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Sperm characterization of the endangered Amazonian fish *Hypancistrus zebra*: Basic knowledge for reproduction and conservation strategies

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ABSTRACT

Hypancistrus zebra is an ornamental fish endemic to the Xingu River (Brazilian Amazon) and is critically endangered by the construction of a hydroelectric plant in its habitat and illegal fishing. In an attempt to create a germplasm bank for conservation purposes, in the present study there was characterization of *H. zebra* sperm for the first time and assessment of sperm quality throughout the year after successive stripping. Semen was collected four times during a year, and there was similar ($P > 0.05$) high quality for all values of sperm variables evaluated. *Hypancistrus zebra* sperm had an average motility rate of 88.60 ± 2.49% and membrane integrity rate of 87.93 ± 1.88%. There was a peculiar characteristic for the species, with an intermediate sperm vigor (3.00 ± 0.13) and a long duration of motility (14.72 ± 1.31 min) which is uncommon for freshwater fish. Semen had an overall mean of 79.13 ± 9.78% normal spermatozoa and 20.96 ± 9.76% of sperm cells with some morphological abnormalities. The most frequent morphological abnormalities were a degenerated head, an isolated head and a coiled flagellum. The collection of good quality semen throughout the year allows for the possible use of artificial reproduction techniques and cryopreservation for development of a germplasm bank that could contribute to successful conservation of this endangered Amazonian fish.

1. Introduction

Brazil has one of the greatest biodiversity ecosystems on the planet, with more than 3000 known fish species, with the natural habitat of most being in the Amazon basin (Winemiller et al., 2017). This biodiversity has, however, been decreasing as a consequence of environmental changes. There has been a marked reduction in habitat for many fish species due to anthropic effects on the aquatic environment.

The growing energy demand in Brazil and its expansive water resources have resulted in the installation of hydroelectric projects. Damming interferes with the original river flow and affects the habitat of several species, causing habitat loss (Agostinho et al., 2008), modifying the capacity of fish to congregate (Barbosa et al., 2015) and negatively affecting reproduction (Röpke et al., 2017). In fact, the international scientific community is concerned about the future of aquatic biodiversity due to the large number of hydroelectric projects under construction and that are planned for establishment in the Amazon Basin (Lees et al., 2016; Latrubesse et al., 2017).

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These detrimental factors affect the capacity for conservation of the zebra pleco *Hypancistrus zebra* (Isbrücker and Nijssen, 1991), a rare ornamental fish, endemic to the Xingu River (Pará State) occupying the lotic microhabitats in rocky outcrops (Lees et al., 2016). The relatively lesser fecundity of this as compared with many other fish species (Roman, 2011) and the mass illegal fishing for the international aquarium trade led to a significant population decrease, that resulted in placement of the *H. zebra* on the Brazilian Red List (ICMBio, 2016) as a critically endangered species. The species has also been added to the list of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2016). The threat is even greater, especially due to the effect of the construction of the Belo Monte Hydroelectric Power Plant on the habitat for this species. The damages to the environment from this Power Plant enterprise are so severe that Belo Monte has been considered one of the most controversial hydroelectric projects in the world (Lees et al., 2016).

Given the circumstances*, ex situ* conservation may be the only alternative to avoid the extinction of this species. Although there have been some captive-born offspring (Ramos et al., 2016), reproduction in captivity has been inconsistent, which contributes to a lack of capacity in controlling reproduction in this species. Having high-quality gametes is very important for reproductive success in fish (Bobe and Labbé, 2010). *H. zebra* gametes have never been evaluated and it is not known whether captive animals will have the capacity to produce viable sperm. Knowing the seminal quality is the first consideration for standardization for *in vitro* manipulation and for development of semen storage protocols. In the present study, the aim was to characterize *H. zebra* semen and to assess semen quality throughout the year after successive stripping.

2. Materials and methods

2.1. Fish care and permits

Adult *Hypancistrus zebra* were kept at a 0.5 g/l stocking density in 140 l aquariums. Each aquarium had three ceramic caves (3.5 cm diameter x19 cm depth) and two ceramic bricks with eight holes of the same size. Fish were fed commercial dry food (Tetra Color Bits™ and Sera Granulat™) and a laboratory produced semi-solid diet containing: 43.2% fresh sardine, 24% boiled carrot, 20.4% peeled shrimp, 8.04% boiled collard greens, 2.87% flavorless gelatin, 0.96% garlic, 0.24% vitamin supplement, 0.24% soybean oil and 0.05% of salt. The proximate composition of the semi-solid diet was: 13% moisture, 63% crude protein and 5% of total lipids.

The physico-chemical variables of water were monitored periodically, with pH and temperature evaluated daily and the other variables were assessed on alternate days. Daily partial water exchange was performed to maintain stable values for variables as follows: temperature: 28 ± 2 °C; dissolved oxygen: 7 ± 1 mg/l; pH: 7.2 ± 0.2; total hardness: 75 ± 25 mg $CaCo_3$/l; conductivity: 75 ± 30 mS/cm; and total dissolved solids (TDS): 70 ± 25 mg / l. The photoperiod was maintained at a 12/12 h (light/dark) cycle.

The experiments were conducted throughout a year, and four semen collections were performed from the same lot of fish that were procured. This study was authorized by the Chico Mendes Institute for Biodiversity Conservation - ICMBio (SISBIO Nº 48459-1) and was consistent with the precepts of the Animal Ethics Committee of Nilton Lins University (Protocol Nº 010/2015). The research was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under protocol N° A429BE3.

2.2. Hormonal induction and semen collection

Males (6.61 ± 0.82 g) (Fig. 1A) were anesthetized in a bath containing eugenol (40 mg/l) and then were administered a single dose of crude carp pituitary extract (3 mg/kg). Semen collection was performed 8.5 h after the hormonal treatment was administered. The urogenital region of each male was dried with paper towel and contamination of sperm with blood, feces, urine or mucus was carefully avoided during stripping. From each fish there was collection of ≈10 µl of sperm using a micropipette (Fig. 1B).

2.3. Sperm quality assessment

A 1 µl semen aliquot from each male was placed on a glass slide and evaluated (400x magnification) using light microscopy (Eclipse E100 Nikon microscope, USA) to ensure the absence of contamination (immotile sperm). The semen sample was then activated using a 0.3% NaCl solution at a 1:10 ratio (semen:activating solution) and evaluated for the following variables:

- Sperm motility rate (%): the percentage of motile cells having forward movement in the optical field (Maria et al., 2006).

- Duration (s) sperm motility was sustained: at the time of sperm cell activation, a timer was started and counting stopped when there was complete cessation of progressive motility for all spermatozoa in the optical field.

- Sperm vigor (score 1–5): rectilinear and very fast progressive movement (score 5); rapid rectilinear progressive movement (score 4); intermediate movement (score 3); slow movement (score 2); and exclusively oscillatory movement (score 1).

To evaluate the integrity of sperm membranes, a smear was prepared using 2 µl of semen and there was staining with 2 µl of Eosin (5%) and 2 µl Nigrosine (10%) (Viveiros et al., 2012) with there being assessment of 100 sperm cells per sample. The cells with the greatest amount of staining were considered to have damaged membranes while those with lesser staining were considered to have an intact membrane.

To assess spermatozoa morphology, 1 µl of semen was diluted in 1000 µl of buffered formal-saline solution. The buffered fixative solution was prepared by dissolving 1.8% NaCl (150 ml), 4.3% Na_2HPO_4 (71.4 ml), 4.5% KH_2PO_4 (28.6 ml), and 37% commercial solution of formaldehyde (62.5 ml) in 500 ml distilled water (Hancoch, 1957). A smear was subsequently prepared and there was staining with Rose Bengal dye (3%) at 3:1 (semen:dye) ratio (Maria et al., 2012). Spermatozoa integrity of the head, mid-piece and

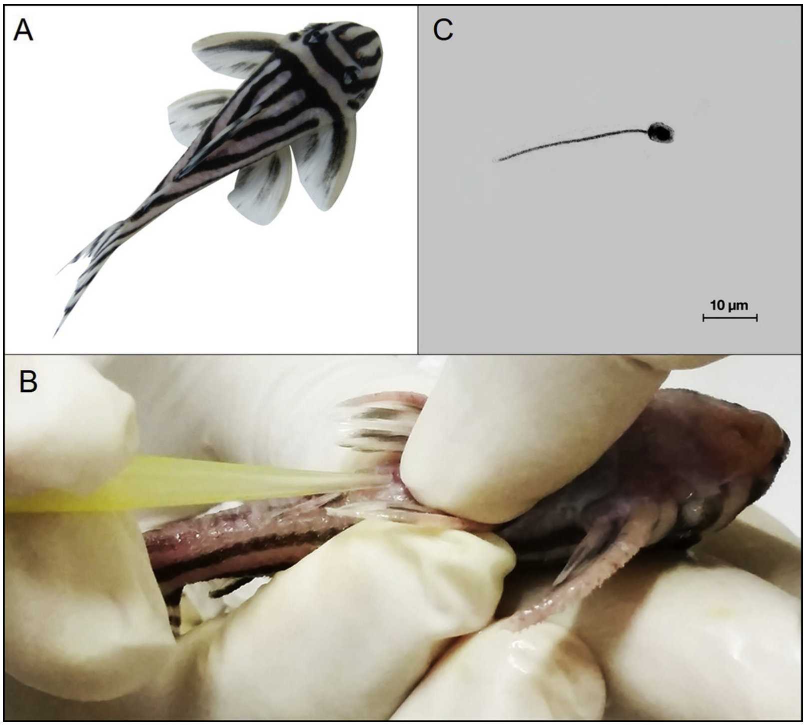

Fig. 1. Adult Hypancistrus zebra male (1A) in which semen was collected four times during a year (1B) to assess sperm quality; Hypancistrus zebra spermatozoon with a total length of 35.00 ± 4.37 µm (1C); (1000x magnification).

flagellum structures as well as a pattern shape (defined in previous examination) were considered to be normal cells. Those cells considered to be abnormal were then categorized to have the following abnormalities (Blom, 1973): Degenerated head (DH), Isolated head (IH), Macrocephaly (Macro), Degenerated mid-piece (DMP), Broken flagellum (BF) and Coiled flagellum (CF), and frequencies of the abnormalities were ascertained. Spermatozoa ($n = 100$) were assessed on each slide using light microscopy (1000x magnification). The values for normal and abnormal spermatozoa were expressed as a percentage of all observed cells.

Spermatozoa morphometry was assessed from 50 spermatozoa images generated from each smear using the Leica DM500 optical microscope with a built-in Leica ICC50 W digital camera (magnifications 400 and 1000x), and later evaluated using the Zeiss ZEN 2 lite software. The diameter of the head, length of the flagellum and total spermatozoon length were determined.

2.4. Statistical analysis

The normal distribution of the data was confirmed using the Shapiro-Wilk and Levene tests. Data are expressed as mean ± standard error of mean (SEM) and sperm morphological abnormalities are expressed as relative frequency of occurrence. Parametric data (motility duration) were analyzed using an one-way ANOVA followed with use of the Tukey's *post hoc* test. Nonparametric data (motility rate, sperm vigor and membrane integrity rate) were analyzed using the Kruskal-Wallis test followed with use of the Dunn's multiple comparisons test. For all comparisons, $P < 0.05$ was considered to indicate statistical significance. BioEstat 5.0 software was used.

3. Results

3.1. Sperm motility, sperm vigor and duration of sustained motility

There was semen collection four times during the year with there being similar ($P > 0.05$) quality for all sperm variables throughout the year (Table 1). Semen of *Hypancistrus zebra* had an average motility rate of 88.60 ± 2.49% ($P = 0.6724$), sperm vigor of 3.00 ± 0.13 ($P = 0.8091$), membrane integrity rate of 87.93 ± 1.88% ($P = 0.0580$) and duration of sustained motility of 14.72 ± 1.31 min ($P = 0.4099$).

The motility rate decreased 55% in the first 5 min after sperm activation (Fig. 2). After 10 min, the rate was 17.58% and in 15 min the motility rate was 3.92%. Sperm cells had some motility for as long as 20 min after activation.

The motility rate and sperm vigor were of a similar pattern, as the motility decreased the sperm vigor also decreased (Figs. 2 and 3). Sperm vigor (Fig. 3) was intermediate (score 2.92) at the time of activation, and there was a marked decrease in the initial 5 min after activation (score 1.83). After 10 min from the time of activation, there was very little sperm vigor with cells having exclusively

Table 1
Quality of *Hypancistrus zebra* sperm throughout the year.

Stripping	Motility rate (%)	Motility duration (min)	Vigor (score 1-5)	Membrane integrity (%)
March/16 *(n = 6)*	85.00 ± 5.00	13.49 ± 2.62	2.83 ± 0.17	89.75 ± 2.61
October/16 *(n = 7)*	90.00 ± 7.45	12.71 ± 3.51	2.86 ± 0.37	89.75 ± 2.61
December/16 *(n = 5)*	90.00 ± 6.90	13.30 ± 1.69	3.20 ± 0.20	95.00 ± 1.34
March/17 *(n = 7)*	89.29 ± 1.70	18.81 ± 1.59	3.14 ± 0.26	76.00 ± 4.09
P-value	0.6724**	0.4099*	0.8091**	0.0580**
Mean value	88.60 ± 2.49	14.72 ± 1.31	3.00 ± 0.13	87.93 ± 1.88

* Parametric data with no significant ($P > 0.05$) difference with assessments using the Tukey's test.
** Non-parametric data with no significant ($P > 0.05$) difference using the Dunn's multiple comparisons test; Data are mean ± standard error of mean.

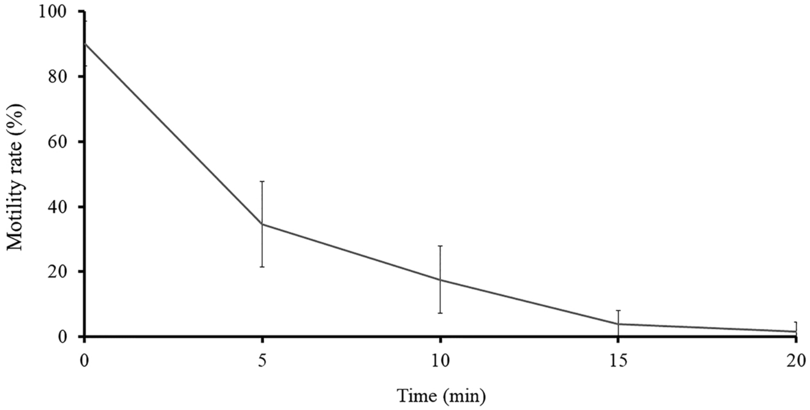

Fig. 2. Spermatozoa motility in *Hypancistrus zeb*ra; Males had semen collected four times during a year to assess sperm quality; Analyses were conducted using a one-way ANOVA followed by use of the Tukey's *post hoc* test; Data are expressed as mean ± standard error of mean.

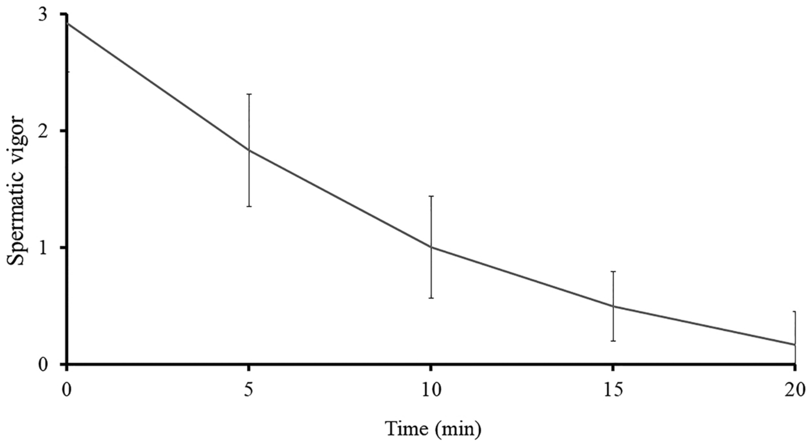

Fig. 3. Sperm vigor in *Hypancistrus zebra*; Males had semen collected four times during a year to assess sperm quality; Score 5 = rectilinear and very fast progressive movement; Score 4 = rapid rectilinear progressive movement; Score 3 = intermediate movement; Score 2 = slow movement; Score 1 = exclusively oscillatory movement; Data were analyzed by Kruskal-Wallis test followed by use of the Dunn's multiple comparisons test; Data are mean ± standard error of mean.

oscillatory movements (Fig. 3).

3.2. Spermatozoa morphology

For the *H. zebra* semen, there was an overall mean of 79.13 ± 9.78% normal spermatozoa ($P = 0.5729$) and 20.96 ± 9.76% of sperm cells with some morphological abnormalities, and there was no difference ($P = 0.5365$) in values for variables among the stripping periods (Fig. 4). Results from morphometry analyses indicate spermatozoa have a head diameter of 4.88 ± 0.84 µm, flagellum length of 30.12 ± 3.97 µm, and a total length of 35.00 ± 4.37 µm (Fig. 1C).

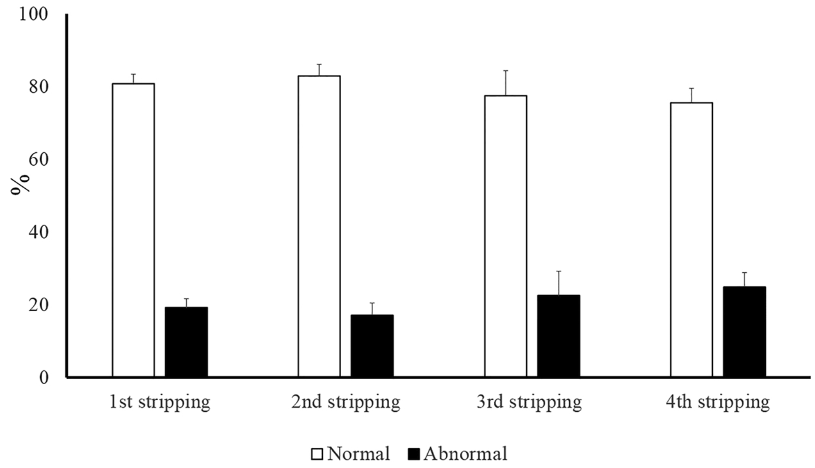

Fig. 4. Percentage of normal and abnormal spermatozoa in *Hypancistrus zebra* semen; Males had the semen collected four times throughout the year: First stripping (March 2016), Second stripping (October 2016), Third stripping (December 2016) and Fourth stripping (March 2017); A smear was prepared and stained with Rose Bengal dye and spermatozoa showing integrity of the head, mid-piece and flagellum structures were considered to be normal cells; Data are mean ± standard error of mean.

The most frequent morphological abnormalities (Fig. 5) in *H. zebra* sperm were a degenerated head, isolated head and coiled flagellum, with an overall frequency of 4.35, 5.17 and 4.39%, respectively. Macrocephaly, degenerated mid-piece and broken flagellum anomalies were of a frequency less than 3%. The anomalies distal and proximal cytoplasmatic droplet were of less than 1% in frequency.

The predominant type of morphological anomaly varied among the stripping periods. At the first stripping, there was a greater frequency of degenerated heads, isolated heads and degenerated mid-pieces (Fig. 5). At the second stripping, the most frequent anomalies were isolated heads and coiled flagella. Isolated heads, macrocephaly and coiled flagellum anomalies were more prevalent at the time of the third stripping, whereas at the last stripping degenerated head (DH), isolated head and coiled flagellum anomalies were more frequent.

4. Discussion

Based on results of the present study, it is possible to collect semen with viable sperm cells from *H. zebra* throughout the year. In addition, there was confirmation that the sperm quality is preserved after successive stripping. There are peculiar sperm motility characteristics for the species, with an intermediate sperm vigor and a longer duration of sustained motility than occurs in sperm of many freshwater fish species, suggesting that the slower flagellar movement allows for energy conservation, allowing for cellular

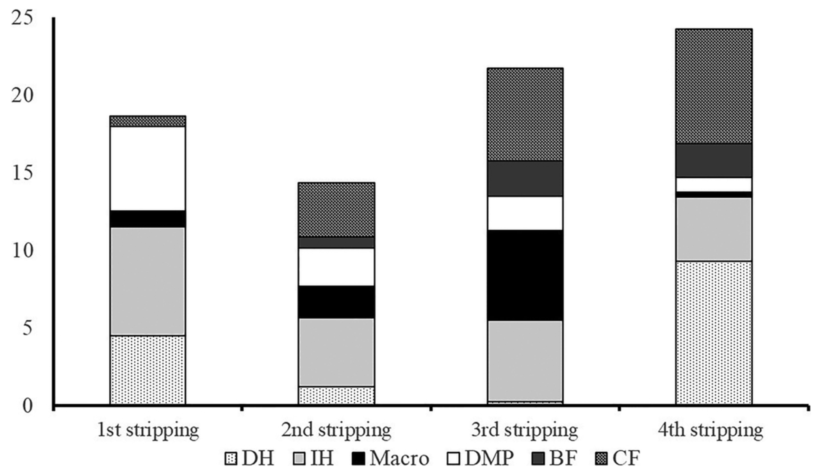

Fig. 5. Frequency of occurrence (%) of morphological abnormalities in *Hypancistrus zebra* semen; Males had the semen collected four times throughout the year: First stripping (March 2016), Second stripping (October 2016), Third stripping (December 2016) and Fourth stripping (March 2017); A smear was prepared and stained with Rose Bengal dye and 100 spermatozoa were assessed on each slide using light microscopy; Abnormalities: Degenerated head (DH), Isolated head (IH), Macrocephaly (Macro), Degenerated mid-piece (DMP), Broken flagellum (BF) and Coiled flagellum (CF); Data are expressed as mean ± standard error of mean (SEM).

motility to be sustained for a longer period. This is a reproductive attribute that could contribute to greater fertilization when using the reproductive technologies for conservation of this species. This pattern of sperm movement may occur due to the relatively lesser rate of flagellar beating and/or reduced magnitude of the movement as compared with sperm of some other fish species (Cachon et al., 1991).

In some Siluriformes fish, an intermediate or slow sperm vigor has been observed, such as a score of 2.0 in *Rhamdia quelen* (Soares et al., 2010), 2.5 in the Amazonian catfish *Leiarius marmoratus* (Galo et al., 2014) and 2.3 in *Pseudoplatystoma reticulatum* (Streit et al., 2012). There are, however, few studies where there was evaluation of the sperm of fish belonging to the family Loricariidae, which does not allow for generalization of seminal characteristics to the fish of the order Siluriformes.

Vigor and duration of sustained motility are sperm quality variables varying among fish species. These characteristics may vary in relation to the different reproductive strategies that ensure reproductive success for the different species. As an analogy to the r/k selection theory for population dynamics (Pianka, 1970), the sperm characteristics may be related to the habitat occupied by the species and related to the environment, being it competitive or not. Differentiated behaviors are observed for fish from lotic or lentic environments, with there being relationships to more or less prolific offspring, with or without investment in parental care for reproductive success (Fogarty et al., 2016). In this sense, *R. quelen* is a Siluriform that releases its gametes in a lentic environment (Soares et al., 2010), which allows for a greater probability of the gametes being deposited in close physical proximity, thus, there not being the need for a great amount of sperm vigor, nor a sustained duration of sperm motility. In contrast, *H. zebra* is a Siluriform from a lotic environment, with a habitat of small caves in rocky outcrops for which there is competition for this specific type of habitat. Considering there is relatively lesser fecundity of this species than many others and that there is competition between males for access to sites were eggs are deposited, the sperm characteristics of this species helps enhance the probability of conservation of the species.

Collazos-Lasso et al. (2018) also observed peculiar characteristics in the semen of sailfin pleco *Pterygoplichthys gibbiceps* (± 600 g) induced to spawn with 4 mg/kg of carp pituitary crude extract. Males released 50 ± 9 µl of semen with 95% of motile cells and the sperm motility lasted for 50 min after collection. Sanches et al. (2011) verified in *Rhinelepis aspera* (± 1150 g) a volume of 9.91 ml of semen and sperm motility rate was 96.5% with the motility not lasting for longer than 90 s after collection. These two representative species of the Loricariidae family, with completely different reproductive strategies and seminal characteristics reinforce the analogy of the r/k selection theory for population dynamics that was described by Fogarty et al. (2016).

In fish with external fertilization, spermatozoa motility depends entirely on energy previously stored or produced by the mitochondria (Dzyuba and Cosson, 2014). There is a rapid ATP utilization after initiation of sperm motility, however, the ATP production by mitochondria is much slower. The energy stores are, therefore, very important and when ATP becomes limiting, sperm motility ceases (Cosson, 2013). The energetic metabolism of spermatozoa can occur in ways that the energy is reserved for flagellar beating by utilization of energy reserves and energy production by the mitochondria.

The flagellum length may affect the ATP distribution and consequently the duration of sustained sperm motility after there was initiation of this process. Cosson (2013) reported that the major energy transport from the mitochondria to the flagellum are in energy-rich molecules such as arginine or creatine-phosphate which are responsible for the homogeneous ATP distribution in the flagellum. Takao and Kamimura (2008) suggested that flagellum lengths shorter than 100 µm may allow for greater efficiency in the ATP distribution because spermatozoa with longer flagella require an alternative means to maintain energy for motility. Thus, the short flagellum of the *H. zebra* spermatozoon (30.12 ± 3.97 µm) is a morphological characteristic that allows for the ATP distribution to be more efficiently distributed along the flagellum, which is a possible explanation for sustaining the relatively longer duration of sperm motility in the species as compared with what occurs in many freshwater fish species.

A long duration of sustaining sperm motility is more common in marine fish than in freshwater species. Marine species such as the turbot and tuna have an average of 10 min of sustained motility duration, whereas most freshwater species have a motility duration lasting no longer than 2 min (Cosson, 2012). Even though there is a longer sustained sperm motility duration in *H. zebra* fresh semen (14.72 ± 1.31 min) there should be a margin of time for management of sperm when manipulations are occurring. It is suggested that the manipulation of semen for artificial reproduction purposes not exceed 5 min at room temperature to avoid loss of fertilization during the period of processing.

The values for seminal variables evaluated indicate that *H. zebra* have a high quality semen, and these characteristics are maintained after successive stripping during the different seasons of the year. Successive stripping from the same male stimulates sperm cell renewal (Kuradomi et al., 2016), allowing for a reservoir of semen with good quality throughout the year. In the specific case of an endangered species such as *H. zebra*, this is particularly important because it allows for several semen collections, which can be cryopreserved and used to develop a germplasm bank for the species.

H. zebra semen had 21% of cells with some abnormalities, and this percentage was constant throughout the year. Even though there are no previous reports regarding the acceptable limit for spermatozoa abnormalities in fresh or chilled fish semen, in the guidelines of the Society for Theriogenology (USA), total spermatozoa morphological abnormalities should not exceed 20% for bulls and boars, and 15% for rams (Ax et al., 2000). This indicates the importance of morpho-pathological examinations of fish sperm in studies, and the effect of morphological abnormalities on fertilization.

In mammals, Blom (1973) classified the spermatozoa morphological abnormalities into major and minor defects. Major defects are those most related to infertility and testicular diseases, while minor defects are related to anomalies with a lesser effect on fertility. Among the sperm abnormalities most frequently detected in *H. zebra* semen throughout the year, degenerated heads are considered a major defect and may originate during spermatogenesis. Pathologies of the spermatozoa head are usually associated with abnormalities in the condensation of sperm chromatin, which may lead to impaired fertilization and early embryonic development, compromising reproductive success (Arruda et al., 2015). Isolated sperm head and coiled flagellum abnormalities are considered minor defects, as these do not interfere with the fertilization process. These abnormalities of the sperm head and flagellum of *H. zebra* had a frequency of occurrence of less than 5%, which should not detract from using this semen for artificial reproduction processes in captivity.

In the present study, the same group of male *H. zebra* had the semen collected four times during a year with there being maintenance of good sperm quality as indicated by there being no differences in values for sperm variables related to fertility among the stripping periods. Although the species have a seasonal profile of reproduction in their natural habitat (Roman, 2011), males kept in captivity under stable conditions of temperature and photoperiod have the capacity for release of semen throughout the year. This characteristic of sustained sperm quality throughout the year allows for the use of semen for artificial reproduction and cryopreservation techniques, which will allow for development of a germplasm bank that can support the conservation of this endangered Amazonian fish.

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