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Evaluation of the semen characteristics after induced spermiation in the bullfrog *Lithobates catesbeianus*

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ABSTRACT. Evaluation of semen characteristics after hormonal induction of the bullfrog could provide valuable information on the gametes of this species, which may be useful for projects related to artificial fertilization, animal improvement, and cryopreservation. Bullfrog males were induced to spermiate with buserelin acetate (GnRHa), and their semen was subsequently analyzed. GnRHa (0.4 μg) was administered to the bullfrog males with secondary sexual characteristics such as weight > 200 g, yellow chin, nuptial callus, and amplexus reflex, being the semen collected after 60 min. The semen volume was 5.76 mL, light-colored. The other characteristics of the semen were: vigor of 4.80, motility of 93%, concentration of 14.24 $\times$ 10^6 mL^{-1}, and content of normal spermatozoa of 70%. The volume, color, vigor, motility, sperm concentration, and content of normal spermatozoa were adequate in these bullfrog semen samples. Evaluation of the bullfrog semen samples based on this set of parameters is essential for decision-making about the quality and destination of the semen.

Keywords: reproduction, semen, spermatozoid, sperm motility.

Características e avaliação do sêmen de rã-touro *Lithobates catesbeianus* induzidos à espermiação

RESUMO. Avaliação de que as características do sêmen, após a indução hormonal da rã-touro, pode fornecer informações valiosas sobre os gametas desta espécie, podendo ser útil para projetos relacionados à fertilização artificial, melhoramento animal, e criopreservação. Machos de rã-touro foram induzidos à espermiação com acetato de buserelina (GnRHa) e o sêmen foi posteriormente analisado. GnRHa (0,4 mg) foi administrado à rã-touro do sexo masculino com características sexuais secundárias, tais como peso superior a 200 g, papo amarelo, calo nupcial, e reflexo amplexo, e seu sêmen foi coletado após 60 min. O sêmen da rã-touro apresentou volume de 5,76 mL, coloração turva, vigor espermático de 4,80; motilidade espermática de 93%, concentração de 14,24 x 10^6 SPTZ mL^{-1} e 70% de espermatozoides normais. O volume, cor, vigor, motilidade, concentração espermática e o número de espermatozoides normais das amostras do sêmen de rã-touro são adequados. O conjunto dos parâmetros para avaliação das amostras de sêmen de rã-touro é indispensável para a tomada de decisão sobre a qualidade e destino do mesmo.

Palavras-chave: reprodução, sêmen, espermatozoide, motilidade espermática.

Introduction

The bullfrog was introduced in Brazil in the 1930s and reared for its meat (BRAGA; LIMA, 2001). Excellent conditions that led to high zootechnical performance were found in some regions (RIBEIRO FILHO et al., 1998). Frog breeding has been improved nationwide, in terms of both rearing environments and rations, but the genetic improvement of frogs in Brazil has been mainly based on the selection of animals that show exceptional weight gain (AGOSTINHO et al., 1991) but the use of artificial fertilization is still inexpressive.

Since the spermiation process is regulated by steroid hormones (OLMSTEAD et al., 2009), these have been used to induce the animals to spermiate (RIBEIRO FILHO et al., 1998; ROSEMBLIT et al., 2006) and to improve semen collection in terms of both quantity and quality (AGOSTINHO et al., 2000). The availability of artificial insemination and cryopreservation techniques enables the storage and transport of genetic material and eases the fertilization among subjects separated by long distances or in cases of rejection between males and females (GOLDBERG et al., 2002; MICHAEL; JONES, 2004). Bullfrog semen has been evaluated for its spermatic concentration (AGOSTINHO et al., 2000;

ROSEMBLIT et al., 2006). The ultrastructure of spermatozoa in anuran amphibians has been examined in phylogenetic studies (AGUIAR JR. et al., 2004, 2006; COSTA et al., 2004; LEE; JAMIESON, 1993; LIPKE et al., 2009; VEIGA-MENONCELLO et al., 2006; ZIERI et al., 2008).

Mammalian spermatozoa are classified into 23 forms (normal or with major or minor defects) (BLOM, 1973), and fish gametes are classified on the basis of their morphology into 18 forms (normal or with primary or secondary anomalies) (MORAES et al., 2004; STREIT JR. et al., 2008).

The morphological evaluation of spermatozoa is important for determining the quality of semen because sperm pathologies can decrease motility and vigor of the sperm (LAHNSTEINER et al., 1998).

The semen of anuran is similar to that of fish, and sperm motility is the main characteristic common to these groups. In both cases, the start depends on the interaction with the aquatic environment (COSSON, 2004).

This study obtained the semen and spermatozoa of the bullfrog after induction with buserelin acetate (GnRHa) and analyzed the characteristics of the semen based on the parameters adopted for fish.

Material and methods

The experiments were conducted at the Laboratory of the Experimental Frog Farm at the Federal University of Viçosa (UFV) from April 2nd to May 3rd, 2009.

Five *L. catesbeianus* Shaw, 1802 studs were collected on April 2nd, 2009, in the weight gain sector of the Experimental Frog farm at UFV. They had the following characteristics: weight > 200 g, yellow chin, nuptial callus, and amplexus reflex.

The weight (determined on a scale accurate to 0.01) and snout-vent length (determined using a caliper accurate to 0.001) of the animals were recorded on the selection day (April 27th, 2009) prior to semen collection (Table 1).

The specimens were placed in an experimental enclosure (1 m height, 1.2 m width, and 0.9 m length) with a 64-L channel and acclimatized at a temperature of 27°C ± 2°C and photoperiod of 12:12 LD. The animals remained in this location for 25 days (adaptation period) prior to the experiment. The water was changed every day, and the studs were fed (40% crude protein) *ad libitum*. The animals were removed from the maintenance location and taken to the laboratory 5 min before induction. They remained in a container (with 0.5 L of water per animal) until semen collection. The bullfrog studs fasted for 48 h (AGOSTINHO et al., 2000) before receiving a 0.1 mL dose of Conceptal® (0.4 μg of GnRHa) in the celomatic cavity to induce spermiation.

Table 1. Weight (g) and snout-vent length (SVL) of breeding *Lithobates catesbeianus* on the days of selection (1) and semen collection (2).

Animal	Weight (1) (g)	SVL (1) (cm)	Weight (2) (g)	SVL (2) (cm)
1	202.34	13.090	204.50	13.193
2	203.90	13.510	204.56	13.656
3	212.93	13.560	218.89	13.723
4	210.79	13.820	217.89	14.119
5	209.00	13.305	213.78	13.379

A 2 mL glass pipette was introduced into the cloaca, and a finger massage was performed simultaneously in the celomatic cavity close to the pelvic region to facilitate semen collection. Bullfrog semen was collected 60 min after hormone administration (AGOSTINHO et al., 2000). Five samples were collected, one per animal.

The collected semen was placed in a 10 mL transparent graduated glass test tube, and the seminal volume was recorded. The color of the semen was classified as transparent (grade 1) or murky (grade 2).

A 50 μL aliquot of semen in natura of each animal was placed on a histological blade, and a coverslip was placed over the aliquot to allow observation in a clear field microscope at 20× magnification. The vigor is defined as the displacement agility of spermatozoa and is classified on a scale of 0–5. The sperm motility is defined as the percentage of mobile spermatozoa in three fields.

The sperm concentration was evaluated in a Neubauer chamber. A 10 μL aliquot of semen was diluted in 50 μL of buffered formaldehyde (10%) and placed in the Neubauer chamber to count five field quadrants of 1 mm^2 each in order to determine the quantity of spermatozoa per milliliter (SPTZ mL^{-1}).

The semen morphology was evaluated in a clear field microscope at 800× magnification. A 10 μL aliquot of semen was fixed with 50 μL of a 10% buffered formaldehyde solution. A 10 μL aliquot of the sample was deposited and fixed on a histological blade and then covered with a small blade. The morphology of 50 spermatozoa, focused in different fields over the blade, was examined. The methodology was similar to the one used for fish (STREIT JR. et al., 2008; MORAES et al., 2004) since the spermatozoa of these organisms use water for fecundation.

The head, intermediary section, and tail of the spermatozoa were analyzed at 800× magnification. The spermatozoa of the semen in natura from bullfrogs were morphologically classified as normal or with major or minor abnormalities. The spermatozoa

considered normal (175 SPTZ) were measured with a graduated ocular lens in a clear field microscope at 800× magnification.

The total length of the cells, head (along with the intermediary section because it was not possible to precisely measure the intermediary section of the spermatozoa), and tail were measured.

The delineation was completely randomized and the values were submitted to the statistical analysis using the program SAEG (2007).

Results and discussion

Semen samples of the bullfrogs were adequate in terms of volume, color, concentration, vigor, and motility (Table 2).

The sperm motility of the bullfrog was 93%, lower than reported for *Bufo baxteri* induced with human chorionic gonadotropin (hCG) with 95% (BROWNE et al., 2006) and that reported for *Litoria peronii* induced with luteinizing hormone releasing hormone (LHRH) with 97% (SHERMAN et al., 2008). On the other hand, the value was higher than reported for *Xenopus tropicalis* induced with hCG (58%) (GYLLENHAMMAR et al., 2009). This was probably due to the reproductive strategy of these species and the differentiated effect of each hormone on each species (CARNEIRO; MIKOS, 2008). It could also be a consequence of the collection methodology (FERREIRA et al., 2001). However values higher than 80%, reported for *Xenopus laevis* (MANSOUR et al., 2009), could be due to the different reproductive strategies employed by the species. The sperm motility in fish is quite varied, with 81.08% for *Oreochromis niloticus* (MATAVELI et al., 2007), 36.72% for *Leporinus elongatus* (STREIT JR. et al., 2008), 73% for *Rhamdia quelen* (FERREIRA et al., 2001), 80% for *Colossoma macropomum* (MENEZES et al., 2008), 74.17% for *Brycon orbignyanus* (MURGAS et al., 2003), and 90.90% for *Brycon insignis* (ANDRADE-TALMELLI et al., 2001). The sperm motility indicates the percentage of mobile spermatozoa in a semen sample, and the higher values for frogs than for some fish species may represent an evolutionary strategy to increase the fecundation of their oocytes. If the semen of *Bufo marinus* is destined to be cryopreserved, it should have motility as high as 76.3 and 34% before and after freezing, respectively (BROWNE et al., 1998). The corresponding values are 80 and 25% for *Colossoma macropomum* (MENEZES et al., 2008), 90 and 46% for *Oreochromis niloticus* (GODINHO et al., 2003), and 69.09 and 23.18% for *Piaractus mesopotamicus* (STREIT JR. et al., 2009).

Table 2. Volume (VE), color (CE), vigor (VI), motility (ME), concentration (CE), and number and percentage of spermatozoa (SPTZ) in the semen samples of *Lithobates catesbeianus*. The spermatozoa were classified as follows: normal (N), major defects (DMA), minor defects (DMN), degenerated head (CABD), degenerated intermediary section (PID), degenerated tail (CADE), fractured (CAUF) or curled (CAUE) tail, macrocephaly (MAE), normal isolated head (CIN), proximal drop (GP) or distal (GD) head, and folded tail (CAUD).

Sample	1	2	3	4	5	Average	SD
VE (mL)	5.4	10.4	7.1	2.5	3.4	5.76	3.148
CE (1-2)	2	2	2	2	2	2	0
VI (1-5)	5	4	5	5	5	4,8	0.447
ME (0-100%)	95	90	95	90	95	93.00	2.738
CE(10^6x SPTZ mL^{-1})	12.2	10.2	8.8	16.8	23.2	14.24	5.850
N (1-50 SPTZ)	38	37	33	34	33	35.00	2.345
N (%)	76	74	66	68	66	70.00	-
DMA (1-50 SPTZ)	9	6	8	6	9	7.60	1.516
DMA (%)	18	12	16	12	18	15.20	-
DMN(1-50 SPTZ)	3	7	9	10	8	7.40	2.701
DMN(%)	6	14	18	2	16	14.80	-
CABD(1-50SPTZ)	1	0	1	0	0	0.40	0.547
PID (1-50 SPTZ)	0	1	0	0	0	0.20	0.447
CADE (1-50SPTZ)	2	2	1	0	2	1.40	0.894
CAUF (1-50SPTZ)	5	1	4	4	4	3.60	1.516
CAUE (1-50SPTZ)	1	2	2	1	3	1.80	0.836
MAE. (1-50 SPTZ)	0	0	0	1	0	0.20	0.447
CIN (1-50 SPTZ)	1	1	1	2	1	1.20	0.447
GP (1-50 SPTZ)	2	2	1	1	2	1.60	0.547
GD (1-50 SPTZ)	0	1	1	1	1	0.80	0.447
CAUD(1-50SPTZ)	0	2	6	6	4	3.60	2.607

The seminal volume was 5.76 mL $bullfrog^{-1}$. The highest volume of 10.4 mL was obtained with only one specimen, higher than other volumes obtained (2.0 to 4.0 mL) from studs of this amphibian (AGOSTINHO et al., 2000). This could be due to the methodology used, since the animals were kept in water between hormonal dosage and seminal collection.

The sperm vigor of the bullfrog was 4.80 (on a scale of 1–5), higher than the value of 3.21 for *O. niloticus* (MATAVELI et al., 2007), 2.37 for *Leporinus elongatus* (STREIT JR. et al., 2008), and 3.45 for *P. mesopotamicus* (STREIT JR. et al., 2009). The fecundation of both amphibians and fish takes place in aquatic environments, but the sperm vigor in water differs between these groups.

The sperm concentration of the bullfrog semen was 14.24×10^6 SPTZ mL^{-1}, higher than the Creole frog (11.00×10^5 SPTZ mL^{-1}) and bullfrog induced with hCG (9.00×10^5 SPTZ mL^{-1}) (ROSEMBLIT et al., 2006). The 10-fold higher values can be explained by the different action of the hormones (GnRHa and hCG) in the animals (CARNEIRO; MIKOS, 2008). Studies on *Bufo marinus* confirmed the hypothesis that hCG had induced more males; possibly due to the affinity of the cell receptors for the hormones (LIMORI et al., 2005). Induction of *Xenopus laevis* by GnRHa resulted in 25×10^6 SPTZ mL^{-1} (MANSOUR et al., 2009), higher than reported in this study. Possibly this species has presented more

receptors for GnRHa. However, the sperm concentration of the bullfrog is within the range of 1.56 × 10^5 to 1.62 × 10^7 SPTZ mL^{-1} proposed for this species (AGOSTINHO et al., 2000). The values for non-induced *Crinia georgiana* were reported to be 8 × 10^6 SPTZ mL^{-1} (SIMMONS et al., 2008) and 2.548 × 10^7 SPTZ mL^{-1} (HETTYEY; ROBERTS, 2007), but the animal had to be sacrificed to remove the testicles. In contrast, hormonal induction allowed collecting the semen with a pipette without euthanizing the animal. In literature, the sperm concentration has varied among different fish species. For example, it was 2.63 × 10^9 SPTZ mL^{-1} for *O. niloticus* (MATAVELI et al., 2007), 7.5263 × 10^9 SPTZ mL^{-1} for *Salminus brasiliensis* (SANCHES et al., 2009), 27.36263 × 10^9 SPTZ mL^{-1} for *Prochilodus lineatus* (MURGAS et al., 2007), 69.9 × 10^6 SPTZ mL^{-1} for *R. quelen* (FERREIRA et al., 2001), 54.42 × 10^6 SPTZ mL^{-1} (LUZ et al., 2001) and 24.76 × 10^6 SPTZ mL^{-1} for *B. insignis* (ANDRADE-TALMELLI et al., 2001). The sperm concentration in fish is higher than in bullfrog, which may be due to the fact that more oocytes are fecundated in fish.

All semen samples obtained from the bullfrog were murky, which is an indicator of the quantity of seminal fluid and concentration of spermatozoa (ANDRADE-TALMELLI et al., 2001).

The number of normal spermatozoa in bullfrog semen samples was higher than in *Prochilodus lineatus* (40.2%), *Leporinus macrocephalus* (49%), and *Cyprinus carpio* (37.6%) (MORAES et al., 2004). It was also higher than registered in *Leporinus elongatus* (54.7%) (STREIT JR. et al., 2008) and *R. quelen* (32.10%) (BOMBARDELLI et al., 2006). Fish has exhibited morphological changes related to hormonal induction for spermiation (KAVAMOTO et al., 1999).

Major abnormalities in spermatozoa were more common (6) than the minor ones (4). This result should be carefully analyzed, because major defects affect fecundation to a greater extent. Furthermore, abnormal spermatozoa are not necessarily unviable for fecundation because the aquatic environment can help in their displacement.

The abnormalities associated with major defects were, in a decreasing order: fractured (3.60 SPTZ), curled (1.80 SPTZ), or degenerated tail (1.40 SPTZ); degenerated head (0.40 SPTZ); degenerated intermediary sector (0.20 SPTZ); and macrocephaly (0.20 SPTZ). The microcephaly abnormality was not detected in the bullfrog semen samples. The abnormalities classified as minor defects in the semen of the bullfrog were as follows: folded tail (3.60 SPTZ), proximal drop (1.60 SPTZ), normal isolated head (1.20 SPTZ), and distal drop (0.80 SPTZ). The abnormalities that affect the tail are the most important since the tail is the specialized organ for locomotion in spermatozoa.

The abnormalities that prevent the fecundation, based on the motility and vigor, were: macrocephaly, degenerated head, degenerated, fractured, or curled tails, normal isolated head, proximal or distal drops, and folded tail.

Folded and fractured tails represented 49.33% of the abnormalities, but these did not impair the fecundation. This explains why the quality of the semen cannot be judged only on the basis of the percentage of normal spermatozoa or on those with major or minor defects.

The spermatozoa from the bullfrog semen exhibited morphologies considered to be abnormal such as degenerated head, degenerated intermediary section, degenerated, fractured, or curled tails, macrocephaly, microcephaly, proximal drop, normal isolated head, distal drop, and folded tail.

The sperm concentration and vigor are also important for evaluating the quality of spermatozoa in bullfrog semen, because those with macrocephaly are mobile but have low vigor, which hinders the fecundation.

The volume, semen color, vigor, motility, concentration, and forms of the spermatozoa are important characteristics that should be examined when choosing bullfrog semen samples for projects of cryopreservation, improvement, or breeding of animals from distant places.

Conclusion

The volume, color, vigor, motility and sperm concentration proved to be useful in comparing different species of frogs and fish. The number of normal spermatozoa or those with major or minor defects is within the acceptable range. The destination of the bullfrog semen can be decided considering these evaluation parameters. The evaluation of bullfrog semen samples should be based on ranges of ideal values for each parameter.

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