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

Marcelo Maia Pereira¹*, Oswaldo Pinto Ribeiro Filho¹, Jose Cola Zanuncio², Allan Reis Troni¹, Rodrigo Diana Navarro³ and Angela Emi Takamura¹

¹Programa de Pós-graduação em Biologia Animal, Departamento de Biologia Animal, Universidade Federal de Viçosa, Av. Peter Henry Rolfs, s/n., 36570-000, Viçosa, Minas Gerais, Brazil. ²Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. ³Universidade de Brasília, Brasília, Distrito Federal, Brazil. *Author for correspondence. E-mail: mmaiap2001@yahoo.com.br

**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 x 10⁶ 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.

### 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 (JUNCA, 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 hypophysial 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 Conceptual® (0.4 μg of GnRHa) (AGOSTINHO et al., 2000) into the celomac 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 celomac 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 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 buffered formaldehyde (10%) and placed in a Neubauer chamber. The sperm concentration was estimated in five quadrants (1 mm²) to determine the amount of spermatozoa per milliliter (SPTZ mL⁻¹). The morphology of the spermatozoa was observed under a clear field microscope at 20× 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 spermate 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>
<thead>
<tr>
<th>Animal</th>
<th>CD (0)</th>
<th>Weight (g) and days in captivity</th>
<th>L (cm)</th>
<th>GW</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9/22</td>
<td>130.10 140.67 135.78 129.01 140.24 128.04 127.90</td>
<td>10.155</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10/5</td>
<td>197.39 203.90 200.89 195.31 188.85 174.09 200.01</td>
<td>11.068</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10/16</td>
<td>220.48 221.90 221.89 216.21 209.00 207.49 221.98</td>
<td>11.350</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>11/2</td>
<td>118.90 112.89 115.89 108.39 102.89 105.27 111.80</td>
<td>9.945</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>11/7</td>
<td>213.89 219.89 217.89 212.33 191.87 N/C 194.00</td>
<td>11.863</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>11/25</td>
<td>169.90 163.89 170.01 177.92 172.35 164.53 190.46</td>
<td>10.752</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>12/6</td>
<td>179.89 176.90 172.90 159.92 165.89 170.20 159.92</td>
<td>11.333</td>
<td></td>
</tr>
</tbody>
</table>

NC= Data not collected.

**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.
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 spermatiation. 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., 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 six are shown in Table 3. Moreover, during the mating of anuran amphibians, protein-containing foam rises, which facilitates chemotaxis and contact of the spermatozoids 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 2. GnRHa-induced spermiation in *Leptodactylus ocellatus*.**

<table>
<thead>
<tr>
<th>Hours</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>97.5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>121.5</td>
<td>-</td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>145.5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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

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 *Bryon 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 10^8 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 x 10^7 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 x 10⁷ SPTZ mL⁻¹) (OLMSTEAD et al., 2009), however, the sperm concentration of the creole frog was within the range for bullfrogs (1.56 x 10⁷ and 1.62 x 10⁷ SPTZ mL⁻¹) (AGOSTINHO et al., 2000). Different sperm concentrations were also observed in fish, with 2.63 x 10⁷ SPTZ mL⁻¹ for Oreochromis niloticus (MATAVELI et al., 2007), 27.362 x 10⁶ SPTZ mL⁻¹ for Prochilodus lineatus (MURGAS et al., 2007), 69.9 x 10⁶ SPTZ mL⁻¹ for Rhamdia quelen (FERREIRA et al., 2001), and 54.42 x 10⁶ SPTZ mL⁻¹ for Steindachneridion scripta (LUZ et al., 2001) and 24.76 x 10⁶ 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), microcephalhy (1.00 SPTZ), macrocephaly (0.28 SPTZ), and degenerated head (0.14 SPTZ). The abnormal morphology of spermatzoa 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

GnRHα can induce spermiation in L. ocellatus. Larger animals with all the required secondary sexual characteristics showed better GnRHα 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.

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