Original article

# Stereological analyses of the annual variation of captive bullfrog adult testes (*Lithobates catesbeianus*, Shaw 1802)

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## Abstract

The occurrence of seasonality in bullfrog (*Lithobates catesbeianus*) spermatogenesis was analyzed by quantifying germ cell populations throughout the year, which were divided into bimesters, from November 2001 to October 2002. Body weight, liver weight, fat body weight and gonadal weight were taken and bimonthly grouped. Using these data, the gonadosomatic, hepatosomatic, and liposomatic indexes were calculated. All animals were kept in the same cement cages in a frog farm, and sampled every month. The cages were under the influence of climate variables changes, such as photoperiod, temperature, rainfall and relative humidity. Body weight was higher during Spring and Summer, and a significant reduction has occurred between July and August. The highest gonadosomatic index values were registered from May to October, coinciding with the period of gonadal preparation for reproduction. Also, the reproduction of bullfrog is directly related to environmental factors, mainly due to Summer and Winter humidity variations.

**Keywords:** amphibia, reproduction, seminiferous tubules, seasonality.

## 1 Introduction

Many environmental factors as temperature, rain, photoperiod, altitude and latitude may influence anuran spermatogenesis (DIAZ-PÁEZ and ORTIZ, 2001), which exhibit different reproductive models: continuous, potentially continuous and discontinuous. In temperate regions, the annual gonadal cycle is determined by the climatic annual changes, occurring potentially continuous and discontinuous spermatogenic cycles (RASTOGI, 1976). Species that inhabit tropical zones, where the climate is mainly warm and wet, show continuous spermatogenesis cycles, with males becoming able to mate during the whole year (RASTOGI, 1976; DIAZ-PÁEZ and ORTIZ, 2001).

The bullfrog (*Lithobates catesbeianus*) is native from the North America and has been used in frog farms, proving to be an excellent source of protein with low fat content. In Brazil, as in other tropical countries, this species has been successfully introduced and exhibits two successive reproduction periods during the raining season (FONTANELLO, SOARES, MANDELLI et al., 1984).

The testes of *L. catesbeianus* are paired ovoid organs surrounded by a layer of fibrous connective tissue (known as *tunica albuginea*) and constituted by seminiferous tubules, which are composed by a germ tissue. The latter holds cysts of germ cells that show a synchronic maturation development and are surrounded and kept by the Sertoli cells (LOFTS, 1974; COSTA, 1992; OLIVEIRA, ZANETONI,ZIERI et al., 2002). The interstitium is lying between the seminiferous tubules and contains a variety of elements, such as blood vessels, Leydig cells, mast cells, lymphatic vessels and spermatic ducts (LOFTS, 1974; KAEFER, BOELTER and CECHIN, 2007). Variations in the cyst's number, size and the distribution of testicular components indicate alterations of the spermatogenic process.

In the present study, we observed the annual biometry and stereological variations of captive bullfrog's testes, regarding specially to the germ cells lineage, in order to investigate the occurrence of seasonality in their reproductive cycle.

## 2 Material and methods

### *2.1 Experimental animals and management*

Ten healthy animals were randomly captured per bimester from an initial group of 240 male bullfrogs kept into cement made cages (UFV Frog Farm, November 2001 to October 2002; –20° 45' 14" S and 42° 52' 55" W). All captured animals were replaced by marked ones (MARTOF, 1953), in order to maintain the population density constant throughout the year. The diet, composed of 40% protein, was offered *ad libitum*. Sampling bimesters were divided as shown on Table 1. In Brazil, Spring starts at September, Summer at December, Autumn at March and Winter at June.

### *2.2 Tissue processing*

All animals were euthanasied after rendered insensible by medullar denervation. Both testes were weighed and cut on the sagittal plane and fixed for 24 hours in glutaraldehyde fixative 3% in sodium phosphate buffer (0.1 mol/L, pH 7.4). Testis fragments were dehydrated (ethanol) and embedded in glycol methacrylate resin. Semi-thin sections (3 μm) were stained with toluidine blue/sodium borate 1%, and analyzed in photomicroscope.

### *2.3 Biometric and stereological parameters*

Based on body and organs weights (liver, fat body and testes), several biometric indexes (BI) were calculated. Gonadosomatic (GSI), hepatosomatic (HSI) and liposomatic (LSI) indexes were obtained using the expression BI = OW/BW × 100 (*OW = organ weight and BW = body weight*). Other measurements as the testicular weight and volume, the volumetric proportion between testicular components, the area of the seminiferous tubules and the diameter of Leydig cells nuclei were also evaluated. The analyses were done following the methods showed by Matta, Vilela, Godinho et al. (2002). Determination of the volumetric proportion of the testicular components was obtained using a 121 points grid, which was coupled to a light microscope ocular; a total of 2000 points were counted per animal. Considering 30 sections of round seminiferous tubules and 30 round Leydig cell nuclei, the average tubular area and the Leydig cell nuclear diameter per animal were calculated, using the Image Pro-Plus Software (Media Cybernetics Inc.). In addition, 20 sections of round seminiferous tubules per animal were used in order to determine the number and type of cysts. The Federal University of Viçosa Meteorology Sector supplied air temperature, photoperiod, humidity and rainfall data (Table 2).

Statistical analyses were performed using Statistica 3.11 for Windows software (StatSoft, Inc., Tulsa, OK). The results from the six groups were tested using ANOVA (Tukey test). Significative data ($P < 0.05$) of cystic population were correlated to meteorological variables (temperature, photoperiod, relative humidity and rainfall) using Pearson Correlation Coefficients. The data were bimonthly grouped.

## 3 Results and discussion

### *3.1 Body and testes weight*

Body weight has been used in several studies for the calculation of different indexes (FIGUEIREDO, AGOSTINHO, BAÊTA et al., 1999; SASSO-CERRI, FARIA, FREYMÜLLER et al., 2004). *Lithobates catesbeianus* presented higher body weight during hot, humid and luminous periods, as found in *R. rugulosa* (KAO, ALEXANDER, YANG et al., 1993). Agostinho, Silva, Torres et al. (1991), studying *Leptodactilus labyrinthicus*, also observed relations among photoperiod, temperature and gonadosomatic indexes of females and males, seeing that the beginning of the reproductive period occurred when those variables presented the higher values. Besides, body weight was related to the liver and fat body weights, showing a negative relation with the testes weight. This pattern was observed in *R. esculenta* (SCHLANGHECKE and BLUM, 1978), in *Bufo canorus* (MORTON, 1981), in *Acris crepitans* and in *Bufo woodhausei* (LONG, 1987).

In our model, the body weight was lower between July and August, known as being colder months. From January to April, during the highest temperature, photoperiod and rainfall period, the animals presented the higher body weight (Table 3). It is well established that physiological alterations, such as the increase of the testes weight, occur due to the preparation for the reproductive season (COSTA, 1992). These events are observed in those animals that do not show a continuous spermatogenic cycle, as *Rana temporaria* (LOFTS, 1974) and *Pachymedusa dacnicolor* (RASTOGI, IELA, DELRIO et al., 1986).

Testicular mass was significantly increased between July and August, while the liver weight was significantly lower (Table 3). It was not observed expressive variations neither in testes weight during other bimesters, nor in fat body weight, the latter probably due to constant food intake throughout the year.

**Table 1.** Sampling months.

| Bimester | Months/Year |
|---|---|
| 1 | November/December/2001 |
| 2 | January/February/2002 |
| 3 | March/April/2002 |
| 4 | May/June/2002 |
| 5 | July/August/2002 |
| 6 | September/October/2002 |

### *3.2 Hepatosomatic (HSI) index*

During spermatogenesis, the liver parenchyma performs several functions such as the production of steroid binding proteins and the formation and storage of lipids and carbohydrates, both used for the metabolic activity of the testes and during the degradation of steroid hormones (KAO, ALEXANDER, YANG et al., 1993). The highest liver weight and HSI were registered in March and April, during Autumn months (Tables 3 and 4). On the other hand, the bullfrog presented a significative reduction of the liver weight and HSI, between July and August (non-reproductive period). The animals were probably using the energetic components from the liver as a source of metabolic energy, thus they can survive during the colder months. The HSI reduction during the Winter time was also observed in *Rana ridibunda* (LOUMBOURDIS and KYRIAKOPOULOU, 1991) and in *R. rugulosa* (KAO, ALEXANDER, YANG et al., 1993).

### *3.3 Liposomatic index (LSI)*

The fat body is a great source of metabolic energy that is used to maintain the testicular activity in normal rates (PANASEN and KOSKELA, 1974; FROST, 1983; COSTA,

**Table 2.** Meteorological data of the Federal University of Viçosa region, from November/2001 to October/2002.

| Bimester/year | Temperature (°C) | Photoperiod (h) | Humidity (%) | Rainfall (mm) |
|---|---|---|---|---|
| Nov./Dec./01 | 22.10 | 4.71 | 81.25 | 7.37 |
| Jan./Feb./02 | 20.48 | 6.94 | 76.96 | 2.68 |
| Mar./Apr./02 | 22.46 | 8.80 | 76.23 | 1.64 |
| May/June/02 | 18.50 | 7.37 | 81.68 | 0.65 |
| July/Aug./02 | 18.40 | 7.22 | 74.40 | 0.03 |
| Sept./Oct./02 | 20.50 | 6.50 | 71.64 | 1.75 |

1992). The loss of such energy source could be harmful for the entire reproductive process, leading to testicular regression, as observed in *Rana esculenta* (CHIEFFI, RASTOGI, ELA et al., 1975). Delgado, Gutierrez and Alonso-Bedate (1989) described significant seasonal variation in fat body weight in *Rana perezi*. In the present study, it was also verified that the higher values occurred between January and April, during the Summer time. In *L. catesbeianus*, the LSI presented a decrease tendency between September and February, with a minimum point in January, as well as observed by Costa (1992) for the same species and by Kao, Alexander, Yang et al. (1993) in *R. rugulosa*. Despite remaining constant during the experimental period, the LSI was higher in July and August.

### *3.4 Gonadosomatic index (GSI)*

GSI was constant along the year, although due to the highest body weight observed on March and April, this index was reduced (Tables 3 and 4). COSTA (1992) found in *L. catesbeianus* a lower gonadosomatic index (GSI) in October and a higher index in July. The increase of the GSI was observed during the hottest and most luminous months. However, we observed a decrease of this index between November and April, when the animals showed significant spermatozoa content inside the tubular lumen, which indicates that the animals were ready to mate. The highest gonadosomatic indexes were observed during the coldest, driest and less luminous months (from May to October) (Table 4).

### *3.5 Testicular parenchyma constituents*

Testicular parenchyma is formed by the seminiferous tubules and the interstitium (MATTA, VILELA, GODINHO et al., 2002). The annual averages of such elements were 81.42% and 19.58%, respectively. The maintenance of these proportions along the year showed that there was absence of seasonality among the parenchyma elements. Besides, the largest tubular areas were associated with the presence of cysts that contained spermatogonia and primary spermatocyte populations. This fact indicates a higher mitotic and meiotic activity from the end of the Sring and the beginning of the Summer, which are humid and hot periods. In temperate countries, large luminal diameters are observed during the Autumn and during this period, a great amount of spermatozoa is found inside the tubules, which will be used during the next reproductive season (LOFTS, 1974).

Despite no alterations in tubular area values (data not shown), the number of primary spermatogonia cysts varied during the year, being lower in the beginning of the summer (November and December) and in May and June. A higher amount ($P < 0.05$) of these cysts was registered between January and February, and from July to October (Figure 1).

The highest values of secondary spermatocytes cysts were registered between September and October. The cyst's number was significative lower from May to August (Table 5). The other cystic populations (spermatocytes I and round spermatids) did not show any change throughout the year (Table 5).

The Pearson's correlation coefficients were calculated, considering the average spermatogonia population along with each of the meteorological variables. It was observed a negative correlation between spermatogonia cystic number and humidity ($r = -0.8455$, $p = 0.0339$). A scatter diagram was built in order to better knowledge where each point was identified according to two months. Therefore, the larger values of humidity, the lower average values for spermatogonia cysts (Figure 1). The spermatocytes cysts variations did not show any correlations with the meteorological variables.

### *3.6 Nuclear diameter of Leydig cells*

The smallest nuclear diameter for Leydig cells was observed between March and April, increasing between September and October, however there was no statistical differences among bimesters (Table 5). During Spring time, it was observed a higher mean nuclear diameter for these

**Table 3.** Biometric values of body and organs weights (testes, liver and fat body) of *Lithobates catesbeianus* adult males.

| Bimester/Year | Body weight (g) | Organs weight (g) | | |
|---|---|---|---|---|
| | | Testes | Liver | Fat body |
| Nov./Dec./01 | 307.73 ± 11.04 | 0.27 ± 0.02 | 13.85 ± 2.19 | 18.11 ± 2.30 |
| Jan./Feb./02 | 324.17 ± 11.83 | 0.22 ± 0.01 | 15.93 ± 1.39 | 20.52 ± 1.89 |
| Mar./Apr./02 | 333.59 ± 19.11 | 0.25 ± 0.02 | 20.44 ± 2.15 | 25.09 ± 2.27 |
| May/June/02 | 298.63 ± 14.72 | 0.26 ± 0.04 | 12.66 ± 1.48* | 18.58 ± 2.18 |
| July/Aug./02 | 250.67 ± 8.62* | 0.43 ± 0.01* | 6.01 ± 0.51* | 18.81 ± 1.01 |
| Sept./Oct./02 | 309.96 ± 14.04 | 0.31 ± 0.03 | 14.80 ± 0.89 | 16.69 ± 2.35 |

Values are mean ± standard error; n = 10. Tukey test *p < 0.05.

**Table 4.** Gonadosomatic index (GSI), hepatosomatic index (HSI), and liposomatic index (LSI) of *Lithobates catesbeianus* adult males.

| Bimester/Year | GSI (%) | HSI (%) | LSI (%) |
|---|---|---|---|
| Nov./Dec./01 | 0.13 ± 0.01 | 4.45 ± 0.69 | 5.86 ± 0.72 |
| Jan./Feb./02 | 0.14 ± 0.01 | 4.83 ± 0.29 | 6.25 ± 0.43 |
| Mar./Apr./02 | 0.10 ± 0.02* | 5.97 ± 0.38 | 7.41 ± 0.39 |
| May/June/02 | 0.17 ± 0.03 | 4.25 ± 0.44 | 6.39 ± 0.69 |
| July/Aug./02 | 0.17 ± 0.01 | 2.38 ± 0.15* | 7.54 ± 0.37 |
| Sept./Oct./02 | 0.20 ± 0.01 | 4.83 ± 0.27 | 5.32 ± 0.60 |

Values are mean ± standard error; n = 10. Tukey test *p < 0.05.

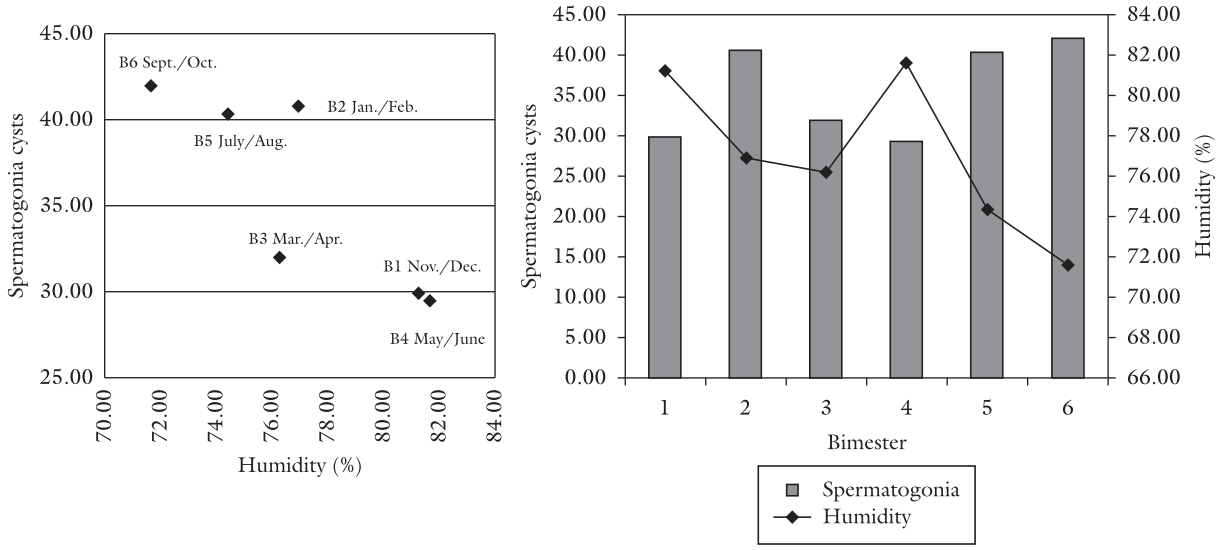


**Figure 1.** Annual variation in the number of spermatogonia cysts in *Lithobates catesbeianus*. A-B. The higher the humidity the lower the number of spermatogonia cysts, mainly observed during the fifth and sixth bimesters.

**Table 5.** Variation of cyst's numbers and Leydig cells nuclear diameter along the year.

| Bimester/year | Number of cysts | | | | Leydig cell nucleus (µm) |
|---|---|---|---|---|---|
| | Gonia | SPTC I | SPTC II | RS | |
| Nov./Dec./01 | 29.90 ± 4.18* | 25.20 ± 3.34 | 1.46 ± 0.44 | 8.70 ± 1.44 | 7.41 ± 0.48 |
| Jan./Feb./02 | 40.80 ± 3.79 | 35.40 ± 2.56 | 1.40 ± 0.59 | 10.20 ± 1.56 | 7.81 ± 0.23 |
| Mar./Apr./02 | 32.00 ± 3.48 | 30.50 ± 3.51 | 1.16 ± 0.46 | 9.90 ± 2.03 | 6.78 ± 0.31 |
| May/June/02 | 29.40 ± 2.54* | 27.00 ± 2.18 | 0.90 ± 0.22* | 6.60 ± 0.93 | 7.12 ± 0.29 |
| July/Aug./02 | 40.30 ± 3.31 | 29.40 ± 2.92 | 0.97 ± 0.31* | 4.50 ± 1.06 | 7.22 ± 0.36 |
| Sept./Oct./02 | 42.00 ± 4.27 | 36.80 ± 4.35 | 1.65 ± 0.76 | 8.30 ± 1.21 | 7.99 ± 0.23 |

Values are mean ± standard error. Number of cysts: n = 10 (*20 sections/animal*); Nuclear diameter: n = 10 (*30 nuclei/animal*). Gonia: spermatogonia, SPTC I: spermatocyte type I, SPTC II: spermatocyte type II, RS: round spermatid. Tukey test *p < 0.05.

cells, which is involved with the production and secretion of testosterone. The secretion of this hormone occurs during the preparation for the mating period (CASTRO, SILVA, SANTOS et al., 2001).

## 4 Conclusions

In the present work, it was demonstrated the direct action of the environmental factors on the reproductive cycle of *L. catesbeianus*. It was clear that, during the two well defined seasons (a hot and humid and a cold and dry), the GSI presented significant differences among the sampled months, defining the reproductive season of this species.

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