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RESEARCH ON THE EVALUATION OF SPERM QUALITY IN THE STURGEON SPECIES *Polyodon spathula* (Walbaum, 1792)

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Abstract

The successful induction of spermiation in sturgeon species is critical for artificial reproduction and aquaculture development. Polyodon spathula (Walbaum, 1792), a commercially valuable fish species, presents challenges in reproductive management under controlled conditions. This study investigates sperm quality parameters in P. spathula males reared in captivity. Five sexually mature males, aged between 9 and 11 years, were selected. Sperm quality was evaluated through macroscopic and microscopic assessments. Hormonal stimulation was performed using Nerestin 5A at doses of 0.05, 0.1, and 0.15 ml/kg body weight in three experimental groups. A fourth male received carp pituitary extract (1.0 ml/kg body weight), and a fifth served as control, receiving physiological saline (0.5 ml/kg body weight). Sperm was collected daily for four consecutive days. The highest sperm yield was recorded with Nerestin 5A at 0.15 ml/kg, producing an average of 2.6 × 10⁹ spermatozoa/kg body weight. These findings underscore the superior efficacy of Nerestin 5A in inducing spermiation in P. spathula, compared to traditional carp pituitary extract or saline solution.

Key words*: carp pituitary extract, Nerestin 5A, Paddlefish, spermiation.*

INTRODUCTION

Fish reproductive technologies are strongly influenced by the quality of seminal products. Sperm quantity and quality (motility, seminal plasma pH, membrane composition and stability, and DNA integrity) can influence fertilization capacity and reproductive success in both natural and artificial reproduction (Kowalski & Cejko, 2019). The sperm of most fish species are immobile in the testis and seminal fluid. Therefore, motility is induced after the sperm are released into the aqueous medium during natural reproduction or into a diluent during artificial reproduction.

The sperm of *Chondrostei* fish are different from those of *Teleostei* fish (Linhart et al., 2006). These differences concern their morphology and physiology, which show a relatively long duration of sperm motility (Linhart et al., 2000a; Linhart et al., 2002).

The quality of sperm is the result of complex interactions between genetic, physiological and environmental factors. These factors influence the different stages of the aquaculture production process, but also the technology of sperm collection and storage in vitro, activation for use in the fertilization and artificial reproduction process. Understanding the factors that influence sperm quality can be useful for their effective regulation and management. These factors have been divided according to the biological characteristics of the breeders (age, weight, length), rearing conditions (water temperature, photoperiod, food, welfare and health of the fish), artificial stimulation of reproduction, breeding season (repeated sperm collection and spermiation interval) and post-milking factors (chemical properties of diluents and short- or long-term storage of sperm).

In aquaculture, different light intensities are used to accelerate or reduce gonadal development, so that fish reproduce at different times of the year, at times desired by fish farmers (Bokor et al., 2008). However, data referring to the role of photoperiod and temperature on sperm quality in fish, especially in the case of species that are commercial, are scarce.

Some studies have shown that temperature influences sperm quality parameters. Hajirezaee et al. (2010) observed that sperm motility (as a percentage and duration)

decreases with decreasing water temperature during the breeding season, while a decrease of only 2ºC has significant influences (this is the end of the breeding season). In contrast, the results obtained in the reproductive process of Siberian sturgeon *Acipenser baerii* (Brandt, 1869), the highest sperm motility was obtained at 10ºC, and the lowest at 17.5ºC (Williot et al., 2000). It is appreciated that the optimal temperature ranges to obtain the best sperm motility should be identical to that in which the species reproduces in the natural environment. The influence of temperature on motility, fertilization percentage and survival rates in scale less carp were highlighted by Bozkurt et al. (2005).

MATERIALS AND METHODS

Research on sperm quality in the sturgeon species *P. spathula* was carried out at the Fish Culture Research and Development Station of Nucet in April 2024, and for this purpose an experimental batch of five males was established. The experimental protocol for sperm analysis and sperm quality assessment involved the following phases: selection of male breeders, breeding stocking, hormonal stimulation, sperm sampling and sperm quality assessment by microscopic and macroscopic analysis.

Selection of male breeders

Five males weighing between 9.620 and 13.400 kg were selected and five experimental groups were formed (Figure 1).

Figure 1. Selection of male breeders (original)

Males were selected according to sexual dimorphism. Thus, males were chosen that had nuptial buttons, a pronounced genital opening, and the degree of maturation was assessed by simple abdominal pressure.

Breeder parking

Parking was done in the breeding and incubation station, in tubs made of tarpaulin that have a useful volume of 4000 L (Figure 2).

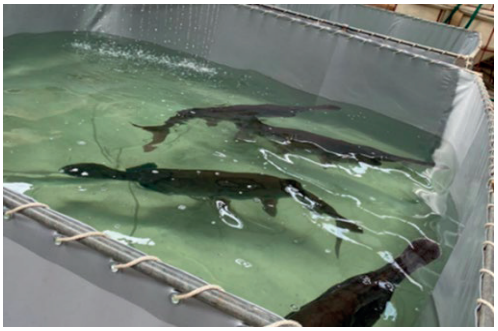

Figure 2. Parking of male breeders (original)

Hormonal stimulation

Hormonal stimulation of *P. spathula* males (Figure 3) was achieved by a dministering a single dose of hormone administered intramuscularly.

Figure 3. Hormonal stimulation of *P. spathula* males (original)

The following doses were used: Nerestin 5A, carp pituitary extract and physiological serum, the doses administered being as follows:

- For male no. 1, Nerestin 5A was used, administered intramuscularly, being injected 0.05 ml/kg body weight;
- For male no. 2, Nerestin 5A was used, administered intramuscularly, being injected 0.1 ml/kg body weight;

- For male no. 3, Nerestin 5A was used, administered intramuscularly, being injected 0.15 ml/kg body weight;
- For male no. 4, carp pituitary extract dissolved in physiological serum at 4 mg/ml and injected intramuscularly at a dose of 4 mg-kg (volume of solution injected 1 ml/kg body weight);
- For male no. 5 (control), physiological saline solution was used, the administered dose being 0.5 ml/kg body weight.

Sperm collection

Sperm was collected after approx. 24 h after the hormonal injection. Dry 20 ml plastic syringes were used, fitted with a 10 cm long cannula made of perfusion tube at the end (Figure 4).

Figure 4. Sperm collection (original)

Sperm was collected over a period of 4 days, every 24 hours. On average, approximately 80-100 ml of sperm were collected from a male during this period. After collection, the sperm was temporarily stored in 25 cm^2 vials placed on wet ice until motility analyses were performed.

Sperm quality assessment

The assessment of sperm quality was carried out by macroscopic and microscopic analysis.

The macroscopic examination involved determining the following: sperm volume, sperm colour, odour, and the presence of sperm waves.

Sperm volume differs depending on the species, and within the species from one breed to another and even from one ejaculate to another in the same breeder. Determination of sperm volume was established in the collecting syringe.

By using sexual stimulation methods, the volume of collected sperm and the number of sperm in the semen can be increased (Linhart et al., 2002). In young males, the volume of ejaculate is lower, compared to that of adult males.

The colour of sperm differs slightly from one species to another, but is closely correlated with the concentration of sperm. Deviations from the appearance and colour considered normal are due to: blood pigments (brown colour); sperm is mixed with urine (yellowish colour); *Pseudomonas spiralis* infections (the colour of the sperm is yellow-green). In all these cases, the sperm cannot be used.

The odour of the sperm is specific and resembles that of starch mucilage. If the sperm has other odours that do not correspond to the mentioned one, this indicates the presence of inflammatory processes in the genital tract and, in this case, the colour also changes.

Assessment of sperm waves in some species, by macroscopic examination, depending on the density and mobility of the sperm, the presence of waves of different intensities caused by the movements of the gametes can be observed. This is visible in thick, freshly harvested sperm, similar to waves, which are shaken in the collecting cup or in a well slide. Medium and rare sperm do not have sperm waves.

By microscopic examination were determined the following parameters:

- Sperm concentration;
- Sperm morphology;
- Sperm motility;
- Sperm viability after sperm activation.

Sperm concentration was determined by the hemocytometric method. The method consists of counting sperm in the squares of the hemocytometric chamber under a microscope and determining the sperm concentration of the seminal material. Sperm concentration was expressed as billions of sperm per millilitres of semen. Sperm volume and sperm count per male were expressed as billions of sperm per male. Sperm volume per kilogram of body weight (ml/kg body weight) and sperm count per kilogram of body weight were expressed as billions of sperm per body weight (kg - 1 b.w.), according to the methods described by Linhart et al., in 1995.

Sperm morphology was assessed immediately after collection by microscopic examination, with or without prior staining and provides important clues regarding the fertilizing

capacity of sperm in the ejaculate. May-Grumwald-Giemsa staining was used to evaluate sperm morphology (Figure 5).

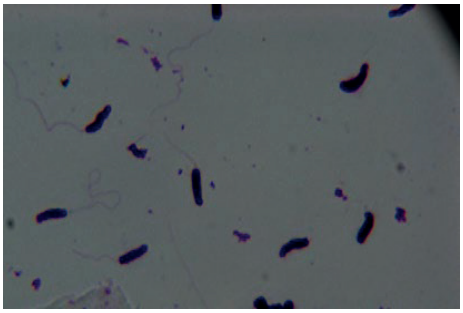

Figure 5. Morphology of *P. spathula* spermatozoa on May-Grunwald-Giemsa stain (original)

Determination of motility was performed using the Axio Vert 1 ZEISS compact inverted microscope. The optical combination used for this control is a 10X eyepiece with a 20X and 40X objective, using a Burker-type gridded slide (Figure 6).

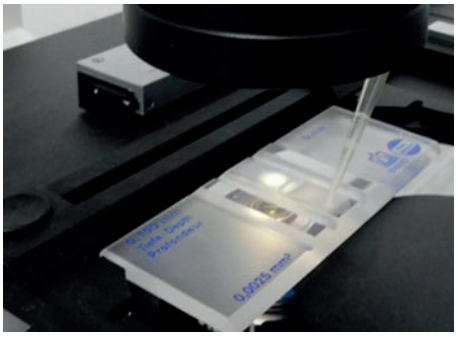

Figure 6. Determination of sperm concentration using a Burker grid slide (original)

For this, the smear was read extemporaneously, using the method used in the practice of artificial reproduction after the sperm was activated with technological water.
Sperm viability was determined by assessing motility. This was done using a compact inverted phase contrast field microscope, the optical combination used is the 10X eyepiece with a 20X or 40X objective, using a gridded slide (Burker). In general, sturgeon sperm has a relatively short viability period after water activation. Therefore, all preparations regarding the operation of activating and diluting the sperm with water must be carried out before fertilization in the incubation station.
After the sperm has been activated, it must be used immediately, within a few seconds, otherwise the chances of producing a good fertilization rate decrease dramatically.

Microscopic examination of semen
Microscopic examination (Figure 7) provides more reliable data on the quality of the ejaculate and allows us to assess: sperm density and concentration, sperm motility, percentage of viable or immature sperm etc.

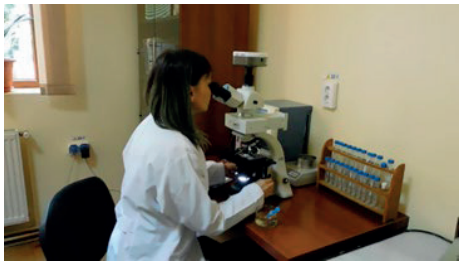

Figure 7. Microscopic examination (original)

The assessment of sperm density was done under a microscope by determining the distance between two spermatozoa.
Determination of sperm concentration was done by the hemocytometric method.
Assessment of spermatozoa mobility was assessed according to their movements, which can be easily followed in the microscopic field. Depending on the appearance of the movements, in a drop of sperm applied to a slide and covered with a coverslip, at physiological temperature, we can encounter several categories of spermatozoa, namely: spermatozoa with forward or rectilinear movement; spermatozoa with circular or "racing" movements; spermatozoa with vibratory or undulating movements; non-motile (dead) spermatozoa.
Determination of the proportion of live and dead spermatozoa - the calculation of this proportion results from counting 100-500 sperm cells and expressing the percentage of live and dead gametes in the respective sample.
Data were obtained in triplicate and statistical significance was assessed using Microsoft Excel '19.

RESULTS AND DISCUSSIONS

Sperm quality in fish is an essential aspect in both natural and artificial reproduction. Sperm quality is influenced by several factors, including sperm count, motility, morphology (shape), and DNA integrity. Nutrition, environment, stress, and other factors can also affect sperm quality.

For the experiment, males were used that were between 9 and 11 years old, weighed between 9.620 kg and 13.420 kg and were between 134.0-148.0 cm length (Table 1).

Table 1. Males used in artificial reproduction experiments in 2024

Male	Age (years)	Weight (g/ex)	Length (cm)
M1	11	13400	148
M2	11	13280	145
M3	9	9830	138
M4	9	9620	134
M5	10	10250	136

The quality of the semen

The main characteristics that define sperm quality and that have been studied are: sperm viability, volume, appearance, sperm concentration and sperm motility. In a study conducted by Linhart et al (2002), who investigated the effects of different environmental conditions on the motility parameters of *P. spathula* sperm, they demonstrated the following characteristics:

- all sperm were motile at 10 s after activation, with a speed of 130-160 microm s^{-1};
- after 2 minutes, the speed decreased to 80-130 microm s^{-1};
- motility was maintained up to 9 minutes.

The characteristics of sperms in this study indicate the following:

- *After one hour of collection*:
  - 2 minutes after activation, approx. 80-90% sperm show forward movements;
  - after 5 minutes, 50% show forward movements;
  - after 10 minutes, 50% show anaemic forward movements (and the rest have pendulum movements);
  - after 15 and 20 minutes, the entire sperm mass was with very weak pendulum movements.
- *After 2 hours after collection:*
  - after activation, about 30% of sperm show vigorous forward movements;
  - after 5 minutes, only 10% show forward movements, 50% have pendulum movements, and the rest of the sperm are motionless.
- *After 4 hours after collection*:
  - sperm show characteristic forward movements, but the vital energy was lower;
  - after 5 minutes activation, only 15-20% of spermatozoa still show pendulum movements, the rest was inert.

At four days post-harvest, spermatozoa of *P. spathula* maintained under refrigeration (4-8°C) demonstrated a marked decline in motility over time following activation. Within the first 5 minutes post-activation, the sperm exhibited distinct progressive motility, characterized by clear forward movement. However, by minute 8 post-activation, only 20–30% of the spermatozoa retained progressive motility, indicating a rapid decline in motile capacity. These observations suggest a limited window of optimal motility for fertilization under the specified storage conditions, emphasizing the need for prompt utilization of sperm post-activation in artificial reproduction protocols.

Volume of sperm collected

The volume of sperm collected from paddlefish male (*P. spathula*) varies depending on several factors: maintenance status, age, body weight, time of year, water temperature and hormonal treatments applied.

According to data from the specialized literature, the volume of sperm collected can vary between 2 and 10 ml per male, depending on age, hormonal treatment and physical condition (Horváth et al., 2006).

In the research carried out, the most effective stimulation of spermiation was obtained with Nerestin 5A compared to carp pituitary glabd extract (CPG) and the saline solution. The highest total sperm volume per male and per kilogram of body weight was obtained during the four days of sperm collection after injection with Nerestin 5A at a dose of 0.15 ml/kg. (Tables 2 and 3).

Table 2. Evolution of *P. spathula* sperm volume obtained after hormonal stimulation

Male	Hormonal Product	Administered dose (ml/kg)	Male weight (kg/ex)	Volume of sperm collected (ml/ex)				
				At 24 hours	At 48 hours	At 72 hours	At 96 hours	Total
M1	Nerestin 5 A	0.05	13.4	23.8	19.5	16.5	12.6	72.4
M2	Nerestin 5 A	0.1	13.28	26.5	21.5	18.2	17.5	83.7
M3	Nerestin 5 A	0.15	9.83	35.6	24.8	22.4	19.5	102.3
M4	CPG	1.0	9.62	18.6	17.5	15.4	10.2	61.7
M5	Saline solution	0.5	10.25	3.4	2.2	0	0	5.6

Table 3. Evolution of *P. spathula* sperm volume per unit body mass after hormonal stimulation

Male	Hormonal Product	Administered dose (ml/kg)	Male weight (kg/ex)	Volume of sperm collected (ml/kg corp)				
				At 24 hours	At 48 hours	At 72 hours	At 96 hours	Average
M1	Nerestin 5 A	0.05	13.4	1.78	1.46	1.23	0.94	1.35
M2	Nerestin 5 A	0.1	13.28	2.00	1.62	1.37	1.32	1.58
M3	Nerestin 5 A	0.15	9.83	3.62	2.52	2.28	1.98	2.60
M4	CPG	1.0	9.62	1.93	1.82	1.60	1.06	1.60
M5	Saline solution	0.5	10.25	0.33	0.21	0.00	0.00	0.14

Total number of sperm collected

Sperm count and quality are widely recognized to determine the success of fertilization in both natural and artificial conditions (Birkhead & Møller, 1998; Locatello, et al., 2020).

A study by Linhart et al. (2000b) investigated the effects of administering a luteinizing hormone-releasing hormone analogue (LHRHa) alongside carp pituitary gland extract. The study found that this combination led to a significant increase in sperm production in male paddlefish. The volume of sperm collected ranged between 0.5 and 5 ml/kg body mass, with a sperm concentration ranging from 5 to 10 billion/ml. The optimal point for sperm collection was identified between 24 and 48 hours after injection, a period in which maximum sperm quality and quantity were observed.

In this research, the total number of sperm collected per male and per kilogram of body weight increased after administration of Nerestin 5A at a dose of 0.15 ml/kg compared to administration of a dose of 0.05 ml/kg and 0.1 ml/kg (Tables 4 and 5).

Table 4. The evolution of the number of spermatozoa in *P. spathula* after hormonal stimulation

Male	Hormonal Product	Administered dose (ml/kg)	Male weight (kg/ex)	The number of spermatozoa collected at n days ($\times 10^9$)				
				At 24 hours	At 48 hours	At 72 hours	At 96 hours	Total
M1	Nerestin 5 A	0.05	13.4	42.9	38.5	28.4	9.8	119.6
M2	Nerestin 5 A	0.1	13.28	46.4	44.3	21.3	5.8	117.8
M3	Nerestin 5 A	0.15	9.83	48.5	44.5	24.5	9.4	126.9
M4	CPG	1.0	9.62	12.8	11.4	5.8	4.6	34.6
M5	Saline solution	0.5	10.25	1.8	8.6	0	0	10.4

Table 5. Evolution of the number of *P. spathula* spermatozoa per unit of body mass after hormonal stimulation

Male	Hormonal Product	Administered dose (ml/kg)	Male weight (kg/ex)	The number of spermatozoa collected at n days ($\times 10^9$)/kg corp				
				At 24 hours	At 48 hours	At 72 hours	At 96 hours	Total
M1	Nerestin 5 A	0.05	13.4	3.62	0.92	0.55	0.38	5.47
M2	Nerestin 5 A	0.1	13.28	3.49	0.95	0.48	0.27	5.20
M3	Nerestin 5 A	0.15	9.83	4.36	0.90	0.74	0.35	6.34
M4	CPG	1.0	9.62	1.33	0.89	0.51	0.79	3.52
M5	Saline solution	0.5	10.25	0.18	4.78	0.00	0.00	4.95

Sperm motility

The most important indicator of sperm quality is the percentage of sperm that are motile and how fast they move. Motility is influenced by: species, temperature, pH, salinity, contaminants and storage time. In fish, sperm become motile upon contact with water, and their movement usually lasts only a few tens of seconds (e.g. 30-60 seconds in most cyprinids) (Linhart et al., 2000a).

Experimental data on sperm motility in *P. spathula* were obtained by microscopic examination of sperm (Figure 8) after hormonal stimulation.

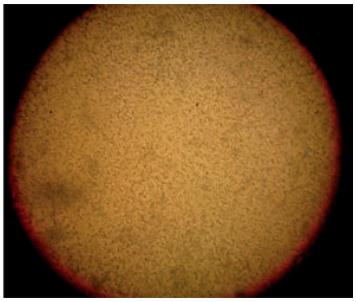

Figure 8. Microscopic examination of *P. spathula* sperm (original)

The research showed that administration of Nerestin 5 A at doses of 0.05 and 0.1 ml/kg resulted in average sperm motility rates of 95.6% and 95.7%, respectively. The 0.15 ml/kg dose had a slightly lower effect, at 88.9%. Treatment with a saline solution (the control group) resulted in very low motility (1.2%) (Table 6).

Thus, it is found that, for 4-5 days after injection, there were no significant differences between the different hormonal treatments applied, in terms of the percentage of motile sperm.

Linhart et al. (2002) observed that the motility of *P. spathula* spermatozoa is influenced by the ionic concentrations in the activation medium. Concentrations of 0.5-5.0 mmol/L of KCl inhibit sperm activation. The addition of $MgCl_2$ significantly improves sperm velocity at 10, 40, 50 and 60 seconds after activation, maintaining a stable velocity of approximately 140 µm/s. Low concentrations of $CaCl_2$ (0.125 mmol/L) combined with 0.5 mmol/l KCl initiate motility in 20% of spermatozoa, while a concentration of 0.25 mmol/L $CaCl_2$ activates all spermatozoa for the entire duration of swimming, with a velocity of approximately 120 µm/s.

Table 6. Evolution of sperm motility in *P. spathula* after hormonal stimulation

Male	Hormonal Product	Administered dose (ml/kg)	Male weight (kg/ex)	Motility of sperm harvested after n days (%)				
				At 24 hours	At 48 hours	At 72 hours	At 96 hours	Average
M1	Nerestin 5 A	0.05	13.4	98.8	98.4	91.1	94.2	95.6
M2	Nerestin 5 A	0.1	13.28	94.2	97.8	94.2	96.4	95.7
M3	Nerestin 5 A	0.15	9.83	91.2	75.4	94.5	94.5	88.9
M4	CPG	1.0	9.62	57.2	94.9	60.2	89.1	75.4
M5	Saline solution	0.5	10.25	0.18	4.78	0	0	1.2

These results suggest that although hormonal stimulation increases the amount of sperm produced, sperm motility is not significantly affected by the type of hormone used. However, environmental conditions, such as ionic concentrations, play an essential role in activating and maintaining sperm motility.

CONCLUSIONS

The use of Nerestin 5A in progressive doses demonstrated superior efficacy to carp pituitary extract, especially at the dose of 0.15 mL/kg, which recommends it as the optimal hormonal agent for inducing spermatogenesis in *P. spathula* under controlled breeding conditions.

The quality of the sperm obtained was closely correlated with the dose of the hormone administered, highlighting the importance of calibrated doses for the effective stimulation of male breeders.

Males of *P. spathula* respond well to hormonal stimulation even after repeated harvests for 4 days.

Microscopic examinations confirmed good morphological integrity and sustained motility of spermatozoa in the first minutes after activation, which is essential for the success of artificial breeding.

The results obtained recommend the use of Nerestin 5A in fish farm managing captive sturgeon stocks, especially to improve the efficiency of artificial breeding and conservation programs.

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