



## Research paper

## Intra-male sperm variability: Is there heteromorphism in scorpions (Arachnida, Scorpiones)?



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## ABSTRACT

There is a natural range of variation in sperm morphology inside males. This variation may be adaptive or a result of poor quality control of sperm production. Sperm aggregates can also show different morphologies inside ejaculates. Preliminary analyses in scorpions suggest the existence of a sperm package morphological variation. It is unclear if this is a true polymorphism. The main purpose of this analysis is to evaluate polymorphism in sperm packages of scorpions. Some species from the order showed width variation or spatial variation that was associated to the number of folds. Some ejaculates of *Bothriurus bonariensis* and *Brachistosternus ferrugineus* males showed width dimorphism, but may be related to the natural variation of sperm width. However, sperm packages in *B. ferrugineus* showed lower counts of spermatozoa and the apparition of these sperm packages seemed to increase at the beginning of the reproductive season in one of the species. A marked width dimorphism of sperm packages appeared inside ejaculates of *Caraboctonus keyserlingi*. The adaptive significance of sperm package differences is not well understood. Differences in width may be a consequence of sperm package production errors, resulting in sperm packages with fewer spermatozoa. Sperm package polymorphism in *C. keyserlingi* may be linked to sperm competition risk and may be associated to other characters and strategies. Alternative hypotheses to the pattern found are further analyzed and discussed within a sexual selection framework.

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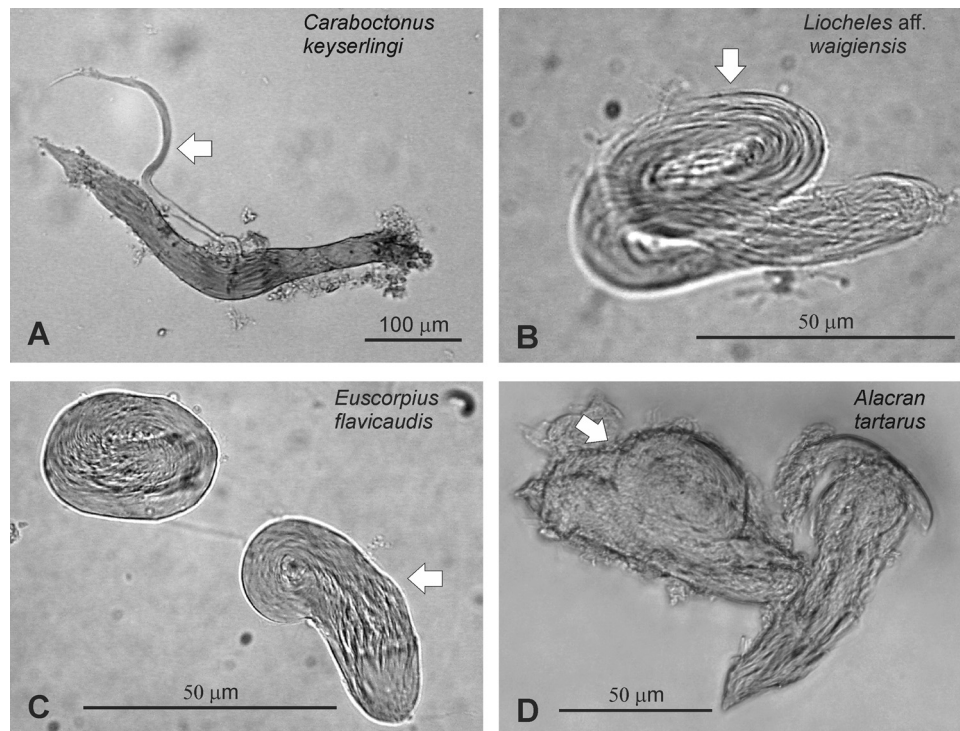
## 1. Introduction

Under sexual selection, sperm competition can shape diverse characters such as genitalia, testes mass, sperm size and morphology, (Simmons, 2001). In many groups, sperm competition commonly influences certain traits of reproductive cells (Birkhead and Møller, 1998). For example, different organisms may show a wide variation in three different traits, spermatozoa length (Hosken, 2003; Birkhead et al., 2009), heteromorphism, that is the production of multiple sperm types within each ejaculate (Snook, 1998; Pitnick et al., 2009; Araújo et al., 2011; Dallai, 2014), and aggregation (Jamieson, 1987; Simmons and Siva-Jothy, 1998; Birkhead et al., 2009; Dallai, 2014). Variations in size and morphology of spermatozoa have been widely studied (Simmons, 2001; Birkhead et al., 2009). Studies have demonstrated that a natural range of variation in sperm morphology exists within males (reviewed in Simmons, 2001 and Calhim et al., 2007). This variation may have an adaptive function or just appear as a result

of a poor quality control of spermiogenesis errors (Cohen, 1967, 1973; Simmons, 2001; Hunter and Birkhead, 2002; Birkhead et al., 2009). Heteromorphism is an example of morphological diversification, where two or more different types of sperm may appear in a single ejaculate (Swallow and Wilkinson, 2002; Pitnick et al., 2009). In fact, different types of spermatozoa can have diverse abilities that can serve various functions when fertilizing the female gamete (Pitnick et al., 2009; Higginson and Pitnick, 2011). Among arthropods, insects show many examples of heteromorphism in spermatozoa (reviewed in Dallai, 2014). Lepidoptera shows a classic example in the form of spermatozoa dimorphism, with morphs called eupyrene and apyrene. The first is a nucleated cell and the second lacks the nucleus and is highly motile (Gage and Cook, 1994; He et al., 1995). For example, eupyrene participates in fertilization whereas apyrene may aid their transport, fertilization, and maintenance, or simply serve as “cheap filler” (Cook and Gage, 1995; Simmons, 2001). Variation in spermatozoa is not only morphological, and can also refer to differences in conjugation, a phenomenon where two or more spermatozoa unite for motility and/or transport (Pitnick et al., 2009). Two types of sperm conjugation exist among arthropods (Higginson and Pitnick, 2011). Primary sperm conjugates originated from a single spermatogonium, where

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**Fig. 1.** Sperm packages morphological variation in different scorpion families. (A) *Caraboctonus keyserlingi* (Luridae) showing average thick sperm package together with a thin sperm package (white arrow). (B) *Liocheles aff. waigiensis* (Hormuridae), regular folded sperm package in the background together with a ring-like package (white arrow). (C) *Euscorpium flavicaudis* (Euscorpidae), average folded sperm package and its morphological variant, a spherical sperm package (white arrow). (D) *Alacran tartarus* (Typhlochactidae), two folded sperm packages appear with one ring like sperm package (white arrow). Arrows indicate morphological variants.

spermatozoa remain grouped, and secondary sperm conjugates originated after separation when spermatozoa are fully individualized during spermiogenesis. The resulting sperm aggregates may also show morphological diversity similar to single sperm cells. For example, Takami and Sota (2007) found size heteromorphism in sperm bundles within ground beetles from the genus *Ohomopterus*, with bigger bundles migrating faster to the spermatheca, but with no clear function for small sperm bundles, despite their rapid diversification.

Both types of conjugates appear among spiders, where spermatozoa are coiled and transferred inside a secretion sheath (Michalik and Huber, 2006; Lipke and Michalik, 2012; Michalik and Ramírez, 2014). Scorpions generally show sperm conjugates in dense aggregates called “bundles” (Jespersen and Hartwick, 1973; Alberti, 1983) or, more precisely, sperm packages (Peretti and Battán-Horenstein, 2003; Michalik and Mercati, 2010; Vrech et al., 2011, 2014), the term we will use in the present study. In scorpions, a sperm package contains a range of 70–400 spermatozoa, depending on the species. For example, Jespersen and Hartwick (1973) found 200–400 spermatozoa in several species of Vaejovidae. Similarly, Peretti and Battán-Horenstein (2003) found between 70 and 100 spermatozoa in eight species of Bothriuridae. Recently, various species from different families have shown nearly 250 spermatozoa per sperm package (e.g., Superfamily Scorpionoidea, which includes Bothriuridae, Scorpionidae and Hormuridae, Michalik and Mercati, 2010; Vrech et al., 2011; Troglotayosicidae, Vignoli et al., 2008).

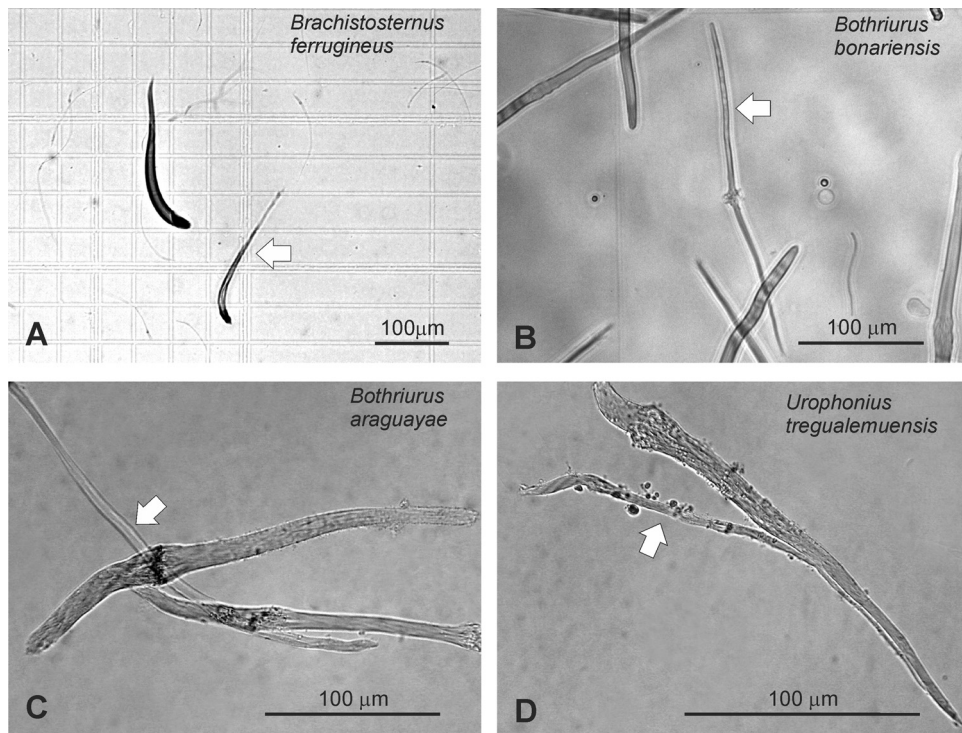
Preliminary analyses in scorpions suggest that sperm package variation could exist within a male's pre-insemination ejaculate (Vrech et al., 2011). However, it is uncertain if such observations correspond to a true heteromorphism, where two or more distinctive types of sperm packages appear systematically within a single pre-insemination ejaculate, or merely to the natural morphological variation. Besides, the distribution of sperm package

heteromorphism among species from different families of scorpions is still unknown. In addition, we lack studies on dissimilarities in sperm packages width, which may be a result of differences in number or width of spermatozoa contained inside each sperm package. Here, we report and evaluate the presence of sperm package heteromorphism in scorpions, including a descriptive comparison among selected scorpion families. First, the morphological variability of sperm packages will be characterized. Second, we will address sperm packages variation in Bothriuridae and Luridae. Finally, the effects of reproductive season and body condition on sperm package morphology will be evaluated for the bothriurid *Brachistosternus ferrugineus* (Thorell, 1876).

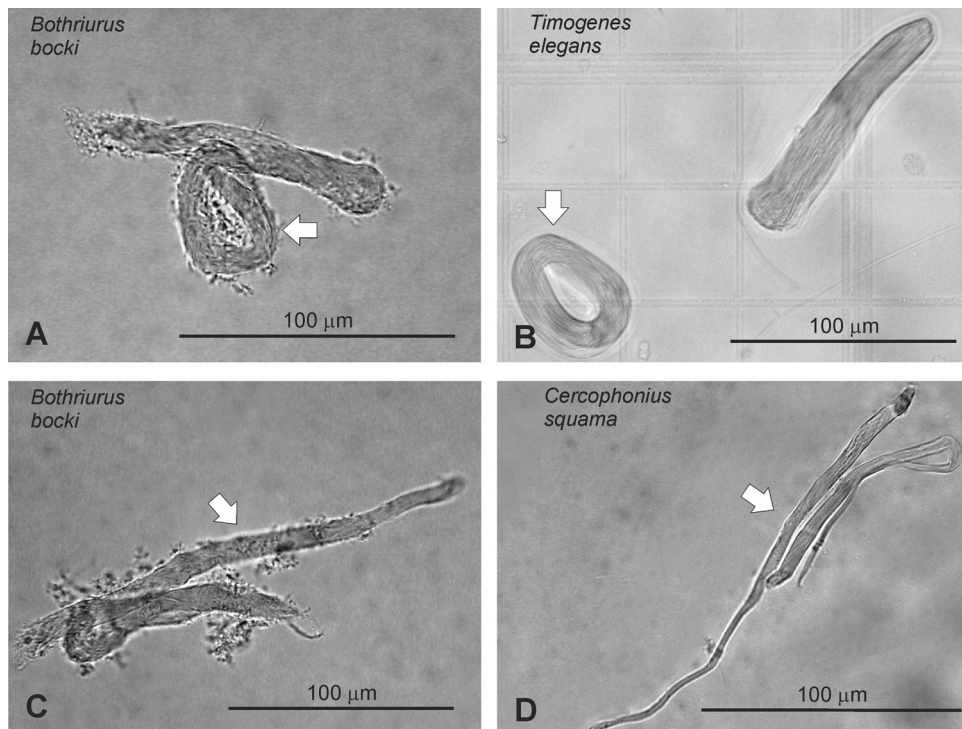
## 2. Material and methods

### 2.1. Descriptive analysis of sperm package variants

We used species belonging to groups where previous observations suggested the occurrence of sperm package variability (see Vrech et al., 2011). We have increased the taxon sampling on the family Bothriuridae because this is a diverse family in the Neotropical region that shows a wide variation in sperm package morphology (Vrech et al., 2011). Samples from other families were included as examples of variability in the order. We used representatives of following families: Bothriuridae Simon, 1880, Euscorpidae Laurie, 1896, Luridae Thorell, 1876, Hormuridae Laurie, 1896, Scorpionidae Latreille, 1802, and Typhlochactidae Mitchell, 1971. For detailed information of the observed specimen (see Supplementary material A). Voucher specimens are deposited in the scorpion collection of the Laboratorio de Biología Reproductiva y Evolución de la Universidad Nacional de Córdoba (IDEA, UNC-CONICET). Since our approach is merely descriptive we number of samples differ often due to difficulties in obtaining specimen of certain species (see Takami and Sota, 2007; Vrech et al., 2011 for



**Fig. 2.** Width variation in different species of Bothriuriidae. (A) *Brachistosternus ferrugineus*, thin sperm package (white arrow) is showed along with its average counterpart. (B) *Bothriurus bonariensis*, thin sperm package (white arrow) is shown among regular sperm packages. (C) *Bothriurus araguayae*, a very thin sperm package (white arrow) is shown below two average sperm packages. (D) *Urophonius tregualemuensis*, thin sperm package variant (white arrow) is next to the average one. Arrows indicate morphological variants.



**Fig. 3.** Spatial variation in different species of Bothriuriidae. (A) *Bothriurus bocki* regular folded sperm packages accompanied by a ring like sperm package (white arrow). (B) *Timogenes elegans* regular folded and variation ring-like packages (white arrow). (C) *Bothriurus bocki* showing folded sperm package and straight sperm package as a variant (white arrow). (D) *Cercophonius squama*, straight (white arrow) and folded sperm packages. Note that folded sperm package in this species is not the same as for example in *Timogenes*. Arrows indicate morphological variants.

a similar criterion). For a general overview of the sperm package morphology in the pre-insemination ejaculate see Figs. 1–3.

## 2.2. Sperm packages in *Bothriuridae* and *Iuridae*

In addition to the preceding descriptive analysis, we performed a detailed study using *B. ferrugineus* ( $N=10$ ), *Bothriurus bonariensis* (Koch, 1842) ( $N=7$ ), and *Caraboctonus keyserlingi* Pocock, 1893 ( $N=5$ ). Commonly, scorpion species may show different levels of sperm competition, and *B. bonariensis* and *C. keyserlingi* are species with very high levels of sperm competition (Vrech et al., 2014; Mattoni and Peretti, pers. obs.).

Specimens from *B. ferrugineus*, were collected using ultra-violet light emission diode (LED) flashlights (Stockmann, 2013) and maintained in the laboratory until euthanized for examination. The other two species were part of the material deposited in the collection of the Laboratorio de Biología Reproductiva y Evolución of the Universidad Nacional de Córdoba (IDEA, UNC-CONICET) and kept in ethylic alcohol 80%. Living organisms were euthanized in a vial containing ether in saturation, prior analysis. These techniques are ideal for our approach as it does not modify sperm package morphology (see Vrech et al., 2011).

To obtain the sperm packages, we dissected males by making an incision in the mesosomal lateral pleura (between segments I and VII) exposing the paraxial organ. Afterwards, the seminal vesicle was removed and torn apart to allow releasing of sperm packages. The packages were transferred to a drop of glycerin for manipulation and imaging. Digital images were taken using a phase-contrast microscope (Nikon Eclipse 50i) with an attached digital camera (Nikon DS-fi1). Finally, sperm packages were measured using ImageJ v.1.49b (Schneider et al., 2012). After characterizing the variability, random pictures from 25 sperm packages were obtained from each different male of *B. bonariensis* (7 males) and *B. ferrugineus* (10 males) (Bothriuridae) and 30 sperm packages from males of *C. keyserlingi* (5 males) (Iuridae). Sperm package width was measured using the software ImageJ (Schneider et al., 2012). We evaluated normality, and bimodality of distributions made from data of width coming from single males and pooled values obtained from these males. In the individual male analysis we investigated 25 sperm packages from each of the 7 males of *B. bonariensis* and each of the 10 males from *B. ferrugineus*, and 30 sperm packages from each of the 5 males from *C. keyserlingi*. Additionally, we analyzed this data pooled per species resulting in  $N=175$  sperm packages in *B. bonariensis*,  $N=250$  in *B. ferrugineus* and  $N=150$  *C. keyserlingi*. For these analyses, we analyzed density curves visually, and statistically. For the latter, we used the Shapiro–Wilks normality test and the Hartigan's dip test for bimodality (Hartigan and Hartigan, 1985) in R (R Core Team, 2012), using the *diptest* package (Maechler, 2014). Density curves graphics of each male are shown in the Supplementary material 3.

### 2.2.1. Transmission electron microscope analysis

Digital images were used for counting the number of spermatozoa that form the sperm packages in *B. ferrugineus* and *B. bonariensis*. This analysis aimed to clarify the source of width variation in these two species. Two males per species were used, and 30 sperm packages were analyzed on each sample. For this ultra-structural analysis, the left seminal vesicles from one male from each species were used for extracting sperm packages. This seminal vesicle 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% using the same buffer. After dehydration in a graded series of acetone, samples were embedded in Araldite and polymerized at 60 °C. Transversal thin sections of sperm packages were obtained with a diamond knife using a JEOL JUM-7 ultramicrotome and analyzed with a Zeiss LEO 906E transmission electron

microscope. Transverse cuts of sperm packages were photographed. Total sperm packages width was measured, and spermatozoa were counted.

## 2.3. Effect of seasonality and body condition on sperm package variation in *Brachistosternus ferrugineus*

Samples were obtained at different periods during the reproductive season, i.e., between November 2010 ( $N=9$ , beginning of the mating season), January ( $N=7$ ) and February ( $N=7$ , end of the mating season), 2011. Body condition was obtained as a body mass index (BMI) combining prosoma length and body weight. The equation used was  $BMI = BW/(PL)^2$ , where BW is the body weight and PL the prosoma length.

Seminal vesicles were removed from the paraxial organ following the procedure explained above. The seminal vesicle was diluted with saline solution (NaCl 0.90 g, Purified water 100 ml) and vortexed (three repetitions of 30 s in 30 Hz). Then, the solution was used for counting sperm packages following the standard procedure for counting large cells with an improved Neubauer counting chamber (Boeco, Hamburg, Germany) (Bastidas, 2010). Sperm packages were counted inside the four big squares from the corners. For full description of the counting procedure, see Supplementary material B. Thin sperm packages were designated as those that were notably thinner compared to the sperm packages of the whole sample (see Vrech et al., 2011 for data of standard width). Firstly, the percentage of males with morphological variability was accounted. Secondly, the effect of the season and body condition over the number of sperm packages with morphological variants was evaluated using a one way ANOVA. Unless explicitly stated, all statistical analyses were performed using the base package of Rv2.15.1 open-source software (R Core Team, 2012).

## 3. Results

### 3.1. Sperm package morphological variation

#### 3.1.1. Interspecific variation

Sperm packages from 17 species of scorpions distributed among Bothriuridae, Euscorpiidae, Hormuridae, Iuridae, Scorpionidae, and Typhlochactidae showed variation in sperm packages morphology within a single pre-insemination ejaculate (Figs. 1A–D, 2A–D, 3A–D).

We observed two basic types of variation in sperm package morphology (see Figs. 2 and 3). The first refers to sperm package width and henceforth this type of variation will be called **width variation** (Figs. 1A, 2A–D). This type seems to be common among species with straight sperm packages. The species showing this type of morphological variation were *Bothriurus araguayae* Vellard, 1934, *B. bonariensis*, *Brachistosternus angustimanus* Ojanguren Affilastro & Roig Alsina, 2001, *B. ferrugineus*, *B. pentheri* Mello-Leitão, 1931, *Cercophonius squama* (Gervais, 1843), *Urophonius tregualemensis* Cekalovic, 1981 (Bothriuridae), and *C. keyserlingi* (Iuridae) (see Figs. 1A, 2A–D).

The second type of morphological variation within a pre-insemination ejaculate encompasses differences in the spatial arrangement and number of bends or folds of the structure of the sperm packages (see Figs. 1B–D, 3A–D). This variant will be referred as **spatial variation**. This type of variant is commonly found in species with folded sperm packages: *Scorpio maurus* Linnaeus, 1758 (Scorpionidae), *Liocheles* aff. *waigiensis* (Hormuridae), *Euscorpius flavicaudis* (DeGeer, 1778) (Euscorpiidae) (Fig. 1B–D), *Bothriurus bocki* Kraepelin, 1911, *B. inermis* Maury, 1981, *C. squama*, *Timogenes elegans* (Mello-Leitão, 1931), *T. dorbignyi* (Guérin Méneville, 1843) and *Vachonia martinezi* Abalos, 1954, (Bothriuridae) (Fig. 3A–D).

**Table 1**  
Summary of standard sperm packages and the main sperm package variants in sampled species of scorpions.

Family	Species	Standard sperm package	Width variation	Spatial variation
Bothriuridae	<i>Bothriurus araguayae</i>	<b>Straight intermediate</b> and uniform head-tail width	x	–
	<i>Bothriurus bocki</i>	<b>Folded uniform</b> head-tail width	–	x
	<i>Bothriurus bonariensis</i>	<b>Straight thin</b> uniform head-tail width	x	–
	<i>Bothriurus inermis</i>	<b>Folded uniform</b>	–	x
	<i>Brachistosternus angustimanus</i>	<b>Cane-like</b> (straight with bent tip, body becomes thinner to the tail)	x	–
	<i>Brachistosternus ferrugineus</i>	<b>Cane-like</b>	x	(x)
	<i>Brachistosternus pentheri</i>	<b>Cane-like</b>	x	–
	<i>Cercophonius squama</i>	<b>Folded not uniform</b> (bent tip, body becomes thinner to the tail)	x	x
	<i>Timogenes dorbignyi</i>	<b>Folded uniform</b>	–	x
	<i>Timogenes elegans</i>	<b>Folded uniform</b>	–	x
	<i>Urophonius brachycentrus</i>	<b>Cane-like</b>	x	(x)
	<i>Urophonius tregualemuensis</i>	<b>Cane-like</b>	x	–
	<i>Vachonia martinezi</i>	<b>Folded uniform</b>	–	x
	Euscorpidae	<i>Euscorpis flavicaudis</i>	<b>Folded uniform</b>	–
Hormuridae	<i>Liocheles</i> aff. <i>waigiensis</i>	<b>Folded uniform</b>	–	x
Luridae	<i>Caraboctonus keyserlingi</i>	<b>Straight thick</b> (slightly thinner to the tail)	x	–
Scorpionidae	<i>Scorpio maurus</i>	<b>Folded uniform</b>	–	x
Typhlochactidae	<i>Alacran tartarus</i>	<b>Folded uniform</b>	–	x

In bold appears the general designation of the sperm package, a short explanation is also added on the first described. Parenthesis suggests the possibility of a putative variation that seems to be uncommon (see Section 3.1.1). For more information see Vrech et al. (2011).

The average and most common sperm package in these species was folded with a bent zone in the middle (e.g., Figs. 1B and D; 3A–D, Table 1). Every analyzed pre-insemination ejaculate showed these folded sperm packages together with ring-like sperm packages. This was common in species like *A. tartarus* (Typhlochactidae) and *L. aff. waigiensis* (Hormuridae) (Fig. 1B), *B. bocki* (Fig. 3A and C), *B. inermis*, *T. dorbignyi* and *T. elegans* (Fig. 3B) (Bothriuridae). Furthermore, straight sperm packages appeared in a lower number (less than 1:100) compared to ring-like sperm packages (Fig. 3B). This case was common in species like *T. dorbignyi*, *T. elegans*, *V. martinezi*, and *B. bocki* (Fig. 3C). The most common sperm packages in *C. squama* were folded, and were also accompanied by straight sperm packages (Fig. 3D). These folded sperm packages differed morphologically from those of *Timogenes* species. *Timogenes* sperm packages were uniform along its width, different from *C. squama*, which showed a thin tail at the end of the sperm package structure (compare Fig. 3B and D). Data from sperm package differences in pre-insemination ejaculates of analyzed species are summarized in Table 1.

Sporadically, spatial variation also appeared in samples of species that had only straight sperm packages, in this case, *B. ferrugineus* (Fig. 4A) and *U. brachycentrus* (Fig. 4B) (Table 1). It is very uncommon for these species to show this type of variant (i.e., bend sperm packages). This was observed only once in three samples (*B. ferrugineus*  $N=2$  males; *U. brachycentrus*  $N=1$  male) collected during the final stage of the reproductive season of these species.

### 3.1.2. Intraspecific and intramale variation

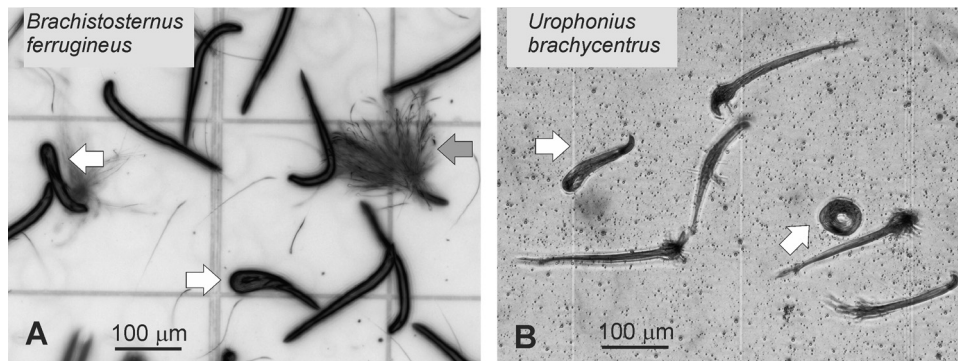
**3.1.2.1. Individual male data analysis.** Detailed pre-insemination ejaculate analysis of sperm packages width on males of *B. bonariensis* showed that 5 out of 7 (71%) males had a normal distribution of the width of their sperm packages (Shapiro–Wilk normality test  $W_{(bb1)}=0.9480$ ,  $p=0.2262$ ;  $W_{(bb3)}=0.9579$ ,  $p=0.3745$ ;  $W_{(bb6)}=0.9739$ ,  $p=0.7442$ ;  $W_{(bbep)}=0.9794$ ,  $p=0.8726$ ;  $W_{(bbqa)}=0.9721$ ,  $p=0.6939$ ) (Fig. 1 Supplementary material). In the remaining two males, distributions were skewed to the left (Shapiro–Wilk normality test  $W_{(bb75)}=0.8770$ ,  $p=0.0060$ ;  $W_{(bbu)}=0.8983$ ,  $p=0.0169$ ) with small peaks in the shoulders of the distribution (Fig. 1 Supplementary material). None of the previous distributions showed bimodality (Hartigan's dip test for bimodality  $p>0.05$ ).

Ejaculate analysis of each male in *B. ferrugineus* showed that sperm package data was normally distributed in 7 out of 10 individuals (70%) (Shapiro–Wilk normality test  $W_{(bf5)}=0.9377$ ,

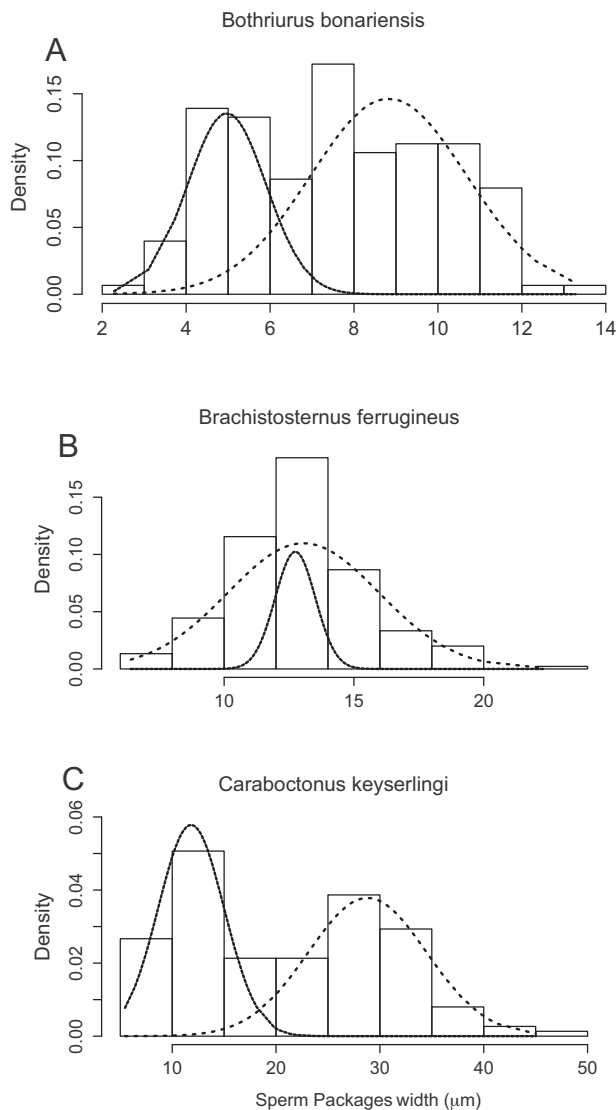
$p=0.1311$ ;  $W_{(bf6)}=0.9776$ ,  $p=0.8346$ ;  $W_{(bf8)}=0.9413$ ,  $p=0.1585$ ;  $W_{(bf9)}=0.9278$ ,  $p=0.0772$ ;  $W_{(bf10)}=0.9574$ ,  $p=0.3652$ ;  $W_{(bf34)}=0.9575$ ,  $p=0.3672$ ;  $W_{(bf02)}=0.9735$ ,  $p=0.7342$ ) (Fig. 2 Supplementary material). Not normally distributed males showed different levels of bimodal distribution (Shapiro–Wilk normality test  $W_{(bf1)}=0.9169$ ,  $p=0.0436$ ;  $W_{(bf2)}=0.8835$ ,  $p=0.0082$ ;  $W_{(bf3)}=0.8937$ ,  $p=0.0134$ ) (Fig. 2 Supplementary material). Dip test of bimodality strongly suggests unimodality in most of the analyzed males (9/10) with just one male showing statistical significance in bimodality (Hartigan's dip test for bimodality  $D_{(bf2)}=0.1101$ ,  $p=0.0095$ ) (Fig. 2 Supplementary material). Visually, three males showed more than one peak (see bf1, bf10, bf3, Fig. 2 Supplementary material).

Finally, pre-insemination ejaculate analysis of sperm packages width on males of *C. keyserlingi* showed that only 1 out of 5 males (20%) showed normal distribution of sperm packages width (Shapiro–Wilk normality test  $W_{(ck4)}=0.9385$ ,  $p=0.0828$ ). Data in the other 4 males showed, visually, a marked bimodal distribution, and normality analysis showed that the data is indeed not normally distributed (Shapiro–Wilk normality test  $W_{(ck1)}=0.8436$ ,  $p=0.0005$ ;  $W_{(ck2)}=0.9164$ ,  $p=0.0217$ ;  $W_{(ck3)}=0.8596$ ,  $p=0.0010$ ;  $W_{(ck5)}=0.8553$ ,  $p=0.0008$ ) (Fig. 3 Supplementary material). However, dip test analysis only supports deviance from unimodality in two males (Hartigan's dip test for bimodality  $D_{(ck1)}=0.1371$ ,  $p<0.01$ ,  $D_{(ck3)}=0.0892$ ,  $p=0.0462$ ). Some males had this marked visual bimodal distribution with a clear cut between putative modes (see ck2, ck5 Fig. 3 Supplementary material). The visual analysis of live sperm packages from this species also supported marked width variation where the average sperm packages were big and thick (Table 1, Fig. 1A), and they were always accompanied by other sperm packages that were notably thinner, which had almost a third of the width of regular sperm packages (see Fig. 1A). Regular sperm packages in this species appeared in a proportion of almost 2:1. Thin sperm packages in the two Bothriuridae species were not as abundant as in *C. keyserlingi*, which is clearly reflected in the pooled data analysis (see below).

**3.1.2.2. Pooled data analysis.** Width data in *B. bonariensis* (Bothriuridae) was not normally distributed (Shapiro–Wilk normality test  $W=0.9715$ ,  $p=0.0012$ ; pooled data of 7 males) (Fig. 5A). Graphically, some peaks can be observed in the distribution (Fig. 5A). However, bimodality test showed that these data are unimodal (Hartigan's dip test for bimodality  $D=0.0329$ ,  $p=0.1969$ ; pooled data of 7 males).



**Fig. 4.** Rare cases of spatial variation within pre-insemination ejaculates of species showing width variation. (A) *Brachistosternus ferrugineus*, shows two folded sperm packages and a sperm package that is releasing its spermatozoa among regular straight sperm packages. (B) *Urophonius brachycentrus*, regular straight sperm packages appear together with one folded sperm package and a ring like one. These variants appeared in very few males at the end of the reproductive season. White arrows show the variants. The grey arrow shows an open sperm package.



**Fig. 5.** Density distribution of sperm packages width in two Bothriuridae and one luridae. (A) *Bothriurus bonariensis*. (B) *Brachistosternus ferrugineus*. (C) *Caraboctonus keyserlingi*. Bars are accompanied by dotted lines that outline normal distributions.

Pooled data in *B. ferrugineus* (Bothriuridae) showed a distribution slightly skewed to the right, and the Shapiro–Wilk normality test supported this by showing that the data was not normally distributed (Shapiro–Wilk normality test  $W=0.9809$ ,  $p=0.0019$ ). Similarly, data from *B. ferrugineus* showed a typical unimodal distribution, with only one marked peak near  $13\ \mu\text{m}$ , (Fig. 5B). The Hartigan’s dip test supported the presence of a unimodal distribution (Hartigan’s dip test for bimodality  $D=0.0144$ ,  $p=0.9909$ ; pooled data of 10 males).

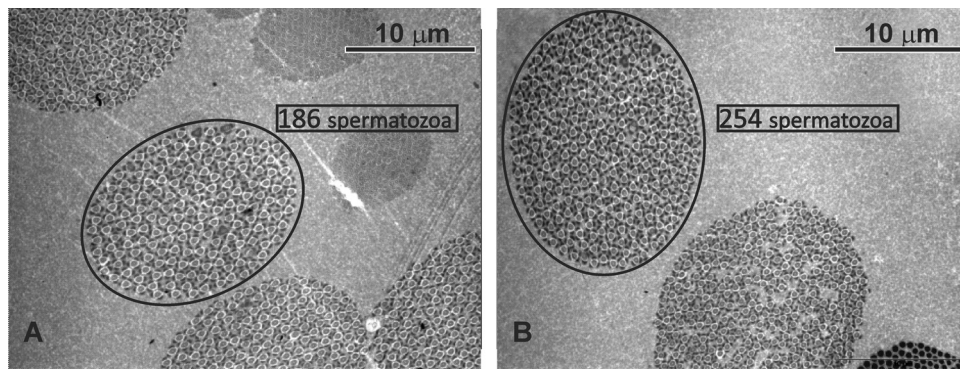
Pooled data in *C. keyserlingi* was not normally distributed, as in the previous two cases (Shapiro–Wilk normality test  $W=0.9809$ ,  $p=0.0019$ ; pooled data of 5 males), but differed in that the distribution showed two distinct peaks near  $10$  and  $30\ \mu\text{m}$  (Fig. 5C). In contrast to the previous results in Bothriuridae, the Dip test showed a clear bimodality in *C. keyserlingi* (Hartigan’s dip test for bimodality  $D=0.0504$ ,  $p<0.001$ ; pooled data of 5 males).

### 3.2. Sperm count in *Bothriurus bonariensis* and *Brachistosternus ferrugineus*

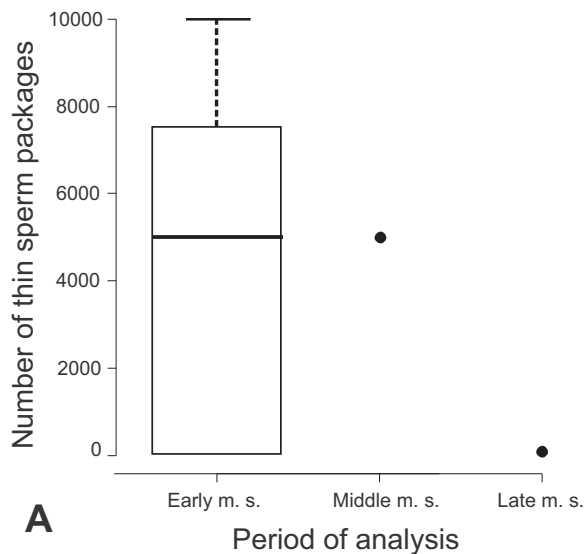
The ultrastructural analysis carried out in *B. ferrugineus* showed that thin sperm packages contained fewer spermatozoa compared to regular ones (Fig. 6A and B). Standard sperm packages in *B. ferrugineus* had approximately  $256 \pm 22$  ( $N=20$  sperm packages, one male) spermatozoa and  $128 \pm 18$  ( $N=20$  sperm packages, one male) in *B. bonariensis*. Thin sperm packages showed  $186 \pm 10$  spermatozoa ( $N=20$  sperm packages, one male) in *B. ferrugineus*, and  $84 \pm 8$  spermatozoa ( $N=20$  sperm packages, one male) in *B. bonariensis*.

### 3.3. Preliminary evaluation of the effect of seasonality and body condition over sperm package variation in *Brachistosternus ferrugineus*

Our results showed a clear effect of the sampling season over the number of sperm package containing width variation in *B. ferrugineus* (ANOVA,  $F_{(2,20)}=6.07$ ,  $p=0.01$ ). The Tukey pos hoc test demonstrated that over the three sampled months, sperm package variation was greater in November compared to January and February (Fig. 7A). In November, 6 out of 9 analyzed males showed thin sperm packages (67%). In January, this percentage dropped to 14% (1 out of 7 analyzed males), and there were no males with thin sperm packages observed in February. However, the effect of the body condition over thin sperm package numbers was not statistically significant (ANOVA  $F_{(1,21)}=1.10$ ,  $p=0.30$ ).



**Fig. 6.** Ultrastructure of width variant in sperm packages from the same male of *Brachistosternus ferrugineus*. The pictures show the comparison of sperm numbers of two sperm packages within a pre insemination ejaculate. The cut in both sperm packages was made through the middle piece of spermatozoa. (A) Standard sperm package. (B) Thin sperm package.



**Fig. 7.** (A) Box plot showing the amount of thin sperm packages among three different months of sampling during *Brachistosternus ferrugineus* reproductive season 2010–2011. The amount of thin sperm packages decreased over the season, and these were totally absent in samples obtained in February. m.s. = Mating season.

#### 4. Discussion

Pre-insemination ejaculates of males from several scorpion species showed variation in sperm package morphology. Two variants turned out to be relevant: one affecting the sperm package width and the other its spatial arrangement. These two variants coexist with the morphological average sperm package in an intra-male scale. It is known that sperm packages extracted from different zones of the seminal vesicle, share the same general morphology (Mattoni, 2003). This fact eliminates the possibility of change in sperm package morphology produced by the movement through the final part of the male's reproductive tract or by different developmental states of sperm packages, as suggested for other arachnids (Moya et al., 2007). *C. keyserlingi* showed evidence of having sperm packages heteromorphism (dimorphism) within a pre-insemination ejaculate. However, this could not be observed in other species. Differences in width are based on variations in sperm number and the reproductive season seemed to have an influence in the generation of sperm packages variation. However, the term heteromorphism is difficult to apply and will be discussed in the following.

#### 4.1. Sperm package heteromorphism in Bothriuridae

Sperm package width in *B. bonariensis* and *B. ferrugineus* was not normally distributed. Both distributions were unimodal, although *B. bonariensis* had a distribution with different peaks. The presence of two or more marked peaks in the distribution may suggest the possibility of size heteromorphism. However, there is no strong support in our data to suggest a width heteromorphism of sperm packages in these species. In general, individual males showed no clear bimodal distribution in concordance to what was shown in the pooled data distribution of the species.

In bothriurids, thin sperm packages appeared in very low numbers (below 15% of the total number of sperm packages counted). Among arthropods, there are some species of insects where the production of a particular sperm package morph is generated in low proportions, even below 10% (e.g., Swallow and Wilkinson, 2002 [Diptera and Lepidoptera]). Thus, sperm package heteromorphism in scorpions may be present, but not in the sense referred to in the previous paragraph of different morphotypes with tightly regulated production as suggested, analogously, for single spermatozoa in other arthropods (e.g., Joly and Lachaise, 1994 [*Drosophila obscura*] or Sasakawa, 2009 [*Bombix mori*]). Although true heteromorphism (sensu, Pitnick et al., 2009; Dallai, 2014) with different sperm functionalities seems to be absent, some interesting variations appeared in width and general morphology. In most of the species with folded sperm packages, these packages were mixed with some variants as ring-like, spherical or straight sperm packages within a single pre-insemination ejaculate. It is still unknown if ring-like sperm packages are derived from folded or straight sperm packages. It is also unknown the modifications involved in this transformation.

Previous analyses in scorpions suggest that, phylogenetically, the most basal form of sperm package seem to be folded and that the derived state may be either straight or spherical/circular (Vrech et al., unpublished data; Vrech, 2013). As an analogy this is also shown in an intramale scale in species with spatial variation, as the main and most abundant form is folded and alternative forms may be straight or circular.

It is currently unknown if the “alternative” morphologies of sperm packages have some adaptive function or show mere teratologies within a male pre-insemination ejaculate (reviewed in Higginson and Pitnick, 2011). Maybe, the presence of fewer spermatozoa within a sperm package is far from determined, and could be based on production failures (Cohen, 1967; Pitnick et al., 2009) or developmental noise (Parker and Begon, 1993), as happens with single spermatozoa. Swallow and Wilkinson (2002) suggested that teratology could explain mammalian sperm deviating from normal morphology (e.g., Cohen, 1973; Harcourt, 1991), but stated

that it may not be the case in most insect sperm polymorphisms. However, data from our analysis may support the teratological explanation, at least for *B. bonariensis* and *B. ferrugineus*. It should be pointed out that teratology should not be understood here as spermatozoa teratology where deleterious failures or shape abnormalities appear affecting single spermatozoa and fecundity as well. We refer to failures in the production of sperm packages with regard to the capacity to “gather” spermatozoa and form the entire package. Precisely scorpions are thought to group mature sperm together at the end of spermiogenesis (Vignoli et al., 2008) and this could be the moment where the failure occurs. However, it would be interesting to address if failures originate at the beginning of sperm production, maybe in mitotic processes or just at the end of the production when mature sperm get together, or maybe a combination of both. The general idea of a teratological origin of variation in sperm packages width in scorpions could be supported by the fact that there was an influence of the reproductive season over the frequency of thin sperm package appearance. In effect, *B. ferrugineus* showed a greater number of thin sperm packages at the beginning of the reproductive season. This could mean that males need to produce a lot of sperm at the beginning of the reproductive season and teratological effects (more aberrant sperm packages or poorer quality control) might appear due to the excessive demand of sperm production. Spermatogenesis is not energetically as cheap as was once thought (see Dewsbury, 1982; Pitnick and Markow, 1994). The excessive energetic requirement during the first part of the reproductive season would have an impact in the production of sperm and may lead to the production of sperm packages with a higher amount of variability. At this point of the reproductive season, females are still virgin (Peretti et al., pers. obs.). In this scenario, males would aim to inseminate the largest amount of females and are subject to sperm competition (Peretti and Carrera, 2005; Peretti, 2010). Some studies among vertebrates suggest the existence of similar teratological effects in gametes, but concentrated outside the reproductive period (Brun and Rimpler, 1990). Bearing this in mind, an alternative hypothesis can be considered: scorpions could produce teratological sperm packages during winter. These packages will then be manifested in pre-insemination ejaculates at the beginning of the reproductive season. In both cases, thin sperm packages in *B. ferrugineus* could give the male a disadvantage in sperm competition with a lower count of thin sperm packages, supposing that a similar number of sperm packages is transferred. Nevertheless, a few facts should be considered. First, in scorpions, sperm package propulsion is not affected as in other arthropods (Mackie and Walker, 1974 [water beetle]; Sivinski, 1980 [insects]; Pizzari and Foster, 2008 [review]; Higginson and Pitnick, 2011 [review]), because sperm packages in scorpions do not move actively inside the female reproductive tract (Peretti and Battán-Horenstein, 2003). In fact sperm packages are absent in the female atrium after an hour, and sperm is stored as single spermatozoa inside the female spermathecae (Peretti and Battán-Horenstein, 2003; Mattoni et al., pers. obs.). Although thin sperm packages showed fewer spermatozoa, the total amount of transferred spermatozoa may not be affected, as males could transfer a higher amount of sperm packages per ejaculate. However, information about this trade-off between sperm number and sperm package number is still lacking in scorpions and should be addressed in the future. On the other hand, males could take the production of thinner sperm packages as a strategy for sperm competition. Males could produce a higher number of thinner sperm packages with fewer spermatozoa, generating sperm packages earlier (as sperm packages with fewer spermatozoa could be energetically cheaper and be ready sooner compared to average sperm packages), and anticipating other males who produce mainly average sperm packages. Thus, hypothetically, these males could begin searching earlier for females to inseminate,

at the expense of a fewer total number of spermatozoa in their pre-insemination ejaculates compared to other males. The early activity of males during the reproductive season would give them an advantage in finding virgin females, and gaining higher reproductive success (e.g., McNamara et al., 2004 [Coleoptera]; Baruffaldi and Costa, 2010 [wolf spider]). Certain characters and behaviors support these ideas. For example, females of different species of scorpions show variable degrees of remating receptiveness (Vrech et al., 2014). Some become less receptive after a successful mating. This behavior is not yet precisely determined in scorpions, but may involve mating plugs, and/or pheromones. (Gaffin and Brownell, 2001; Mattoni and Peretti, 2004; Contreras-Garduño et al., 2006; Althaus et al., 2010; Peretti, 2010; Vrech et al., 2014). Besides behavior, the morphology of the spermathecae suggests that, if sperm stratification occurs, there could be a first male sperm precedence (Volschenk et al., 2008). Thus contemplating post-copulatory female reluctance and spermathecal structure, males that copulate first with virgin females may have an advantage in sperm competition. Nevertheless, studies of the frequency of occurrence of the sperm package variants throughout the year are needed, as well as sperm precedence analyses in some of these species. An alternative hypothesis may suggest that differences in sperm package width could be the result of sperm package being activated (i.e., sperm package being dissociated by active movement of individual spermatozoa) prematurely. Previous analyses shown that sperm packages in this situation are much wider than the average ones as sperm starts to separate from each other (Peretti and Battán-Horenstein, 2003; Vrech pers. obs.). Furthermore, this process could produce the separation of some spermatozoa bundles from the entire sperm package and leave thinner “sub sperm packages”. However, this would result in sperm packages with a wide variety of spermatozoa numbers, which is disproved in our analysis. In fact, as the activation is very common under saline solution (Peretti and Battán-Horenstein, 2003), we have controlled this possible source of bias by using ethanol and glycerin.

#### 4.2. Sperm package dimorphism in *Caraboctonus keyserlingi* (Luridae)

*C. keyserlingi* (Luridae) showed a clear sperm package dimorphism. The average morph is much thicker compared to the “alternative” one. Males of *C. keyserlingi* have huge seminal vesicles and long straight sperm packages, as happens in other Luridae (Vrech et al., 2011; Mattoni pers. obs.). Both characters, together with the presence of dimorphic sperm packages, may suggest the existence of a strong sperm competition risk among males. Bearing this in mind, the presence of dimorphic sperm packages may not be the result of production failure as we have previously argued for Bothriuridae. Various authors have analyzed individual sperm heteromorphism, mainly on butterflies (reviewed in Simmons, 2001; and Birkhead et al., 2009). In these and other insects, teratological effects are hardly responsible for spermatozoa heteromorphism, (Swallow and Wilkinson, 2002; Simmons, 2001; Birkhead et al., 2009). In an evolutionary scenario, sperm package heteromorphism is suggested to be subjected to sexual selection through sperm competition (Snook, 1998; Holman and Snook, 2006), and this is marked in species with specimen morphotypes that bear different types of spermatozoa (reviewed in Swallow and Wilkinson, 2002). There are also some evidence for sperm packages where both morphotypes produce fertile spermatozoa (eusperm–eusperm) (e.g., Joly and Lachaise, 1994; Snook, 1998; Takami and Sota, 2007) as may be the case in Luridae. However, if this trait is adaptive, the true advantage of heteromorphic sperm packages in *C. keyserlingi* males is still unknown. Some hypotheses suggest that sperm competition in males may produce additional types of spermatozoa that can facilitate viable spermatozoa to achieve ova fertilization



(e.g., Simmons, 2001; Wigby and Chapman, 2004; Higginson and Pitnick, 2011). It is currently unknown if spermatozoa from thin sperm packages in *Iuridae* have the same fertilization capabilities as those contained inside average sperm packages. This matter should be assessed in the future using nuclear staining techniques. Furthermore, it should be explored whether the variation in sperm package morphology is a strategy for overcoming sperm competition, as suggested for insects in similar situations (e.g., Wigby and Chapman, 2004; Takami and Sota, 2007). Meanwhile, if this is true, the precise mechanism of this strategy should be characterized, as there is little knowledge of how sperm packages really behave in vivo. In addition, studies of the reproductive behavior in scorpions are needed to understand the influence of sperm competition over general morphological variables, together with the possible appearance of polymorphic sperm packages in species with a high sperm competition level.

Finally, we hope that this analysis could be the first step for future studies linking sperm packages and sperm polymorphism with mating strategies (in the form of sperm competition and/or cryptic female choice).

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcz.2015.11.002>.

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