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Chemical caressess: geographical variation of male sexual signals in a Neotropical scorpion

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Abstract

Differences in sexual signals among species are common, and may influence mate recognition and reproductive isolation. In scorpions, behavioural mechanisms and other sexual signals involved in mate selection and reproductive isolation have been scarcely studied. In this paper, we compare different male sexual signals between two distant populations of the scorpion *Bothriurus bonariensis*, one located in Uruguay and the other in Central Argentina. We compare sexual behaviours from intra-populations and inter-populations matings. In addition, we extent this comparison to the secreted compounds and morphology of the exocrine glands located on the dorsal side of the telson in this species. Males of *B. bonariensis* performed stimulatory behaviours to the female with different frequency of occurrence and duration in the two populations. Chemical analyses of the glandular extracts showed that Uruguayan males present compounds which are absent in males of Argentinian population. In addition, we observed that in inter-population matings, stimulatory behaviours had intermediate patterns to intra-population matings. However, males failed to achieve a successful sperm transfer with females of different population. Mechanisms of sexual isolation between these two distant populations of *B. bonariensis* apparently seem to have evolved due to divergence in allopatry. The differences in stimulatory levels during courtship between the two populations studied here give evidence for an early behavioural divergence promoted by sexual selection.

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Keywords
scorpiones, courtship, chemical signals, behavioural signals.

1. Introduction

There are plenty of studies — involving both vertebrates and invertebrates — that have shown clear relationships between mating success and quality of various sex-specific traits, presumably driven by sexual selection (Andersson, 1994; Kokko et al., 2002, 2006; Mead & Arnold, 2004). Male sexual behaviour can be stimulatory when female resistance acts as a discriminatory filter for choosing the better partners. Indeed, in many species females are able to control the different phases of mating and, therefore, males have to perform persuasive behavioural patterns to be accepted (Eberhard, 2003).

Divergence of male sexual signals and the corresponding female preferences may contribute to reproductive isolation (Lande, 1981; Panhuis et al., 2001; Pizzari & Snook, 2003; Arnqvist & Rowe, 2005). Courtship commonly involves the exchange of signals spanning several sensory modalities (e.g., vibratory, visual, acoustic, chemical) and transmission of information about quality and species identity (Simmons, 1988; Choe & Crespi, 1997; LeMaster & Mason, 2001; Candolin, 2003; Melville et al., 2003; Scheuber et al., 2003; Virant-Dobertlet & Cokl, 2004; Hebets & Papaj, 2005). Chemical communication is used by a wide range of animals, due to the variety of information transmitted (species, sex, age, sexual receptivity) and the low cost of the emission (Redondo, 1994). Though much attention has been given to pheromone signalling in insects (Kaissling, 1997; Wyatt, 2003), there are only few studies about such signalling in arachnids (Foelix, 1982; Sonenshine, 1985; Gaskett, 2007).

In scorpions, little is known about chemical communication. Some studies focused on responses of individuals to conspecifics from the same population, show that males exhibited positive responses to areas previously exposed to conspecifics females (Gaffin & Brownell, 1992, 2001; Melville et al., 2003). Another study on the Vaejovidae scorpion *Paruroctonus boreus* Girard, 1854 suggested the existence of sex-specific responses to pheromones that could be population-specific (Miller & Formanowicz, 2010). Furthermore, in related species of the family Bothriuridae, the presence of cuticular pheromones in females has been reported and appears to be very important in intra-specific recognition (Peretti, 1995). Also, in numerous species of this family, males have external secretory glands on the dorsal

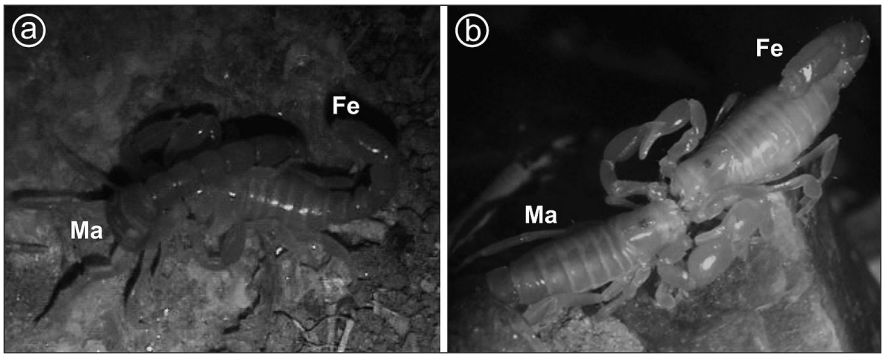

Figure 1. (a) Mating pair of *Bothriurus bonariensis* of Uruguayan population during 'Rubbing with telson' behaviour. (b) Mating pair of *Bothriurus bonariensis* of Argentinian population during the course of 'Pull and approximation' behaviour. Fe, female; Ma, male.

side of the telson or the fifth metasomal segment that shows numerous micropores, in which a whitish substance accumulates (De la Serna de Esteban, 1977, 1978). Peretti (1993) originally suggested that males of some species of bothriuridae, performed a behaviour named 'Rubbing with telson' (RT: Figure 1a; Video 1 in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x), in which the product of this gland is spread on the female body (ventral part of metasoma and mesosoma, including pectines). Females detect this exudate through the pectines, two ventrolateral, mechanoreceptive and chemosensory appendages (Gaffin & Brownell, 1992; Gaffin et al., 1992; Brownell, 1998), but principally through chemosensory setae, that are scattered all over the animal's body, including mesosoma and metasoma (Foelix & Mueller-Vorholt, 1983; Farley, 2000; Gaffin & Brownell, 2001).

In *Bothriurus bonariensis* (Koch, 1842), Peretti (1997) has demonstrate that the gland secretion have a sexual function and causes an increase in female receptivity during courtship. These conclusions were obtained after experiments in which the opening area of the gland was totally masked with paraffin in one group of males, to avoid the exudate of any secretion. Another group of males was remained with the gland unmasked. The results showed that, after RT behaviour, males with unmasked glands obtained significantly more receptivity from females than males with masked glands.

These results suggest that there is a relationship between the male gland secretion and the increase of female receptivity during courtship. However, up to date, the chemical composition of the gland secretions involved in this

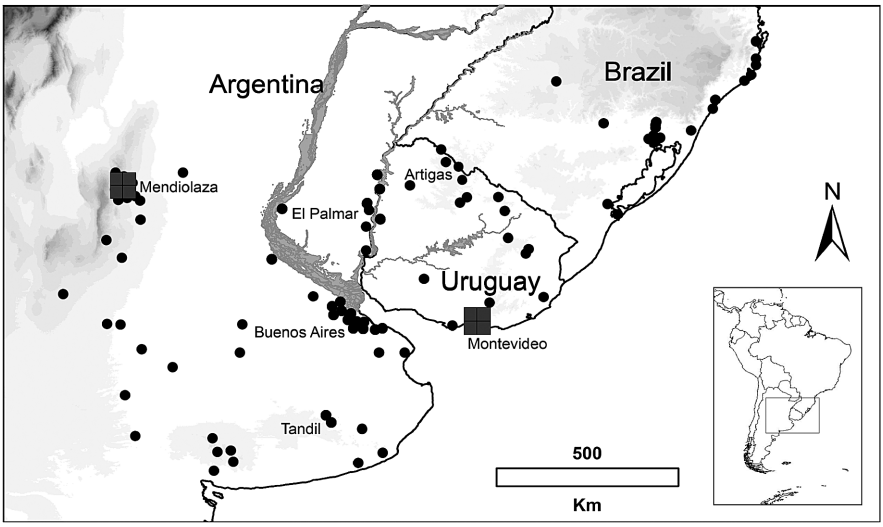

Figure 2. Geographic distribution of *Bothriurus bonariensis*. Black circles indicate the total distribution of the species known to date; grey grid squares show the populations used in this study.

male behaviour remains unknown. *B. bonariensis* has a wide distribution in South America, ranging from Southeast Brazil to Central Argentina. Argentinian populations are separated of Uruguay and Brazil populations by the geographical barrier imposed by the Parana and Uruguay rivers (Figure 2). Recent studies have demonstrated a certain degree of divergence in morphological patterns such as body size, coloration, and some allometry and asymmetry indexes among distant populations (e.g., Uruguay vs. central Argentina) (Ojanguren-Afilastro, 2005; Olivero et al., 2012, 2014; Olivero, 2014). In this context, data from sexual behaviour, including a first characterization of the involved chemicals, would be very relevant to evaluate the possibility of an incipient stage of speciation (Shine et al., 2002; Gabirot et al., 2012).

Males of this species perform ritualized behaviours during courtship that could function as stimulation for the female (Peretti, 1993). RT behaviour is one of the most frequent stimulatory behaviours performed by the male during courtship. However, potential differences in patterns of frequency of occurrence and duration of RT as well as in chemical compositions have not been explored between populations. Besides RT, males perform other frequent stimulatory behaviour, called 'Pull and approxi-

mation' (PA: Figure 1b; Video 2 in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x) (Peretti, 1993). Its name comes from the movements performed by the male, who pulls slightly the female and approaches her, reaching her mouth region. Then he takes distance and repeats all the sequence. This behaviour has not been analysed between different populations of the species.

The aim of this study was to compare two sexual behavioural patterns, including RT (which involves exudate of chemical substances) and PA (which does not involve any additional signal), between two distant populations of *B. bonariensis*, one from Uruguay and the other from Central Argentina. For that purpose, we compared these behaviours not only from intra-populations matings but also from inter-populations matings. This last option is essential in order to determine whether males exhibit capacity of modulating courtship behaviours according to frequencies and/or durations of these behaviours in the respective populations of courted females. In addition, we included an analysis and comparison of exocrine glands associated with the RT behaviour. For that aim, we analysed the external morphology of the glands as well as the chemical composition of their secretions.

2. Materials and methods

2.1. Populations studied

We used males and females of *B. bonariensis* collected at Central Argentina (Mendiolaza, Córdoba) and Uruguay (Piedras de Afilar, Canelones) populations (Figure 2). Individuals of these two populations have significant morphological differences (Mattoni, 2003; Ojanguren Affilastro, 2005; Olivero et al., 2012): In Central Argentina, the specimens are smaller and present slimmer and more delicate structures (i.e., hands and telson). Males from this population have shorter hemispermatophores but with broader laminas. Scorpions from the Uruguay population are more robust and present more spherical structures (i.e., hands and telson). The hemispermatophores of the males are larger and possess slimmer and longer laminas (Olivero et al., 2012). Although these morphological differences between the populations are well known and probably the populations could be considered as different species, this separation is not yet confirmed. For this reason, we consider them as one species.

2.2. Collection and rearing

Bothriurus bonariensis adult males and females were collected from November 2009–2012 to December 2009–2012. Animals were kept individually in plastic cages and were fed with larvae of *Tenebrio molitor* (Insecta, Coleoptera) once a week. Each cage included one moist cotton ball to provide humidity. The temperature varied from 28 to 34°C during the period of breeding. All the animals were kept under a 12:12 h light: dark cycle.

2.3. Observation and analysis of sexual behaviours

Male–female pairs were placed in a mating arena (18 × 30 × 30 cm) with soil as substrate, stones and pieces of tree bark from the capture sites. We placed the females in the arena 20 min prior to the males. We performed the observations during four mating seasons, from 2009 to 2012. We recorded mating sequences with a digital video-camera (Sony DCR-TRV 351) equipped with +4 close up lenses and used 'night shot' function. We obtained and recorded 13 complete matings of pairs from Argentinian population of a total of 45 experiments (Table 1). In pairs from the Uruguay population, 14 complete matings of a total of 45 experiments were obtained and recorded. We also obtained and recorded six inter-population matings (couples using males of Uruguayan population and females of Argentinian population) of a total of 46 experiments. Because to the difficulty in obtaining complete inter-population matings (due to morphological incompatibilities between individuals of different populations), we could not analyse couples with males of Argentina and females of Uruguay. We analysed the observations with JWatcher 1.0 program (©2000 D.T. Blumstein, C.S. Evans & J.C. Daniel). We performed all the statistical analyses with the PC program NCSS 2007 (E. Hintze, 2007). We analysed absolute and relative frequency and duration of the behavioural patterns 'Rubbing with telson' and 'Pull and Approximation' (RT and PA) with analysis of variance tests (ANOVA or Kruskal–Wallis one-way ANOVA, depending of the normality of the data) and post hoc test (Tukey–Kramer or Dunn).

2.4. Morphological analysis of male caudal glands

We measured the opening area of the glands of 10 males of Argentinian population and 10 males of Uruguay population. To compare the relative size of the gland, the dorsal area of the telson and the prosoma length of each individual were also measured (Figure 3). The measurements were taken

Table 1.
Complete, failed and null mating experiences observed in inter-populations and intra-population crosses.

Population	Mating experiment			
	Started		Null	Total
	Completed	Failed		
Argentina	11	2	29	45
Uruguay	11	3	31	45
Inter-population	1	5	40	46

Inter-population, Uruguay males and Argentina females; null, couples that did not begin courtship.

with a Nikon SMZ1500 stereomicroscope with ocular micrometer, equipped with a Nikon Sight DS-Fi1 digital camera. Digital pictures were analysed with the Image Tool 3.0 measuring software (© UTHSCSA 1996–2002). The measurements of the two populations were compared with a *t*-test or Mann–Whitney *U*-test. The normality of the data and homogeneity of variances were determined with Kurtosis Normality test and Modified Levene Equal Variance test.

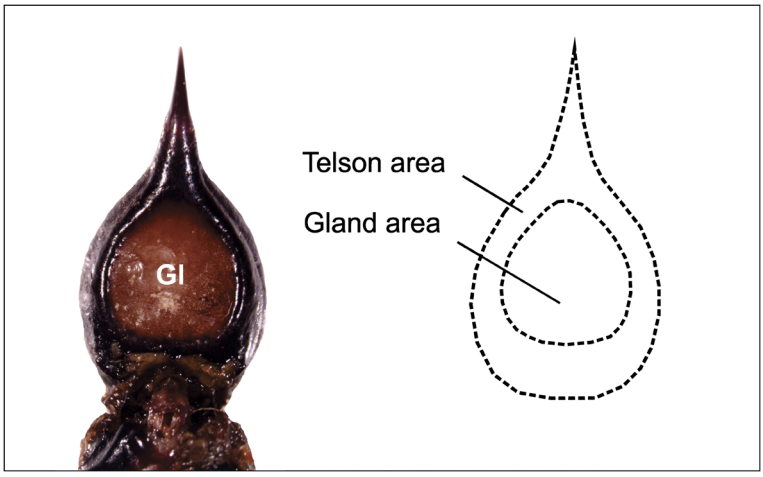

Figure 3. Measures obtained in telson and caudal glands of analysed *Bothriurus bonariensis* males of Uruguay and Argentina populations. Gl, area of the gland. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.

2.5. Chemical analysis of the glandular extract

We extracted exudates of glands from 20 males of the Argentinian population and 20 males from the Uruguayan population and we analysed them as two separate samples (one joined sample for each population, to obtain a higher concentration of the extract). The waxy gland exudates were scraped from the dorsal side of the telson and transferred to glass vials. The samples were extracted with 0.5 ml of dichloromethane in an ultrasonic bath for 10 min, and analysed by gas chromatography coupled to mass spectrometry (GC-MS), using a Shimadzu QP-2010 GC-MS. The GC was equipped with a RTX5-MS capillary column (30 m; 0.25 mm i.d.; 0.25 μm film thickness). The oven temperature was heated (10°C/min) from an initial temperature of 70 (1 min) to 150°C (1 min), and subsequently at 5°C/min to 300°C (5 min). The temperature of the injector was set at 250°C and the MS interphase at 310°C. A sample (1 μl) was injected in the splitless mode. Electron impact ionization was achieved at 70 eV, and mass spectra were collected in the scanning mode from 30 to 550 amu. Compounds were tentatively identified by matching their gas chromatographic retention times and mass spectra with the NIST mass spectra database.

3. Results

3.1. Sexual behavioural sequences

The general analysis of behavioural sequences showed that, regarding intra-population matings, in Argentinian pairs, 81% of matings (11/13) were completed (i.e., sperm transfer occurred) and in the pairs belonging to the Uruguayan population, 80% (11/14) were successful (Table 1). The main behavioural differences between individuals from different populations appeared during courtship. Males from both populations performed RT and PA behaviours in all the examined courtship sequences. However, we found some differences in these behaviours between Argentina and Uruguayan populations. Regarding RT behaviour, a higher frequency of occurrence ($F = 7.96,\ p = 0.001$) and a higher duration ($H = 14.22,\ p = 0.008$) was observed in Uruguayan compared to Argentinian pairs. Conversely, the frequency of occurrence ($H = 8.17,\ p = 0.017$) and duration of PA ($H = 7.89,\ p = 0.019$) was higher in Argentinian pairs (Figure 4).

Regarding inter-population matings, only one (7%, Table 1) was completed and sperm transfer occured. In all the inter-population experiments,

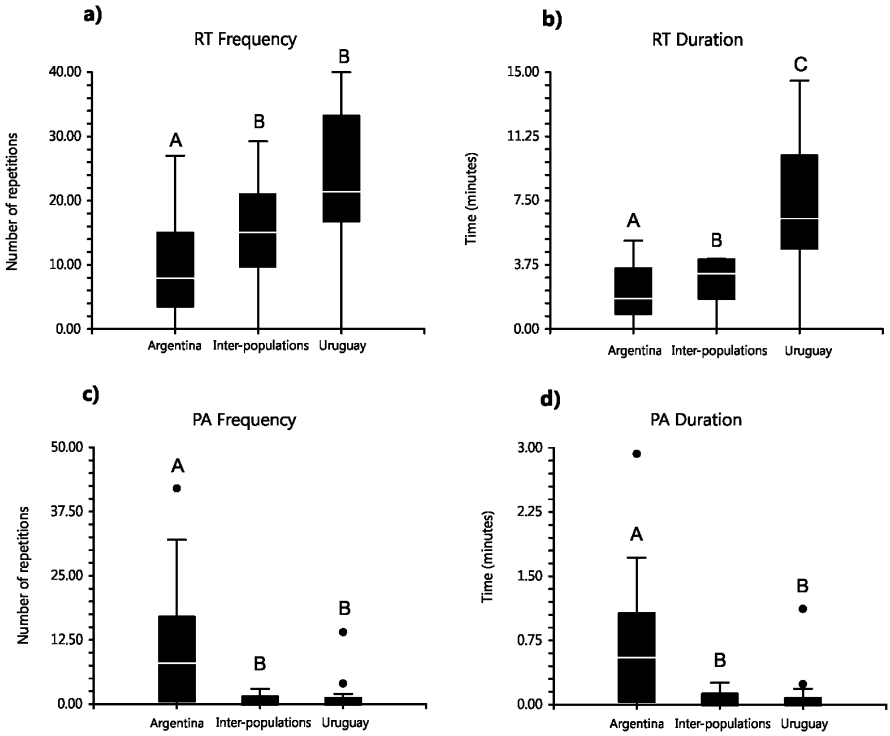

Figure 4. (a) Box plot of frequency of 'Rubbing with telson' behaviour in the two studied populations and inter-population matings. (b) Box plot of duration of 'Rubbing with telson' behaviour in the two studied populations and inter-population matings. (c) Box plot of frequency of 'Pull and approximation' behaviour in the two studied populations and inter-population matings. (d) Box plot of duration of 'Pull and approximation' behaviour in the two studied populations and inter-population matings. Letters in box plots indicate the grouping and separation of populations by Tukey–Kramer and Dunn tests.

females offered resistance to males. Interestingly, RT occurred with intermediate and significantly different duration in comparison to intra-population matings (Figure 4b).

3.2. Morphological analysis of the caudal glands

Males of the Uruguay population showed glands with higher absolute size than males of Argentinian population (Figure 5a: $t = -4.647$, $p < 0.0001$). In the same direction, body indices showed a higher relative size of glands in males of the Uruguayan population (Figure 5b: $t = -4.521$, $p < 0.0001$; Figure 5c: $Z = -4.189$, $p < 0.0001$).

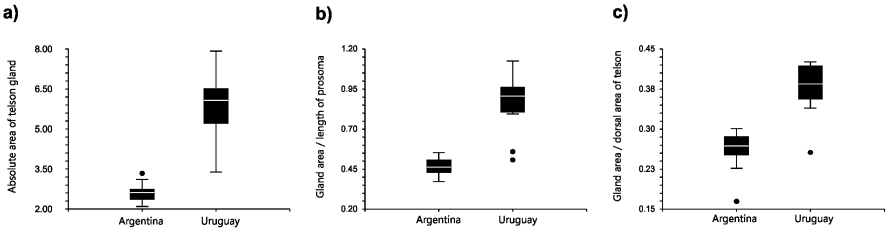

Figure 5. (a) Significant differences in the absolute area of the caudal gland (mm^2) in males of Argentina (Córdoba) and Uruguay (Uruguay) populations. (b) Significant differences between males of Argentina and Uruguay populations in the relation of the gland area (mm^2)/length of prosoma (mm). (c) Significant differences between males of Argentina and Uruguay populations in the relation of the gland area (mm^2)/dorsal area of telson (mm^2).

3.3. Chemical analysis of the glandular extract

Gland extracts from Uruguayan males showed a more complex chemistry than those from Argentinian males, with more compounds as well as a greater amount compounds shared between the two populations (Figure 6a, b). Some of the more volatile compounds could be identified on the bases of their mass spectra and retention times as n-tetradecanal, -pentadecanal (t_R: retention times 14.3 and 16.3 min, respectively) and decanoic acid (t_R: 9.8 min) (Figure 6a and Appendix). Three less volatile compounds were found exclusively in the gland extracts from the Uruguayan population (arrows, Figure 6b), and could not be identified based on their mass spectra. In general, heavier components were shared between both populations, although they occurred in larger amounts in the Uruguayan population. While these heavier components could not be identified, their mass spectra resembled those from long-chain branched and/or hydroxylated esters.

4. Discussion

The results of this work show that, even though males of *B. bonariensis* always perform RT and PA during courtship, they perform these behaviours with different frequency of occurrence and duration in two populations. The differences in RT and PA behaviours between populations may occur because males of higher quality can afford more expensive behaviours than males of lower quality (Zahavi, 1975; Nur & Hasson, 1984; Grafen, 1990; Rowe & Houle, 1996). Males of Uruguay population are larger, have more robust structures and live in environments with temperature and humidity

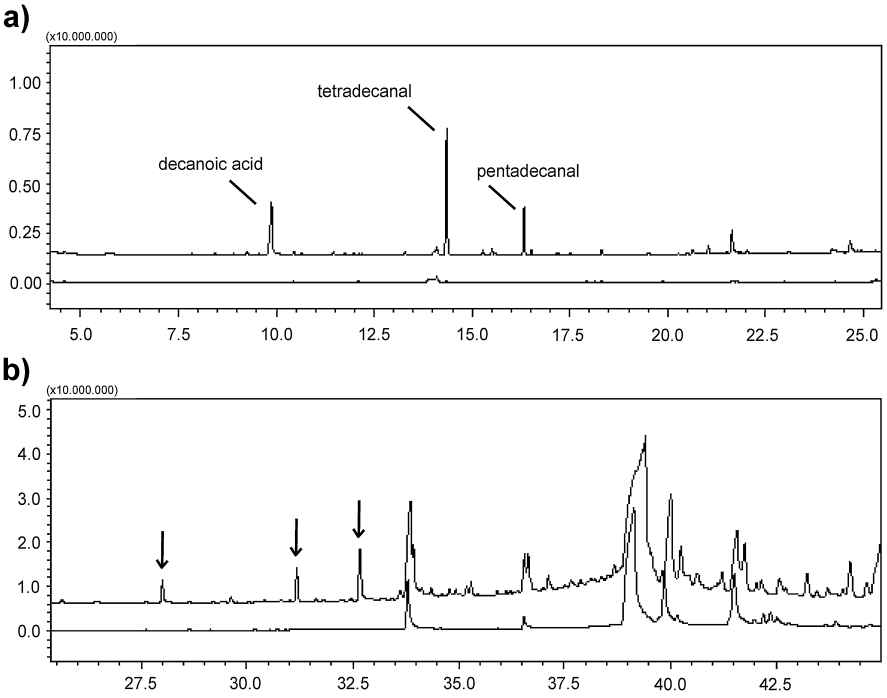

Figure 6. Chromatogram of the extracts of the caudal gland of Uruguay males (upper line) and Argentina males (bottom line). (a) From the start to 25 min; (b) from 25 min to the end.

more favourable to the group (Mattoni, 2003; Olivero et al., 2012, 2014). In addition, caudal glands of these males are larger than caudal glands of males of Argentinian population. Therefore, it is possible that they are able to allocate more energy during courtship, involving more movement and the production of more substances exuded by the caudal gland (Blaul & Ruther, 2012). The chemical signals could reflect individual's quality, such as symmetry (Thornhill, 1992; Martin & Lopez, 2000), fertility (Marco et al., 1998; Van Dongen et al., 1998), attractiveness (Schlyter & Zhang, 1996; Jones et al., 1998) and 'good genes' (Droney & Hock, 1998; Reusch et al., 2001). If the signal of quality is honest, there is a correlation between individual's quality and performance. Performance can be costly, and it will be more efficient for the animal to realize the same action in a more cheaply way (Számadó, 2011). Males from Argentina live on less favourable environments; also they are smaller and have smaller telson glands. For these features, it is possible that those males performed less frequently RT behaviour, and prob-

ably this behaviour was replaced by other stimulatory behaviour such as PA, which could represent a lower cost for males.

On the other hand, it is known that high quality females are more selective, for example because they have more discriminatory preference functions or sample more males before making a mate choice (Cotton et al., 2006). This relationship can arise because higher quality females are better able to pay the costs of preference or gain greater benefits from discrimination (Iwasa & Pomiankowski, 1994, 1999). Low quality females should suffer higher costs of mate preference than higher quality females making the same mating decision, or higher quality females may be more discerning for the same cost than poorer quality females (Cotton et al., 2006). In this regard, females of Uruguay population could have more requirements for mate choice. Probably due to these preferences of females, males of Uruguay population perform behaviours that imply higher energy expenditure with females of their population.

Regarding inter-population matings, we observed that RT behaviour had an intermediate and significantly different duration to which was observed in intra population matings. About this, males of Uruguay population (who performed RT behaviour more frequently and with greater duration with females of their own population) could decrease the performance of this behaviour because it would be unnecessary for the acceptance of Argentinian females. The variation of the behaviour in this case, could demonstrate that males of *B. bonariensis* can modulate their stimulatory sexual behaviour, in front of females that expect a different 'level' of stimulatory courtship. These results are consistent with other studies in scorpions and other animal species which show a modulated response of males in courtship display intensity, determined by female behaviour (Patricelli et al., 2002; Peretti & Carrera, 2005). Although males of *B. bonariensis* modulated their behaviour in inter-population matings, female's selectivity was very high. In addition, there are some mechanical incompatibilities between males and females of different populations due to size of the pedipalp hands (Olivero et al., 2012; Olivero, 2014). Hence, the success of these matings was low and sperm transfer did not occur in most experiences.

Correlating to the RT behaviour, as it was mentioned above, it is known that there is a relationship between the exudate of the caudal glands and the increase of the receptivity of the females during courtship. Females with aggressive tendencies showed an increase of its receptivity when they were rubbed with the caudal gland by males (Peretti, 1997). In the present work

it was observed that, in males of the Uruguayan population, chemical analysis of the glandular extracts showed that those males showed compounds that did not occur in males of the Argentinian population, such as linear saturated aldehydes and decanoic acid. These compounds are volatile and may have a signalling function over short to medium distances. Previous studies on the role of chemical signals in the courtship behaviour of scorpions suggest that they may indeed be relevant for species recognition, mate recognition and attractiveness. For example, cuticular pheromones deposited by *Hadrurus arizonensis* (Ewing, 1928) females on the soil were attractive to males, which preferred substrates previously exposed to conspecific females (Melville et al., 2003). Compounds that are not volatile at ambient temperature, also present in the caudal gland secretion extracts of *B. bonariensis*, may also bear signalling value. While they could not be identified, these compounds appear to be long-chain aliphatic esters with hydroxyl and/or alkyl substituents, and both qualitative and quantitative differences were found between the two populations included in this study. Non-volatile sex pheromones have been reported both in vertebrates and invertebrates, either deposited by one sex on the substrate (Fauvergue et al., 1995; Nakashima & Hirose, 1999; LeMaster & Mason, 2001), or sensed by antennal contact of the cuticle of a potential mate (Kim et al., 1992; Hanks et al., 1996; Wang et al., 1996; Lingafelter, 1998; Dejia et al., 1999). Contact pheromones have been reported in arachnids, for instance in the silk of spider species that use non-volatile carboxylic acids as female pheromones (Trabalon & Bagnères, 2010). Finally, potential secretion contents that cannot be analysed by gas chromatography should not be ruled out, and the use of other analytical techniques may be needed to further describe the chemical composition of the caudal gland secretion of *B. bonariensis*. To the best of our knowledge, this study is the first analysis of potential contact chemicals signals exuded by male caudal glands in scorpions.

In the case of inter-population crosses analysed here, the decrease in the RT behaviour of Uruguay males with Argentinian females could occur because these females expect a lower level of chemical stimuli to be receptive to mating. Therefore, males of Argentina have smaller glands and also generate less quantity and variety of compounds. Actually, males in the population of Argentina, as previously stated in this work, perform other behaviour (PA) that could function to replace the low frequency of RT, and possibly females of this population have a higher requirement for this behaviour. However,

although males of Uruguay population decrease RT behaviour with females of Cordoba, this was not enough to reach a successful mating.

In conclusion, in inter-population matings males of *B. bonariensis* modulated their sexual behaviour and stimulatory signals to reach the stimulatory level requested by females. However, males of Uruguay failed to achieve successful sperm transfer with females of Argentina. Mechanisms of sexual isolation between these two distant populations of *B. bonariensis* apparently seem to have evolved due to divergence in allopatry. The differences in stimulatory levels during courtship between the two populations studied here, give evidence for an early behavioural divergence promoted by sexual selection (Peretti & Carrera, 2005). In future works, it would be important to perform a comparison of sexual behaviour in other allopatric populations of the species. This would allow studying the changes in sexual behaviour of males along the distribution of the species, and thus confirming the divergence of sexual behaviour in allopatric populations.

Finally, it is known from previous works that there are other species of Bothriuridae and even species of other families in which males have caudal glands in telson or fifth metasomal segment (De La Serna de Esteban, 1977, 1978; Acosta, 1989; Peretti, 1993, 1997; González-Santillán & Prendini, 2013). Some of these species perform RT during courtship behaviour too (Peretti, 1993, 1995, 1996, 1997). However, in other species the response of females to RT sexual behaviour or the composition of the extracts of caudal glands are unknown. This work represents a first step to the analysis about RT behaviour and caudal glands in scorpions, and signifies a contribution to the knowledge of reproductive behaviour in this group.

Video 1. Recording of a mating pair of *Bothriurus bonariensis* of Uruguayan population all through 'Rubbing with telson' behaviour.

Video 2. Recording of a mating pair of *Bothriurus bonariensis* of Argentinian population during 'Pull and aproximation' behaviour.

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Appendix: Chemical caressess: geographical variation of male sexual signals in a Neotropical scorpion

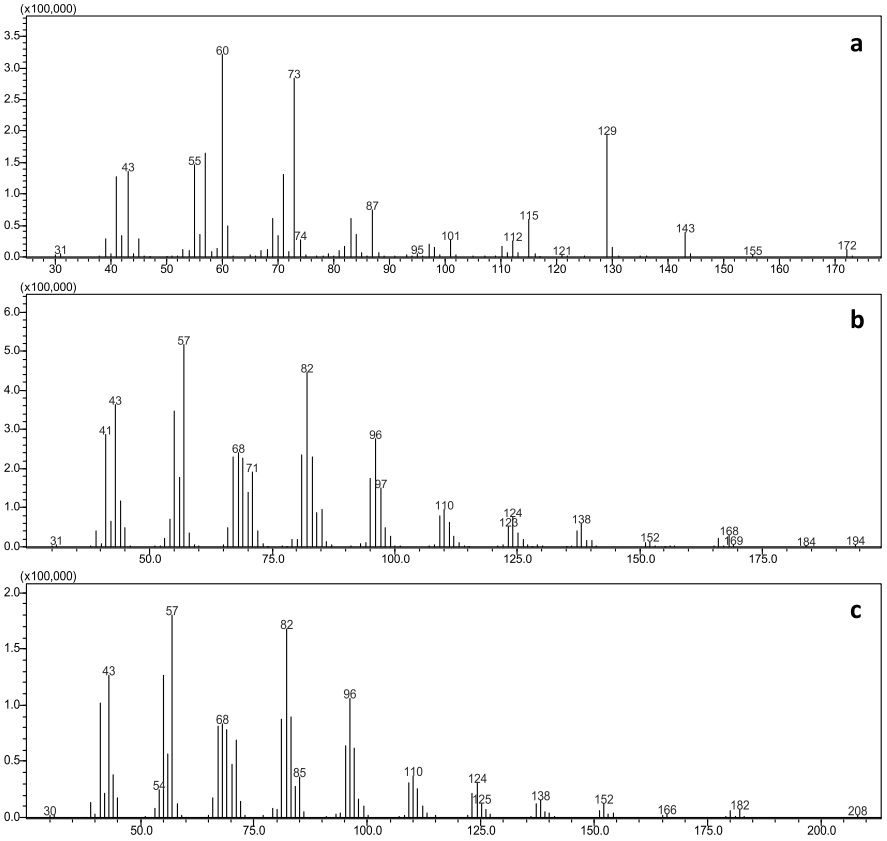

Figure A1. Electronic impact (EI) mass spectrum (MS) of decanoic acid (a), n-tetradecanal (b) and n-pentadecanal (c) in the gland exudate extracts of *B. bonariensis* (Uruguayan population). The spectra correspond to the chromatographic peaks eluting at retention times 9.8, 14.3 and 16.3 min, respectively (Fig. 6a). Similarity with the corresponding compounds in the NIST08 database: (a) 96%, (b) 96%, (c) 94%.