# Strong functional integration among multiple parts of the complex male and female genitalia of stink bugs

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Genitalia are among the most studied phenotypes because they exhibit high anatomical diversity, experience fast evolutionary rates and may be shaped by several evolutionary mechanisms. A key element to uncover the mechanisms behind such impressive diversity is their copulatory function. This topic has been overlooked, especially concerning structures not directly involved in sperm transfer and reception. Here, we conduct a hypothesis-driven experimental study to elucidate the operation of various external genital parts in five species of stink bugs with differing levels of phylogenetic relatedness. These insects are unique because their male and female genitalia are externally well developed, rigid and composed of multiple components. In contrast with their anatomical complexity and diversity, we show that genital structures work jointly to perform a single function of mechanical stabilization during copula. However, distinct lineages have evolved alternative strategies to clasp different parts of the opposite sex. In spite of a high functional correspondence between male and female traits, the overall pattern of our data does not clearly support an intersexual coevolutionary scenario. We propose that the extraordinary male genital diversity in the family is probably a result of a process of natural selection enhancing morphological accommodation, but we consider alternative mechanisms.

ADDITIONAL KEYWORDS: coevolution – *Edessa* – *Euschistus* – functional morphology – *Mormidea* – *Podisus* – sexual selection – sperm competition.

## INTRODUCTION

Extraordinarily divergent genitalia are ubiquitous across animal taxa with internal fertilization. The evolutionary forces behind this trend have sparked heated debate over the last decades, but most models of natural and sexual selection proposed have been at least partially supported (Hosken & Stockley, 2004; Masly, 2012; Brennan & Prum, 2015; Firman *et al.*, 2017). Distinguishing among these models in a particular group can be challenging because genitalia may exhibit similar patterns of differentiation and coevolution under different pressures. Thus, uncovering the origins of genital diversification is paramount to discern among alternative evolutionary mechanisms. In this sense, a key question is how different genital parts engage during copula and how morphology relates to function (Jagadeeshan & Singh, 2006; Simmons, 2014; Wulff & Lehmann, 2016). In the taurus scarab beetle (Ontophagus taurus), two distinct functionalities have been described to four male genitalic sclerites: three sclerites act directly in sperm transfer comprising an integrated unit, while the other acts as a holdfast structure (Werner & Simmons, 2008). Such findings are crucial to explain how different parts are able to influence paternity or stabilize the genitalia in copula (Werner & Simmons, 2008), illustrating the importance of studies on functional morphology to detect sources of selection. The scarcity of studies on functional morphology of genitalia has been repeatedly pointed as a key obstacle that hinders the progress on this research field (Simmons, 2014; Brennan & Prum, 2015). Although functional integration between male and female is usually thought as a major source of coevolution, evidence for such correlation is yet limited. In

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fact, the most comprehensive study dealing with this subject has found weak evidence to such correlation (Richmond, Park & Henry, 2016).

Insects are probably the most representative organisms in studies on genital evolution. Assessments of their genital functionalities have revealed peculiar and unique modes of operation such as traumatic insemination (Tatarnic, Cassis & Hochuli, 2006; Kamimura, Tee & Lee, 2016), mating plugs (Baer, Morgan & Schmid-Hempel, 2001; Seidelmann, 2015), sonorous genitalia (Sueur, Mackie & Windmill, 2011) and female penises (Yoshizawa et al., 2014). Three major biases may be identified in studies with insect genitalia. First, thorough investigations covering both functional and evolutionary aspects have been mostly conducted with a few model groups such as flies (e.g. Eberhard & Ramirez, 2004), beetles (e.g. Hotzy et al., 2012) and water striders (e.g. Fairbairn et al., 2003). Second, the historical male bias that characterizes the research on genital evolution as a whole (Ah-King, Barron & Herberstein, 2014) also seems to apply to insects. Third, given the growing acceptance of sexual selection as a preponderant mechanism, studies examining structures associated to sperm transfer and sensory communication are increasingly predominant in relation to those examining structures with secondary sexual functions. However, recent studies provide unequivocal evidence that male and female genitalia may be shaped by alternative processes other than the traditional cryptic female choice and sexual antagonistic coevolution (e.g. Wojcieszek et al., 2012; House et al., 2013; Anderson & Langerhans, 2015; Varcholová et al., 2016). This raises the question of whether these mechanisms of sexual selection are indeed overwhelmingly prevalent as usually thought, especially considering our elusive knowledge on the function and diversity of genitalia in numerous understudied groups.

Among insects, stink bugs (Hemiptera: Pentatomidae) stand out for particularities in male and female genitalia. Both sexes exhibit highly complex and well-developed internal and external genital parts (Sharp, 1890; Marks, 1951) with presumable diverse functionalities (Genevcius, Caetano & Schwertner, 2017). While a couple of studies with pentatomids have linked their intromittent genitals to a complex system of sperm selectivity, transfer, regulation and storage (Adams, 2001; Stacconi & Romani, 2011), the function of non-intromittent external parts in copula remains virtually unknown. The non-intromittent part of the male organ (=pygophore, male external genitalia herein) is characterized by extraordinary diversity and species specificity, being consistently the most decisive characteristics in taxonomic studies and showing strong phylogenetic structure at different levels (e.g. Grazia, Schuh & Wheeler, 2008;

Ferrari, Schwertner & Grazia, 2010; Genevcius, Grazia & Schwertner, 2012). The structure comprises a capsule and associated structures that can take the form of folds, projections and hooks, originated from a series of modifications and fusions between the ninth and tenth abdominal segments (Bonhag & Wick, 1953; Schaefer, 1977). The female external genitalia is composed of various flattened plates that cover the genital opening, derived from the eighth, ninth and tenth segments (Scudder, 1959). A recent study has found an evolutionary correlation between the pygophore and a pair of female plates, but the functional significance of this trend remains to be investigated (Genevcius et al., 2017). Although our knowledge on how these structures operate is vague, their remarkable diversity and species specificity suggest an important sexual and evolutionary role in the family which has never been scrutinized.

In this study, we examined the role of the genital parts that presumably interact externally during copula in Pentatomidae. Given the morphology of the external genitalia of its members, the group offers an interesting model to study the interplay between genitalia function, complexity and evolution in structures disassociated to sperm transfer. We reviewed the literature and compiled a series of testable hypotheses of functional mechanics in the group (Table 1). We performed mating trials for five species showing varying degrees of phylogenetic relatedness and conducted a series of detailed morphological observations to address the following questions: (1) How do the external parts of the male and female genitalia interact with one another during copula? (2) Do the modes of operation vary across species of different lineages of the family? Our results revealed an entangled mechanism of functional integration in which several parts of the genitalia operate in a cooperative fashion to provide stabilization during copula. Furthermore, we show significant among-species variation in the attachment mechanism, suggesting distinct evolutionary strategies to clasp the opposite sex exhibited by different lineages. We discuss how our data adequate to the functional hypotheses, the evolutionary implications of the genital interactions observed and possible underlying mechanisms.

## MATERIAL AND METHODS

## MORPHOLOGY AND TERMINOLOGY OF GENITAL PARTS

The terminology used to refer to the male genital components in Heteroptera has been historically inconsistent. Schaefer (1977) compiled and discussed the contrasting classification in Pentatomomorpha (which includes Pentatomidae and related families), proposing a unified terminology. Here, we followed his

Structure	Taxon	Functional hypothesis	Reference	Support
Parameres	Pentatomidae	H1. 'The functions of the <b>claspers</b> [] to assist in separating the genital sclerites of the female, and to assist as clasping organs during copulation'.	Baker (1931)	Corroborated
Parameres	Hemiptera	H2. 'Also, it appears [] that the <b>parameres</b> do operate to some extent in keeping apart the gonapophyses which hide the female gonopore'	Singh-Pruthi (1925)	Rejected
Parameres	Piezodorus lituratus (Pentatomidae)	H3. 'During copulation in Pentatominae the male <b>gonopods</b> are pressed against the outside of the 2nd valvifers of the female'	Leston (1955)	Rejected
Ventral rim of pygophore	Geocorisae (Terrestrial Heteropterans)	H4. 'the <b>infolded portion of the ventral</b> <b>rim</b> , [] presumably share the function of holding and guiding the aedeagus during copulation.'	Schaefer (1977)	Partially rejected
Ventral rim of pygophore	Geocorisae (Terrestrial Heteropterans)	H5. 'These structures [the <b>infolded portion</b> <b>of the ventral rim</b> ] appear to have limited functional significance, because they are usually immovable and not provided with muscles; they may provide tactile clues to the female and/or provide support to the various movable structures during copulation.'	Schaefer (1977)	Partially rejected
Pygophore	Pentatomidae	H6. 'The aesthetic aspect of the arrangement [of the <b>genital chamber</b> ] in many of the higher species, [], is very remarkable, but I do not think there is at present evidence that would justify us in attaching any special biological importance to it.'	Sharp (1890)	Partially rejected

**Table 1.** Hypotheses of functional morphology of the external genitalia compiled from literature with reference to the taxon to which each hypothesis has been proposed

Column 'structure' refers to the terminology used here, while the original terminology is indicated in bold within the hypothesis quote. Column 'support' denotes whether the hypothesis was supported herein.

terminology with a few additions of other recent studies (Genevcius *et al.*, 2012).

The male genitalia is roughly a tube-like sclerotized capsule (=pygophore) with associated structures (e.g. a pair of claspers) and an internal phallus. Although some authors refrain to use the terms 'external' and 'internal' genitalia, we designate internal genitalia as the movable intromittent parts that penetrate the female internal tract, whereas the capsule itself, the parameres and the tenth segment are considered as external genitalia. The pygophore can be divided into a dorsal and a ventral wall. Since it remains twisted in 180° inside the male's body while in rest position, the ventral and the dorsal sides are opposite to the body's plans (Schaefer, 1977). All structures can be seen in dorsal view, including the posterior extremity of the ventral wall, denominated ventral rim (Fig. 1). We follow Grazia *et al.* (2008) to the female parts, which compiled the nomenclature and reviewed homology statements. The morphology of the female genitalia is relatively simpler, comprising a series of soft tubes and chambers (the internal genitalia) covered by various sclerotized plates (the external genitalia). The opening of the female internal tract gets covered by the larger genital plates, the gonocoxites 8 (Fig. 1E, F). The terminology of all genital parts and respective abbreviations used in this work are described in Figure 1 and Table 2.

#### SPECIES CHOICE, COLLECTION AND REARING

We investigated the functional morphology of male and female external genitalia in five species of Pentatomidae in a hypothesis-driven approach



**Figure 1.** Male (A–D) and female (E, F) external genitalia of the studied genera, with terminology and abbreviations indicated. Female genitalia are represented with the internal tract exposed (E) and unexposed (F). A and F = *Euschistus heros*; B and E = *Mormidea v-luteum*; C = *Edessa meditabunda*; D = *Podisus nigrispinus*. Scale bar is 0.25 mm.

(Table 1). To examine whether the general system of attachment between the genitalia vary within the family, we chose species with different levels of relatedness. Even though a complete phylogeny of the family does not exist, different phylogenetic studies support the recognition of different groups within Pentatomidae (Gapud, 1991; Bistolas *et al.*, 2014; Banho, 2016; Wu *et al.*, 2016) with certain congruence with the current taxonomic classification in tribes and subfamilies (Rider *et al.*, 2017). The five species studied herein represent three of the four major and most diverse lineages of Neotropical pentatomids (i.e. Asopinae, Discocephalinae, Edessinae and Pentatominae).

We selected two species from the same genus, Mormidea v-luteum (Lichtenstein) and Mormidea maculata (Dallas), and a third species from the same tribe as the Mormidea, Euschistus heros (Fabricius). The three species belong to the group of the Neotropical Carpocorini (subfamily Pentatominae). The fourth and the fifth species belong to other subfamilies: Podisus nigrispinus (Dallas) (Asopinae) and Edessa meditabunda (Fabricius) (Edessinae). We manually collected specimens in the municipality of Diadema, São Paulo,

Abbreviation	Structure	
Female		
gcx8	Gonocoxite 8	
gcx9	Gonocoxite 9	
gnp8	Gonapophysis 8	
ltg8	Laterotergite 8	
ltg9	Laterotergite 9	
Male		
e.d.r	Extension of dorsal rim	
m.e.	Median excavation	
p.l.a.	Posterolateral angle	
Par	Paramere	
Pyg	Pygophore	
s.p.	Superior process	

**Table 2.** Abbreviations of the genital parts used in text

 and figures

Brazil (-23.7204, -46.6276) and maintained them in laboratory inside plastic cages of 2 L. Males were reared separately from females prior to the experiments using the following conditions to all species:  $26 \pm 2^{\circ}$ C,  $70 \pm 10\%$  relative humidity and photophase of 14 L:10 D. Individuals of *E. heros* and *Ed. meditabunda* were fed on bean pods (*Phaseolus vulgaris*) and peanut seeds (*Arachis hypogaea*), *M. v-luteum* and *M. maculata* on branches of *Brachiaria* sp. and *P. nigrispinus* on larvae of *Tenebrio molitor*.

### EXPERIMENTAL APPROACH

We randomly formed couples which were maintained in separate cages during the mating trials. The number of couples observed per species (n) varied from three to 12 (E. heros = 12, Ed. meditabunda = 3, M. v-luteum = 10,M. maculata = 8, P. nigrispinus = 3). All observations were consistent showing no differences among pairs of the same species. Mating pairs were frozen in copula in a -20°C freezer. Because pentatomids commonly tend to copulate for several hours (McLain, 1980; Rodrigues et al., 2009), we were able to wait several minutes after copula had started to guarantee that genitalia were properly coupled. After 20 min in the freezer, mating pairs were pinned and promptly analysed in a stereomicroscope Leica MZ205C. Photographs were taken firstly of the attached genitalia and secondly after slight manipulations, using a Leica DFC450 and the Leica Application Suite software with Z-stacking acquisition.

## RESULTS

The arrangement between male and female genitalia from a dorsal view of the pygophore was similar in all species. Left and right gcx8 were the only mobile structures of the female genitalia. They touch the dorsal side of the pygophore and are pressed against the lateral rim (Fig. 2) by the parameters internally (Fig. 3). This connection apparently comprises the tightest point of attachment between the two genitalia. In P. nigrispinus, the gcx8 is also grasped externally by the superior processes (=genital plates according to some authors). The parameters and the superior processes function as tweezers to keep the gcx8 opened (Fig. 3). The opening angle of the gcx8 differed slightly among species. In M. v-luteum and Ed. meditabunda, the gcx8 remains virtually parallel to the male's body plan (Fig. 2C, D), whereas the angle is around 45° in the remaining species (Fig. 2A, B). In all five species, the connection between genitalia is probably mediated by several sensory setae mostly concentred on the e.d.r. and p.l.a. of males and on the internal angles of the gcx8 of females (Fig. 3).

The ventral rim of the pygophore makes direct contact with the female plates in all species except *P. nigrispinus*. However, we found three different modes of accommodation between these two traits, each mode corresponding to one genus. In E. heros, the ventral rim of the pygophore is pronouncedly differentiated to engage with the female plates (Fig. 4A); the posterolateral angles fit between the ltg8 and ltg9 while the sinuosity of the ventral rim matches the ltg9 and tenth segment (Fig. 4A). In the Mormidea, the ventral rim of the pygophore is less modified showing only a simple v-shaped median excavation (Fig. 1B); the m.e. fits the gcx9, whereas the ltg8, ltg9 and tenth segment remain untouched by the pygophore (Fig. 4B, C). In Ed. meditabunda, the p.l.a. of the pygophore makes contact with the outer side of the ltg8 (Fig. 4D). In such species, both the ltg9 and tenth segment lie in the median excavation of the pygophore (Fig. 4D), and the tenth segment is untouched by the ventral rim. We could not visualize whether the gcx9 engages with a specific portion of the male genitalia in E. heros and Ed. meditabunda because it was covered by the pygophore ventrally and by the gcx8 dorsally. In P. nigrispinus, the ventral rim of the pygophore is not well developed and does not engage with any of the female plates. In this species, the attachment between the genitalia is mediated exclusively by the parameres, lateral rim and superior processes (Fig. 3A).

In the Carpocorini (i.e. *E. heros*, *M. v-luteum* and *M. maculata*), the e.d.r. of the pygophore is well developed and bifurcated (Fig. 1A, B). This structure is used to accommodate the gnp8 (Fig. 5A), which is covered by the gcx8 while in rest position (Fig. 1E, F). In these three species, the bifurcation of the e.d.r. fits thoroughly the median longitudinal elevation of the gnp8 (Fig. 5A). In *P. nigrispinus* and *Ed. meditabunda*, the



**Figure 2.** Attached genitalia after 20 min in copula, dorsolateral perspective of the pygophore. Male traits are highlighted in green and female traits in pink. A = *Euschistus heros*; B = *Podisus nigrispinus*; C = *Edessa meditabunda*; D = *Mormidea v-luteum*. Scale bar is 0.4 mm.

e.d.r. is vestigial and do not participate in the connection with the gnp8 (Fig. 3A). We could also visualize the interaction between some anatomical parts that were not focus of our study but can be relevant to interpret mechanisms of evolution (see 'Discussion' section). In Ed. meditabunda, the last pre-genital abdominal segment (i.e. the seventh segment) is strongly extended and thickened. The male projections of the seventh segment anchor on the inner side of the female projections (Fig. 2C). Such anchoring may be important to avoid the rotation of the individuals in copula. After slight manipulation to decouple the mating pairs, we could visualize the intromittent male genitalia (=phallus) inflated inside the female tract (Fig. 5B). While the external parts could be easily untied, this internal connection was much tighter.

In summary, both the dorsal rim of the pygophore and the parameres work jointly to support and keep

the gcx8 opened in all species (Figs 2, 3). In addition, the similarity among all species (except *P. nigrispinus*) was the perpendicular connection between the pygophore and the female genitalia in which the female plates accommodate the ventral rim of the pygophore. However, each genus exhibited a different pattern with respect to which plates engage with the curvatures of the ventral rim and in which portion of the ventral rim the plates get supported (Fig. 4). In E. heros, the ventral rim touches all the unmovable plates; in the Mormidea, only the gcx9 interacts with the ventral rim; in Ed. meditabunda, the ltg9 and the tenth segment lie in the median excavation and the p.l.a. touches the outer side of the ltg8; in *P. nigrispinus*, the ventral rim does not touch the female genitalia at all. Furthermore, the Carpocorini (i.e. Euschistus and Mormidea) showed an additional point of stabilization, between the e.d.r. and the gnp8.



**Figure 3.** Genitalia of *Podisus nigrispinus* during copula (A) and after a slight manipulation, with the structures hidden by the gcx8 indicated (B). Scale bar is 0.2 mm.

#### DISCUSSION

Our study revealed a unique pattern of strong functional integration among multiple parts of male and female external genitalia. Several male parts, mostly located in the dorsal face, accommodate one or more parts of the female external genitalia. Some of such male structures are apparently modified and specialized to this function. Interestingly, the mechanism of attachment between the genitalia varied among the lineages once certain homologous parts of the male genitalia in different species engage with different parts of the female genitalia. Below we discuss how our data fit the functional hypotheses derived from literature, the evolutionary trends of the genitalia and the probable underlying mechanisms.

#### HYPOTHESES OF FUNCTIONAL MORPHOLOGY

Sharp (1890) suggested that the pygophore does not participate directly in the copulatory process and it should instead function to protect the internal parts (H6; Table 1). Although it is not possible to discard this 'protective hypothesis' with our data, we uncovered an important role of accommodation of the female parts by the pygophore, rejecting his hypothesis at least partially. The most explicit fastening structure of the male genitalia was the ventral rim of the pygophore, which fits either the ltg8, ltg9 and the tenth segment or the gcx9. The ventral rim has apparently evolved to retract in its parts that touch the female plates. Since the female genitalia is being pushed towards the outside by the parameres, such fit between the ventral rim and the female plates probably helps to avoid the male capsule do slide laterally. Particularly in P. nigrispinus, where the ventral rim does not participate in the genital attachment, the superior processes appear to perform this function. These results are to a certain extent in disagreement with Schaefer's (1977) hypotheses that the ventral rim has limited functionalities and should mainly support the internal parts of the male genitalia (H4 and H5). Although the ventral rim per se is clearly engaged with the female genitalia externally, it is possible that certain structures derived from the ventral rim (e.g. the cup-like sclerite) interact with the internal parts during and after intromission. Unfortunately, we were not able to visualize the operation of the internal parts because they were completely covered by the male capsule and the female plates.

We showed that the parameres operate in holding the female gcx8 opened to provide access of the phallus to the internal female genitalia. This result is in line with Baker's (1931) hypothesis (H1) and with the operation mode observed in true bugs of other families (e.g. Moreno-García & Cordero, 2008). However, the parameres keep the gcx8 separate by pressing their inner surface, contrary to Leston's (1955) hypothesis which suggest contact with the outer surface of the gcx8 (H3). Our results also refute Singh-Pruthi's (1925) hypothesis (H2) by showing that the female gonapophyses 8 are supported by the e.d.r. of the pygophore and not by the parameres. In summary, we fully rejected H2 and H3, partially rejected H4, H5 and H6 and corroborated H1.

### FUNCTIONAL INTEGRATION AND GENITAL EVOLUTION

Anatomically diverse genitalia are usually thought to be also diverse in function (Huber, 2004; Song & Wenzel, 2008), implying that distinct selective pressures should operate within a single genitalia (Rowe & Arnqvist, 2012). This has been shown true even to structures that are physically connected (Song & Wenzel,



**Figure 4.** Attachment between the ventral rim of the pygophore (green) and the female plates (pink) from ventral (A–C) and ventrolateral (D) perspective of the pygophore. A = *Euschistus heros*; B = *Mormidea maculata*; C = *Mormidea v-luteum*; D = *Edessa meditabunda*. Scale bar is 0.5 mm.

2008). In contrast with this general view, we show that the multiple components of the Pentatomidae external genitalia are integrated to function exclusively as anchoring structures. The ventral rim of the pygophore is the most obvious example since it interacts with at least three of the five female external parts in most species (i.e. ltg8, ltg9 and tenth segment). The female gcx8 is analogous and shows a similar level of integration, interacting simultaneously with the lateral rim, e.d.r and the parameres. These results indicate that the external genitalia of the Pentatomidae comprise a system of strong level of functional integration, which means that their parts are prone to vary in a combined and coordinated manner. Accordingly, we suggest that virtually all external genital parts studied here should be directly or indirectly integrated to each other to some degree, a process similar to the one shown in a dung beetle (House & Simmons, 2005; Werner & Simmons, 2008).

In systems as such, it is intuitive to predict that changes in one component would entail changes in another to maintain the coordination integrity among the parts (Klingenberg, 2014). For instance, as the gcx8 is supported on one side by the parameres and by the lateral rim on the other, some level of evolutionary correlation among these three traits would be expected. Nevertheless, our data are limited in supporting an intersexual coevolutionary process between the genitalia. While various anchoring parts of the male genitalia are morphologically peculiar and species specific, the female plates were relatively more constant among the species we studied. For example, the e.d.r. of E. heros, M. v-luteum and M. maculata is differentiated to grasp the female gnp8, but the gnp8 is mostly invariable among all species we analyzed. Several other structures of the male genitalia seem much more diverse among species than the female plates such as the parameres, the tenth segment and the ventral rim,



**Figure 5.** Genitalia of *Mormidea maculata* in copula (A) illustrating the connection between the e.d.r. of the pygophore (green) and the female gnp8 and gcx8 (pink); connection of the internal genitalia of *Mormidea v-luteum* exposed after manipulation (B). Scale bar is 0.3 mm.

what is consistently observed across the taxonomic literature (e.g. Ferrari *et al.*, 2010; Genevcius *et al.*, 2012). These observations suggest that a probable process of selection enhancing the mechanical fitness of the genitalia should be acting essentially or predominantly over male genitalia, while female genitalia should be subjected to a weaker selective pressure (Genevcius *et al.*, 2017). Alternatively, female genitalia may be constrained due to other processes such as intersexual differences in gene expression and regulation during the developmental process (Aspiras, Smith & Angelini, 2011).

Although we found no explicit evidence of coevolution, it should be noted that our approach only allows for examination of qualitative variation exhibited by the genitalia. Thus, we cannot rule out the hypothesis that female genitalia may evolve in response to male genitalia in a small scale, detectable only by approaches that take continuous variation into account. This hypothesis is somewhat in line with a recent study with stink bugs which shows lesser changes in female genitalia compared to fast-evolving male genitalia in a coevolutionary scenario (Genevcius et al., 2017). Because rates of genitalia change have rarely been quantified to males and females simultaneously, similar scenarios with other groups are unknown and we are not able to speculate about its prevalence across animals. We believe that various structures of the Pentatomidae genitalia are candidate to be tested for coevolution using continuous data: the margins of the gcx8 and the curvatures of the dorsal rim of the pygophore, the shape of the parameres and the concavity of the gcx8, the length of the spines of the seventh segment, among others. Future fine-scale studies will allow one to test whether different levels of functional integration exhibited by different lineages are good predictors of evolutionary correlation.

## CONVERGENCE AND EVOLUTIONARY TRENDS OF THE PENTATOMIDAE GENITALIA

The overall taxonomic literature of stink bugs documents high levels of pygophore species specificity. However, if pygophore conformation has fitness consequences and female plates are more evolutionarily conserved, one would expect the repeated evolution of certain male shapes across different lineages. Within Euschistus, the biconvex ventral rims of the pygophore in several species are similar to the observed to E. heros, for instance in E. atrox, E. acutus, E. cornutus, E. emoorei, E. irroratus, E. nicaraguensis, E. schaffneri and E. stali (Rolston, 1974; Bunde, Grazia & Mendonca-Junior, 2006). As at least four of these species belong to well-separated lineages (Weiler, Ferrari & Grazia, 2016; Bianchi et al., 2017), we may presume at least four episodes of convergent evolution within this genus. By briefly analyzing the taxonomic literature, we found five other species belonging to other tribes and subfamilies that exhibit similar ventral rims: Acledra spp. (Faúndez, Rider & Carvajal, 2014), Cahara incisura (Fan & Liu, 2013), Braunus sciocorinus (Barão et al., 2016), Edessa puravida (Fernandes et al., 2015), Mecocephala bonariensis (Schwertner, Grazia & Fernandes, 2002). This series of potential convergences reinforce that shape changes of the pygophore in the parts that touch the female plates (and vice versa) are advantageous strategies to perform an effective genital coupling in Pentatomidae.

Interestingly, our analyses revealed that pygophores of different species have evolved in distinct directions to achieve morphological stability in copula. This idea is supported by the fact that certain male parts in different species engage with different parts of the females. For instance, the ventral rim of the pygophore engages with the female ltg8, ltg9 and tenth segment in E. heros, with the gcx9 in the Mormidea spp., with the ltg8 in Ed. meditabunda and does not engage with the female genitalia at all in *P. nigrispinus*. We observed certain particularities in the modes of interaction among male and female parts to the four genera studied here, despite the fact that female genitalia are relatively similar in these species. This raises the intriguing question of how many other modes of morphological correspondence exist within Pentatomidae. We believe the extraordinary diversity of pygophores and the existence of peculiar female plates across several lineages of Pentatomidae (Schuh & Slater, 1995; Rider et al., 2017) suggest the existence of a high diversity of alternative mechanisms of genital coupling.

Another intriguing implication of our results concerns the use of genital characters in phylogenetic analyses of pentatomids. We hypothesize that several genital structures of the Pentatomidae, if not all, may be more phylogenetically dependent among each other than previously thought due to a mechanism favouring their morphofunctional integrity. This raises the question of whether using disproportional amounts of genital characteristics in phylogenetic reconstructions may result in strongly genitalia-biased phylogenies that rely on few dependent evolutionary processes. We are not arguing that genital characters should be rejected *a priori*, especially considering their proven phylogenetic usefulness in insect systematics (Song & Bucheli, 2010). However, since character independence is basically a presumption of the majority of phylogenetic methods (O'Keefe & Wagner, 2001), this issue should be considered with caution.

## WHICH EVOLUTIONARY MECHANISMS ARE MOST LIKELY?

The majority of studies on sexual behaviour of pentatomids report long copulations, sometimes spanning several days. Such mechanism of prolonged copulation seems to be controlled by the male to avoid male-male competition for mates and thereby avoid sperm competition (McLain, 1980; Wang & Millar, 1997). The mechanism employed by males to hold the females is unknown to date, but our results shed some light on this topic. We showed that the external structures of the genitalia are not associated to sperm transfer/ storage and thereby should not influence paternity because they interact externally and work as anchoring structures. By manipulating the genitalia to investigate the tightest points of attachment between the

individuals, we observed that the external connection was relatively fragile and could be easily untied. On the other hand, the attachment between the inflated phallus and the female internal tract was much stronger (Fig. 5B), indicating that such internal connection might be the determinant mechanism to avoid female access to other males. It should be considered the possibility that while individuals were alive, the parameres could be boosted by muscles to hold the females and the external connection could be actually stronger than we observed with recently dead specimens. However, a functional study with other terrestrial true bug with relatively similar genitalia suggest a passive mode of operation of the male parts coupled with a cooperative movement of the female plates (Moreno-García & Cordero, 2008). While it remains to be tested whether the inflated phallus has a role in physically displacing rival sperm, our study suggest that they participate at least indirectly in the avoidance of sperm competition by holding females and preventing them from subsequent copulations.

Our results coupled with other experiments with true bugs suggest that both sperm transfer/storage and female holding are performed by interactions of the internal genitalia (Moreno-García & Cordero, 2008; Stacconi & Romani, 2011; Genevcius et al., 2017). Accordingly, the external traits are probably disassociated to any function that may directly influence paternity and intersexual conflict for the control of mating. The apparent absence of male-female coevolution and damaged genitalia in museum collections. as well as the passive mating behaviours exhibited by pentatomids (e.g. Wang & Millar, 1997), provide additional support for this hypothesis. Therefore, we believe our data are more indicative of a scenario of natural selection to the external genitalia, which could happen essentially via pure morphological accommodation or species specificity reinforcement (Brennan & Prum, 2015). Since different studies with pentatomids report viable copulation between species with differentiated external genitalia (Foot & Strobell, 1914; Kiritani, Hokyo & Yukawa, 1963), we believe selection favouring the interlocking effectiveness of genitalia rather than species reinforcement is more plausible (Richmond et al., 2016). However, because we do not know whether and how the external genitalia may interact with the internal parts, an additional aspect should be considered. If the pygophore is used to provide support to the movable internal structures as hypothesized by Schaefer (1977), the morphological diversity exhibited by the external genitalia may have arisen also as a by-product of sexual selection acting on the shape of internal parts. Because most of these mechanisms are not mutually exclusive, discerning among them will be possible through an examination

of the internal and external parts using histological and micro-computed tomography techniques.

## CONCLUSIONS

Our study revealed an interesting interlocking genital system in which male and female external structures are functionally integrated to stabilize the genitalia during mating. Furthermore, species from different lineages have evolved to engage with distinct parts of the opposite sex. Because female genitalia are greatly more constant than male genitalia when comparing species, the processes that lead to such morphological stability are certainly more directed to the male parts. Literature data and our observations indicate neither intersexual conflict nor a direct participation of the external parts in sperm transfer and storage. Accordingly, we believe the genital traits we studied here are more prone to a process of natural selection, most probably enhancing the morphological accommodation rather than species reinforcement. To discern among mechanisms of evolution, further studies should attempt to determine why selection should favour a stable and strong coupling, which may be either cooperative or conflicting. Fine-scale analyses using histology and micro-CT scan techniques will make possible to investigate the functioning of the internal parts and to test whether these parts are functionally integrated to the external genitalia.

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