Spermatozoa characterization in the one-sided livebearing *Jenynsia multidentata* (Cyprinodontiformes: Anablepidae)

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Abstract: Several sperm parameters have been employed as useful tools to evaluate fish fertility. Within teleosts, approximately 3% of fish species are known to be viviparous. The Order Cyprinodontiformes includes several species with internal fertilization, and within this group most of the studies about sperm quality have been mainly focused on the Poeciliidae family. The livebearing fish *Jenynsia multidentata* (Anablepidae) inhabits an extensive area of the Neotropical region and it has been used as a useful fish laboratory model to evaluate the effects of xenobiotics through different biomarkers. The present work characterized the sperm of this species through a simple protocol of semen collection. Sperm population showed linearity greater than 89% and 70% of fish have a straight line and curvilinear velocity valued between 50 and 100µm/s. Although 85% of individuals showed a proportion of live sperm higher than 60%, the male population had a high degree of heterogeneity in its sperm count. Morphometry analyses showed a total sperm and head lengths of 46.66±2.06µm and 3.46±0.41mm, respectively. A rather long midpiece region (9.12±0.65µm) was registered, which may indicate high energy-producing capabilities of the spermatozoa. This study established basic parameter values which could be useful for evaluating reproductive potential of *J. multidentata* populations. Rev. Biol. Trop. 62 (3): 997-1006. Epub 2014 September 01.

Key words: sperm parameters, morphometry, sperm motility, sperm viability, viviparous fish, *Jenynsia multidentata.*

Spermatozoa structure in teleost species reveals a high diversity, mainly at the family level. The spermatozoa of internal and external fertilizers differ in their organization. External fertilizers have a simpler organization, showing an ovoid or spherical nucleus, and a small midpiece containing only few mitochondria, whereas species with internal fertilization have an elongated nucleus and a relatively bigger midpiece (Lahnsteiner & Patzner, 2008; Jamieson, 1991, 2009).

Within teleosts, approximately 3% of fish species are known to be viviparous (Wourms, 2005). Particularly, the order Cyprinodontiformes includes several species with internal fertilization (Parenti, 2005). Within this order, most of the studies about sperm characteristics have focused on the family Poeciliidae (Grier, 1973, 1975; Constantz, 1984; Meffe & Snelson, 1989; Meyer & Lydeard, 1993; Evans, Pilastro, & Ramnarine, 2003), whereas within the Anablepidae family, the few existing studies have focused on sperm ultrastructure only (Dadone & Narbaitz, 1967; Greven & Schmahl, 2006). Thus, spermatozoa

morphometry and dynamic parameters within this family are still unknown.

The one-sided livebearing fish, *Jenynsia multidentata* (Jenyns 1842) (Anablepidae) has a wide distribution in an extensive area of the Neotropical region (Ghedotti, 1998) inhabiting both polluted and non-polluted areas (Hued & Bistoni, 2005). It presents sexual dimorphism; males are smaller than females and have a modificated anal fin, called gonopodium (Galindo-Villegas & Sosa-Lima, 2002). Mating behavior is coercive; males approach females from behind and try to thrust their copulatory organ in the female genital pore (Bisazza, Manfredi, & Pilastro, 2000). It is important to note that this species has been used as a useful fish laboratory model to evaluate the effects of xenobiotics through different biomarkers (Cazenave et al., 2008; Amé, Galanti, Bocco, & Wunderlin, 2009; Hued, Oberhofer, & Bistoni, 2012). On the other hand, this fish is considered a useful species in controlling mosquito populations since *J. multidentata* feeds on mosquito larvae (Ringuelet, Aramburu, & de Aramburu, 1967; Marti, Azpelicueta, Tranchida, Pelizza, & Garcia, 2006).

Spermatozoa characterization offers a useful tool to evaluate the fertility potential of male fish. It has been demonstrated that sperm quality could be determined by sperm count, motility, viability and morphology (Kime & Nash, 1999; Burness, Casselman, Schulte-Hostedde, Moyes, & Montgomerie, 2004; Gage et al., 2004; Rurangwa, Kime, Ollevier, & Nash, 2004; Snook, 2005). The main goal of the present study was to characterize the spermatozoa of *J. multidentata* by dynamic parameters, sperm count, viability and morphometry, through a simple protocol of semen collection and to establish basic parameter values for the evaluation of the reproductive potential of *J. multidentata* populations.

MATERIALS AND METHODS

Semen collection: Forty-five adult males of *J. multidentata* (mean standard length: 28.83±3.41mm; mean body weight: 0.479±0.131g) were captured by a backpack electrofisher from a site on Yuspe River, Córdoba, Argentina (31°14'1"8 S - 64°31'14" W), during a reproductive season (September to April) (Mai, Garcia, Vieira, & Mai, 2007; Bianco, Guyón, & Bistoni, 2011). Fish were transported to the laboratory in plastic water tanks (20L). Samplings were performed every two months. Males were acclimated during two weeks under controlled laboratory conditions (temperature at 21ºC; light/dark cycle of 12:12 h) and were fed daily with commercial fish food (TetraMin®). At the end of the acclimatization period, each male was anaesthetized in a water solution of MS-222 (5g /L) (Tricaine methanesulfonate; Sigma Aldrich). The gonopodium was swung forward and introduced in a capillary tube. In order to release sperm, gentle pressure was applied to the side of the abdomen using a cotton tip. The sperm was collected at the base of the gonopodium. The spermatozoa of this species are not packaged as spermatozeugmata or spermatophores but they are released as clumps within mucilaginous material (Grier, Burns, & Flores, 1981). This action was repeated five times for each fish to ensure that all available sperm had been collected. Sperm samples were suspended in 80μL of HAMF-10 culture medium, at pH 7.4 (Invitrogen, Argentina) and sperm separation was achieved by mixing the suspension with a micropipette. All measurements were carried out at room temperature (21±2°C).

Sperm dynamic parameters: Immediately after semen extraction, 12μL aliquot of diluted sperm suspension was placed on a glass slide. The samples were recorded at 100x magnification during four minutes, with a random change of the microscope field every ten seconds. Sperm analysis was carried out with a videomicroscopy system consisting of a phase microscope (Olympus® CX41) and a digital camera (ICAM 1500; Labomed). One hundred and fifty individual cells were tracked per sample. Each track was followed for one second divided in seven steps. Sperm dynamic parameters were analyzed with two softwares.

The ImageJ (NIH, USA) plugin MTrackJ (ver.191.1.0, Eric Meijering; www.imagescience.org/meijering/software /mtrackj/) was used to obtain the X and Y coordinates of each track. On the other hand, each track was analyzed by the Spermtrack IV (Centre for Cell and Molecular Biology, University of Cordoba) to calculate the following kinetic parameters: (i) Straight line velocity (VSL) (μm/s): Straight distance traveled by the spermatozoon from the beginning to the end of its track over time, (ii) Curvilinear velocity (VCL) (μm/s): Length of the spermatozoon track over measurement time and (iii) Linearity (LIN): The quotient between VSL and VCL as an adimensional value that indicates the grade of straightness of a track (expressed in percentage), where values near 100% represent a linear movement and values near 0%, a more erratic path.

Viability and sperm count: Fifteen minutes after sample collection, sperm viability was measured using the eosin-Y staining test (WHO, 2010). Eosin works by penetrating the head membrane of dead cells, which then have pink heads (live cells appear unstained). An amount of 10μL of the sample was mixed on a microscope slide with 1μL of Eosin-Y stain (0.5% wt/vol). Within 1-2 minutes after addition of the stain the sample was covered with a coverslip and examined under a light microscope at 1 000x magnification. One hundred cells were randomly chosen in order to register the number of unstained mobile and immobile cells and stained spermatozoa (died cells). From this, the percentage of spermatozoa viability was estimated for each male.

The volume of semen obtained from each individual, determined by observation of fluid height in the capillary, was approximately the same (about 2μL). A sperm sample dilution of 1:10 was prepared in distilled water and placed in an "improved Neubauer chamber" haemocytometer in order to register the sperm count, measured by duplicate samples. The total amount of spermatozoa was calculated by microlitre of the sample.

Sperm morphometry: 20μL of sperm sample were fixed with 2% formaldehyde and then stained with Coomassie-blue (220% wt/vol). The microphotographs were taken at 1 000x magnification using a light microscope (Olympus® CX31) and a digital camera (Moticam 2300). The total sperm length (TSL), head length (HL) and midpiece length (MPL) were measured using the software Image J (version 1.42q, NIH, USA). A mean value per individual was calculated for each parameter (20 spermatozoa per male).

In order to corroborate the spermatozoa lengths, gonopodium of five males were fixed with 2% of glutaraldehyde and 4% formaldehyde in 0.1M cacodylate buffer for 2h, and then post-fixed with osmium tetroxide at 1% in the same buffer, dehydrated and embedded in Araldite. Thin sections were cut with a diamond knife on a JEOL JUM-7 ultramicrotome, mounted on nickel grids, contrasted with alcoholic uranyl acetate followed by lead citrate, and examined in a Zeiss LEO 906E electron microscope. Microphotographs of ten spermatozoa per male were taken. To ensure a more accurate measurement, only spermatozoa with flagellum insertion site in the head were considered.

Descriptive statistical measures were obtained through the software package InfoStat (2011). Values are presented as means±standard deviation (SD).

RESULTS

Forty-five males presented mean values of VSL and VCL of 81.67μm/s (SD=3.53) and 85.73mm/s (SD=3.55), respectively (Fig. 1A, Fig. B). A linear pattern of movement greater than 89% were observed in all sperm samples, showing a linearity between 94 and 98% in 75% of samples. A proportion of live spermatozoa higher than 60% was registered in 85% of the individuals (Fig. 1C). The mean percentage of live mobile and immobile spermatozoa were 68.28% (SD=8.32%) and 9.46% (SD=4.56%), respectively. On the other hand, the spermatozoa count showed a high

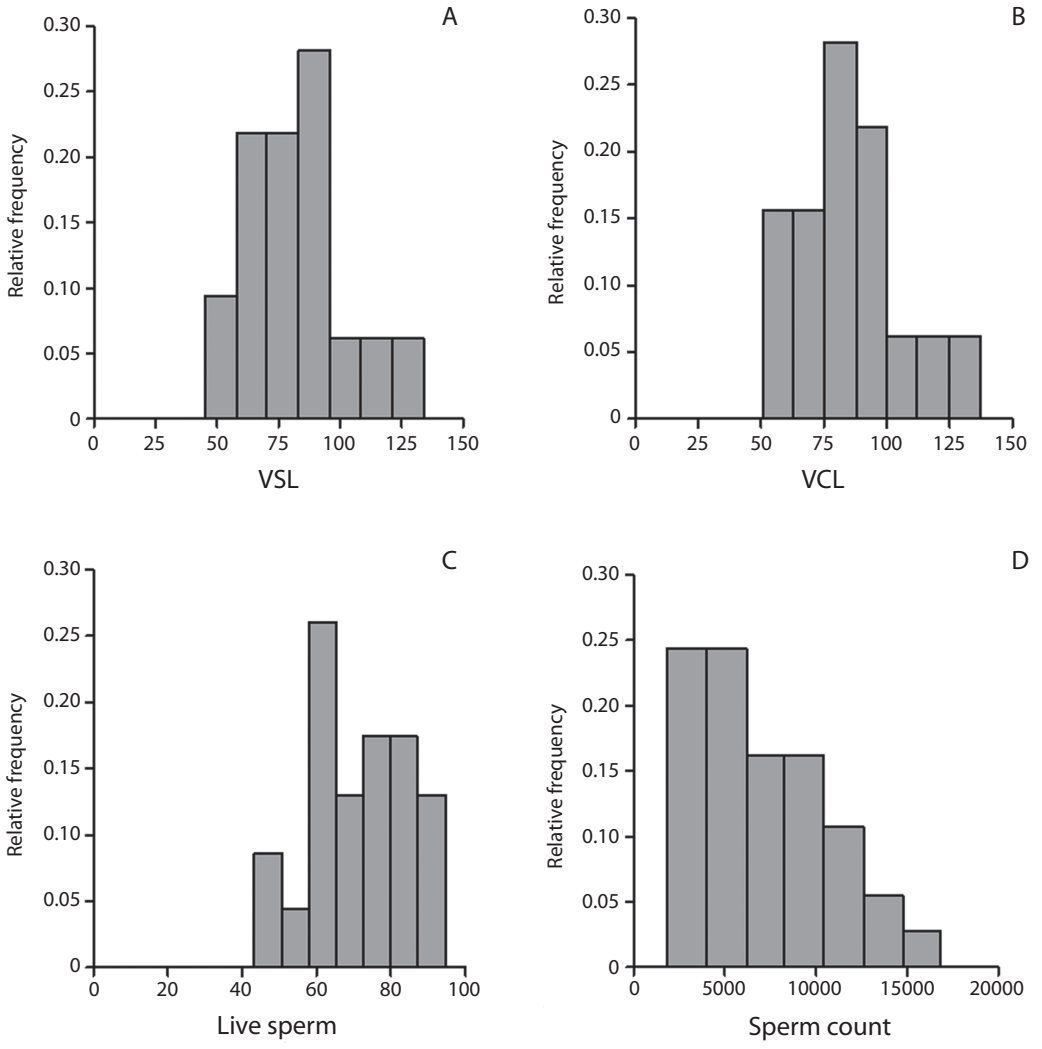

Fig. 1. (A) Relative frequency distribution of straight line velocity (μm/s); **(B)** Relative frequency distribution of curvilinear velocity (mm/s); **(C)** Percentage of live spermatozoa; **(D)** Spermatozoa count (cells/μL sample).

variability between males, being the average value of the 5 524cells/μL sample (SD=728) (Fig. 1D).

The morphometry analyses showed a total sperm length of 46.66μm (SD=2.06), a head length of 3.46μm (SD=0.41) and a midpiece region of 9.12μm (SD=0.65) (Table 1; Fig. 2A, Fig. 2C). The relative frequency of morphometrical values are shown in figure 3. The 65% of the individuals showed TSL values between 46 and 49μm (Fig. 3A). A midpiece region

TABLE 1
Morphometric parameters of
Jenynsia multidentata spermatozoa

Spermatozoa Region (mm)	Mean+S.D	Min	Max
Total length	46.65+2.06	42.20	50.00
Head length	3.46+0.41	2.32	4.59
Midpiece length	9.12+0.65	8.33	10.7

Data are expressed in μm and derived from 20 spermatozoa per individual (n=45). (Mean±S.D, Min, minimum value; Max, maximum value)

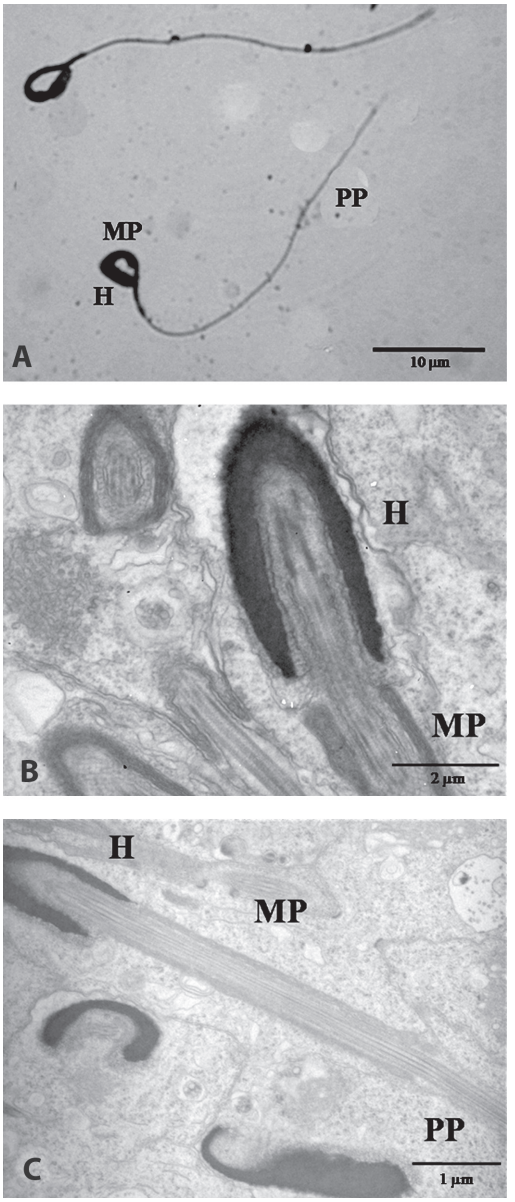

Fig. 2. Spermatozoa of *Jenynsia multidentata* **(A)** Micrograph of spermatozoa showing its head, midpiece and principal piece. The spermatozoa were stained with Coomassie-blue and examined in a light microscope **(B)** Transmission electron micrograph of head; **(C)** Transmission electron micrograph of midpiece region. H, head; MP, midpiece; PP, principal piece.

length values between 9 and 10µm were registered in 50% of males (Fig. 3C).

DISCUSSION

The present work characterized the spermatozoa of *J. multidentata* through a simple protocol of semen collection. The procedure proposed in this work is not invasive, causing no further stress to the individual beyond that of the temporary immobilization and avoid the contamination of the ejaculate with faecal material.

Males of *J. multidentata* present tubular gonopodium, an enclosed tube that enables sperm transfer during copulation (Turner, 1950; Grier et al., 1981; Malabarba, Reis, Vari, Lucena, & Lucena, 1998). Therefore, spermatozoa are not packaged as spermatozeugmata or spermatophores as occurs in Poeciliidae, but they are released as clumps within mucilaginous material (Grier et al*.,* 1981). Although the sperm ultrastructure has been described by Dadone & Narbaitz (1967), these authors did report neither morphometrical values nor dynamic parameters.

It is known that the spermatozoa of inseminating fish present some differences when compared with externally fertilizing species, which appear to be correlated with the mode of insemination (Jamieson, 1991, 2009; Burns & Weitzman, 2005). Species with internal fertilization have a more complex sperm organization, an elongated sperm nucleus and a relatively larger midpiece region compared to externally fertilizing fishes (Jamieson, 1991; Mattei, 1991; Lahnsteiner & Patzner, 2008).

Spermatozoa of *J. multidentata* share a similar morphology with the general model described for inseminating fish. We registered a total length of spermatozoa of around 46.66µm. Comparing with other viviparous species within the same order, the total length is longer than in *Anableps anableps* Linnaeus, 1758 (40µm) (Greven & Schmahl, 2006) but shorter than in *Poecilia reticulata* Peters, 1859 (54.56µm) (Skinner & Watt, 2007) and *Xiphophorus nigrensis* Rosen, 1960 (57.7µm) (Smith & Ryan, 2010). Similar length (around 50µm) has been registered in viviparous fish of another order such as *Cymatoguster aggregata*

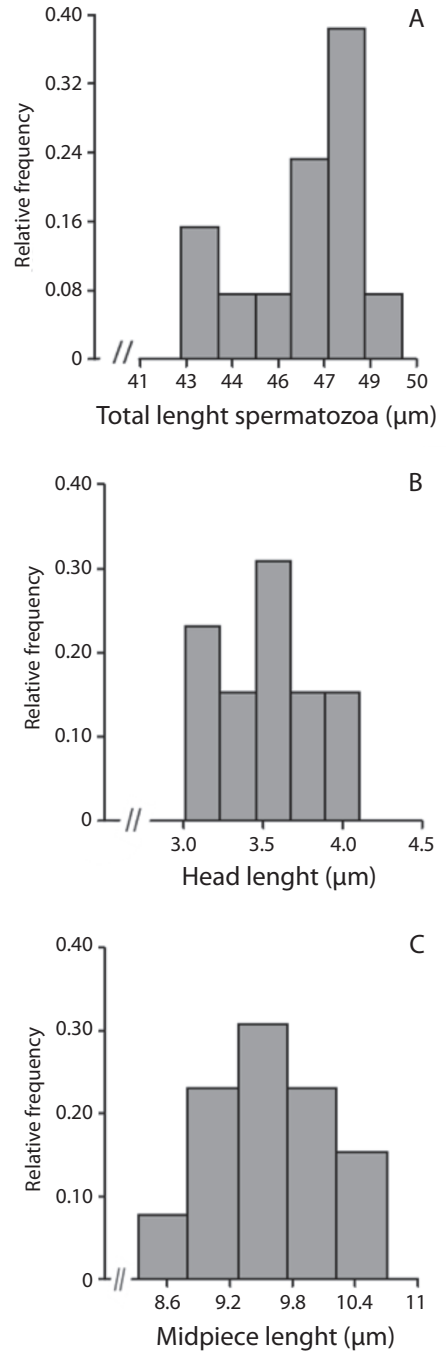

Fig. 3. (A) Relative frequency distribution of Total length spermatozoa (µm); **(B)** Relative frequency distribution of head length spermatozoa (µm); **(C)** Relative frequency distribution of midpiece length spermatozoa. Values were obtained from 20 spermatozoa per male (n=45).

(Perciformes, Embiotocidae) (Gardiner, 1978). On the contrary, in external fertilization fish, it has been registered a high heterogeneity in this parameter, having a shorter length in this kind of fish (Jamieson, 1991, 2009; Burns & Weitzman, 2005; Lahnsteiner & Patzner, 2008).

The head length of *J. multidentata* spermatozoa presents a similar value (around 3.5µm) to the above-mentioned viviparous species, except for *X. nigrensis* which has a shorter head length (2.70µm approximately). Also, this parameter is longer than most of the external fertilization fishes which present a head length lesser than 2µm (Hadi-Alavi et al., 2009; Lahnsteiner & Patzner, 2008). An elongated head, such as the recorded in *J. multidentata*, is a common feature shared by most fishes with internal fertilization. This characteristic gives the sperm certain advantages to move into the female reproductive tract, such as the increase of the side-to-side alignment which enables the clumping of the cells allowing spermatozoa to flow together, and the increase of the directionality of cell movement (Ginzburg, 1968; Gardiner, 1978).

The midpiece of *J. multidentata* (9.12µm) is similar to that registered in *X. nigrensis* (8.94µm approximately) (Smith & Ryan, 2010) but is longer than the values recorded for *P. reticulata*, and *A. anableps* (4.79 and 3.9µm, respectively) (Greven & Schmahl, 2006; Skinner & Watt, 2007). However, the differences are noticeable when comparing with *C. aggregate* (approximately 2µm) (Gardiner, 1978) and external fertilization fishes, where the midpiece is less than 2µm (Lahnsteiner & Patzner, 2008; Hadi-Alavi et al., 2009). In viviparous fish, it has been proposed that an enlarged midpiece increases the capacity of the sperm's energy-generating mechanism and might help to prolong the life-span of the spermatozoa during storage in the ovary, as well as it may provide energy for sperm dispersal throughout the ovary (Fawcett, 1970; Pecio & Rafinsrisky, 1994; Yao, Emerson, & Crim, 1995).

Sperm fertility has been related to sperm motility in several fish species. Sperm motility, evaluated as the sperm velocity and the

percentage of motile spermatozoa, is an integrative quality parameter which combines the quality of several cellular compartments responsible for motility activation and progressive sustained movement. This parameter is extensively used to compare different experimental conditions such as collecting procedures, sperm dilution medium, sperm storage condition and assessment of the effect of xenobiotic on sperm quality (Bobe & Labbé, 2010; Kime & Nash, 1999). Although it is known that anesthesia impacts on sperm motility (Wagner, Arndt, & Hilton, 2002; Dietrich et al., 2005), in the present study, fishes were previously anesthetized to allow the survival of individuals in order to obtain sperm samples and to continue other studies.

The high sperm linearity observed in *J. multidentata* is similar to other fish species with either external or internal fertilization (Lahnsteiner & Patzner, 2008). These authors proposed that the shape of head/midpiece complex has no effect on the swimming pattern of spermatozoa, since comparing the motility pattern of many species with a wide diversity of sperm forms, spermatozoa are predominant linearly motile. It is known that the swimming pattern is mainly modulated by the symmetry of the wave of flagellar beating (Cosson, Dreanno, Billard, Suquet, & Cibert, 1999), and the flagellum is very constant in this construction (in general it is ten times longer than the head-midpiece complex) (Lahnsteiner & Patzner, 2008). The high motility percentage exhibited by J. *multidentata* could be an adaptation to sperm competition pressures. Therefore, in internally fertilizing species with female sperm storage, sperm motility would be important in determining paternity because more motile sperm can remain longer in the female tract (Snook, 2005; Evans, Pilastro, & Schlupp, 2011).

The sperm count recorded in *J. multidentata* presented a great variation among individuals. Our results were in agreement with the high heterogeneity reported by Rurangwa et al. (2004) in external fertilization fishes such as *Oncorhynchus mykiss, Cyprinus carpio* and *Acipenser fluvescens*. These authors pointed out that sperm concentration is not a sensitive or specific measure of sperm fertilizing capacity, as the concentration can vary greatly within a fish species and across the reproductive season. Copulation in poeciliids is rapid (<1s) and does not involve male mounting or clasping that may increase male control over sperm transfer (Birkhead & Møller, 1998). In this regard, *J. multidentata* also presents the same behavior. These results suggest that female behavior is often effective in limiting the size of the ejaculate transferred by finishing the copulation early. Therefore, it has been proposed that traits that increase sperm quality, such as viability and motility (e.g. spermatozoa velocities), as well as ejaculate size or the number of sperm produced might evolve in species in which males have little control over the amount of sperm inseminated (Pilastro, Gasparini, Boschetto, & Evans, 2008; Gasparini, Simmons, Beveridge, & Evans, 2010; Smith & Ryan, 2010; Evans et al., 2011).

Several of the parameters discussed above have been used as useful tools to evaluate fish fertility (Billard & Cosson, 1992; Kime & Nash, 1999; Rurangwa et al., 2004). The evaluation of seminal quality constitutes a critical step in species management and conservation. The results of our work have established the basic parameter values to be in use in the evaluation of the reproductive potential of *J. multidentata*. Since this species is widely distributed in both polluted and non-polluted sites and has been used as a bioindicator in water quality assessment, the most of the sperm parameters characterized in the present work could be used as a sensitive set of indirect biomarkers that could provide early warning signal of reproductive alterations in polluted freshwater systems of an extensive area of the Neotropical region where this species occurs.

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RESUMEN

Caracterización de parámetros espermáticos del pez vivíparo *Jenynsia multidentata* (Cyprinodontiformes: Anablepidae). Diversos parámetros espermáticos han sido utilizados para evaluar la fertilidad de peces. Dentro de los peces teleósteos, aproximadamente el 3% de las especies son vivíparas. El orden Cyprinodontiformes incluye varias especies con fecundación interna. Dentro de este orden la mayor parte de los estudios sobre la calidad del esperma se han centrado principalmente en la familia Poeciliidae. El pez vivíparo *Jenynsia multidentata* (Anablepidae) habita una extensa área de la región Neotropical y ha sido utilizado como un exitoso modelo de laboratorio. El objetivo del presente trabajo fue caracterizar los espermatozoides de esta especie a través de un simple protocolo de recolección de esperma. La población de espermatozoides mostró una linealidad superior al 89% y el 70% de los peces tienen una velocidad lineal y curvilineal entre 50 y 100µm/s. Aunque el 85% de los individuos mostró una proporción de espermatozoides vivos de más del 60%, se observó una alta heterogeneidad en el recuento espermático. Los análisis morfométricos mostraron una longitud total de espermatozoides de 46.66±2.06µm y una longitud de la cabeza de 3.46±0.41µm. Los espermatozoides presentan una pieza media larga (9.12±0.65µm) lo que puede indicar una alta capacidad de producción de energía. El presente estudio establece valores básicos de parámetros que pueden ser útiles para evaluar el potencial reproductivo de las poblaciones de *J. multidentata*.

Palabras clave: parámetros espermáticos, morfometría, motilidad espermática, peces vivíparos, *Jenynsia multidentata.*

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