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The giant and complex genital plug of the *asper* group of *Bothriurus* (Scorpiones, Bothriuridae): morphology and comparison with other genital plugs in scorpions

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Abstract

The genital plugs of two species of the *asper* group of *Bothriurus* (Scorpiones: Bothriuridae) are described and compared with other genital plugs reported in the family Bothriuridae. In both species, *B. asper* and *Bothriurus* sp., the genital plug is cone-shaped and formed by fusion of the basal lobes of the hemispermatophore. Fusion is complete in *B. asper* and the surface of the plug has many microspines that anchor it to the female genital atrium. In *Bothriurus* sp., the basal lobes are partially fused, but free on the dorsal side, and the plug has a smooth surface with a dorsal curvature. Both genital plugs completely fill the genital atrium of inseminated females, pressing against the cuticular wall of the atrium. Given the large size and complex shape, the genital plug of the *B. asper* group is unique, not only among Bothriuridae, but in the order Scorpiones. This new type of genital plug resembles the genital plugs of the scorpion families Urodacidae and Vaejovidae. A comparison of the four major types of genital plugs reported in *Bothriurus* species and some other bothriurids is provided, as well as a comparison with other genital plugs reported in more distantly related families of scorpions.

Keywords: Spermatophore; Basal lobes; Sperm competition

Introduction

The structure and function of genital or "mating" plugs have been explored in diverse animal groups, including many arthropods such as insects and spiders (Elgar 1998; Simmons and Siva-Jothy 1998). In general, male genital plugs have been interpreted as a mechanism to prevent or reduce sperm competition (Parker 1970, 1984). By intromission of a plug inside the female gonopore, a male prevents or reduces the entry of sperm from males that attempt to copulate subsequently with the same female (Simmons and Siva-Jothy 1998; Simmons 2001). Therefore, the presence of a male genital plug occluding the female genital gonopore and/or atrium directly signifies competition among males for insemination of females. In addition, the sexual conflict hypothesis (Chapman et al. 2003) predicts that some types of genital plug could have evolved as a result of male–female conflict over control of fertilization. For example, when a male plug completely blocks the gonopore or genital atrium of an inseminated female, she will be deprived of the opportunity to select among

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the sperm of multiple males. However, an exclusively ''male origin'' for the genital plug has been questioned in some cases, given that glandular secretions of the female could also be involved in its construction (Eberhard 1996, 1998, pers. comm 2003; Méndez and Eberhard in preparation). Furthermore, the role of the female in maintaining and/or degrading the male genital plug is almost unknown (Peretti 2003).

In scorpions, sperm is transferred to the female by a single spermatophore composed of two, generally symmetrical halves, called hemispermatophores, tath become joined together just as the spermatophore emerges from the male gonopore (Hjelle 1990; Farley 2001). In most scorpion families, there is a middle region in the spermatophore called the capsule (composed of several folds, the lobes), an invagination of the sperm duct that is everted during the sperm transfer phase, ejecting sperm into the female atrium (Peretti 1993, 2003).

The most well-known (but nevertheless poorly studied) example of a genital plug originating from a male scorpion is found in the family Vaejovidae. Typically, a vaejovid genital plug is formed by two hemi-genital plugs (corresponding to a part of the lobes of each hemispermatophore) that join together inside the spermatophore prior to sperm transfer (Stockwell 1989; Contreras-Garduño and Peretti unpublished data). Indeed, these hemi-genital plugs offer good morphological characters for taxonomy (e.g., Sissom 1992, 1993; Sissom and Stockwell 1992). Each hemi-genital plug possesses a 'distal barb' (naked or with small-teeth) that enhances the subsequent anchorage of the plug to the cuticular wall of female genital atrium once sperm transfer has concluded (Contreras-Garduño and Peretti unpublished data).

In the family Bothriuridae, the presence of genital plugs has been observed in species of the following genera: *Bothriurus* Peters, *Urophonius* Pocock, *Phoniocercus* Pocock, and *Brachistosternus* Pocock (Castelvetri and Peretti 1999; Peretti 2003; Mattoni unpublished data). With the exception of *Timogenes* Simon, a hard-amorphous plug was always found partially occluding the genital atrium of inseminated females of all bothriurid species examined (Castelvetri and Peretti 1999; Peretti 2003). This type of genital plug could be similar to the ''spermatocleutrum'' described by Pavlovsky (1924) in gravid females of some scorpions. Recent studies suggest that hard-amorphous genital plugs of bothriurids originate from seminal secretions contained in the spermatophore, possibly produced by a gland from the male reproductive system (Peretti and Battán-Horenstein 2003).

The present contribution describes a new type of genital plug from the order Scorpiones: the giant plug of *Bothriurus asper* Pocok and of its as yet undescribed sister species. Here we describe the structure of the genital plug and the hemi-genital plugs and provide a discussion and comparison of the major types of genital plugs thus far examined in the family Bothriuridae.

Material and methods

Study taxa

B. asper is common scorpion in inhabiting the Caatinga region northeastern Brazil (Cabrera and Willink 1980; Maury 1982). This *Bothriurus* species is related to those of the *araguayae*, *rochai* and *bonariensis* groups (Mattoni 2003). *B. asper* males show a particular hemispermatophore morphology (Maury 1982). The form of the lobes is very complex. The basal lobe is divided into two parts: a curved dorsal portion and a spatulate ventral portion. Maury (1982) considered these features, together with other morphological characteristics, and proposed the ''*asper*'' group, initially including only one species, *B. asper*. However, recent taxonomic study (Mattoni 2003) found a similar hemispermatophore morphology in a closely related and as yet undescribed species from the State of Bahia, Brazil.

Structural analysis of the genital plug

The morphology of the genital plug was studied in 25 adult females of *B. asper* and three adult females of *Bothriurus* sp.; the hemi-genital plugs were analysed in six adult males of *B. asper* and in five adult males of *Bothriurus* sp. The individuals were captured at different localities in the Brazilian states of Bahia, Alagoas, Ceará, and Pernambuco, preserved in 80% ethanol and deposited in the following collections: Museu de Zoologia de la Universidade de São Paulo, Brazil (MZUSP); Museo Nacional de Rio de Janeiro, Brazil (MNRJ); Laboratorio de Animais Peçonhentos de la Universidade Federal de Bahia, Salvador de Bahia, Brazil (UFBA-ESC:); Laboratório de Artrópodos Peçonhentos del Instituto Butantán, São Paulo, Brazil (IBSP-SC) and Cátedra de Diversidad Animal I, Universidad Nacional de Córdoba, Argentina (CDA). Several have been deposited as voucher specimens in the MZUSP (*B. asper*: 1 male, 1 female; *Bothriurus* sp.: 1 male, 1 female), IBSP-SC (*B. asper*: 12 females and 3 young), and at the UFBA-ESC (*Bothriurus* sp.: 1 female).

The male paraxial organs were dissected to examine in both hemispermatophores all parts involved in the formation of a genital plug. The number and position of genital plugs inside the female genital atrium was observed under a light microscope. The genital atrium was dissected to observe the general morphology and to

determine whether the surface of the plug made contact with the cuticular wall of the atrium. Genital plugs broken or altered during these manipulations were discarded. Seminal receptacles were also dissected in some females to observe the presence or absence of spermatozoa as an indication of whether they had been recently inseminated. Light and scanning electron microscopes (SEM) were used to observe, measure and describe the fine structure of the hemi-genital plugs obtained from males and the genital plugs removed from the female genital tract.

Results

Genital plug: structure and position in the female genital atrium

Bothriurus asper

In total, 15 of 25 females studied, displayed a complete genital plug, one showed only small fragments of a plug, and nine did not possess plugs. The genital plug of this species is a sclerotized, conical structure, slightly curved, with a wide trapezoidal base, that completely fills the female genital atrium (Figs. 6, 7, 9, 10 and 16). The average length of the genital plugs was 1.08 ± 0.15 mm (range 0.94–1.36 mm, $N = 10$). The plug also obliterates the two ducts that allow communication between the genital atrium and the seminal receptacles. No females presented more than one genital plug ($N = 15$). The basal part of the plug can be observed by moving the genital operculum with a pair of forceps. In three females, the operculum was slightly lifted by the basal part of the plug.

The basal part exhibits a large expansion (Fig. 16) representing the limit of intromission of the base of the plug inside the female atrium. This expansion is produced by fusion of the dorsal parts of the two basal lobes. The interior of the plug is hollow and the lumen is filled with a granular substance anteriorly and medially (Fig. 16). An amorphous-sclerotized substance obliterates the posterior part (Fig. 5). The surface of the plug is covered with thick retrograde microspines (Figs. 9, 10, 16 and 17), the tips of which are directed to the external opening of the gonopore, thereby anchoring the genital plug to the wall of the female atrium (Figs. 6 and 7). The retrograde arrangement of these microspines prevents its complete removal (i.e. without breaking the plug) from the genital atrium using forceps. This is possible only by dissecting the entire cuticular wall of the atrium. Despite this strong anchorage, the cuticular wall of the genital atrium shows no evidence of lesions (Fig. 8).

Eight of 15 females dissected presented a complete genital plug inside the genital atrium. The seven females without a genital plug displayed empty seminal receptacles. When a genital plug was found in the genital atrium of a dissected female ($N = 8$), sperm was usually present in both seminal receptacles. For example, four females without embryos presented full seminal receptacles and a fresh light brown plug in the atrium. The other four females exhibited developing embryos in the ovariuterus and contained less or no sperm (2/2) in the seminal receptacles. In these females the genital plug was blackish, apparently degraded (in two cases, the plug was covered by a granular substance similar to vegetable coal), and easily broken during manipulation. A female with fully developed embryos demonstrated only small black fragments of the genital plug.

Observations of fine structure indicate that the genital plug of *B. asper* is formed by two halves, the hemi-genital plugs, fused to each other (sometimes weakly at the distal and basal tips). Each hemi-genital plug corresponds to the dorsal portion of a basal lobe of the hemispermatophore (Figs. 1–3, 9, 10 and 16): colour,

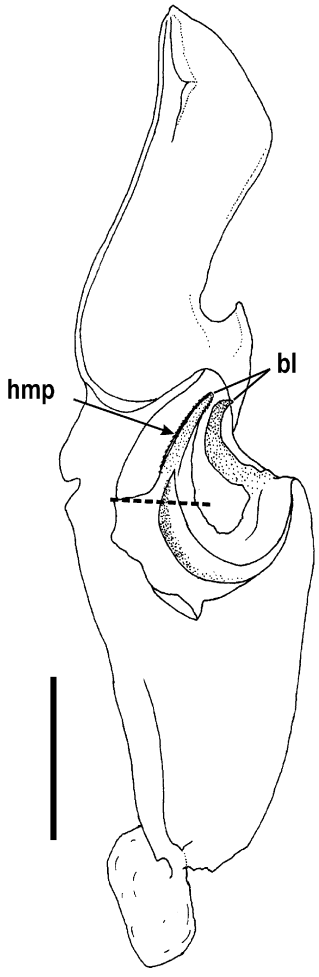
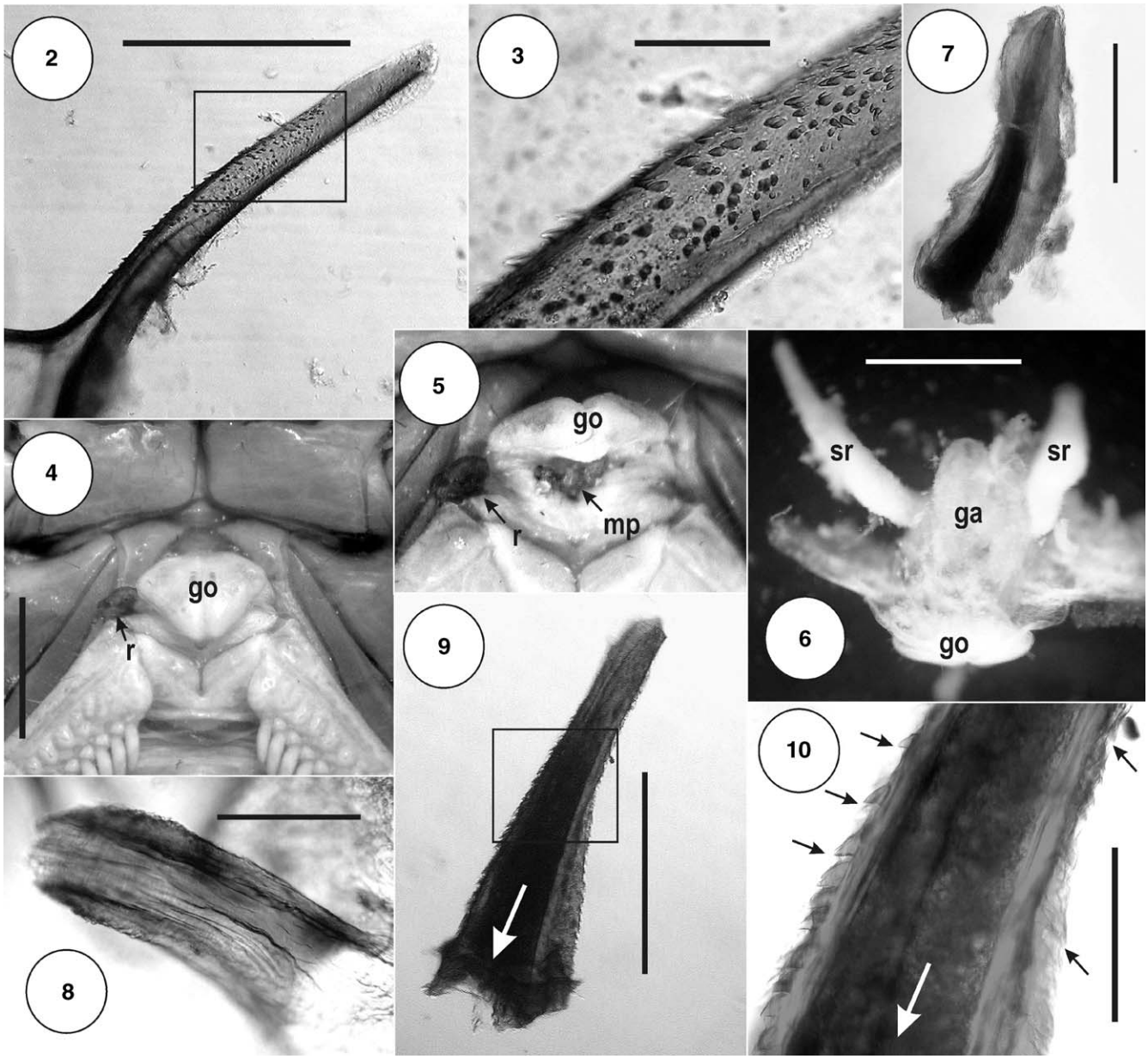


Fig. 1. Internal view of left hemispermatophore of *B. asper* showing region of the basal lobe that is produced into a hemi-genital plug (dots indicate the point of detachment of the lobe). Abbreviations: hmp = hemi-genital plug; bl = basal lobe. Scale: 1 mm.

length and microstructure of that part of the basal lobe (Figs. 1–3) are identical to the longitudinal half of the genital plug. Furthermore, both basal lobes present a defined point of detachment at the base (Fig. 1). They are easily detached during manipulation of the hemispermatophore.

***Bothriurus* sp.**

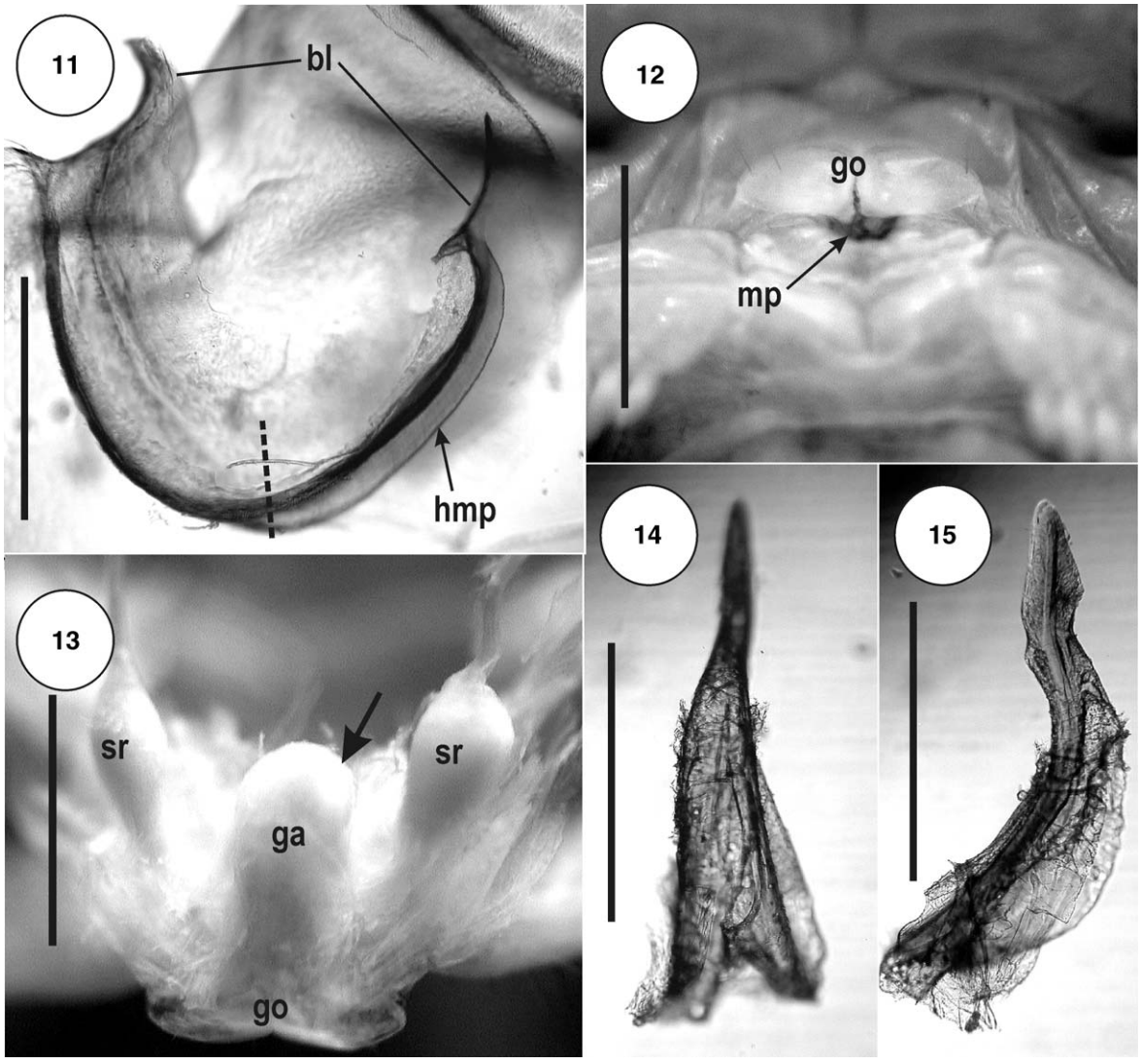
A genital plug, strongly anchored to the cuticular wall, was found in the genital atrium of two out of three females examined during this study. One of these females displayed one genital plug in her atrium; the other had two. The average length of the genital plugs



Figs. 2–10. Genital plug of *B. asper*: 2–3. External view of dorsal portion of basal lobe of right hemispermatophore: 2. General view. 3. Detailed view. 4–5. Ventral view of external opening of female gonopore: 4. Gonopore containing residue of a genital plug on left side. 5. Gonopore with genital operculum lifted to reveal the base of an introduced genital plug. 6. External view of female genital atrium and seminal receptacles containing sperm (the former appears expanded due to presence of a genital plug inside). 7. A dissected female atrium showing, by transparency, a genital plug completely occluding its lumen (the plug is partially broken medially). 8. View of female atrium after removal of a genital plug, showing its internal cuticular wall without visible lesions. 9–10. Genital plug (white arrows indicate position of female gonopore): 9. General view. 10. Detailed view of external surface of the plug, note presence of abundant microspines (black arrows). Abbreviations: go = genital operculum; mp = genital plug; r = residues of plug; ga = female genital atrium; sr = seminal receptacle. Scales: 1 mm in Figs. 4–6; 0.5 mm in Fig. 2 and Figs. 7–9; 0.1 mm in Figs. 3 and 10.

was similar to that of *B. asper*: 0.95 ± 0.03 mm ($N = 2$). Likewise, the basal part of the plug was visible from the external opening of the female gonopore (Fig. 12). However, the genital plug of *Bothriurus* sp. did not exhibit a basal expansion, indicating the limit of intromission in the female atrium (Figs. 14, 15 and 18). Although, in this species, the plug is also formed by combination of the dorsal portions of the basal lobes of both hemispermatophores (Fig. 11), fusion is less complete than in *B. asper*. The dorsal surfaces of the medial-basal and distal regions of the plug were not fused (Figs. 14, 15, 18 and 19). The two females had full seminal receptacles and sperm was also found in the internal portion of the genital atrium (Fig. 13).

Although the lobes of the hemispermatophore of *Bothriurus* sp. are very similar to those of *B. asper*, differences were observed on the surface of the dorsal portion of the basal lobe. In *Bothriurus* sp. this region of the hemispermatophore does not display microspines; only a large spine is evident at the distal tip (Fig. 11). This distal spine was absent in the genital plugs obtained from the genital atrium of the females studied, although dorsal curvature was observed (see Figs. 15 and 18). This conspicuous curvature (together with the irregular



Figs. 11–15. Genital plug of *B.* sp. 11. Internal view of right hemispermatophore showing region of the basal lobe that is produced into a hemi-genital plug (dots indicate the point of detachment of the lobe). 12. Ventral view of female gonopore with a genital plug occluding entrance to the atrium. 13. External view of female genital atrium and full seminal receptacles (black arrow indicates presence of sperm in internal part of the atrium). 14. Dorsal view of a genital plug. 15. Lateral view of a genital plug. Abbreviations: hmp = hemi-genital plug; bl = basal lobe; go = genital operculum; mp = genital plug; ga = genital atrium; sr = seminal receptacle. Scales: 0.5 mm in Figs. 11, 14 and 15; 1 mm in Figs. 12 and 13.

Table 1. Types of genital plugs observed in 18 species of Bothriuridae

Species	Mating plug type					
	A *(hard-amorphous)*	B *(membranous)*	C *(filamental)*	D *(cone-shaped)*		E *(mixed)*
				D-1 (smooth;partially fused lobes)	D-2 (many lateral spines; fused lobes)	
Bothriurus:						
B. prospicuus	●					
B. cordubensis	●					
B. noa	●					
B. flavidus	●	●				
B. sp. (*flavidus* group)	●	●				
B. olaen	●					
B. sp. (*burmeisteri* group)	●					
B. bonariensis	●		●			
B. chacoensis	●					
B. asper	**?**				●	
B. sp. (*asper* group)	**?**			●		
Urophonius:						
U. brachycentrus	●					?
U. iheringii	●					?
U. tregualemuensis	●					**?**
Phoniocercus:						
P. pictus	●					●
P. sanmartini	●					●
Brachistosternus:						
B. ferrugineus	●					
B. pentheri	●					
Total species:	16	2	1	1	1	2

More details in the text and in Castelvetri and Peretti (1999), Peretti and Battán-Horenstein (2003) and Peretti (2003).

dorsal edges of the plug) may facilitate anchorage to the genital atrium (Figs. 11–15).

Comparison with other genital plugs

The genital plug of the *B. asper* group is completely different from the hard-amorphous plug found in inseminated females of most other bothriurid species (Table 1). These plugs are formed by the combination of a granular substance and hard elements (not sperm packages or free spermatozoa) that could be observed inside a fresh spermatophore (Peretti 2003). Typically, a hard-amorphous plug appears in the centre of the female genital atrium lumen and increases in size with further matings. The granular substance is apparently secreted by the medial accessory gland of the paraxial organ (Peretti and Battán-Horenstein 2003). Clear internal obstructions were observed only in female *B. bonariensis* (Table 2), where a hard-amorphous plug has been observed to block the spermathecal ducts (Castelvetri and Peretti 1999; Peretti 2003).

Genital plugs originating from spermatophore structures have been observed in the *B. flavidus* group and in *B. bonariensis* (Types B, membranous, and C, filamental, in Tables 1 and 2). The membranous genital plug occurs near the genital opening of female *B. flavidus*. The position of the plug only partially prevents the intromission of new spermatophores (Peretti 2003). The plug could be formed by membraneous components that are in close contact with the region of the spermatophore where the foramen will appear immediately after capsular eversion (Peretti 2003).

The filamental plug of *B. bonariensis* is formed by a thin filament extending from the capsular foramen of the spermatophore that is broken during sperm transfer and remains in the genital atrium, together with previous hard-amorphous plugs (Peretti 2003). This filamental plug does not affect the eversion process of a new spermatophore. However, because of its more

Table 2. Origin and position in the female of the main types of the bothriurid genital plugs

Origin		Mating plug type					
		A *(hard-amorphous)*	B *(membranous)*	C *(filamental)*	D *(cone-shaped)*		E *(mixed)*
					D-1	D-2	
Male	From accesory glands	●					●
	A part of the		●	●	●	●	●
Female	spermatophore	●[a]					
Position in the female							
Genital atrium	Full	●[b]			●	●	●
	Partial	●	●[c]	●			
Seminal receptacle duct	Full	●[b]					
	Partial						
Seminal receptacles	Full						
	partial	●[b]					

More details in the text and in Castelvetri and Peretti (1999), Peretti and Battán-Horenstein (2003) and Peretti (2003).

[a]The female may contribute with atrial secretions.

[b]Found in *B. bonariensis*.

[c]Type B only occupies the first part of the female atrium, near the genital aperture.

internal position in the female genital atrium, this type of plug may block the initial portion of the spermathecal ducts and ensure that the sperm of a second male remains in the genital atrium.

The genital plug observed in the genus *Phoniocercus* (Type E, mixed, in Tables 1 and 2) is formed by the fusion of some hemispermatophore detachable structures (associated with the basal lobe) with a hard sclerotized substance that resembles the hard-amorphous plug (Mattoni unpublished data). Inseminated females of *Urophonius* species present a plug that is similar to type E (Tables 1 and 2). Nevertheless, the composition of the different parts of this plug is uncertain (Moyano and Peretti unpublished data).

Owing to its large size, unique anchorage mechanism and the magnitude of its obstruction of the female genital atrium, the plug of the *B. asper* group described here represents a type not previously reported in scorpions. It is comparable in part to the plugs observed in the family Vaejovidae which originate from the capsular lobes of both hemispermatophores (Stockwell 1989; Sissom 1992, 1993; Sissom and Stockwell 1992) and, after detaching from the everted capsule, remain in the female atrium blocking the entry of new spermatophores (Contreras-Garduño and Peretti unpublished data). However, a typical vaejovid genital plug is nowhere near the size observed in the plugs of the *B. asper* group.

In the family Urodacidae there is a big structure in the hemispermatophore lobes that has been referred as a hemi-mating plug (Stockwell 1989) and that could have a similar function to the plugs described here (E. Volschenk, pers. com. 2004).

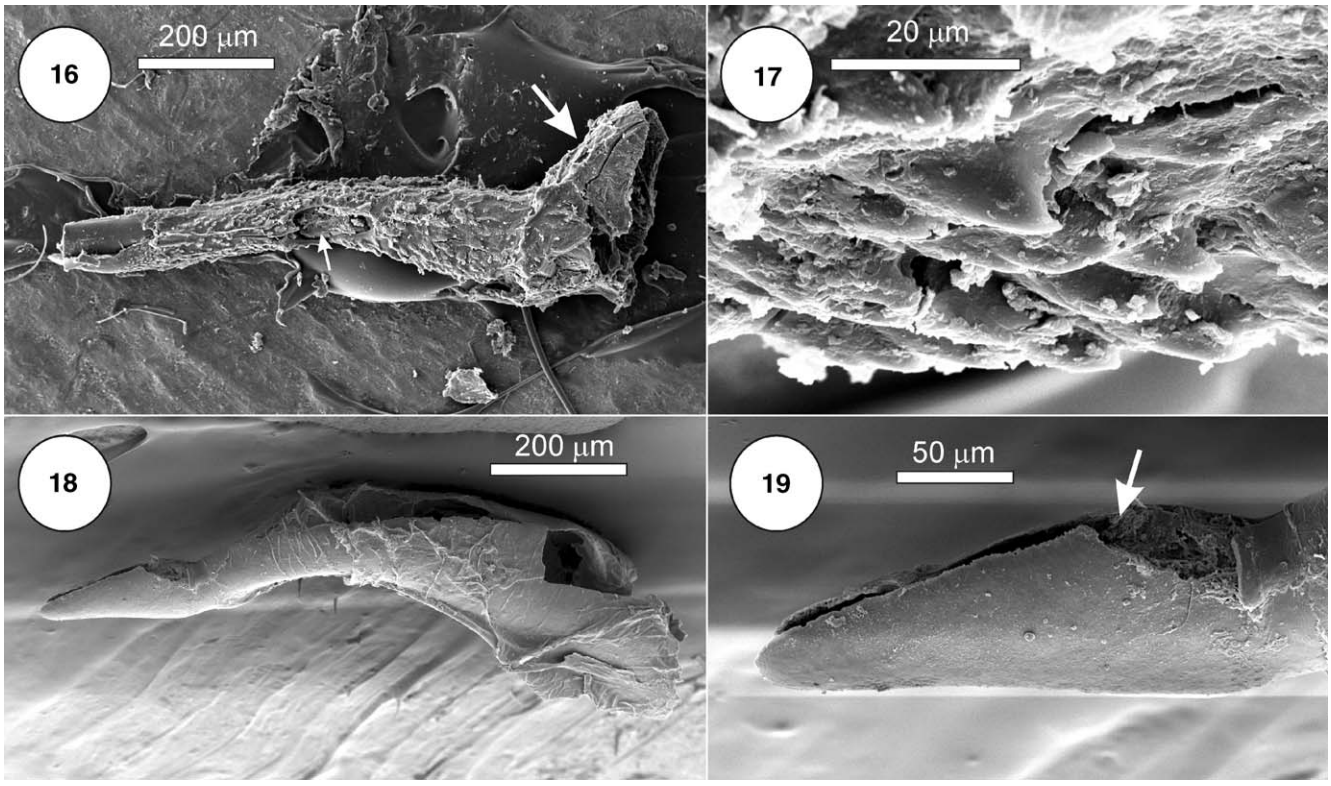
Discussion

Interpretation of genital plug morphology

Bothriurus asper group

In *B. asper*, the concave surfaces of the two basal lobes of both hemispermatophores fuse to create the genital plug, with a hollow interior observed in tranverse sections. The spiny convex parts of each basal lobe form the external surface of the plug, facilitating its anchorage to the cuticular wall of the female genital atrium. The weakness observed at the base of each basal lobe probably assists in detachment of the genital plug from the spermatophore during, or immediately after, sperm injection. The absence of lesions in the female atrium, caused by the spiny surface of the genital plug, may be attributed to the presence, in all bothriurids, of a folded atrial epithelium, covered by a thick cuticular wall, perhaps to avoid injury during capsular evertion of the spermatophore (Peretti 2003).

Both the presence of sperm in the seminal receptacles and of fully developed embryos in the ovari-uterus in all *B. asper* females containing a genital plug in their atria indicate that they had been previously inseminated. As no females exhibited more than one genital plug, it seems probable that the plug and the sperm found in each female originated from the same spermatophore. Owing to its large size (filling the whole atrium) and secure anchorage mechanism, this type of genital plug may prevent or retard intromission of spermatophores during subsequent matings, as observed in *Vaejovis punctatus* Karsch, 1879 (Vaejovidae) (Contreras-Garduño and Peretti unpublished data) although, in this



Figs. 16–19. SEM micrographs of genital plugs of *Bothriurus* from *asper* group. 16–17. Plug of *B. asper*: 16. Lateral view (large white arrow indicates the basal expansion; small arrow shows interior of plug, filled with a granular substance). 17. Detailed view of ventral portion of plug, showing retrograde microspines. 18–19. Plug from *Bothriurus* sp.: 18. Lateral view. 19. Detail of tip, showing unfused dorsal surface (white arrow).

species, the plug is smaller than that observed in *B. asper*. As with the unfolded basal process of the plug observed in *V. punctatus* (Contreras-Garduño and Peretti unpublished data), the large basal portion of the plug of *B. asper* may function to block the external opening of the female gonopore. The presence of a broken and deteriorated, blackish genital plug in gravid females suggests that the plugs could be progressively degraded by the environment of the genital atrium. This also appears to be the case in *V. punctatus* (Contreras-Garduño and Peretti unpublished data), where the genital plug degrades gradually after mating, is reduced to small fragments during gestation and disappears a few months before parturition.

Although the formation and subsequent detachment of the genital plug of *Bothriurus* sp. appears to be similar to that of *B. asper*, fusion of the basal lobes is incomplete in this species and the plug remains open in the dorsal part due to the absence of a concavity in both lobes. The edges of the dorsal parts are irregular and acute. This feature, together with the sinuous shape of the apical part of the plug, may facilitate its anchorage within the atrium. In contrast to the plug of *B. asper*, there is no structure in the basal part of the plug of *Bothriurus* sp. that would serve to delimit its intromission in the female genital atrium. This characteristic, together with the reduced fusion between both hemi-genital plugs, could allow the spermatophore of a new male to enter the female atrium, as observed in the female containing two genital plugs. Therefore, the genital plug of *Bothriurus* sp. might be less effective in preventing sperm transfer than that of *B. asper*. However, behavioural observations are necessary to assess this possibility.

Genital plugs in other scorpions

Genital plugs reported in other Bothriuridae (Table 1) serve a similar function: partial or complete prevention of intromission of a new spermatophore or its sperm to the female genital atrium after an initial mating. However, these types of genital plugs do not appear to be homologous, considering their disparate morphologies (e.g. hard-amorphous, membranous, filamental, cone-shaped and mixed), apparently different composition, and the different structures from which they originate.

Stockwell (1989) postulated that amorphous (or "gelatinous spermatocleutra") genital plugs may represent a plesiomorphic character, given that this type is common inside the genital atrium of inseminated females of many scorpions. New comparative data from Bothriuridae (Table 1) confirm this supposition for the

hard-amorphous plugs, which have been found in most species in which mating behaviour and/or reproductive morphology have been examined. Therefore, this plesiomorphic type of genital plug is usually shown by species that also contain other, more elaborate genital plugs (Table 1).

Other types of genital plugs (B: membranous, C: filamental and D: cone-shaped), represent apomorphies of some *Bothriurus* species: type B for the *B. flavidus* group, type D for the *B. asper* group, and type C for *B. bonariensis* (Mattoni 2003); and the genital plug type E could be apomorphic for the genus *Phoniocercus*.

The complex morphology of the genital plug of the *B. asper* group and its origin from detachable basal lobes of the spermatophore are novel features in the Bothriuridae. Only the "sclerotized genital plug" described in species of the families Vaejovidae and Urodacidae (Stockwell 1989; Prendini 2000) shows such structural complexity and origin. However, these functional and morphological similarities are evidently not homologous and must have evolved independently. All three families are distantly related phylogenetically and the conspicuous character "presence of a sclerotized genital plug" shows little tendency to disappear, occurring in all members of Urodacidae and most Vaejovidae (Stockwell 1989; Prendini 2000).

Further experimental studies are needed on the genital plugs of the *B. asper* group. The presence of this large and complex plug suggests that sperm competition is prevented since it blocks the female atrium and its external opening, preventing intromission of a new spermatophore. In addition, it appears that degradation of these plugs may occur in the female atrium. Because the atrial epithelium of bothriurid females is well supplied with glandular cells with abundant secretions (Peretti 2003; Peretti and Battán-Horenstein 2003), it is possible that active or passive degradation of the genital plug could be favoured by the female. Observations of sperm transfer are necessary to confirm or reject this hypothesis.

More data are required to measure the effect of the large genital plug in preventing intromission, or female use of sperm from new males, in accordance with the classical sperm competition approach (Parker 1979; Simmons 2001). An assessment should also be made of the consequences of such a large and complex plug on the subsequent reproductive life and viability of the female, according to current hypotheses of sexual conflict (Chapman et al. 2003; Eberhard and Cordero 2003).

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