Study of the Effect of Dilution on the Quality of Milt of *Clarias gariepinus* Broodstock in Artificial Reproduction in Mali

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Abstract: *Clarias gariepinus* is one of the species of farmed fish that has been studied and popularized in Mali. The difficulties of his reproduction in captivity have led researchers to conduct studies on the effects of thinners on the quality of his milt. The objective of this study is to test the effectiveness of *Clarias gariepinus* seed mobility from diluents in Mali. It was conducted on 3 batches of 9 males and 12 females from the fish farm of Bathily in Sikoulou. The average weights in each batch were respectively 846.25±2.35 (batch 1); 849.22±1.22 (batch 2); 861.40±2.68 (batch 3) male, and respectively 1190.23±53.44 (batch 1); 1167.33 ±34.15 (batch 2) and 1200.18±40.81 (batch 3) for female. The results on the volumes of milt collected gave values of 0.17g, 0.7g and 0.9g. The sperm concentration was the same for all beaches and the motility durations varied respectively during 12h. The value of pH varied between 6.73 ± 0.06 and 6.75 ± 0.04 . All the sperm characteristics revealed a good quality of the seeds of the *Clarias gariepinus* strain from the farm of Bathily. Only physiological serums and drilling waters allowed fertilization after activation of the spermatozoa by the diluents. However, the results obtained with these diluents after 12 hours of storage suggest a possibility of improving their effectiveness for the artificial reproduction of *Clarias gariepinus*.

Keywords: Broodstock; Clarias gariepinus; Fish Milt; Diluent; Artificial Reproduction.

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I. INTRODUCTION

Aquatic food products are an important component of healthy and balanced diets. Even in small amounts, they can have a very positive nutritional impact by providing essential nutrients that are insufficient in plant-based diets. The fisheries and aquaculture sector plays an important role in achieving Sustainable Development Goal 14 to "conserve and sustainably use the oceans and seas for sustainable development"(FAO,2020). Globally, aquaculture is booming (FAO, 2022) but the development of fish production is based on only a few domesticated species. Of these, *Oreochromis niloticus* and *Clarias gariepinus* are the main species used in fish farming in African continental waters.

Mali is a continental country watered mainly by two major rivers (the Niger River and the Senegal River), with their multiple tributaries that make the country a suitable area for the development of fish farming (DNP, 2022). In Mali, total fish production was 124,953 tons in 2023 with 8,981 tons for fish farms (DNP, 2022). For the development and practice of fish farming in Mali, several species of fish are used, including: *Oreochromis niloticus, Clarias gariepinus, Heterobranchus bidorsalis, Heterotis niloticus,* because of their enormous potential to meet the criteria of choice of fish farmers. However, *Clarias gariepinus,* which is an endemic species of Africa, is by far the hardiest with better growth.

Clarias broodstock do not breed in captivity but can produce large amounts of eggs and sperm year-round by artificial methods that are not well managed in hatcheries in Mali. On the other hand, this species is easy to feed, as it accepts a wide variety of cheap artificial foods, tolerates high densities under rearing conditions and tolerates poor environmental conditions. It is a species that is particularly popular with consumers and tastes good when smoked. To succeed in developing fish farming in all its forms, it will be necessary to ensure a regular supply of quality and quantity fingerlings. To this end, our efforts have focused on reliable hormonal reproduction methods on the one hand, and on the other hand, affordable collection and storage methods at a lower cost in order to increase the income of fish farmers. Volume 10, Issue 4, April – 2025

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Thus, with a view to the sustainable management of Clarias broodstock stocks, this study was initiated. The dilution of the milt of *Clarias gariepinus* broodstock will allow it to be used after storage in the short and medium term.

II. LITERATURE REVIEW

Clarias gariepinus or African catfish is a species resistant to pathologies. It is highly appreciated by fish farmers because of its predisposition to adapt to abiotic factors, its speed of growth and the quality of its flesh with the few bones it contains. However, the natural reproduction of this species in most African countries takes place during the rainy season, hence the need for artificial breeding.

In Mali, the artificial reproduction of this species encounters a number of problems, including the low survival rate of larvae after hatching and also the absence of gonads in many male broodstock that have reached reproductive age, hence the need to preserve the gonads of the best specimens for several uses with diluents. Cryopreservation is the ex-situ preservation of gametes by means of freezing at very low temperatures.

The success of cryopreservation depends on mastery of the use of appropriate diluents and cryoprotectants as well as freezing and thawing procedures (Maisse et al., 1998). The collected seeds must first be diluted in a physiological medium (diluent) before freezing. This freezing medium plays a major role in protecting the cellular functions of spermatozoa. The pH and osmotic pressure of the medium should be close to those of the seminal plasma. It must also contain nutrients and cryoprotective elements to limit cell damage during freezing and above all to keep spermatozoa immobile.

Two types of cryoprotectants can be considered (Maisse et al., 1998). Permeable cryoprotectants or internal cryoprotectants are those that can cross the plasma membrane and provide isotonicity between the intra- and extracellular environment during freezing. The choice of the permeable cryoprotectant must be appropriate to the species (Maisse et al., 1998). The freezer diluter should be brought to the same temperature as the semen (4°C) before mixing to avoid thermal shock. Several dilution ratios (semen volume / diluter volume) have been tested in fish. It is suggested that dilution ratios of 1:10 should not be exceeded (Suquet et al., 2000).

The freezing of the seed depends on the method of use. Thus, there is short-term freezing and long-term freezing. Short-term freezing takes place at temperatures around 0°C (Carpentier and Billard, 1978) and is generally intended to ensure a wide diffusion of milt during artificial fertilization.

Rapid thawing of frozen seeds is necessary to prevent recrystallization. The flakes removed from the liquid nitrogen are thawed at temperatures that vary depending on the species (Suquet et al., 2000). For freshwater species, the range of thawing temperatures is 30 to 80°C (Rana, 1995a).

Many studies have focused on the freezing of the sperm of teleost fish. However, these studies have mainly been carried out on European aquaculture species such as salmonids (Maisse et al., 1998). Interest in this family was focused very early on because of its importance in European aquaculture and the many achievements that already existed on the testicular physiology and biology of their spermatozoa (Maisse et al., 1998). On the other hand, for most species such as tilapia and clarias, cryopreservation studies are still very rare.

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III. MATERIALS AND METHODS

A. Materials

Description of the Study Area

This study was carried out at the IPR/IFRA in Katibougou. Fish of the improved strain of *Clarias gariepinus* were reared in tanks for seed collection to see dilute and undiluted sperm motility. Some of the broodstock was used during the experiment to see the viability of the spermatozoa at the National Center for Animal Artificial Insemination (CNIA). The collection phase was carried out in the seed conservation laboratory, INSP in Bamako.

The three broodstock lots were kept in a closed circuit of a 2000 l tank with a continuous water renewal system.

➤ Animal Material

The material made available is composed of twenty-one broodstock of *Clarias gariepinus* including: 9 males of 11 months with an average weight of 846.25 ± 2.35 g respectively (lot 1); $850 \pm g$ (lot 2) and 861.40 ± 2.68 g (lot 3); 12 females of 11 months old whose average weight varied between 1167.33 ± 34.15 g to 1200.18 ± 40.81 g all from the fish farm of Bathily in Sikoulou

Sperm Measurement Equipment

The count was made using a hematmeter which is made up of a glass slide hollowed out with a small basin whose bottom is lined with a grid that allows the count of living and dead spermatozoa and the total numbers of spermatozoa in 1ml of semen.

B. Methods

> Experimental Design

The set-up of this Fisher block experiment (3 blocks), each corresponding to a processing of three replications.

> Treatments

For this study, three treatments were implemented by diluting each batch of spawning milt in a given diluent. The 12 female and 9 male broodstock were divided into the following three treatments:

- T1: Milt of 3 males without diluent,
- T2: Milt from 3 males used in physiological serum,
- T3: Milt of 3 males diluted in borehole water.

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For each batch, corresponding to a given treatment, three samples of milt were observed and used for artificial reproduction.

> Selection of Broodstock

The selection of the 9 males and 12 females was made according to age and weight. The 9 males had an average weight of 846.25 ± 2.35 g respectively (lot 1); $850\pm$ g (lot 2); 861.40 ± 2.68 g (lot 3); The 12 females had an average weight ranging from 1167.33 ± 34.15 g to 1200.18 ± 40.81 g respectively.

Marking of Broodstock

For the identification of the broodstock, each batch of males was marked at the level of the caudal peduncle by a different cut mark (horizontal section of the bottom half of the peduncle for batch 1, vertical section of the top half of the peduncle for batch 2, horizontal section of the top half of the peduncle for batch 3). After marking, the wound was disinfected with Betadine. The broodstock were then fed to apparent satiety (3 times a day) with imported feed (repro-kudjis) dosing 40% crude protein. The physical chemical properties of the water, such as temperature and pH, were measured in the morning and evening using a multifunctional pH meter.

➤ Milt Collection

To collect milt, each male is captured with a landing net and placed on a wet towel. The broodstock is then placed on its dorsal fin and immobilized, the head covered by another towel by an operator's hand. With the help of a towel - the entire genital area of the fish is cleaned and dried.

Each male underwent surgery; which consisted of an incision and suturing of the abdomen to remove the gonads,
The testicular lobes are cleaned with paper towels, then incised with a blade to collect the sperm in a beaker jar.

Conservation of the Gonads of Clarias gariepinus Broodstock

Diluted and undiluted semen were put in 15ml beaker jars and stored at 4°C in a refrigerator before use.

> Milt analysis

For the analysis of sperm characteristics, two phase contrast microscopes were used to observe sperm motility and to do the count. A haematometer (enhanced Neubauer cell) was used to determine the sperm concentration of the milt.

➢ Microscopic Observation of Milt

Several parameters are used to assess sperm quality. Among these parameters we have the motility of spermatozoa; sperm concentration, percentage of living and dead cells; the normal sperm count.

Spermatozoa are observed under a microscope (x100) immediately after gonad collection in males. The sperm is stored in a container (beaker jar) well dried before immobilization in order to prevent activation by water and serum.

A drop of semen is placed on a slide and then mixed under a microscope with a 0.01ml drop of water of serum to wake up the spermatozoa. The times corresponding to 100%, 50% and 0% of sperm motility are then recorded. For each semen sample, three measurements were made.

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For the first male, a volume of 1 ml of semen was observed under a binocular microscope after activation in serum physiology of 0.01 ml at 0.9°/0. The motility of spermatozoa was observed in waves under the microscope. For the second male, 1 ml of sperm from the broodstock was observed under a dry binocular microscope without activation with water or serum physiology. No sperm movement was observed. For the third male, 1ml of semen was observed under a binocular microscope after activation in borehole water of an amount of 0.01 ml. Weak sperm movements were observed.

> Dose of Hormone Injected into Females

The females received an injection of hormone in proportion to their weight to ensure the final maturation of the oocytes, this dose of hormone is 0.7ml/kg of body weight.

Harvesting Eggs from Females

At the indicated time, the females are removed one after the other in the order of induction for oocyte extraction.

The female's head is covered with a wet towel to keep her quiet.

The first operator lifts it by the head and puts it against his side, then with the help of the other hand, massages the abdomen (from the pectoral fins to the anal fin), a second operator holds its tail. The oocytes are expelled in jets through genital orifice, collected in a container held by a third operator.

After collecting as many oocytes as possible, the female is put back in the packaging tank while waiting to bring her back to the pond.

> Fertilization and Artificial Incubation of Eggs

This involves mixing sperm and oocytes for fertilization, which is the fusion between a spermatozoon and an oocyte to give an egg.

- The oocytes were divided into 6 parts of the same weight, mixed with the corresponding volume of semen using a syringe.
- Stirring the mixture slightly, add a little water as well.
- The fertilized eggs are well spread out on the incubation sieves
- As soon as they hatch, the larvae pass through the mesh of the sieve to end up in the tank.
- When the maximum number of larvae descends into the tank, the sieve is shaken and removed from the water.
- The valve of the circuit is opened to let in water and ensure its renewal, this marks the beginning of larval rearing.

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Removal of Incubation Baskets

The incubation period depends on the temperature of the water in the incubators. The higher the temperature of the water in the incubators, the faster the hatching. The larvae will then pass through the meshes of the incubation baskets. Once the majority of the larvae have descended, the baskets are removed.

> Larval Breeding

This is undoubtedly the most delicate and difficult step in the artificial reproduction of Clarias. Indeed, the eggs being very small, their yolk reserve is very low. When they hatch, they look like thin filaments, with a small green sphere, the umbilical vesicle. In three days, the yolk vesicle will be absorbed and the larvae, having become fresh, will begin to look for food. At the first intake of food, it should be ensured that more than 80% of the population swims, this makes it possible to avoid heterogeneity and therefore the development of cannibalism.

As the larvae are quite fragile at this stage, the weighing was not carried out to determine a daily ration. The feed was dispensed in a quantity of three pinches of pro powder late every four hours, from 8 a.m. to 4 p.m.

From 14 days, the starter feed pros tard 0.1mm was replaced by pros tard 0.3mm distributed at the same

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frequency until the 20th day which is the end of larval rearing.

Larval rearing was carried out in incubators filled with water and well oxygenated at an average temperature of 28° C.

C. Statistical Analysis of the Data

Data concerning the weight of the spawners (males and females), the weight of their gonads, the characteristics of the milt of the males (sperm concentration, sperm motility) and the parameters of artificial reproduction were processed with the XL-STAT version software.

The one-way simple analysis of variance (ANOVA) was performed, as well as the Fisher test.

IV. RESULT AND DISCUSSION

A. Result

> Water quality parameters

Water quality parameters were measured periodically. The data concerning these parameters during the experiment are mentioned in the table below.

Treatments	Temperature	рН
T1 (I1, I2)	27,40±0,08a	6,73±0,06a
T2 (I3, I4)	27,37±0,17a	6,67±0,02a
T3 (I5, I6)	27,35±0,18a	6,7535±0,04a
F de Fisher	0,06	1,91
Pr>F	0,95	0,23
Signification	NS	NS

Characteristics of Gonads and Milt

The characteristics of the gonads and milt are shown in the table below.

	Table 2: Gonad	weights and	d properties	s of milt
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Males	broodstock weight	Gonads weight	Concentration	Nbr of spz	Alive Spz	Death Spz
	(g)	(g)	(%)	$(x \ 10^6)$	(%)	(%)
T1	846,25±2,35 b	2,63±0,84	100	2060	71	29
T2	849,22±1,22 b	2,00±0,26	100	2220	67	33
T3	861,40±2,68 a	$1,99\pm0,58$	100	3000	74	26
F de Fisher	27,26	0,74	ND	ND	ND	ND
Pr>F	0,001	0,52	ND	ND	ND	ND
Sig	S	NS	ND	ND	ND	ND

The highest number of live spermatozoa was observed in the males of batch 3 with 74 spermatozoa and the lowest number in the males of batch 2 with 67 spermatozoa. The highest numbers of dead sperm were observed in the males of batch 2 with 33 sperm and the lowest number in the males, lot 3 with 26 sperm. The concentration was identical in all males with 100% mobility. The highest total sperm counts were observed in the males of batch 3 with 3005x105 spermatozoa and the lowest in the males of batch 3 corresponding to 2060x106 spermatozoa.

Sperm Movements

After sampling the gonads, the milt observed under the microscope made it possible to determine these characteristics, including the movements of the spermatozoa in the milt samples. The different movements of the spermatozoa are noted in the table below.

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Table 3. Results of Binocular Microscopic Observations

Tuble 5. Results of Billocular Microscopic Observations				
Male broodstock	Diluent	Observation		
M1 (males of lot 1)	Without diluent	All sperm cells are immobile		
M2 (males of lot 2)	Physiological serum	All sperm move vigorously; impossibility of fixing the view on one of them.		
M3 (males of lot 3)	borehole water	Spermatozoa exhibit three behaviours (in roughly equal numbers):		

➢ Incubation and Hatching of Eggs

Each treatment received an incubator with the same amount of eggs (80g), data regarding egg hatching rate and larval survival are noted in the table below.

Treatments	Incubators	Incubating eggs (g)	Larvae on day 3 (n)	Hatching rate (%)
T1: Without diluent	I_1	80	30000	75
	I_2	80	30400	76
T2: Physiological serum	I_3	80	16000	40
	I_4	80	16800	42
T3: borehole water	I_5	80	25000	64
	I_6	80	25000	64

Table 4:	Hatching rate	s of Eggs	in Incubators
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The T1 treatment had the best hatching rates with 76% and 75%, followed by the T3 treatment with a hatching rate of 64% and the T2 treatment with an hatching rate of 42% and 40%. As for larval survival rates, the results are shown in Table 5 below

Table 5: Larval Survival Rate at Day 15					
Treatments	Incubators	Larvae on day 3 (n)	Larvae on day 15 (n)	Survival rate (%)	
T1: Without diluent	I_3	16000	3 860	24,13	
	I_4	16800	3 902	23,23	
T2: Physiological serum	I_1	30400	5 600	18,42	
	I 2	30000	5 450	18,16	
T3: borehole water	I_5	25000	4 300	17,20	
	I_6	25000	4 380	17,52	

The highest number of larvae on day 15 was obtained at the level of the T2 treatment, corresponding to incubators I_1 (5,600), I_2 (5,450); followed by the T3 treatment represented by the I_6 (4,380), I_5 (4,300) incubators and finally the T1 treatment, corresponding to the I_4 (3,902) and I_3 (3,860) incubators, on the other hand the survival rate is higher in T1 with the I_3 incubators (24.13%), I_4 (23.23%); followed by the T2 treatment of the I_1 incubators (18.42%), I_2 (18.16%) and finally the T3 treatment corresponding to the I6 incubators (17.52%),I5 (17.20%).

B. Discussion

The present study showed a non-significant difference between morning and evening temperatures. The same significance was observed with the variation in pH.

At the level of incubator, the highest temperature was observed in the incubators: I_1 and I_2 with 27.40±0.08°C and the lowest temperature in the I_5 and I_6 incubator with 27.35±0.18°C.

As for pH, the highest pH was observed in the incubators: I_5 and I_6 corresponding to 6.75 ± 0.04 and the lowest pH in the incubator I3-I4 with 6.67 ± 0.02 . Thus, we note that these values are within the range of the standards indicated by AYOOLA (2017), Chukwukadibia (2016) and TAPSOBA (2017) at the International Center for Research and Development on Livestock in Subhumid Areas

(CIRDES) where the values were between $27.42 \pm 0.54^{\circ}$ C at the 4th week and a maximum of $28.80 \pm 0.88^{\circ}$ C at the 1st week for temperature and 7.36 ± 0.70 to 7.99 ± 0.26 for pH.

The variations in average temperatures and pH in the morning follow those observed in the evenings and there is no significant difference between the temperatures and pH in the morning and evening, which reflects a certain thermal and pH homogeneity during the day ; which confirms the present study.

Several parameters are used to assess sperm quality. Among these parameters we have the motility of spermatozoa; sperm concentration, percentage of living and dead cells; the normal sperm count.

Regarding sperm motility, the results of the present study showed that sperm storage alters sperm motility (the motility percentage of 100% in fresh spermatozoa drops to 74-71% in canned sperm), however it does not deteriorate its fertilizing power.

The count was made using a hematmeter which is made up of a glass slide hollowed out with a small basin whose bottom is lined with a grid that allows the count of living spermatozoa, dead spermatozoa and the total numbers of spermatozoa to 1ml of semen. The highest numbers of live sperm were observed in the males of batch 3 with 74% sperm Volume 10, Issue 4, April – 2025

and the lowest number in the males of batch 2 with 67 spermatozoa. The highest dead sperm counts were observed in batch 2 males with 33% of sperm and the lowest number in batch 3 males with 26% of sperm. The concentration was identical in all males with 100% mobility. The highest total sperm counts were observed in males in batch 3 with 3000,106 sperm and lowest in males in batch 1 with 2060,106 sperm; this was confirmed by TAPSOBA (2017) who indicated that the mechanism of motility appears complex and differs depending on the species. In general, it has been shown that sperm activation is due to a variation in the osmotic pressure of the surrounding sperm environment (Maisse, 1990, Sadiqul Islam, 2011). In marine fish, it is the passage through a hypertonic environment that causes the spermatozoa to move. In freshwater fish, it is the passage through a hypotonic environment that activates spermatozoa (Maisse, 1990). However, osmolarity in itself does not seem to have a regulatory role in the activation of motility.

Regarding concentration, the author of this study indicates that concentration expresses the number of spermatozoa per milliliter of semen. It can be determined directly by sperm count using a hematometer or indirectly by spectrophotometry, flow cytometry, and spermatocrit value (Fauve et al. 2010). Concentration determination by spectrophotometry or flow cytometry consists of observing variations in the optical density of the absorption spectra of a sperm suspension at appropriate wavelengths (Billard et al. 1971; Fauve et al. 2010). The spermatocrit value method consists of determining the sperm density after centrifugation (Fauve et al. 2010). Each of these techniques has drawbacks (Fauve et al. 2010). However, the basic method for determining the concentration is counting with a hematisometer (Fauve et al. 2010). It consists of counting the number of spermatozoa on a grid area of the hematimeter and determining the concentration by a calculation formula established according to each hematimeter. This method requires prior dilution of the semen in a solution capable of fixing the spermatozoa and spreading over the entire surface of the counting area (4% formalized physiological solution or 8‰ NaCl physiological water solution (Billard et al. 1971)). The dilution rate varies depending on the authors and the haematmetric cells used; which confirms the present study.

Regarding larval hatching and survival rates, T1 treatment had the best hatching rates with 76% and 75% followed by T3 treatment with a hatching rate of 64% and T2 treatment with a hatching rate of 42% and 40%. Post-hatch larval survival rates were very low during the experiment. It ranged between 24.13 and 17.20. The best rate was obtained with the diluent-free treatment followed by the saline treatment and the lowest rate was that of borehole water. These hatching, larval and fry survival rates are low compared to those reported by Viveen et al, 1985 in "Manuel pratique de pisciculture de poisson chat Africain, *Clarias gariepinus*".

The difference in the results at the level of the different treatments may be in the application of maintenance care and also in the inadequate sorting to detect jumpers. Indeed, cannibalism carried out by jumpers (fast-growing fry), which consume other normal fry, can be a cause of the difference in the survival rate of the fry.

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V. CONCLUSION AND RECOMMENDATION

The study of the dilution effect on the quality of milt of *Claria gariepinus* broodstock in artificial reproduction demonstrated a non-significant difference between morning and evening temperatures and pH.

The diluents of the physiological serum and water also allowed sperm motility with high resuscitation rates. However, the results obtained with these diluents after 12 hours of storage suggest a possibility of improving their effectiveness for the artificial reproduction of *Clarias gariepinus*, once the dilution process has been mastered.

We recommend further replication of this study in order to be able to better use the results at the level of fingerling producers for the development of fish farming in Mali.

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