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Morphological description and comparison of sperm from eighteen species of cricetid rodents

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Analyses of the dimensions and morphology of spermatozoa can be useful in the identification of mammalian species. We compared and contrasted sperm morphology and dimensions in 9 genera and 18 species of the family Cricetidae. Spermatozoa were obtained from the cauda epididymes of animals previously fixed in 10% formalin, and stained with Giemsa or silver-nitrate staining methods. At least 50 spermatozoa from different specimens were examined for each species. Discriminant function analysis was used to distinguish between the spermatozoa of different species and to identify the best discriminating characteristics. MANOVA revealed that differences between species were significant. Species in the same genus tended to group together. Qualitative characteristics that discriminate between species are discussed.

El análisis de las dimensiones y la morfología de los espermatozoides puede ser útil en la identificación de especies de mamíferos. La morfología y dimensiones de espermatozoides (largo y ancho de la cabeza, longitud de la pieza intermedia, pieza principal con la pieza final y longitud total) de 9 géneros y 18 especies de cricétidos fueron comparados y contrastados. Los mismos se obtuvieron de la cola del epidídimo de animales previamente fijados en formalina al 10%, y fueron teñidos con Giemsa o con nitrato de plata. Al menos 50 espermatozoides (de diferentes especímenes) por especie fueron estudiados. El análisis de la función discriminante se utilizó para distinguir entre los espermatozoides de las diferentes especies e identificar las mejores características discriminantes. Una prueba de MANOVA reveló que las diferencias entre especies son altamente significativas. Las especies del mismo género tienden a agruparse juntas. También se discuten las características cualitativas que ayudaron a discriminar entre especies.

Key words: Cricetidae, rodents, spermatozoa, sperm morphology, taxonomy

Spermatozoa are likely under intense selective pressure given their crucial role in reproduction. Many studies have evaluated the phylogenetic relationships and evolution of vertebrate and invertebrate spermatozoa (Austin and Bishop 1958; Baccetti and Afzelius 1976; Jamieson 1987; Roldan et al. 1992; Cétic a et al. 1997; Gage 1998; Swallow and Wilkinson 2002; Breed et al. 2014). Although the sperm cells of all mammalian species possess the same basic structural components, they can vary widely in size and morphology (Cummins and Woodall 1985; Pitnick et al. 2009). The evolution of features such as sperm shape, size, and count is probably the result of natural or sexual selective forces (Roldan et al. 1992). Mammalian spermatozoa are diverse in form and size across species (Roldan et al. 1992).

Members of the order Rodentia exhibit the greatest interspecific variability of any mammalian taxa and account for the highest species richness of all mammals (Burgin et al. 2018). Their sperm are complex and show considerable differences in terms of head shape (e.g., ovoid to falciform, with 1 or more apical hooks, and with the presence of nuclear caudal extensions) and measurements (e.g., total length 34.64 and 258.32 µm in *Myocastor coypus* and *Cricetulus griseus*, respectively—Roldan et al. 1992; Gallardo et al. 2002; Breed et al. 2014).

Understanding the diverse morphology and dimensions of spermatozoa can provide valuable information for taxonomic studies (Rouse and Robson 1986; Harding et al. 1987; Roldan et al. 1992). The accurate identification of rodent species is problematic: dental, cranial, and external morphology cannot always resolve cryptic yet genetically distinct species (Roldan et al. 1985). Unequivocal species identification is essential when studying rodent ecology, especially when some sympatric species host different zoonotic diseases (e.g., Junin virus, hosted in *Calomys musculinus*; Machupo virus hosted in *Calomys callosus*; or hantavirus hosted in *Oligoryzomys*), while similar taxa pose no health risk (Weissenbacher et al. 1990). Accurate identification is also essential in biodiversity and conservation studies, especially at greater biogeographical scales (de la Sancha 2014; de la Sancha et al. 2014). The utility of information about sperm morphology is clear when comparing sperm of *Calomys hummelincki*, which has sperm with a hooked head and an eccentrically inserted tail, with those of *Calomys laucha*, which has sperm with a hookless head and a centrally inserted tail (Pérez Zapata et al. 1987). Some cryptic rodent species are known to have very different spermatozoa (Gordon and Watson 1986; Pérez Zapata et al. 1987; Roldan et al. 1992).

Sigmodontinae is the most diverse subfamily of the Cricetidae in South America (Reig 1986). They are found over most of South American, in most habitats, and extend as far north as southern North America (D'Elía and Pardiñas 2015). There is some debate about which species should be included in the Sigmodontinae. Should the subfamily include only South American cricetids with a complex penis morphology, or should it also include North American forms with a single-pronged baculum, namely the subfamilies Neotominae and Tylomyinae that include neotomine-peromyscines (D'Elía and Pardinas 2015). Regardless, all complex-penis cricetids form a monophyletic group with neotomine-peromyscines as outgroups (Steppan and Schenk 2017). Currently, complex-penis sigmodontines are divided into 9 tribes, including Oryzomyini, Thomasomyini, Wiedomyini, Ichthyomyini, Abrotrichini, Reithrodontini, Sigmodontini, Phyllotini, and Akodontini (D'Elía and Pardinas 2015). However, clear affinities for many of these groups are not yet resolved (D'Elía 2015).

In this study, we surveyed sperm morphology and provided morphometric comparisons across 9 genera and 18 species of South American sigmodontines, representing the tribes Akodontini, Oryzomini, and Phyllotini sensu D'Elía and Pardiñas (2015). While the total number of sigmodontine species is unclear (D'Elía and Pardiñas 2015), we include descriptions for 3 major tribes. This adds considerably to our understanding of sigmodontine spermatozoid morphology, and to rodent spermatozoid morphology as a whole. Our results show that these characteristics are useful in making taxonomic distinctions and can be used to infer phylogenetic relationships when combined with biogeographical, morphological, chromosomal, or genetic data.

Materials and Methods

Animals used in this study (n = 58; see Supplementary Data SD1 for species [as identified by their original collectors] and sample sizes) were trapped following protocols and guidelines established by the American Society of Mammalogists (Sikes et al. 2016). The Argentinean specimens (n = 29) belong to the mammal collection of the Museo Lorenzo Scaglia, Mar del Plata, and contain karyotypic and cytotaxonomic descriptions (Vitullo et al. 1982, 1983). The Paraguayan specimens (n = 29) were collected in 4 forest reserves in eastern Paraguay. Their capture was approved by the Texas Tech University Animal Care and Use Committee (reference number 07045-10) for the provision of voucher specimens for identification purposes. These specimens are now deposited at the Field Museum of Natural History (FMNH), Chicago, Illinois; the Natural Science Research Laboratory, Texas Tech University (TTU), Lubbock, Texas; and at the Colección de Zoología, Facultad de Ciencias Naturales y Exactas (CZ), Universidad Nacional de Asunción, San Lorenzo, Paraguay (for details, see de la Sancha 2014).

All samples were obtained from testes fixed in 10% formalin. We dissected the cauda epididymis to obtain tissue, and these were macerated in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4) and centrifuged at 800 rpm. The supernatant was discarded. The pellet was then resuspended in PBS and passed through a series of filters to remove large epididymal debris. A drop of the final suspension was spread on a microscope slide, air-dried, and stained either with modified Giemsa (Watson 1975) or via the silver-nitrate staining method (Cétika et al. 1997). This made it possible to clearly identify the differentiated structure of the sperm (Cétika et al. 1997; Andraszek and Smalec 2011). The morphology and linear dimensions of at least 50 spermatozoa per species were then measured from their digitized images: total length (from tip of the head to the lowest point of the tail), length and width of the head (across the longest and widest parts of the head), length of the midpiece (from the base of the head to the beginning of the principal piece), and combined length of the principal and end piece (from the beginning of the principal piece to the end of the end piece). The morphology of the sperm heads was characterized as pyriform (pear-shaped and the base of the head is concave), polygonal (3 or more usually straight sides and the base of the head is flat), or oval (a hookless head in the shape of an egg). Insertion of the tail in the base of the head was characterized in all species (centrally or eccentrically). Only spermatozoa that were complete, including the end piece, were used.

To study the location of the nucleus within the sperm head, spermatozoa in a second drop of prepared sperm suspension were stained with DAPI (4′,6-diamidino-2-phenylindole, 0.2 µm/ml in McIlvaine's buffer pH 6.8) for 5 min, mounted in glycerol, viewed under a Leitz DMRB epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) (magnification 1,000×), and photographed using a Leica DFC 300 FX digital camera (Leica Microsystems). Images were processed using Leica Application Suite v.3.6.0 software (Leica Microsystems).

Species-specific descriptive statistics were computed for each sperm characteristic. Discriminant function analysis (DFA—Strauss 1985; dos Reis et al. 1990) was used to assess morphological differences and to maximize group discrimination (Strauss 2010). We plotted DF1 and DF2 scores to show group discrimination. Multivariate normality was assessed with the Mardia normality test using PAST software (Hammer et al. 2001). Because the data were not normally distributed, we used a nonparametric approach to assess significance of differences between spermatozoa of rodent species following the protocol of Hernández et al. (2017) to improve confidence in our results. Thus, the DFA was followed by a nonparametric MANOVA with 10,000 permutations to determine significance with Wilks' lambda using Matlab functions "Dfa" and "MANOVA" (Strauss 2015). We analyzed differences between groups for the entire sample of species, and for pairwise comparisons between all 18 species.

Finally, we used the recent rodent phylogeny by Steppan and Schenk (2017) to create a trimmed phylogeny with the taxa used in our study to test if rodent sperm shape has a phylogenetic signal. We used our class mean DF1 and DF2 scores as a proxy for shape and then used the R package picante function "phylosignal" to calculate the *K* statistic for phylogenetic signal and test significance based on the variance of phylogenetically independent contrasts relative to tip-shuffling randomization (Kembel et al. 2010).

Results

Differences in total length and size of the sperm head, midpiece, and principal piece + end piece were seen between most species (Table 1). Sperm length varied from 59.35 ± 3.33 µm (*SE*) in *C. laucha* to 99.13 ± 3.43 µm in *Scapteromys aquaticus* (Table 1). DFA discriminated between the tribes and genera examined. Species of *Calomys* were completely separated from all others except *C. musculinus* (Fig. 1). *Akodon* spp. and *S. aquaticus* grouped together and were distintic from all other species (Fig. 1). Other Akodontines examined, including *Necromys lasiurus*, *Oxymecterus rufus*, and *Thaptomys nigrita* completely discriminated from the genus *Akodon*. However, *Necromys lasiurus* and *O. rufus* grouped with *Orizomines*, but not *Sooretamys angouya* (Fig. 1). DF1 accounted for 53.97% of the variation in morphometric measurements for the spermatozoa, and was associated primarily with total tail length (Fig. 1; $\rho = 0.75$), while DF2 accounted for 39.09% of the variation and was strongly most associated with the midpiece (Fig. 1; $\rho = 0.94$; Supplementary Data SD2). MANOVA revealed differences between rodent species were significant (Wilks' $\lambda = 0.001$, $F_{85,4.12} = 501.00$, $P < 0.01$). Pairwise comparisons confirmed significant differences between all pairs of species (Supplementary Data SD2). Sperm morphology can be used as a diagnostic character that clearly discriminates between species. This idea is supported by the fact that DF1

Table 1.—Summary statistics $\bar{X} \pm SE$ for the 5 characters used to describe the morphology of spermatozoa for each cricetid species evaluated.

Tribe	Species	Head		Midpiece (µm)	Principal piece + end piece (µm)	Total length (µm)
		Length (µm)	Width (µm)			
Phyllotini						
	Calomys callidus	6.1 ± 0.30	2.1 ± 0.16	19.4 ± 0.25	59.7 ± 1.38	85.7 ± 1.74
	C. laucha	6.7 ± 0.08	2.4 ± 0.02	17.8 ± 0.08	37.6 ± 1.64	59.3 ± 3.33
	C. musculinus	6.5 ± 0.31	2.1 ± 0.17	15.2 ± 0.47	57.1 ± 1.58	72.6 ± 2.06
	C. venustus	7.2 ± 0.18	3.0 ± 0.10	21.3 ± 0.26	56.3 ± 1.01	87.2 ± 1.47
Akodontini						
	Akodon azarae	6.5 ± 0.31	2.6 ± 0.15	15.2 ± 0.44	77.3 ± 1.35	98.2 ± 1.25
	A. dolores	6.4 ± 0.23	2.7 ± 0.14	15.1 ± 0.29	72.1 ± 0.93	93.5 ± 1.18
	A. albiventer	5.9 ± 0.24	2.7 ± 0.19	15.2 ± 0.24	70.7 ± 2.34	89.6 ± 1.41
	A. montensis	6.3 ± 0.44	2.3 ± 0.12	15.5 ± 0.59	71.9 ± 1.09	93.7 ± 2.26
	A. paranaensis	7.1 ± 0.27	3.2 ± 0.16	15.7 ± 0.20	63.4 ± 1.43	87.7 ± 1.43
	Oxymycterus rufus	6.3 ± 0.18	2.2 ± 0.16	13.5 ± 0.49	53.8 ± 1.73	73.9 ± 1.73
	Scapteromys acuaticus	5.9 ± 0.49	2.5 ± 0.39	13.6 ± 1.10	74.9 ± 3.41	99.1 ± 3.43
	Thaptomys nigrita	6.1 ± 0.22	2.4 ± 0.13	14.6 ± 0.27	62.5 ± 1.94	77.3 ± 1.32
	Necromys lasiurus	5.8 ± 0.27	2.9 ± 0.06	11.3 ± 0.73	56.6 ± 3.46	71.7 ± 2.63
Oryzomini						
	Oligoryzomys flavescens	7.0 ± 0.27	2.1 ± 0.19	12.2 ± 0.31	53.3 ± 1.85	70.1 ± 1.76
	O. longicaudatus	6.2 ± 0.34	2.4 ± 0.23	12.5 ± 0.40	54.3 ± 2.00	75.6 ± 1.93
	O. nigripes	7.4 ± 0.14	2.1 ± 0.20	12.9 ± 0.32	52.2 ± 1.35	72.2 ± 2.03
	Hylaeamys megacephalus	5.9 ± 0.38	2.2 ± 0.33	13.8 ± 1.26	52.6 ± 2.66	72.1 ± 1.60
	Sooretamys angouya	6.1 ± 0.30	2.7 ± 0.31	14.81 ± 0.39	62.0 ± 1.98	83.1 ± 1.73

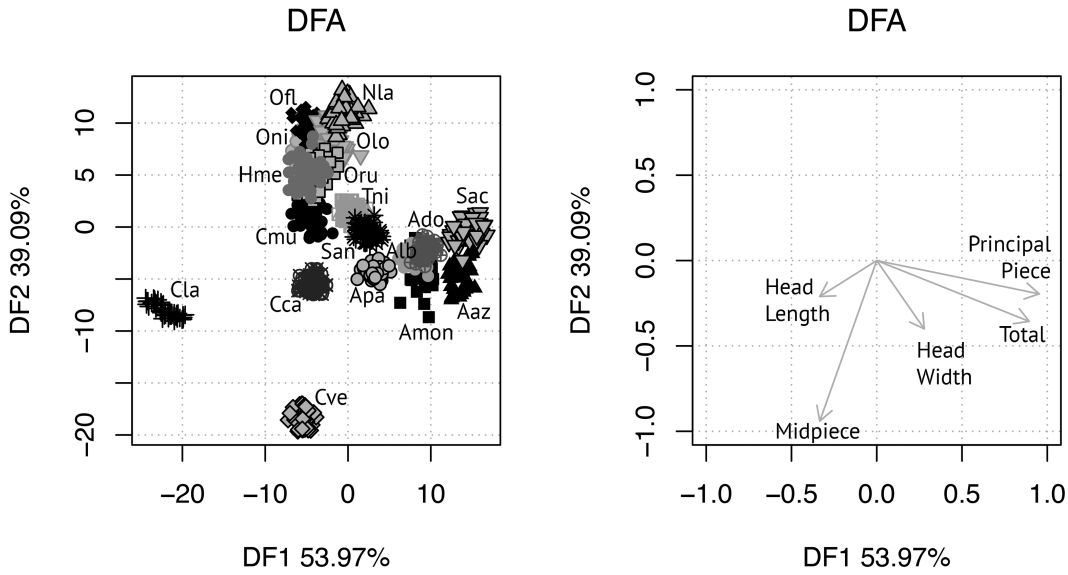

Fig. 1.—Morphometric analysis. Discriminant function analysis (DFA) scatter- and bi-plots for the examined species: *Calomys callidus* (Cca), *C. laucha* (Cla), *C. musculinus* (Cmu), *C. venustrus* (Cve), *Akodon albiventer* (Aal), *A. azarae* (Aaz), *A. dolores* (Ado), *A. montensis* (Amo), *A. paranaensis* (Apa), *Oxymycterus rufus* (Oru), *Necromys lasiurus* (Nla), *Scapteromys acuaticus* (Sac), *Thaptomys nigrita* (Tni), *Oligoryzomys flavescens* (Ofl), *O. longicaudatus* (Olo), *O. nigripes* (Oni), *Hylaeamys megacephalus* (Hme), *Sooretamys angouya* (San).

has a significant phylogenetic signal ($K = 0.94301$, $P = 0.002$, Z-score = −1.529).

Most species (16 of 18) had sperm heads with a single hook in the apical portion of the head (Figs. 2–4), while 2 species (*T. nigrita* and *C. laucha*) had oval heads. The apical hook observed in most species consisted mainly of acrosomal material, with the nucleus terminating near the base (observable with DAPI staining).

Tribe Phyllotini.—The spermatozoa of *C. musculinus*, *C. callidus*, and *C. venustus* had hooked heads. However, in *C. callidus* and *C. venustus*, the sperm head was pyriform and the tail centrally inserted (Fig. 2), while in *C. musculinus* the sperm head was polygonal and the tail inserted eccentrically (Fig. 2). The sperm heads of *C. laucha* were hookless and the tail inserted centrally into the flat base of the head (Fig. 2). Considerable variation was recorded in sperm cell dimensions across *Calomys* (Table 1), with a large difference seen between sperm cell total length in *C. laucha* (59.35 ± 3.33 µm; the shortest sperm cells) and *C. venustus* (87.25 ± 1.47 µm; the longest). Sperm length variables on both discriminant axes separated these species completely (Fig. 1; Table 2).

Tribe Akodontini.—Sperm heads of *Akodon* spp. had an apical hook (Fig. 3A). However, morphological differences were seen in terms of sperm cell head shape, differences in the base of the head, and the point of tail insertion. *Akodon montensis* and *A. azarae* had pyriform sperm heads, and showed central insertion of the tail at the base (Fig. 3A). In *A. paranaensis*, *A. dolores*, and *A. albiventer*, the sperm heads were polygonal (Fig. 3A). The location of tail insertion differentiated *A. paranaensis* (eccentric) from *A. dolores* and *A. albiventer* (central). Total sperm length across the genus ranged from 87.75 ± 1.43 µm to 98.21 ± 1.25 µm (Fig. 1). No significant differences were seen in length of the midpiece between any members of *Akodon*. Although sperms cells of *A. azarae* and *A. montensis* showed no morphological differences, they were

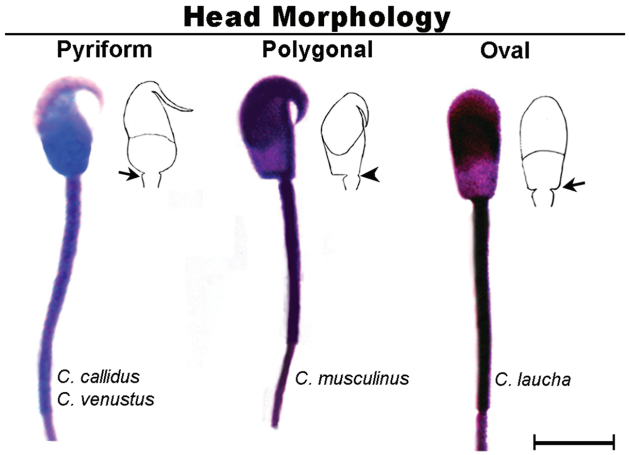

Fig. 2.—Sperm heads in the tribe Phyllotini, genus *Calomys*: *C. callidus* and *C. venustus* showed sperm heads with a single hook and pyriform head shape, *C. musculinus* showed sperm heads with a single hook and polygonal head shape, and *C. laucha* (Cla) sperm heads were oval and hookless. Arrow: sperm cell with a centrally inserted tail. Arrowhead: sperm cell with an eccentrically inserted tail. Scale bar: 5 µm. Sperm stained with modified Giemsa.

Table 2.—Summary of discriminant function analysis (DFA) of 5 linear measurements of spermatozoa from 18 cricetid rodent species.

Variable	DF1	DF2
Head length	−0.142	−0.024
Head width	0.069	−0.130
Midpiece	−0.320	−0.940
Principal piece + end piece	0.751	−0.105
Total length	0.624	−0.312

distinguishable by overall sperm cell length. Indeed, *A. azarae* had the longest spermatozoa of all *Akodon* species studied, and were much longer than the sperm cells of *A. montensis*

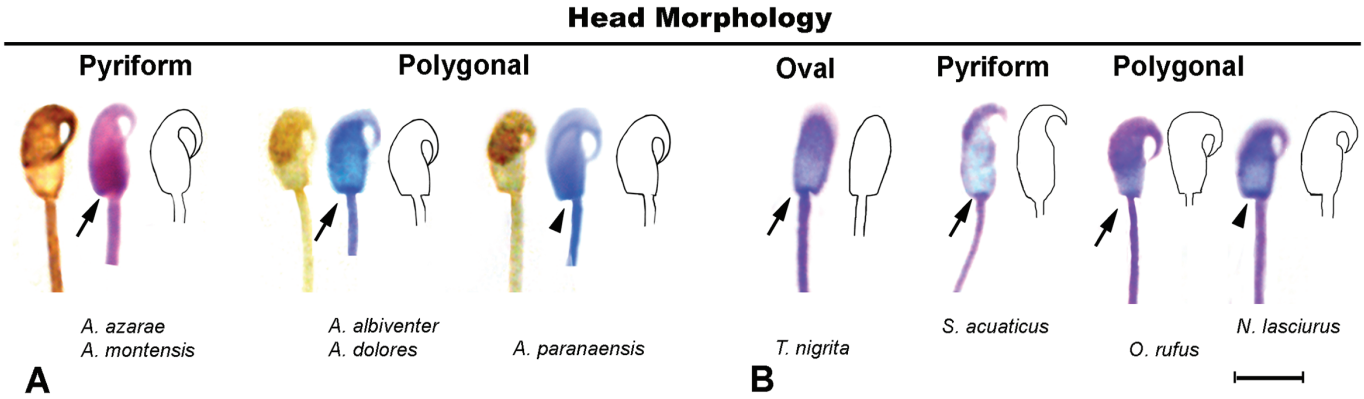

Fig. 3.—Sperm heads in the tribe Akodontini. A) Sperm heads in *Akodon*. Species with pyriform head shapes: *A. azarae* and *A. montensis*; species with polygonal sperm heads: *A. paranaensis*, *A. dolores*, and *A. albiventer*. B) Sperm heads of members of other genera in the tribe Akodontini. *Thaptomys nigrita* with oval head shapes, *Scapteromys acuaticus* with pyriform sperm heads, *Oxymycterus rufus* and *Necromys lasiurus* with polygonal sperm heads. Arrow: sperm cell with a centrally inserted tail. Arrowhead: sperm cell with an eccentrically inserted tail. Scale bar: 5 µm. Sperm stained with modified Giemsa and silver nitrate.

(98.21 ± 1.25 and 93.73 ± 2.26 µm, respectively). *Akodon dolores* and *A. albiventer* had similar spermatozoa, and no consistent differentiating characteristics were found. *Oxymycterus rufus*, *N. lasiurus*, and *T. nigrita* were clearly distinguishable from all other *Akodon* species (Fig. 1). *Thaptomys nigrita* had an oval sperm head like that of *C. laucha*, and the insertion of the tail was central in both (Fig. 3B). However, total sperm length of *T. nigrita* was longer (77.26 ± 1.32 µm) than that of *C. laucha* (57.35 ± 3.33 µm; Table 1). *Scapteromys aquaticus* had a pyriform sperm head (Fig. 3B) and the longest sperm cells of all species studied (99.13 ± 3.43; Table 1). *Oxymecterus rufus* and *N. lasiurus* were characterized by spermatozoa with polygonal heads, but they showed a central and an eccentric insertion of the tail, respectively (Fig. 3B).

Tribe Oryzomini.—The spermatozoa of *O. nigripes*, *O. longicaudatus*, and *O. flavescens* were similar. All showed polygonal heads and centrally inserted tails (Fig. 4A). The sperm morphometrics of *Oligoryzomys* spp. overlapped considerably (Table 1); DFA did not completely discriminate between them (Fig. 1). The remaining Oryzomini had sperm cells with hooked heads. The heads of *Hylaeamys megacephalus* sperm were pyriform and the tail centrally inserted, while those of *S. angouya* showed a polygonal head with a centrally inserted tail (Fig. 4B). Additionally, the sperm of *S. angouya* was longer than that of any other member of the tribe (Table 1). With the exception of some overlap between *Hylaeamys* and *Oligoryzomys*, there was little overlap among other species of the tribe (Fig. 1).

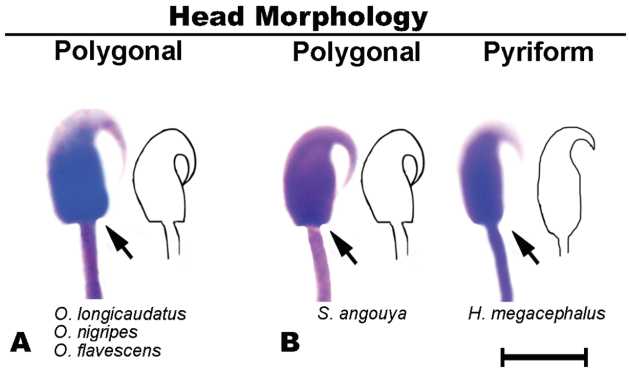

Fig. 4.—Sperm heads in the tribe Oryzomini. A) Sperm head morphology in members of the genus *Oligoryzomys*. All specimens showed polygonal sperm heads. B) Sperm heads of other members of the tribe Oryzomini. Polygonal sperm head of *Sooretamys angouya* and the pyriform sperm head of *Hylaeamys megacephalus*. Arrow: sperm cell with a centrally inserted tail. Scale bar: 5 µm. Sperm stained with modified Giemsa.

Discussion

Of the 18 species studied, 16 showed a characteristic hooked sperm head and 2 (*C. laucha* and *T. nigrita*) had spermatozoa with oval heads. It has been postulated that species with a sperm head showing an apical hook (that largely contains acrosomal material) generally have longer sperm tails, and that species with oval sperm heads have shorter tails (Ding et al. 2010). While this may be true for *C. laucha*, it appears not to apply to *T. nigrita* or the rest of the species examined. Our results also show that South American cricetid species are more likely to have apically hooked sperm heads than oval heads. Roldan (1992) concluded that oval sperm heads were the ancestral condition for *Calomys*, but we did not evaluate this hypothesis.

The genus *Calomys* showed the greatest variation in sperm dimensions and head morphology. Roldan et al. (1992) described a hooked heads in *C. callidus* and *C. musculinus*, while the sperm heads of *C. laucha* are hookless. The sperm of these 3 species differ in their linear dimensions. Because earlier studies presented no measurements of the head, midpiece, or tail (Pérez Zapata et al. 1987; Roldan et al. 1992), it is difficult to provide a more comprehensive comparison with earlier studies.

Differences between the sperm cells of *Akodon* spp. are not as evident as in *Calomys*. The DFA did not separate most *Akodon* species, and only the spermatozoa of *A. azarae* can be easily differentiated in terms of length from those of the others. The size of the midpiece was similar (ca. 15 µm) in all *Akodon*

sperm cells; it may represent a conserved character within the genus. However, no midpiece measurements have been reported for any other members of this genus. The hooked heads of *Akodon* spermatozoa are consistent with those described by Roldan et al. (1992). *Thaptomys nigrita* is phylogenetically close to the genus *Akodon*, and in fact was once referred to as a subgenus of *Akodon* (Cabrera 1961; Reig 1987). However, our work shows that sperm of these taxa can be clearly distinguished by their different head morphologies (oval with no apical hook in *T. nigrita* versus nonovoid and with a hook for members of *Akodon*). The DFA separated *T. nigrita* completely from *Akodon*, and the other Akodontines examined (*N. lasiurus*, *O. rufus*).

The phylogenetic position of species within the genus *Oligoryzomys* has long been unclear. Indeed, several authors have questioned the distinctions between different species of *Oligoryzomys* (Carleton and Musser 1989; Andrades Miranda et al. 2001; Musser and Carleton 2005; Weksler and Bonvicino 2005; Frances and D'Elía 2006). Our study shows that sperm dimensions in this genus are very similar. Thus, sperm dimensions do not provide an easy tool to separate *Oligoryzomys* species that are difficult to identify.

We show that sperm cell morphology and morphometrics provide a valuable tool for identifying some cricetid rodents, which reinforces the idea that sperm morphology and dimensions likely have a strong phylogenetic signal. Future work should examine spermatozoa of other species in an effort to assess the mapping of sperm morphology on phylogenetic history. Sperm morphology also should be considered in concert with biogeographic, chromosomal, and genetic data so that comprehensive evolutionary analyses of related species can be performed. We hope that the ideas developed here will stimulate further research in this field, and perhaps ultimately suggest new approaches.

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Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Specimen information for species used in this study.

Supplementary Data SD2.—MANOVA (permutated 10,000 times) pairwise comparisons between Cricetidae spermatozoa; Wilks' λ (above) and *F*-values with corresponding degrees of freedom (below). All pairwise comparisons were significant with $P < 0.001$.

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