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Testicular Histology and Sperm Morphometrics of the Bird-voiced Treefrog, *Hyla avivoca* (Anura: Hylidae), from Arkansas

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Running Title: Testicular Histology and Sperm Morphometrics in *Hyla avivoca*

Abstract

We examined the testicular histology and spermatozoal dimensions of the bird-voiced treefrog, *Hyla avivoca* (Anura: Hylidae), from samples collected in May, June, and July from localities in three counties (Calhoun, Conway, and Little River) in Arkansas. Calling frogs were necropsied in the lab, and testes were prepared for light and scanning electron microscopy. Spermatocysts within seminiferous tubules of all males contained large aggregates of spermatozoa. Primary spermatogonia, the largest of all germ cells, ranged from 13.3 – 17.8 µm in diameter ($\overline{x}$ = 15.37 ± 1.22; n = 20). Measurements of sperm dimensions yielded the following length parameters (range, mean ± standard deviation, sample size): acrosome, 2.10 – 3.37 µm ($\overline{x}$ = 2.58 ± 0.40; n = 11); nucleus, 10.22 – 13.71 µm ($\overline{x}$ = 11.70 ± 0.86; n = 65); acrosome, nucleus, midpiece complex (ANM) in three frogs, 14.87 – 23.98 µm ($\overline{x}$ = 19.62 ± 2.72; n = 17), 18.83 – 26.96 µm ($\overline{x}$ = 22.92 ± 2.26; n = 17), 17.40 – 26.96 µm ($\overline{x}$ = 23.92 ± 3.27; n = 11); principal piece, 24.36 – 27.68 µm ($\overline{x}$ = 25.98 ± 1.19; n = 14); total tail length (endpiece intact), 29.87 – 39.00 µm ($\overline{x}$ = 33.37 ± 2.63; n = 23); and total sperm length, 51.02 – 62.98 µm ($\overline{x}$ = 54.63 ± 3.54; n = 20). Our sperm morphometric findings complement previously published data on this species and fill in gaps that may aid in future intra- and interfamilial comparisons.

Introduction

The bird-voiced treefrog, *Hyla avivoca*, is a small hylid species that is found primarily in cypress/tupelo swamps, swampy floodplains, and swampy rivers, lakes and ponds in the southeastern and southcentral United States (Dodd 2013). The species' range extends northward into extreme southern Illinois, southeastern Missouri, and western portions of Kentucky and Tennessee. Western populations are scattered in parts of the Arkansas Valley and Ouachita River basin in Arkansas (Fulmer and Tumlison 2004; Trauth et al. 2004). The biology of this species was recently summarized by Dodd (2013).

The sperm morphology/ultrastructure of relatively few species of the 40 or so genera of hyline frogs (Vitt and Caldwell 2014) has been investigated (Scheltinga et al. 2002; Scheltinga and Jamieson 2003). For example, other than a light microscopic analysis by Delahoussaye (1966), who reported on the sperm structure in 10 hylid species from Louisiana (including *Hyla avivoca*), only one other North American hylid, *Pseudacris regilla*, has received any detailed attention regarding sperm structure (Scheltinga 2002). Moreover, Delahoussaye (1966) did not provide any descriptive accounts of testicular microanatomy on any of the species he examined and, more importantly, he was unable to measure several dimensions of sperm morphology in most species due to the limitations in his light micrographic techniques.

In the present study, we report on the testicular histology of the bird-voiced treefrog, *Hyla avivoca*, and provide additional morphometric information on sperm structure obtained using scanning electron microscopy that is not available in the study by Delahoussaye (1966).

Materials and Methods

Calling frogs were collected in several counties (Calhoun—7 May 2013 [n = 1], 22 July 2014 [n = 4]; Conway—9 June 1991 [n = 17], and Little River—18 June 1997 [n = 8]) of central and southern Arkansas. Live specimens were returned to the Electron Microscope Facility at Arkansas State University (ASU) and processed for histological analyses. The frogs were sacrificed by submersion into a dilute chloretone solution, and the testes prepared for both light and scanning electron microscopy (LM and SEM, respectively). Snout-vent length (SVL) was measured

to the nearest mm. Voucher specimens of the frogs were deposited in the ASU herpetological collection (ASUMZ), whereas reproductive tissues embedded in plastic resin were stored in the histo-herpetological collection of the senior author.

For LM, we fixed plastic-embedded testes in 2% glutaraldehyde (GTA). Testes were then dehydrated in a graded series of increasing ethanol solutions (50-100%), placed in a 50/50% acetone/plastic mixture for overnight infiltration, and embedded in Mollenhauer's Epon-Araldite #2 (Dawes 1988). For thick sectioning (approximately 1 µm in thickness) and staining, we used glass knives on an LKB Ultrotome (Type 4801A) with Ladd® multiple stain (LMS), respectively. For photomicroscopy, we utilized a Nikon Eclipse 600 epi-fluorescent light microscope with a Nikon DXM 1200C digital camera (Nikon Instruments Inc, Melville, NY).

For SEM, we fixed sperm samples on cover slips (18 mm X 4 mm) in a 2% GTA solution buffered with 0.1 M sodium cacodylate at a pH of 7.2 for a minimum of 2 h. The cover slips, previously coated with Poly-l-lysine, were dehydrated in a graded series of increasing ethanol solutions (50-100%), followed by several fluid exchanges in 100% ethanol. An Autosamdri-815 critical point drier (Tousimis Research Corporation, Rockville, MD) was used (31°C, 1072 psi, ventilation rate ~100 psi/min) to remove excess ethanol. Cover slips were adhered to copper boats and then mounted on 25.4 mm aluminum pin stub specimen mounts and coated with gold using a Cressington 108 sputter coater (Cressington Scientific Instruments Ltd, Watford, UK). Sperm samples were analyzed both qualitatively and quantitatively with a Vega TS 5136XM digital scanning electron microscope (Tescan USA Inc., Cranberry Township, PA) at 19.5 kV.

For the descriptive terminology of testicular histology, we followed Rastogi et al. (1988), Pudney (1995) and Scheltinga and Jamieson (2003). For describing the sperm ultrastructure, we followed Scheltinga and Jamieson (2003). Measurements of sperm structure for both LM and SEM images were obtained using ImageJ software (National Institutes of Health). Descriptive statistics included means ± 1 standard deviation.

Results

Testicular Histology and Spermatogenesis

As with all anamniotes, the testes of *Hyla avivoca* exhibit cystic spermatogenesis. The testes are organized into numerous seminiferous tubules, whose seminiferous epithelia possess capsular-like follicles, called spermatocysts. Numerous Leydig cells (Fig. 1) are situated within the interstitial spaces along the periphery of each tubule. There can be as many as 20-25 spermatocyts observed in any one cross-sectional histo-section through a single tubule (Fig. 1). In addition, the spermatocysts appear to be aligned with one another in one or more layers extending away from the basement membrane (Figs. 1B and 2). Spermatocysts originate from a single large primary spermatogonium (Fig. 2 A and B), also called a primordial germ cell, which undergoes several mitotic

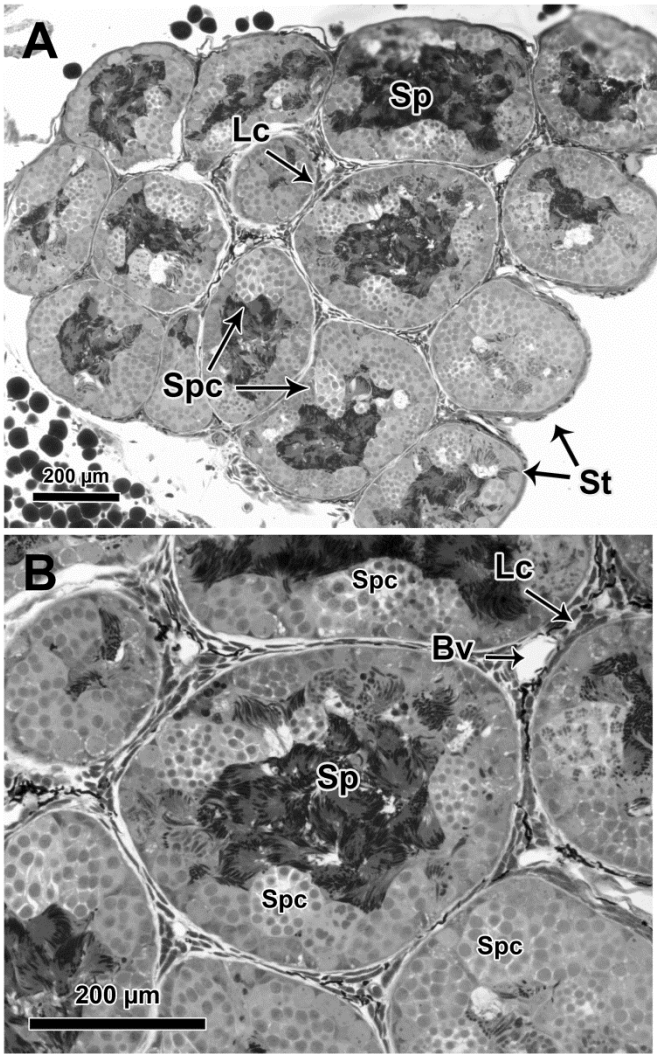

Figure 1. Light micrographs of the seminiferous tubules of a plastic-embedded testis of *Hyla avivoca*. A. Transverse section through a testis from a late July specimen (ASUMZ 33250) revealing sperm (Sp) aggregates within most lumina of seminiferous tubules (St); Lc = Leydig cells. Individual spermatocysts (Spc), which line the basement membrane are also evident. B. Magnification of A showing spermatocysts in different cell stages of spermatogenesis. Bv = blood vessel.

divisions to eventually yield clonal aggregates of germ cells (secondary spermatogonia to mature spermatozoa) through the process of spermatocytogenesis. Primary spermatogonia, the largest of all germ cells, ranged from 13.3 – 17.8 µm in diameter ($\bar{x}$ = 15.37 ± 1.22; n = 20).

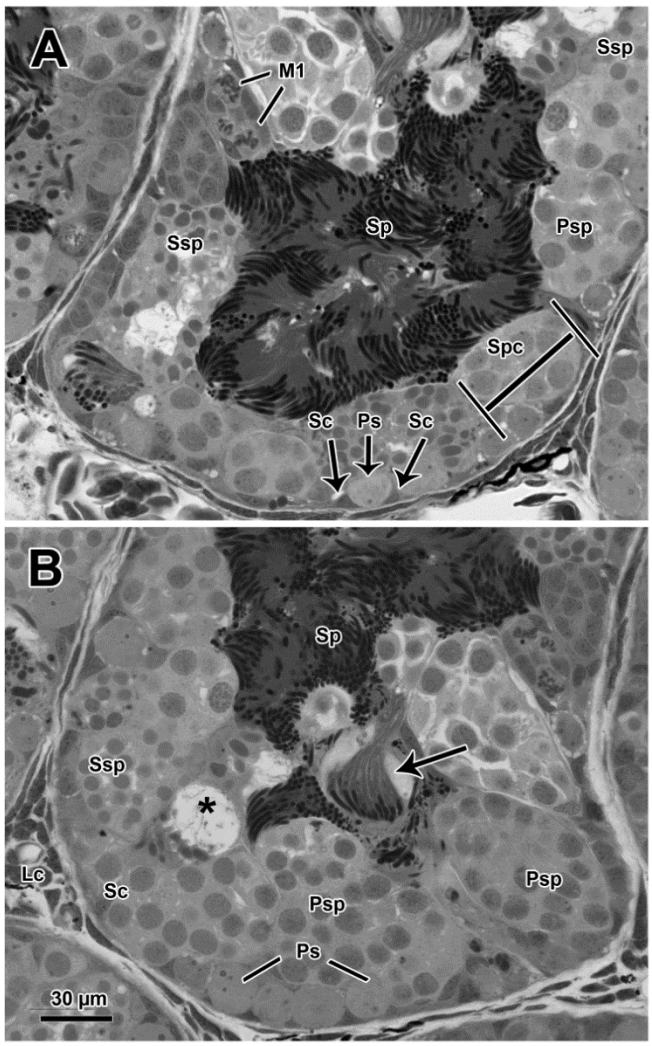

Figure 2. Light micrographs of the seminiferous tubules of a plastic-embedded testis of *Hyla avivoca* (from section in Fig. 1A). A. Transverse section through a portion of a seminiferous tubule showing spermatocyts (Spc) with germ cell clusters in different stages of development including primary spermatocytes (Psp) and secondary spermatocytes (Ssp). Mature sperm (Sp) dominate the tubular lumen. Nuclei of Sertoli cells (Sc) are positioned basally between primary spermatogonia (Ps) along the basement membrane. Spermatocytes of meiotic cells (M1) are also shown. B. Adjacent portion of tubule shown in A showing additional spermatocysts. Interstitial Leydig cells (Lc) can be seen bordering tubule at lower left. A cluster of mature sperm (arrow) is seen exiting a spermatocyst. Cellular debris resides in a spermatocyst devoid of sperm (asterisk). Abbreviations are the same as in A. Scale bar the same for A.

As testicular activity continues, spermatocysts rapidly increase in size and produce a variety of spermatogenic cell stages (Fig. 2). Most testes measured 3 – 4 mm in length. Metaphase of mitotic divisions of secondary spermatogonia within a spermatocyst is shown in Fig. 2B. Several of these spermatogenic stages (e.g., primary spermatocytes, secondary spermatocytes, and spermatids) are also shown in Fig. 2. The nuclei of Sertoli cells (= sustentacular cells) are prevalent along the basement membrane as they flank each primary spermatogonium (Fig. 2); on the other hand, the cytoplasm of Sertoli cells may extend well into the lumen of each tubule. Clonal clusters of sperm remain together as they exit rupturing spermatocysts (Fig. 2B).

Sperm Morphometrics

The morphology of a mature hylid spermatozoon consists of an acrosome, nucleus, midpiece, and tail (principal piece and endpiece) and was illustrated most recently by Scheltinga and Jamieson (2003). In the present study, measurements of the lengths of the nucleus (Fig. 3) were accomplished using LM, whereas lengths of individual acrosomes, the acrosome, nucleus and midpiece complex (ANM) as well as the principal piece, endpiece, and total sperm length were best achieved using SEM (Fig. 4).

The acrosome ranged from 2.10 – 3.37 µm in length ($\bar{x}$ = 2.58 ± 0.40; n = 11), whereas the nucleus ranged from 10.22 – 13.71 µm in length ($\bar{x}$ = 11.70 ± 0.86; n = 65). The ANM complex varied somewhat among three males collected on 7 May 2013. For example, in ASUMZ 32704 (SVL = 37 mm), the ANM ranged from 14.87 – 23.98 µm in length ($\bar{x}$ = 19.62 ± 2.72; n = 17). In ASUMZ 32705 (SVL = 38 mm), ANM ranged from 18.83 – 26.96 µm in length ($\bar{x}$ = 22.92 ± 2.26; n = 17), and lastly ASUMZ 32706 (SVL = 40 mm), the ANM ranged from 17.40 – 26.96 µm in length ($\bar{x}$ = 23.92 ± 3.27; n = 11).

Tail length measurements pose a problem in hylid frogs due to the fact that the endpiece is fragile (Delahoussaye 1966). During the preparatory process for SEM in the present study, many of the sperm tails examined did not retain their endpieces. Consequently, tail length measurements consisted of two sets of values: one was considered principal piece only, whereas the other was total tail length (endpiece intact). The former ranged from 24.36 – 27.68 µm in length ($\bar{x}$ = 25.98 ± 1.19; n = 14), and the latter value ranged from 29.87 – 39.00 µm in length ($\bar{x}$ = 33.37 ± 2.63; n = 23). By noting the size difference between total tail length and principal piece length, a rough

estimate of the endpiece length is around 8 µm. Total sperm length was calculated using only sperm that appeared to possess an intact endpiece; these data yielded a range of 51.02 – 62.98 µm in length ($\overline{x}$ = 54.63 ± 3.54; n = 20).

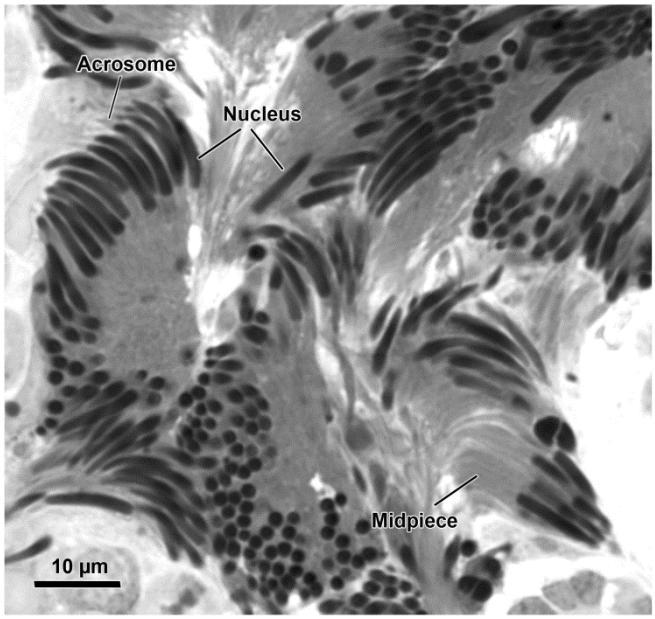

Figure 3. Light micrograph within the central luminal region of a seminiferous tubule showing clonal clusters of mature sperm. The acrosome, nucleus, and midpiece are easily discernible.

Discussion

Mature sperm dominated the luminal interior of seminiferous tubules in all frogs examined during this study. It has been estimated that there are 7 – 8 divisions occurring from each primary spermatogonium in some frog species (Rastogi et al. 1988, Takamure et al. 1995), which yield large numbers (2^8) of primary spermatocytes.

Morphometric comparisons between our sperm data and those reported by Delahoussaye (1966) revealed several differences. He found a smaller average acrosome length (2.1 µm ± 0.2; range, 1.9 – 2.4), and his average ANM (18.6 µm ± 1.1; range, 17.1 – 20.6) was also smaller than our calculations on three males. Moreover, he was unable to accurately determine tail length and, therefore, reported no total sperm length values. Our results suggest that total sperm length for most sperm will fall within 50 – 60 µm. Scheltinga (2002) reported a total sperm length in *Pseudacris regilla* to be 50 µm. Of the 36 species of Australian hylid frogs examined by Scheltinga (2002), 16 (44.4%) exhibited total sperm lengths within the 50 – 60 µm range.

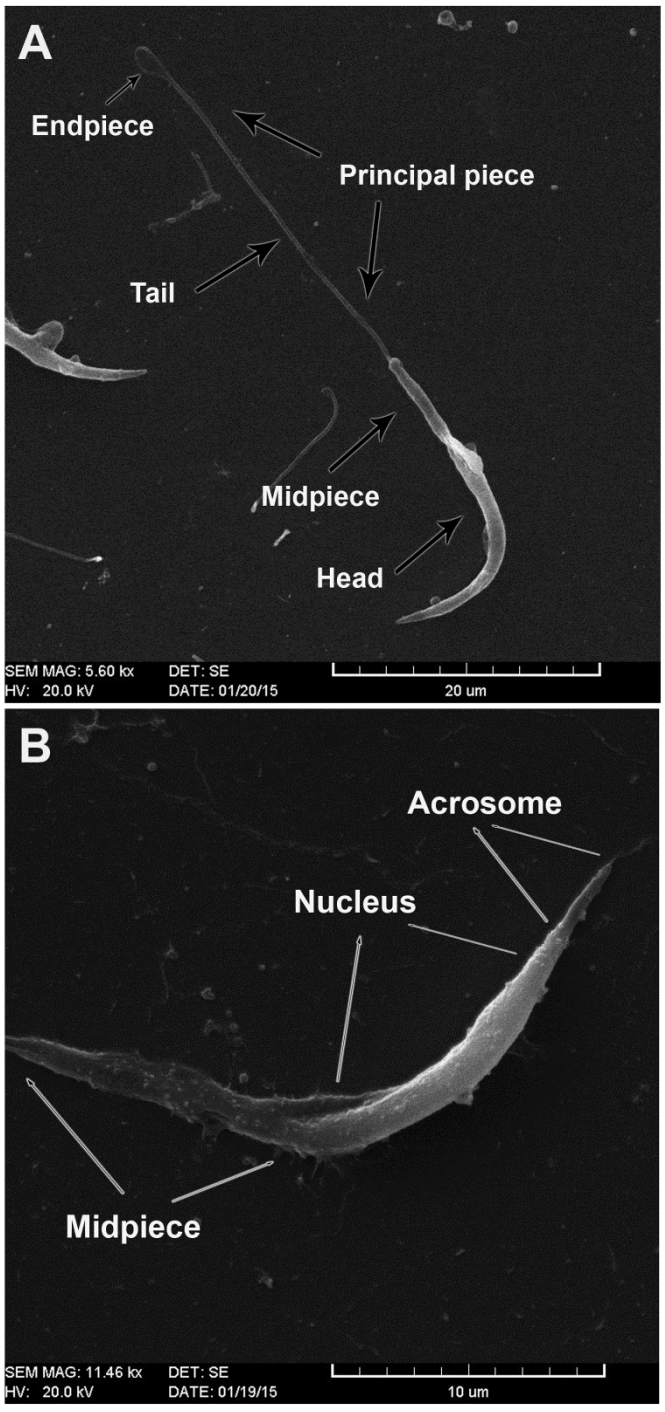

Figure 4. Scanning electron micrographs of mature sperm of *Hyla avivoca* (ASUMZ 32706). A. Spermatozoon (total length = 60.84 µm) showing anatomical regions including the head, midpiece, and tail (principal piece and endpiece). B. The acrosome, nucleus, and midpiece complex and their distinct morphologies.

Future studies on anuran sperm morphometrics will benefit greatly by incorporating SEM analyses into their techniques. This will allow for useful comparisons to be made for a better understanding of sperm structure.

Acknowledgments

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