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Seasonal testicular changes in *Dendropsophus minutus* Peters, 1872 (Anura, Hylidae)

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ABSTRACT: The reproductive cycle in anurans may be either continuous or discontinuous. These differences may be connected to seasonal climate changes and/or to anthropic activity. Forty adult male individuals of the *Dendropsophus minutus* species were collected during one year, in the municipality of Chapada dos Guimarães (Mato Grosso, Brazil). The testicles were studied under light and transmission electron microscopy. No variations were observed when the diameter of the seminiferous tubules and the thickness of the interstitial tissue were studied. However, changes in spermatogenesis were conspicuous and indicated that the reproductive cycle of *D. minutus* in Chapada dos Guimarães is discontinuous and seems related to variations in air temperature and rainfall.

Introduction

Breeding activity of most anurans is associated with the rainy season both in the temperate zone (Wiest, 1972; Salvador and Carrascal, 1990) and the tropics (Heyer, 1973; Barbault and Rodrigues, 1978; Hoogmoed and Gorzula, 1979; Toft and Duellman, 1979; Aichinger, 1987; Gascon, 1991; Duellman, 1995). Yet there are no descriptions of anuran reproductive activity in the Brazilian Cerrado (savanna) bioma.

Dendropsophus minutus (Hylidae) is a small size anuran measuring 20-25 mm (crown-rump length). Its geographical distribution ranges from the lowlands up to 2000 meters in altitude, from low areas to the east of the Andes in Colombia, passing through Venezuela and Trinidad and, moving south, through Ecuador, Peru and Brazil as far as Bolivia, Uruguay and Argentina (Ribeiro *et al*., 2005).

Chapada dos Guimarães comprises a wide plateau with an altitude varying between 600 and 850 meters. In Mato Grosso, where the most elevated areas are formed by plateaus, the altitude associated with ventilation constitutes the geographical factor that most influences the variations in temperature and rainfall (Maitelli, 2005). Chapada dos Guimarães is located in the center-south of the state of Mato Grosso and is a watershed between the Platina and Amazon basins (Maitelli, 2005). During the year there are two notably different seasons, with regard to pluviometric precipitations: the rainy season (spring and summer) and the dry season (autumn and winter). This seasonal variation may be characterized by six rainy months and six hot months, with oscillations between extreme heat and dry cold (Fig. 3).

The anuran testicles show a seminiferous epithelium with cysts formed from the association of germ

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cells with Sertoli cells, and all cells present in the same cyst are at the same stage of differentiation (Ferreira *et al.* 2008; 2009).

Material and Methods

Study area

Animals were collected in the permanent Monjolinho stream on the Buriti farm (15°36'S; 56°03'W) in the municipality of Chapada dos Guimarães, state of Mato Grosso, Brazil. This area belongs to the biogeographical domain of the Cerrado (Maitelli, 2005). The average monthly averages of temperature and rainfall during 2006 (Fig. 3), were supplied by INMET (Instituto Nacional de Metereologia - 9th District of the municipality of Cuiabá, Mato Grosso State).

Light microscopy

Adult male individuals were collected monthly from July 2004 to June 2005 (N=50). They were measured and fixed in 10 per cent formalin, preserved in 70 per

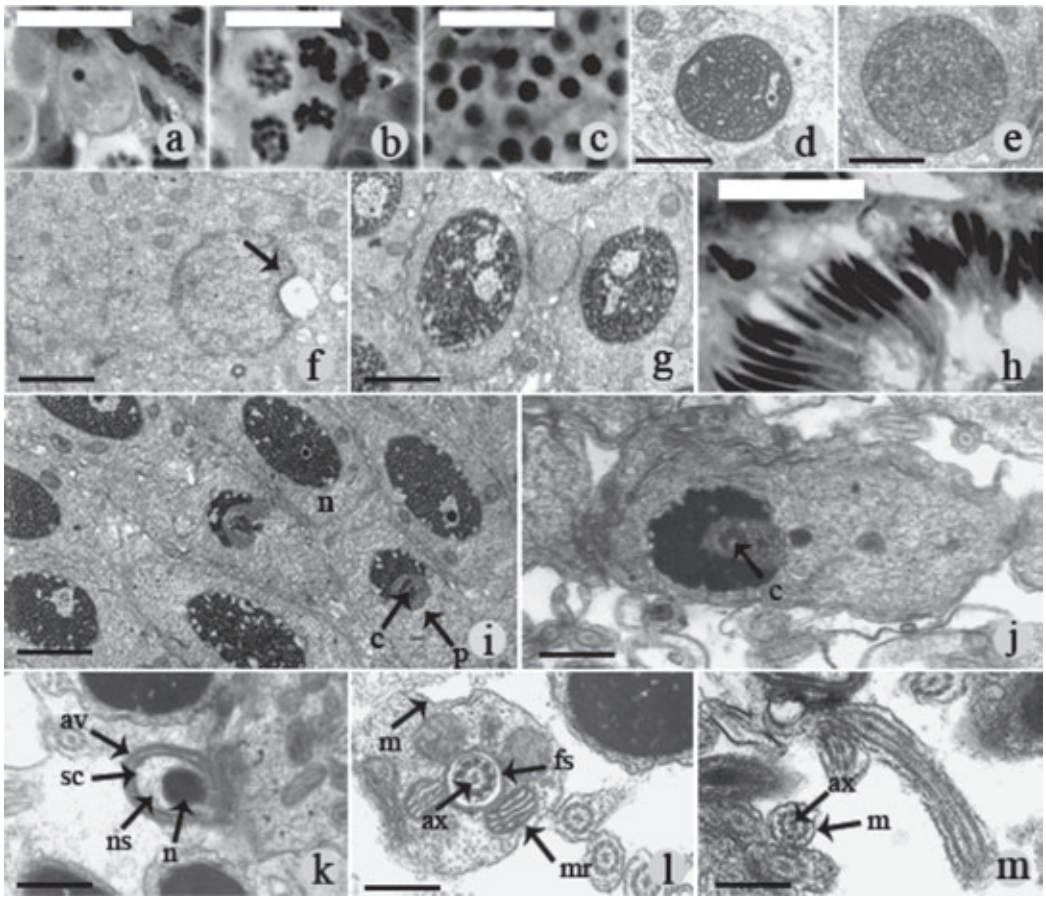

FIGURE 1: Spermatogenesis stages of *Dendropsophus minutus*. **a-c.** Light microscopy. Bar is 20μm. **a.** Primary spermatogonia. **b.** Primary spermatocyte. **c.** Secondary spermatocyte. **d-g.** Transmission electron microscopy. **d.** Primary spermatocyte. Bar is 580 nm. **e.** Secondary spermatocyte. Bar is 580 nm. **f.** Early spermatid, the arrow indicates the pro-acrosomal vesicle. Bar is 750 nm. **g.** Early spermatid in phase of compacting chromatin. Bar is 580 nm. **h.** Light Microscopy, late spermatids with nuclear elongate and compacting chromatin. Bar is 20 μm. **i-m.** Transmission electron microscopy. **i.** Late spermatid, in phase compacting chromatin and still evident nucleolus (n), formation of nuclear fossa is the implantation of the midpiece with centriole (c) and showing initial dense bodies (p). Bar is 1 μm. **j.** Late spermatid with more accented cromatin compactation and implantation nuclear fossa of the midpiece, with central centriole (c). Bar is 580 nm. **k.** Acrosome apical, nucleus (n), subacrosomal cone (sc), nuclear space (ns) and acrosomal vesicle (av). Bar is 430 nm. **l.** Middle piece, axoneme (ax), fibrous sheath (fs), mitochondrial ring (mr). Bar is 310 nm. **m.** End piece, axoneme (ax) and plasma membrane (m). Bar is 310 nm.

cent alcohol and deposited in the Zoological Collection of Vertebrates of the Mato Grosso Federal University. One testicle was obtained from each animal, and was fixed in 5% paraformaldehyde, dehydrated in increasing graduations of ethanol, infiltrated and embedded in a methacrylate glycol resin; 3µm sections were obtained and stained with 1% toluidine blue.

Transmission electron microscopy

Testes were fixed overnight at 4ºC in a 0.1M sodium cacodylate buffer solution (pH = 7.2) containing 2.5% glutaraldehyde. After, they were fixed following the above protocol, they were post-fixed for 2 h in 0.1M sodium cacodylate buffer solution (pH = 7.2) contain-

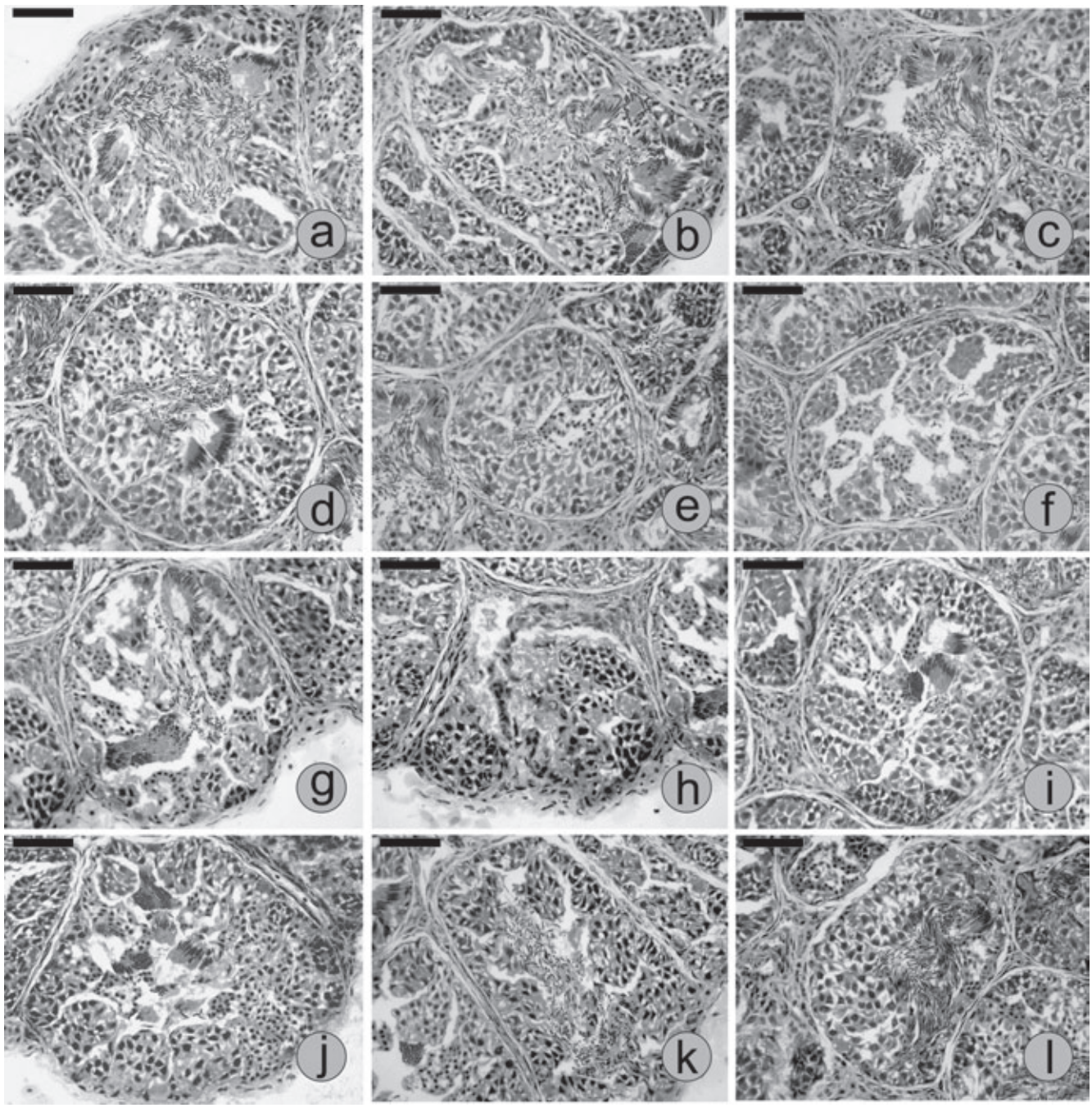

FIGURE 2. a1. Histological images showing the general aspect of the seminiferous tubule of *Dendropsophus minutus* throughout the year. **a.** January. **b.** February. **c.** March. **d.** April. **e.** May. **f.** June. **g.** July. **h.** August. **i.** September. **j.** October. **k.** November. **l.** December. Bar is 20µm.

ing 1% osmium tetroxide. Next, they were dehydrated in acetone and embedded in LR White resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Zeiss, Leo 906).

Results

The testis of *Dendropsophus minutus* are round and whitish, without noticeable variations in their macroscopical appearance throughout the year.

The structural and ultrastructural analysis of germ cells showed that the primary spermatogonia is an isolated cell that does not form a cyst and is larger in size than the other cells and is located close to the seminiferous tubule wall. Its cytoplasm possesses a great affinity for staining, enabling good visualization of cell limits under light microscopy (Fig. 1a). Secondary spermatogonia are smaller cells organized in cysts of a dark appearance. The primary spermatocyte has a large nucleus with loose chromatin and a thread-like appearance (Figs. 1 b and d). Secondary spermatocytes are smaller than the primary ones, with a roundish and strongly colored nucleus, the chromatin of which is much more compact (Figs. 1 c and e). Spermatids are found in two stages of morphological differentiation: roundish or early and elongated or late. The early spermatids have a spherical nucleus, with relatively compact chromatin, while the elongated ones have a longitudinally extended nucleus (Figs. 1 f and g). The ultrastructure shows the formation of a pre-acrosomal vesicle in one of the nuclear poles of the round early spermatids (Fig. 1f). Following cell differentiation, the nucleus of the late spermatid shows an increasingly more compact chromatin, elongating itself and transforming itself into spermatozoid, which appears strongly colored, and thinner than the spermatid (Figs. 1 h-j). Sperm have a head composed of a nucleus whose chromatin is highly compacted, with few electron-lucent spaces, and an acrosome with an acrosomal vesicle (Fig. 1k). The middle piece is implanted in the nucleus through the penetration of a pair of perpendicular centrioles surrounded by groups of electron-dense granules (Figs. 1 i and j). This piece is constituted by a mitochondrial collar (4 to 8 mitochondria) and a typical axoneme (9+2) with associated dense fibers (Fig. 1l). Between the axoneme and the mitochondria there is a ring of dense fibers (Fig. 1l). The flagellum is simple, consisting of a typical axoneme and the plasma membrane (Fig. 1m).

Upon analyzing the structural variations in spermatogenesis throughout the year, we observed that there occurs a subtle, but noticeable difference in the proportion of germ cells. However, no variation was noted in the interstitial tissue. All germ cell stages are found in every month of the year (Fig. 2). However, both elongated spermatids and spermatozoa are more frequent than the other cell types from January to April (Figs. 2 a-d) and an appreciable reduction in frequency of these elongated cells was observed from May to September, when spermatogonia and spermatocytes were more frequently found (Figs. 2 e-i). In October to December a subtle increase in the proportion of elongated cells recommenced (Figs. 2 j-l).

The presence of sperm in the lumen of the seminiferous tubules enabled us to identify the period of reproductive activity (November to February) which was coincident with the highest rainfall sperm (Fig. 3). In the following months there was a tendency for spermatogenesis to decrease and about half of the tubules show no sperm in the lumen in August, just before the increase in rainfall (Fig. 3). However, no obvious changes in the diameter of the seminiferous tubules or in the extent of the interstitial tissue were observed in the course of the year.

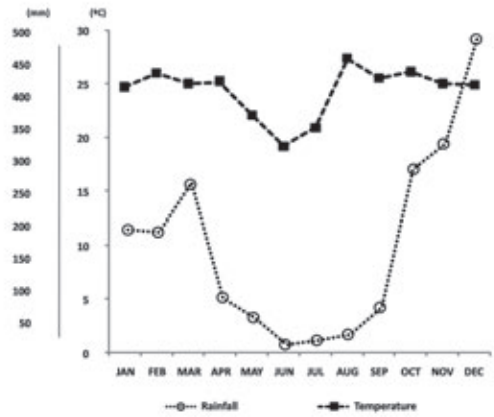

FIGURE 3. Average monthly variations in temperature and rainfall during the studied period.

Discussion

The seminiferous tubules of anurans showing continuous cycles, present cysts at various stages of maturation, with sperm production throughout the year, as it is frequently found in tropical and subtropical species (Saidapur, 1983). However, germ cells mature uniformly in each tubule in anuran species exhibiting discontinuous cycles. Notwithstanding, males of some anuran species of the temperate zone enter a sexually quiescent

period after the reproductive period, and spermatogenesis is interrupted for some months (Lofts *et al.*, 1972).

All differentiation stages of spermatogenesis are present in *Dendropsophus minutus* in October, reaching maximum development in February. This is similar to observations in *Hyla pulchella andina* (Montero and Pisanó, 1992). In April, when the dry season starts, we observed that spermatogenesis decreases in comparison with the preceding months, and this tendency continues until reaching a peak in August, when secondary spermatogonia become the predominant cells. This pattern of spermatogenesis is very similar to the spermatogenic cycle of *Telmatobius pisanoi* (Montero and Pisanó, 1990), which halts the spermatogenic process in August, when secondary spermatogonia predominate in the seminiferous tubules. A reactivation of spermatogenesis is indicated by an increase in spermatocyte number in September.

Up to now, the spermatogenic process has received little attention in anurans. The initial phases have only been described in *Xenopus laevis* (Pipidae) (Kalt, 1976). Similar findings are reported here for *D. minutus*, mainly in relation to spermatogonia and spermatocyte morphology, and we have added a few more cytoplasmatic characteristics.

Among vertebrates, amphibians present an enormous diversity in their reproductive strategies (Duellman and Trueb, 1994), and this is reflected as large differentiation and variability of the caudal filament, including the protean appendages and membranous parts that this region (Taboga and Dolder, 1993).

The caudal filament presents a typical axoneme (9+2) in *D. minutus.* Similar axonemes without any caudal annex have been observed in *R. pipiens* and *R. clamitans* (Poirier and Spink, 1971) and some Microhylidae (Scheltinga *et al*., 2002), or with two axonemes in *Megophrys montana* (Asa and Phillips, 1988) and *Lepdobatrachus laevis* (Waggener and Carrol Jr., 1998). The caudal filament with axoneme, an undulating membrane and an absent axial rod occur in *Pachymedusa dacnicolor* (Rastogi *et al*., 1988) and *Bufo gargarizans* (Kwon and Lee, 1995). The tail of *Hyla japonica* consists of an axoneme and an axial rod, but does not show an undulating membrane (Kwon and Lee, 1995). In *D. minutus* no appendage to the axoneme was observed, except for the fibrous ring at the midpiece region.

Global diversity is changing rapidly as a complex response to various environmental changes caused by man (Vitousek *et al.,* 1997). Globally, intact ecosystems are being degraded at an average rate of 1.2% each year, with the greatest threat to biological diversity and the loss of habitats (Primack and Rodrigues, 2001; Balmford *et al*., 2002). The Cerrado has for some time suffered high rates of biodiversity loss due to human occupation and agricultural expansion (Klink and Moreira, 2002). For this reason, urgent conservation strategies are important for this bioma.

The decline of amphibian populations, mainly in Australia, Central America and the Pacific north-east of the United States, has been the focus of research projects over the last 12 years (Lanoo, 2005) but amphibian populations in the tropics (as in Ecuador and Brazil) have been insufficiently monitored (Stuart *et al*., 2004). The causes of amphibian populations' decline, both within and outside protected areas, are still uncertain, but a consensus exists that four main factors may be involved: climate change, pollution, increase in ultraviolet B (UVB) radiation and infectious diseases (Lanoo, 2005). These factors render the group wanting in terms of swift action for conservation strategies.

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