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Bundles of Sperm: Structural Diversity in Scorpion Sperm Packages Illuminates Evolution of Insemination in an Ancient Lineage

DAVID E. VRECH,[1] ALFREDO V. PERETTI,[1] LORENZO PRENDINI,[2]
AND CAMILO I. MATTONI[1]

ABSTRACT

The spermatozoa of scorpions are often bundled together, forming a type of sperm conjugation known as a sperm package. Sperm packages may be found inside the testes and seminal vesicles but vanish in the female atrium, leaving free spermatozoa. Previous studies, based on a limited number of taxa, suggested a diversity of sperm package morphology across the order Scorpiones C.L. Koch, 1850. However, the sperm packages of most scorpion taxa remained unknown. The present study provides the first systematic survey of sperm package morphology across the order, covering 89 exemplar species in 66 genera and 19 families representing all suprafamilial ranks, with a more detailed investigation of the family Bothriuridae Simon, 1880. Whereas all exemplar species of scorpions exhibit sperm packages, Buthida Soleglad and Fet, 2003, including Chaerilidae Pocock, 1893, and most Buthidae C.L. Koch, 1837, present unorganized sperm or loosely organized bundles. Although the details vary, three main types of sperm packages may be recognized in all other families: single folded; straight; and multiple folded. Subtypes may be identified according to general shape and folding patterns, mainly among sperm packages that are folded multiple times. Single-folded sperm packages are the most common type observed in the order. Although most sperm packages lack a covering, a conspicuous secretion sheath may be evident, e.g., in some Chactidae Pocock, 1893. Sperm

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[1] Laboratorio de Biología Reproductiva y Evolución, Instituto de Diversidad y Ecología Animal, CONICET – FCEFyN, Universidad Nacional de Córdoba, Córdoba, Argentina.

[2] Arachnology Lab and Scorpion Systematics Research Group, Division of Invertebrate Zoology, American Museum of Natural History, New York.

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packages vary in length from 112–354 μm and bent sperm packages are not necessarily longer than straight sperm packages. Four exemplar species of Bothriuridae reveal that variation in sperm count within a single sperm package is consistent with the count derived in spermatogenesis. The diversity of sperm packages suggests a path from free spermatozoa, via bent sperm packages, to other forms. Sperm packages may aid in the transport, cooperation, competition, and survival of spermatozoa. The diverse morphology, function, and evolution of sperm packages merit further investigation.

INTRODUCTION

The spermatozoa of scorpions are morphologically rather uniform (André, 1959, 1963; Hood et al., 1972; Cruz-Landim and Ferreira, 1971, 1973; Jespersen and Hartwick, 1973; Phillips, 1974; Alberti, 1983). The spermatozoa are filiform and flagellate, comprising a head, middle piece, and flagellum (Jespersen and Hartwick, 1973; Alberti, 2000, 2005; Michalik and Mercati, 2010). Structural differences in the axoneme or the acrosomal complex, between Buthidae C.L. Koch, 1837, and other extant families, suggest variation in the morphology of scorpion sperm may be phylogenetically informative (Vignoli et al., 2008; Michalik and Mercati, 2010; Peretti, 2010).

Scorpion spermatozoa are often gathered, or bundled, forming sperm conjugations in which spermatozoa are held together during the final phase of spermatogenesis (Pitnick et al., 2009; Higginson and Pitnick, 2011; Dallai, 2014). Sperm conjugates in scorpions are formed by grouping spermatozoa with an undetermined electrodense substance, lacking a secretion sheath (Vignoli et al., 2008; Michalik and Mercati, 2010; Vrech et al., 2011). Previous studies indicated that sperm conjugates may contain 70–250 spermatozoa per package (Peretti and Battán-Horenstein, 2003; Vignoli et al., 2008; Michalik and Mercati, 2010; Vrech et al., 2016).

The terminology describing sperm conjugates in animals is heterogeneous and inconsistently applied (Pitnick et al., 2009). In the present study, "sperm package" refers to the sperm conjugates of scorpions, following recent scorpion literature (Vignoli et al., 2008, Michalik and Mercati, 2010; Vrech et al., 2011, 2016) and is synonymous with "sperm bundle," widely used for insects (e.g., Virkki, 1969; Mojica and Bruck, 1996; Sasakawa and Toki, 2008; Higginson and Pitnick, 2011; Hodgson et al., 2013) as well as in the older literature on scorpions (e.g., Jespersen and Hartwick, 1973; Alberti, 1983).

Many early studies of scorpion sperm were conducted on taxa of the family Buthidae, with detailed data presented on spermiogenesis and spermatozoa ultrastructure (Wilson, 1931; Cruz-Landim and Ferreira, 1971, 1973; Riess et al., 1978a, b; Alberti, 1983). Jespersen and Hartwick (1973) were the first to record the presence of sperm packages in four nonbuthid taxa from North America, i.e., *Anuroctonus phaiodactylus* (Wood, 1863), *Hadrurus arizonensis* Ewing, 1928, *Uroctonus mordax* Thorell, 1876, and *Paravaejovis puritanus* (Gertsch, 1958) (as *Vejovis puritanus* Gertsch, 1958), currently assigned to three chactoid families, Chactidae Pocock, 1893, Hadruridae Stahnke, 1974, and Vaejovidae Thorell, 1876. Unfortunately, Jespersen and Hartwick (1973) provided few data on the morphology of the sperm packages in these taxa.

The presence of sperm packages in four genera and eight species of another nonbuthid family, Bothriuridae Simon, 1880, was reported 30 years later (Peretti and Battán-Horenstein, 2003). In addition to describing the male genitalia and measuring sperm packages, Peretti and Battán-Horenstein (2003) discussed sperm package dynamics inside the female genital tract for the first time. Sperm packages disaggregate inside the female genital atrium, spermatozoa then separate and are stored freely inside the female spermathecae.

Vignoli et al. (2008) presented the first description of the sperm packages of the troglomorphic chactoid, *Belisarius xambeui* Simon, 1879, currently accommodated in Belisariidae Lourenço, 1998. The sperm of *B. xambeui* formed elongate, oval packages containing 150 spermatozoa and lacking a secretion sheath.

Michalik and Mercati (2010) subsequently described the sperm packages of *Opistophthalmus penrithorum* Lamoral, 1979, the first such example for the family Scorpionidae Latreille, 1802, and superfamily Scorpionoidea Latreille, 1802. The sperm packages of *O. penrithorum* resembled those of *B. xambeui* described by Vignoli et al. (2008) but contained 250 spermatozoa. Additionally, Michalik and Mercati (2010) reviewed and summarized the appearance, shape, sperm arrangement, and number of sperm packages, together with ultrastructural characters of the spermatozoa, in 15 species representing six families of scorpions. Building on the work of Peretti and Battán-Horenstein (2003), Michalik and Mercati (2010) discussed the evolution of sperm packages in scorpions, hypothesizing that more compact sperm packages may have been selectively advantageous.

Bent sperm packages were later described in *Euscorpius italicus* (Herbst, 1800), representing another chactoid family, Euscorpiidae Laurie 1896, by Althaus et al. (2010). Vrech et al. (2011) updated previous studies with the addition of more species, focusing on Bothriuridae. For the first time, Vrech et al. (2011) analyzed various measurements of individual sperm packages, investigated intraspecific variation, and discussed the utility of sperm packages as characters for scorpion systematics. Based in part on differences in sperm package morphology, Vrech et al. (2011) recommended elevating the subgenus *Andibothriurus* Maury, 1975, long recognized based on other characters (Mattoni, 2003; Peretti, 2010), to the rank of genus. Vrech et al. (2011, 2016) also described abnormalities in the shape of sperm packages and the number of spermatozoa inside male ejaculates.

The above-mentioned studies demonstrated that sperm packages are widely distributed across the order Scorpiones C.L. Koch, 1850 (Mattoni, 2003; Michalik and Mercati, 2010; Vrech et al., 2011), to date reported in 54 species, representing 32 genera, seven families, and three superfamilies of Iurida Soleglad and Fet, 2003: Belisariidae (Vignoli et al., 2008); Bothriuridae (Peretti and Battán-Horenstein, 2003; Vrech et al., 2011); Chactidae (Jespersen and Hartwick, 1973); Euscorpiidae (Althaus et al., 2010), Hadruridae (Jespersen and Hartwick, 1973); Scorpionidae (Michalik and Mercati, 2010); and Vaejovidae (Jespersen and Hartwick, 1973).

At present, sperm packages have not yet been reported in Buthidae (Cruz-Landim and Ferreira, 1973; Peretti and Battán-Horenstein, 2003; Vrech et al., 2011) or Chaerilidae Pocock, 1893 (Vrech et al., 2011), both assigned to the suborder Buthida Soleglad and Fet, 2003, an early branch of the order (Lamoral, 1980; Stockwell, 1989; Sissom, 1990; Prendini, 2000, 2003; Coddington et al., 2004; Prendini et al., 2006; Volschenk et al., 2008). Alberti (1983) suggested that the loosely aggregated spermatozoa of the buthid, *Buthus occitanus* (Amoreux, 1789), represent sperm bundles. The presence of sperm packages also remains unknown in Pseudochactidae Gromov, 1998, considered closely related to Chaerilidae, in superfamily Chaeriloidea Pocock, 1893 (Prendini et al., 2006, 2021). Data on sperm packages are also lacking for another 12 families, notably the basal nonbuthid family Iuridae Thorell, 1876, of superfamily Iuroidea Thorell, 1876; five chactoid families, Caraboctonidae Kraepelin, 1905, Scorpiopidae Kraepelin, 1905, Superstitioniidae Stahnke, 1940, Troglotayosicidae Lourenço, 1998, and Typhlochactidae Mitchell, 1971; and six scorpionoid families, Diplocentridae Karsch, 1880, Hemiscorpiidae Pocock, 1893, Heteroscorpionidae Kraepelin, 1905, Hormuridae Laurie, 1896, Rugodentidae Bastawade et al., 2005, and Urodacidae Kraepelin, 1905.

The precise function of sperm packages in scorpions is unclear. The aggregation of sperm may aid their protection during sperm transfer (Peretti and Battán-Horenstein, 2003; Peretti, 2010). Comparisons among exemplar species of Buthidae and Bothriuridae revealed that male buthids, with bundles of loosely aggregated spermatozoa, inseminate females rapidly, usually within a few seconds, whereas insemination takes longer in bothriurids, which possess sperm packages. Furthermore, compact sperm may be delivered more easily and in greater quantity, without substantial loss (Peretti, 2010; Vrech et al., 2011). Althaus et al. (2010) demonstrated that sperm packages play a role in forming a temporary sperm plug in the female genital opening, after sperm transfer.

Despite an accumulation of observations and hypotheses concerning the sperm packages of scorpions in recent decades, knowledge of their diversity, function, and evolution remains limited, in part due to an absence of data for many scorpion taxa (Vignoli et al., 2008; Vrech et al., 2011). The present study provides the first systematic survey of sperm package morphology across the order, covering 89 exemplar species in 66 genera and 19 families representing all suprafamilial ranks, with a more detailed investigation of the family Bothriuridae, in which the greatest diversity of sperm packages is observed (Vrech et al., 2011). The general morphology of sperm packages is described and illustrated, sperm package length is measured and analyzed statistically, and sperm counts are presented for four exemplar species of Bothriuridae. Comparison of the diversity in sperm package morphology across the order provides new insights into the evolution of sperm packages in scorpions, and their potential utility as characters for scorpion systematics.

Material and Methods

Taxon Sampling and Material Examined: Adult males of 89 exemplar species, representing 66 genera and 19 families, were examined (table 1). Buthida were represented by 17 species in 16 genera and three families, representing both superfamilies Buthoidea C.L. Koch, 1837, and Chaeriloidea. Iurida were represented by 72 species in 50 genera and 16 families, representing all superfamilies (Bothriuroidea Simon, 1880; Chactoidea Pocock, 1893; Iuroidea; Scorpionoidea). Three families were omitted: Belisariidae, for which the sperm packages of *B. xambeui* were described by Vignoli et al. (2008); Microcharmidae Lourenço, 1996, synonymized with Buthidae by Volschenk et al. (2008) and considered a junior synonym thereof, in the present investigation; and Rugodentidae, for which material was unavailable for study.

The greatest sample of genera (13) and species (33) was examined in Bothriuridae (table 1), and *Bothriurus* Peters, 1861, in particular (16 species), on account of the diversity of sperm package morphology in these taxa (Mattoni, 2003; Ojanguren-Affilastro, 2005; Vrech et al., 2011).

Sperm packages were dissected from adult males deposited in the following collections (appendix 1): AMNH, American Museum of Natural History, New York; AVP, Alfredo V. Peretti Private Collection, Universidad Nacional de Córdoba, Argentina; CAS, California Academy of Sciences, San Francisco; CIM, Camilo I. Mattoni Private Collection, Universidad Nacional de Córdoba, Argentina; DEV, David E. Vrech Private Collection, Universidad Nacional de Córdoba, Argentina; IBSP, Instituto Butantan, São Paulo, Brazil; MACN, Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Buenos Aires, Argentina; MCZ, Museum of Comparative Zoology, Harvard University, Cambridge, MA; MPUJ, Museo Javeriano de Historia Natural "Lorenzo Uribe S.J.," Pontificia Universidad Javeriana, Bogotá, Colombia; MZUSP, Museu de Zoología da Universidade de São Paulo, Brazil; MZUC, Museo de Zoología, Universidad de Concepción, Chile; UFBA, Universidade Federal da Bahía, Salvador da Bahía, Brazil; ZMB, Museum für Naturkunde, Berlin, Germany.

The scarcity of material prevented the examination of more than one male for most taxa. However, intraspecific variation in sperm package morphology was assessed in a few species by observations within one or more individuals from the same or different populations. Polymorphism in sperm packages from a single individual was investigated by Vrech et al. (2016).

DISSECTION, MICROSCOPY, AND IMAGING: Incisions were made in the pleural membrane of each specimen, and the sinistral paraxial organ removed using fine forceps. Sperm packages were extracted for light microscopy by dissecting the seminal vesicle over a microscope slide. Twenty-five exemplar species were examined with scanning electron microscopy (SEM) and the rest with light microscopy.

Seminal vesicles containing sperm packages used for SEM were dehydrated in progressively increasing concentrations of ethanol, from 70% to 100%. Two desiccation techniques, critical-point drying (CPD) and Hexamethyldisilazane (HMDS) (Nation, 1983), were investigated and neither was found to affect the morphology of the sperm packages (Bray et al., 1993). Desiccated samples were mounted and coated with gold (Au) and palladium (Pd) in a DENTON Desk II Sputter Coater (Moorestown, NJ). Samples were then analyzed using a Hitachi S-4700 Field Emission SEM (Tokyo, Japan). Sample processing and imaging was conducted at the AMNH Microscopy and Imaging Facility and the Laboratorio de Análisis de Materiales por Espectometría de Rayos X (LAMARX-FaMAF), Universidad Nacional de Córdoba (UNC), Argentina.

A sample of sperm packages was mixed in liquid glycerin and scattered in an Improved Neubauer counting chamber (Boeco, Wuppertal, Germany) to enable posterior measurements using the chamber's grid. Digital images were taken using a Nikon DIGITAL SIGHT DS-fi1 camera (Tokyo, Japan) attached to a Nikon *eclipse*50i phase contrast light microscope. Exposure to ethanol and glycerin did not affect the sperm packages, which maintained their integrity after a year in glycerin. Sperm packages were found to be generally well preserved by standard methods of scorpion specimen preservation, allowing their extraction and examination from museum material.

TABLE 1. Count of exemplar species representing major groups of scorpions in which sperm packages were examined.

Suborder	Superfamily	Family	Genera	Species
Buthida Soleglad and Fet, 2003	Buthoidea C.L. Koch, 1837	Buthidae C.L. Koch, 1837	14	14
	Chaeriloidea Pocock, 1893	Chaerilidae Pocock, 1893	1	2
		Pseudochactidae Gromov, 1998	1	1
Iurida Soleglad and Fet, 2003	Bothriuroidea Simon, 1880	Bothriuridae Simon, 1880	13	33
	Chactoidea Pocock, 1893	Caraboctonidae Kraepelin, 1905	2	2
		Chactidae Pocock, 1893	7	7
		Euscorpiidae Laurie, 1896	2	3
		Scorpiopidae Kraepelin, 1905	3	3
		Superstitioniidae Stahnke, 1940	1	1
		Troglotayosicidae Lourenço, 1998	1	1
		Typhlochactidae Mitchell, 1971	2	2
		Vaejovidae Thorell, 1876	7	7
	Iuroidea Thorell, 1879	Iuridae Thorell, 1876	1	1
	Scorpionoidea Latreille, 1802	Diplocentridae Karsch, 1880	2	2
		Hemiscorpiidae Pocock, 1893	1	1
		Heteroscorpionidae Kraepelin, 1905	1	1
		Hormuridae Laurie, 1896	4	4
		Scorpionidae Latreille, 1802	2	2
		Urodacidae Pocock, 1893	1	2
Total		19	66	89

Analysis of Shape and Length: The diversity in shape of sperm packages was assessed from images obtained with light microscopy and SEM. The structure of the sperm packages observed in each family was compared and described, allowing general patterns to be observed and different types of sperm packages to be identified across the order. Type describes the general pattern. Shape describes the form observed, for example in sperm packages with many folds, the shape may be a slender bar or an ellipsoid. The term spherical was applied if the ellipsoid resembled a sphere. Where possible, patterns of sperm package folding were also described. Sometimes, folding was obscured by secretions. Folding terminology was adopted from the brochure and leaflet industry (ALPHA2OMEGA, n.d.; APS, 2022). Images that exemplified the major types were presented. Descriptions of sperm packages from species of the family Bothriuridae follow Vrech et al. (2011).

When comparing sperm packages, the main type is described first, followed by variation in shape, and finally, when possible, the manner in which the sperm was folded within (type, shape, and folding; table 2). For example, a sperm package that is folded multiple times may present an ellipsoidal shape, and the sperm within presenting an open gatefold folding, in which the tips of the sperm package arms bend inward.

Where possible, the length and area of the sperm packages were calculated from the images using Image J 64-bit image-processing software (Schneider et al., 2012). Length was assessed to be a more precise measurement than area, as it was less affected by the state of preservation of the material. Length was measured with the straight tool, along the midline of the sperm package from the anterior part, which contains the nuclei of the spermatozoa, to the posterior part, which contains the flagella of the spermatozoa.

When the sperm package was not entirely straight or presented folds, the number of folds were counted, and the length estimated by multiplying the length measured by the number of branches produced by the folds (fig. 1A, C). For example, if a bend in the middle of the sperm package produced a single fold, the length measured was multiplied by two because a single fold produces two branches (fig. 1A). When two folds were observed, as in round sperm packages, the length measured was multiplied by four. Length was not estimated in sperm packages with other types of folds, which were difficult to quantify.

A one-way ANOVA with a Tukey post hoc test (α = 5%) was performed to compare length among sperm packages with a single fold, the most common type among the taxa examined.

Counts of Spermatozoa in Bothriuridae: Four species of Bothriuridae, *Bothriurus bonariensis* (C.L. Koch, 1842), *Brachistosternus ferrugineus* (Thorell, 1876), *Timogenes elegans* (Mello-Leitão, 1931), and *Urophonius brachycentrus* (Thorell, 1876), were investigated in more detail. The reproductive biology of these species has been extensively studied (Vrech, 2013; Vrech et al., 2014, 2018, 2019; Romero-Lebrón et al., 2019; Oviedo-Diego et al., 2019, 2020; Olivero et al., 2015, 2019, 2021) and fresh material was more readily available compared to other taxa.

One seminal vesicle of each species was fixed in 4% formaldehyde 2% glutaraldehyde in cacodylate buffer (pH 7.3, 0.1 M) for 2 hours, and then transferred to 1% osmium tetroxide in the same buffer. After dehydration in progressively increasing concentrations of acetone, samples were embedded in Araldite and polymerized at 60° C. Transverse thin sections of sperm packages were prepared with a diamond knife on a JEOL JUM-7 ultramicrotome (Tokyo, Japan) and imaged with a Zeiss LEO 906E (Jena, Germany) transmission electron microscope (TEM). Spermatozoa were counted from the TEM images (n = 15 sperm packages per species). For consistency, spermatozoa were counted from sperm packages only in the medial zone of the image, as spermatozoa at the periphery could be slightly out of phase. The number of mitochondria and the form of the axoneme were also examined. Sample processing and imaging was conducted in the Centro de Microscopía Electrónica, Facultad de Ciencias Médicas, UNC.

RESULTS

Major Types of Sperm Packages in Scorpions: Sperm packages were present in 17 (89%) of the 19 families studied (table 2). Except for three exemplar species of Buthidae, all Buthida lacked sperm conjugation, possessing free, disordered spermatozoa.

The sperm packages of Iurida were highly variable. Three major types were recognized (figs. 1A–F): straight sperm packages; single-folded sperm packages; and multiple-folded sperm

TABLE 2. Presence and qualitative differences in structure of sperm packages among exemplar species representing major groups of scorpions. Sources: [1] Jespersen and Hartwick (1973), refers to “bundles”; [2] Alberti (1983), refers to “sperm bundles”; [3] Peretti and Battán-Horenstein (2003); [4] Vignoli et al. (2008); [5] Michalik and Mercati (2010); [6] Althaus et al. (2010); [7] Vrech et al. (2011); [8] this study. Mt = mitochondria.

Classification	Species	Type	Shape	Folding	Sheath	Mt	Axoneme	Source
Buthida								
Buthoidea								
Buthidae	*Ananteris arcadioi*	absent[1]	(block)					[8]
	Babycurus jacksoni	absent	free/loosely aggregated sperm					[8]
	Buthus occitanus	absent	free/loosely aggregated sperm			2	9 + 2	[2]
	Buthus paris	absent	free/loosely aggregated sperm			2		[8]
	Hottentotta conspersus	absent	free/loosely aggregated sperm					[8]
	Isometrus maculatus	absent	free/loosely aggregated sperm					[8]
	Lychas obsti	absent	free/loosely aggregated sperm					[8]
	Mesobuthus eupeus thersites	absent	free/loosely aggregated sperm					[8]
	Microtityus waeringi	absent	free/loosely aggregated sperm					[8]
	Parabuthus granulatus	fusiform[1]	(fusiform)					[8]
	Pseudolychas ochraceus	folded[1]	(folded)					[8]
	Reddyanus assamensis	absent	free/loosely aggregated sperm					[8]
	Teruelius ankarana	absent	free/loosely aggregated sperm					[8]
	Tityus elii	absent	free/loosely aggregated sperm					[8]
	Zabius fuscus	absent	free/loosely aggregated sperm					[3, 7]
Chaeriloidea								
Chaerilidae	*Chaerilus julietteae*	absent	free/loosely aggregated sperm					[8]
	Chaerilus variegatus	absent	free/loosely aggregated sperm					[6]
Pseudochactidae	*Troglokhammouanus steineri*	absent (?)[2]	N/A					[8]

TABLE 2 *continued*

Classification	Species	Type	Shape	Folding	Sheath	Mt	Axoneme	Source
Iurida								
Bothriuroidea								
Bothriuridae	*Bothriurus araguayae*	straight	straight		absent			[7, 8]
	Bothriurus asper	straight/single folded	straight/bent	half fold	absent			[7, 8]
	Bothriurus bocki	single folded/straight	bent/straight	half fold	absent			[7, 8]
	Bothriurus bonariensis	straight	sraight		absent		9 + 0	[3, 7, 8]
	Bothriurus burmeisteri	straight	canelike		absent			[7, 8]
	Bothriurus chacoensis	straight	Straight		absent			[3, 7, 8]
	Bothriurus cordubensis	straight	canelike		absent			[3, 7, 8]
	Bothriurus coriaceus	straight	canelike		absent			[7, 8]
	Bothriurus flavidus	straight	canelike		absent			[3, 7, 8]
	Bothriurus inermis	straight	canelike		absent			[7, 8]
	Bothriurus keyserlingi	straight	canelike		absent			[7, 8]
	Bothriurus noa	straight	canelike		absent			[7, 8]
	Bothriurus olaen	straight	canelike		absent			[7, 8]
	Bothriurus rochai	straight	canelike		absent			[7, 8]
	Bothriurus rochensis	straight	canelike		absent			[7, 8]
	Bothriurus sp.	straight	straight		absent			[7, 8]
	Brachistosternus angustimanus	straight	canelike		absent			[7, 8]
	Brachistosternus ferrugineus	straight	canelike		absent	3	9 + 0	[3, 7, 8]
	Brachistosternus pentheri	straight	canelike		absent			[7, 8]
	Centromachetes obscurus	straight	canelike		absent			[7, 8]
	Centromachetes pocockii	straight	canelike		absent			[7, 8]
	Cercophonius squama	single folded/ straight canelike	bent/straight	half fold	absent			[7, 8]
	Lisposoma josehermana	straight	straight		absent			[7, 8]
	Orobothriurus tamarugal	straight	straight		absent			[7, 8]
	Phoniocercus pictus	straight	canelike		absent			[7, 8]
	Rumikiru lourencoi	straight	straight		absent			[7, 8]
	Tehuankea moyanoi	straight	canelike		absent			[7, 8]

TABLE 2 *continued*

Classification	Species	Type	Shape	Folding	Sheath	Mt	Axoneme	Source
Iurida								
Bothriuroidea								
Bothriuridae	*Thestylus aurantiurus*	straight	straight		absent			[7, 8]
	Timogenes dorbignyi	single folded	bent	half fold	absent			[3, 7, 8]
	Timogenes elegans	single folded	bent	half fold	absent	3	9 + 0	[3, 7, 8]
	Urophonius brachycentrus	straight	canelike		absent	3	9 + 0	[3, 7, 8]
	Urophonius tregualemuensis	straight	canelike		absent			[7, 8]
	Vachonia martinezi	single folded	bent	half fold	absent			[7, 8]
Chactoidea								
Belisariidae	*Belisarius xambeui*	single folded	bent	half fold	absent	3, 4, 6	9 + 0	[4]
Caraboctonidae	*Caraboctonus keyserlingi*	straight	straight		absent			[7, 8]
	Hadruroides lunatus	straight	straight		absent			[8]
Chactidae	*Anuroctonus phaiodactylus*	single folded	bent	half fold	absent	4, 7	9 + 0	[1, 5]
	Brotheas sp.	multiple folded	ellipsoidal/double bent	open/closed gatefold	present			[8]
	Broteochactas nitidus	multiple folded	double bent	open gatefold	present			[8]
	Chactas aequinoctialis	multiple folded	ellipsoidal/spherical	open gatefold/ roll fold?	present			[7, 8]
	Chactopsoides anduzei	multiple folded	ellipsoidal	open gatefold?	present			[8]
	Nullibrotheas allenii	multiple folded	ellipsoidal	open gatefold?	present			[8]
	Teuthraustes sp.	multiple folded	spherical/ellipsoidal	open/closed gatefold?	present			[8]
	Uroctonus mordax	single folded	bent	half fold	absent	4, 5, 6, 7	9 + 0	[1, 5, 8]

TABLE 2 *continued*

Classification	Species	Type	Shape	Folding	Sheath	Mt	Axoneme	Source
Iurida								
Chactoidea								
Euscorpiidae	*Megacormus gertschi*	multiple folded	spherical/ellipsoidal	closed gatefold/ roll fold	present			[7, 8]
	Megacormus sp.	ellipsoidal	spherical/ellipsoidal	closed gatefold/ roll fold	present			[7, 8]
	Euscorpius italicus	single folded	bent	half fold	present			[6]
	Euscorpius sicanus	single folded	bent	half fold	present	6, 7, 8	9 + 0	[5]
	Tetratrichobothrius flavicaudis	multiple folded	ellipsoidal/bent	open gatefold/ annular twisted/closed gatefold	present			[7, 8]
Hadruridae	*Hadrurus arizonensis*	straight?	straight?		absent	5	9 + 0	[1, 5]
Scorpiopidae	*Euscorpiops longimanus*	multiple folded	ellipsoidal	roll fold	present			[8]
	Scorpiops zubairi	single folded	bent	half fold	absent			[8]
	Troglocormus ciego	multiple folded	ellipsoidal	roll fold	absent			[8]
Superstitioniidae	*Superstitionia donensis*	straight	straight		absent			[8]
Troglotayosicidae	*Troglotayosicus humiculum*	single folded	bent	half fold	absent			[8]
Typhlochactidae	*Alacran tartarus*	single folded	bent	half fold	absent			[8]
	Typhlochactas mitchelli	single folded	bent	half fold	absent			[8]
Vaejovidae	*Graemeloweus glimmei*	single folded	bent	half fold	absent			[8]
	Mesomexovis variegatus	single folded	bent	half fold	absent			[8]
	Paravaejovis puritanus	single folded	bent	half fold	absent	4, 5, 6, 7	9 + 0	[1, 5]
	Paravaejovis spinigerus	single folded	bent	half fold	absent			[5, 8]
	Smeringurus grandis	single folded	bent	half fold	absent			[8]
	Syntropis williamsi	single folded	bent	half fold	absent			[8]
	Vaejovis mexicanus	single folded	bent	half fold	absent			[8]
	Vejovoidus longiunguis	single folded	bent	half fold	absent			[8]
Iuroidea								
Iuridae	*Protoiurus asiaticus*	straight	straight		absent			[8]

TABLE 2 *continued*

Classification	Species	Type	Shape	Folding	Sheath	Mt	Axoneme	Source
Iurida								
Scorpionoidea								
Diplocentridae	*Diplocentrus lindo*	multiple/single folded	ellipsoidal/bent	roll fold?/half fold	absent			[8]
	Nebo hierichonticus	single folded	bent	half fold	absent			[8]
Hemiscorpiidae	*Hemiscorpius lepturus*	single folded	bent	half fold	absent			[8]
Heteroscorpionidae	*Heteroscorpion goodmani*	single/multiple folded	bent/double bent	half fold/open gatefold	present			[8]
Hormuridae	*Hadogenes troglodytes*	multiple folded	annular/ellipsoidal	annular/roll fold	absent			[8]
	Hormurus sp.	multiple folded	annular/annular twisted/double bent	annular twisted	absent			[7, 8]
	Opisthacanthus capensis	multiple folded	ellipsoidal	roll fold	absent			[7, 8]
	Opisthacanthus valerioi	multiple folded	ellipsoidal	roll fold	absent			[7, 8]
Scorpionidae	*Opistophthalmus penrithorum*	single folded	bent	half fold	absent	5, 6	9 + 0	[5]
	Pandinus imperator	single folded/straight	bent/canelike	half fold	absent			[7, 8]
	Scorpio fuliginosus	multiple folded	ellipsoidal/spherical	double parallel fold/roll fold	absent			[7, 8]
Urodacidae	*Urodacus butleri*	multiple folded	ellipsoidal/spherical	double parallel fold/roll fold	present			[8]
	Urodacus planimanus	multiple folded	ellipsoidal/spherical	double parallel fold/roll fold	present			[8]

[1] May exhibit some form of sperm conjugation, see text.

[2] Not analyzed.

packages. The third category included sperm packages with annular, double-folded, or ellipsoidal shapes (fig. 1C, D). In many sperm packages, the folding was difficult to describe either because of its intricate configuration or because a sheath obscured the coiling of the sperm inside. A sheath was observed in Chactidae, Euscorpiidae, Heteroscorpionidae, Scorpiopidae, and Urodacidae but appears to be uncommon in the order.

Sperm packages folded once only were termed bent sperm packages. Bent sperm packages presented limited variation except, occasionally, in the symmetry of their arms. Variation was common among straight sperm packages and sperm packages with multiple folds, however. Straight sperm packages presented different widths (e.g., some bothriurids) and angles in the anterior part (fig. 1F). Among the sperm packages with multiple folds, some sperm packages were folded medially, resembling bent sperm packages, but were also folded in one or both tips of their arms (fig. 1C, bottom). These were termed double-bent sperm packages as they were longer than ellipsoidal sperm packages and differed from bent sperm packages.

The bent sperm package, characterized by a single fold that bends the package medially (fig. 1A, E) was the most abundant type, observed in 11 (58%) of the families and 35% of the exemplar species. Although observed in 38% of the exemplar species, straight sperm packages (fig. 1B, E, F) were concentrated in four (21%) families (Bothriuridae, Caraboctonidae, Iuridae, and Superstitioniidae). Sperm packages that folded multiple times, including different shapes such as annular, ellipsoidal, and double-bent sperm packages (fig. 1C, D), were observed in seven (37%) of the families and 21% of the exemplar species. In some cases, different shapes occurred in the same taxon. For example, annular sperm packages were commonly associated with bent sperm packages (fig. 1D), and sometimes also with straight sperm packages in a sample extracted from a single male of *Timogenes elegans*. Similarly, straight sperm packages were sometimes present together with bent sperm packages (fig. 1E; table 2). There was great variation in sperm package morphology within the family Bothriuridae, representing all three main categories of sperm packages (single folded, straight, and multiple folded; fig. 1D–F).

In general, the spermatozoa forming sperm packages were usually twisted along the entire length of the sperm package, resembling the inside of a copper-wire cable, an arrangement which appears to provide structural cohesion to the sperm package.

Comparison of Sperm Packages among Scorpion Families: Sperm packages were examined in 14 exemplar species of Buthidae. Eleven (79%) species exhibited free spermatozoa with no apparent aggregation inside the seminal vesicle (figs. 1G–L, 2A, B). There were three exceptions to this general pattern, however (fig. 2C–E). *Parabuthus granulatus* (Ehrenberg, 1831) possessed loosely aggregated sperm packages, approximately 150–200 μm in length, with a fusiform shape, resembling the sperm packages of Iurida (fig. 2C). Spermatozoa in these arrangements were aligned, but not densely packed as in other families (see below). Similarly, *Pseudolychas ochraceus* (Hirst, 1911) exhibited sperm arrangements of 100 μm length (fig. 2D) ellipsoidal in shape, but it is unclear whether this is comparable to the ellipsoidal sperm packages of Iurida. Finally, *Ananteris arcadioi* Botero-Trujillo, 2008, exhibited large sperm masses resembling blocks of spermatozoa (fig. 2E).

Chaerilidae exhibited the same general organization of free, irregularly dispersed spermatozoa observed in most buthid taxa. Both exemplar species, *Chaerilus julietteae* Lourenço, 2011, and *Chaerilus variegatus* Simon, 1877, lacked identifiable sperm packages, the spermatozoa instead forming a loose, disordered mass inside the seminal vesicle (fig. 2F).

Sperm packages also appear to be absent in Pseudochactidae, but this could not be definitively confirmed in the material examined of the exemplar species, *Troglokhammouanus steineri* Lourenço, 2007.

Protoiurus asiaticus (Birula, 1903), the sole exemplar species of Iuridae, possessed straight, slender sperm packages that may be curved anteriorly in the first third of the sperm package, in the area where the nuclei of the spermatozoa are clustered (fig. 2G). This anterior part is curved and appears aligned and attached to the rest of the sperm package and twisted around it.

The sperm packages of Euscorpiidae and the related Scorpiopidae are folded multiple times and highly variable in shape (fig. 2H–L). This variation is complex due to the number and position of folds along the body of the sperm package, resembling a straight sperm package bent at the extremes of its length like an office staple (double bent), and termed open gatefolding. This kind of folding may resemble single folded sperm packages (fig. 2H, lower left corner). The sperm package may exhibit an annular twisted or lemniscate shape, resembling an infinity sign, ∞ (fig. 2H, lower right corner). This twisted ring may bend again medially, becoming smaller, more compact, and rounded, resembling a sphere or ellipsoid (fig. 1C, 2I). This folding is termed a double parallel fold.

The euscorpiid, *Tetratrichobothrius flavicaudis* (DeGeer, 1778), presents ellipsoidal sperm packages with various types of folding, e.g., an open gatefold, double parallel fold, roll fold (similar to a spiral coiling), and annular twisting (figs. 1C, 2H). Sometimes, this species presents sperm packages like the typical folded packages of Bothriuridae and some Vaejovidae (see below), with a long slender ellipsoidal appearance (fig. 2H, lower left corner), however, these are not folded once only as in the latter families. The sperm package of *T. flavicaudis* may also exhibit a second fold in one of the two branches (fig. 1C: lower sperm package, denoted by the number 1).

A more compact type of folding is observed in *Megacormus gertschi* Diaz Nájera, 1966, and *Megacormus* sp., and also appears in *T. flavicaudis* (fig. 1C: upper sperm package). *Megacormus* Karsch, 1881, possess ellipsoidal, almost spherical sperm packages in which the spermatozoa appear to be folded many times, the single fold packages arcing again medially (double parallel fold), or folding spirally, to produce a short, roundish sperm package (fig. 2I). This spherical shape and compactness resembles that of the chactid, *Nullibrotheas allenii* (Wood, 1863) (see below). A covering or sheath over the spermatozoa was not observed in *Megacormus*, although the sperm packages of this genus resemble those of other taxa, in which a sheath is present (table 2), e.g., Chactidae and the euscorpiids *Euscorpius italicus* and *Euscorpius sicanus* (C.L. Koch, 1837).

Slightly ellipsoidal sperm packages with multiple folds are evident in the scorpiopid, *Troglocormus ciego* Francke, 1981 (fig. 2J). The sperm packages of *T. ciego* are very similar to those of *Megacormus*, similarly lacking evidence of a sheath covering the spermatozoa. *Scorpiops zubairi* Kovařík, 2020, possessed a single-folded sperm package, bent medially (fig. 2K). The ellipsoidal sperm packages of *Euscorpiops longimanus* (Pocock, 1893) were wrinkled anteriorly (fig. 2L), the entire sperm conjugation coiled spirally. Unlike other exemplar species of Scorpiopidae, a sheath covered the spermatozoa of the entire sperm package in *E. longimanus* (fig. 2L). This character was also observed in Chactidae, Euscorpiidae, and Urodacidae (see below), but appears to be uncommon in the order.

The caraboctonid, *Caraboctonus keyserlingi* Pocock, 1893, possessed broad, thick, straight sperm packages (fig. 3A). The anterior part of the sperm package, approximately one-third of its length, appeared to be folded toward the posterior part, creating a rounded appearance. This part was also attached to the main body of the package and twisted around it, resembling the sperm package of the iurid, *P. asiaticus* (fig. 2G). Sperm packages of another caraboctonid, *Hadruroides lunatus* (L. Koch, 1867) (fig. 3B), were straight and slender, also resembling those of *P. asiaticus*. Similar slender sperm packages were also observed in *C. keyserlingi*, but in far lower numbers.

Superstitionia donensis Stahnke, 1940, the only species of Superstitioniidae, exhibited straight sperm packages (fig. 3C), differing from those observed in Iuridae and all exemplar species of Bothriuridae, except *Lisposoma josehermana* Lamoral, 1979, in widening anteriorly where the nuclei of the spermatozoa are gathered, and narrowing posteriorly where the flagella of the spermatozoa occur.

Although the sperm was poorly preserved in the exemplar species of Typhlochactidae, *Alacran tartarus* Francke, 1982, and *Typhlochactas mitchelli* Sissom, 1988, and no further material was available, the images suggest the sperm packages are folded in these taxa.

Species of Chactidae possess sperm packages that are folded many times. The general shapes range from ellipsoidal to almost spherical (fig. 3D–K). *Nullibrotheas allenii* possessed an almost spherical and ellipsoidal sperm package (fig. 3D, E) that appeared to be folded more than once and also twisted (fig. 3E), but it was difficult to determine the folding pattern. A thin sheath appeared to cover the entire sperm package of *N. allenii*, but the undulations of individual spermatozoa could be identified (fig. 3E). The multiple-folded sperm package of *Brotheas* sp. exhibited different shapes in the same sample, including ellipsoidal (fig. 3F), double-bent (fig. 3G), and almost spherical sperm packages with a spiral or double gatefold; a clear smooth sheath was also noticeable (fig. 3F, G). The sperm packages of *Broteochactas nitidus* Pocock, 1893, also appeared to be folded multiple times (fig. 3H) and were similar in shape to the double-bent sperm package with open gatefold of *Brotheas* sp. (fig. 3G). The sperm packages of *Chactopsoides anduzei* (González-Sponga, 1982) resembled those of *B. nitidus*, exhibiting a double-bent shape with patterns of lines medially (fig. 3I). The unusual shape of the sperm package of this species prevented assessment of how the sperm folds inside, but it is assumed to be an open gatefolding, similar to *B. nitidus*. The covering sheath of these latter species was difficult to identify under light microscopy but is assumed to be present (fig. 3G). The sperm packages of *Chactas aequinoctialis* (Karsch, 1879) were ellipsoidal to almost spherical (fig. 3J), sometimes resembling the spherical sperm packages of *Brotheas* sp. and the euscorpiid, *Megacormus* (fig. 2I). As in other chactids, C. *aequinoctialis* presented a secretion sheath. An ellipsoidal sperm package was also observed in *Teuthraustes* sp., but its shape and size were more variable than in *C. aequinoctialis*, and a sheath covered the entire sperm package (fig. 3K).

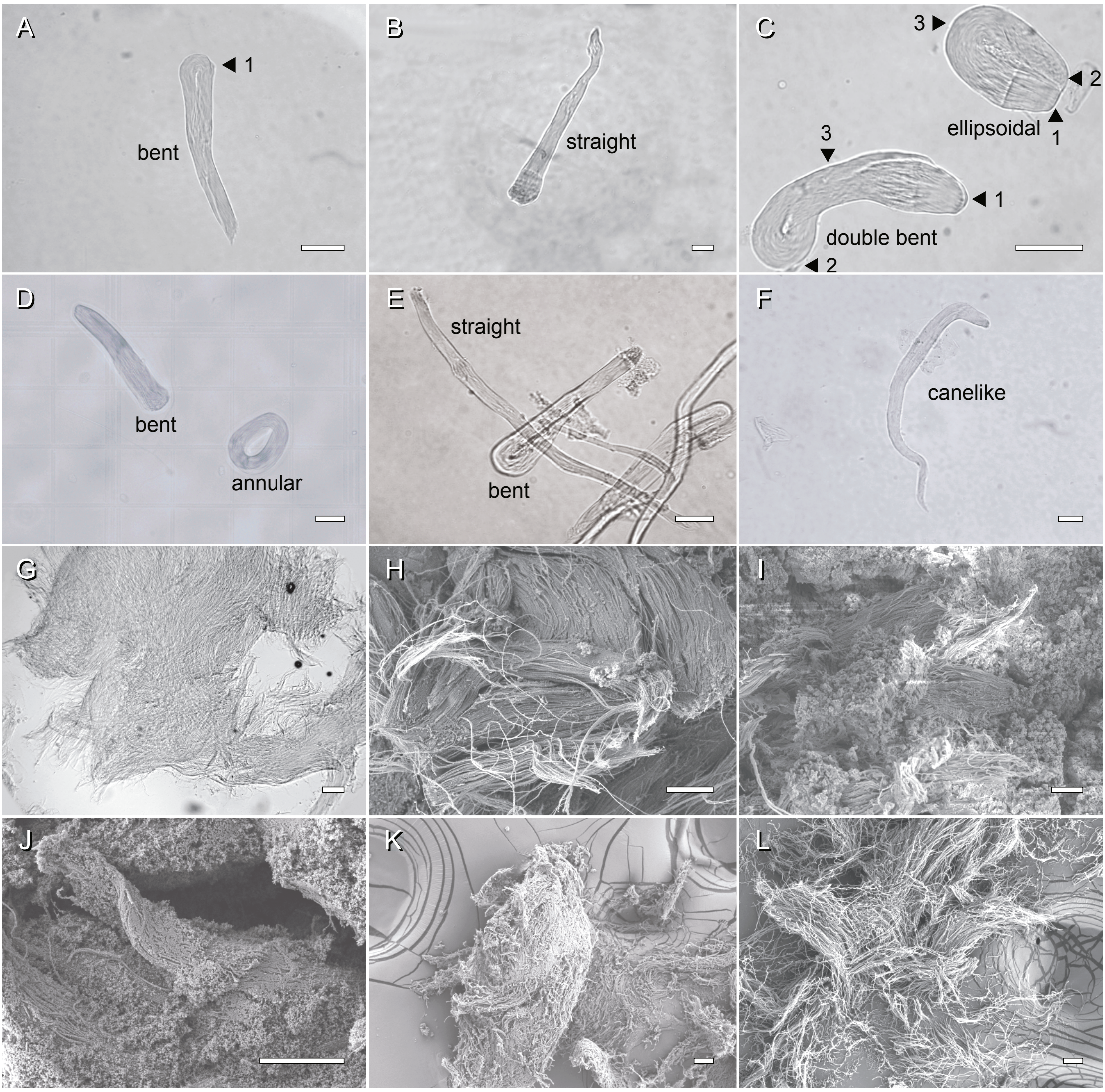

FIGURE 1. Sperm packages of Bothriuridae Simon, 1880 (**A, D, E, F**), Superstitioniidae Stahnke, 1940 (**B**), Euscorpiidae Pocock, 1893 (**C**), and Buthidae C.L. Koch, 1837 (**G–L**), imaged with light microscopy (**A–F**) or scanning electron microscopy (**G–L**). **A, D, E.** *Timogenes elegans* (Mello-Leitão, 1931): bent, annular, straight. **B.** *Superstitionia donensis* Stahnke, 1940: straight. **C.** *Tetratrichobothrius flavicaudis* (De Geer, 1778): double bent/ellipsoidal; numbers represent folding that causes differences in shape. **F.** *Brachistosternus ferrugineus* (Thorell, 1876): straight. **G.** *Zabius fuscus* (Thorell, 1876): absent. **H.** *Buthus paris* (C.L. Koch, 1839): absent. **I.** *Teruelius ankarana* (Lourenço and Goodman, 2003): absent. J. *Hottentotta conspersus* (Thorell, 1876): absent. **K.** *Babycurus jacksoni* (Pocock, 1890): absent. **L.** *Lychas obsti* Kraepelin, 1913: absent. Arrows indicate folding of sperm packages described in text, including variations in conspecifics. Scale bars: 25 μm.

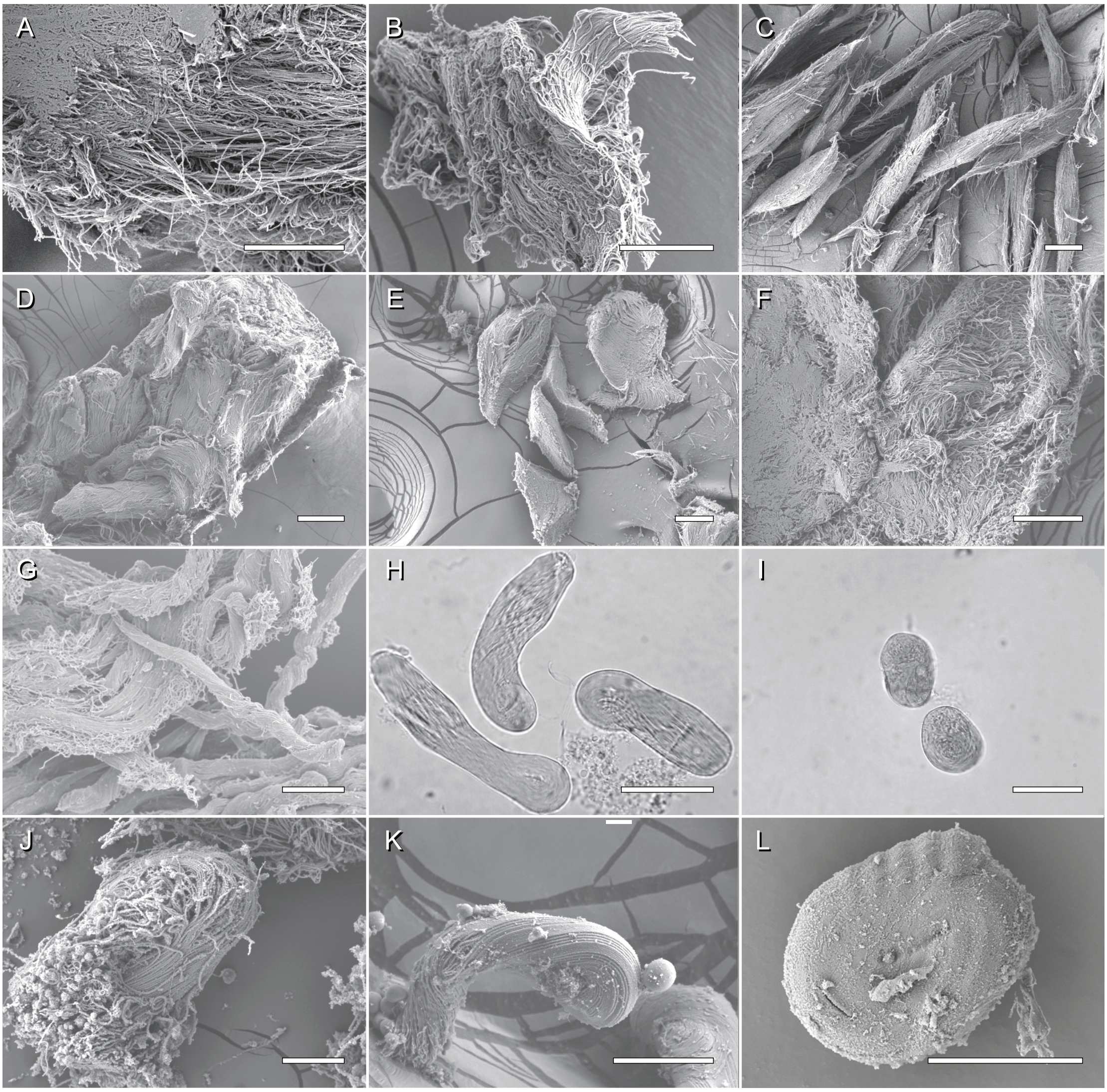

FIGURE 2. Sperm packages of Buthidae C.L. Koch, 1837 (**A–E**), Chaerilidae Pocock, 1893 (**F**), Iuridae Thorell, 1876 (**G**), Euscorpiidae Laurie, 1896 (**H, I**), and Scorpiopidae Kraepelin, 1905 (**J–L**), imaged with scanning electron microscopy (**A–G, J–L**) or light microscopy (**H, I**). **A.** *Tityus elii* Armas and Marcano Fondeur, 1992: absent. **B.** *Isometrus maculatus* (DeGeer, 1778): folded. **C.** *Parabuthus granulatus* (Ehrenberg, 1831): fusiform. **D.** *Pseudolychas ochraceus* (Hirst, 1911): folded. **E.** *Ananteris arcadioi* Botero-Trujillo, 2008: block. **F.** *Chaerilus julietteae* Lourenço, 2011: absent. **G.** *Protoiurus asiaticus* (Birula, 1903): straight. **H.** *Tetratrichobothrius flavicaudis* (De Geer, 1778): double bent. **I.** *Megacormus* sp., Veracruz, Mexico: spherical. **J.** *Troglocormus ciego* Francke, 1981: spherical. **K.** *Scorpiops zubairi* Kovařík, 2020: bent. **L.** *Euscorpiops longimanus* (Pocock, 1893): spiral. Scale bars: 25 μm.

Scanning electron micrographs of *Brotheas* sp., *N. allenii*, and *Teuthraustes* sp. revealed the presence of a sheath that covered the spermatozoa, providing a smooth appearance to the sperm package (fig. 3F, G, K). The different zones of the sperm package (head, middle zone, and flagellar zone) were difficult to differentiate, however, due to the bending and sheath (when present). The covering sheath appeared to be transparent under light microscopy, revealing the sperm inside. A similar pattern was evident in Euscorpiidae (see above).

The sperm obtained from the exemplar species of Troglotayosicidae, *Troglotayosicus humiculum* Botero-Trujillo and Francke, 2009, was also in poor state of preservation. The images available and previous literature suggest this species possesses folded sperm packages (fig. 3L).

All exemplar species of Vaejovidae exhibited bent single folded sperm packages (fig. 4A–F), lacking a covering sheath and with few other differences (any differences observed appear to be related to the state of preservation). The sperm packages of vaejovids were the typical folded type, bent medially but also twisted along the length of the branches delimited by the anterior and posterior ends of the package (e.g., fig. 4D, E).

Four subtypes of sperm package were observed in Bothriuridae: typical bent (single folded) (fig. 1A, D, E); among straight sperm packages there were straight (fig. 1E) and canelike shapes (fig. 1F); multiple-folded sperm packages were represented by annular or ringlike shapes (fig. 1D). The canelike shape (fig. 1F) resembled the straight shape (fig. 1B) but with its anterior part bent 45°–90°. This was the most common type, observed in 35% of the exemplar species analyzed. Bent sperm packages were observed in 26% of the exemplar species, i.e., both species of *Timogenes* Simon, 1880, *Vachonia martinezi* Abalos, 1954, and some species of the nominal subgenus of *Bothriurus*. Annular sperm packages were also observed in the same taxa, but appeared to be more common in some species, e.g., *Bothriurus inermis* Maury, 1981, than others.

Heteroscorpion goodmani Lourenço, 1996, the sole exemplar species of Heteroscorpionidae, possessed typical bent sperm packages. A few double-bent sperm packages were also observed in this species and appeared to be covered by a thin sheath (fig. 4G, H), as in the chactid, *N. allenii* (fig. 3E, F).

Hemiscorpius lepturus Peters, 1861, the sole exemplar species of Hemiscorpiidae, was poorly preserved but possessed bent sperm packages and some double-bent and ellipsoidal sperm packages. The sperm packages appear to lack a covering sheath (fig. 4I).

Mostly annular sperm packages were observed among the exemplar species of Hormuridae (fig. 4J–L). The annular sperm package appears to be achieved by coiling the straight package, and sometimes further twisting the ring into a lemniscate shape (fig. 4L). Exemplar species of *Opisthacanthus* Peters, 1861, also possessed ellipsoidal sperm packages, but the poor preservation of the material prevented their structure from being properly determined.

The sperm packages of the two exemplar species of Urodacidae were ellipsoidal to almost spherical with a sheath (fig. 5A, B) that appeared to be much thicker than observed in Chactidae (fig. 3E–K).

Among the exemplar species of Diplocentridae, both bent and ellipsoidal sperm packages were observed in *Diplocentrus lindo* Stockwell, 2001 (fig. 5C) whereas only bent sperm packages were observed in *Nebo hierichonticus* (Simon, 1872) (fig. 5D). The ellipsoidal shape of the sperm packages of *D. lindo* appears to be achieved by spiral coiling of the sperm package (fig. 5C). No sheath was observed covering the spermatozoa in either of the diplocentrid exemplars.

Among the exemplar species of Scorpionidae, *Pandinus imperator* (C.L. Koch, 1841) exhibited straight (fig. 5E), and bent sperm packages (table 1), whereas *Scorpio fuliginosus* (Pallary, 1928) exhibited ellipsoidal sperm packages (fig. 5F), along with some folded sperm packages. The material, particularly for *P. imperator*, was not well preserved.

Diversity of Sperm Packages in Bothriuridae: All three exemplar species of *Brachistosternus* Pocock, 1893, possessed straight sperm packages with a canelike shape (fig. 5G, H). The sperm packages of *Brachistosternus angustimanus* Ojanguren Affilastro and Roig Alsina, 2001, were longer than those of the other two species (table 3). Narrower sperm packages were observed in some samples of *B. ferrugineus*. The same pattern was evident in the caraboctonid, *Caraboctonus keyserlingi*.

Lisposoma josehermana exhibited thick, straight sperm packages with a straight, pointed head (fig. 5I), unusually long, relative to the length of the sperm package. In most of the sperm packages, the anterior zone always appeared swollen, with the spermatozoa loosely arranged and separated from each other. The shape of this sperm package resembled that of the superstitioniid, *Superstitionia donensis* (fig. 3C).

The sperm packages of *Orobothriurus tamarugal* Ochoa et al., 2011, were short, straight, and uniform from the head to the medial zone, tapering terminally (fig. 5J). These were the narrowest sperm packages of the family and, together with those of *Phoniocercus pictus* Pocock, 1893, *Rumikiru lourencoi* (Ojanguren Affilastro, 2003), and *Thestylus aurantiurus* Yamaguti and Pinto-da-Rocha, 2003, among the shortest (table 3). The sperm packages of *R. lourencoi* were short and slender, with a long head relative to the length, tapering terminally (fig. 5K), as in *O. tamarugal*. The short, narrow sperm packages of *T. aurantiurus* were more uniform along their length than those of *O. tamarugal* and *R. lourencoi*, and not tapering as abruptly terminally (fig. 5L).

In all exemplar species of *Urophonius* Pocock, 1893, the head of the sperm package was angled 45°–90° to the body (fig. 6A, B). The body of the sperm package tapered terminally. An undulated area was sometimes evident medially on the sperm package of *U. brachycentrus* (fig. 6B).

The sperm packages of *Cercophonius squama* (Gervais, 1843) resembled those of *P. pictus* and *Urophonius*. The head zone was short and the head-body angle gentle (fig. 6C). Some sperm packages appeared to be single folded medially (table 2). The bending resembled that observed in other families, e.g., Vaejovidae, but the general shape was different as the sperm package was not uniform in width, and the head was angled approximately 90°, differing from the straight head zone associated with the folded sperm packages of other species in the family.

The general form of the sperm packages of *P. pictus* resembled those of *C. squama* and *Urophonius*, but the sperm packages of *P. pictus* were shorter (table 3), and the tips usually sharper (fig. 6D).

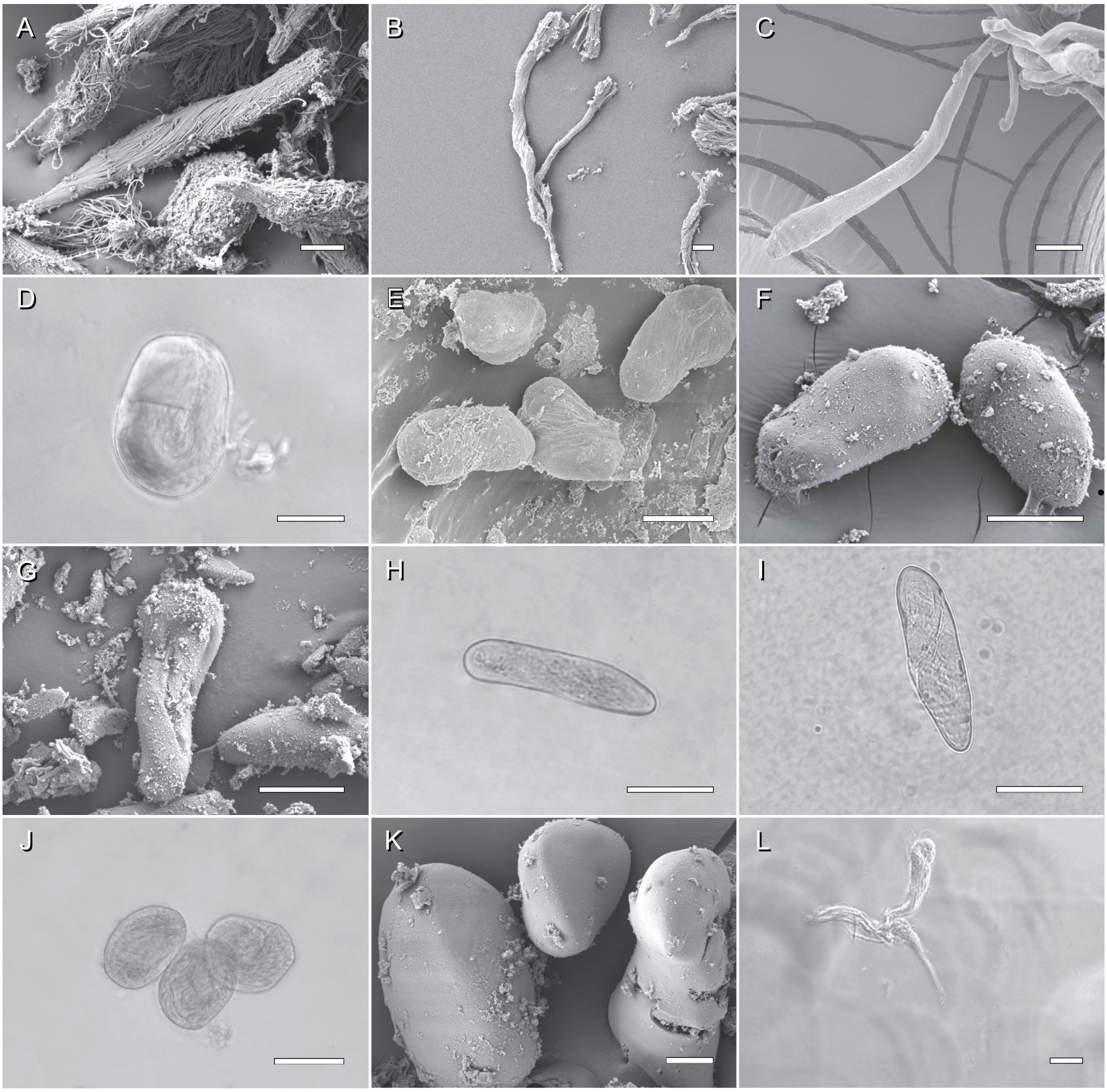

FIGURE 3. Sperm packages of Caraboctonidae Kraepelin, 1905 (**A, B**), Superstitioniidae Stahnke, 1940 (**C**), Chactidae Pocock, 1893 (**D–K**), and Troglotayosicidae Lourenco, 1998 (**L**) imaged with scanning electron microscopy (**A–C, E–G, K**) or light microscopy (**D, H–J, L**). **A.** *Caraboctonus keyserlingi* Pocock, 1893: straight. **B.** *Hadruroides lunatus* (L. Koch, 1867): straight. **C.** *Superstitionia donensis* Stahnke, 1940: straight. **D, E.** *Nullibrotheas allenii* (Wood, 1863): ellipsoidal, double bent. **F, G.** *Brotheas* sp., Bartica District, Guyana: ellipsoidal, double bent. **H.** *Broteochactas nitidus* Pocock, 1893: double bent. **I.** *Chactopsoides anduzei* (González-Sponga, 1982): double bent. **J.** *Chactas aequinoctialis* (Karsch, 1879): ellipsoidal, spherical. **K.** *Teuthraustes* sp., Aguay Province, Ecuador: ellipsoidal. **L.** *Troglotayosicus humiculum* Botero-Trujillo and Francke, 2009: bent. Scale bars: 25 µm.

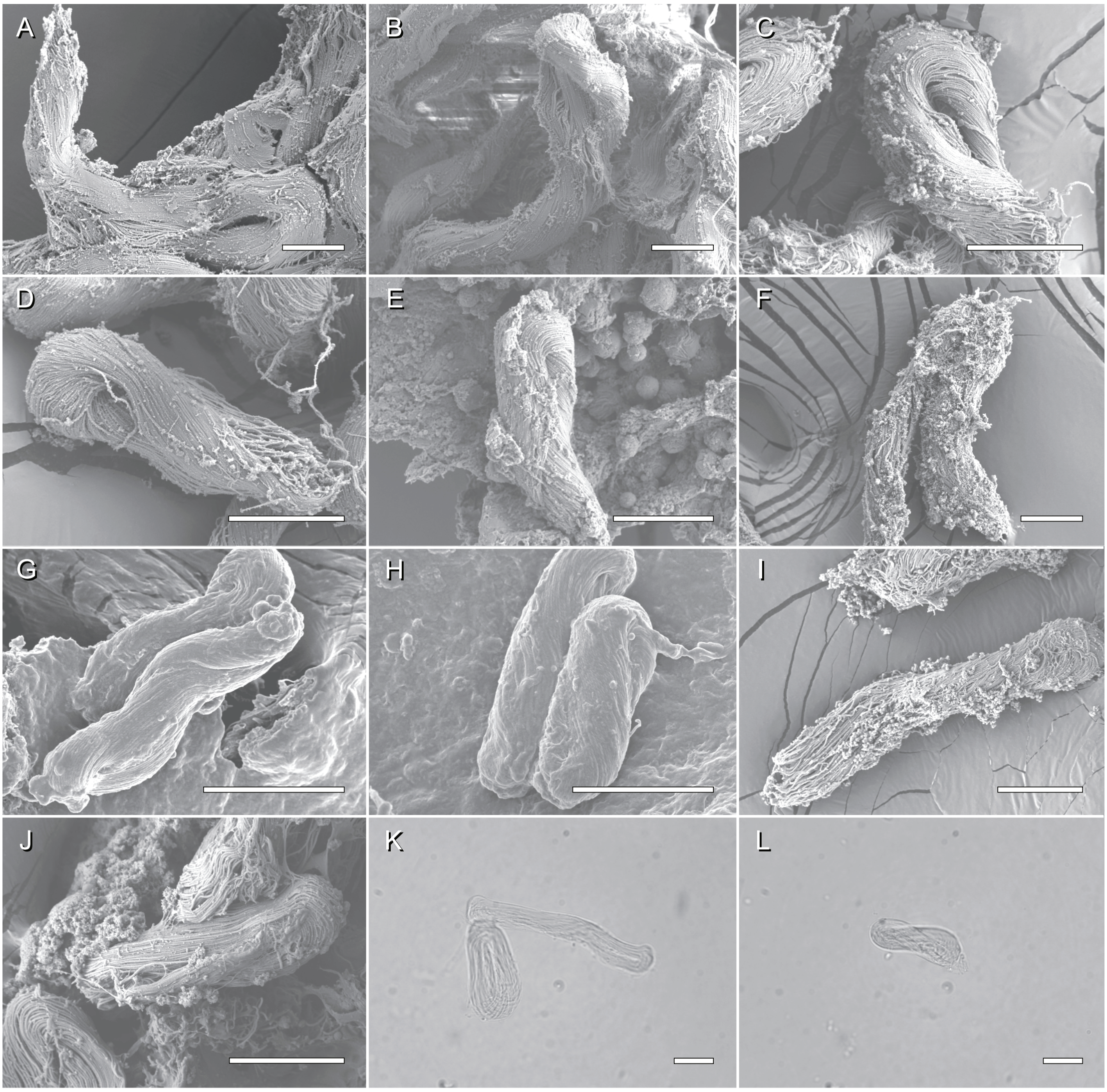

FIGURE 4. Sperm packages of Chactidae Pocock, 1893 (**A, B**), Vaejovidae Thorell, 1876 (**C–F**), Heteroscorpionidae Kraepelin, 1905 (**G, H**), Hemiscorpiidae Pocock, 1893 (**I**), and Hormuridae Laurie, 1896 (**J–L**) imaged with scanning electron microscopy (**A–J**) or light microscopy (**K, L**). **A, B.** *Uroctonus mordax* Thorell, 1876: bent. **C, D.** *Paravaejovis spinigerus* (Wood, 1863): bent. **E.** *Graemeloweus glimmei* (Hjelle, 1972): bent. **F.** *Vejovoidus longiunguis* (Williams, 1969): bent. **G, H.** *Heteroscorpion goodmani* Lourenco, 1996: bent/double bent. **I.** *Hemiscorpius lepturus* Peters, 1861: bent. **J.** *Hadogenes troglodytes* (Peters, 1861): annular. **K, L.** *Hormurus* sp., Queensland, Australia: annular, double bent. Scale bars: 25 μm.

TABLE 3. Length (mean ± standard deviation, in μm) of sperm packages among exemplar species representing major groups of scorpions.

Suborder	Superfamily	Family	Species	Length
Buthida	Buthoidea	Buthidae	*Ananteris arcadioi*	152 ± 19
			Parabuthus granulatus	230 ± 28
Iurida	Bothriuroidea	Bothriuridae	*Bothriurus araguayae*	199 ± 5
			Bothriurus bocki	159 ± 5
			Bothriurus bonariensis	249 ± 11
			Bothriurus burmeisteri	287 ± 19
			Bothriurus chacoensis	226 ± 11
			Bothriurus cordubensis	302 ± 3
			Bothriurus coriaceus	329 ± 7
			Bothriurus flavidus	212 ± 23
			Bothriurus keyserlingi	300 ± 14
			Bothriurus noa	332 ± 16
			Bothriurus olaen	328 ± 9
			Bothriurus rochai	201 ± 33
			Bothriurus rochensis	269 ± 15
			Brachistosternus angustimanus	253 ± 1
			Brachistosternus ferrugineus	233 ± 8
			Brachistosternus pentheri	205 ± 13
			Centromachetes obscurus	354 ± 10
			Cercophonius squama	227 ± 20
			Lisposoma josehermana	288 ± 8
			Orobothriurus tamarugal	193 ± 5
			Phoniocercus pictus	165 ± 6
			Rumikiru lourencoi	158 ± 3
			Tehuankea moyanoi	324 ± 6
			Thestylus aurantiurus	162 ± 7
			Timogenes dorbignyi	227 ± 14
			Timogenes elegans	240 ± 17
			Urophonius brachycentrus	311 ± 7
			Urophonius tregualemuensis	264 ± 4
			Vachonia martinezi	200 ± 9
	Chactoidea	Caraboctonidae	*Caraboctonus keyserlingi*	323 ± 6
		Chactidae	*Brotheas* sp.	210 ± 8
			Chactas aequinoctialis	144 ± 4
			Chactopsoides anduzei	244 ± 6
			Nullibrotheas allenii	231 ± 3
			Teuthraustes sp.	246 ± 27
			Uroctonus mordax	203 ± 9

TABLE 3 *continued*

Suborder	Superfamily	Family	Species	Length
Iurida	Chactoidea	Euscorpiidae	*Megacormus gertschi*	230 ± 2
			Megacormus sp.	217 ± 4
			Tetratrichobothrius flavicaudis	112 ± 5
		Scorpiopidae	*Euscorpiops longimanus*	166 ± 3
			Scorpiops zubairi	193 ± 19
			Troglocormus ciego	130 ± 17
		Superstitioniidae	*Superstitionia donensis*	231 ± 46
		Troglotayosicidae	*Troglotayosicus humiculum*	145 ± 3
		Typhlochactidae	*Alacran tartarus*	177 ± 21
		Urodacidae	*Urodacus planimanus*	282 ± 35
		Vaejovidae	*Vaejovis mexicanus*	160 ± 7
			Vejovoidus longiunguis	187 ± 4
	Iuroidea	Iuridae	*Iurus dufoureius*	335 ± 14
	Scorpionoidea	Diplocentridae	*Diplocentrus lindo*	192 ± 7
		Hemiscorpiidae	*Hemiscorpius lepturus*	195 ± 12
		Heteroscorpionidae	*Heteroscorpion goodmani*	171 ± 14
		Hormuridae	*Hormurus* sp.	146 ± 4
			Opisthacanthus capensis	151 ± 8
		Scorpionidae	*Pandinus imperator*	158 ± 8
			Scorpio fuliginosus	149 ± 4

The sperm packages of *Tehuankea moyanoi* Cekalovic, 1973, were longer than similar sperm packages in other bothriurids, gradually tapering terminally (fig. 6E). The heads of the sperm package were very short compared with the length, and a conspicuous angle was evident between the head and the body of the sperm package (fig. 6E).

The sperm packages of the two exemplar species of *Centromachetes* Lönnberg, 1897, were long and uniformly straight, with a short head zone (fig. 6F).

Among the 16 exemplar species of *Bothriurus*, the shapes of the sperm packages were diverse in some species groups and uniform in others. A marked difference was evident between the two subgenera. Straight sperm packages, taking the form of a cane, were common in subgenus *Andibothriurus*, comprising 50% of the exemplar species (table 2). Canelike sperm packages are straight, with the frontal part slightly bent at an angle greater than 90° from the body of the sperm package (fig. 1F). Sometimes, however, the anterior zone may also be straight (e.g., fig. 6G). This zone is wide and tapers terminally in the zone bearing the spermatozoa flagella. This canelike subtype of sperm package was observed only in Bothriuridae and mostly among exemplar species of *Andibothriurus*. Minor variation was observed in the length of canelike sperm packages, but the shape was generally similar among species.

Exemplar species of the nominal subgenus *Bothriurus* displayed a wider variety of sperm packages than observed in *Andibothriurus* (e.g., fig. 6H, I). The sperm packages observed in exemplar species of subgenus *Bothriurus* were straight and uniform in width along the entire length. These packages appeared annular or single folded in some species (fig. 6I). However, the packages of *Bothriurus araguayae* Vellard, 1934, were straight, with the zone of sperm heads long and markedly defined, compared with other species. Similarly, both *Bothriurus bonariensis* and *Bothriurus chacoensis* Maury and Acosta, 1993, possessed straight sperm packages (fig. 6H), like those of *B. araguayae*, but slightly slenderer in *B. chacoensis*. *Bothriurus bocki* Kraepelin, 1911, and *B. inermis* displayed bent sperm packages, resembling those of other families (table 2; fig. 6I), together with some annular sperm packages, and straight sperm packages, as in *B. araguayae*. All three types of sperm packages (single folded, straight, and multiple folded) are commonly observed together in many species in which the single-folded sperm package is the most common type. Annular sperm packages, formed from multiple-folded sperm packages, were more abundant in *B. inermis* than other species in which both folding types were observed, and exhibited variation within individuals.

The sperm packages of exemplar species of subgenus *Bothriurus*, i.e., *Bothriurus asper* Pocock, 1893, and *Bothriurus rochai* Mello-Leitão, 1932, were difficult to interpret due to poor preservation. These species appear to possess straight sperm packages, uniform in width along the entire length, like *B. araguayae* and *B. bonariensis*. The zone of the head of these sperm packages appeared to be short compared to the sperm packages of *B. araguayae* and *B. bocki. Bothriurus asper* exhibited aggregations that resemble straight sperm packages. The sperm packages of *B. rochai* were wider anteriorly than posteriorly, but the sperm heads could not be easily identified.

The typical bent sperm package was abundant in both exemplar species of *Timogenes* (fig. 6J). The width of the sperm package was uniform, and its head appeared to be long, as in *Bothriurus araguayae*. Two other types of sperm packages were also observed in *Timogenes* but were far less abundant than the typical folded sperm packages: annular sperm packages (fig. 6K), resembling those of *Bothriurus inermis*, and straight sperm packages, resembling those of *B. araguayae* or the *bonariensis* group of *Bothriurus*. The sperm packages of *Vachonia martinezi* closely resembled the single-folded, bent sperm packages of *Timogenes* (fig. 6L).

Analysis of Sperm Package Length across Scorpions: The mean length of the sperm packages of all exemplar species was 203.95 ± 56.54 μm. Species values are summarized in table 3. The shortest sperm packages were observed among Euscorpiidae, e.g., *Tetratrichobothrius flavicaudis* (112 ± 5 μm), and Scorpiopidae, e.g., *Troglocormus ciego* (130 ± 17 μm). The longest sperm packages, observed in Bothriuridae, e.g., *Centromachetes pocockii* (Kraepelin, 1894) (354 ± 10 μm), were almost three times greater.

Among all sampled species, straight sperm packages were longer than bent sperm packages, the only exception being the urodacid, *Urodacus planimanus* Pocock, 1893, the bent sperm package of which was similar in length to the straight sperm package of, e.g., Iuridae. Variation in the length of sperm packages, ranging from 143 μm to 223 μm (183 ± 40 μm), was observed among families with bent sperm packages (table 3). A one-way ANOVA confirmed a statistical difference in length among the species ($F_{(11; 54)} = 13.71$, $p << 0.001$). Three groups were identified by the Tukey HSD test (fig. 7): *U. planimanus* (Urodacidae) with the greatest mean value; an intermediate group including *Alacran tartarus* (Typhlochactidae); the smallest mean value in *T. flavicaudis* (Euscorpiidae). The longest sperm packages in the dataset, observed in Iuridae, were straight (table 3).

Among Bothriuridae, straight, canelike sperm packages varied in length from 212 ± 2 µm (*Bothriurus flavidus* Kraepelin, 1911) to 332 ± 7 µm (*Bothriurus noa* Maury, 1984), and across the entire family, straight packages varied in length from 158 ± 3 µm (*Rumikiru lourencoi*) to 354 ± 10 µm (*Centromachetes obscurus* Mello-Leitao, 1932) (table 3). Among straight sperm packages, the mean value of the exemplar species of this family was 254 ± 52 µm.

Counts of Spermatozoa per Sperm Package in Bothriuridae: Transmission electron microscopy of four species of Bothriuridae revealed that spermatozoa were clustered adjacent to one another and surrounded by an electrodense substance without a covering sheath (fig. 8A–H). Sections at different levels along the sperm package indicated distinctive parts of individual spermatozoa (fig. 8A–D). A section across the anterior part of the sperm package, the zone corresponding to the spermatozoa nucleus, appeared electrodense (fig. 8A, B). The arrangement of the mitochondria and disposition of the microtubules in the axoneme can be identified in the medial zone of the spermatozoa (fig. 8C). The flagellar zone reveals the 9 + 0 arrangement of microtubules without a central pair (fig. 8D).

Although spermatozoa are aligned in the sperm package, the alignment is imperfect at the ends, i.e., the zone of the nuclei and the zone bearing the flagella, where the sperm counts are always lower than the counts in the medial zone. This mismatch is consistent with the tapering of the sperm packages at either end (fig. 1). It is evident that only two mitochondria are present on either side of the axoneme in *Bothriurus bonariensis* (fig. 8E), unlike the other three species studied, in which three mitochondria are present (fig. 8F–H).

The lowest count of spermatozoa, fewer than 100 spermatozoa per sperm package (96 ± 16 spermatozoa), was observed in *B. bonariensis* (fig. 8E), compared with 244 ± 35 spermatozoa per sperm package in *Timogenes elegans* (fig. 8F), 260 ± 25 spermatozoa in *Brachistosternus ferrugineus* (fig. 8G), and 169 ± 19 spermatozoa in *Urophonius brachycentrus* (fig. 8H). Several electrodense drops, noticeable with optical microscopy and TEM, accompanied the sperm packages of *U. brachycentrus* (fig. 8H, I) but were not consistently observed in the other species. The droplets appear somewhat transparent and refringent using optical microscopy, suggesting a lipid, but their chemical composition is unknown.

In the four species assessed, the width of the sperm package is a function of the number of spermatozoa rather than their width, which is quite uniform. When comparing the numbers of spermatozoa and the size of the sperm package, there is a slight tendency for short sperm packages to contain more spermatozoa among the four species studied. However, this could not be tested statistically because of the small sample size.

DISCUSSION

Sperm Package Morphology: The general absence of sperm packages in Buthidae is well known (Cruz-Landim and Ferreira, 1973; Peretti and Battán-Horenstein, 2003; Michalik and

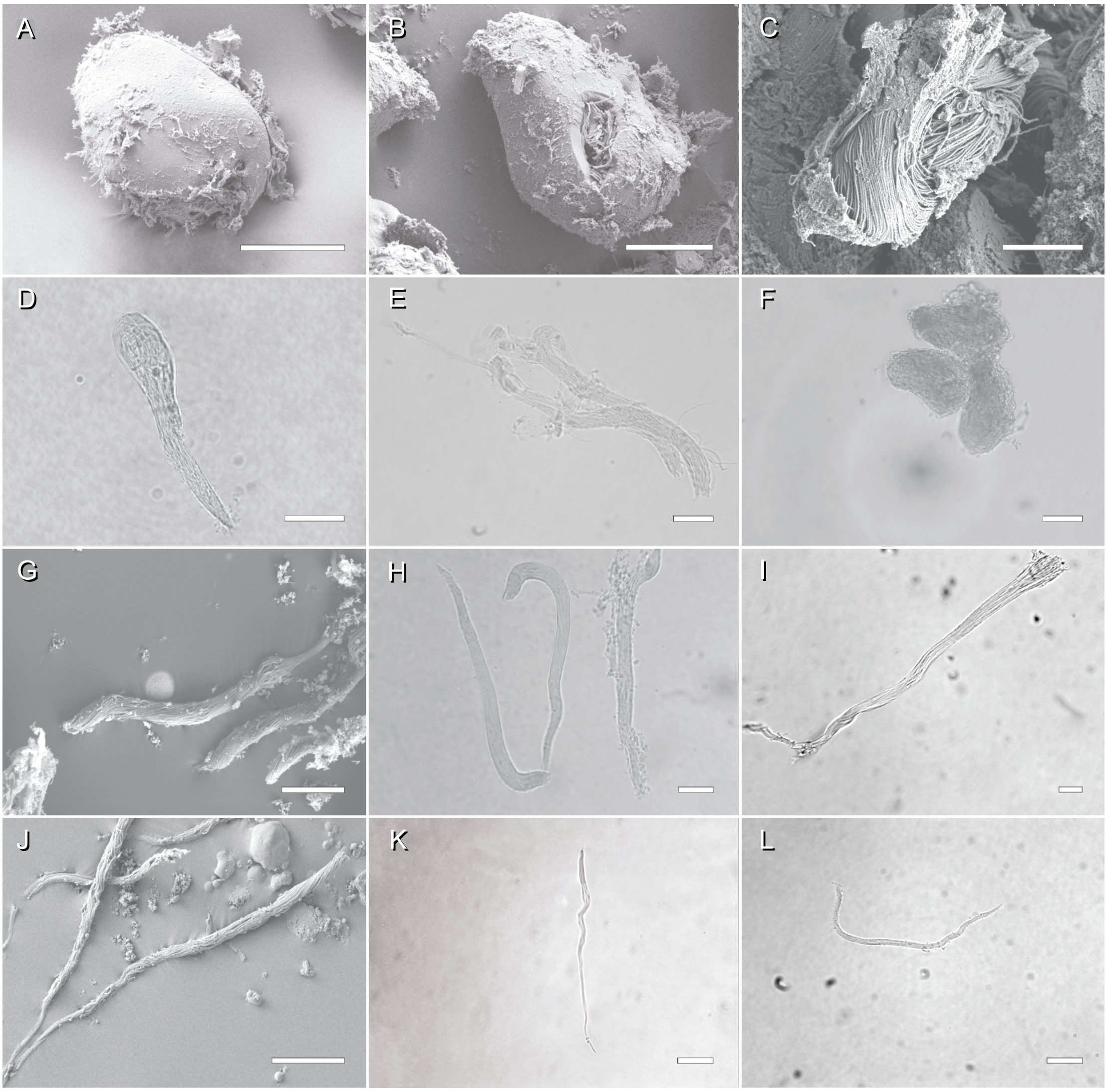

FIGURE 5. Sperm packages of Urodacidae Pocock, 1893 (**A, B**), Diplocentridae Karsch, 1880 (**C, D**), Scorpionidae Latreille, 1802 (**E, F**), and Bothriuridae Simon, 1880 (**G–L**) imaged with scanning electron microscopy (**A–C, G, J**) or light microscopy (**D–F, H, I, K, L**). **A, B.** *Urodacus planimanus* Pocock, 1893: ellipsoidal/spherical. **C.** *Diplocentrus lindo* Stockwell and Baldwin, 2001: spiral/spherical. **D.** *Nebo hierichonticus* (Simon, 1872): bent. **E.** *Pandinus imperator* (C.L. Koch, 1841): straight. **F.** *Scorpio fuliginosus* (Pallary, 1928): ellipsoidal. **G.** *Brachistosternus ferrugineus* (Thorell, 1876): canelike. **H.** *Brachistosternus pentheri* Mello-Leitão, 1931: canelike. **I.** *Lisposoma josehermana* Lamoral, 1979: straight. **J.** *Orobothriurus tamarugal* Ochoa et al., 2011: straight. **K.** *Rumikiru lourencoi* (Ojanguren Affilastro, 2003): straight. **L.** *Thestylus aurantiurus* Yamaguti and Pinto-da-Rocha, 2003: straight. Scale bars: 25 μm.

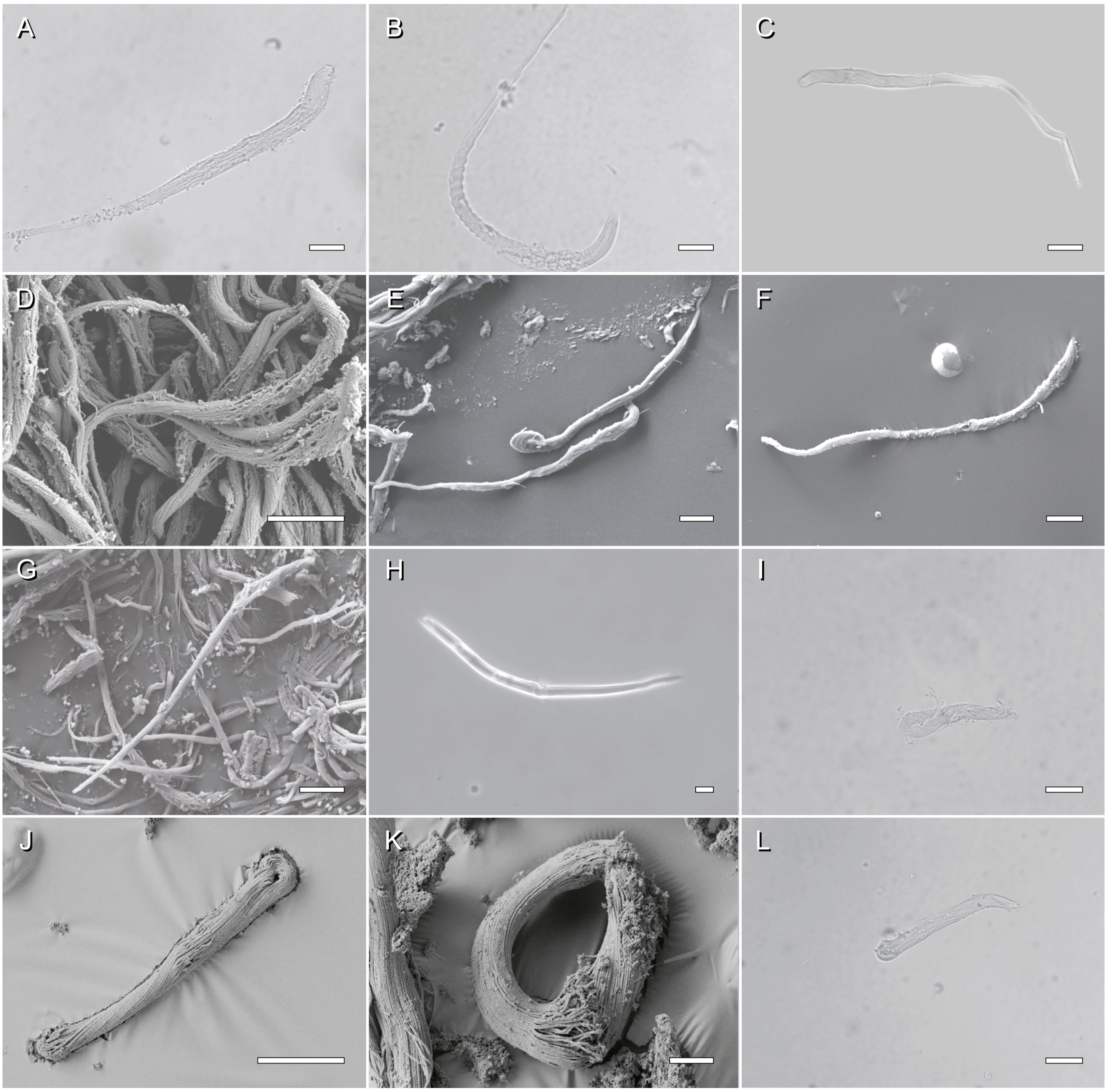

FIGURE 6. Sperm packages of Bothriuridae Simon, 1880 imaged with light microscopy (**A–C, H, I, L**) and scanning electron microscopy (**D–G, K**). **A.** *Urophonius tregualemuensis* Cekalovic, 1981: canelike. **B.** *Urophonius brachycentrus* (Thorell, 1876): canelike. **C.** *Cercophonius squama* (Gervais, 1843): canelike. **D.** *Phoniocercus pictus* Pocock, 1893: canelike. **E.** *Tehuankea moyanoi* Cekalovic, 1973: canelike. **F.** *Centromachetes pocockii* (Kraepelin, 1894): straight. **G.** *Bothriurus cordubensis* Acosta, 1995: canelike. **H.** *Bothriurus bonariensis* (C.L. Koch, 1842): canelike. **I.** *Bothriurus bocki* Kraepelin, 1911: bent. **J, K.** *Timogenes elegans* (Mello-Leitão, 1931): bent, annular. **L.** *Vachonia martinezi* Abalos, 1954: bent. Scale bars: 25 μm.

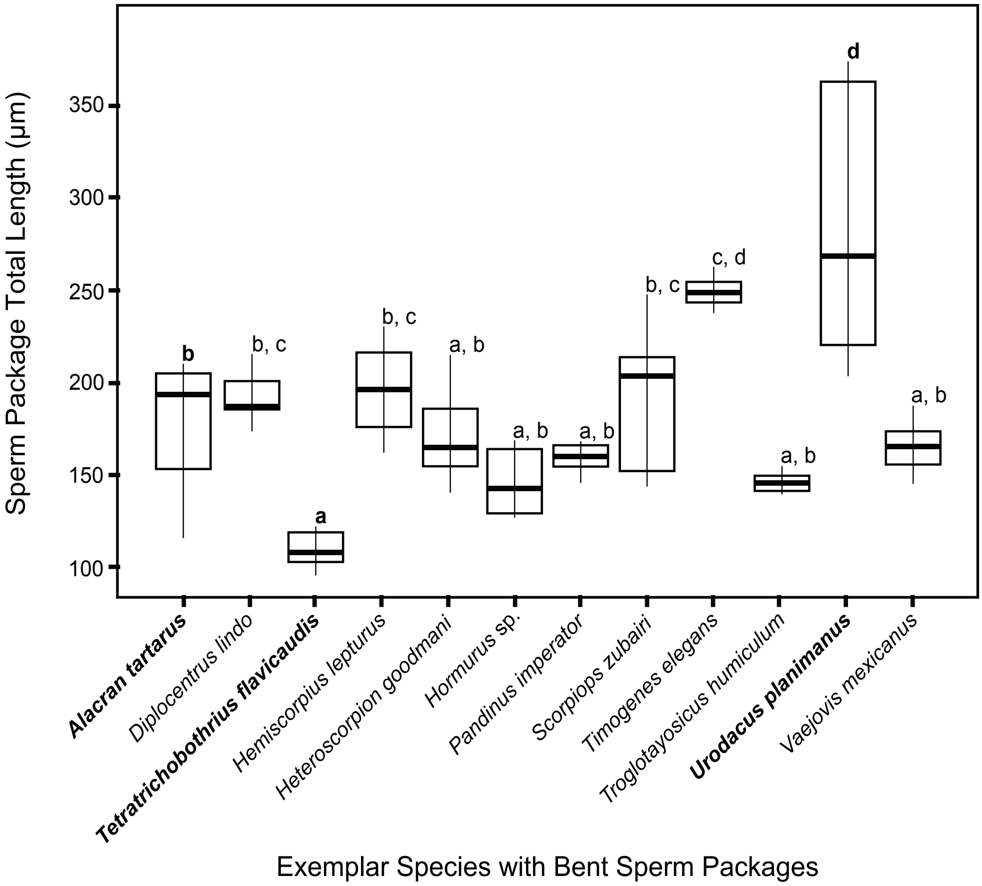

FIGURE 7. Boxplot of Tukey HSD test illustrating three major length types (a–c) of single folded sperm packages in Scorpiones. Representatives of the three groups in boldface (see text).

Mercati, 2010; Peretti, 2010; Vrech et al., 2011; but see Alberti, 1983). For example, Peretti and Battán-Horenstein (2003) reported the absence of sperm conjugation in *Zabius fuscus* (Thorell, 1876) and suggested a hypothesis, concerning a difference in sperm transfer between Buthidae and Bothriuridae, which could account for the absence or presence of sperm packages, respectively. Insemination is faster in buthids than bothriurids, hence sperm do not need to be grouped together, suggesting that the purpose of sperm conjugation in scorpions is to maintain the viability of spermatozoa during sperm transfer. Although Vignoli et al. (2008) and Michalik and Mercati (2010) described the sperm packages of Buthidae as loosely clustered sperm cells or loose bundles, Vrech et al. (2011) formalized the character state of Buthidae as the absence of sperm packages and reported it also in Chaerilidae. The general absence of sperm conjugation in both families was confirmed in the present investigation (table 2). The basal phylogenetic position of these families (Prendini and Wheeler, 2005; Prendini et al. 2006) supports the hypothesis that free spermatozoa represent the plesiomorphic state in scorpions (Vignoli et al., 2008; Michalik and Mercati, 2010; Vrech et al., 2011). This claim is also supported by the fact that there are no sperm packages or bundles of spermatozoa in arachnid orders closely related to the order Scorpiones (Lozano-Fernandez et al., 2019; Howard et al., 2020).

Nevertheless, it is important to note that some kind of sperm gathering, or conjugation, was observed in three exemplar species of Buthidae in the present study: *Ananteris arcadioi*, *Parabuthus granulatus*, and *Pseudolychas ochraceus*. The spermatozoa of these taxa appeared to be loosely aggregated, creating the appearance of sperm packages less tightly grouped than in other, nonbuthid taxa. Although it is possible that these cases are artifactual, this seems unlikely, especially as the two African taxa are known to be phylogenetically related, and some reconstructions have suggested a relationship to the South American genus *Ananteris* Thorell, 1891 (Coddington et al., 2004; Prendini, 2004). It is important to note that ordered structures, i.e., conjugated spermatozoa, are seldom observed in Buthidae, and such structures are unlikely to arise by chance. Besides, sperm conjugation was already reported in another buthid, *Buthus occitanus*, using the more general term, sperm bundle, with sperm heads embedded in an electrodense substance that may give consistency to the structure (Alberti, 1983). Unfortunately, sperm conjugates were not described in more detail, nor was an illustration of the sperm package or bundle provided, preventing a determination as to whether the conjugation noted by Alberti (1983) resembles those observed in the present study. In conclusion, whereas the absence of sperm conjugation is typical in Buthida, at least based on the exemplar species of Buthidae and Chaerilidae studied to date (table 2), the presence of conjugation in some taxa should not be disregarded. Further investigation, including a broader sample of taxa in both families, may reveal additional examples of conjugation in Buthida.

Conjugation is assumed to be absent in Pseudochactidae, given its close phylogenetic relationship to Chaerilidae (Prendini et al., 2006, 2021). Unfortunately, this could not be verified in the pseudochactid exemplar species, *Troglokhammouanus steineri*, as the seminal vesicle could not be located in its greatly reduced genitalia, which are markedly different from those of other scorpions (Prendini et al., 2006, 2021). Further investigation of the genitalia and sperm structure of this family are important to formulate a more precise hypothesis of sperm package evolution in scorpions.

The present study revealed that sperm conjugation is ubiquitous in Iurida, the group comprising all other nonbuthid scorpion families, although marked differences are evident among some. Bent sperm packages were observed in most families (table 2), consistent with the observations of Michalik and Mercati (2010). The present investigation also supports the evolutionary transformation from free spermatozoa via straight sperm packages to single-folded and ultimately multiple-folded sperm packages, following successive folding events (Vignoli et al., 2008; Vrech et al., 2011).

The first conclusive evidence is presented of sperm packages that evolved a sheath or capsule covering the entire conjugate. Previous studies suggested sperm packages generally lack a sheath (Vignoli et al., 2008; Michalik and Mercati, 2010; Vrech et al., 2011), the only exceptions being an observation in the euscorpiid *Euscorpius sicanus* (Michalik and Mercati, 2010) and the bothriurid taxa studied by Peretti and Battán-Horenstein (2003). The present study revealed the absence of any kind of sheath in Bothriuridae and the presence of a true sheath in five families (Chactidae, Euscorpiidae, Heteroscorpionidae, Scorpiopidae, and Urodacidae) from

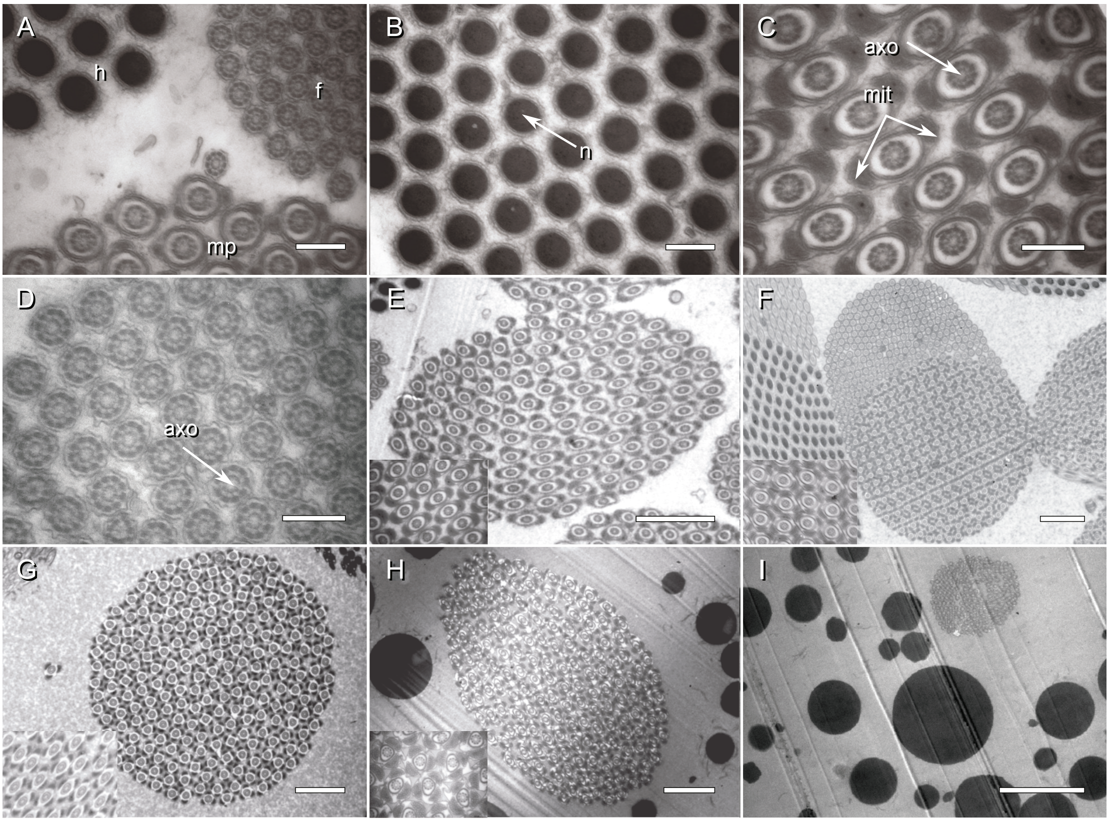

FIGURE 8. Transmission electron micrographs of spermatozoa from sperm packages of the bothriurid, *Bothriurus bonariensis* (C.L. Koch, 1842), sectioned at different points along an axis (**A–D**), and of sperm packages of four species of Bothriuridae Simon, 1880 sectioned in the middle (**E–H**). **A.** Head, middle piece, and flagella of different sperm packages. **B.** Nuclei of heads from different spermatozoa. **C.** Middle piece of different spermatozoa with paired mitochondria. **D.** Flagella of different spermatozoa. **E.** *Bothriurus bonariensis*. **F.** *Timogenes elegans* (Mello-Leitão, 1931). **G.** *Brachistosternus ferrugineus* (Thorell, 1876). **H, I.** *Urophonius brachycentrus* (Thorell, 1876). Inset in **E–H** illustrates details of axoneme. Abbreviations: axo, axoneme; f, flagellum; h, head; mit, mitochondria; mp, middle piece; n, nucleus. Scale bars: 0.5 µm (**A–D**); 2.5 µm (**E–H**).

two superfamilies, suggesting this character arose independently on multiple occasions. This may also be suggested for the single-folded sperm packages, which appear to have evolved independently in different taxa. For example, the bent shape observed in some *Bothriurus*, *Timogenes*, and *Vachonia*, relatively distal taxa within Bothriuridae (Prendini, 2003; Mattoni and Prendini, 2007), is very similar to the shapes observed in other distantly related families like Scorpionidae and Vaejovidae.

Despite the apparently high levels of convergence, sperm package morphology is potentially informative for scorpion systematics, as noted by others (Vignoli et al., 2008; Michalik and Mercati, 2010; Vrech et al., 2011). For example, sperm packages suggest that *Bothriurus* may not be monophyletic (Vrech et al., 2011), in agreement with previous studies based on other characters (Prendini, 2000, 2003; Mattoni, 2003; Mattoni and Prendini, 2007). Three species groups are identified in *Bothriurus* based on sperm package morphology. The first group includes species of the nominal subgenus with straight sperm packages, such as *B. araguayae*, *B. bonariensis*, and *B. chacoensis*; the second includes species of the nominal subgenus with folded sperm packages similar to *Timogenes* and *Vachonia*, such as *B. bocki*, and *B. inermis*; and the third includes the species of subgenus *Andibothriurus*, characterized by canelike sperm packages. Preliminary analyses by Vrech et al. (2011) suggested the absence of sperm packages in some *Bothriurus* species, i.e., *B. asper* and species of the *rochai* group. During the present investigation, however, the presence of sperm packages was verified in these species (table 2), albeit with difficulty due to poor preservation of the material examined. Preservation is evidently important for the successful study of sperm packages.

Sperm Package Metrics: Considerable variation in the length of the sperm packages was observed across the order and in the family Bothriuridae. Bent sperm packages were not necessarily longer than straight sperm packages, suggesting that folding of the sperm package is independent of length, and that long sperm packages can be successfully transferred without folding. Chactidae and Urodacidae, in which long sperm packages were folded at least once, sometimes with the presence of a sheath rendering them even more compact, were notable exceptions. An overestimation of the total length of the sperm packages of Chactidae could not be disregarded due to the complexity of the folding, however. Although the length of a sperm package does not appear to induce folding, as some of the longest sperm packages were observed in taxa with straight sperm packages, folded sperm packages appear to be more easily transferred through the male genital tract (Vrech et al., 2011). Similarly, some insects, including fruit flies of the genus *Drosophila* Fallén, 1823, produce coiled sperm bundles that facilitate sperm transport through the testes (Mojica and Bruck, 1996). Sperm package length resembles sperm length except in extremely coiled structures. Sperm length in insects may be related either to sexual selection (Gage, 1994; Morrow and Gage, 2000; Pitnick et al., 2009) or to allometry and its relationship with male body size (Gage, 1994; Pitnick, 1996; Pitnick et al., 2009; Vrech et al., 2014). Also, sperm length is highly correlated with the female inner reproductive structures in some insect taxa (Pitnick et al., 2009; Higginson and Pitnick, 2011).

Several studies of insects suggest a correlation between sperm length or sperm bundles and sperm storage or associated structures of the female (Sasakawa, 2007; Pitnick et al., 2009). Although such relationships have not yet been investigated in scorpions, it is noteworthy that sperm packages disaggregate inside the female genital tract, hence interactions with the female reproductive structures would involve only individual spermatozoa (Peretti and Battán-Horenstein, 2003).

Transmission electron microscopy indicated material of unknown chemical composition, binding the spermatozoa together (fig. 8). The spatial organization of the spermatozoa revealed that individual spermatozoa were aligned in the same direction. Although imperfect, the alignment allowed the length of the spermatozoa to be estimated precisely from the length of the sperm package (Peretti, 2010).

Axonemal organization in four exemplar species of Bothriuridae, *Bothriurus bonariensis*, *Brachistosternus ferrugineus*, *Timogenes elegans*, and *Urophonius brachycentrus*, was consistent with the

9 + 0 pattern described for other Iurida by Michalik and Mercati (2010). This pattern differs from the 9 + 2 axonemal pattern observed in Buthidae (Michalik and Mercati, 2010; but see Hood et al., 1972), which is the most common pattern among animals (Mitchell, 2004; Pitnick et al., 2009), and is therefore assumed to be the plesiomorphic state in scorpions (Michalik and Mercati, 2010).

Counts of spermatozoa in the sperm packages were generally consistent with the $2^n \times 4$ formula where n is the number of premeiotic mitotic divisions (Higginson and Pitnick, 2011) or any of its modifications (Virkki, 1969; Phillips, 1970; Sivinski, 1980; Jurečić, 1988; Schiff et al., 2001; Dias et al., 2013). *Brachistosternus ferrugineus* and *Timogenes elegans* appear to conform with counts close to 256, implying almost six premeiotic mitotic divisions to form the sperm package ($2^6 \times 4 = 256$). However, the counts were less precise in other species, e.g., *Bothriurus bonariensis*, with close to 128 spermatozoa, implying approximately five mitotic and two meiotic processes ($2^7 = 128$), but usually fewer than 100 spermatozoa, and *Urophonius brachycentrus*, with counts between 128 and 256 spermatozoa. It is not uncommon for some species to present an intermediate or slightly variable count of spermatozoa (Nur, 1962; Schärer et al., 2008; Higginson and Pitnick, 2011). The counts of spermatozoa for bothriurids obtained in the present study were generally greater than those for bothriurids cited by Peretti and Battán-Horenstein (2003) but similar to those for scorpionids cited by Michalik and Mercati (2010).

Hypotheses for the Evolution and Function of Sperm Conjugation: A new hypothesis is proposed herein for the origin of sperm packages. Based on the arachnid phylogeny (Lozano-Fernandez et al., 2019; Howard et al., 2020), free sperm would be the plesiomorphic state in scorpions. A change in spermiogenesis would have caused individual spermatozoa to cluster together as conjugated sperm instead of separating into free spermatozoa as in the families of Buthida (Buthidae, Chaerilidae, and presumably, Pseudochactidae). Sperm would have become loosely aggregated into bundles in some Buthidae. Apparent conjugation of sperm in buthids like *Parabuthus granulatus* would represent convergence, assuming it represents true conjugation. Conjugation of spermatozoa would have initially produced single-folded bent sperm packages instead of straight sperm packages, accounting for their widespread presence in Iurida. These bent sperm packages could subsequently evolve in two directions, either unfolding to produce straight sperm packages or continuing to fold multiple times, producing ellipsoidal, spherical, or annular sperm packages. Straight sperm packages appear to have evolved independently at least four times, in Iuridae, Caraboctonidae, Bothriuridae, and Superstitioniidae. The canelike sperm packages of some *Bothriurus* are presumably derived from straight sperm packages, which appears to be the plesiomorphic state in Bothriuridae. The annular and multiple-folded sperm packages appear to be autapomorphic or synapomorphic for particular taxa, e.g., annular in *Bothriurus inermis* and *Hormurus* sp., or multiple folded in *Megacormus*, *Troglocormus* and some Chactidae. The hypothesized evolutionary transformation series of sperm package shape is summarized in figure 9.

The change in spermiogenesis that allowed individual spermatozoa to remain together could have been adaptive or nonadaptive, derived from cyst cells failing to individualize their products (Higginson and Pitnick, 2011; Burnett and Heinze, 2014), raising the question as to why sperm conjugation arose? This question has been repeatedly asked, and some current hypotheses were reviewed by Pitnick et al. (2009) and Higginson and Pitnick (2011). Although data remain limited, three hypotheses may be suggested to account for the occurrence of sperm conjugation in scorpions:

(1) Sperm transport and movement: According to the classical hypothesis, sperm conjugation facilitates sperm transport (Mojica and Bruck, 1996, Pitnick et al., 2009; Higginson, 2011; Higginson and Pitnick, 2011; Gage, 2012; Higginson and Henn, 2012). This may be true in scorpions as spermatozoa are long and conjugation may aid transport. Perhaps the widespread presence of single-folded sperm packages and the appearance of multiple folded sperm packages resulted from a selective advantage to enhancing sperm transfer by males (Vrech, 2011), facilitating insemination and avoiding stagnation. It has been suggested that sperm packages with increased compression, caused by folding, would be easier to transfer (Michalik and Mercati, 2010; Vrech et al., 2011). Studies of insects suggest the interaction between spermatozoa and the male genital tract favors bending in sperm aggregates (Mojica and Bruck, 1996, 2003; Joly et al., 2003; Joly et al., 2008), especially for the transport of long sperm groups (sperm bundles) through the testes, a hypothesis supported by Bruck (1978) and Virkki and Bruck (1994). The present study suggests that folding is maximized in ellipsoidal sperm packages, with the compression reinforced by the addition of a sheath covering the spermatozoa.

It has also been suggested that sperm packages could be related to the type of spermatophore (Peretti and Battán-Horenstein, 2003), the structure used by scorpions to indirectly transfer sperm to the female (Francke, 1979). Sperm packages are mostly absent in Buthida (Buthidae, Chaerilidae and Pseudochactidae), in which the spermatophores are rather simple, whereas sperm packages are present in Iurida, in which the spermatophores are more complex (Francke, 1979; Monod et al., 2017). The simple flagelliform spermatophores of Buthidae transfer sperm more rapidly than the complex lamelliform spermatophores of Bothriuridae (Peretti and Battán-Horenstein, 2003). As such, the correlation between the presence or absence of sperm packages and the complexity of the spermatophore may be related to the time required for insemination. Sperm packages may provide a measure of protection for sperm during the additional time required for transfer to the female genital atrium.

Related to sperm transport is the hypothesis of sperm cooperation in which the spermatozoa work together to enhance motility and strength of movement (Pitnick et al., 2009). Bothriurid spermatozoa show movement that allows separation from one another on contact with saline solution (Peretti and Battán-Horenstein, 2003), but there is no direct evidence for active directional movement of sperm packages, at least in vitro. Indeed, movement to the female genital tract is passive, aided by spermatophore action. The sperm package could be viewed as a mechanism for transporting spermatozoa in an inactive state (Alberti, 1983), preventing the active movement of spermatozoa across the walls of the male genital tract as happens in some insects (Werner and Simmons, 2008) and resembling the sperm bundles of others (Robison, 1966; Ross and Robison, 1969; Nur, 1962). There is no evidence of spermatozoa cooperation to enhance swimming in scorpions, as suggested for insects (Werner and Simmons, 2008; Pitnick et al., 2009). Spermatozoa grouped inside a sperm conjugation could dissociate inside the female's storage or fertilization tracts (Higginson and Pitnick, 2011) but there is evidence that sperm dissociate before entry in some taxa (Buckland-Nicks et al., 1999; Hayashi and Kamimura, 2002a, 2002b). Studies of scorpions suggest this may be the mechanism as the spermatozoa appear to be dissociated prior to entering the female spermathecae (Peretti and Battán-Horenstein, 2003; Althaus et al., 2010). Perhaps the spermatozoa become active once freed inside the female genital tract, as suggested by Alberti (1983) and Althaus et al. (2010).

(2) Sexual selection: Sperm conjugation may also enhance fertilization success. By enhancing the swimming capabilities of individual spermatozoa, sperm conjugation may aid sperm competition (Simmons, 2001; Pitnick et al., 2009). This may not be the only benefit of sperm conjugation, which is sexually selected, however. Females may evaluate and favor some males by exerting cryptic female choice on their sperm packages (Eberhard, 1996; Peretti and Aisenberg, 2015). Sperm length correlates with the length of the female genital tract in many taxa of vertebrates and invertebrates (Cummins and Woodall, 1985; Briskie and Montgomerie, 1992, 1993, Simmons, 2001; Pitnick et al., 2009). Females may also favor larger sperm packages (Higginson and Pitnick, 2011). For example, in carabid beetles, sperm conjugate size correlates positively with spermathecal length (Sasakawa, 2007). Sperm conjugation could represent a mechanism to increase size with minimal energy expenditure (Higginson and Pitnick, 2011). Besides, males may use conjugation to compactly transfer more sperm in an environment with sperm competition. As such, sperm packages may offer a means to transfer more sperm without producing larger sperm (Higginson and Pitnick, 2011).

Males of the caraboctonid, *Caraboctonus keyserlingi*, produce large sperm packages stored inside seminal vesicles, so large that the hemispermatophores are sometimes difficult to find (unpubl. data); the spermathecae of females are similarly enormous compared to other scorpion taxa (Volschenk et al., 2008). The almost negligible volume of sperm contained in the spermatophore compared to what is stored in the seminal vesicle suggests male *C. keyserlingi* may copulate repeatedly which, together with the presence of large sperm packages, and polymorphism, with broad and slender packages in the same individual (Vrech et al., 2011, 2014), are indicative of alternative strategies related to high levels of sperm competition as observed in insects (Simmons, 2001; Shuker and Simmons, 2014; Pitnick et al., 2009; Higginson and Pitnick, 2011).

Data presented herein demonstrate that short sperm packages tend to contain more spermatozoa among four exemplar species of Bothriuridae. Although only a few species were compared, this observation suggests a direct trade-off between the length of spermatozoa and the number of spermatozoa contained in a sperm package (Pitnick, 1996; Oppliger et al., 1998; Gage and Morrow, 2003; Pitnick et al., 2009). Assuming sperm packages are affected by sperm competition, such a trade-off might be expected as suggested by classical models (Parker et al., 2010). Some studies on insects assessed the relationship between the count of spermatozoa per sperm bundle and the phylogenetic position of the taxon (White, 1955; Virkki, 1969; Jurečić, 1988; Schiff et al., 2001). These studies suggest that species placed relatively basal in the phylogeny possessed a greater number of spermatozoa per sperm bundle. The few scorpion species assessed in the present study are consistent with this pattern, except for *Timogenes elegans*. However, further investigation with more exemplar species is needed to test this hypothesis more rigorously in scorpions.

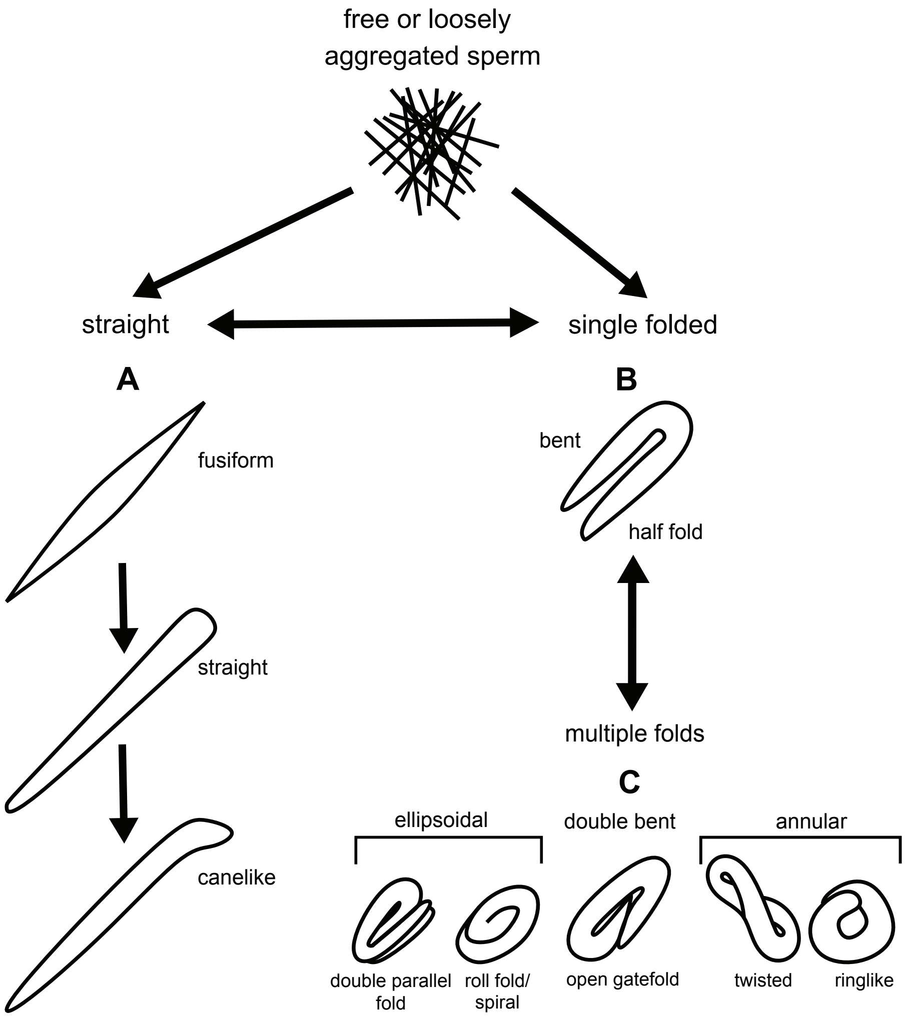

FIGURE 9. Schematic illustration summarizing the major types, shapes, and folding of sperm packages in Scorpiones with hypothesized evolutionary transformation from absence (free sperm), e.g., Buthida Soleglad and Fet 2003: **A.** Straight: fusiform, e.g., *Parabuthus granulatus* (Ehrenberg, 1831) (Buthidae C.L. Koch, 1837); straight, e.g., Iuridae Thorell, 1876, and Superstitioniidae Stahnke, 1940; or canelike, e.g., Bothriuridae Simon, 1880. **B.** Single fold: bent in half, e.g., Vaejovidae Thorell, 1876, *Timogenes* and *Vachonia* Abalos, 1954 (Bothriuridae). **C.** Multiple folds: ellipsoidal: double parallel fold, e.g., *Tetratrichobothrius flavicaudis* (De Geer, 1778), *Nullibrotheas allenii* (Wood, 1863); spiral, e.g., *Euscorpiops longimanus* (Pocock, 1893); double bent open gatefold, e.g., *Broteochactas* Pocock, 1893; annular: twisted, e.g., Hormuridae Laurie, 1896; or ringlike, e.g., *Bothriurus* Peters, 1861, and *Timogenes* Simon, 1880 (Bothriuridae).

Sperm packages may also assist with avoiding sperm competition in some scorpion taxa. For example, sperm packages serve two functions in the euscorpiid *Euscorpius italicus* (Althaus et al., 2010). In addition to their primary function in assisting with fertilization, sperm packages, together with ejaculate coagulates, form a genital plug in the female genital orifice. Genital plugs are structures of variable composition that reduce sperm competition by preventing the female from remating (Chapman et al., 2003; Shuker and Simmons, 2014). Although widespread among animals (Shine et al., 2000), genital plugs are rarely composed mainly of sperm. The genital plugs of scorpions may comprise spermatophore structures (Mattoni and Peretti, 2004; Contreras-Garduño et al., 2006), ejaculate substances (Castelvetri and Peretti, 1999), a mixture of both (Mattoni and Peretti, 2004), or a combination of substances from the male and female (Oviedo-Diego et al., 2020). In *E. italicus*, however, sperm forms a permanent hard structure (Althaus et al., 2010) that appears to be effective in restraining female mating, as females appear to mate only once (Benton, 1992).

(3) Protection from harsh environments: Sperm conjugation may provide physical protection against unfavorable environments and may assist molecular exchange among sperm cells. Preliminary observations of sperm viability in scorpions suggest that grouping may aid in retaining the viability of spermatozoa for a longer period (unpubl. data). Spermatozoa on the external surface of the sperm package may shield spermatozoa inside, helping the great majority of spermatozoa to remain viable until reaching the female genital tract or specific sites inside it. Such sperm cooperation or altruism has been described in other taxa such as insects, rodents, or mollusks (Pizzari and Parker, 2009). Sperm conjugation may also assist in avoiding premature acrosome reactions and maintain the integrity of most of the spermatozoa (Pitnick et al., 2009; Higginson and Pitnick, 2011). Additionally, the impact of forces associated with the mechanical action of a lamelliform spermatophore, which could be detrimental to isolated spermatozoa, may be lessened by their occurrence within the supportive framework of a sperm package. This hypothesis also awaits more rigorous scrutiny.

In addition to potentially improving sperm transfer, the presence of a covering sheath gives the sperm package additional protection against potentially harmful agents, which spermatozoa could encounter immediately upon immediate entry to the female genital tract after insemination (Pitnick et al., 2009). The presence of a protective cover to encapsulate sperm is common in other arachnids such as spiders, mites, and solifuges (Alberti, 2000; Klann et al., 2005; Michalik and Ramírez, 2014). Sperm conjugation in spiders is characterized by capsules that protect the sperm inside. Such capsules, also observed in some solifuges, such as Galeodidae Sundevall, 1833, are termed coenospermia (Klann et al., 2005; Michalik and Ramírez, 2014). The female genital tract of spiders is considered unfavorable for sperm viability, so the protection of a capsule is a useful protective trait (Michalik and Lipke, 2013). From this point of view, sperm protection appears to avoid sexual conflict (Birkhead et al. 1993; Birkhead and Møller, 1993). Little is known about this aspect in scorpions, however.

Finally, the presence of a sheath could confer auxiliary protection from environmental factors other than those directly related to sexual conflict that also decrease the viability of spermatozoa, such as water loss during deposition inside the spermatophore, indirect transfer, or similar factors (Peretti and Battán-Horenstein, 2003; Peretti, 2010; Michalik and Ramírez, 2014; Cargnelutti et al., 2019). Further investigation is needed into the mechanisms by means of which sperm packages may confer protection to spermatozoa.

CONCLUSIONS

The present study surveyed sperm packages in the largest taxon sample of scorpions to date, along with a comprehensive sample representing most genera of Bothriuridae. Data from classical morphometry were combined with qualitative assessments of shape to enable the identification of general patterns across the order. Despite the morphological diversity of sperm packages, single-folded sperm packages are the most common form. Basal scorpion lineages lack sperm packages or present loosely aggregated forms, and some taxa independently developed sheaths covering the sperm packages. In addition to being phylogenetically informative, sperm packages enhance the understanding of spermatozoa production, storage, and transfer in scorpions. Future research should investigate and describe the sperm packages of additional taxa, analyze the ultrastructure of sperm packages, and further investigate the evolutionary role of sperm packages from the perspectives of reproductive strategies and sexual selection.

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APPENDIX 1

MATERIAL EXAMINED FOR STUDY OF SPERM PACKAGES IN THE ORDER SCORPIONES C.L. KOCH, 1850

Abbreviations for natural history collections as follows: AMNH, American Museum of Natural History, New York; AVP, Alfredo V. Peretti Private Collection, Universidad de Córdoba, Argentina; CAS, California Academy of Sciences, San Francisco, CA; CIM, Camilo I. Mattoni Private Collection, Universidad de Córdoba, Argentina; DEV, David E. Vrech Private Collection, Universidad de Córdoba, Argentina; IBSP, Instituto Butantan, São Paulo, Brazil; MACN, Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Buenos Aires, Argentina; MCZ, Museum of Comparative Zoology, Harvard University, Cambridge, MA; MPUJ, Museo Javeriano de Historia Natural "Lorenzo Uribe S. J.," Pontificia Universidad Javeriana, Bogotá, Colombia; MZUSP, Museu de Zoología da Universidade de São Paulo, Brazil; MZUC, Museo de Zoología, Universidad de Concepción, Chile; UFBA, Universidade Federal da Bahía, Salvador da Bahía, Brazil; ZMB, Museum für Naturkunde, Berlin, Germany.

Family Bothriuridae Simon, 1880

Bothriurus araguayae Vellard, 1934: BRAZIL: *São Paulo*: Estaçao Ecológica de Itirapina, Itirapina, 12.ix.2000, G. Machado, 10 ♂ (CIM).

Bothriurus asper Pocock, 1893: BRAZIL: *Bahía*: 5 ♂ (UFBA).

Bothriurus bocki Kraepelin, 1911: BOLIVIA: *Potosí Department*: Mojotorillo, 5 km E of Betanzos, 19°34′40.9″S 65°24′57.9″W, 3217 m, 9.i.2005, C. Mattoni, A. Ojanguren and J. Ochoa, 1 ♂ (CIM).

Bothriurus bonariensis (C.L. Koch, 1842): ARGENTINA: *Córdoba Province*: Córdoba, 2 ♂ (AVP), Mendiolaza, 1 ♂ (DEV). URUGUAY: *Rivera Department*: Route 30 km 233, ca. 100 km SE of Artigas, 01°08′25.629″S 55°55′11.28″W, 345 m, 13.xii.2005, C. Mattoni, A. Ojanguren and F. Labarque, 2 ♂ (CIM).

Bothriurus burmeisteri Kraepelin, 1894: ARGENTINA: 1 ♂ (AVP). *Mendoza Province*: Reserva Natural Bosque Telteca, 32°22.967′S 68°03.309′W, 580 m, 19.xi.2003, C. Mattoni, J. Ochoa and L. Prendini, 3 ♂ (CIM).

Bothriurus chacoensis Maury and Acosta, 1993: ARGENTINA: *Córdoba Province*: Córdoba, 3 ♂ (AVP).

Bothriurus cordubensis Acosta, 1995: ARGENTINA: *Córdoba Province*: Córdoba, 4 ♂ (AVP).

Bothriurus coriaceus Pocock, 1893: CHILE: *Región IV* (*Coquimbo*): Limarí Province: Parque Nacional Fray Jorge, hillside below Bosque Fray Jorge, 30°39.124′S 71°40.645′W, 500 m, 4.xi.2003, L. Prendini, C. Mattoni and J. Ochoa, 4 ♂ (CIM).

Bothriurus flavidus Kraepelin, 1911: ARGENTINA: *Córdoba Province*: Córdoba, 2 ♂ (AVP); Pampa de Olaen, Capilla de Olaen, ca. 11 km W of Molinari, 31°09′44.46″S 64°36′24.336″W, 1096 m, 29.xii.2005, C. Mattoni, A. Peretti, P. Carreras, M. Zerda and D. Vrech, 4 ♂ (AVP).

Bothriurus inermis Maury, 1981: BRAZIL: *Rondônia*: Km 53 on BR 364, 8.xi.1985, 1 ♂ (MZUSP 18995).

Bothriurus keyserlingi Pocock, 1893: CHILE: *Región V* (*Valparaíso*): Quillota Province: Parque Nacional La Campana, Palmas de Ocoa, Sendero Quillay trail from campsite, 32°56.048′S 71°04.562′W, 494 m, L. Prendini, C. Mattoni and J. Ochoa, 12.xi.2003, 1 ♂ (CIM).

Bothriurus noa Maury, 1984: ARGENTINA: *La Rioja*: El Cantadero, 16 km from La Rioja, 27.xi.1994, A. Peretti, L. Acosta, C. Mattoni and A. Martínez, 1 ♂ (AVP).

Bothriurus olaen Acosta, 1997: ARGENTINA: *Córdoba Province*: Vaquerías, 21.xii.1994, A. Peretti and A. Martínez, 2 ♂ (AVP).

Bothriurus rochai Mello-Leitão, 1932: BRAZIL: *Piauí*: Estação Ecológica Uruçui, Una, 08°52′S 44°57′W, 19–29.i.2001, C.G. Martingelli, 5 ♂ (MZUSP 18999).

Bothriurus rochensis San Martín, 1965: URUGUAY: 2 ♂ (AVP). *Rocha Department*: Santa Teresa Park, road to camping close to Picada de los Cuervos, 34°00′36.072″S 53°33′20.772″ W, 48 m, 11.xii.2005, C. Mattoni, A. Ojanguren and F. Labarque, 1 ♂ (CIM).

Bothriurus sp.: BRAZIL: *Bahía*: Ceraíma Municipality: Guanambí, 7 km S, 14°17′05.6″S 42°47′02.2″W, 533 m, 24.i.2007, C. Mattoni, R. Pinto-da-Rocha and H. Yamaguti, 3 ♂ (CIM).

Brachistosternus angustimanus Ojanguren Affilastro and Roig Alsina, 2001: ARGENTINA: *Neuquén Province*: Picún Leufú, 25.i.2005, M. Magnanelli and G. López, 3 ♂ (CIM).

Brachistosternus ferrugineus (Thorell, 1876): ARGENTINA: *Córdoba Province*: Parque Provincial Chancaní, 20 and 23.xi.2001, C. Mattoni and J. Ochoa, 10 ♂ (CIM).

Brachistosternus pentheri Mello-Leitão, 1931: ARGENTINA: *Mendoza Province*: Reserva Ecológia de Biosfera Ñacuñan, 20.xi.2003, C. Mattoni, J. Ochoa and L. Prendini, 5 ♂ (CIM); Reserva Natural Bosque Telteca, 32°22.967′S 68°03.309′W, 580 m, 19.xi.2003, C. Mattoni, J. Ochoa and L. Prendini, 5 ♂ (CIM).

Centromachetes obscurus Mello-Leitao, 1932: CHILE: *Region IX* (*La Araucanía*): Malleco Province: Parque Nacional Nahuelbuta, near campsite, 37°49.744′S 73°00.418′W, 1126 m, 14.xi.2003, C. Mattoni, L. Prendini and J. Ochoa, 5 ♂ (CIM).

Centromachetes pocockii (Kraepelin, 1894): CHILE: *Region VII* (*Maule*): Malleco Province: Trail to Salto Rayén, next to Monumento Natural Contulmo, 38°01′07.896″S 73°09′54.720″W, 210 m, 10–11.i.2006, C. Mattoni and M. Vivanco, 1 ♂ (CIM).

Cercophonius squama (Gervais, 1843): AUSTRALIA: *Tasmania*: Mt. Barrow, 570 m, 15–17.ii.1980, A. Newton and M. Thayer, 3 ♂ (AMNH).

Lisposoma josehermana Lamoral, 1979: NAMIBIA: *Oshikoto Region*: Tsumeb District: Farm Varianto on Elandshoek 771, 19°22.773′S 17°44.456′E, 1500 m, 4.i.2004, L. Prendini, E. Scott, T. and C. Bird, Q. and N. Martins, 1 ♂ (CIM).

Orobothriurus tamarugal Ochoa et al., 2011: CHILE: *Region I* (*Tarapacá*): Tarapacá Province: La Tirana, 2 km W, 20°19′59.8″S 69°40′07.6″W, 999 m, 18. i.2005, C. Mattoni, A. Ojanguren and J. Ochoa, 2 ♂ (AMNH, MACN).

Phoniocercus pictus Pocock, 1893: CHILE: *Region La Araucanía*: Cautín Province: Fundo las Selva, N of Temuco and NW of Nueva Imperial, 750 m, 16–20.ii.1981, L.E. Peña, 1 ♂ (AMNH).

Rumikiru lourencoi (Ojanguren Affilastro, 2003): CHILE: *Region III* (*Atacama*): Huasco Province: Parque Nacional Llanos de Challe, near 'Administración', 28°09′39.8″S 71°03′20″W, 205 m, 25.i.2005, C. Mattoni and A. Ojanguren, UV full moon, 1 ♂ (CIM).

Tehuankea moyanoi Cekalovic, 1973: CHILE: *Región VIII* (*Bio Bío*): Arauco Province: Ramadilla, 18.i.1971, R. Donoso Barros, holotype ♂ (MZUC 567).

Thestylus aurantiurus Yamaguti and Pinto-da-Rocha, 2003: BRAZIL: *São Paulo*: Parque Estadual da Serra da Cantareira, 22–30.xi.2004, S. Favorito et al., 1 ♂ (MZUSP).

Timogenes dorbignyi (Guérin Méneville, 1843): ARGENTINA: *Córdoba Province*: Parque Provincial Chancaní, 20–23.xi.2001, C. Mattoni and J. Ochoa, 6 ♂ (CIM); Salinas Grandes, iii.2007, C. Mattoni, A. Peretti and D. Vrech, 4 ♂ (CIM).

Timogenes elegans (Mello-Leitao, 1931): ARGENTINA: *Córdoba Province*: Parque Provincial Chancaní, 1–25.iii.1994, C. Mattoni, 5 ♂ (CIM), 2004, M. Izquierdo, 3 ♂ (CIM).

Urophonius brachycentrus (Thorell, 1876): ARGENTINA: 13.vi.1993, S. Castelvetri, 2 ♂ (AVP).

Urophonius tregualemuensis Cekalovic, 1981: CHILE: *Region VII* (*Maule*): Cauquenes Province: Reserva Nacional Los Ruiles, NW of Cauquenes, 37°49.744′S 73°00.418′W, 146 m, 13.xi.2003, C. Mattoni, J. Ochoa and L. Prendini, 5 ♂ (CIM).

Vachonia martinezi Abalos, 1954: ARGENTINA: *Río Negro Province*: Balneario el Cóndor, S of Viedma, 5.v.2003, M. Magnanelli, 4 ♂ (CIM).

Family Buthidae C.L. Koch, 1837

Ananteris arcadioi Botero-Trujillo, 2008: COLOMBIA: *Meta Department*: Hacienda Con Esto Tengo, between Villavicencio and Restrepo, 04°11.855′N 73°35.620′W, 407 m, 7.ix.2008, R. Botero-Trujillo and J.A. Ochoa, 3 ♂ (AMNH).

Babycurus jacksoni (Pocock, 1890): TANZANIA: *Rufiji District*: Kichi Hills Forest Reserve, 08°14.333′S 38°39.014′E, 7.iii.2008, P. Hawkes, 1 ♂ (AMNH).

Buthus paris (C.L. Koch, 1839): ALGERIA: El Guetna, NW of Mascara, 550 m, 24.v.1981, E.S. Ross, 1 ♂ (CAS).

Hottentotta conspersus (Thorell, 1876): NAMIBIA: *Kunene Region*: Opuwo District: Blue Drum, 5 km SE on S bank of Ondondujengo River, 17°48.894′S 12°25.626′E, 841 m, 15.i.2004, L. Prendini, E. Scott, T. and C. Bird, Q. and N. Martins, 3 ♂ (AMNH).

Isometrus maculatus (DeGeer, 1778): KENYA: *Coast Province*: Kilifi District: Watamu Beach, 25.vii/8.viii.2008, S. Mwangi, 1 ♂ (AMNH).

Lychas obsti Kraepelin, 1913: KENYA: *Eastern Province*: Kibwezi County: Mtito Andei Division, near Tsavo National Park, 25.vii/8.viii.2008, S. Mwangi, 1 ♂ (AMNH).

Mesobuthus eupeus thersites (C.L. Koch, 1839): UZBEKISTAN: *Bukhara Area*: Gizhduvan District: SW foothills of Karatau Mountain Range, 14.5 km N of Kanimekh, 40°24.851′N 65°08.955′E, 396 m, 5.vi.2003. L. Prendini and A.V. Gromov, 1 ♂ (AMNH).

Microtityus waeringi Francke and Sissom, 1980: U.S.A.: *U.S. Virgin Islands*: St. John, Virgin Islands National Park, Lameshur Bay trail, parking lot and first few hundred meters of trail, 18°19.242′N 64°43.641′W, 22 m, L. Prendini and J. Huff, 17.x.2009, 1 ♂ (AMNH)

Parabuthus granulatus (Ehrenberg, 1831): SOUTH AFRICA: *Northern Cape Province*: Gordonia District: Farm Boksputs 462, 25 km SE Lutsputz on road to Keimoes, 28°33.368′S 20°50.427′E, 869 m, 8.i.2004, I. Engelbrecht and B. Watkins, 1 ♂ (AMNH).

Pseudolychas ochraceus (Hirst, 1911): SOUTH AFRICA: *Limpopo Province*: Potgietersrus District: Makapansgat, 1981, A. Harington, 1 ♂ (AMNH [AH 2263]).

Reddyanus assamensis (Oates, 1888): INDIA: *West Bengal*: 25 mi. from Calcutta, 11.vi.1966, N.K. Paul, 1 ♂ (CAS [Stahnke coll. 66-862]).

Teruelius ankarana (Lourenço and Goodman, 2003): MADAGASCAR: *Antsiranana Province*: Andrafiabe, 2.6 km E, 12°57.523′S 49°07.189′E, 4/6.i.2007, A.H. Kirk-Springs, 3 ♂ (AMNH).

Tityus elii Armas and Marcano Fondeur, 1992: DOMINICAN REPUBLIC: *La Vega Province*: Loma Casabito, Reserva Científica Ébano Verde, along road to transmitter station, 19°02′15.3″W 70°31′06.5″W, 1478 m, 18.vii.2004, E.S. Volschenk and J. Huff, 3 ♂ (AMNH).

Zabius fuscus (Thorell, 1876): ARGENTINA: *Córdoba Province*: Cuesta Blanca, 3 ♂ (AVP).

Family Caraboctonidae Kraepelin, 1905

Caraboctonus keyserlingi Pocock, 1893: CHILE: *Region IV* (*Coquimbo*): Elqui Province: Monte Grande, Elqui Valley, 27.ii.2004, J. Pizarro, J. Ochoa and C. Mattoni, 1 ♂ (AVP), 2 ♂ (CIM).

Hadruroides lunatus (L. Koch, 1867): PERU: *Lima Department*: Lima Province: Cieneguilla, 6 km W, ca. 400 m, 30.xii.1975, O.F. Francke, 4 ♂ (AMNH).

Family Chactidae Pocock, 1893

Brotheas sp.: GUYANA: *Bartica District*: Kartabo, 1922, 1 ♂ (AMNH).

Broteochactas nitidus Pocock, 1893: TRINIDAD AND TOBAGO: Blanchisseuse valley, northern Trinidad, 1.xi.1979, R. Mendez and J. Boos, 1 ♂ (AMNH).

Chactas aequinoctialis (Karsch, 1879): COLOMBIA: Sierra Nevada de Santa Marta, San Sebastián de Rabago, 2000 m, 1–10.iv.1968, B. Malkin, 1 ♂ (AMNH).

Chactopsoides anduzei (González-Sponga, 1982): VENEZUELA: *Amazonas*: Autana Municipality: Isla Ratón (S part), Orinoco River, 05°03.882′N 67°49.000′W, 81 m, 7.viii.2009, F. Rojas-Runjaic, A. Ferrer and J.A. Ochoa, 1 ♂ (AMNH).

Nullibrotheas allenii (Wood, 1863): MEXICO: *Baja California Sur*: Sierra de la Laguna nucleus zone, 23°33′16″N 109°59′27.4″W, 1800 m, 20.vii.2004, E. González, A. Valdez, W. Savary and O. Francke, ♂ (AMNH).

Teuthraustes sp.: ECUADOR: *Aguay Province*: Susudel, ca. San Felipe de Oria, 03°24.339′S 79°10.581′W, 2324 m, 11.ii.2008, 1 ♂ (AMNH).

Uroctonus mordax Thorell, 1876: U.S.A.: *Oregon*: Douglas County: Canyonville, 4 mi. S, 4.v.1936, G. Ferguson, 1 ♂ (AMNH).

Family Chaerilidae Pocock, 1893

Chaerilus julietteae Lourenço, 2011: VIETNAM: *Binh Thuan Province*: Ham Thuan Nam District: Ta Cu/Ta Kou Mountain Nature Reserve, Nui Ta Cu/Ta Kou below summit, above reclining Buddha, 10°48′58″N 107°53′46.1″E, ca. 500 m, 17.xi.2012, L Prendini, 1 ♂ (AMNH).

Chaerilus variegatus Simon, 1877: INDONESIA: Java, 1 ♂ (MACN).

Family Diplocentridae Karsch, 1880

Cazierus garridoi Armas, 2005: U.S.A.: *Puerto Rico*: Isla Mona, Trail #1 to Punta Capitan from Sardiniera, 18.08823°N 67.93815°W, L. Esposito and H. Yamaguti, 19.x.2008, 1 ♂ (AMNH).

Diplocentrus lindo Stockwell, 2001: U.S.A.: *Texas*: Jeff Davis County: David Mountains State Park, Limpia Canyon Campground, 5.vi.1974, L. Draper, M.A. Cazier and O.F. Francke, 1 ♂ (AMNH).

Nebo hierichonticus (Simon, 1872): ISRAEL: *Haifa District* (*Mehoz*): Mount Carmel National Park, Ha'Agam Campsite, upper Nahal Oren, 32°43′24″N 35°00′33″E, 219 m, 22.viii.2011. L. Prendini, T.L. Bird and E. Gefen, 1 ♂ (AMNH).

Family Euscorpiidae Laurie, 1896

Megacormus gertschi Diaz Nájera, 1966: MEXICO: 1 ♂ (AMNH).

Megacormus sp.: MEXICO: *Veracruz*: road to Tlaquilpan, 18°38.507′N 97°06.425′W, 2125 m, 19.vii.2002, L. Prendini and O.F. Francke, 1 ♂ (AMNH).

Tetratrichobothrius flavicaudis (DeGeer, 1778): FRANCE: *Hérault*: Lodève, 1969, B. Monroy, 1 ♂ (MACN).

Family Hemiscorpiidae Pocock, 1893

Hemiscorpius lepturus Peters, 1861: IRAN: *Khoozestan Province*: Baghmalek, Karbalai Ghasem village, 31°27′24″N 49°57′37″E, 285 m, vii.2007, Salari, 1 ♂ (AMNH [LP 9711]).

Family Heteroscorpionidae Kraepelin, 1905

Heteroscorpion goodmani Lourenço, 1996: MADAGASCAR: *Toliara Province*: Parc National Andohahela, Tonatana, 33.3 km NW Tolagnaro, 24°45′31″S 46°51′13″E, 275 m, 22.xi.2006, B.L. Fisher, BLF 15101, 1 ♂ (CAS ENT 9026922).

Family Hormuridae Laurie, 1896

Hadogenes troglodytes (Peters, 1861): MOZAMBIQUE: *Tete Region*: Moatize District: Kapanga, hills overlooking village on S bank Rovubwe River, 06°07′15″S 33°39′48″E, 212 m, 10.xii.2007, L. Prendini and W.R. Schmidt, 1 ♂ (AMNH).

Hormurus sp.: AUSTRALIA: *Queensland*: Mt. Tozer Range, northern foot, 1 ♂ (MACN).

Opisthacanthus capensis Thorell, 1876: SOUTH AFRICA: *Western Cape Province*: 1 ♂ (CIM).

Opisthacanthus valerioi Lourenço, 1980: COSTA RICA: Isla de Coco, 4.iii.2002, A. Peretti, 1 ♂ (AVP).

Family Iuridae Thorell, 1876

Protoiurus asiaticus (Birula, 1903): 1 ♂ (ZMB).

Family Pseudochactidae Gromov, 1998

Troglokhammouanus steineri Lourenço, 2007: LAOS: *Khammouane Province*: Boualapha District: Hin Namno National Biodiversity Conservation Area, Tham Xe Bang Fai (Xe Bang Fai River Cave), left bank of Xe Bang Fai River (coming from downstream entrance), 17°22′23.6″N 105°50′11.8″E, 159 m, 19–21.ii.2012, L. Prendini, P. Kanyavong and W. Phimmathong, 1 ♂ (AMNH).

Family Scorpionidae Latreille, 1802

Pandinus imperator (C.L. Koch, 1841): pet trade, 1 ♂ (AVP).

Scorpio fuliginosus (Pallary, 1928): MOROCCO: *Marrakech Province*: Arbalow Ourika, 1000 m, 14–18.v.1975, B. Malkin, 1 ♂ (AMNH).

Family Scorpiopidae Kraepelin, 1905

Euscorpiops longimanus (Pocock, 1893): 1 ♂ (AMNH).

Scorpiops zubairi Kovařík, 2020: (Gervais, 1843): PAKISTAN: Murree, 33°55′N 73°726′E, 7500 ft, 29. vi.1967, B.L. Haines, 1 ♂ (MCZ)

Troglocormus ciego Francke, 1981: MEXICO: *San Luis Potosí*: Cueva de Elias, 13 km N Agua Buena, 3.viii.1975, D. McKenzie, holotype ♂ (AMNH).

Family Superstitioniidae Stahnke, 1940

Superstitionia donensis Stahnke, 1940: U.S.A.: *Nevada*: Clark County: 8.7 mi. SW Hwy 95 on Hwy 156, on E slopes of Spring Mountains, 1800 m, 14.viii.1991, S.C. Williams, V.F. Lee and R.C. Bechtel, UV, 1 ♂ (CAS).

Family Troglotayosicidae Lourenço, 1998

Troglotayosicus humiculum Botero-Trujillo and Francke, 2009: COLOMBIA: *Nariño Department*: Ricaurte Muncipality: Vereda Alto Cartagena, Finca Nueva Estrella, 01°13′15.7″N 77°58′08.6″W, 1617 m, 12.ix.2008, R. Botero-Trujillo, J.P. Botero, and J.A. Ochoa, 1 ♂ (MPUJ ENT 46835 old SCO 399).

Family Typhlochactidae Mitchell, 1971

Alacran tartarus Francke, 1982: MEXICO: *Oaxaca*: Sotano Li Nita, Huautla de Jimenez, 812 m, 29. iii.1980, B. Steele and S. Zeman, 1 ♂ (AMNH).

Typhlochactas mitchelli Sissom, 1988: MEXICO: *Oaxaca*: Município de San José Tenango: Cerro Ocote [18°08′57.5″N 96°43′59.1″W], 5 mi. S San José de Tenango, iv.1987, A. Grubbs, A. Cressler and P. Smith, holotype ♂, paratype ♂ (AMNH).

Family Urodacidae Pocock, 1893

Urodacus butleri Volschenk et al., 2012: AUSTRALIA: *Western Australia*: Barrow Island, 20°46′S 115°24′E, 9.ii.1977, W.H. Butler, K. Edwards and N. Gunawardene, paratype ♂ (AMNH).

Urodacus planimanus Pocock, 1893: AUSTRALIA: *Western Australia*: Jarrahdale, 20.iii.1998, E.S. Volschenk, under stone, 1 ♂ (AMNH).

Family Vaejovidae Thorell, 1876

Graemeloweus glimmei (Hjelle, 1972): U.S.A.: *California*: Lake County: ca. 5 mi. N Rayhouse Rd, at junction of David and Cacje Creeks, 900 m, 15.vi.1969, J.T. Hjelle and M. Bolander, 1 ♂ (CAS).

Mesomexovis variegatus (Pocock, 1898): MEXICO: *Morelos*: Xochitepec, 20.x.1974, O. Martínez, 1 ♂ (MACN).

Paravaejovis spinigerus (Wood, 1863): U.S.A.: *Arizona*: Pima County: Madera Canyon, Santa Rita Mountains, 14.vii, M.A. Cazier and J. Bigelow, roadcuts, 1 ♂ (AMNH).

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