggs (Urbanyi *et al*., 1999). The experiment conducted on *Labeobarbus brevicephalus* resulted highest equilibration (87.3±1.5%) and post-thaw (83.0±1%) motility from the diluent, Extender 3 plus DMSO 10%, that proved its suitability for the preservation of sperm (Abdissa *et al.*, 2022).

Apart from the right choice of the cryoprotectant and extender, cryopreservation success depends also on the freezing and thawing protocols used, the concentration of cryoprotectant, exposure to cryoprotectant prior to freezing, and duration of exposure are also important (Christensen and Tiersch, 1996). At a slower freezing rate, larger ice crystals that potentially can damage the cell membrane can be formed and faster freezing may result in a cold shock (Leung and Jaemison 1991; Linhart *et al*., 1993; Mongkonpunya *et al*., 1995; Padhi and Mandal, 1995; Rana, 1995; Viveiros *et al*., 2000).). The use of a freezer with a programmable control rate is advised to achieve consistent and predictable freezing rates (Steyn, 1993). Studies confirmed that sperm of *C. gariepinus* cryopreserved for 7 months in liquid Nitrogen and diluted more than 40 times with the extender is viable (Steyn, 1993; Omitogun *et al*., 2012).

The fertilization and hatching rates of African catfish vary with the concentration and type of cryoprotectants used. According to Muchlisin *et al*. (2015), sperm preserved in 10% DMSO for 45 days has given the same fertilization rate (> 91%) as that of non-preserved (fresh semen) indicating the possibility of preserving semen for more than a month without losing its fertilizing ability. The fertilization and hatching rate of cryoprotected semen of African catfish using different cryoprotectant is summarized in Table 1.

Table 1: Study results on hatching and fertilization rates of cryopreserved African catfish semen

Cryoprotectant	Freezing rate	Rates		Reference
		Fertilization	Hatching	
10%DMSO	-11 to -80^{0}C/min; LN_2	Not indicated	96%	Urbanyi *et.al.,* 1999
10%DMSO	-4 to -79^{0}C/5min; LN_2	91%	31%	Muchlisin *et al*., 2015
15%DMSO	-4 to -79^{0}C/5min; LN_2	66%	27%	Muchlisin *et al*., 2015
11% glycerol, 5% glucose extender	-11 to -70^{0}C/min; LN_2	Not indicated	51%	Steyn and van Vuren, 1987
5% Glucose	-4 to -79^{0}C/5min; LN_2	59%	16%	Muchlisin *et al*., 2015
10% glucose	-4 to -79^{0}C/5min; LN_2	65.33%	17%	Muchlisin *et al*., 2015
5% Egg yolk	-4 to -79^{0}C/5min; LN_2	80.67%	24.33%	Muchlisin *et al*., 2015
10% Methanol in ringer extender	Not indicated	90%	88%	Viveiros *et al*., 2000
Fresh sperm	-	95.67%	68.63%	Muchlisin *et al*., 2015

5. Fish Sperm Collection and Management

Controlled reproduction in fish includes the possibility of manipulation or preservation of gametes and their optimum utilization by artificial insemination. Artificial insemination consists of the manual mixing of the freshly spawned eggs with sperm, adding appropriate water (Müller *et al*., 2019) and keeping the mix in a container with a recommended temperature. For further improvement, a mixture of NaCl and urea is used to remove the sticky layer of eggs in fish (Adebayo, 2006). In addition, the solution of NaCl and urea also extends the ability of the sperm to fertilize an egg (Billard, 1978a). Lack of synchronization in male and female gonadal maturation hampers the artificial propagation of fish. The cryopreservation of male gametes enables to fertilization of eggs at any time convenient and allows the banking of semen that could be used in the manipulation of spawning efforts in hatcheries (Billard, 1978b). Attempts made to transfer this technology to the preservation of fish sperm showed difficulties because techniques developed for mammalian sperm were not compatible with the physiological peculiarities of fish semen (Harvey and Ashwood-Smith, 1982). Fish spermatozoa can be refrigerated successfully at 0 - 4°C for several days and can also be extended at above zero temperatures by providing adequate airspace above the semen sample or by storing semen under oxygen (Truscott *et al*., 1968; Hoyle *et al.*, 1968; Graybill and Horton, 1969). Many investigations and magnificent efforts have been done over the last many decades and found various success rates in the cryogenic preservation of fish semen (Viveiros *et al*., 2000; Kamaruding *et al*., 2012; Omitogun *et al*., 2012; Muchlisin *et al*., 2015; Bøe, *et al*., 2021; Abdissa *et al*., 2022).

Artificial insemination in fish works well as sperm from one male can fertilize eggs from 3 to 4 females (Petit *et al*., 1974). However, through the use of an isotonic salt solution as an extender, one male sperm can be used to fertilize 20 females in the artificial insemination of *C. gariepinus* (Rurangwa *et al*., 2001). Osmotic pressure and pH are the most important factors for fertilization and studies confirmed that the percentage of fertilized eggs increased at the osmotic pressure of 250 mOsmol/kg and 9.0 pH (Alavi *et al*, 2004). To set up a reliable technique of artificial insemination and achieve a higher percentage of fertilization, the buffer and optimum gametes to diluent ratio need to be fixed (Rurangwa *et al*., 2004). Mixing eggs from several females or sperm from several males during artificial insemination does not affect the percentage of fertilization (Rurangwa *et al*., 2004; Adebayo, 2006; Omitogun *et al*., 2012).

To maximize the use of a single male *C. gariepinus* sperm, optimization of sperm; egg insemination ratio is very important (Bobe and Labbe, 2009). The optimal ratio of fresh spermatozoa to egg to achieve up to 67% hatching is 15,000: 1 for artificial insemination of *C. gariepinus* (Rurangwa *et al*., 1998). However, 49 x 10^3 live frozen-thawed spermatozoa of *C. gariepinus* is required to fertilize

an egg and achieve a hatching rate of 51.2% (Steyn, 1993). Tiersch *et al*. (1994) reported that 50 x 10^6 frozen-thawed spermatozoa per 0.5 ml straw enabled to fertilization of 250 Channel catfish eggs. However, a minimum of 13 x 10^6 frozen-thawed spermatozoa per egg is required to achieve 54% fertilization in blue catfish, *Ictalurus furcatus* (Bart and Dunham, 1996). In Striped catfish, 1.89 × 10^6 fresh spermatozoa was required to fertilize a fresh egg while more cryopreserved sperm (6.94 × 10^6) was required to fertilize an egg and achieve the same rate of fertilization (Ponchuchoovong and Bart, 2008).

In artificial insemination, the sperm-to-egg ratio should be optimized and should not be excessive (Padhi and Mandal, 1995). Using excess spermatozoa for fertilization obviously masks the quality of cryopreserved spermatozoa as cryopreservation provokes both structural and biochemical damages to spermatozoa, which may lead to impairment (Omitogun *et al*., 2012). Nevertheless, due to the higher percentage of spermatozoa that die during the freezing and thawing processes and for effective insemination, it is advisable to increase the ratio of frozen spermatozoa (Omitogun *et al*, 2012).

6. Conclusion

The *C. gariepinus* fish is becoming a very good candidate fish for food and income, as it is easier for smallholder farmers to manage it, grow better and yield more biomass, and to get good market value. Linked to its unique reproduction behavior and cycle, there exists a higher scarcity of fingerlings in this fish species. Multiplying this fish in a hatchery by artificial means is crucial as milt from a single male can fertilize eggs stripped from 3 to 4 fishes. Since it is not possible to get milt by stripping, it is important to use techniques and possibilities that enable to collect and preserve the germplasm from selected males. By doing so, a hatchery can reduce the number of male parents that need to be kept on a farm and minimize cost through optimizing the use of cryopreserved semen. Apart from the differences in success rates, it is possible to use cryopreserved semen for artificial insemination of *C. gariepinus* the scientific findings confirmed that eggs fertilized by cryopreserved semen can give higher rates of fertilization and hatching in *C. gariepinus* depending on the management, duration and application of the cryopreserved semen.

Having all the techniques and procedures so far developed by different scientists and reviewed in this paper, it is possible to adopt and update the protocols and approaches to collect the selected male gamete (while in excess), store it in an appropriate freezing temperature and preserved in liquid nitrogen till been used. Through harnessing the necessary processes and protocols investigated to preserve and inseminate *C. gariepinus*, the hatcheries in developing countries could effectively produce *C. gariepinus* fingerlings. Hence, it is suggested that the hatchery operators need to practice the research results and facts reviewed and compiled here in this article as a source of information for the success of their business and alleviation of the apparent scarcity of fingerlings.

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