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Sperm package morphology in scorpions and its relation to phylogeny

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Sperm packages are widespread in the order Scorpiones, but absent in the family Buthidae. The morphology of sperm packages is diverse and apparently has phylogenetic information. The objectives of this work were to show diversity of sperm packages and to provide a quantitative basis for using sperm packages' morphology as a taxonomic character. For this, we conducted a morphological analysis and comparison of the different sperm packages of species of the family Bothriuridae. The seminal content from males of species of Bothriuridae was studied. Specimens from Iuridae, Buthidae, Euscorpiidae, Liochelidae, Scorpionidae, Vaejovidae, Chaerilidae, and Chactidae were used for comparison. Digital images of sperm packages were measured and statistically analysed based on the following variables: total length, head width, head–body angle, total area, and head length. Pairs of variables were also contrasted, and all the variables were correlated with the current phylogenetic hypothesis for Bothriuridae. High morphological diversity and variability in measures was observed. In general, measurements were similar within each genus, but differed amongst genera. Cane-like sperm packages are very common in species of the family Bothriuridae. Species from *Bothriurus* show a wide range of sperm package shapes, some of them shared with *Timogenes* and *Vachonia* species, supporting the idea of nonmonophyly of the genus. Many species showed sperm package dimorphism inside a single male. Some of the analysed features fit well with the phylogenetic hypothesis in Bothriuridae, and the general package shape shows high correlation with scorpion phylogeny in other families. Bent and round packages are the most common amongst the different families. Sperm packages are not developed in Chaerilidae, as in Buthidae. This is the first morphological and comparative analysis of sperm packages in scorpions, and reveals much greater diversity in this trait than previously known. Our results reinforce the idea that the study of morphology of sperm packages would contribute characters for scorpion phylogeny at different levels.

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ADDITIONAL KEYWORDS: Bothriuridae – character variation – consistency index – distance method – evolutionary trends – morphological comparison – polymorphic characters – reproductive biology – sperm aggregation.

INTRODUCTION

Scorpion spermatozoa present a very similar morphology amongst species (André, 1959, 1963; Cruz Landin & Ferreira, 1972, 1973; Hood *et al*., 1972; Jespersen & Hartwick, 1973; Phillips, 1974). They show a filiform-flagellate and elongated organization divided into three parts (head, middle piece, and flagellum; see Jespersen & Hartwick, 1973; Alberti, 2000; Michalik & Mercati, 2010). All these parts show structural differences, such as the organization of the axoneme or the acrosomal complex (Michalik & Mercati, 2010). Besides, there seem to be differences in the morphology of the flagellar axonemes between Buthidae and other analysed families (Vignoli, Klann & Michalik, 2008; Michalik & Mercati, 2010; Peretti, 2010).

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Many scorpion species present spermatozoa gathered in sperm packages in the male genital system (end of testes tubes and seminal vesicle) and in the spermatophores (Peretti & Battán-Horenstein, 2003; Vignoli *et al*., 2008; Michalik & Mercati, 2010). The presence of sperm packages was first described by Jespersen & Hartwick (1973) in *Hadrurus arizonensis* Ewing, 1928 (Iuridae). Unfortunately, very little information was provided in that contribution. Thirty years later Peretti & Battán-Horenstein (2003) reported their presence in some species of Bothriuridae. Recent studies indicate that sperm packages are widely distributed in the order Scorpiones (Mattoni, 2003; Michalik & Mercati, 2010). Their presence has been shown in species of a wide variety of families; e.g. Bothriuridae (Peretti & Battán-Horenstein, 2003), Troglotayosicidae (Vignoli *et al*., 2008), Scorpionidae (Michalik & Mercati, 2010), and Euscorpiidae (Althaus *et al.,* 2010).

To date, sperm packages have not been observed in the family Buthidae (Cruz Landin & Ferreira, 1973; Alberti, 1983; Peretti & Battán-Horenstein, 2003). Buthidae is considered a basal family in the order (Stockwell, 1989; Sissom, 1990; Prendini, 2000; Coddington *et al*., 2004; Prendini *et al*., 2006), without any closely related families (Prendini, 2000, 2003; Volschenk, Mattoni & Prendini, 2008). It is unknown if sperm packages are present in Pseudochactidae and Chaerilidae, two other families also considered basal (Stockwell, 1989; Coddington *et al*., 2004; Prendini *et al*., 2006). Data are also lacking from other scorpion families (e.g. Chactidae, Vaejovidae, and Liochelidae).

The morphology of sperm packages is highly variable and apparently has phylogenetic information in the family Bothriuridae Simon, 1880 (Mattoni, 2003) and some other families (Vignoli *et al*., 2008; Michalik & Mercati, 2010). Studies have shown that bothriurids have either curved, coiled, or cane-like sperm packages and these differences appear mainly amongst species (Mattoni, 2003; Peretti & Battán-Horenstein, 2003). Spermatozoa seem to be clustered together in a gathering matrix of not-yet-determined material, with no secretion sheath around them (Vignoli *et al*., 2008; Michalik & Mercati, 2010). Various authors have claimed that these packages contain around 70, 100 (Peretti & Battán-Horenstein, 2003), 150 (Vignoli *et al*., 2008), or 250 spermatozoa (Michalik & Mercati, 2010).

The exact function of sperm packages is still unknown. Peretti & Battán-Horenstein (2003) suggested that the presence of sperm packages may be related to the method of transferring sperm. Althaus *et al*. (2010) found sperm packages to be the structural material of genital sperm plugs in Euscorpiidae.

Despite these studies, the knowledge of sperm packages in scorpions is remarkably poor (Vignoli *et al*., 2008). In general, little is known about true dimensions, variability, and detailed morphology (sizes, angles, etc.) of sperm packages. The main goal of this comparative study was to make the first morphological analysis of sperm packages in scorpions, focused mostly on Bothriuridae, but also analysing and comparing other families. To accomplish this task the general morphology of sperm packages was described in selected species from the Bothriuridae family.

The main forms and designs were identified and described, and qualitative and quantitative analyses of data were performed. In addition, we report the presence and shape of sperm packages in species of Vaejovidae, Euscorpiidae, Chactidae, Iuridae, Liochelidae, and Chaerilidae, in some of these species for the first time. We also present new information to evaluate the monophyly of the genus *Bothriurus*, and assess if sperm package shape could be species-specific.

MATERIAL AND METHODS

STUDIED SPECIES

Sperm packages of 11 genera of Bothriuridae were analysed (32 species), as well as members of several scorpion families. In the genus *Bothriurus* Peters we increased the taxon sampling as this is the most morphologically diverse genus of the family Bothriuridae (Maury, 1980, 1981; Mattoni, 2003; Ojanguren Affilastro, 2005). Species from different subgenera and species groups were observed. Species of nonbothriurid families were used as outgroups to compare general shape of sperm packages, and to provide a first look at presence of sperm packages and diversity of shapes in different families.

Analysed specimens (classification according to Prendini & Wheeler, 2005)

Family Bothriuridae: *Bothriurus* (*Bothriurus*) *araguayae* Vellard, 1934, *Bothriurus* (*Bothriurus*) *asper* Pocock, 1893, *Bothriurus* (*Bothriurus*) *bonariensis* (C. L. Koch, 1842), *Bothriurus* (*Bothriurus*) *chacoensis* Maury & Acosta, 1993, *Bothriurus* (*Bothriurus*) *rochai* Mello-Leitão, 1932, *Bothriurus* (*Bothriurus*) sp., *Bothriurus* (*Bothriurus*) *bocki* Kraepelin, 1911, *Bothriurus* (*Bothriurus*) *inermis* Maury 1981, *Bothriurus* (*Andibothriurus*) *coriaceus* Pocock, 1893, *Bothriurus* (*Andibothriurus*) *keyserlingi* Pocock, 1893, *Bothriurus* (*Andibothriurus*) *rochensis* San Martín, 1965, *Bothriurus* (*Andibothriurus*) *flavidus* Kraepelin, 1911, *Bothriurus* (*Andibothriurus*) *cordubensis* Acosta, 1995, *Bothriurus* (*Andibothriurus*) *noa* Maury, 1984, *Bothriurus* (*Andibothriurus*) *burmeisteri* Kraepelin, 1894, *Bothriurus* (*Andibothriurus*) *olaen* Acosta, 1997, *Brachistosternus* (*Leptosternus*) *angustimanus* Ojanguren Affilastro & Roig Alsina, 2001, *Brachistosternus* (*Leptosternus*) *pentheri* Mello-Leitão, 1931, *Brachistosternus* (*Ministernus*) *ferrugineus* Thorell, 1876, *Centromachetes obscurus* Mello-Leitão, 1932, *Centromachetes pocockii* (Kraepelin, 1894), *Cercophonius squama* (Gervais, 1843), *Lisposoma josehermana* Lamoral, 1979, *Orobothriurus* sp., *Orobothriurus lourencoi* Ojanguren Affilastro, 2003, *Tehuankea moyanoi* Cekalovic, 1973, *Thestylus aurantiurus* Yamaguti & Pinto-da-Rocha, 2003, *Timogenes dorbignyi* (Guérin Méneville, 1843), *Timogenes elegans* (Mello-Leitão, 1931), *Urophonius brachycentrus* (Thorell, 1876), *Urophonius tregualemuensis* Cekalovic, 1981, *Vachonia martinezi* Abalos, 1954; family Euscorpiidae: *Megacormus gertschi* Díaz Najera, 1966, *Megacormus* sp., *Euscorpius* (*Tetratrichobothrius*) *flavicaudis* (DeGeer, 1778); family Iuridae: *Caraboctonus keyserlingi* Pocock, 1893; family Buthidae: *Zabius fuscus* (Thorell, 1876); family Chactidae: *Chactas aequinoctialis* (Karsch, 1879); family Chaerilidae: *Chaerilus variegatus* Simon, 1877; family Liochelidae: *Liocheles* sp., *Opisthacanthus capensis* Thorell, 1876, *Opisthacanthus valerioi* Lourenço, 1980; family Scorpionidae: *Pandinus* (*Pandinus*) *imperator* (C. L. Koch, 1841), *Scorpio maurus* Linnaeus, 1758; family Vaejovidae: *Vaejovis spinigerus* (Wood, 1863), *Vaejovis variegatus* Pocock, 1898.

Scorpion specimens collected personally were found by turning stones during the day or by ultraviolet (UV) light detection at night (Stahnke, 1972). Portable UV lamps with mercury vapour tubes or Maglite torches with an UV light emitting diode attachment were used for the fieldwork. Full lists of the material analysed and the collections are shown in the Appendix.

General procedure for description of sperm packages

The seminal content was extracted from the seminal vesicle of the left paraxial organ of each male in media containing 80% ethanol (Peretti & Battán-Horenstein, 2003). Temporary microscope slide preparations using glycerine were created by carefully disaggregating the seminal content with fine-tip forceps and histological needles. We used glycerine because sperm packages were easier to disaggregate and the media did not dry easily as with alcohol. It is very important to note that glycerine and alcohol do not affect the general shape of sperm packages. Comparisons between fresh and preserved material were carried out for *Ti. elegans*, *Ti. dorbignyi*, *Br. ferrugineus*, *Br. pentheri*, *Bo. bonariensis*, and *Bo. cordubensis*, and no differences were found. All specimens studied for this paper were previously fixed in 80% ethanol. This is very relevant because some of the specimens were from collections; some of them had been fixed for many years. This is a potent tool to compare amongst different species, some of which can be very difficult to find alive. Pictures were taken under a light microscope using a Sony Cyber-shot DSC-S40 digital camera and analysed with the computer program Image Tool 3.0 (UTHSCSA, 2002). Digital pictures of ten sperm packages per male were taken to examine the main traits included for general description and subsequent analyses of measurements. The following terminology was used for the sites measured: for the head we took into account the frontal border, dorsal arcs (convex or not), head–body angle, and frontal border angle (Fig. 1B).

In addition, one seminal vesicle of *Ti. elegans* was fixed in 4% formaldehyde-2% glutaraldehyde in cacodylate buffer (pH 7.3, 0.1 M) for 2 h, and then post-fixed with osmium tetroxide at 1% in the same buffer. After dehydration in increasing concentrations of acetone, samples were embedded in Araldite and polymerized at 60 °C. Transversal thin sections of sperm packages were obtained with a diamond knife on a JEOL JUM-7 ultramicrotome and photographed in a Zeiss LEO 906E transmission electron microscope. Transverse cuts of sperm packages were photographed and spermatozoa were counted.

Analysis of sperm package measurements

Intraspecific and interspecific variability

The following five variables were examined: total length, head width, head–body angle, area, and head length (Fig. 1A). For the head we used the definitions in Figure 1B for the general description in Table 1. A mean value of each variable was taken per male ($N = 10$ sperm packages per individual) to measure intraspecific population variability. These mean values were also used to examine differences amongst species in the species with five or more males analysed. One-way ANOVA was used to test both types of differences. Significant differences between means were analysed by a Tukey's a posteriori test. *Bothriurus asper* and *Bo. rochai* were not included in the analyses because they lack sperm packages. *Bothriurus inermis* and *Bo. bocki* packages were not measured because the bent shape of the packages prevented determination of where the head and/or the tail ended as well as the total length. In *Timogenes* packages, only the unfolded ones were measured. All the statistical analyses were performed with NCSS 2007 (http://www.ncss.com/).

Bivariate correlations

The following four sets of variables were compared: (1) package total length vs. package area; (2) package

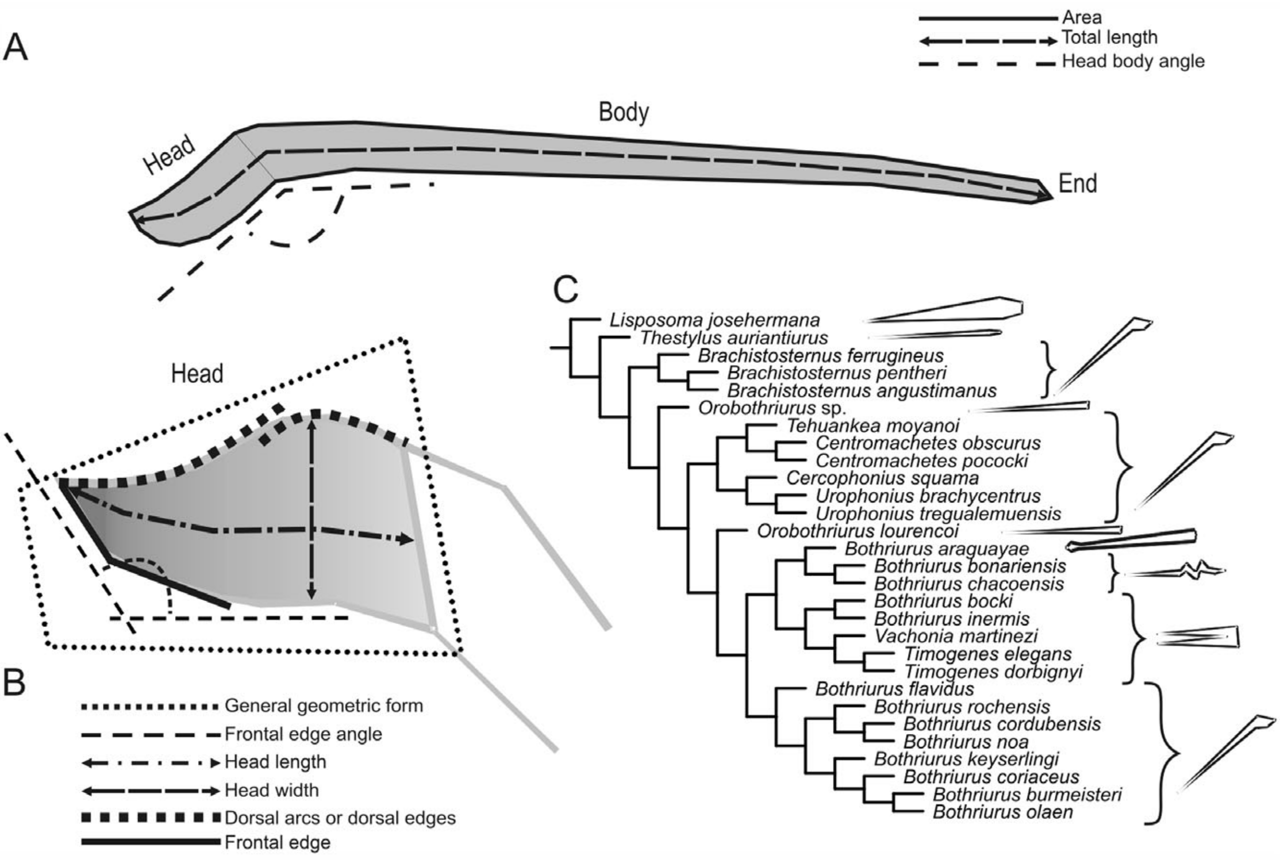

Figure 1. Measures taken from sperm packages of scorpions (Bothriuridae). A, schematic drawings of a general complete sperm package showing body measures used in quantitative analysis. B, schematic drawing of a head of a sperm package indicating general measures from both quantitative and qualitative analysis. C, phylogenetic organization of shapes of sperm packages found in analysed species from the Bothriuridae family (based on Mattoni, 2003; Mattoni & Prendini, 2007). On the right side the general geometric form is represented.

total length vs. male size (using the prosomal length as an indicator of body size); (3) package total length vs. package head length; and (4) ratio of package head length to package total length. In all of these comparisons, a Spearman correlation test was used, except for the last set, in which a simple division between package head length and package total length was performed. All the examined variables were $\log_{10}$ transformed prior to analysis in order to make magnitudes comparable.

Cluster analysis

The average per species for the variable was used to group species in a cluster analysis using 'group average linkage' with the Euclidean distance method. Besides, the range scaling method was used to form comparable units without need of mathematical data transformation. A qualitative nominal variable was added symbolizing sperm package general geometric form (Fig. 1C).

Fit to the phylogenetic hypotheses

Using the current phylogenetic hypotheses available (Mattoni, 2003; Mattoni & Prendini, 2007), a cladogram was built for the Bothriuridae species studied. For each variable we created intervals (mean ± one standard deviation), as recommended in Goloboff, Mattoni & Quinteros (2006), for all analysed species of this family. As explained above, species from *Bo. asper*, *Bo. rochai*, *Bo. inermis*, and *Bo. bocki* were excluded. These obtained values (as continuous characters) and the cladogram were entered into TNT 1.1 phylogenetic software (Goloboff, Farris & Nixon, 2008). The values were optimized over the cladogram using parsimony (Farris, 1970) and the hypothetical values were registered for internal nodes (hypothetical ancestors) (Goloboff *et al.*, 2006). The level of adjustment of each variable to the cladogram was measured by the consistency index (CI). The index ranges from 0 (poor adjustment) to 1 (perfect adjustment), and it is calculated dividing the minimum

Table 1. Qualitative general characteristics of sperm packages from analysed species of the Bothriuridae family

	General shape	Head shape	Dorsal border	Frontal border	Frontal border angle	Body	Polymorphism	Particularities
Brachistosternus ferrugineus	Cane	Knife-like	Straight or slightly concave	Roundish or inclined	> 90°	Thick it thins smoothly	Width	Division between head and body is in front of the head body angle place
Brachistosternus pentheri	Cane	Undulated, fine tip	Dorsal arcs concave and slightly convex or totally straight	Slightly inclined pointed end	> 90°	Thick it thins smoothly	Width	Division between head and body is in front of the head body angle place
Brachistosternus angustimanus	Cane	Trapezoidal	Straight or slightly convex	Short and vertical	~90°	Long it thins smoothly	Width	–
Centromachetes	Cane	Trapezoidal	Convex. The concavity is absent or is reduced to a notch in the tip	Lacking round end	> 90°	Long	–	Showed longest packages of all analysed species
Urophonius	Cane	Undulated, round tip	Dorsal arcs concave & convex forming an ‘S’	Roundish or inclined upwards	> 90°	Long thins smoothly. *U. brachycentrus* has it first part undulated	Width	*U. tregualemuensis* has thicker and more compact heads than *U brachycentrus*
Tehuankea moyanoi	Cane	Trapezoidal	Dorsal arcs slightly concave and convex or straight border	Roundish	> 90°	Long	–	–
Bothriurus (Andibothriurus)	Cane	Trapezoidal or triangular	Dorsal arcs not very marked or with convex dominant and poor notorious concave	Inclined	> 90°	Thins sharply at the end	–	–
Bothriurus araguayae	Straight thick	Thick rectangular	Straight	Roundish	> 90°	Thick	Width	Tail with pentagonal form end
Bothriurus bonariensis	Helicoidally in first third of body	Thin rectangular	Straight	Inclined	> 90°	Fine and uniform	–	–
Bothriurus inermis	Middle bent, circular	Thick rectangular	Straight	Roundish	> 90°	Thick	Shape	Packages similar to *Timogenes* in shape but smaller
Bothriurus rochai and *asper*	Lacks sperm packages	–	–	–	–	–	–	–
Timogenes, Vachonia	Middle bent, straight, circular	Thick rectangular	Straight	Roundish	> 90°	Very thick	Shape	Roundish package end
Cercophonius squama	Straight, bent dorsally	Bullet head like	Straight or slightly concave	Roundish	> 90°	Wide	Both	Division between head, body and tail very notorious
Lisposoma josehermana	Straight, very thick	?	?	?	?	Thick	–	Head shape unknown. It always appeared broken
Orobothriurus lourencoi	Straight thin	Squared	Straight	Straight	= 90°	Fine	–	Very little with not very notorious head
Orobothriurus sp.	Straight thin	Lanceolated	Straight or with slight dorsal arcs	Slightly inclined	> 90°	Fine	–	–
Thestylus aurantiurus	Straight thin	Fine straight	Convex	Inclined	> 90°	Uniformly fine	–	–

possible number of transformations of a character in a cladogram by the number actually seen on it (Kluge & Farris, 1969).

In addition, an analysis of the evolution of characters was performed, analysing species and the changes or patterns of change of these characters seen in the optimization of the values in the cladogram.

RESULTS

Morphology of sperm packages

Qualitative description

The cane-like form of sperm package is the most common shape amongst all the species studied in the family Bothriuridae (55%; Figs 1C, 2A–I). This type of sperm package has a head–body angle between 90 and 140°. Another common type is the folded package seen in *Timogenes*, *Vachonia*, and some *Bothriurus* (approximately 26% of studied Bothriuridae species; Figs 1C, 2J, N). These species also have ring-like packages that seem to be more common in some species than in others (e.g. in *Bo. inermis*; Fig. 2K). This type of sperm package is also common in other families as seen below. The main characteristics of the sperm packages in Bothriuridae species are summarized in Table 1.

Other families have packages that are folded in the middle, similar to those of *Timogenes* and related species cited before (see below for more information). In *Ti. elegans* the sperm packages have approximately 250 (244 ± 35, $N = 14$ packages) spermatozoa (Fig. 3). Figure 3A shows a *Ti. elegans* sperm package in cross section, clearly revealing the alignment of the spermatozoa, with all the nuclei together (in dark black) and the end pieces with axonemes in the other half. Figure 3B exhibits a section of the middle pieces with the axonemes surrounded by mitochondria, these being more developed in one half of the package than in the other, perhaps this developed half contains the ends of the middle pieces. Finally, Figure 3C displays a section of the middle pieces that are bent in the sperm package.

Another common shape was the spherical shape (round or ovoid) that could possibly be derived from the folded package (Fig. 4A, B). *Chactas aequinoctialis* (Chactidae) has oval sperm packages; some of them resemble a lemon in shape (Fig. 4A). *Megacormus gertschi* and *Megacormus* sp. (Euscorpiidae) show only spherical packages (Fig. 4B). The other analysed Euscorpiidae, *Euscorpius flavicaudis,* has two types: spherical and folded packages, the latter with a broad and trapezoidal head (Fig. 4C). The iurid *Ca. keyserlingi* has straight, long, and broad sperm packages (Fig. 4D). The exact shape of the head was not completely visible because it always appeared broken or opened (probably the process of spermatozoa separation was beginning there). The first third of the package (behind the open head) is smoother and more compact. The body in general seems wider than the neck (zone that follows the opened anterior portion of the package), and the tail looks like an arrow tip. Many very thin packages are usually observed accompanying the broader ones (see below).

In Liochelidae, *Opisthacanthus* shows folded packages and *Liocheles* sp. has folded and ring-like packages as well (Fig. 4E). The appearance of both ring-like and folded packages is repeated in many species with folded packages, like for example *Timogenes* and associated species. In the scorpionids, *Scorpio maurus* has folded packages, uniform in width, very similar to those of *Timogenes*, but *Pandinus imperator* also presents straight slim packages, apparently as a result of unfolding (Fig. 4F). *Vaejovis variegatus* has folded packages, with rounded heads, with the last third of the body smoother and slightly slimmer than the first part, which appears to be broader (Fig. 4G). *Zabius fuscus* (Buthidae) and *Chaerilus variegatus* (Chaerilidae) lack sperm packages (Fig. 4H, I). In these species all spermatozoa are free and form a single sperm mass. The same occurs in the bothriurids *Bo. rochai*, *Bo.* sp., and *Bo. asper.*

Sperm package polymorphism in single males

Males from 12 species possessed different sperm packages in the same specimen. Some species presented differences in sperm package width: *Br. ferrugineus, Br. angustimanus, Br. pentheri, Bo. araguayae, Cercophonius squama, Urophonius tregualemuensis,* and *Ca. keyserlingi* (Fig. 5 A–D). By contrast, *Ti. elegans, Ti. dorbignyi, Vachonia martinezi, Bo. bocki,* and *Bo. inermis* presented differences in shape or bending (Fig. 5E a, b, c). Species that showed differences in their width had some packages markedly thinner than the regular ones (Fig. 5A–D).

Timogenes dorbignyi, *Ti. elegans*, *Bo. bocki*, *Bo. inermis*, and *V. martinezi* showed variability in package bending position inside the sperm vesicle of the same male (Fig. 5E). There were three different shapes: folded in the middle, very common in *Timogenes, V. martinezi*, and *Bo. inermis*, rare in *Bo. bocki* (Figs 2J, 2N, 5E a); straight, unfolded, present in low numbers in all these species (Fig. 5E b); and circular, ring-like, common in *Bo. bocki*, rare in the other species (Figs 2K, 5E c).

The bothriurid *Cer. squama* has both straight and bent sperm packages in the same male. A lower number of packages are straight, and the majority of them are bent in the middle (Fig. 2P). *Timogenes* sperm packages gave the impression of having almost

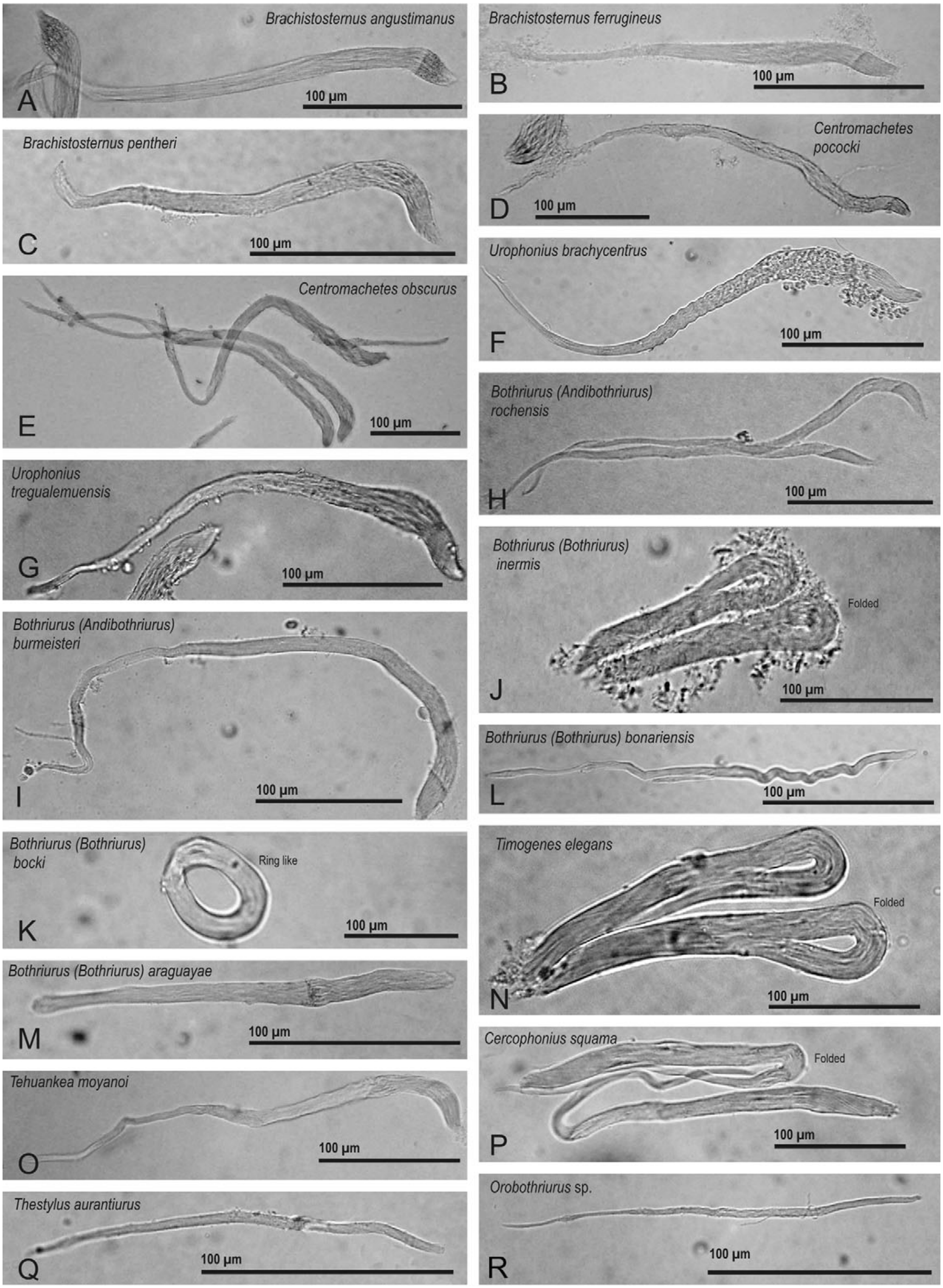

Figure 2. Sperm packages of different species of the family Bothriuridae. A, *Brachistosternus* (*Leptosternus*) *angustimanus*; B, *Brachistosternus* (*Ministernus*) *ferrugineus*; C, *Brachistosternus* (*Leptosternus*) *pentheri*; D, *Centromachetes pococki*; E, *Centromachetes obscurus*; F, *Urophonius brachycentrus*; G, *Urophonius tregualemuensis*; H, *Bothriurus* (*Andibothriurus*) *rochensis*; I, *Bothriurus* (*Andibothriurus*) *burmeisteri*; J, *Bothriurus* (*Bothriurus*) *inermis*; K, *Bothriurus* (*Bothriurus*) *bocki*; L, *Bothriurus* (*Bothriurus*) *bonariensis*; M, *Bothriurus* (*Bothriurus*) *araguayae*; N, *Timogenes elegans*; O, *Tehuankea moyanoi*; P, *Cercophonius squama*; Q, *Thestylus aurantiurus*; R, *Orobothriurus* sp.

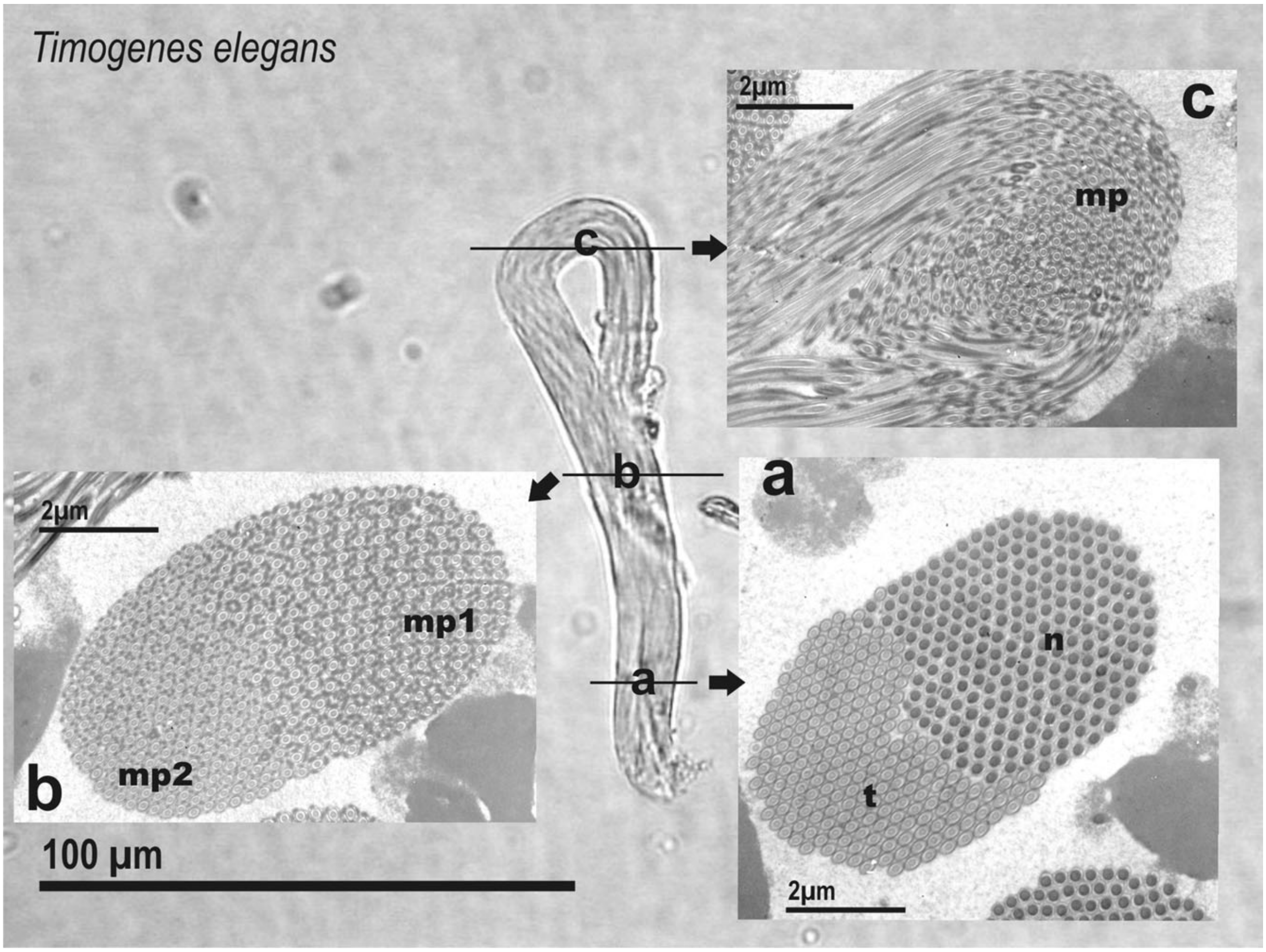

Figure 3. Sperm package of *Timogenes elegans.* Light microscope photograph showing the approximate sections of the transmission electronic micrographs. A, head and tail; B, middle pieces and limit between middle pieces and tails; C, bending point. Abbreviations: mp, middle pieces; mp1, first portion of middle pieces; mp2, final part of middle pieces; n, nuclei; t, tails.

equal width along the whole package, but in *Cer. squama* the body width becomes much thinner towards the distal end of the package.

Variables and main measures in Bothriuridae

Some variables show more variability than others (Fig. 6A–E), some with great SD (Fig. 6C). Contrary to *Timogenes* and *Centromachetes*, which have sperm packages of almost the same total length amongst species within each genus, *Bothriurus* and *Brachistosternus* species exhibit great variability in total sperm package length. For example, *Bo. noa* has the longest sperm packages whereas *Bo. araguayae* has the shortest of all analysed *Bothriurus*, the former being approximately 1.70 times longer. Nevertheless, *Bo. araguayae* possesses the longest head of all analysed *Bothriurus* species, at almost one third of its total size. This also occurs in *Timogenes* and *Vachonia* species, which are phylogenetically related to *Bo. araguayae* (Mattoni & Prendini, 2007). Total length ranged from 158.07 µm (*O. lourencoi*) to 358.88 µm (*Cen. pocockii*). The general mean for the studied species was 254.24 ± 51.81 µm.

Orobothriurus sp. showed packages with the smallest head length (7.68 µm) whereas *Timogenes*, *Vachonia*, and *Bo. araguayae* had the largest, being approximately one third of the total sperm package length. The head of *Orobothriurus* sp. was about eight times smaller than that of *Bo. araguayae*. There were substantial differences in overall area of the bothriurid sperm package: *Cen. pocockii* packages had an area of 3416.58 µm^2 whereas packages in *O. lourencoi* were only about 10% of that area (355.38 µm^2). Although these values differed by an order of magnitude, males from both species show a similar size range.

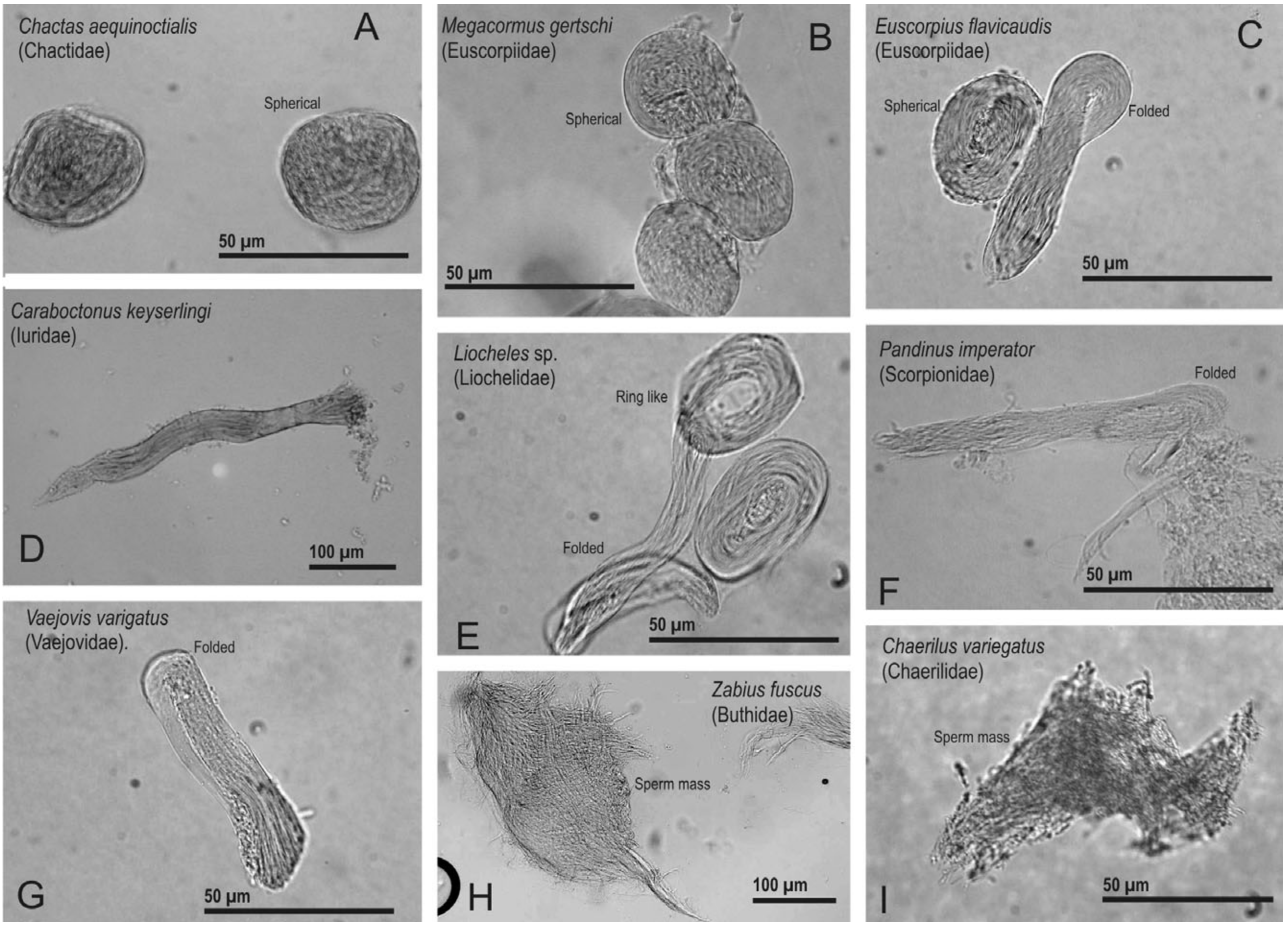

Figure 4. Sperm packages from different families of the order Scorpiones. A, *Chactas aequinoctialis* (Chactidae); B, *Megacormus gertschi* (Euscorpiidae); C, *Euscorpius flavicaudis* (Euscorpiidae); D, *Caraboctonus keyserlingi* (Iuridae); E, *Liocheles* sp. (Liochelidae); F, *Pandinus imperator* (Scorpionidae); G, *Vaejovis varigatus* (Vaejovidae); H, *Zabius fuscus* (Buthidae); I, *Chaerilus variegatus* (Chaerilidae).

The head–body angle of the packages varied greatly amongst bothriurid species, averaging 150°. The species with smaller angles were *Thestylus aurantiurus* and *Brachistosternus* of subgenus *Leptosternus* (*Br. angustimanus* and *Br. pentheri*), which averaged 120°. Sperm packages with bigger angles were present in *Lisposoma josehermana, Ti. dorbignyi, Ti. elegans*, and *Bo. bonariensis* with values near 180°, meaning that they bear straight packages or they bend in other places. Finally, *Li. josehermana* also had the widest sperm package, but the spermatozoa in the head was loosely aggregated and looked like it was separating. We need to investigate further to determine the actual size of the head of the sperm package. For this reason, *Br. angustimanus* (13.85 μm) had the package with the widest head in this study. At the opposite end of the spectrum is *Orobothriurus* sp., showing a head width of 2.74 μm.

Quantitative comparison of sperm packages within and amongst species and grouping analyses

Intraspecific variation

With the exception of marked polymorphism of sperm packages in a few species, the overall shape of the sperm packages was similar amongst males within a given species. However, the measurements of sperm packages from males within the same species differed statistically in all or nearly all analysed variables (Table 2). An exception is the head–body angle, which did not show significant differences in five out of eight studied species (Table 2). Only males within two species (*Cen. obscurus* and *U. tregualemuensis*) showed little significant intraspecific differences in many of their parameters (Table 2).

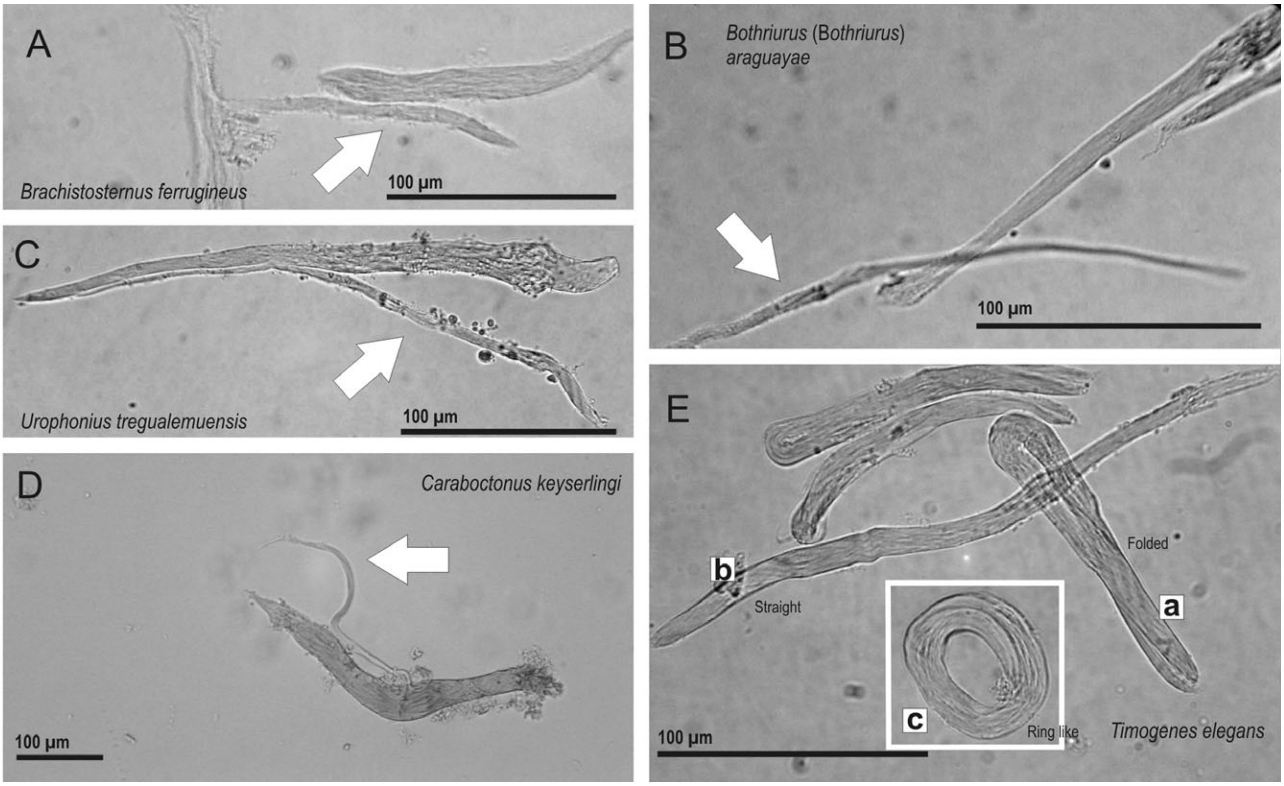

Figure 5. Sperm package differences inside a male. A, *Brachistosternus ferrugineus*; B, *Bothriurus araguayae*; C, *Urophonius tregualemuensis*; D, *Caraboctonus keyserlingi* (Iuridae); E, *Timogenes elegans*: a, folded packages; b, straight packages; c, round packages.

Variation amongst species

Species differed significantly in overall shape of the sperm packages (Fig. 2) and in all variables measured (Table 3). The relative size of sperm packages differed substantially amongst species. Measurements in some species were even two or three times the measures of others, as observed in the length of sperm packages in *Cen. obscurus* and *Bo. araguayae*, as well as in head length of the packages in *Bo. araguayae* and *Bo. flavidus* (with a great difference between end values –see Table 3).

Cluster analysis

The analysis of similarity of sperm packages showed that variables taken into account can separate analysed species quite well (Fig. 7). *Bothriurus* (*Andibothriurus*) species were not grouped together into a cluster as they should be (mainly because of size) as the packages are very much alike within the same genus. The upper group of *Bothriurus* has longer packages. This is why they are more similar to *Centromachetes* species and *Te. moyanoi*. All *Bothriurus* (*Andibothriurus*) as well as the *Centromachetes* and *Tehuankea* species have a similar cane-like shape. This is also shared by *Urophonius* and *Brachistosternus* species but differences in the head can be spotted. This is why these species group with greater dissimilarity. *Timogenes* species have a similar pattern of bending and are similar to *Bo. araguayae* in the total size of the sperm package. The gnarled, helicoidally anterior part of the sperm packages in the *Bo. bonariensis* group (*Bo. bonariensis* and *Bo. chacoensis*) seem to distinguish these species, because if this character is not considered, the groups split and their species join other groups. Although the two species of *Orobothriurus* studied here apparently belong to different phylogenetically groups, they have very similar sperm packages (thin and straight). Therefore, they group together in the cluster analysis.

Fit to the phylogenetic hypothesis

According to the consistency index, the variables that better fit to the phylogenetic hypothesis were head–body angle (CI = 0.5420) and head length (CI = 0.4419). The other variables showed a consistency index lower than 0.32 (total length = 0.2820; head width = 0.3145; area = 0.3137). The total sperm package length (Fig. 8A) tended to be longer in more derived species. An exception occured for *Li. josehermana*. Although this species is considered basal

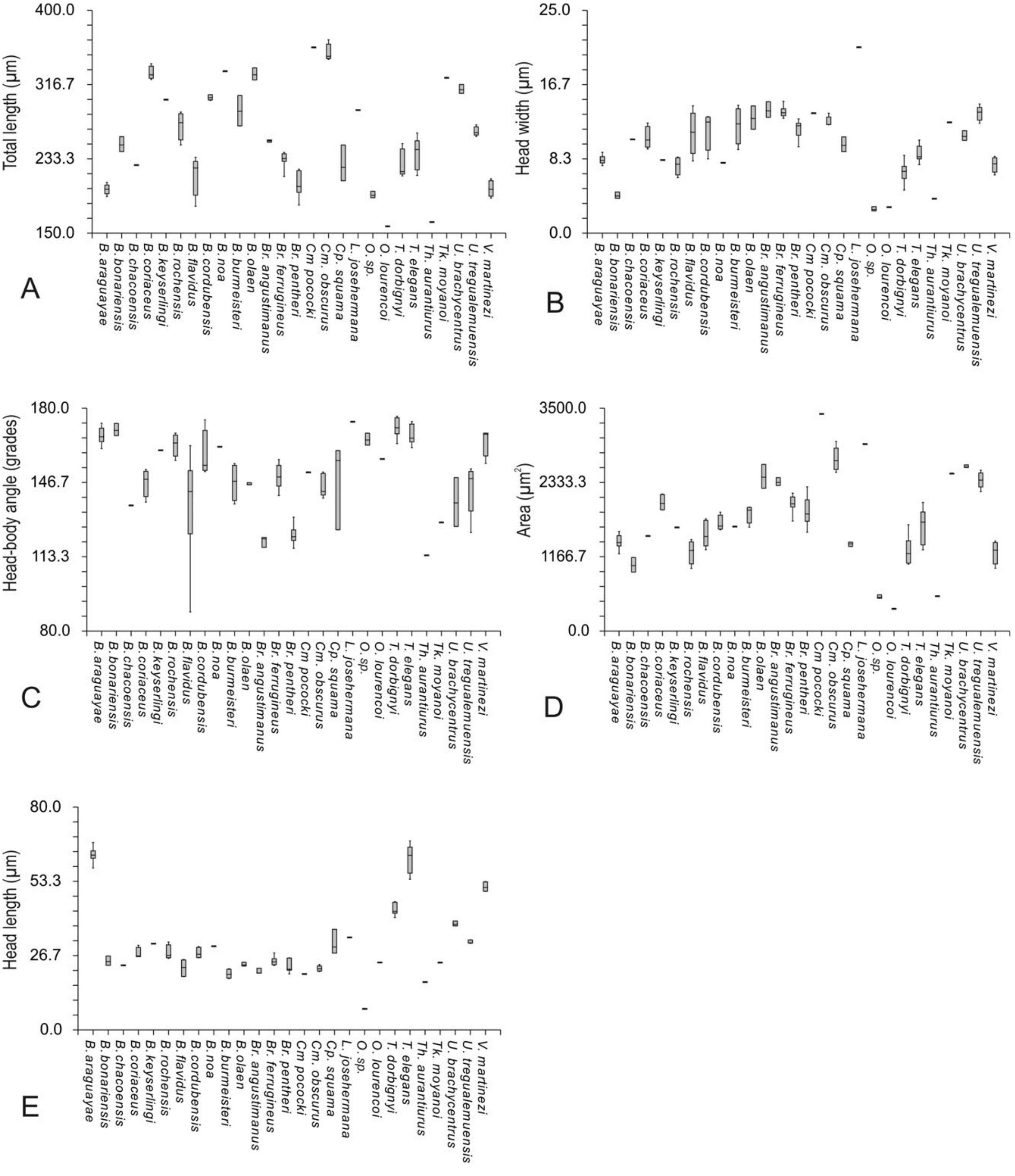

Figure 6. Summary of measures from sperm packages in scorpion species of the Bothriuridae family. A, total length; B, head width; C, head–body angle; D, area; E, head length. All of the data obtained are outlined in the box plots with the respective means, standard deviations, the points within which 75% of the data are grouped, and the end values. The *x*-axis shows all of the species studied. Abbreviations: B., *Bothriurus*; Br., *Brachistosternus*; Cm., *Centromachetes*; Cp., *Cercophonius*; L., *Lisposoma*; O., *Orobothriurus*; T., *Timogenes*; Th., *Thestylus*; Tk., *Tehuankea*; U., *Urophonius*; V., *Vachonia*. Species are shown in alphabetical order along the *x*-axis.

Table 2. Analysis of the intraspecific variance in the measurements of the sperm packages from Bothriuridae scorpions. ANOVA results from species with five or more analysed males

	Brachistosternus ferrugineus	*Brachistosternus pentheri*	*Timogenes dorbignyi*	*Timogenes elegans*	*Bothriurus flavidus*	*Bothriurus araguayae*	*Centromachetes obscurus*	*Urophonius tregualemuensis*
Total length	$F = 12.30$	$F = 66.67$	$F = 24.29$	$F = 97.28$	$F = 58.96$	$F = 10.96$	$F = 4.59$	$F = 1.29$
	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P = 0.0037}$	$P = 0.2077$
Head width	$F = 1.60$	$F = 5.81$	$F = 3.84$	$F = 2.35$	$F = 24.25$	$F = 1.81$	$F = 1.07$	$F = 1.09$
	$P = 0.1264$	$\mathbf{P < 0.0001}$	$\mathbf{P = 0.0008}$	$\mathbf{P = 0.0396}$	$\mathbf{P < 0.0001}$	$\mathbf{P = 0.0773}$	$P = 0.3837$	$P = 0.3748$
Angle	$F = 3.34$	$F = 0.69$	$F = 1.85$	$F = 1.17$	$F = 28.32$	$F = 1.44$	$F = 0.66$	$F = 11.73$
	$\mathbf{P = 0.0015}$	$P = 0.7173$	$P = 0.0793$	$P = 0.3400$	$\mathbf{P < 0.0001}$	$P = 0.1824$	$P = 0.6212$	$\mathbf{P < 0.0001}$
Area	$F = 6.00$	$F = 13.98$	$F = 14.26$	$F = 4.71$	$F = 9.13$	$F = 3.84$	$F = 2.94$	$F = 0.67$
	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P = 0.0005}$	$\mathbf{P < 0.0001}$	$\mathbf{P = 0.0004}$	$P = 0.0314$	$P = 0.6169$
Head length	$F = 2.90$	$F = 8.85$	$F = 4.81$	$F = 18.62$	$F = 14.91$	$F = 8.60$	$F = 1.57$	$F = 0.63$
	$\mathbf{P = 0.0047}$	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$\mathbf{P < 0.0001}$	$P = 0.2018$	$P = 0.6434$
N	10	10	10	8	6	10	5	5

Probability values in bold indicate significant differences (alpha < 0.05) amongst specimens in the species.

Table 3. Analysis of the interspecific variance in the measurements of the sperm packages from Bothriuridae scorpions. ANOVA results in species of the Bothriuridae family

Total length (μm)	*Bothriurus araguayae* 199.21 ± 5.37 A	*Brachistosternus pentheri* 205.66 ± 12.95 AB	*Bothriurus flavidus* 212.12 ± 23.05 BC	*Timogenes dorbignyi* 227.33 ± 14.53 CD	*Brachistosternus ferrugineus* 216.19 ± 24.33 DE	*Timogenes elegans* 240.60 ± 17.39 E	*Urophonius tregualemuensis* 264.50 ± 4.99 F	*Centromachetes obscurus* 353.12 ± 9.03 G	ANOVA $F = 86.30$ $P < 0.0001$
Head width (μm)	*Timogenes dorbignyi* 6.87 ± 1.06 A	*Bothriurus araguayae* 8.29 ± 0.49 B	*Timogenes elegans* 8.91 ± 0.91 B	*Bothriurus flavidus* 11.04 ± 2.02 C	*Brachistosternus pentheri* 11.57 ± 0.88 C	*Centromachetes obscurus* 12.55 ± 0.54 CD	*Brachistosternus ferrugineus* 12.85 ± 1.78 D	*Urophonius tregualemuensis* 13.45 ± 0.81 D	ANOVA $F = 18.15$ $P < 0.0001$
Angle (°)	*Brachistosternus pentheri* 123.90 ± 4.73 A	*Bothriurus flavidus* 131.62 ± 24.49 B	*Urophonius tregualemuensis* 143.36 ± 11.52 BC	*Centromachetes obscurus* 145.32 ± 5.14 BC	*Brachistosternus ferrugineus* 146.12 ± 7.10 C	*Bothriurus araguayae* 167.87 ± 3.63 D	*Timogenes elegans* 168.06 ± 4.23 D	*Timogenes dorbignyi* 171.17 ± 4.12 D	ANOVA $F = 32.05$ $P < 0.0001$
Area (μm^2)	*Timogenes dorbignyi* 1261.17 ± 190.96 A	*Bothriurus araguayae* 1400.97 ± 106.61 AB	*Bothriurus flavidus* 1472.63 ± 176.42 BC	*Timogenes elegans* 1651.13 ± 277.17 C	*Brachistosternus pentheri* 1855.10 ± 246.51 D	*Brachistosternus ferrugineus* 1875.22 ± 395.30 D	*Urophonius tregualemuensis* 236.09 ± 125.82 E	*Centromachetes obscurus* 2703.04 ± 185.63 F	ANOVA $F = 45.59$ $P < 0.0001$
Head length (μm)	*Bothriurus flavidus* 21.76 ± 2.54 A	*Centromachetes obscurus* 22.08 ± 1.03 A	*Brachistosternus pentheri* 23.16 ± 2.10 A	*Brachistosternus ferrugineus* 23.98 ± 1.69 A	*Urophonius tregualemuensis* 31.69 ± 0.56 B	*Timogenes dorbignyi* 43.33 ± 1.94 C	*Timogenes elegans* 61.46 ± 5.20 D	*Bothriurus araguayae* 62.89 ± 2.36 D	ANOVA $F = 383.01$ $P < 0.0001$

Values are shown as mean ± SD. Capital letters (A–G) symbolize different groups obtained with a Tukey's a posterior test.

relative to other bothriurids (Prendini, 2003), it has ‘derived’ sperm package characters (such as larger size and total length). In the optimization, the variable head–body angle did not show a notable increase of values in the internal nodes, as these values remain generally constant (Fig. 8B). Some clades show a tendency to increase or decrease compared with ancestral nodes for the head–body angle and head length variables (arrows in Fig. 8A, B).

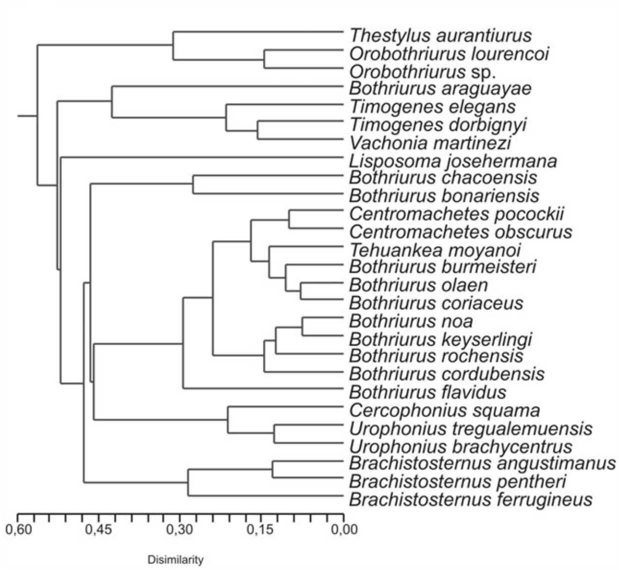

Figure 7. Cluster analysis of the sperm package variables in Bothriuridae scorpions. Dendrogram created using group average linkage with the Euclidean distance method.

RELATIONS BETWEEN PAIRS OF VARIABLES

Sperm package length vs. area

Six out of eight analysed species (75%) did not show a linear association between these two variables (Table 4). Two species showed a significant correlation (*Br. pentheri* and *Ti. dorbignyi*), with the first one showing a higher correlation index ($r_s = 0.9394$; $P = 0.0001$). In these two species, sperm packages that are greater in area are also longer.

Sperm package length vs. male size

These two variables were not linearly associated (Table 4). Correlation coefficients (r_s) were always low. The length of sperm packages was not related to male size. Bigger males did not necessarily have bigger sperm packages; in three species, bigger males tended to show shorter sperm packages (Table 4 –see the negative correlation coefficients).

Sperm package length vs. head length

Only two species, *Bo. araguayae* and *Br. pentheri*, showed significant correlation of these two variables. Nevertheless, in the remaining species there were two (*Bo. flavidus* and *U. tregualemuensis*) with high r_s values and low significance ($P > 0.05$). These four species showed longer heads in longer sperm packages.

Proportion of head length to total body length

When head length was divided by total length, proportions ranged from 3.97 (*O. lourencoi*) to 31.57%

Table 4. Simple linear regression of variables from sperm packages and prosoma length in species of Bothriuridae scorpions. Variables were correlated using mean values

Species	*Brachistosternus ferrugineus*		*Brachistosternus pentheri*		*Timogenes dorbignyi*		*Timogenes elegans*	
Correlation	r_s	P	r_s	P	r_s	P	r_s	P
AR vs. TL	0.4134	0.2351	**0.9394**	**0.0001**	**0.7091**	**0.0217**	0.0952	0.8225
TL vs. PL	0.4723	0.1680	0.3891	0.2665	0.0305	0.9334	–0.2275	0.5878
HL vs. TL	0.3587	0.3088	**0.7842**	**0.0072**	0.4904	0.1497	0.6905	0.0580

Species	*Bothriurus flavidus*		*Bothriurus araguayae*		*Urophonius tregualemuensis*		*Centromachetes obscurus*	
Correlation	r_s	P	r_s	P	r_s	P	r_s	P
AR vs. TL	0.4857	0.3287	0.4182	0.2291	0.4000	0.5046	0.3000	0.6238
TL vs. PL	–0.5218	0.2883	0.2595	0.4691	–0.2052	0.7406	–0.0513	0.9347
HL vs. TL	0.6571	0.1562	**0.6970**	**0.0251**	0.7000	0.1881	0.2000	0.7441

Values in bold indicate significant correlation (alpha ≤ 0.05). Abbreviations: TL, total length; AR, area; PL, prosoma length; HL, head length.

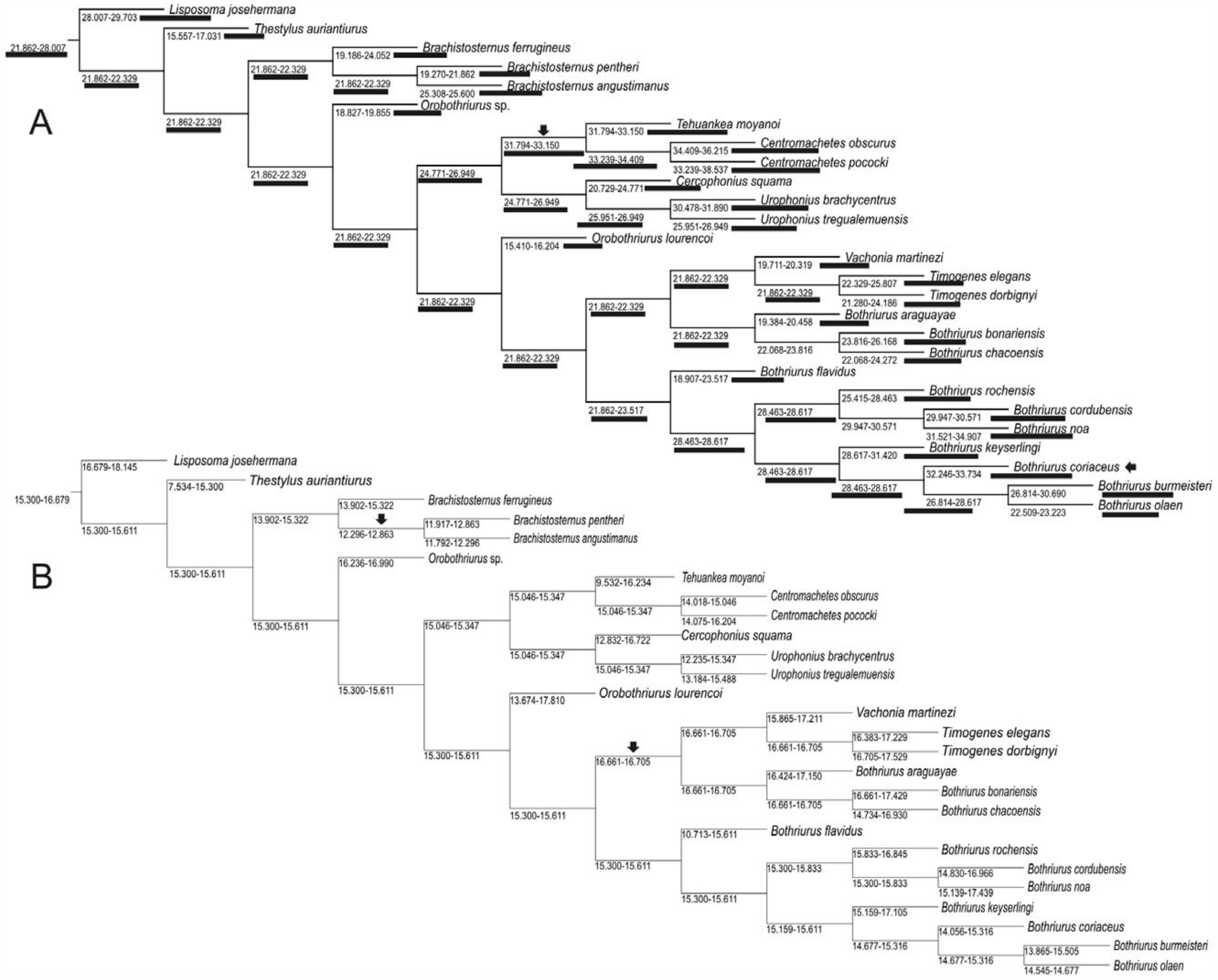

Figure 8. Optimization of variables of the sperm packages from Bothriuridae scorpions into a phylogenetic (partial) hypothesis for the family. Values under each terminal branch correspond to the values observed in the species, divided by 10. Values under the internal branches are optimal values for hypothetical ancestors. A, optimization of total length (in μm/10) of sperm packages; lengths of the bars next to the measures are proportional to the values. B, optimization of head–body angle (in degrees/10). Arrows show changes or peculiarities of species or phylogenetic branches (see text).

(*Bo. araguayae*). However, most data fitted from 10 to 12% (mean value = 11.96%).

DISCUSSION

For the first time, a detailed morphological analysis of sperm packages with measurements of different variables was conducted. In this work, a quantitative basis was presented for using sperm packages as a morphological character for taxonomic research. It was noted in this work that the general pattern for the Bothriuridae family was the cane-like package. However, when considering the different analysed families, the most common shape found was the bent package, with different degrees of bending as stated by Vignoli *et al*. (2008). In addition, we made the first observations of the absence of sperm packages in Chaerilidae, a basal family other than Buthidae, and in some *Bothriurus* (Bothriuridae). It is important to notice that in all the species that have sperm packages there was never a case in which a single individual lacked sperm packages. This gives support to the statements made on these species.

In general we found that sperm package morphology was consistent within species, although some measures varied substantially. We spotted for the first time some differences in sperm packages inside a male. Differences were not only in width but also in spatial arrangement or bending. Our cluster analysis resembles the phylogeny quite well and reinforced the idea that sperm packages can be a good character for taxonomical analysis. Relation between variables did not show a general pattern. Nevertheless *Br. pentheri* and *Ti. dorbignyi* showed longer packages with greater area. In addition, we found no relation between package length and male size.

SPERM PACKAGE MORPHOLOGY AMONGST SCORPIONS

Substantial differences appear at different levels. As expected, family differences are evident (Michalik & Mercati, 2010). Differences amongst some genera and species were observed as stated by Peretti & Battán-Horenstein (2003) but, by means of a substantially wider taxonomic coverage, we showed here that there is a great diversity of package types (e.g. amongst genera) and subtypes (e.g. within some species). The absence of sperm packages in Buthidae (Alberti, 1983; Peretti & Battán-Horenstein, 2003) and Chaerilidae suggests that the plesiomorphic state of sperm in scorpions could have been the absence of sperm packages, the spermatozoa being free or loosely aggregated inside the seminal vesicle (see also Michalik & Mercati, 2010). All the other families analysed here showed species with some kind of sperm aggregation, as was observed in previous studies (Peretti & Battán-Horenstein, 2003; Vignoli *et al*., 2008; Michalik & Mercati, 2010). Nevertheless, we found exceptions to this pattern in species of *Bothriurus* from the *Bo. rochai* group and *Bo. asper* (Bothriuridae) that apparently do not have sperm packages. These species could have lost this trait secondarily.

Data from the analysed species show an interesting pattern of packaging. In general, folded packages are widespread in scorpions, being found in all the analysed families that present sperm packages. In Bothriuridae, with an increased taxon sample, other forms could be observed, dominated by the straight, cane-like package. However, even this family has species with bent packages in some genera (*Timogenes, Vachonia*, and some *Bothriurus*). Other families also have round ball-like packages (Chactidae, Euscorpiidae), thought to be derived from a folded sperm package that has been bent several times (Michalik & Mercati, 2010). The widespread presence of folded and/or round packages can perhaps be explained by the need to improve sperm transfer in males. There is a tendency to increase the packing of sperm (Michalik & Mercati, 2010), making it easier to manage and transfer. Loosely aggregated packages could have derived in straight, long sperm packages that could have bent one or several times, resulting in densely packed, easy-to-move spermatozoa (Vignoli *et al*., 2008). Coiling could be giving an advantage, not only in transport (as coiled packages could be easier to carry, presenting less stagnation while moving through spermatophore tubes) but also in enhancing storage capacity (Michalik & Mercati, 2010). As mentioned before, the spermatozoa are not packaged inside the seminal receptacle of bothriurid females (Peretti & Battán-Horenstein, 2003), so the packages are not involved at all in the fecundation process.

It is important to point out that the spermatophores of buthid and chaerilid scorpions (called ‘flagelliform’ and ‘fusiform’ spermatophores, respectively) are always considered simpler in the order than the ‘lamelliform’ ones, present in all the remaining families (Stockwell, 1989; Sissom, 1990; Prendini, 2000; Coddington *et al*., 2004). In addition, the amount of sperm transferred inside ‘flagelliform’ and ‘fusiform’ spermatophores could be much less than in the ‘lamelliform’ ones (Peretti, 2010), as indirectly evidenced by the size of the female spermathecae (Volschenk *et al*., 2008). The folded sperm packages have evolved at least twice in scorpions: at the base of the ‘lamelliform’ families, and once again inside the Bothriuridae family. The straight packages evolved twice independently: in Iuridae and in Bothriuridae.

The cluster analysis and the phylogenetic tree for bothriurid species showed some similarities, although they group things in very different ways. It seems that the sperm package measures reflect current phylogenetic hypotheses (Stockwell, 1989; Prendini, 2000, 2003; Coddington *et al*., 2004). When identifying the different shapes in the cladogram, it seems that the straight or cane-like shape may represent the ancestral form of sperm packages for this family (see Fig. 1C). Other forms, such as coiled or folded sperm packages, are restricted to certain clades, and could be cited as potential synapomorphies for these groups.

BOTHRIURUS MONOPHYLY

Our results support a nonmonophyletic genus *Bothriurus* as suggested by Prendini (2000, 2003), Mattoni (2003), and Mattoni & Prendini (2007). Four different groups can be recognized in the analysed species of this genus. The first is composed of members of the *Bothriurus* subgenus (*Bo. araguayae, Bo. bonariensis*, and *Bo. chacoensis*) with packages that are either thick and straight or slim and coiled anteriorly. Another group is formed by *Bo. bocki* and *Bo. inermis*, with packages similar to those of *Timogenes* and *Vachonia*. The remaining *Bothriurus* species (subgenus *Andibothriurus*) are characterized by cane-like sperm packages. *Bothriurus* from the *Bo. rochai* group and *Bo. asper* (both belonging to the *Bothriurus* subgenus) have loosely aggregated spermatozoa and do not show ordered sperm packages.

SPERM PACKAGE DIFFERENCES INSIDE A SINGLE MALE

Polymorphism or differences in shapes have been cited mainly for sperm (e.g. Swallow & Wilkinson, 2002; Wigby & Chapman, 2004; Holman & Snook, 2006; Moya *et al*., 2007) but few references for sperm packages are found (e.g. Takami & Sota, 2007). Sperm polymorphism is thought to be under sexual selection control via sperm competition where males can introduce additional types of sperm that help the true viable sperm in different ways to reach the set of ova (e.g. Wigby & Chapman, 2004).

Polymorphic sperm packages were observed in various members of *Bothriurus* (*Bothriurus*) (*Bo. inermis, Bo. bocki, Bo. araguayae*) and in its sister group, which includes *Ti. elegans, Ti. dorbignyi*, and *V. martinezi*. Interestingly, the *Bothriurus* species from the *Bo. bonariensis* group (*Bo. bonariensis* and *Bo. chacoensis,* closely related to *Bo. araguayae*) do not show polymorphism in sperm packages. Other species that, in general, do not show important changes in sperm package shape within a single male are those belonging to the *Bothriurus* (*Andibothriurus*) subgenus. It was seen that activation of sperm is not responsible for these polymorphisms. Sperm packages do not totally unbend, neither do they bend in a circle when in contact with phosphate Buffer or saline, as seen in *Timogenes* (Peretti & Battán-Horenstein, 2003) and related species (D. Vrech, unpublished data). In addition, sperm packages taken from different places in the seminal vesicle always showed the same morphology, ruling out the possibility of shape changes because of movement of sperm inside the passage. Besides, no isolated spermatozoa were seen around to think that thinner sperm packages could be activated and have already lost some spermatozoa.

It is unknown if the differences shown represent a true polymorphism of sperm package structures or solely the natural wide range of shape variation amongst sperm packages in a single male. However, some species had huge differences between two specific forms (e.g. *Ca. keyserlingi, Br. pentheri*) that were surely not a result of manipulation as sperm packages did not show any damage and loose sperm was not found around them. The unusual sperm packages were generally not very abundant, but in the case of *Ca. keyserlingi* thinner packages appeared in greater numbers. This variability could be the result of a teratology originated by failures during formation of sperm packages in the testes (e.g. deficient nutrient supply or endogenous problems) as packages that differed from the standard ones seemed to have lower numbers of spermatozoa. Normal sperm packages in Bothriuridae generally bear around 70, 100, or 250 spermatozoa depending upon the species (Peretti & Battán-Horenstein, 2003; Michalik & Mercati, 2010; present work). This variation in sperm packaging may also be a strategy for sperm competition (e.g. Wigby & Chapman, 2004; Takami & Sota, 2007). This issue should be revisited in the future in order to define whether this is a true polymorphism or solely teratology in sperm packages. The influences of the male's age and production of spermatophores on sperm package polymorphism will be examined in future studies.

INTRASPECIFIC AND INTERSPECIFIC VARIABILITY

The differences in several variables observed amongst specimens from a single species can be explained by natural variation amongst populations, as males belonged to different localities. Accordingly, in the species that showed less variability in this study (*U. tregualemuensis* and *Cen. obscurus*), all the dissected specimens came from single capture events. Differences amongst analysed species were expected, as the species compared belonged to different lineages, some very distant from a phylogenetic point of view (Stockwell, 1989; Prendini, 2000, 2003; Mattoni, 2003). The relative plasticity seen in different species of some of the sperm package variables (total length, head–body angle, head length, general morphology, etc.) may suggest that the sperm packages are under sexual selection pressure, which tends to produce great divergence in genitalia and associated characters including sperm (Eberhard, 1985; Arnqvist, 1998; Møller, 1998). However, the possible mechanisms of sexual selection controlling this divergence should be studied to elucidate this matter.

ASSOCIATIONS AMONGST THE MAIN VARIABLES OF SPERM PACKAGES

Although more specimens should be included to examine deeper these relationships, some clear patterns can be identified from our current results. The fact that some correlations were not significant does not mean that the variables were not related at all. Recent preliminary analyses of plots gave us the idea that some relationships differed from straight lines (some curves appeared). Further studies are needed in order to evaluate the biological significance of these curves. What resulted clearly from this set of data is that the total size of sperm package varied independently from male body size. Nevertheless, the appearance of negative correlations in some cases surely calls for further studies in which larger numbers of specimens should be included to evaluate this tendency. The comparison between the total length and head length of packages was ambiguous, but in general there was no tendency for longer sperm packages to have longer heads. As a matter of fact some, such as *Bo. araguayae*, showed really long heads for their package length, whereas others, like *Centromachetes,* had tiny heads and great sperm package length. The scorpion spermatozoa studied so far the head averaged 12% of the total body size (Peretti, 2010). Sperm packages also showed this average in the species analysed in the present article. Thus, it can be hypothesized that measures from the spermatozoa could be positively correlated with some measures of the sperm packages.

PROSPECTS

In resume, our results strongly support the idea that the study of the morphology of sperm packages can contribute with characters for scorpion phylogeny at different levels (family to species) as also shown for spermatozoa (Michalik & Mercati, 2010). In contrast, the study of the function of sperm packages is still incipient. The most plausible hypothesis to explain the formation of sperm packages (but not their high diversity) suggests that aggregation of spermatozoa in sperm packages may allow the organism to transfer a higher number of sperm by joining a group of spermatozoa together to avoid the sperm getting lost, harmed, or inactivated (Guinzburg, 1968). In fact, species such as buthids that have quick sperm transfer lasting only a few seconds (Peretti & Battán-Horenstein, 2003) lack sperm packaging, whereas species such as bothriurids that have slow transfer (a few minutes) have sperm packaging (Peretti & Battán-Horenstein, 2003; present work). Exceptions are the *Bo. rochai* group and *Bo. asper*, but the reproductive biology of these species is almost unknown. Another plausible, but non-unique, explanation is that the packages may also serve in producing the genital plug found in the female's genital atrium of some scorpion species (e.g. in *Euscorpius* -Althaus *et al*., 2010).

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APPENDIX

SPECIMENS EXAMINED

Museum or collection abbreviations are as follows: AMNH, American Museum of Natural History, New York, USA; AVP, A. V. Peretti private collection, Universidad de Córdoba, Argentina; CIM, C. I. Mattoni private collection, Universidad de Córdoba, Argentina; IBSP, Instituto Butantan, São Paulo, Brazil; MZUSP, Museu de Zoología da Universidade de São Paulo, Brazil; MZUC, Museo de Zoología, Universidad de Concepción, Chile; UFBA, Universidade Federal da Bahia, Brazil.

Bothriuridae Simon, 1880

Bothriurus araguayae Vellard, 1934: 10 ♂ (CIM), Estaçao ecológica de Itirapina, Itirapina, SP, Brazil, 12.ix.2000, G. Machado. *Bothriurus asper* Pocock, 1893: 5 ♂ (UFBA. *Bothriurus bonariensis* (C. L. Koch, 1842): 2 ♂ (CIM), Route 30 km 233, around 100 km SE of Artigas, 1°8′25.629″S 55°55′11.280″W, 345 m, Rivera department, Uruguay, 13.xii.2005, C. Mattoni, A. Ojanguren, F. Labarque; 2 ♂ (AVP), Córdoba, Argentina [no further data]. ***Bothriurus chacoensis*** Maury & Acosta, 1993: 3 ♂ (AVP), Córdoba, Argentina [no further data]. ***Bothriurus rochai*** Mello-Leitão, 1932: 5 ♂ (MZUSP 18999), Estação ecológica Uruçui, 8°52′S, 44°57′W, Una, Piauí State, Brazil, 19–29.i.2001, C. G. Martingelli. ***Bothriurus* sp.**: 3 ♂ (CIM), 7 km S of Guanambí, 14°17′5.6″S, 42°47′2.2″W, 533 m, Município Ceraíma, Bahia State, Brazil, 24.i.2007, UV sampling on modified savannah, cloudy and raining, C. Mattoni, R. Pinto-da-Rocha, H. Yamaguti. ***Bothriurus bocki*** Kraepelin, 1911: 1 ♂ (CIM), Mojotorillo, 5 km E of Betanzos, 19°34′40.9″S, 65°24′57.9″W, 3217 m, Potosí department, Bolivia, 9.i.2005, C. Mattoni, A. Ojanguren, J. Ochoa. ***Bothriurus inermis*** Maury, 1981: 1 ♂ (MZUSP 18995), Km 53 on BR 364, Rondônia State, Brazil, 8.xi.1985. ***Bothriurus coriaceus*** Pocock, 1893: 4 ♂ (CIM), Parque Nacional Fray Jorge, Hillside below Bosque Fray Jorge, 30°39.124′S, 71°40.645′W, 500 m, Provincia de Limarí, Chile, Región IV (Coquimbo), 4.xi.2003, UV detection and diurnal rock rolling on hillside, L. Prendini, C. Mattoni & J. Ochoa. ***Bothriurus keyserlingi*** Pocock, 1893: 1 ♂ (AVP), Parque Nacional La Campana, Palmas de Ocoa, Sendero Quillay trail from campsite, 32°56.048′S, 71°04.562′W, 494 m, Provincia Quillota, Región V (Valparaíso), Chile, 12.xi.2003, rock-rolling and UV detection, L. Prendini, C. Mattoni, J. Ochoa. ***Bothriurus rochensis*** San Martín, 1965: 1 ♂ (CIM), Santa Teresa Park, road to camping close to Picada de los Cuervos, 34°0′36.072″S, 53°33′20.772″W, 48 m, Rocha department, Uruguay, 11.xii.2005, under stones, mixed Eucalyptus and natural forest, C. Mattoni, A. Ojanguren, Labarque; 2 ♂ (AVP), Uruguay [no further data]. ***Bothriurus flavidus*** Kraepelin, 1911: 4 ♂ (AVP), Pampa de Olaen, Capilla de Olaen, around 11 km W of Molinari, 31°9′44.46″S, 64°36′24.336″W, 1096 m, Córdoba Province, Argentina, 29.xii.2005, grassland with granitic rocks, under stones and UV detection, C. Mattoni, A. Peretti, P. Carreras, M. Zerda, D. Vrech; 2 ♂ (AVP), Córdoba, Argentina, [no further data]. ***Bothriurus cordubensis*** Acosta, 1995: 4 ♂ (AVP), Córdoba, Argentina [no further data]. ***Bothriurus noa*** Maury, 1984 1 ♂ (AVP), El Cantadero (16 km from La Rioja), La Rioja, Argentina, 27.xi.1994, UV detection, A. Peretti, L. Acosta, C. Mattoni, A. Martinez. ***Bothriurus burmeisteri*** Kraepelin, 1894: 3 ♂ (CIM), Reserva Provincial Telteca, Province of Mendoza, Argentina, 19.xi.2003, C. Mattoni, J. Ochoa, L. Prendini; 1 ♂ (AVP) [no further data]. ***Bothriurus olaen*** Acosta, 1997 2 ♂ (AVP), Vaquerías, Province of Córdoba, Argentina, 21.xii.1994, A. Peretti, A. Martinez. ***Brachistosternus* (*Leptosternus*) *angustimanus*** Ojanguren Affilastro and Roig Alsina, 2001: 3 ♂ (CIM), Picún Leufú, Neuquén, Argentina, 25.i.2005, M. Magnanelli, López. ***Brachistosternus* (*Leptosternus*) *pentheri*** Mello-Leitão, 1931: 5 ♂ (CIM), Reserva de la Biosfera Ñacuñan, Province of Mendoza, Argentina, 20.xi.2003, C. Mattoni, J. Ochoa, L. Prendini; 5 ♂ (CIM), Reserva de Flora y Fauna Telteca, Province of Mendoza, Argentina, 19.xi.2003, C. Mattoni, J. Ochoa, L. Prendini. ***Brachistosternus* (*Ministernus*) *ferrugineus*** Thorell, 1876: 10 ♂ (CIM), Parque Provincial Chancaní, Province of Córdoba, Argentina, 20, 23.xi.2001, C. Mattoni, J. Ochoa. ***Centromachetes obscurus*** Mello-Leitão, 1932: 5 ♂ (CIM), Parque Nacional Nahuelbuta, Chile, 14.xi.2003, C. Mattoni, L. Prendini, J. Ochoa. ***Centromachetes pocockii*** (Kraepelin, 1894): 1 ♂ (CIM), trail to Salto Rayén, next to Monumento Natural Contulmo, under stones and U.V., *Nothofagus* forest, 38°1′7.896″S, 73°9′54.720″W, 210 m, VIII Region, Chile, 10–11.i.2006, C. Mattoni, M. Vivanco. ***Cercophonius squama*** (Guervais, 1843): 3 ♂ (AMNH), Mt. Barrow 570 m, Tasmania, Australia, 15–17.ii.1980, A. Newton M. Thayer. ***Lisposoma josehermana*** Lamoral, 1979: 1 ♂ (CIM), Farm Varianto on Elandshock 771, 19°22.773′S, 17°44.456′E, 1500 m, Oshokoto region, Namibia, 4.i.2004, L. Prendini, E. Scott, T. & C. Bird, Q. & N. Martins. ***Orobothriurus* sp.**: 2 ♂ (AMNH, MACN), 2 km W of Tirana, 20°19′59.8″S, 69°40′07.6″W, 999 m, Tarapacá Region (I), Chile, 18.i.2005, UV sampling on *Prosopis tamarugo* forest, C. Mattoni, A. Ojanguren, J. Ochoa. ***Orobothriurus lourencoi*** Ojanguren Affilastro, 2003: 1 ♂ (CIM), Parque nacional Llanos de Challe, 28°09′39.8″S, 71°03′20″W, 205 m, Atacama Region, Chile, 25.i.2005, near ‘Administración’ UV full moon, C. Mattoni, A Ojanguren. ***Tehuankea moyanoi*** Cekalovic, 1973: 1 ♂ (MZUC 567, Holotype), Ramadilla, Arauco Province, Región VIII (Biobío), Chile, 18.i.1971, R. Donoso Barros. ***Thestylus aurantiurus*** Yamaguti & Pinto-da-Rocha, 2003: 1 ♂ (MZUSP), Parque Estadual da Serra da Cantareira, São Paulo, Brazil, 22–30.xi.2004, S. Favorito *et al*. Legit ***Timogenes dorbignyi*** (Guérin Méneville, 1843): 6 ♂ (CIM), Parque Provincial Chancaní, Province of Córdoba, Argentina, 20–23.xi.2001, C. Mattoni, J. Ochoa; 4 ♂, Salinas Grandes, Province of Córdoba, Argentina, iii.2007, C. Mattoni, A. Peretti, D. Vrech. ***Timogenes elegans*** (Mello-Leitão, 1931): 5 ♂ (CIM), Parque Provincial Chancaní, Province of Córdoba, Argentina, 1–25.iii.1994, pitfall trap, C. Mattoni; 3 ♂, Parque Provincial Chancaní, Province of Córdoba, Argentina, 2004, pitfall trap, M. Izquierdo. ***Urophonius brachycentrus*** (Thorell, 1876) 2 ♂ (AVP), 13.vi.1993, S. Castelvetri [no further data]. ***Urophonius tregualemuensis*** Cekalovic, 1981: 5 ♂ (CIM), Reserva Nacional Los Ruiles, Chile, 13.xi.2003, C. Mattoni, J. Ochoa, L. Prendini. ***Vachonia martinezi*** Abalos, 1954: 4 ♂ (CIM), Balneario el Cóndor, S of Viedma, Province of Río Negro, Argentina, 5.v.2003, M. Magnanelli.

Euscorpiidae Laurie, 1896
Megacormus gertschi Díaz Najera, 1966: 1 ♂ (AMNH), Mexico [no further data]. ***Megacormus* sp.**: 1 ♂ (AMNH), road to Tlaquilpan 18°38.507′N, 97°06.425′W, 2125 m, Veracruz, Mexico, 19.vii.2002, open pine forest; in crevices in limestone outcrops covered by moss, L. Prendini, O. Francke. ***Euscorpius flavicaudis*** (DeGeer, 1778): 1 ♂ (MACN), Lodève, Herault, France, 1969, B. Monroy.

Iuridae Thorell, 1876
Caraboctonus keyserlingi Pocock, 1893: 2 ♂ (CIM), Monte Grande, Elqui Valley, Province of Elqui, Coquimbo, Chile, 27.ii.2004, J. Pizarro, J. Ochoa, C. Mattoni. 1 ♂ (AVP), Chile, [no further data].

Buthidae C. L. Koch, 1837
Zabius fuscus (Thorell, 1876): 3 ♂ (AVP), Cuesta Blanca, Córdoba, Argentina, [no further data].

Chactidae Pocock, 1893
Chactas aequinoctialis (Karsch, 1879): 1 ♂ (AMNH), Sierra Nevada de Santa Marta, San Sebastián de Rabago, 2000 m, Colombia 1–10.iv.1968, under logs and stones and in rotten ground banana foliage, B. Malkin.

Chaerilidae Pocock, 1893
Chaerilus variegatus Simon, 1877: 1 ♂ (MACN), Java [no further data].

Liochelidae
Liocheles* sp.**: 1 ♂ (MACN), Mt. Tozer Range north foot, Queensland, Australia [no further data]. ***Opisthacanthus capensis Thorell, 1876: 1 ♂ (CIM), South Africa [no further data]. ***Opisthacanthus valerioi*** Lourenco, 1980: 1 ♂ (AVP), Isla de Coco, Costa Rica, 4.iii.2002, A. Peretti.

Scorpionidae Latreille, 1802
Pandinus imperator (C. L. Koch, 1841): 1 ♂ (AVP), pet trade specimen. ***Scorpio maurus*** Linnaeus, 1758: 1 ♂ (AMNH), Arbalow Ourika, 1000 m, Marakesch province, Morocco, 14–18.v.1975, B. Malkin.

Vaejovidae Thorell, 1976
Vaejovis spinigerus (Wood, 1863): 1 ♂ (AMNH), Roadcuts, Madera Canyon, Santa Rita Mnt., Pima-Santa Cruz Co., Arizona, USA, 14.vii.?, Coli, Cazier, Bigelow. ***Vaejovis variegatus*** Pocock, 1898: 1 ♂ (MACN), Xochitepec, Morelos, Mexico, 20-X-74, O. Martinez.