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Sperm evaluation in strains of Nile tilapia, *Oreochromis niloticus*

Diones Bender Almeida[1]*, Marco André Paldês da Costa[1], Liane Ney Bassini[1], Cecilia Irene Pérez Calabuig[1], Carla Giovane Ávila Moreira[1], Marília Danyelle Nunes Rodrigues[1], Antonio Sergio Varela Junior[2], Carine Dahl Corcini[3], Maria Eduarda Bicca Dode[1], Heden Luiz Marques Moreira[1]

1Animal Genetic Engineering Laboratory, Department of Zoology and Genetics, Federal University of Pelotas, Campus Universitário Capão do Leão s/n°, caixa postal 354, CEP: 96010-900, Pelotas, Rio Grande do Sul, Brazil.

2Biological Sciences Institute, Federal University of Rio Grande, Campus Carreiros s/n - 96201-900 - Rio Grande - RS - Brazil

3Animal Reproduction Laboratory, College of Veterinary Medicine, Federal University of Pelotas, Campus Universitário Capão do Leão s/n°, caixa postal 354, CEP: 96010-900, Pelotas, Rio Grande do Sul, Brazil

*Corresponding author: E-mail: diones_almeida@yahoo.com.br

ABSTRACT

The reproductive characteristics of tilapia species used for large-scale production is critically important for breeding management. Although Nile tilapia (Oreochromis niloticus) is one of the most cultivated species globally, little is known about its sperm characteristics. Therefore, the objective of this study was to evaluate and compare the reproductive parameters of three tilapia strains before and after cryopreservation. Nile tilapia specimens from three strains, Supreme (SUP), Premium Aquabel (PA), and Chitralada (CHI) (90 specimens in total, 30 from each strain), were examined. All morphometric measurements were highest in PA. CHI had the highest sperm motility and speed, although less than half the sperm concentration of SUP. PA had a similar sperm speed and concentration to CHI, although its sperm motility was similar to that of SUP. SUP had a relatively high sperm viability and higher percentages of sperm without dislocation than PA and CHI. However, all strains showed similar rectilinear dislocation. Except for PA DNA integrity, SUP showed the best results before cryopreservation. From the results it was not possible to identify an ideal marker for semen quality, due to the differences observed between specimens from the same fish. However, the results regarding efficient sperm cryopreservation in the present study are important for the genetic screening and development of these strains in aquaculture.

Indexing terms/Keywords

Cryopreservation; production; reproduction; tilapia.

Academic Discipline And Sub-Disciplines

Aquaculture.

SUBJECT CLASSIFICATION

Pisciculture; Production; Reproduction; Genetics.

TYPE (METHOD/APPROACH)

Original research work.

INTRODUCTION

The Nile tilapia, Oreochromis niloticus, has suitable characteristics for farming, such as high growth rate, adaptability to various farming conditions, and positive acceptance by the consumer market (Turra et al. 2010). These characteristics make tilapia the second most farmed fish species globally, with more than 3.7 million tons produced in 2010 (FAO 2010). In Brazil, tilapia farming is growing exponentially, with average growth exceeding 105% between 2003 and 2009 (MPA 2012).

This growth in productivity is primarily due to the introduction of selectively bred tilapia strains to Brazil including Chitralada (CHI), introduced in 1996 (Kubitza 2000; Lovshin 2000) and Supreme (SUP), introduced in 2002 (Zimmermann 2003). New strains, such as Premium Aquabel (PA), have been cultivated at the Aquabel Pisciculture Station for approximately 3 years. These strains are important for improving the genetic quality of tilapia and are essential to assure the future of tilapia farming (Li et al. 2006).

Several studies have been conducted to improve productivity (Fülber et al. 2009; Ponzoni et al. 2005), genetics (Fortes-Silva et al. 2010; Khaw et al. 2012), nutrition (Nakaghi et al. 2009; Tsadik and Bart 2007), and reproductive characteristics (Almeida et al. 2013; Mataveli et al. 2007) in tilapias. Reproductive characteristics are fundamental for the fertilization success in tilapia breeding; however, there is limited information in this regard.

Parameters describing semen quality can benefit broodfish selection (Beirão et al. 2009). However, the number of external factors influencing semen quality is unknown, and these factors are thought to be extremely variable. Divergent results

can be explained by the influence of external factors, which justifies the extensive search for more efficient semen quality indicators (Bobe and Labbé 2010; Hafez and Hafez 2003).

Identifying animals capable of producing higher quality semen is important to improve the reproductive rate of a species. Differences in reproductive processes among strains directly influence semen quality, which is a main factor that can limit cryopreservation biotechnology. Therefore, numerous post-freezing evaluations of semen such as membrane and DNA integrity, and mitochondrial functionality, which are already widely used in other animal species, are being developed and used for fish (Li et al. 2010). These evaluations permit the assessment of specific cell structures, structural integrity (Celeghini et al. 2005), and the simultaneous evaluation of multiple cellular compartments (Celeghini et al. 2007), thereby complementing subjective evaluations (Mataveli et al. 2007). Since studies evaluating sperm quality in tilapia strains are limited, the contribution of such studies can greatly improve the results of assisted reproduction.

The aim of the present study was to evaluate and compare the reproductive properties in three tilapia strains that are currently cultivated in Brazil by evaluating sperm quality and the effect of cryopreservation.

2. MATERIALS AND METHODS

The present study was divided into the following three modules: 1) specimen selection, semen collection, morphometric measurements, and the measurement of semen and papilla color and pH; 2) analysis of semen velocity, type of movement, motility, and concentration; and 3) analysis of cryopreserved sperm including sperm viability, membrane and DNA integrity, and mitochondrial functionality.

2.1. Study Sites

The first module of the study was performed at the Aquabel Pisciculture Station (Rolândia, Paraná, Brazil; 23°17' 03.89" S and 51°23' 32.93" W) between November 2009 and November 2010, the second module was performed at the Animal Genetic Engineering Laboratory (Laboratório de Engenharia Genética Animal – LEGA), and the third module was performed at the Animal Reproduction Laboratory (ReproPel) of the Federal University of Pelotas (Universidade Federal de Pelotas – UFPel).

2.2. Module I

Thirty males of each strain cultivated at Aquabel (SUP, PA, and CHI) were selected separately, and were microchipped for future identification.

One week before semen collection, the individuals from each strain were randomly distributed into five nylon net enclosures (hapas) measuring 3 × 2 × 1 m (length × width × depth), with each hapa containing 6 males and 15 females (1 male:2.5 females).

During the week preceding the collection of semen samples, temperature and dissolved oxygen varied from 23.2 to 29.5°C and 4.3 to 5.6 mg/L, respectively. We fed the animals twice daily with feed containing 36% crude protein and 8% lipids.

The fish were conditioned in water boxes with oxygen before morphometric measurements and semen were collected. Clove oil (Eugenol®, 120 ppm) was added to reduce stress and avoid handling mortality, according to Moreira et al (2010).

Total length, standard length, and body depth were measured using an ictiometer (mm) and a ruler (cm). Each animal was weighed on an electronic digital scale (0.1 g). The urogenital papilla color was evaluated on a scale from one to three (one for white, two for red, and three for yellow).

After drying the genital and anal fin region using paper towel, the abdominal region was compressed in the anterior posterior direction (Billard et al. 1995). Semen was collected using a 1 mL syringe. Three aliquots per fish were obtained: one was used for fresh evaluation, one was maintained in buffered saline formol solution (4%), and one was used for the freezing process.

For freezing, the semen was mixed with Beltsville thawing solution (BTS) diluent in a 1:9 ratio using 5% DMSO as an intracellular cryoprotectant, and then placed in 0.25 mL plastic straws (Paillettes Crystal 133 mm; Cryo Bio System, France). The plastic straws were stored in racks inside a dry shipper (CryoPort Systems, model PSX1-A5 - 04284) during the freezing curve. After 3 h, the samples were transferred to a liquid nitrogen container (MVE, model CP-34) for storage at -196°C.

For the measurement of pH, 250 µL of fresh semen was applied to a strip of pH-FIX 0-14 litmus paper (Macherey-Nagel®).

The color of fresh semen was evaluated on a scale from one to three: one for a milky white color, two for a watery white color, and three for a citric yellow color.

Water was added at a 2:98 (µL) ratio (semen:distilled water) to activate the fresh semen and evaluate motility, velocity, and movement type. The semen was observed and filmed for 1 min under a bright field microscope with a 40× objective lens. These analyses were also performed in module II of the study.

In addition, 2 µL of semen from each animal was diluted in an Eppendorf tube containing 98 µL of saline-buffered formol solution (Hancock 1957). After storing the semen in this solution, its concentration was evaluated in module II.

2.3. Module II

Forty-four spermatozoa per individual were randomly chosen and individually evaluated for velocity and type of movement. The average velocity was calculated in µm/s using the software Measurement in Motion (Learning in Motion 2004), and the type of movement for each spermatozoon (stopped, in circles, or rectilinear) was evaluated during 100 frames (± 3.3 s). Each trial was recorded using a digital camera (JVC Everio GZ-MG505; 30 frames/s) positioned above an Olympus optical microscope. Spermatozoa were analyzed under 100 × magnification.

After evaluating the video recordings, a score of 0 to 100% was used to calculate the percentage of sperm moving for the semen motility analysis.

For sperm concentration, the number of spermatozoa was calculated using a Neubauer chamber and a phase contrast microscope with a 40 × objective lens. We counted the number of spermatozoa in the five largest squares of the 1 mm2 field.

2.4. Module III

To thaw the semen collected in module I, the samples were immersed in a 50°C water bath for 10 s, and the following variables were evaluated.

Nigrosin/eosin dye was added to the semen to determine sperm viability. Spermatozoa with damaged plasma membranes are stained red whereas those with intact cell membranes remain white (Maria et al. 2012).

Membrane integrity was measured using the fluorescent probes, carboxyfluorescein diacetate (C5041- CFDA) and propidium iodide (P4170 - PI) (Varela Junior et al. 2012), and an epifluorescence microscope (40 ×) with a 525 nm wavelength excitation filter. On each slide, 200 spermatozoa were classified based on their color as intact (green) or injured (red or green and red).

DNA integrity was estimated using the probe, acridine orange (A6014), under an epifluorescent microscope (400 ×) with a 525 nm wavelength excitation filter (Varela Jr et al. 2012). Green fluorescence in the head of the sperm determined those that were normal (double-stranded DNA), and those with red or yellow fluorescence were considered denatured (single-stranded DNA).

For the assessment of mitochondrial functionality, the spermatozoa were stained with rhodamine 123 fluorescent dye and evaluated using an epifluorescent microscope (400 ×) with a 525 nm wavelength excitation filter. Cells were classified based on the presence of intact (green) or injured (transparent) mitochondria, and the data were expressed as the percentage of cells with intact mitochondria (He and Woods 2004).

2.5. Statistical Analysis

The results are expressed as mean ± standard deviation (SD). After verifying normality using the Shapiro-Wilk test, the data that exhibited a normal distribution (standard length and body depth) were submitted to an analysis of variance (ANOVA) using Tukey's post-hoc tests. The data that did not exhibit a normal distribution (weight, total length, concentration, velocity, motility, pH, sperm viability, membrane and DNA integrity, and mitochondrial functionality) were analyzed using the Kruskal-Wallis H-test. When the data represented categorical variables (color of the semen and color of the papilla), the chi-squared ($\chi 2$) test was used. We tested for correlation between variables, with all strains combined (n = 90), using Pearson's correlation coefficient. All analyses were performed using the Statistix® program (Statistix 2008).

3. RESULTS

Although all fish were reared under the same conditions, we found significant differences in the morphometry between the three strains by the end of the trial (Table 1). PA and CHI differed significantly ($P < 0.05$) in all the growth measurements and the values of SUP were intermediary between the PA and CHI strains. No significant differences were observed between SUP and other strains.

The papilla color did not differ between the strains ($P > 0.05$); 58% PA and 53% CHI exhibited white papillae, while 63% SUP exhibited red papillae.

Differences in semen color were not statistically significant between the strains ($P > 0.05$). A watery white color semen was exhibited in 61% PA and 68% CHI, which was higher than in SUP (44%).

In addition, semen pH was not significantly different between the strains ($P > 0.05$) (Table 1).

Table 1 - Macroscopic morphologic characteristics and analyses of fresh semen from three Nile tilapia broodfish strains (means ± SD).

	Supreme	Premium Aquabel	Chitralada
Weight (g)	644.65 ± 25.77^{ab}	719.43 ± 22.74^{a}	579.45 ± 20.39^{b}
Total length (cm)	31.84 ±0.32^{ab}	32.94 ± 0.34^{a}	32.04 ± 0.10^{b}
Standard length (cm)	26.74 ± 0.29^{ab}	27.51 ± 0.26^{a}	26.39 ± 0.28^{b}
Body depth (cm)	9.95 ± 0.14^{ab}	10.27 ± 0.14^{a}	9.66 ± 0.13^{b}
pH	7.70 ± 0.06^{a}	7.8 ± 0.06^{a}	7.80 ± 0.06^{a}
Sperm motility (%)	89.06 ± 2.63^{b}	90.32 ± 1.99^{b}	97.94 ± 0.70^{a}
Sperm velocity (μm/^{s}-1)	48 ± 3^{b}	63 ± 5^{a}	67 ± 5^{a}
Sperm concentration (X 10^{9}/ml)	7.76 ± 1.36^{a}	3.29 ± 0.71^{b}	3.82 ± 1.01^{b}

Note: Different lowercase letters in the same row indicate a significant difference (P < 0.05).

CHI semen exhibited the greatest number of spermatozoa with rectilinear movement (61%); however, statistically, their velocity was only significantly greater than that of SUP (Table 1). In general, the spermatozoa from all three strains exhibited more rectilinear movements than other types of movements. CHI had the smallest percentage of immobile cells (5%). Although SUP and CHI had the same percentage of cells with circular movements (34%), SUP released a greater proportion of sperm cells that were immobile (9%) after stimulation with water.

The semen motility of CHI was significantly higher than that of SUP and PA (P < 0.05) (Table 1).

SUP had statistically higher values than PA and CHI for sperm concentration, sperm viability, membrane integrity, and mitochondrial functionality (P < 0.05) (Tables 1 and 2).

Table 2 - Sperm viability, membrane and DNA integrity and mitochondrial funcionality of cryopreserved spermatozoa from three Nile tilapia strains (means ± SD).

	Supreme	Premium Aquabel	Chitralada
Sperm viability (%)	26.21 ± 0.68^{a}	7.84 ± 0.81^{b}	8.96 ± 0.66^{b}
Membrane Integrity (%)	86.00 ± 3.18^{a}	8.92 ± 0.91^{b}	5.38 ± 0.74^{b}
Mitochondrial Funcionality (%)	75.90 ± 2.85^{a}	18.24 ± 4.24^{b}	16.11 ± 4.87^{b}
DNA Integrity (%)	98.35 ± 0.32^{b}	99.60 ± 0.21^{a}	14.91 ± 3.85^{c}

Note: Different lowercase letters in the same row indicate a significant difference (P < 0.05).

The DNA integrity of CHI was extremely low (Table 2), lower than that of SUP, which was in turn was lower than that observed in PA.

A number of estimations of semen quality exhibited important and significant correlations (P < 0.05) (Table 3), thus demonstrating the efficiency of the evaluations used in the present study. In general, spermatozoa with good motility were also fast but exhibited low DNA integrity and mitochondrial functionality. In addition, velocity correlated negatively with sperm viability, membrane integrity, and mitochondrial functionality, whereas these properties correlated positively with sperm concentration. A greater DNA integrity corresponded with enhanced sperm viability, membrane integrity, and mitochondrial functionality. The greatest correlations occurred between sperm viability and membrane integrity and mitochondrial functionality, and there was a high correlation between membrane integrity and mitochondrial functionality (Table 3).

Table 3: Correlation between the principal evaluated reproductive parameters before and after the cryopreservation process.

	DNA Integrity	Semen Concentration	Sperm Motility	Membrane Integrity	Mitochondrial Funcionality	Sperm Velocity
Semen Concentration	**0.15**					
Sperm Motility	**-0.30***	**-0.03**				
Membrane Integrity	**0.48***	**0.31***	**-0.14**			
Mitochondrial Funcionality	**0.40***	**0.31***	**-0.28***	**0.83***		
Sperm velocity	**-0.12**	**-0.12**	**0.37***	**-0.36***	**-0.37***	
Sperm viability	**0.31***	**0.31***	**-0.23**	**0.90***	**0.78***	**-0.36***

***P < 0.05**

4. DISCUSSION

When breeding tilapia, it is a challenge to combine growth characteristics that produce the highest meat yield with reproductive characteristics that produce high fertilization rates in a relatively shorter time. Therefore, it is fundamental to understand the physiology and biology of these organisms, and use tools to selectively breed strains that reconcile both reproductive and growth aspects.

Several seminal characteristics such as color, pH, sperm concentration, sperm velocity, sperm viability, membrane integrity, DNA integrity, and mitochondrial functionality are used to assess semen quality. Color is a good indicator of semen quality because it indicates the quantity of seminal fluid, which directly influences the sperm concentration (Andrade-Talmelli et al. 2001). This observation explains the lower sperm concentration found in the semen of PA and CHI that was frequently a white-aqueous color in the current study, although there were no significant differences between the strains. Similarly, pH is considered an important characteristic of the seminal plasma that influences sperm motility (Chereguini et al. 2001; Lahnsteiner et al. 1997). In the present study, pH did not differ between the strains. These results are similar to those reported by Mataveli et al (2007).

Several factors such as temperature, nutrition, sanitation, analysis conditions, and species influence sperm motility (Murgas et al. 2011). Spermatozoa are highly susceptible to oxidative stress because of the large amounts of polyunsaturated fatty acids in their membranes (Alvarez and Storey 1992) and the low cytoplasmic concentration of antioxidant enzymes (Sharma and Agarwal 1996). Oxidative stress causes reduced motility, and the ability to combat stress depends on the quantity of cellular antioxidants (Silva and Gadella 2006). These factors may have reduced sperm motility in both SUP and PA in relation to CHI.

The multifactorial influence and difficulty of measuring reproductive characteristics (Silva et al. 2005) make it challenging to obtain accurate results in most studies. Sperm velocity and type of movement may be useful as tools for evaluating semen quality. When more than one male tilapia broodfish is placed next to many females for breeding purposes, competition to penetrate the oocyte micropyle can occur between the sperm from different fish (Bobe and Labbé 2010). Therefore, sperm velocity and type of movement are important because faster sperm yield higher fertilization rates (Billard et al. 1980). Although it is not possible to explain the low sperm velocity observed in SUP in the present study, or if this influences reproductive rates, this objective measurement allowed the different strains to be separated into two groups (higher or lower sperm velocity). It is possible that the higher sperm concentrations in SUP compensate for slower sperm.

High sperm concentrations also modify motility and velocity estimates because excess cells make movement difficult (Geffen and Evans 2000; Williot et al. 2000). Although the motility and velocity data for the sperm did not show a significant inverse correlation with the sperm concentration in the current study, a trend was observed (see Table 1). On average, SUP had a greater concentration of sperm with lower motility and velocity, whereas CHI had a lower concentration of sperm with greater motility and velocity than the other strains. The sperm concentrations observed in these strains were within the range described for Nile tilapia, which extended from 2.63 to 8.19 x 109 sperm/mL (Bombardelli et al. 2010; Mataveli et al. 2007). Another important observation of the present study was that motility was directly related to velocity.

These divergent results may have occurred because of the selective breeding processes practiced in these strains. SUP, after more than 20 years of selection, is derived from the largest, most expensive, and longest breeding program, the genetically improved farmed tilapia (GIFT), developed by Genomar, Norway (Zimmermann 2003). CHI was developed in Japan, improved in Thailand, and officially introduced to Brazil in 1996 by the Asian Institute of Technology (AIT), Thailand (Kubitza 2000). In contrast to CHI and SUP, PA does not have a defined breeding trajectory because it is still undergoing genetic improvement. This demonstrates the necessity of considering the breeding trajectory of strains when developing

genetic and reproductive evaluations in the population under selection (Granda and Aguirre 2007). A complete understanding of all of the variables involved in the production of one definitive strain is necessary, including information on other internal variables such as cryopreservation techniques, which are becoming more commonly used and have been improved resulting in reliable selection.

The storage of reproductive cells for long periods helps to conserve genetic diversity and maintain genetic variability in breeding programs for various fish species (Streit Jr et al. 2009). The availability of gametes on demand facilitates the development and application of methodologies that aim to control reproduction, thus allowing the selection of breeding stocks and the reduction of male stocks (Silveira 2007).

The quality of cryopreserved sperm determines the success of oocyte fertilization (Johnson et al. 2000); therefore, the composition of the diluting solution used for cryopreservation is fundamental to its efficiency (Cloud 2000). Cryopreservation can cause extensive cell damage (Rana 1995), resulting in reduced sperm motility and the loss of sperm viability (Salamon and Maxwell 2000). Despite using a standard protocol for fish, cryopreservation significantly affected the sperm quality of all the strains analyzed in the current study, because the protocol was not appropriate for these species.

Even though the evaluation of the fertilization rate of the present strains was not the main purpose of the present study, it is known that DNA integrity is correlated with fertility and may cause a decrease in fertilization rate, hatching, and the final number of progeny produced. Therefore, the low value found for DNA integrity (14.91%) in CHI may have affected fertilization, although this was not evaluated.

In the current study, cryopreservation significantly reduced the values of the variables measured in in vitro samples, especially in PA and CHI. However, this reduction does not imply that cryopreservation affected sperm quality, because, despite being low, these indices may have been sufficient for fertilization to occur. Further studies are needed to improve the cryopreservation methods to determine the best possible protocol.

Some estimates of the quality of broodfish semen obtained in the present study differed from those observed in other studies (Bobe and Labbé 2010; Butts et al. 2011; Li et al. 2010). These differences may be due to various factors such as not using hormones to induce spermiation (Ferreira et al. 2001), the use of successive semen collections (Kavamoto et al. 1997), the time of year (Borges et al. 2005), or the size of the fish (Rurangwa et al. 2004). As we did not use any stimulation for sperm collection, there were differences in ejaculation volumes; however, this parameter was not considered in the analysis because the quantity of semen collected could not be considered as the total volume (Ferreira et al. 2001).

It is known that estimates of genetic correlations are essential to define selection strategies in breeding programs. However, it is very difficult to correlate a particular parameter of semen quality with fertilization rates because oocyte fertilization requires only a single healthy sperm among millions. Nevertheless, selecting animals with enhanced semen quality may improve their reproductive success. The results obtained in the correlation analysis of the current study (Table 3), especially for in vitro parameters, should not be used entirely to estimate the fertilization ability of sperm (Bobe and Labbé 2010).

Most selection programs are limited almost exclusively to growth rates (Ponzoni et al. 2005). However, selection based on reproductive characteristics, mainly in larviculture, must also be considered, even though these characteristics have a relatively low heritability (Silva et al. 2005).

Differences in sperm quality observed between strains are common within (Piironen 1985) and between (Bozkurt et al. 2011; Routray et al. 2007) species. As shown in the present study, substantial differences in sperm quality are observed within a species, which makes the search for a single tracer of sperm quality more difficult. This indicates that it is still necessary to understand the biological features of several strains of tilapia found on the market. Studies of this type can effectively support the selection of strains with greater economic importance, which will serve as reference points for new research. These results are also useful for the establishment of breeding programs that consider production characteristics for the improvement of different tilapia strains. However, before these programs are established, it is important to define the characteristics that should be improved (Rezk et al. 2009).

Although it is not possible to characterize all reproductive properties among different strains of tilapia by simply assessing semen freezability, identifying the most efficient sperm cryopreservation techniques enables enhanced genetic selection and culture of such strains, facilitating the development of the aquaculture industry. Thus, the characteristics of effectively cryopreserved sperm can be used for strain selection in breeding programs. Several studies have focused on methods of improving gamete cryopreservation. In fish, enhanced viability of male gametes enables higher genetic variability (Bobe and Labbé 2010).

The present study shows that for fish, it is important to select a particular sperm quality to be applied in the breeding industry. Based on a review published by Cabrita et al (2014), the identification of sperm quality tracers in fish is vital to accurately estimate sperm quality. It is also important to correlate these quality estimators with the capacity of these cells to reach the oocyte and fertilize the egg, and for successful initial embryonic development to further guarantee a high quality progeny. In this way, better clarifications for the sperm quality of each fish species will be reached. Our results demonstrate the necessity for additional studies (for example, evaluation of the fertilization success of these strains), and serve as a qualitative tool in breeding research for the production of tilapia species.

5. CONCLUSION

Despite SUP presenting the best in vitro results, it is unknown whether this strain will have better fertility rates in vivo, as correlations were not made between sperm characteristics and fertilization data. However, the results clearly show that even within the same species, there is a substantial difference between strains, making it more challenging to find a single tracer for seminal quality. However, based on the results regarding semen cryopreservation, further studies may elucidate an ideal tracer for selective breeding programs.

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