Article

Cryopreservation of *Potamotrygon* Stingrays' Semen: Enhancing One Conservation Effort

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Abstract: This pioneering study aimed to evaluate the cryopreservation of semen from *P. falkneri* (n = 4) and *P. motoro* (n = 4), maintained *ex situ* at the Sao Paulo Aquarium, Brazil. For this purpose, the animals were physically restrained, biometric data of the disc and clasper were obtained, and semen was collected through manual massage. Total motility and progressive motility parameters were evaluated using Computer-Assisted Sperm Analysis (CASA) with IVOS II equipment and Animal Breeders II software. The semen extenders INRA 96 and OptiXcell were used to assess their efficacy in sperm cryopreservation. INRA required the addition of 5% dimethyl sulfoxide (DMSO) as a cryoprotectant. The results indicated that there was no difference in semen motility values before and after freezing with INRA + DMSO (p = 0.6226). On the other hand, samples cryopreserved with OptiXcell showed a difference in semen motility post-thaw (p = 0.0156). These findings contribute to a broader study on optimizing cryopreservation protocols to ensure long-term viability and fertility of semen, enhancing genetic diversity and supporting wild population restoration. A multidisciplinary approach integrating reproductive biology, ecology, physiology, and assisted reproduction technologies, aligned with the One Conservation concept, is essential for advancing conservation and management strategies for these threatened species.

Keywords: species conservation; reproductive biology; cryoprotectants; assisted reproduction technologies; genetic diversity; freshwater elasmobranchs; sperm motility; wild population management; biobanking

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1. Introduction

Freshwater stingrays, belonging to the genus *Potamotrygon*, are unique to South America. They cannot survive in saltwater environments and prefer dwelling in the riverbed, often burying themselves [1,2]. These behaviors, coupled with the turbidity of their habitats, pose significant challenges for direct observation and in-depth *in situ* study of these animals [3]. Moreover, the discovery of new species within this genus is a relatively recent phenomenon [4], particularly in the Amazon Basin, known for its flooded areas and locations that are difficult to access. Despite these challenges, *Potamotrygon* sp. populations face increasing threats from river pollution, predatory fishing, and wildlife trafficking, putting them at risk of extinction before being fully studied [5].

Maintaining specimens in captivity emerges as a valuable strategy to support and leverage the *in situ* conservation of these stingrays, facilitating access to biological material for studies and allowing detailed observation of their behavior. In this context, *ex*

situ breeding plays a crucial role, requiring a deep understanding of these animals' reproductive characteristics for the effective development and application of reproductive biotechnologies, in alignment with the One Conservation concept [6,7].

Although the practice of breeding freshwater stingrays in captivity is a reality among aquarists and specialized institutions, academic research in this area remains nascent with scarce publications. Only a limited number of studies, employing techniques such as ultrasonography, hormonal analyses, and sperm quality assessment, have shown progress in assisted reproduction of elasmobranchs, a class that includes stingrays and other cartilaginous fishes [8–12]. However, these methodologies have not been fully adapted or validated for Potamotrygonids, which exhibit significant physiological and ecological differences compared to their marine counterparts. Therefore, it is imperative to conduct further research focused on the essential reproductive aspects of these stingrays to establish effective assisted reproduction techniques specific to the *Potamotrygon* genus.

This study evaluated and compared motility patterns of cooled sperm from two *Potamotrygon* species, using two different semen extenders over a set period. Furthermore, it evaluated the effectiveness of these extenders in sperm cryopreservation, thereby contributing to the development of more efficient conservation and assisted reproduction practices for these endemic species.

2. Materials and Methods

This study was conducted at the Sao Paulo Aquarium (Sao Paulo, SP, Brazil; 23°35′36.5″ S 46°36′51.1″ W) in June 2021 and was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA/FMVZ—protocol # 2227140521). The genetic heritage accessions of the animals were also recorded in the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under the protocol number AB3A771. All adopted procedures followed ethical principles and animal welfare [13], being monitored by veterinarians and biologists before, during, and after each procedure.

2.1. Experimental Design

This study utilized eight male stingrays from the genus *Potamotrygon*, comprising four individuals of *P. falkneri* (n = 4; F1 to F4) and four of *P. motoro* (n = 4; M1 to M4) identified by microchips.

These stingrays were all housed at the Sao Paulo Aquarium, maintained following previously established protocols for exhibit disinfection, feeding, environmental conditions, and health management. They were accommodated in three single-sex tanks: M4 alone, M2 and M3 together, and M1 along with all four *P. falkneri*. Water temperatures were maintained between 26.5 °C and 27.1 °C, with a pH level around 7.0.

2.2. Biometrics and Semen Collection

Animals were contained and handled by the same team on the same day to ensure consistency in procedures and data collection. Experienced staff from the aquarium physically restrained the animals, taking precautions to prevent accidents safely isolating the stinger. The stingrays were removed from their tanks using a fishnet, weighed, and then placed in a supine position on a plastic tray with a wet foam base to prevent slipping caused by their body mucus. Biometric data analyzed are presented in Figure 1. Measurements of disc length and width, along with clasper length, were taken using a measuring tape, as per Rosa [14]. The degree of clasper calcification was assessed (non-calcified, partially calcified, and calcified) following the methodology described by Pedreros-Sierra and Ramírez-Pinilla [15]. Additionally, clasper diameter measurements were obtained using a calibrated caliper at the thickest part of the copulatory organ.

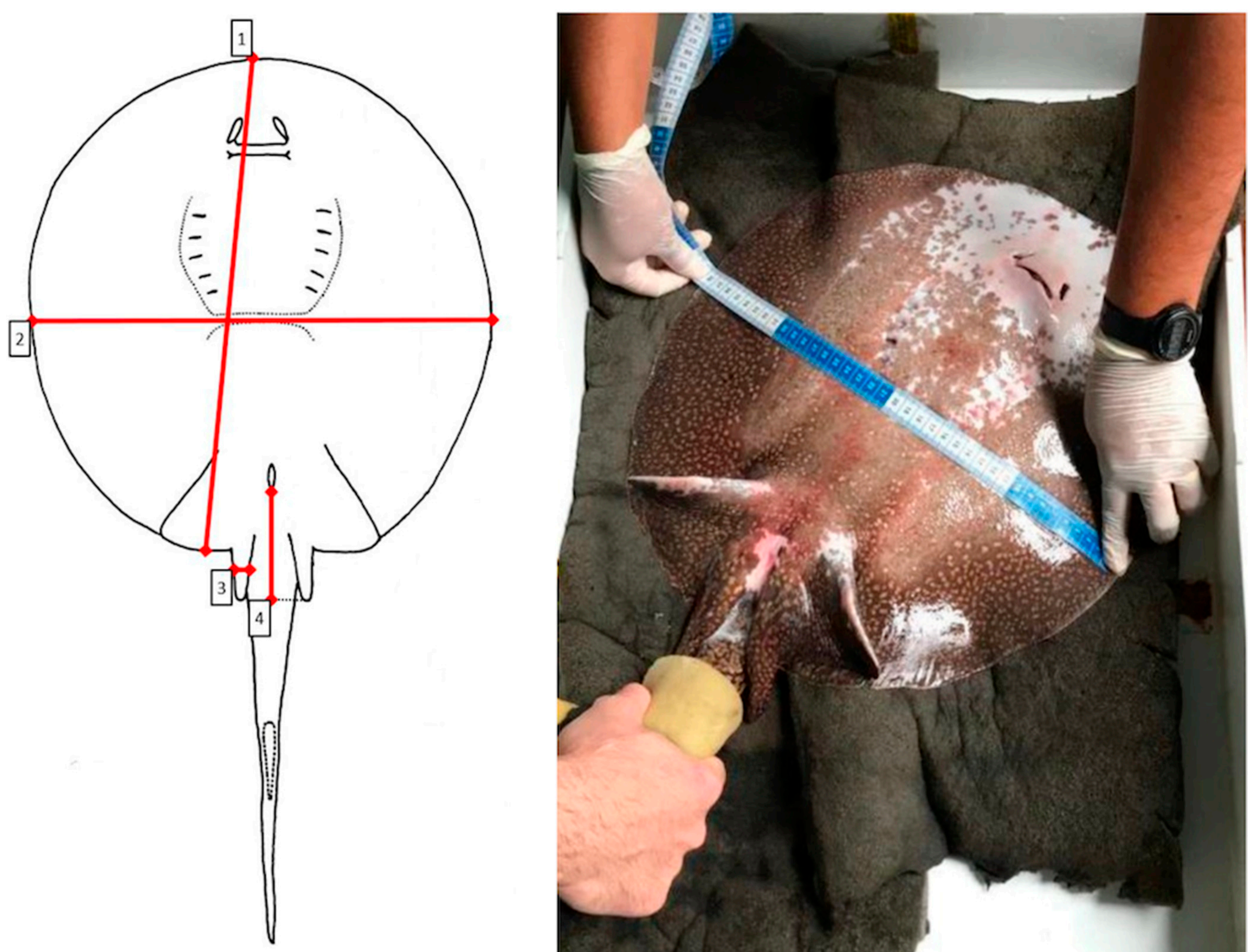

Figure 1. Biometric Data: Measurements of the disc's length (1) and width (2) and the clasper's diameter (3) and length (4).

For sperm collection, the cloacal region of each physically restrained stingray was cleaned and dried with absorbent paper. Subsequently, the region of the seminal vesicles was massaged in a craniocaudal direction to facilitate collection, as described by Padilha et al. [9]. During the procedure, the animals were continuously misted with water from their tank in order to maintain their well-being. Optimization of the biometrics and sperm collection procedure ensured that animals were not restrained for more than five minutes, allowing for their prompt return to their tanks.

2.3. Sperm Evaluation

Collected sperm samples were stored unprocessed and refrigerated in a Styrofoam container with ice for approximately 4 h before being transported to the IMV Technologies Brazil headquarters (Campinas, SP; 22°54′59.8″ S 47°03′40.9″ W) for an 18-day evaluation period. Samples were stored at 4 °C until the moment of each sperm analysis.

Two extenders were evaluated: INRA 96 (IMV Technologies, L'Aigle, France), a milk-type extender based on native casein primarily used for chilling equine sperm, and OptiXcell (IMV Technologies, France), an extender composed of liposomes in a tris-citrate-fructose solution with 6.4% glycerol, designed for the cryopreservation of mammalian sperm. Samples were diluted with either INRA 96 or OptiXcell at ratios of 1:100 (M3; v/v), 1:400 (F3; v/v), and 1:200 (all other males; v/v). This dilution was based on visual assessment, aiming to achieve an optimal concentration of 20×10 sperm/mL (~60 spermatozoa

per system screen), thereby minimizing cell overlap and enhancing the accuracy of the analysis [16]. After dilution, the samples were homogenized for 5 s by vortexing, and 3 µL was placed onto a 4-chamber Leja slide (IMV Technologies, France).

Total motility and progressive motility parameters were assessed using Computer Assisted Sperm Analysis (CASA) with IVOS II equipment (version MK5; Hamilton Thorne, Beverly, MA, USA) and Animal Breeders II software (version 1.11.9; Hamilton Thorne, Beverly, MA, USA). The analysis followed the base setup for elasmobranchs, as previously described by Jorge-Neto et al. [16]. Analysis was performed immediately after dilution at room temperature and again 30 min post-incubation at 37 °C, with the latter time point designated as D0. On subsequent working days, samples were consistently analyzed each 24 h after a 30 min incubation at 37 °C.

2.4. Sperm Cryopreservation

OptiXcell and INRA extenders were also used for cryopreservation. Unlike OptiXcell, which already contains glycerol, INRA required the addition of 5% dimethyl sulfoxide (DMSO) as a cryoprotectant. The semen was diluted at a ratio of 1:3 (semen: extender; v/v). This was extrapolated from studies on teleost fish, where this ratio is generally adopted, as the spermatozoa of some species can be sensitive to the dilution ratio and the components of the extender [17].

Once diluted at a 1:3 (v/v) ratio with each semen extender, the semen was filled into mini straws (IMV Technologies, France) and arranged horizontally on a mini straw freezing rack (IMV Technologies, France). The cryopreservation process occurred on D1 by placing the straws 5 cm above liquid nitrogen within a Styrofoam container for 20 min. Subsequently, they were immersed in liquid nitrogen for storage in a cryogenic tank for future evaluation, as per the methodology described by Araujo et al. [18].

Fifty-seven days post-freezing, three straws from each batch and animal were thawed at 37 °C for 60 s using a semen thawer. The straws were completely emptied with a mandrel, combined into a microtube, and homogenized using a vortex. CASA assessment was performed with a dilution of thawed samples at a 1:1 (v/v) ratio with EasyBuffer B (IMV Technologies, France), except for F3 (1:2 v/v; highly concentrated) and M3 (undiluted; low concentration). The appropriate dilution ratio should not exceed 30×10^6 sperm/mL or fall below 10×10^6 sperm/mL [16]. The samples were then incubated at 37 °C for 30 min before undergoing analysis as previously described.

2.5. Statistical Analysis

Descriptive statistical analysis was conducted for the biometric data, which are presented as mean and standard deviation. Sperm evaluation and cryopreservation data were first assessed for normality using the Shapiro–Wilk test. For hypothesis testing, the Student’s t-test was used for parametric data, while the Wilcoxon test was applied for non-parametric data. The significance level used to reject H0 (null hypothesis—no difference in semen motility between species and between extenders) was 5%. To analyze the correlation between motility and the biometric variables of the studied animals, the Spearman’s test was performed. Both species were grouped for cryopreservation analysis, allowing a more robust assessment of post-thawed sperm.

3. Results

3.1. Biometrics

Reproductively active males of *P. falkneri* exhibited a disc diameter between 35.4 and 37.6 cm, while the disc diameter of *P. motoro* ranged from 29.6 to 33.6 cm. The complete biometric data collected are presented in Table 1.

Table 1. Biometric data.

Measures	*P. falkneri* (n = 4)	*P. motoro* (n = 4)
Weight (kg)	2.5 ± 0.30	1.5 ± 0.37
Disc length (cm)	39.5 ± 1.28	32.5 ± 2.61
Disc width (cm)	36.5 ± 0.96	31.1 ± 1.81
Diameter of the right clasper (cm)	1.8 ± 0.53	1.6 ± 0.13
Diameter of the left clasper (cm)	1.8 ± 0.40	1.7 ± 0.26
Length of the right clasper (cm)	10.1 ± 0.75	9.6 ± 0.67
Length of the right clasper (cm)	9.9 ± 0.29	9.4 ± 0.70

Average values and standard deviation for biometric data obtained for *P. falkneri* and *P. motoro*.

3.2. Sperm Evaluation

The average sample volume collected per animal was 5 mL. Generally, sperm exhibited a viscous consistency, except for individual M3, which produced semen with a liquid-like appearance, and M4, whose sperm coagulated in one of the microtubes.

When correlating motility in the extenders on Day 0 (D0) with the weight and disc width of the individuals, the Spearman test did not identify a correlation between these variables for the extenders (INRA—Spearman test: p = 0.1077; r = 0.6107894; OptiXcell—Spearman test: p = 0.06939; r = 0.6904762).

Sperm samples assessed 30 min after incubation at 37 °C on D0 showed no differences in motility compared to those analyzed immediately after dilution for both extenders (INRA—Shapiro–Wilk test: p = 0.0003; Wilcoxon test: p = 1; OptiXcell—Shapiro–Wilk test: p = 0.0011; Wilcoxon test: p = 0.25; Figure 2). Motility values showed no difference between extenders (Shapiro–Wilk test: p = 0.0292; Wilcoxon test—p = 0.6406). The 18-day average sperm motility values for the INRA and OptiXcell extenders are listed in Table 2 with the corresponding motility curves displayed in Figure 3.

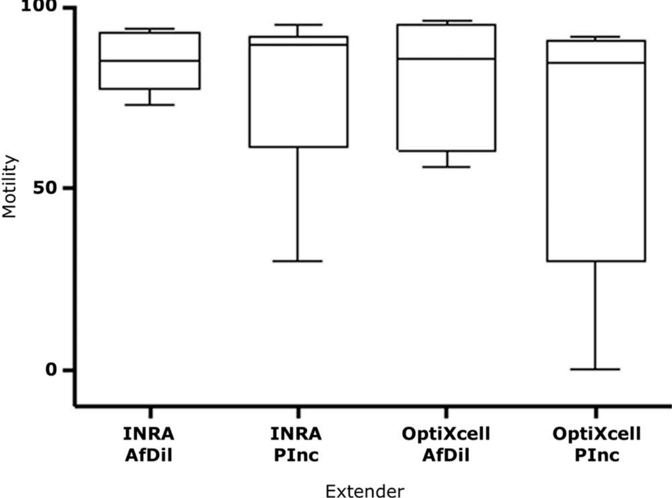

Figure 2. Distribution of sperm motility values for *Potamotrygon falkneri* and *P. motoro* on the first day of the experiment, using INRA 96 and OptiXcell diluents, analyzed immediately after dilution (AfDil) at room temperature and 30 min post-incubation (PInc) at 37 °C (D0). For both extenders, the Shapiro–Wilk test indicated a non-normal distribution (INRA: p = 0.0003/OptiXcell: p = 0.0011), and the Wilcoxon test showed no significant differences in motility between AfDil and PInc (INRA: p = 1/OptiXcell: p = 0.25).

Table 2. Daily average motility (%).

Days	INRA %		OptiXcell %	
	P. falkneri n = 4	*P. motoro* n = 4	*P. falkneri* n = 4	*P. motoro* n = 4
D0	88.0 ± 6.41	82.0 ± 9.24	85.0 ± 13.71	74.2 ± 19.70
D1	91.5 ± 3.88	74.0 ± 14.85	74.8 ± 27.35	77.1 ± 21.84
D2	-	-	72 ± 32.69	68.1 ± 18.06
D3	87.2 ± 5.62	47.1 ± 29.02	85.8 ± 6.31	57.1 ± 30.09
D5	64.4 ± 24.42	59.9 ± 19.14	73.4 ± 28.50	65.4 ± 21.78
D6	67.8 ± 14.93	60.4 ± 21.41	66.7 ± 23.36	54.7 ± 34.11
D8	60.0 ± 22.19	51.0 ± 26.77	57.5 ± 31.75	51.4 ± 31.17
D9	57.3 ± 20.77	41.6 ± 29.91	50.3 ± 32.32	50.6 ± 38.76
D10	51.8 ± 27.98	52.9 ± 23.43	49.9 ± 32.76	57.4 ± 26.95
D11	44.3 ± 20.76	48.6 ± 25.91	36.4 ± 24.21	49.7 ± 31.67
D12	36.9 ± 26.30	46.4 ± 17.60	37.2 ± 27.41	40.2 ± 28.05
D14	45.4 ± 20.11	42.9 ± 20.08	40.5 ± 27.41	26.8 ± 18.40
D15	32.7 ± 17.53	37.3 ± 23.78	31.6 ± 25.38	26.5 ± 16.58
D16	32.0 ± 23.51	29.1 ± 29.20	24.4 ± 23.61	28.1 ± 27.35
D17	31.7 ± 30.27	32.8 ± 27.63	15.8 ± 17.12	20.4 ± 27.09
D18	20.8 ± 18.36	22.9 ± 22.34	17.4 ± 27.45	18.4 ± 18.93

Mean values and standard deviations for the daily motility data of *P. falkneri* and *P. motoro*.

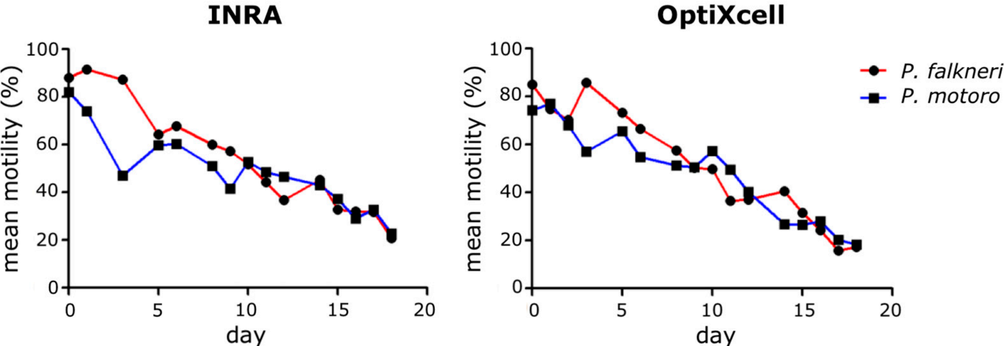

Figure 3. Daily average values of sperm motility for *P. falkneri* and *P. motoro* in INRA 96 and OptiXcell extenders over an 18-day evaluation period, using the CASA system. For both extenders, the Shapiro–Wilk test indicated a normal distribution (INRA: $p = 0.1804$; OptiXcell: $p = 0.195$), and the Student's *t*-test showed no significant differences in motility between species (INRA: $p = 0.09701$; OptiXcell: $p = 0.2282$).

Although no statistical differences were found in the daily motility averages between species for both extenders (INRA: Shapiro–Wilk test: $p = 0.1804$; Student's *t*-test: $p = 0.09701$; OptiXcell: Shapiro–Wilk test: $p = 0.195$; Student's *t*-test: $p = 0.2282$), Figure 3 shows a slightly higher motility average for *P. falkneri* in the first quarter of the study, especially under the use of INRA. Further studies are needed to confirm this differentiation between the species.

3.3. Sperm Cryopreservation

Post-thaw sperm from individuals F3, M1, and M3 exhibited clotting within the straw. In spite of that, after transferring to a microtube, homogenizing, and diluting, the CASA analyses remained unaffected. There was no difference in sperm motility values before and after freezing with INRA + DMSO (Shapiro–Wilk test: $p = 0.0532$; Student's *t*-test: $p = 0.6226$). Conversely, samples cryopreserved with OptiXcell showed a difference in sperm motility post-thaw (Shapiro–Wilk test: $p = 0.0059$; Wilcoxon test: $p = 0.0156$). Pre-freezing and post-thaw motility data for each extender are presented in Figure 4.

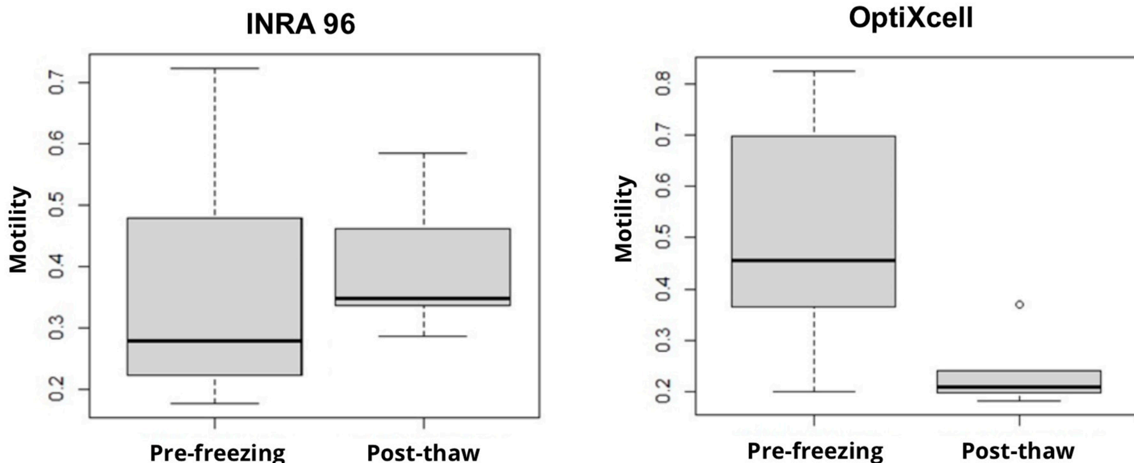

Figure 4. Distribution of average sperm motility values for INRA 96 and OptiXcell analyzed before freezing (Pre-freezing) on the second day (D1) of the experiment and post-thawing (Post-thaw).

4. Discussion

To the best of our knowledge, this study is the first to perform sperm cryopreservation from living *Potamotrygon*. The development of this technology paves the way for the addition of new genetic material from *in situ* males to *ex situ* females [6,7]. Reproductively active males of *P. falkneri* used in this study exhibited disc diameter values exceeding the 26 cm minimum indicated for the onset of sexual activity [19,20], while disc diameter values of *P. motoro* were below the 39 cm [21] but above the 23 to 27 cm parameters [19,20,22] indicated for the species. Therefore, all males used met the reproductive standards predominantly indicated by the literature.

Considering the possible trend in the correlation analysis results observed between the animal's size and weight and sperm motility with INRA and OptiXcell extenders, it is possible that a positive correlation between these variables can be established with the evaluation of a larger number of animals in the future. Since the animals used were close to this maximum size limit to consider the reproduction maturity classification, correlation analysis between size and sperm parameters could be more evident when assessed throughout the male maturation process. The animal with the most diluted ejaculate (M3) was the smallest specimen in terms of weight, disc size, and the length and diameter of the right clasper, despite presenting fully calcified claspers and biometric values consistent with those reported in the literature for a reproductively active male.

Sperm motility analysis after dilution presented no difference on both extenders, however, the results show extremely low minimum values (Figure 2), indicating the limited motility of the unincubated samples. A possible sperm activation by the incubation could explain the observed average increase in sperm motility, with no difference in daily motility curves for INRA and OptiXcell extenders (Figure 3).

The extended survival period of the refrigerated spermatozoa was an aspect that drew considerable attention. Jones et al. [23] reported that chilled semen from broadnose sevengill sharks (*Notorynchus cepedianus*) maintained good motility for one week. Our observation of motile sperm 18 days after collection is unprecedented. It is possible that this period would be longer, as samples still presented an average total motility of 17 to 22% after the evaluation period. The ionic environment and presence of hexose appear to play an important role in the maintenance of cartilaginous fish sperm [24]; nevertheless, further studies on the composition of elasmobranchs sperm are needed for a better understanding of its extensive durability under refrigeration at 4 °C.

The fact that no difference was observed in sperm motility over the 18-day period for *P. falkneri* and *P. motoro* with the extenders tested, indicates that both INRA and OptiXcell can be good extender options for sperm evaluation of these species. Taking these results

into consideration, both species were grouped for cryopreservation analysis, allowing a more robust assessment of post-thawed sperm.

The results of the pre- and post-cryopreservation analysis indicated that the INRA-DMSO showed an increase in sperm motility after thawing in most samples. Although OptiXcell maintained viable and motile cells, it may appear to be an inferior option. However, it is important to consider that elasmobranch spermatozoa remain quiescent in the testis, regulated by ion concentration, pH, or osmolarity of the gonadal environment [25]. This phenomenon varies among different species and, in batoids, is influenced by clasper and alkaline glands. The alkaline glands, which contain highly alkaline fluid, may be involved in the propulsion or motility of semen. Female elasmobranchs possess the nidamental or oviducal gland [26], which can store spermatozoa; however, it is uncertain whether Potamotrygonids have this capability [22,27,28]. While kinetic aspects and progressive motility are highly relevant in mammals, what is ideal for elasmobranchs is not yet well understood. Therefore, the increase in post-thaw motility may not necessarily be desirable for freshwater stingrays, and further studies should evaluate the importance of kinetic and motility parameters in elasmobranchs.

The findings obtained in this study underscore the need for further evaluations on freshwater stingray sperm composition, especially because studies on elasmobranch sperm constitution and its activation [24,29] are only related to marine animals.

Sperm motility of a potamotrygonid in the presence of different fluids was studied by Dzyuba et al. [30], using samples from euthanized animals. Their findings were important to improve the knowledge of seminal fluid composition before ejaculation, without considering, however, the water role.

Despite the fact that elasmobranchs have internal fertilization, few studies discuss the theory that sperm transfer during copulation could involve the water role in facilitating sperm entry into the oviducts [24,31–33]. Carrier et al. [32] suggested that the formation of sperm clusters could serve as protection against sperm loss upon contact with water during copulation. However, if the water also acts on sperm activation, the cluster formation could prevent direct contact of a large number of sperm cells with water.

The possibility that the tank water could play a role in freshwater stingray sperm activation, as demonstrated in marine rays [25], indicates the importance of preventing sperm contact with water during collections used for artificial insemination or cryopreservation. The sperm collection performed in this study was made with care to avoid water contact, drying the cloaca region with absorbent paper, and, at any sign of feces and urine elimination, the collection tube was moved away to avoid contact with the contaminants, as recommended by Cosson et al. [34]

Regarding the sperm motile assessment, the use of counting chambers (Leja) on the IVOS II CASA equipment, utilizing the specific setup for elasmobranchs developed by Jorge-Neto et al. [16], proved to be very effective with high accuracy, allowing the cells free movement without external mechanical interferences that could alter motility. The use of this technique is crucial, considering that the use of cover slips that press the deposited material onto the slide by their own weight can compromise the sperm movement [35]. Moreover, the sperm assessment with counting chambers and the CASA system allows the replicability of studies and comparison between different research efforts, which is crucial for advancing knowledge in the field of elasmobranch reproduction, especially for freshwater species such as *Potamotrygon*.

The findings from this study contribute to a better understanding of reproductive biology in freshwater elasmobranchs, a group for which reproductive information is scant and often extrapolated from marine counterparts. The observed longevity of sperm motility far exceeds previously reported durations, suggesting that *Potamotrygon* species may possess unique physiological adaptations conducive to extended sperm storage. This revelation has profound implications for the development of reproductive technologies and conservation strategies, particularly for species where *in situ* conservation efforts are critical. Furthermore, the successful cryopreservation and subsequent thawing of semen without a

significant loss in motility and viability underscore the potential for implementing artificial insemination and genetic management practices in *ex situ* conservation programs.

5. Conclusions

The successful cryopreservation of *Potamotrygon* semen represents a milestone in the field of freshwater elasmobranch conservation biology. This method has the potential to facilitate the exchange of germplasm between captive and wild populations. The extender INRA 96, with the addition of the cryoprotectant DMSO, was effective in cryopreservation, showing post-thaw motility similar to pre-freezing levels. It was possible to maintain the semen refrigerated for an extended period, with sperm motility ranging from 17% to 22% for both species after 18 days of refrigeration. Further investigations are necessary to verify the fertility of both refrigerated and cryopreserved semen, including the production of offspring resulting from artificial insemination. Through such endeavors, the conservation community can better harness the power of reproductive technologies to safeguard the genetic heritage and biodiversity of aquatic ecosystems worldwide.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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Conflicts of Interest: P.N.J.-N. and F.M.F. were employed by the company IMV Technologies. All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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