Buserelin acetate-induced spermiation in *Leptodactylus ocellatus* and evaluation of semen characteristics

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ABSTRACT. By understanding the hormonal action behind the induction of spermiation in *Leptodactylus ocellatus* can help in the collection of semen for artificial insemination and cryopreservation. The experiments were conducted at the Experimental Frog Farm of the Federal University of Viçosa. Seven *L. ocellatus* males with the following secondary sexual characteristics were selected: black thorns, developed forelimbs, and amplexus reflex. Spermiation was induced in these animals by applying daily 0.4 µg buserelin acetate hormone (GnRHa) for 7 days, being the semen collected 90 min after the application. The volume, color, vigor, motility, sperm concentration, and spermatozoa morphology were subsequently evaluated. Three animals responded to GnRHa induction, enabling the collection of seven samples of semen, and the first sample was collected 97.5h after the first application. The semen of the creole frog presented the following characteristics: an average volume of 0.38 mL, murky color, sperm vigor of 3.71, sperm motility of 77.14%, sperm concentration of 6.60×10^6 SPTZ mL^{-1}, and 69% of normal sperm. GnRHa can induce spermiation in the creole frog. Although the volume of collected semen was low, the color, vigor, motility, concentration, and spermatozoa content showed to be adequate.

Keywords: creole frog, GnRHa, semen, spermatozoon, sperm motility.

Espermiação com acetato de buserelina em *Leptodactylus ocellatus* e avaliação de características seminais

RESUMO. O conhecimento da ação de hormônios na indução à espermiação de *Leptodactylus ocellatus* pode permitir a coleta de sêmen para estudos de fertilização artificial e criopreservação. O experimento foi realizado no Ranário Experimental da Universidade Federal de Viçosa, onde sete machos de *L. ocellatus* com as características sexuais secundárias: acúleos negros, membros anteriores desenvolvidos e reflexos ao amplexo foram induzidos à espermiação com aplicação do hormônio acetato de buserelina (GnRHa) na dosagem de 0,4 µg, durante sete dias a cada 24h e o sêmen coletado após 90 min. O volume, cor do sêmen, vigor espermático, motilidade espermática, concentração espermática e morfologia dos espermatozoides foram avaliados. Três animais responderam à indução pelo acetato de buserelina permitindo a coleta de sete amostras de sêmen, a primeira após 97,5h da primeira aplicação. O sêmen da rã-manteiga apresentou volume médio de 0,38 mL, coloração turva, vigor espermático de 3,71; motilidade espermática de 77,14%, concentração de $6,60 \times 10^6$ SPTZ mL^{-1} e 69% de espermatozoides normais. O acetato de buserelina pode induzir à espermiação da rã-manteiga. O volume seminal coletado foi baixo, mas a coloração, vigor, motilidade, concentração e número de espermatozoides de *L. ocellatus* foram adequados.

Palavras-chave: rã-manteiga, GnRH, sêmen, espermatozóide, motilidade espermática.

Introduction

Degradation of natural ecosystems by human activities (YOUNG et al., 2000), introduction of exotic species such as the bullfrog, and hunting are reasons for the decline in amphibian population in different parts of the world (JUNCÁ, 2001). The rearing of native species like *Leptodactylus ocellatus* for return to the wild can help in lessening this problem. Understanding the process of reproduction and the action of reproductive hormones is important when using induction-based methods to obtain gametes for cryopreservation and artificial fertilization.

Fertilization is an important tool in aquaculture that facilitates the storage and transport of gametes (GOLDBERG et al., 2002). However, due to the lack of studies on *L. ocellatus* biology, especially on its reproductive physiology and gametes, it becomes difficult to study this organism; which reinforces the need for further research on this Brazilian species.

Spermiation inducers, hormone doses, and semen collection time are important factors for both induced reproduction and for obtaining tadpoles in captivity for repopulation. Injection of hypophyseal raw extract (EBH) induced the mating in bullfrogs, with each female laying 24,310 oocytes (RIBEIRO FILHO et al., 1998).

Hormones can induce mating in bullfrog individuals with secondary sexual characteristics (RIBEIRO FILHO et al., 1998). Moreover, artificial fertilization with hormone analogues (LHRH) can stimulate gonadal activity and gamete production in this frog under favorable conditions of temperature, photoperiod, and relative humidity (AGOSTINHO et al., 2000). Bullfrogs and creole frogs can spermiate upon induction with hCG, in the absence of complementary hormones (ROSEMBLIT et al., 2006).

The ultrastructure of spermatozoids in anuran amphibians was studied for phylogenesis (AGUIAR JR. et al., 2004, 2006; COSTA et al., 2004; LEE; JAMIESON, 1993; VEIGA-MENONCELLO et al., 2006). Studies were conducted on the gametes of these animals to improve the reproductive techniques used in frog farming (RIBEIRO FILHO et al., 1998; AGOSTINHO et al., 2000).

Anuran semen was classified on the basis of its concentration (ROSEMBLIT et al., 2006; AGOSTINHO et al., 2000) and motility (BROWNE et al., 2006). Mammalian spermatozoa are classified into 23 forms (normal or with major or minor defects) (BLOM, 1973), and fish gametes are classified into 18 forms based on their morphology (normal or with primary or secondary anomalies) (MORAES et al., 2004; STREIT JR. et al., 2008). Morphological evaluation of sperm is important for determining the quality of semen because sperm pathologies can decrease motility and sperm vigor (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. The initiation of sperm motility depends on the interaction with the aquatic environment (COSSON, 2004).

This study developed a hormonal induction technique for *L. ocellatus* by applying the methodology used in bullfrogs. Moreover, the semen and spermatozoa of these frogs were analyzed using the parameters adopted for fish.

Material and methods

The experiments were conducted at the Experimental Frog Farm of the Federal University of Viçosa (UFV) between January 27th and May 3rd, 2009.

Thirty-four *L. ocellatus* specimens were collected from a natural environment (field) over a period of 34 days. In addition, 30 were collected from the Municipality of Viçosa, 2 from Vieiras, and 2 from Coimbra. All of these places are within the State of Minas Gerais. The animals were collected under license number 17.252-1 (Ibama/Sisbio) from September 18th, 2008, to March 4th, 2009, between 19:00 and 23:00 hours using a funnel net after detection with a flashlight. The specimens were put into transparent plastic bags immediately after capture, and then transferred to a canvas pack for transportation. The temperature at the animal's collection site was measured with a digital thermometer and varied from 20 to 26°C. The collected animals were taken to the laboratory of the Frog Farm at UFV, weighed on a digital scale (accurate to 0.01 g), and measured with digital calipers (accurate to 0.001 cm).

Male frogs were isolated in white plastic boxes (0.40 m in height, 0.50 m in width, and 0.60 m in length) in an acclimatized room (temperature 29.5°C ± 1.5°C and photoperiod of 12:12 LD). Each box contained 3 kg of soil from the B horizon of a red-yellow latosol distributed throughout the base and a 1-L round glass container with water. A mesh was placed over the boxes to prevent escape, and they remained in the room during the biometric experiments.

Male creole frogs with the following characteristics were selected: weight > 100 g, black thorns, developed forelimbs, and amplexus reflex. The animals were selected on April 27th, 2009, for hormonal induction and semen collection. Seven out of 27 collected male animals were used for the experiment. Thirteen animals had died during the period of adaptation or acclimatization, 6 did not present the required characteristics, and 1 was injured. The selected animals were fed for 138 days of acclimatization period prior to the experiment.

From the selected animals, numbers one, two, four, six, and seven were collected from Viçosa, number three was from Vieiras, and number five came from Coimbra. The water was changed daily, and the studs were fed with froglets and tadpoles of bullfrog.

For hormonal induction, each male creole frog received a daily dose of 0.1 mL Conceptal® (0.4 μg of GnRHa) (AGOSTINHO et al., 2000) into the celomatic cavity for 7 consecutive days. To extract the spermatozoa, a 2 mL glass pipette was introduced into the animal's cloaca 90 min. after hormone application (AGOSTINHO et al., 2000). The collector carefully finger massaged the celomatic cavity near the pelvic region without excessive pressure to avoid collection of feces and

organisms (worms) along with the semen. Sperm were daily collected.

Semen drops of each sample were placed on histological blades and visualized under a clear field microscope. Samples containing spermatozoa were considered to be positive.

The semen of the animals was placed into a 10 mL test tube, and samples that tested positive for spermatozoa were later examined.

The volumes of the semen samples were verified using a graduated test tube, and the color was classified as either 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 sample to allow observation under a clear field microscope at 20× magnification. The vigor of the spermatozoa was classified on a scale of 0-5. Sperm motility was defined as the average percentage of mobile spermatozoa in three fields.

A 10 µL aliquot of semen was diluted into 50 µL of buffered formaldehyde (10%) and placed in a Neubauer chamber. The sperm concentration was estimated in five quadrants (1 mm^2) to determine the amount of spermatozoa per milliliter (SPTZ mL^{-1}). The morphology of the spermatozoa was observed under a clear field microscope at 800× magnification.

A 10 µL aliquot of semen was diluted into 50 µL of 10% buffered formaldehyde, and a 10 µL aliquot was placed on a histological blade and covered with a small blade. The morphology of 50 spermatozoa was observed in several fields over the blade. The methodology used here is the same as that for fish (MORAES et al., 2004; STREIT JR. et al., 2008) because amphibians also use water for fecundation. The heads and tails of the spermatozoa were observed at 800× magnification, but the intermediary section was not examined due to difficulties in visualization. The spermatozoa in the semen aliquots were classified depending on the presence of major or minor abnormalities. Major abnormalities of the spermatozoa in the *L. ocellatus* semen included degenerated head, macrocephaly, microcephaly, degenerated tail, fractured tail, and curled tail. Minor abnormalities included normal

isolated head, proximal drop, distal drop and folded tail. The abnormalities were decreasing order classified (MORAES et al., 2004; STREIT JR. et al., 2008).

The motility and vigor were used to determine the quality and descending sequence of the abnormalities in terms of the capacity of the forms to hinder fecundation. The sequence of the abnormalities was as follow: macrocephaly, degenerated head, degenerated tail, fractured tail, microcephaly, curled tail, normal isolated head, proximal drop, distal drop, and folded tail.

The experimental design was completely randomized, and the values evaluated with the SAEG (2007) statistical program.

Results and discussion

The temperature at the collection sites varied from 20 to 26°C.

Four of the selected animals showed weight gain, while three lost weight from the time of collection to the day of selection biometry (Table 1). Only seven samples contained semen with spermatozoa, and the collection yield was 14.3% (Table 2).

Three animals responded to GnRHa application (42.9%), one on the fifth, sixth, and seventh days; another on the fifth and seventh days; and the third on the sixth and seventh days. Four animals did not spermiate upon GnRHa induction (57.1%), although they presented the same secondary sexual characteristics (black thorns, amplexus reflex, and well-developed forelimbs) and had been subjected to the same treatment in terms of temperature, photoperiod and humidity.

Animals number two, three, and six showed positive responses to the treatment. Moreover, only one individual lost weight between the collection and selection biometry days. The results from the three heaviest animals indicated that they were older and physiologically better prepared for reproduction. One possible reason for obtaining fewer animals with a positive response could be the difficulty in maintaining their body weight during captivity.

Table 1. Number of animals, collection date (CD), weight, weight gain (GW) until the selection day (138 days) and length (L) of the *Leptodactylus ocellatus* specimens after 138 days.

Animal	CD (0)	Biometrics								L (cm)
		Weight (g) and days in captivity								
		0	1	30	60	90	120	138	GW	
1	9/22	150.10	140.67	135.78	129.01	140.24	138.34	127.80	-22.30	10.155
2	10/5	19739	203.90	200.89	195.31	188.85	174.09	200.01	2.71	11.068
3	10/16	220.48	221.90	221.89	216.21	209.00	207.49	221.98	1.50	13.100
4	11/2	118.90	112.89	115.89	108.39	102.89	105.27	111.80	- 7.10	9.945
5	11/7	213.89	219.89	217.89	212.35	191.87	NC	194.00	- 19.89	11.863
6	11/25	169.90	163.89	170.01	177.92	172.35	164.53	190.46	20.56	10.752
7	12/6	179.89	176.90	172.90	165.89	170.20	159.92	181.59	1.70	11.533

NC= Data not collected.

Table 2. GnRHa-induced spermiation in *Leptodactylus ocellatus*.

Hours	Animals						
	1	2	3	4	5	6	7
97.5	-	-	+	-	-	+	-
121.5	-	+	+	-	-	-	-
145.5	-	+	+	-	-	+	-

(-) Negative response, absence SPTZ; (+) Positive response, presence of SPTZ.

The 42.9% response of the animals to the treatment reinforced the idea that the spermatogenesis of amphibians depends on androgynous hormones (CALLARD, 1992). Frog *Rana nigromaculata* (KOBAYASHI et al., 1993) and *Bufo arenarum* (POZZI; CEBALLOS, 2000) induced with human chorionic gonadotropin hormone (hCG) responded positively to spermiation. However, each hormone elicits different responses according to species (CARNEIRO; MIKOS, 2008).

The animals were fed on tadpoles and froglets of *L. catesbeianus*; which differs from the feed in their natural habitat, where they prey on a variety of insects and small-sized amphibians (FRANÇA et al., 2004; MANEYRO et al., 2004). This could explain the low spermiation rate. The semen of the creole frog was collected after 97.5h, while that of the bullfrog was collected after 1h (AGOSTINHO et al., 2000); however, these time periods may vary depending on the species, feeding, domestication, temperature, and photoperiod.

The volume, color, motility, concentration, vigor, and morphology of the spermatozoa in the semen from samples one, three, and five of animal three; two and six from animal two; and seven from animal six are shown in Table 3.

The average semen volume was 0.38 mL because the studs were fed during the experimental period, which increases the probability of contamination of the semen by feces during collection. The volume collected from the bullfrog ranged from 2 to 4 mL (AGOSTINHO et al., 2000), but these animals had fasted for 48h. In this study, the animals had not fasted, once the action time of the hormone was unknown.

The sperm vigor of the creole frog was 3.71 on a scale of 1-5, higher than the value of 3.21 for *Oreochromis niloticus* (MATAVELI et al., 2007), 2.37 for *Leporinus elongatus* (STREIT JR. et al., 2008), and 3.45 for *Piaractus mesopotamicus* (STREIT JR. et al., 2009). These differences, even when fecundation takes place in the same environment, are an indicator of the reproductive strategy for each species. Moreover, during the mating of anuran amphibians, protein-containing foam rises, which facilitates chemotaxis and contact of the spermatozoid for fecundation. This also influenced the initiation of motility and the swimming speed of

mobile spermatozoids in frog *Crinia georgiana* (SIMMONS et al., 2008).

Table 3. Volume (VE), color (CE), vigor (VI), motility (ME), concentration (CE), and number and percentage of spermatozoa (SPTZ) in the semen samples of *Leptodactyllus ocellatus*. The spermatozoa were classified as follows: normal (SN), major defects (DMA), minor defects (DME), degenerated head (CABD), degenerated tail (CADE), fractured (CAUF) or curled (CAUE) tail, macrocephaly (MAE), microcephaly (MIE), normal isolated head (CIN), proximal drop (GP) or distal (GD) head, and folded tail (CAUD).

Sample	Animals							AV	SD
	1	2	3	4	5	6	7		
VE (mL)	0.2	0.2	0.4	0.4	0.8	0.2	0,5	0.38	0.219
CE (1-2)	2	2	2	2	2	2	2	2	0
VI (1-5)	2	4	5	5	2	4	4	3.71	1.253
ME (0-100%)	60	80	80	90	70	80	80	77.14	9.511
CE (x 10^6 SPTZ mL^{-1})	2.2	11.4	12.6	4.0	4.0	8.8	3.2	6.60	4.248
SN (1-50 SPTZ)	35	30	35	30	38	36	36	34.50	3.093
SN (%)	70	60	70	60	76	72	72	69.00	6.187
DMA (1-50 SPTZ)	10	15	8	13	5	4	6	8.50	4.151
DMA (%)	20	30	16	26	10	8	12	17.00	8.303
DME (1-50 SPTZ)	5	5	7	7	7	10	8	7.00	1.732
DME (%)	10	10	14	14	14	20	16	14.00	3.464
CABD (1-50 SPTZ)	0	1	0	0	0	0	0	0.14	0.377
CADE (1-50 SPTZ)	2	4	1	0	1	2	1	1.57	1.272
CAUF (1-50 SPTZ)	6	7	4	4	2	1	3	3.85	2.115
CAUE (1-50 SPTZ)	1	1	2	4	2	1	2	1.85	1.069
MAE (1-50 SPTZ)	0	0	0	2	0	0	0	0.28	0.755
MIE (1-50 SPTZ)	1	2	1	3	0	0	0	1.00	1.154
CIN (1-50 SPTZ)	0	0	1	4	1	2	2	1.42	1.397
GP (1-50 SPTZ)	2	0	1	0	1	4	1	1.28	1.380
GD (1-50 SPTZ)	1	3	3	2	4	2	4	2.71	1.112
CAUD (1-50 SPTZ)	2	2	2	1	1	2	1	1.57	0.354

AV= Average; SD=Standard deviation.

The sperm motility of the creole frog was 77.14%, lower than observed in toad *Bufo baxteri* induced with hCG (95%) (BROWNE et al., 2006). This can be due to differences in the reproductive strategies for this species. Different values of sperm motility were also observed in fish, with 81.08% for *Oreochromis niloticus* (MATAVELI et al., 2007), 96.38% for *Salminus brasiliensis* (SANCHES et al., 2009), 36.72% for *Leporinus elongatus* (STREIT JR. et al., 2008), 73% for *Rhamdia quelen* (FERREIRA et al., 2001), and 90.90% for *Brycon insignis* (ANDRADE-TALMELLI et al., 2001). These results indicated that fish species exhibit a wide array of responses (CARNEIRO; MIKOS, 2008). Semen for cryopreservation should have high motility. For example, in *Bufo marinus* (BROWNE et al., 1998), the motility should be 76.3 and 34% before and after freezing, respectively, and 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).

The sperm concentration in the creole frog semen was 6.60 x 10^6 SPTZ mL^{-1}, comparable to the value of 11.00 x 10^5 SPTZ mL^{-1} for the species

induced with hCG and 9.00×10^5 SPTZ mL^{-1} for bullfrog induced with hCG (ROSEMBLIT et al., 2006). These results pointed out that the sperm concentration depends on the type of hormone used (CARNEIRO; MIKOS, 2008; ROSEMBLIT et al., 2006). However, this value of the sperm concentration was lower than of *Xenopus tropicalis* (2.5×10^7 SPTZ mL^{-1}) (OLMSTEAD et al., 2009), however, the sperm concentration of the creole frog was within the range for bullfrogs (1.56×10^5 and 1.62×10^7 SPTZ mL^{-1}) (AGOSTINHO et al., 2000). Different sperm concentrations were also observed in fish, with 2.63×10^9 SPTZ mL^{-1} for *Oreochromis niloticus* (MATAVELI et al., 2007), 27.362×10^9 SPTZ mL^{-1} for *Prochilodus lineatus* (MURGAS et al., 2007), 69.9×10^6 SPTZ mL^{-1} for *Rhamdia quelen* (FERREIRA et al., 2001), and 54.42×10^6 SPTZ mL^{-1} for *Steindachnerdion scripta* (LUZ et al., 2001) and 24.76×10^6 SPTZ mL^{-1} for *Brycon insignis* (ANDRADE-TALMELLI et al., 2001). The responses from different fish species depend on the hormone used (CARNEIRO; MIKOS, 2008) and the reproductive strategy of each species. All of the samples of creole frog semen were murky, and this color indicates higher or lower quantity of seminal fluid and the concentration of spermatozoa (ANDRADE-TALMELLI et al., 2001).

The proportion of normal spermatozoa (SPTZ) in 50 SPTZ of bullfrog semen samples was 34.50 (69%). The proportion of abnormal spermatozoa in the semen (31.00%) found in the present study was lower than in *Rhamdia quelen* (32.10%) (BOMBARDELLI et al., 2006), curimbatá (40.2%), *Leporinus macrocephalus* (49%), *Cyprinus carpio* (37.6%) (MORAES et al., 2004), and *Leporinus elongatus* (54.7%) (STREIT JR. et al., 2008). The morphology of the spermatozoa in fish differs depending on the hormones used and the time until spermiation (KAVAMOTO et al., 1999).

The quantitative decreasing sequence of major defects with the number of spermatozoa in the semen sample of the creole frog was as follows: fractured tail (3.85 SPTZ), curled tail (1.85 SPTZ), degenerated tail (1.57 SPTZ), microcephaly (1.00 SPTZ), macrocephaly (0.28 SPTZ), and degenerated head (0.14 SPTZ); macrocephaly had a greater effect during visualization. On the other hand, the sequence for the minor defects were distal drop (2.71 SPTZ), folded tail (1.57 SPTZ), normal isolated head (1.42 SPTZ), and proximal drop (1.28 SPTZ). The abnormal morphology of spermatozoa with a fractured tail (3.75 SPTZ) was more common than those with a degenerated head (0.14 SPTZ). The quantitative order showed a higher frequency of abnormalities with a lower impact on the fecundation of spermatozoa of creole frogs. In other words, only a few abnormalities were harmful to fecundation. The classification of spermatozoa of this frog in terms of major or minor defects allows a better qualification and use of sperm. Although the occurrence of major defects (8.50 SPTZ) was more frequent than minor defects (7.00 SPTZ) in the semen samples, most of them do not affect fecundation. That is, abnormal spermatozoa can be viable for fecundation in an aquatic environment where the females can enhance their movement by moving their legs during mating. Thus, even when the number of normal spermatozoa (69.28%) is low, the fecundation rate of the creole frog might be acceptable.

Conclusion

GnRHa can induce spermiation in *L. ocellatus*. Larger animals with all the required secondary sexual characteristics showed better GnRHa induction to spermiation. The difficulties associated with the collection and breeding of the creole frog in captivity can hinder work with this species. The semen color, vigor, motility, and concentration were similar to those of other amphibians and fish, although the semen volume was low. The presence of normal spermatozoa with major or minor defects in the creole frog is within an acceptable range.

Acknowledgements

References

AGOSTINHO, C. A.; WECHSLER, F. S.; NICTEROY, P. E. O.; PINHEIRO, D. F. Indução á ovulação pelo uso de LHRH análogo e fertilização em rã-touro (*Rana catesbeiana*). **Revista Brasileira de Zootecnia**, v. 29, n. 3, p. 1261-1265, 2000.

AGUIAR JR., O.; LIMA, A. P.; BÀO, S. N.; RECCO-PIMENTEL, S. M. Sperm ultrastructure of the Brazilian Amazon poison frogs *Epipedobates trivittatus* and *Epipedobates hahmeli* (Anura, Dendobratidae). **Acta Zoologica**, v. 85, n. 1, p. 21-28, 2004.

AGUIAR JR., O.; GIARETTA, A. A.; RECCO-PIMENTEL, S. M. The sperm of hylodinal species (Anura, Leptodactylidae): Ultrastruture characteristics and their relevance to interspecific relationships. **Journal of Biosciences**, v. 31, n. 3, p. 379-388, 2006.

ANDRADE-TALMELLI, E. F.; KAVAMOTO, E. T.; FENERICHI-VERANI, N. Características seminais de

piabanha, *Brycon insignis* (Steindachner, 1876), após estimulação hormonal. **Boletim do Instituto de Pesca**, v. 27, n. 2, p. 149-154, 2001.

BLOM, E. The ultrastructure of some characteistic sperm defects and a proposal for a new classification of the bull spermgram. **Nordisk Veterinarer Medicine**, v. 25, n. 7, p. 383-391, 1973.

BOMBARDELLI, R. A.; MÖRSCHBÄCHER, E. F.; CAMPAGNOLO, R.; SANCHES, E. A.; SYPERRECK, M. A. Dose inseminate para fertilização artificial de ovócitos de junadiá cinza, *Rhamdia quelen* (Quoy e Gaimardm, 1824). **Revista Brasileira de Zootecnia**, v. 35, n. 4, p. 1251-1257, 2006.

BROWNE, R. K.; CLULOW, J.; MAHONY, M.; CLARK, A. Successful recovery of motility and fertility of cryopreserved cane toad (*Bufo marinus*) sperm. **Cryobiology**, v. 37, n. 4, p. 339-345, 1998.

BROWNE, R. K.; SERATT, J.; VENCE, C.; KOUBA, A. Hormonal priming, induction of ovulation and in-vitro fertilization of the endangered Wyoming toad (*Bufo baxteri*). **Reproductive Biology and Endocrinology**, v. 4, n. 34, p. 1-11, 2006.

CALLARD, G. V. Autocrine and paracrine role during spermatogenesis: studies in *Squalus acanthias* and *Necturus maculosus*. **Journal of Experimental Zoology A**, v. 261, n. 2, p. 132-142, 1992.

CARNEIRO, P. C. F.; MIKOS, J. D. Gonadotrofina coriônica humana e hormônio liberador de gonadotrofina como indutores de reprodução do jundiá. **Acta Scientarium. Animal Sciences**, v. 30, n. 3, p. 345-350, 2008.

COSSON, J. The ionic and osmotic factors controlling motility of fish spermatozoa. **Aquaculture International**, v. 12, n. 1, p. 69-85, 2004.

COSTA, G. C.; GARDA, A. A.; TEIXEIRA, R. D.; COLLI, G. R.; BÀO, S. N. Comparative analysis of the sperm ultrastructure of three species of *Phyllomedusa* (Anura, Hylidae). **Acta Zoológica**, v. 85, n. 4, p. 257-262, 2004.

FERREIRA, A. A.; NUÑER, A. P. O.; LUZ, R. K.; TATAJE, D. A. R.; ESQUIVEL, J. R.; RESTREPO, J. B. Avaliação qualitativa do sêmen do jundiá, (*Rhamdia quelen*). **Boletim do Instituto de Pesca**, v. 27, n. 1, p. 57-60, 2001.

FRANÇA, L. F.; FACURE, K. G.; GIARETTA, A. A. Trophic and spacial niches of two large-sized species of *Leptodactylus* (Anura) in Southeastern Brazil. Studies on **Neotropical Fauna and Environment**, v. 39, n. 3, p. 243-248, 2004.

GODINHO, H. P.; AMORIM, V. M. C.; PEIXOTO, M. T. D. Criopreservação do sêmen de tilápia *Oreochromis niloticus*, var. chitralada: crioprotetores, soluções ativadoras e refriogeradores criogênico. **Revista Brasileira de Zootecnia**, v. 32, n. 6, p. 1537-1543, 2003.

GOLDBERG, R. S.; ALBUQUERQUE, F. T.; OSHIRO, L. M. Y. Criopreservação de material genético do camarão-de-água-doce *Macrobrachium rosenbergii*. **Revista Brasileira de Zootecnia**, v. 29, n. 6, p. 2157-2161, 2002.

JUNCÁ, F. A. Declínio mundial das populações de anfíbios. **Sitientibus Série Ciências Biológicas**, v. 1, n. 1, p. 84-87, 2001.

KAVAMOTO, E. T.; BARNABE, V. H.; CAMPOS, B. E. S.; ANDRADE-TALMELLI, E. F. Anormalidades morfológicas nos espermatozóides do curimbatá, *Prochilodus scrofa* (Steidachner, 1881) (Osteichthyes, Characiformes, Prochilodontidae). **Boletim do Instituto de Pesca**, v. 25, n. 2, p. 61-66, 1999.

KOBAYASHI, T.; SAKAI, N.; ADACHI, S.; ASAHINA, K.; IWASAWA, H.; NAGAHAMA, Y. 17α, 20α-dihydroxy-4-pregnen-3-one is the naturally occuring spermiation-inducing hormone in the testis of frog, *Rana nigromaculata*. **Endocrinology**, v. 133, n. 1, p. 321-327, 1993.

LAHNSTEINER, F.; BERGER, B.; WEISMANN, T.; PAZTER, R. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. **Aquaculture**, v. 163, n. 1, p. 163-181, 1998.

LEE, M. S. Y.; JAMIESON, B. G. M. The ultrastructure of the spermatozoa of bufonid and hilid frogs (Anura, Amphibia): implication for phylogeny and fertilizantion biology. **Zoologica Scripta**, v. 22, n. 3, p. 309-323, 1993.

LUZ, R. K.; FERREIRA, A. A.; REYNALTE, D. A. T.; ZANIBONI FILHO, E. Avaliação qualitativa do sêmen de suruvi, *Steindachneridion scripta* (Pimolodidae). **Boletim do Instituto de Pesca**, v. 27, n. 1, p. 39-42, 2001.

MANEYRO, R.; NAYA, D. E.; ROSA, I.; CANAVERO, A.; CAMARGO, A. Diet of the South American frog *Leptodactylus ocellatus* (Anura, Leptodactylidae) in Uruguay. **Iheringia, Série Zoológica**, v. 94, n. 1, p. 57-61, 2004.

MATAVELI, M.; MORAES, G. V.; STREIT JR., D. P.; VARGAS, L. D. M.; SAGAGUTI, E. S.; TONINATO, J. C.; BARBOSA, R. C.; MERLINI, L. Avaliação da qualidade do sêmen de tilápia-do-nilo (*Oreochromis niloticus*), linhagem chiltralada, suplementada com diferentes concentrações de vitamina C. **Boletim do Instituto de Pesca**, v. 33, n. 1, n. 3, p. 1-7, 2007.

MENEZES, J. T. B.; QUEIROZ, L. J.; DORIA, C. R. C.; MENEZES JR, J. B. Avaliação espermática pós-descongelamento em tambaqui, *Colossoma macropomum* (Cuvier, 1818). **Acta Amazônica**, v. 38, n. 2, p. 365-368, 2008.

MORAES, G. N.; STREIT JR., D. P.; RIBEIRO, R. P.; SAKAGUTI, E. S.; SOUZA, E. D.; POVH, J. A. Ação de diferentes indutores reprodutivos hormonais no aparecimento de anormalidades morfológicas em espermatozóides de piauvuçu (*Leporinus macrocephalus*), curimbatá (*Prochilodus lineatus*) e carpa comum (*Cyprinus carpio*). **Boletim do Instituto de Pesca**, v. 30, n. 3, p. 109-116, 2004.

MURGAS, L. D. S.; MILIORINI, A. B.; FREITAS, R. T. F.; PEREIRA, G. J. M. Criopreservação do sêmen de curimba (*Prochilodus lineatus*) mediante adição de diferentes diluidores, ativadores e crioprotetores. **Revista Brasileira de Zootecnia**, v. 36, n. 4, p. 526-531, 2007.

OLMSTEAD, A. W.; KORTE, J. J.; WOODIS, K. K.; BENNETT, B. A.; OSTAZESKI, S.; DEGITZ, S. J. Reproductive maturation of the tropical clawed frog:

Xenopus tropicalis. **General and Comparative Endocrinology**, v. 160, n. 2, p. 117-123, 2009.

POZZI, A. G.; CEBALLOS, N. R. Human chorionic gonadotropin-induced spermiation in *Bufo arenarum* is not mediated by steroid biosynthesis. **General and Comparative Endocrinology**, v. 119, n. 2, p. 164-171, 2000.

RIBEIRO FILHO, O. P.; LIMA, S. L.; ANDRADE, D. R.; SEIXAS FILHO, J. T. Estudo da desova de rã-touro, *Rana catesbeiana*, mediante indução do acasalamento. **Revista Brasileira de Zootecnia**, v. 27, n. 2, p. 216-233, 1998.

ROSEMBLIT, C.; POZZI, A. G.; CEBALLOS, N. R. Relationship between steroidogenesis and spermiation in *Rana catesbeiana* and *Leptodactylus ocellatus*. **Journal of Comparative Physiology (B)**, v. 176, n. 6, p. 559-566, 2006.

SAEG. **Sistema para Análises Estatísticas**. Versão 9.1: Fundação Arthur Bernardes. Viçosa: UFV, 2007.

SANCHES, E. A.; BOMBARDELLI, R. A.; BAGGIO, D. M.; SOUZA, E. S. Dose inseminante para fertilização de ovócitos de dourado. **Revista Brasileira de Zootecnia**, v. 38, n. 4, p. 2091-2098, 2009.

SIMMONS, L. W.; ROBERTS, J. D.; DZIMINSKI, M. A. Egg jelly influences sperm motility in the externally fertilizing frog, *Crinia georgiana*. **Journal of Evolutionary Biology**, v. 22, n. 1, p. 225-229, 2008.

STREIT JR., D. P.; SIROL, R. N.; RIBEIRO, R. P.; MORAES, G. C.; VARGAS, L. D. M.; WATANABE, A. L. Qualitative of the piapara semen (*Leporinus elongatus valenciennes*, 1850). **Brazilian Journal of Biology**, v. 68, n. 2, p. 373-377, 2008.

STREIT JR., D. P.; OLIVEIRA, A. C.; RIBEIRO, R. P.; SIROL, R. N.; MORAES, G. V.; GALO, J. M.; DIGMAYER, M. Motilidade, vigor e patologia seminal *in natura* e pós criopreservação de *Piaractus mesopotamicus*. **Boletim de Instituto de Pesca**, v. 35, n. 1, p. 159-167, 2009.

VEIGA-MENONCELLO, A. C. P.; LIMA, A. L.; RECCO-PIMENTEL, S. M. Sperm morphology of five species of *Colostethus* (Anura, Dendrobatidae) from Brazil, with phylogenetic comments. **Acta Zoologica**, v. 87, n. 2, p. 147-157, 2006.

YOUNG, B. E.; LIPS, K. R.; REASER, J. K.; IBAÑES, R.; SALAS, A. W.; CEDEÑO, J. R.; COLOMA, L. A.; RON, S.; MARCA, E.; MEYER, J. R.; MUÑOZ, A.; BOLAÑOS, E.; CHAVES, G.; ROMO, D. Population declines and priorities for amphibiam conservation in Latin America. **Conservation Biology**, v. 15, n. 5, p. 1213-1223, 2000.