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Authors: Olguín-Pérez, Laura, Francke, Oscar F., and Carbajal-Saucedo, Alejandro

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Evidence of piercing and sexual differences in venom composition in a sexual stinging scorpion (Scorpiones: Euscorpiidae)

Laura Olguín-Pérez[1,2], **Oscar F. Francke**[2] and **Alejandro Carbajal-Saucedo**[3]: [1]Posgrado de Ciencias Biológicas, UNAM. [2]Colección Nacional de Arácnidos, Departamento de Zoología, Instituto de Biología, Apartado Postal 70-153, UNAM, C. P. 04510 CDMX, Mexico; E-mail: laura.olguinp@ciencias.unam.mx [3]ISBI Biosciences S.C. C.P. 62510 Mor., Mexico

Abstract. The males of the Mexican species *Megacormus gertschi* Díaz-Najera, 1966 (Scorpiones: Euscorpiidae: Megacorminae) sting the female repeatedly in the pedipalp tibia-patella intersegmental membrane (TPIM) during the initiation of the *promenade a deux*. It has been suggested that the male's venom introduced during this "sexual sting" behavior could generate some sedative effect and reduce the possibility of being cannibalized by the female. However, this is unsupported by evidence regarding venom transference. Here, we provide evidence of perforation of the TPIM by the male aculeus and venom transfer during sexual sting performance. We also provide the first venom characterization of this species and show that it has a sexually dimorphic composition. These results, in combination with observations that the sexual sting is displayed in successful matings with non-defensive females, lead us to consider the pre-insemination sexual stinging as a non-genitalic sexual interaction with a potential role as a courtship element.

Keywords: Coercive sexual behavior, female choice, *Megacormus gertschi*, scorpion venom

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Among intersexual mechanisms of interest are the dominant behaviors expressed by males, where female choice is seemingly suppressed (Krebs & Davies 1993; Parker 2006). These dominance displays involve energy expenditure and can vary from non-invasive acts of persuasion, a certain degree of coercion where conditional struggle may occur, or even domination using force. It is suggested that certain benefits of these displays balance the costs in terms of survival, nutrition, prevention of subsequent copulation, and fecundity, among others (Eberhard 1996; Arnqvist & Rowe 2005). All these processes result in sex-dependent differential reproduction.

Differential reproduction is also affected by the performance of courtship behavior and the response to it by the opposite sex. Behavioral patterns displayed as courtship favor access to a partner and maintenance of the interaction long enough to complete sperm transfer, and it is closely correlated with reproductive success (Ryan & Cummings 2013). There are sexual displays where the male's displays were once considered to be dominance behavior but were reconsidered when analyzed under courtship hypotheses. Those displays involve chemical, mechanical, and visual signals, with some notable examples in which the arthropods are the study system. Such is the case of the intimidating courtship displayed by water strider males (Insecta: Hemiptera), that increases the risk of predation on the females. In these insects that glide by means of water surface tension, the male performs tapping in the water with his legs while riding on the female. These vibrations can attract underwater predators to which the female is more exposed. The male stops the tapping once the female's genitalia are protruded and copulation is begun. Initial studies considered that the female yielded to coercive action, avoiding predation costs. Subsequent studies obtained evidence that females have control over the protrusion of their genitalia and therefore control over copulation. In a predator-free environment, males that do not insert the genitalia could be easily knocked down. So, the delay in the protrusion of the genitalia can be interpreted as resistance to the coercive behavior of the male, but it can also be interpreted as a female choice that favors males with more intense courtships, which the authors call "audacious males" (Han & Jablonsky 2009; Han et al. 2015).

Other examples are male structures that attach to the female during mating, as observed with claspers in dragonflies (Odonata) (Córdoba-Aguilar et al. 2015), and butterflies (Lepidoptera) (Koshio et al. 2007), as well as with pedipalp chelae in decapods (Subramoniam 2017) and scorpions (Carrera & Peretti 2005). In such examples, it has been determined that these non-genital structures can be involved in courtship behaviors rather than in dominance displays. These structures are of interest because of the potential mechanisms to which males most likely resort in trying to stimulate, persuade, or force some reciprocal behavior in the female. The participation of these non-genitalic structures in a sexual context results in the exchange of signals between the sexes while the reproductive organs or sexual structures are not involved. However, the effects of this exchange of signals can hardly be differentiated from those stimuli generated by the copulating organs as they frequently act simultaneously. For this reason, the interaction of non-genitalic structures has been poorly explored due to the lack of better controls on possible artifacts derived from the method of analysis or behaviors that mask the true participation of non-genitalic structures (Eberhard 2015).

To assess whether the observed displays constitute a situation of dominance or courtship behaviors, it is necessary that the study system allows the differentiation between sperm transfer and the action of non-genitalic structures in such a behavioral context. Scorpions are ideal organisms to analyze the role of non-genitalic structures during the mating display, because these arachnids have indirect insemination via a spermatophore that is deposited on the substrate and subsequently activated in the female's genital atrium (Polis & Sissom 1990; Peretti 2001). The mating display in scorpions

consists of three main phases with differentiable behavioral phases: (a) initiation or introductory, (b) central or *promenade a deux*, and (c) sperm transfer (Polis 1990; Peretti et al. 2000; Peretti 2001).

During the mating season, the male initiates courtship by approaching the female while executing vibrations with his pedipalps and grabbing the female's pedipalp chelae with his own, performing a type of ritualized dance known as *promenade a deux*, until the time when a suitable place to attach the spermatophore is found. The male then guides the female over the spermatophore in order to introduce and activate it inside her genital atrium (Francke 1979; Polis 1990; Carrera et al. 2009; Toscano-Gadea 2010; Rein 2019). During the pre-insemination courtship, prior to or at the beginning of the *promenade a deux,* males of some scorpion species insert the aculeus (stinger) into different membranous regions of the female's body, a behavior known as 'sexual stinging', and then proceeds with physical contact behaviors until the sperm transfer is completed. Performance of the sexual sting has been recorded in at least 20 species belonging to 6 of the 16 currently recognized families of scorpions (Polis & Sissom 1990; Peretti 2001, 2013; Jiao & Zhu 2010). Among them is the Mexican species *Megacormus gertschi* Díaz-Najera, 1966 (Scorpiones: Euscorpiidae: Megacorminae) (Fig. 1a,b), for which Francke (1979) first reported the sexual sting. Males of this species sting the female repeatedly in the tibia-patella intersegmental membrane (TPIM) of both pedipalps in alternation. When a female is receptive, pedipalpal grasp is allowed immediately (Fig. 1c). Sexual stinging is performed during the initiation of the *promenade a deux*, with the female exhibiting a clearly non-defensive posture, indicated by her metasoma resting parallel to the substrate (Fig. 1c). The sexual sting lasts for up to 50 minutes in the single longest penetration (personal observation during the main ongoing ethological project). Males have been observed to sting either the left or right intersegmental membrane of pedipalp tibia-patella joint (Fig. 1d). Occasionally, two stings with different duration occur in the same courtship event. Once the male has removed the sting, he starts a sequence of vibrations and juddering, while the female remains passive, allowing chelicer al massage and ventral stimulation by the male's first pair of legs. Spermatophore deposition is evident when the male's mesosoma is lifted, and the female is pulled forward by the male towards the spermatophore, promoting its activation (Fig. 1e). Post-mating escape is usually performed by the male, whereas the female can consume the spermatophore, or run away also.

It has been speculated that sexual stinging results in venom injection (Polis & Sissom 1990). Arachnologists have debated the potential role or effect of this behavior in the female's reproductive choices; it has been discussed in a sexual conflict context, speculating that the male's venom could generate some sedative effect and thus reduce the possibility of being cannibalized by the female (Arnqvist & Rowe 2005). However, this is unsupported by evidence regarding venom transference, and even sting penetration has been questioned by some authors (refs. Toscano-Gadea 2010; Peretti 2013). Unlike other examples of non-genitalic interaction cited in a dominant behavior context with an ambiguous aggressive role, the use of stinging behavior in scorpions outside this context is always aggressive in predation or defense; thus, it has been assumed to imply a negative input in the balance of the costs and benefits for the female in a sexual context. However, the possibility that the sexual sting may be a stimulatory inducement in the context of male courtship, without a cost for the female cannot be ruled out. Therefore, it is important to assess first the extent of the sexual sting in terms of both actual aculeus penetration into the female's body cavity, and secondly whether venom injection occurs or not.

The aims of this study were (1) to confirm or disprove the puncturing of the female body by the male aculeus during the sexual sting. If the female's membrane is punctured, (2) to confirm or reject the injection of venom. Finally, if venom is injected, (3) to test the hypothesis that venom composition is sexually dimorphic, thus potentially playing a coercive or stimulatory role in the female's choice of mates. We present the comparative venom analysis of both sexes to show similarities as well as the main differences. To test the possible selective pressures over venom composition, a set of specific experiments including the characterization of biological activities of the whole venom, as well of individual toxins, should be required and is being considered for future studies.

METHODS

Scorpions.—Specimens of different instars of *Megacormus gertschi* scorpions (Fig. 1a, adult male; 1b, adult female) were collected during 2014 to 2018, under scientific permit FAUT-0175 (from Secretaría de Medio Ambiente y Recursos Naturales, SEMARNAT, to OFF) from three nearby localities around the type locality of the species, in the state of Hidalgo, Mexico: Rancho Manzanal (RM), Municipio de Meztitlán, 20.68° N, 98.71° W; Piedra Blanca (PB), Municipio de San Agustín Mezquititlán, 20.60° N, 98.63° W); and Tianguistengo (T), Municipio de Zacualtipan. The habitat was pine-oak woodlands at elevations ranging from 1900–2200 m.a.s.l. Specimens were housed individually in the laboratory (CNAN, UNAM), in 60 mL plastic containers with suitable substrate at room temperature (15 ± 5 °C) and fed twice a month with crickets (*Achaeta domestica*), until the mating season. Animal care and handling procedures were adapted following Mexican protocol for animal care, handling and housing NOM-062-ZOO-1999.

Evidence of sexual sting.—The immune system response to pathogens or physical injury in arthropods is the encapsulation and melanization of the affected area (Schmid-Hempel 2003, 2005). The degree of melanization depends on the area of affected tissue and the time elapsed since injury (Bilandžija et al. 2017). In scorpions, penetration of the stinger produces a perforation and the subsequent development of melanization at the site of injection, which is the reason why melanized marks in the integument serve as indicators of the injury. Preliminary observations of artificial sexual sting were made (protocol adapted from Oviedo et al. 2019) in the females' dorsal membranous region, including specimens of *M. gertschi, Bothriurus bonarensis* (CL Koch, 1842) and *Zabius fuscus* (Thorell, 1876), to assess the utility of the melanization process in the stung area as evidence of perforation. It was noted that the injuries were not immediately evident, i.e., the initial perforation is not seen even in cases involving

Figure 1.—Adult *Megacormus gertschi*. (a) male; (b) female; (c) courtship during *promenade a deux*; (d) sexual sting performance, representative specimens from locality "Rancho Manzanal"; (e) newly attached and activated spermatophore.

hemolymph release at the site of injury, and the color change due to the melanization process can be noticed beginning at the 7-10th day after the injury.

The search for sexual sting evidence in *M. gertschi* was performed with a binocular microscope, examining TPIM (right and left sides) of 85 live specimens of both sexes from the three localities (females $n = 60$; males $n = 25$). In order to establish whether the melanized marks in this area are found only in adult females (collected gravid from the field and having offspring born in captivity, and individuals not gravid nor with offspring born in captivity), adult males and sub-adults of both sexes were also included (see Table 1 for total specimens examined per instar).

Specimens were immobilized with Parafilm® attached to a glass slide to stretch out the pedipalp under light microscope,

Table 1.—Summary of *Megacormus gertschi* material examined for melanized marks. Number of individuals with or without melanic marks observed per sex per instar. "Os", "no Os": indicate with or without offspring born in captivity, respectively.

	Males			Females			
					Adult		
	Sub-adult	Adult		Sub-adult	Os	no Os	
Marks	0	5	**5**	0	45	10	**55**
without marks	2	18	**20**	4	1	0	**5**
Total	2	23	**25**	4	46	10	**60**

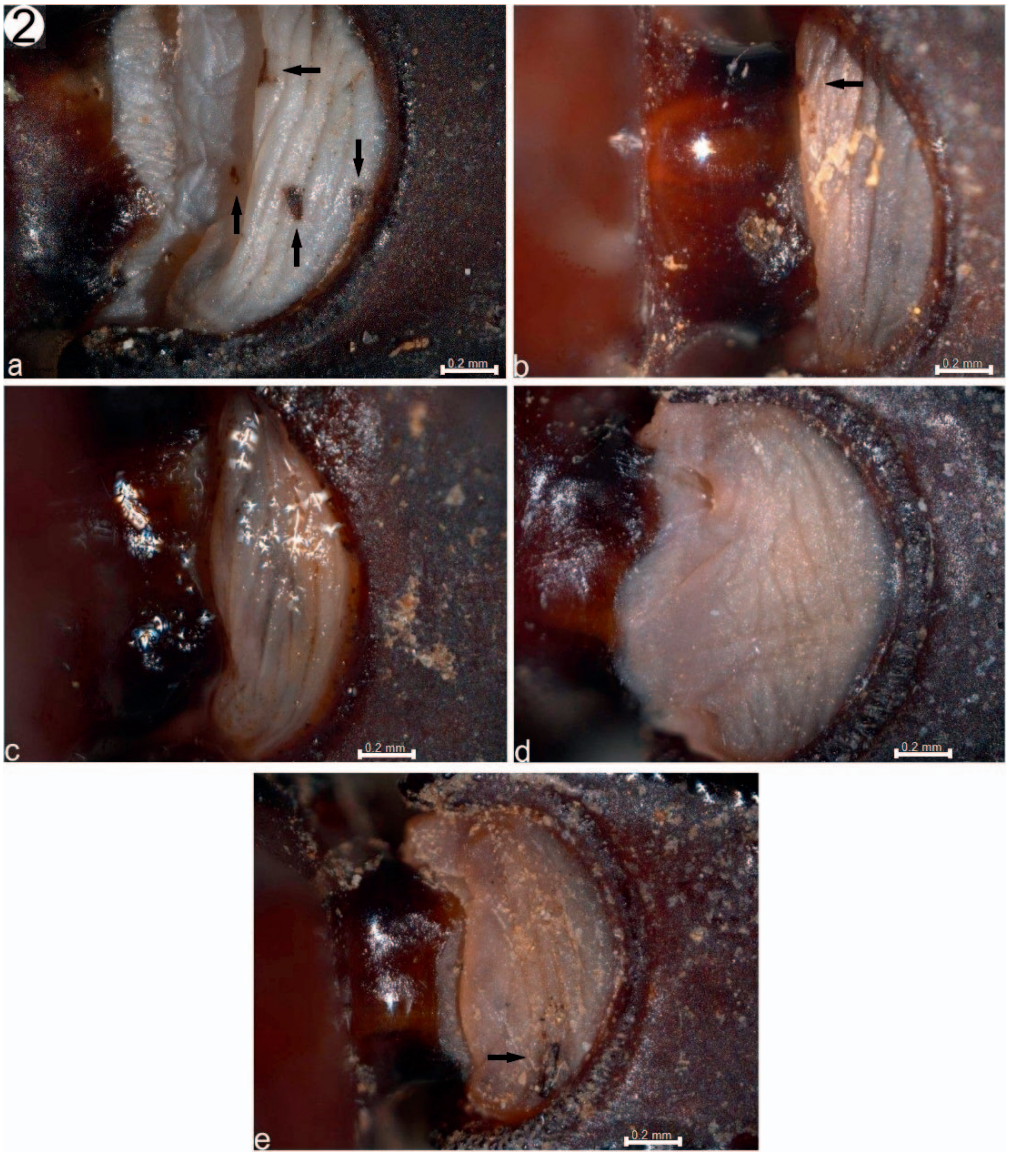

Figure 2.—Analysis of melanized marks. Tibia-patella palpal membrane of preserved specimens. (a) adult female, Rancho Manzanal; (b) adult female, Piedra Blanca; (c) subadult female; (d) adult male; (e) adult male with melanized mark. Scale bars = 2.0 mm. Black arrows indicates the sites with melanized marks.

and the number of melanized marks was recorded. Adults used in staged mating experiments were explored after the experimental procedure to prevent unnecessary stress prior to the behavioral observations, except for three adult females that were examined before and after mating presentations to assess the time of appearance of the melanization response.

Photographs of preserved specimens, as illustrated in Fig. 2a–e, were obtained with light microscope imaging with a digital camera Leica DFC490 (8 mp) attached to a Leica Z16 APOA microscope and processed using Leica Application Suite (LAS) version 4.3.0. Images were saved as color TIFF files and managed with Adobe Photoshop CS6.

A Chi-square test was performed to analyze whether only adult field-collected females would have melanized marks related to their reproductive status. This analysis compared the proportions of females with or without marks against the proportions of males with and without marks, under the hypothesis that only females that have mated would have melanized markings in the tibia-patella joint of the pedipalp (regardless of the number or size of the marks).

Evidence of venom sexual dimorphism.—Animal venoms, including those of scorpions, consist of complex mixtures of components of diverse molecular nature and size, as well as diverse biological activities. Venoms in general are analyzed through methods that determine their characteristics in terms of physical (absorbance profile, viscosity) and biochemical (composition, component proportion) properties, among others. The presence of sexual stinging behavior in *M. gertschi* led to the hypothesis that there might be differences in the properties of male and female venoms because natural selection should favor females that do not spend energy to produce unnecessary venom components; whereas males

Table 2.—Results of the detection of male *Megacormus gertschi* venom in female hemolymph. Abbreviations: R.T. – retention time. AUC – area under the curve. µg corresponds to venom equivalent quantity expressed in total micrograms. SS – sexual sting. Freq – frequency, total number of times such behavior was executed. Dur – duration, total duration of execution of such behavior, expressed in minutes. ND – not detected signal. N/A – not applicable. e.t. – elapsed time, hemolymph sampling time elapsed since the end of the mating event, expressed in minutes. Vol. – volume, female hemolymph sample volume. * – Samples of non-mated in captivity adult females.

				SS		Sampling	
Sample	R.T.	AUC	Venom (µg)	Freq	Dur	e.t.	Vol. (µL)
"Natural"							
264	32.09	1821	0.12	1	20	10	1
284	32.42	2087	0.14	2	20	10	10
249	32.36	4909	0.25	2	21	120	10
244	32.39	16956	0.72	1	20	30	10
242	32.05	22117	0.92	2	14	30	10
239	32.82	93778	3.71	1	46	60	10
"Stingless"							
233	32.00	ND	ND	0	0	30	10
282	32.00	ND	ND	0	0	10	10
248	32.00	ND	ND	0	0	25	10
Controls							
Negative*							
266	32.00	ND	ND	N/A	N/A	N/A	10
285	32.00	ND	ND	N/A	N/A	N/A	20
292	32.00	ND	ND	N/A	N/A	N/A	10
Positive (reference levels)							
Low	32.01	2562	0.1	N/A	N/A	N/A	N/A
Medium	32.05	22860	1	N/A	N/A	N/A	N/A
High	31.95	1280720	50	N/A	N/A	N/A	N/A

might be injecting some energy-expensive component(s) into the female's body to increase their own reproductive fitness. Venom from field collected specimens from the three localities was biochemically analyzed in this study to evaluate possible dimorphism in the venoms of *M. gertschi* males and females, using the following procedures.

To obtain venom samples, the living specimens were milked directly into capillary tubes by electrical stimulation (Meadows & Russell 1970), and the samples were pooled by sex and locality (RM: ♀ = 20, ♂ = 24; PB: ♀ = 8, ♂ = 11; T: ♀ = 9, ♂ = 4). Venom samples were stored at -80° C until analysis, when they were reconstituted in MilliQ water.

Protein content was measured by spectrophotometric absorbance at 280 nm, assuming that one absorbance unit corresponds to 1 mg/ml of protein, using Nanodrop (NanoDrop 2000, Thermo Scientific). Males' and females' venoms were measured separately.

The separation of venom components was performed using two complementary methodologies: gel electrophoresis, which favors the separation of proteic components based on molecular weight, and liquid chromatography, which adds information about proteic and non-proteic components separated by their hydrophobicity.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation of venoms was performed by loading samples of 20 µg of each venom sample into a 17% polyacrylamide gel, to allow separation of low molecular weight components, under reduced (addition of 2-mercapto ethanol) and non-reduced conditions. Molecular weight calibration markers (3 µL; Precision Plus, #1610374, BIORAD) were used as controls. Protein bands were stained with Coomasie Brilliant Blue R-250.

High performance liquid chromatography (HPLC) allows separation of venom components based on their differential desorption from a hydrophobic column; the higher the hydrophobicity of the column matrix, the higher the resolution between two components. Separation of venom samples was performed according to Arnaud-Franco et al. (2018), by loading 200 µg of each pooled venom sample into a Vydac 218 TP-C18 (250 × 4.6 mm, 5 µm) column (C18 column) in an Agilent 1260 HPLC system (Agilent, CA) using two-solvent system: (A) H_2O + 0.05% trifluoroacetic acid (TFA) and (B) Acetonitrile + 0.05% TFA at 0.75 ml/min. The column was equilibrated in 2% solvent B. Linear increment of solvent B from 0 to 60 % in 60 minutes (1% per minute), was performed five minutes after venom injection into the column. Elution of venom components was monitored at 220 nm.

Evidence of venom injection.—To assess whether venom transfer is associated with sexual stinging, venom components were separated from female's hemolymph using an *in vitro* analysis performed by HPLC that allows the separation and identification of components of low molecular weight or low concentration.

As part of the parallel ethological studies, the hemolymph of females with a staged mating event in the laboratory were sampled after finishing (see Table 2 for specific volumes and time of sampling). Two adult female groups were mated in captivity: females receiving sexual sting during the mating event and a "stingless" group. The latter corresponds to an experimental group of males in which the stinger was mechanically blocked with a small, temporary drop of wax on its tip to prevent venom injection into the female during the performance of the sexual sting behavior. Hemolymph of three females from this group was also analyzed ("Stingless" group on Table 2). Hemolymph of adult females not mated in captivity were used as controls.

The specimen handling for hemolymph sampling of adult females was performed as described in the previous section. Hemolymph samples (see Table 2 for specific volumes) were obtained by dorsal puncture with a sterile insulin needle in the intersegmental membrane between mesosomal tergites IV-V or V-VI. The hemolymph samples were recovered individually with micropipette tips, centrifuged and kept frozen at -80 °C until analysis when they were reconstituted in MilliQ water.

To detect male venom in female hemolymph, a C3 column was used, unlike the C18 column chromatographic method used for venom dimorphism characterization. High molecular weight components, such as hemocyanin (390 kDa), that are present in hemolymph are poorly resolved in C18 columns (used for venom characterization). Thus, a column with shorter hydrophobic arm (C3 column) was used for separation of venom (low molecular weight components, <15 kDa) from hemolymph components (high molecular weight components, >300 kDa).

HPLC was performed in an Agilent Sorbax 300SB-C3 (250 × 4.6 mm, 5 µm; SN 002759) column (C3 column) in a Varian Pro Star (Varian) equipment using a two-solvent system: (A)

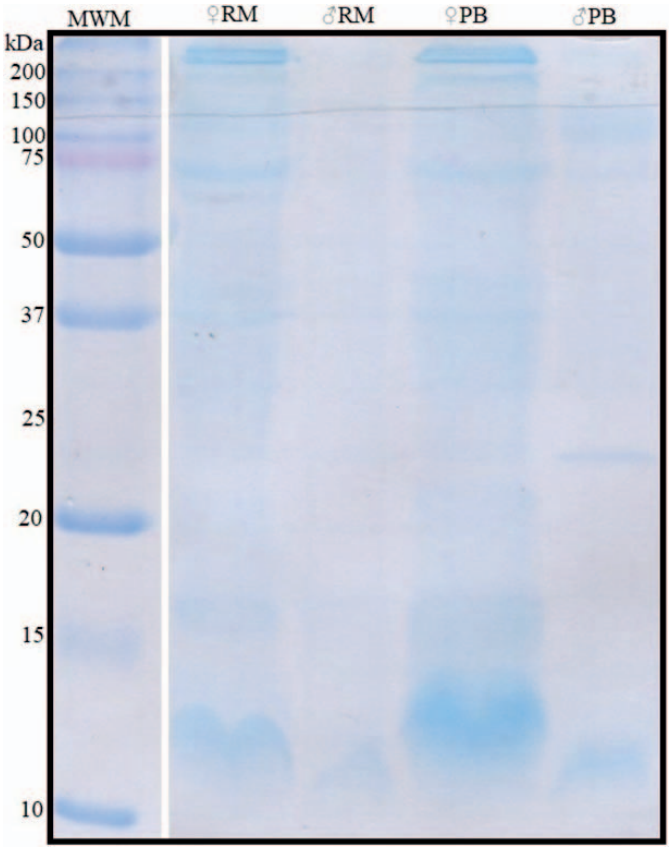

Figure 3.—Analysis of venom proteins. Representative electrophoretic pattern (SDS-PAGE) of *M. gertschi* venoms. Samples of 20 µg venom per lane. ♀, ♂: indicate females, males. RM: "Rancho Manzanal" locality ($n = 17$ ♀ and 16 ♂). PB: "Piedra Blanca" locality ($n = 8$ ♀ and 7♂). MWM: molecular weight markers expressed in kilo daltons (kDa).

H_2O + 0.05% trifluoroacetic acid (TFA) and (B) CH_3CN + 0.05% TFA at 0.75ml/min. Five minutes after injection, linear increase of solvent B was performed for 60 minutes and components monitored at 215 nm.

Hemolymph samples from unmated adult females in captivity were used to establish reference levels as well positive controls. Quantities of venom of known concentrations (0.1, 0.5, 1, 50 and 100 µg; $n = 4$ per dose level) were pre-incubated with constant hemolymph volumes (5 µl) and injected in a total volume of 1 ml using MilliQ water. The obtained recovery value was within the acceptance range (80–120% C.V.). Detection limit was 0.1 µg, interval confidence set at 40%, which allows an approximation of the amount of venom present in each sample (See positive controls in Table 2). Because the major venom signal corresponds to the peak near retention time (RT) 32 min, we decided to use it as reference to establish venom presence in mated females' hemolymph samples. For negative controls, samples of captive non-mated adult female hemolymph were injected independently.

Data from HPLC profiles were compared against standard curve to calculate venom recovery using GraphPad Prism® software version 6.0. Results are presented as mean ± standard deviation of three replicates.

RESULTS

Evidence of sexual sting: melanized marks.—Adult females collected gravid from the field and having offspring born in captivity ($n = 46$), as well as females not gravid nor with offspring in captivity ($n = 10$) show melanized marks in both patella-tibia joints (Fig. 2a,b, black arrows) in variable quantity and size (up to nine marks in one specimen). Two females without evident melanized marks gave birth in captivity. Sub-adult females, presumably "virgins" (i.e., without mating experience) ($n = 4$) did not present melanized marks on the expected site (Fig. 2c). Three females belonging to the group of gravid from field and giving birth in captivity were examined before their subsequent exposure to a mating event in captivity, finding the presence of previous melanized marks (1, 2 and 5, respectively), expected from one or more previous matings in the wild before being collected. The same three females were examined 10–20 days after the mating event in captivity took place, finding new, smaller and paler melanized marks (3, 3 and 6, respectively), as would be expected due to the difference in time of the wild and the captive matings.

In the case of males, melanized marks were absent on 18 adult and two sub-adult specimens (Fig. 2d). The presence of melanized marks in five adult males, one of them with four marks in the referred site (Fig. 2e, black arrow) is noteworthy and could be result of either male-male competition, or aggression on the male by an unreceptive female. The proportion of field collected females with melanized marks is statistically significantly higher than the proportion of field collected males with melanized marks $\chi^2_{1,85} = 43.6569$, $P < .00001$ (see Table 1 for total specimens examined per sex).

Evidence of venom sexual dimorphism on venom composition.—Venom volumes expelled by females during the milking process were higher than in males (not accurately measured), and the differences could be clearly observed in the columns in the capillary tubes used. Comparison of pooled venom by sex, from the three different localities, revealed that female venoms were opaque and viscous, with a high absorbance signal at 280 nm, and spectral profile (200–400 nm) as expected for peptidic material; whereas male's venoms were clear and barely viscous, showing two major peaks between 290 and 320 nm, suggesting the presence of non-peptidic saturated cyclic molecules at those peaks. Pooled venoms separated by sex and locality were analyzed by SDS-PAGE (Fig. 3), showing similar electrophoretic profiles between samples of the same sex but major differences between sexes. Females' venom profile shows most prominent bands near to 12–15 kDa in which ion-channel toxins are commonly found (Rodríguez de la Vega et al. 2003) as well as two high molecular weight components at 75 and 150 kDa, with diffused area near at 37 kDa. Even when the most prominent bands in male's venoms are also those at 12–15 kDa, they show comparatively fewer bands along the gel. Sex-related protein content could be responsible of this electrophoretic behavior but also, presence of non-proteic, highly A280nm absorptive material could provoke overestimation of protein concentration.

Chromatographic separation by analytical HPLC (Fig. 4) confirmed the existence of several components evenly distributed along the profiles in both venoms. Major differences between venoms are evident in the peak eluted at 18.1 min,

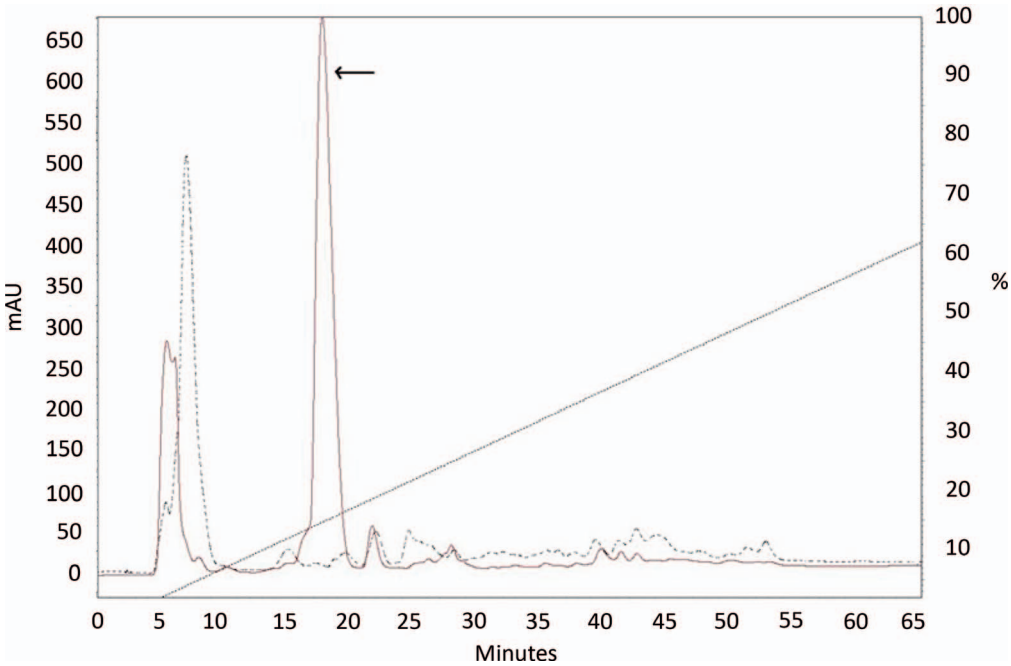

Figure 4.—Analysis of venom sexual dimorphism. Comparative chromatographic profiles of female (dotted line) and male (solid line) *M. gertschi* venoms in C18 column. The major difference is observed in the peak at ~ 18 min in male venom (black arrow) which is absent in female's venom. Peaks were followed at 215 nm., gradient is depicted as dotted line. mAU: milli absorbance units. %: % solvent B (acetonitrile + 0.05% TFA).

that accounts for more than 50% of absorbing material in the male's venoms and is completely absent in the female's venom. The chemical nature of this peak was not studied; nevertheless, it could correspond to an alkaloid component reported by Banerjee et al. (2018). Female venom shows a prominent peak around 7.6 min that is completely absent in males' venom. Components eluting from the beginning of the gradient could correspond to low mass non-proteic or blocked material previously reported for this venom (Santibañez et al. 2017). According to Santibañez et al. (2017) scorpion toxins elute from the middle to the end of the chromatographic gradient; in this study, other differences between venoms could also been observed at 24.9–26.5 min, 43.5–45 min and 51–53 min, where females' venoms show various peaks that are absent in the male's venom.

Evidence of venom injection: male venom components in female hemolymph.—To establish the elution profile of male venom from female hemolymph, three independent runs of each type of biological sample were performed in C3 RP-HPLC column. Retention times (RT) for male venom components ranges from 27–35 min, constituted by five defined peaks, with peak 4 being the most abundant with RT = 32.3903 ± 0.779 min (Fig. 5, horizontal arrow). Meanwhile, RT for female hemolymph components ranged from 39 to 60 min, with a minor peak around 40 min, and the main peaks from 53 to 60 min (Fig. 5, horizontal square bracket and asterisk).

Table 2 shows the results of the HPLC analysis of hemolymph samples from females ($n = 9$) involved in sexual interactions during the last mating season (April to October 2018), indicating number (frequency) and duration of sexual stings performed by males in each couple, as well as sampling details (i.e., hemolymph sampling time elapsed since the end of the mating event, as well as the sample volume). Male venom signal was detected in six of the nine samples, with a range from 0.1 to 3.97 μg, corresponding to females that received at least one sexual sting ("Natural" samples on table 2), confirming the transference of venom during sexual stinging in *M. gertschi*. The highest amount of male venom recovered from a mated female's hemolymph (3.97 μg) was from a female that received a single sexual sting with a duration of 42 minutes.

The hemolymph sampled from females of the Stingless experimental group, whose partners displayed normal courtship behavior including sexual stinging attempts with the blocked aculeus tip, have no trace of the male's venom in the expected retention time ("Stingless" samples on Table 2).

To assess differential responses of venom versus hemolymph components, interference assays were carried out by adding known quantities of venom into raw female's hemolymph. Male venom and female hemolymph profiles show no overlapping, as seen on figure 5. To quantify the venom a reference curve was constructed by adding 0.1, 1 y 50 μg of male venom to raw females' hemolymph (see "Positive controls" levels on Table 2).

DISCUSSION

The presence of melanized marks in the membranous region of *M. gertschi* adult females' pedipalps provides anatomical evidence regarding the perforation produced by the male's sting during sexual stinging behavior. Some authors refer to the sexual sting as the "apparent" sting made with the male's aculeus in the female's body during mating. It is mentioned in

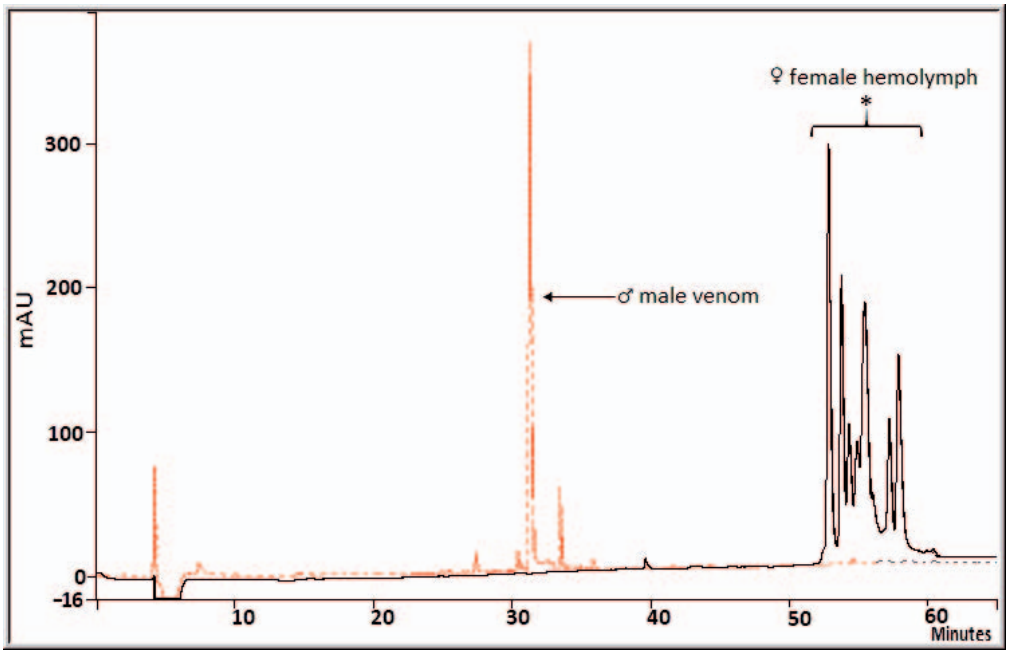

Figure 5.—Representative profile showing presence of male venom in female hemolymph. Separation of male *M. gertschi* venom (red line) from female hemolymph (black line) by HPLC in C3 column. Main components of adult male venom elute between 30 and 35 minutes, indicated by the horizontal arrow. Main components of the female's hemolymph elute between 50 to 60 minutes, indicated by the horizontal bracket and asterisk; there is no signal above noise level between 30 and 35 minutes. The presence of a distinguishable signal at RT = 32.39 ± 0.78 min (horizontal arrow) in a female's hemolymph was considered evidence of venom transference. Peaks were followed at 215 nm. See Table 2 for details of venom quantitation on other female's hemolymph samples.

various descriptions of South American and European species' sexual behavior that sexual stinging is a "ritual" of sorts, suggesting that during the display of this behavior, the male only places the stinger on the female's body without achieving perforation (Carrera et al. 2009; Toscano-Gadea 2010; Peretti 2013).

In *M. gertschi* the sexual sting is consistently displayed in successful matings, both as a behavioral unit as well as mechanical interaction (personal observation, manuscript in preparation). The evidence of melanized marks may serve as a starting point to question the behavior's ritualization theory. Jiao & Zhu (2010) report sexual sting display in another species of the family Euscorpiidae, in the same anatomical area targeted by *M. gertschi*, speculating that although a perforation is made (authors do not provide evidence of puncture), the male could "decide" whether to transfer venom or not, depending on the female's behavioral response (no venom transference assessment evidence is provided either).

The observation of melanized marks as perforation evidence suggests that this behavior may be at least a tactile stimulus of greater intensity than when it is only a superficial contact on the female's body, such as rubbing (i.e., "telson rubbing") as described in various *Bothriurus* species (Peretti et al. 2000; Peretti 2001; Toscano-Gadea 2010). Considering that stinging behavior in scorpions usually takes place in a context of attack/defense against prey and predators, there is the potential for the male to paralyze or even kill the female when stinging her in a sexual context. The execution of the sexual sting would imply fitness repercussions for both sexes. In the female's case, two types of costs would be present: survival, because the perforation site is a potential entry for pathogens, and reproductive success, if the sexual sting promotes the acceptance of a suboptimal mating partner.

Intersexual communication is a mechanism for mate choice as well as an opportunity for the opposite sex's manipulation and sensory exploitation (Shaw 1995). Both mechanisms assume the existence of some signal exchange prior to mating. In the context of intersexual communication, individuals establish diverse communication channels such as tactile, chemical, and vibratory, among others (Rendall et al. 2009; Patricelli & Hebets 2016; Scott et al. 2018). The interaction of the male's stinger in the tibia-patella membrane of the female's pedipalp during the sexual sting represents an opportunity for signal exchange. Given that the intersegmental membrane is vulnerable and susceptible to perforation, the act of touching this area with the sting could be considered *per se* a mechanical signal measurable by the female and used as such to assess the potential mates' quality. Additionally, the perforation could establish another type of more invasive and even chemical communication pathway if there is venom transference.

The situation that leads to consideration of an alternative stimulation pathway in the case of perforation is the fact that offensive/defensive stinging strikes are of short duration (milliseconds) (Edmunds & Sibly 2010; Meijden et al. 2015), whereas sexual stinging lasts several minutes in the case of bothriurids (Toscano-Gadea 2010), as well as in *M. gertschi* (Francke 1979; L. Olguin-Pérez, pers. obs.).

Given that scorpions have muscular control over their venom gland (Nisani & Hayes 2011), stinging behavior is not necessarily expected to be associated with venom transfer. But, when the act of stinging is associated with venom inoculation, differences between the venom amounts in a female receiving a

sexual sting and the amounts in a prey receiving an offensive sting are expected, in terms of dosage and biological activity or presence in the hemolymph. Detection of venom in hemolypmph samples by HPLC assays is evidence of actual venom transference, but it remains unknown whether there is actually transference of only specific venom fractions during sexual stinging, although this alternative is unlikely, given the anatomy of the scorpion venom glands with a single storage space leading to the venom gland conducts.

Transfer of the male's venom opens another communication pathway with potential effect on the female's reproductive decisions, as has been observed in some arthropods that transfer bioactive molecules in nuptial gifts, both orally and genitalically (deCarvalho & Shaw 2010). These effects could be in terms of courtship or manipulation.

The transference of venom to the female of *M. gertschi* during sexual stinging could be beneficial to the female in terms of her reproductive success. These would be direct benefits if they were related to biological activities triggered by one or more components of the male's venom (e.g., antibiotic protection or immunostimulant action), or indirect if her offspring have the ability to successfully perform this behavior and/or if they inherit a 'seductive' venom from their father.

From the males' perspective, this behavior in a sexual conflict context would convey a different type of cost. On one hand, sexual stinging may have detrimental effects on the female's survival or on their offspring's quality. In addition, the use of energy-expensive venom in a sexual encounter may restrict further feeding or defensive uses by the male, thus affecting his fitness. However, sexual stinging may be correlated with the fertilization of the stung female's oocytes or may serve as a criterion for female choice as an honest signal of the male's quality; since it occurs before insemination, the female can still reject the male's attempt to guide her over the spermatophore if she doesn't receive the proper chemical signal from the inoculation of male venom. Under such scenario, we also would predict that venom sexual dimorphism would evolve, as found in this study.

The main finding of this study is the anatomical evidence of intersegmental membrane perforation during performance of the sexual stinging behavior, something that many authors have questioned. Furthermore, the evidence obtained of the male's venom transference during sexual stinging, in addition to the observed differences between both sex's venoms, leads to consideration of pre-insemination sexual stinging as a non-genitalic sexual interaction, due to its potential role as a courtship element.

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LITERATURE CITED

Arnaud-Franco G, Cordero-Tapia A, Ortíz-Avila V, Moctezuma-González CL, Tejocote-Pérez M, Carbajal-Saucedo A. 2018. Comparison of biological and biochemical characteristics of venom from rattlesnakes in the southern Baja California Peninsula. *Toxicon* 148:197–201.

Arnqvist G, Rowe L. 2005. Sexual Conflict. Princeton University Press, NJ, USA.

Banerjee S, Gnanamani E, Lynch SR, Zamudio Zuñiga F, Jiménez-Vargas JM, Possani LD et al. 2018. An alkaloid from scorpion venom: chemical structure and synthesis. *Journal of Natural Products* 81:1899–1904.

Bilandžija H, Laslo M, Porter ML, Fong DW. 2017. Melanization in response to wounding is ancestral in arthropods and conserved in albino cave species. *Scientific Reports* 7:17148.

Carrera PC, Peretti AV. 2005. Female control of mating sequences in the mountain scorpion *Zabius fuscus*: males do not use coercion as a response to unreceptive females. *Animal Behaviour* 69:453–462.

Carrera PC, Mattoni CI, Peretti AV. 2009. Chelicerae as male grasping organs in scorpions: sexual dimorphism and associated behavior. *Zoology* 112:332–350.

Córdoba-Aguilar A, Vrech DE, Rivas M, Nava-Bolaños A, González-Tokman D, González-Soriano E. 2015. Allometry of male grasping apparatus in odonates does not suggest physical coercion of females. *Journal of Insect Behavior* 28:15–25.

deCarvalho TN, Shaw KL. 2010. Elaborate courtship enhances sperm transfer in the Hawaiian swordtail cricket, *Laupala cerasina*. *Animal Behaviour* 79:819–826.

Eberhard WG. 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton New Jersey.

Eberhard WG. 2015. Cryptic female choice and other types of post-copulatory sexual selection. Pp. 1–26. *In* Cryptic Female Choice in Arthropods: Patterns, Mechanisms and Prospects (Peretti A, Aisenberg A, eds.). Springer, Heidelberg.

Edmunds MC, Sibly RM. 2010. Optimal sting use in the feeding

behavior of the scorpion *Hadrurus spadix*. *Journal of Arachnology* 38:123–125.

Francke OF. 1979. Observations on the reproductive biology and life history of *Megacormus gertschi* Diaz (Scorpiones, Chactidae, Megacorminae). *Journal of Arachnology* 7:223–230.

Han CS, Jablonski PG. 2009. Female genitalia concealment promotes intimate male courtship in a water strider. *PLoS One* 4(6):e5793.

Han CS, Jablonski PG, Brooks RC. 2015. Intimidating courtship and sex differences in predation risk lead to sex-specific behavioural syndromes. *Animal Behavior* 109:177–185.

Jiao GB, Zhu MS. 2010. Courtship and mating of *Scorpiops luridus* Zhu Lourenço & Qi, 2005 (Scorpiones: Euscorpiidae) from Xizang province, China. *Journal of Venomous and Animal Toxins including Tropical Diseases* 6:155–165.

Krebs J, Davies N. 1993. Sexual Conflict and Sexual Selection. An Introduction to Behavioral Ecology. Blackwell, Oxford.

Koshio C, Muraji M, Tatsuta H, Kudo S. 2007. Sexual selection in a moth: effect of symmetry on male mating success in the wild. *Behavioral Ecology* 18:571–578.

Meadows PE, Russell FE. 1970. Milking of arthropods. *Toxicon* 8:311–312.

Meijden A, Coelho P, Rasko M. 2015. Variability in venom volume, flow rate and duration in defensive stings of five scorpion species. *Toxicon* 100:50–55.

Nisani Z, Hayes WK. 2015. Venom-spraying behavior of the scorpion *Parabuthus transvaalicus* (Arachnida: Buthidae). *Behavioural Processes* 115:46–52.

Oviedo-Diego MA, Mattoni CI, Peretti AV. 2019. Specificity of the female's local cellular immune response in genital plug producing scorpion species. *PLoS ONE* 14(2):1–24.

Parker GA. 2006. Sexual conflict over mating and fertilization: an overview. *Philosophical Transactions of the Royal Society B: Biological Sciences* 361:235–259.

Patricelli G, Hebets EA. 2016. New dimensions in animal communication: the case for complexity. *Current Opinion in Behavioral Sciences* 12:80–89.

Peretti A. 2001. Patrones de Resistencia femenina y respuesta del macho durante el apareamiento en escorpiones Bothriuridae y Buthidae: qué hipótesis puede explicarlos mejor? *Revista de Etología* 3:25–45.

Peretti A. 2013. Sexual selection in Neotropical species: Rules and exceptions. Pp. 33–52. *In* Sexual Selection: Perspectives and Models from the Neotropics. (Macedo RH, Machado G, eds.). Elsevier, USA.

Peretti AV, Acosta L, Martínez M. 2000. Comportamiento de apareamiento en tres especies de *Bothriurus* del grupo prospicuus: estudio comparado y su relación con *Bothriurus flavidus* (Scorpiones, Bothriuridae). *Revue Arachnologique* 13:73–91.

Polis GA. 1990. The Biology of Scorpions, Stanford University Press, Palo Alto.

Polis GA, Sissom WD. 1990. Life history. Pp. 81–111. *In* The Biology of Scorpions. (G.A. Polis, ed.). Stanford University Press, Palo Alto.

Rein JO. 2019. The scorpion files. Norwegian University of Science and Technology. http://www.ntnu.no/ub/scorpion-files/.

Rendall D, Owren MJ, Ryan MJ. 2009. What do animal signals mean? *Animal Behaviour* 78:233–240.

Rodríguez de la Vega RC, Merino E, Becerril B, Possani LD. 2003. Novel interactions between K+ channels and scorpion toxins. *Trends in Pharmacological Sciences* 24:222–227.

Ryan MJ, Cummings ME. 2013. Perceptual biases and mate choice. *Annual Review of Ecology, Evolution, and Systematics* 44:437–459.

Santibañez-Lopez CE, Cid-Uribe JI, Zamudio FZ, Batista CVF, Ortiz E, Possani LD. 2017. Venom gland transcriptomic and venom proteomic analyses of the scorpion *Megacormus gertschi* Díaz-Najera, 1966 (Scorpiones: Euscorpiidae: Megacorminae). *Toxicon* 133:95–109.

Schmid-Hempel P. 2003. Variation in immune defense as a question of evolutionary ecology. *Proceedings of the Royal Society of London B* 270:357–366.

Schmid-Hempel P. 2005. Natural insect host-parasite systems show immune priming and specificity: puzzles to be solved. *Bioassays* 27:1026–1034.

Scott CE, Anderson AG, Andrade MCB. 2018. A review of the mechanisms and functional roles of male silk use in spider courtship and mating. *Journal of Arachnology* 46:173–206.

Shaw K. 1995. Phylogenetic tests of the sensory exploitation model of sexual selection. *Trends in Ecology and Evolution* 10:117–120.

Subramoniam T. 2017. Mating systems. Pp. 105–130. *In* Sexual Biology and Reproduction in Crustaceans (Subramoniam T. ed.). Academic Press.

Toscano-Gadea CA. 2010. Sexual behavior of *Bothriurus buecherli* (Scorpiones: Bothriuridae) and comparison with the *B. prospicuus* group. *Journal of Arachnology* 38:360–363.

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