

# Mosaic evolution of grasping and genitalic traits in two sympatric scorpion species with reproductive interference

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## Research Article

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# Abstract

Reproductive interference (RI) can occur when two related species coexist in sympatry, involving sexual attraction, mating, and even hybridization between heterospecifics. Consequently, reproductive key characters of these species may suffer morphological shifts in sympatry to avoid the success of heterospecific sexual interactions, a phenomenon known as reproductive character displacement (RCD). RCD can be promoted by natural selection, although sexual selection pressures can act synergistically or agonistically so that phenotypic variation can respond in different directions and magnitudes to these forces. In turn, the size and shape of characters may respond differentially (mosaic evolution) to these pressures, so the analysis of multiple dimensions in traits is essential to understand the complexity of their phenotypic variability. To date, there are no studies evaluating this topic in scorpions, and two species (*Urophonius brachycentrus* and *U. achalensis*) sympatric and synchronous with RI represent an ideal model to evaluate the phenotypic variation and occurrence of RCD. In addition, the populations of these species are found in an altitudinal cline, so environmental factors may also be responsible for explaining their morphological variation. We compared the intra-specific variation, the size and shape of multiple characters involved in courtship, and sperm transfer in individuals from sympatric and allopatric populations using geometric morphometrics. We found asymmetric RCD of several sexual characters for courtship success (grasping structures) and sperm transfer (genital characters). This would evidence the action of natural selection pressures and the existence of a possible mechanism to avoid heterospecific mating success. In addition, we found a pattern of asymmetric morphological variation where one species in the sympatric zone suffered an increase in size in several characters due to environmental factors (pattern of morphological convergence). The convergence of characters combined with RI and a scramble competition mating system could intensify sexual selection pressures on specific characters, which was reflected in their high coefficients of variation. Our results suggest that in this sympatric zone, several selective regimes act differentially on various dimensions of the characters evaluated, which would support a possible mosaic evolution. This comprehensive study illuminates the complexity inherent in the evolution of multi-functional traits in a previously unexplored model, providing novel insights for evaluating traits under multiple selective pressures in animal systems experimenting RI.

## 1. Introduction

Determining the factors underlying phenotypic variation in natural populations is important for comprehending the evolution of species and their biological diversity and is a fundamental task of evolutionary biology (Coyne & Orr, 2004). Morphological characters are shaped by multiple selective pressures, especially those involved in various components of the life history of organisms. Secondary sexual characters undergo relatively fast evolutionary divergence due to sexual and natural selection (Svensson & Gosden, 2007). Natural selection favors morphological traits linked to growth, reproduction, and survival resulting in greater reproductive success for certain environments. In contrast, sexual selection underlies morphological changes that favor reproductive success through intra-sexual competition, inter-sexual mate choice, or post-copulatory processes (Kraaijeveld et al., 2011; Maan & Seehausen, 2011; Safran et al., 2013).

The study of interspecific interactions is crucial for understanding sex-linked ecological and evolutionary patterns (Cothran, 2015). Reproductive interference (henceforth referred as 'RI') is defined as any type of interspecific interaction between sympatric species associated with their mating systems caused by incomplete recognition between species (Gröning & Hochkirch, 2008; Burdfield-Steel & Shuker, 2011). This process may negatively affect the reproductive success of at least one species (Hochkirch et al., 2007). RI between species can lead to the displacement of key characters in reproductive interactions (i.e., reproductive character displacement - henceforth referred as 'RCD') (Howard, 1993), which generally results in a divergence of these characters alleviating RI and thus reinforcing reproductive isolation (Servedio & Noor, 2003; Coyne & Orr, 2004; Kyogoku, 2015). Characters of coexisting species should be more divergent in sympatry than in allopatry. The more similar the characters of interacting species are in sympatry, the greater the consequences of RI on reproductive success (Pfennig & Pfennig, 2010; Konuma & Chiba, 2012). In turn, the degree and direction of divergence of sexual characters may differ according to their function or the moment of the reproductive event in which RI occurs (Gröning & Hochkirch, 2008). In other cases, adaptive promiscuity may exist, and competition for exploitation (for females beyond their species) is propitiated, which may prevent divergence and even generate convergence of sexual characters (Grant, 1972; Grether et al., 2009; Tobias et al., 2014; Drury et al., 2015; Sobroza et al., 2021) with consequent maintenance or intensification of RI (Takakura et al., 2015; Wheatcroft, 2015; Yamaguchi & Iwasa, 2015).

In sympatric areas, intraspecific sexual selection pressures may join interspecific interactions generating a mosaic of selective pressures with different outcomes in terms of morphological variation (Grether et al., 2009). Secondary sexual characters may play a role in specific recognition, so their divergence can be explained by natural selection (Mayr, 1963; Bennet-Clark & Ewing, 1970). However, it has been postulated that mate choice and specific recognition are part of a continuum and that sexual selection may also lead to

reinforcement or RCD (Ryan & Rand, 1993; Boake et al., 1997; Liou & Price, 1994; Mendelson & Shaw, 2012). In cases where the female is the selective sex, it is hypothesized that female choice that promotes isolation will result in the divergence of male sexual characters to avoid RI or hybridization (Butlin, 1987; Gröning & Hochkirch, 2008; Hoskin & Higgie, 2010). RCD has been reported for body size (Ding et al., 2018; Sağlam et al., 2019), characters for grasping the female and genital characters (Kawano, 2002; Kameda et al., 2009; Anderson & Langerhans, 2015; Kosuda et al., 2016; Sağlam et al., 2019; Nishimura et al., 2022) or other types of characters (Marsteller et al., 2009; Kawakami & Tatsuta, 2010; Roth-Monzón et al., 2017). In many works where RCD is evaluated, one or a few characters linked to sexual reproduction are analyzed. However, phenotypic divergence can occur due to selective pressures along multiple phenotype axes simultaneously so that divergence can be multidimensional (Haines et al., 2021; White & Butlin, 2021; Vega-Sánchez et al., 2022).

Animal genitalia, especially in the male, can exhibit relatively complex morphologies and show fast and divergent evolutionary changes compared to other body parts (Tuxen, 1970; Eberhard, 1985; Leonard & Córdoba-Aguilar, 2010). Sexual selection may play a key role in the evolution of genitalia (Eberhard, 1985, 2010; Hosken & Stockley, 2004; Simmons, 2014). In turn, genital divergence can be explained by natural selection, as it contributes to reproductive isolation among species by promoting speciation (Eberhard, 1985, 2010; Masly, 2012; Wojcieszek & Simmons, 2012; House et al., 2013). Phenomena such as RCD may contribute to differences in genitalia between species in sympatric zones, whereby mechanical or interlocking incompatibilities between male and female genitalia may be frequent (Masly, 2012). The relative importance of natural and sexual selection in genitalia evolution continues under discussion (Jennions & Kelly, 2002; Eberhard, 2010; Simmons, 2014; Brennan & Prum, 2015; Eberhard & Lehmann, 2019; Sloan & Simmons, 2019), although there is evidence that multiple selective pressures may be determinant in the morphological evolution of the genitalia (Langerhans et al., 2005; Song & Wenzel, 2008; McPeck et al., 2009; Simmons et al., 2009; House et al., 2013; Simmons, 2014; Frazee & Masly, 2015). This multiplicity of selective regimes can cause what is known as "mosaic evolution", where different portions of the same structure can respond in a mixed manner to concordant or antagonistic selective pressures (due to their multi-factorial nature) and where even shape and size of the same structure can diverge differentially (House & Simmons, 2005; Song & Wenzel, 2008; Werner & Simmons, 2008).

Morphological variation in non-genital contact characters used during pre-copulatory or copulatory mating can be explained by some of the natural or sexual selection hypotheses that may generate genital morphological diversity (Robson & Richards, 1936; Eberhard 1985, 2004, 2010). These characters also possess a pattern of rapid evolutionary divergence and generally have the function of grasping or grasping the female during mating by resembling functionally genital "claspers" (Eberhard, 1985, 2010). The intra-specific phenotypic variation can be considered as the raw material on which selection acts (West-Eberhard, 2005; Eberhard, 2009), and the patterns of variation are helpful in understanding the evolution of different morphological characters. In general, sexually selected display traits show high within-species phenotypic variation (Cuervo & Møller, 1999; Eberhard et al., 1998; Eberhard, 2009). High values of coefficient of variation (CVs) indicate directional selective forces, while low values of CVs are associated with stabilizing selective pressures (Eberhard et al., 1998; Eberhard, 2009).

Phenotypic plasticity refers to the ability of organisms of a species to change their morphology, behavior, or physiology in response to environmental variation (Stearns, 1989; West-Eberhard, 2003; Whitman & Agrawal, 2009). When characters express some degree of phenotypic plasticity, environmentally based phenotypic differences among species and populations can underlie the patterns of morphological variation (Jennions & Kelly, 2002; Garnier et al., 2005; Song & Wenzel, 2008). The case of morphological divergence in environmental gradients deserves particular mention. In these cases, morphological differences between populations may be due to selective pressures for species differentiation and morphological changes linked to an environmental cline (Goldberg & Lande, 2006). Therefore, among the requirements for testing RCD, it is necessary to separate allopatric/sympatric context effects from other ecological effects. The environment can directly or indirectly influence genetic and phenotypic variation. Therefore, geographic variation among different populations is expected (Sota et al., 2000; Kosuda et al., 2016). Controlling for the effects of correlation between phenotype and environmental or geographic clines allows for finding patterns of divergence that might otherwise be undetectable (Goldberg & Lande, 2006). Variation in latitude or altitude is mainly linked to changes in temperature, an abiotic factor that affects animal growth, causing a substantial impact on the observed phenotypic variation results (Bergmann, 1847; Allen, 1877; Rensch, 1938; Atkinson, 1994).

Examples of this RI exist in many animal and plant groups (e.g., Levin, 1970; Armbruster & Herzig, 1984; Hettyey & Pearman, 2003; Dame & Petren, 2006; Gröning & Hochkirch, 2008; Matsumoto et al., 2010), and among them, arthropods have been shown to provide interesting models for studying this phenomenon (Shuker & Burdfield-Steel, 2017). Although some cases of ecological character displacement have been described in insects and arachnids, there are fewer examples of RCD in these taxa due to the difficulty of empirically evidencing this process (Waage, 1979). However, in arthropods, evidence of RCD was found in pre-copulatory characters used during courtship (Marshall & Cooley, 2000; Jang & Gerhardt, 2006; Kronforst et al., 2007; Dyer et al., 2014; Rundle & Dyer, 2015; Yukilevich, 2021) and there are also examples of RCD in genital characters (Kawano, 2002; Kawakami & Tatsuta, 2010; Kosuda et al.,

2016; Nishimura et al., 2022). In arachnids, there are some suggestions that RCD might be occurring between species in sympatry (Barth, 1990; Stratton, 1997; Agnarsson et al., 2016; Muster & Michalik, 2020), as is the case of genital characters between *Paratrechalea* spider species with RI (Costa-Schmidt & de Araújo, 2010).

The study of phenotype variation and its causes may be complicated because adaptation can be viewed as a multivariate process acting on sets of characters (Lande & Arnold, 1983; Schluter & Nychka, 1994; Blows, 2007). Organisms can be interpreted as composite objects, with characters not necessarily independent of each other that respond in complex and different magnitudes and directions to different selective pressures (Klingenberg, 2009). Geometric morphometrics (GM) helps address the inherent complexity of characters separating their size and shape to evaluate the effect of selective pressures on these two dimensions of the phenotype (Bookstein, 1998; Adams et al., 2004; Zelditch et al., 2004). Indeed, shape metrics are better descriptors of genital morphology diversity, containing more information than size measures (Slice, 2007; Shen et al., 2009). These type of studies are ideally performed in species where the function of the characters to be assessed is well-known. Arachnids have proven to be exceptional models, although morphological quantification techniques have generally been applied mainly in systematic or ecomorphological studies (Costa-Schmidt & de Araújo, 2010; Kallal et al., 2019; Santibáñez-López et al., 2021; Wilson et al., 2021; Bellvert et al., 2022). Some studies have demonstrated the usefulness of these techniques in addressing sexual dimorphism (Fernández-Montraveta et al., 2017; Kallal et al., 2019), as well as the combination with other approaches such as the analysis of phenotypic variation (by the coefficient of variation -CV) of certain characters in some arachnids (Eberhard et al., 1998; Peretti et al., 2001; Calbacho-Rosa et al., 2019; Lai et al., 2021).

Although studies applying fine morphological quantification methodologies in scorpions are scarce, these organisms appear to be excellent models for this type of analysis (Bechara & Liria, 2012; Santibáñez-López et al., 2017). It is known that different selective pressures act on specific scorpion characters (e.g., pedipalps, pectines, chelicerae) as demonstrated in studies that have evaluated their CV, allometric patterns, or where selection pressures behind dimorphic characters have been explored (Peretti et al., 2001; Carrera et al., 2009; Fox et al., 2015; Santibáñez-López et al., 2017; Visser & Geerts, 2021). Furthermore, during an elaborate courtship, both sexes displayed unique characters with functional roles such as stimulation or increased female receptivity with non-genital contact structures (e.g., the caudal gland in 'rubbing with telson', the sting in 'sexual sting') or grasping characters to overcome female resistance (e.g., apophyses in pedipalps, chelicerae) (Polis & Sissom, 1990; Carrera et al., 2009; Peretti, 2001). In particular, these characters were extensively studied in the family Bothriuridae in the evolutionary framework of sexual selection (Peretti et al., 2001; Carrera et al., 2009; Olivero et al., 2014, 2019; Peretti, 2010). Lastly, scorpions present indirect sperm transfer via a sclerotized spermatophore deposited in the substrate (Weygoldt, 1990; Proctor, 1998). This spermatophore is regenerated each time the male mates from two chitinous halves (i.e., hemispermaphores) produced in internal glandular structures called paraxial organs (Polis & Sissom, 1990). These genital characters are incredibly complex and can be divided into subunits offering interesting opportunities for studying the evolution of genitalia (Peretti et al., 2001; Peretti, 2003, 2010; Mattoni et al., 2012; Monod et al., 2017). For example, some characters follow a distinctive pattern of characters under sexual selection pressures (i.e., evolve rapidly and divergently), while others show only minor variations coinciding with what is predicted for characters under natural selection, such as structures with mechanical constraints or with key reproductive functions such as sperm passage (Peretti, 2010; Mattoni et al., 2012). The morphological diversity of sexual characters and spermatophores of scorpions responds to diverse (and not mutually exclusive) evolutionary hypotheses (Peretti, 2010). These mixed patterns result from complex synergistic or antagonistic interactions between different selective pressures (Peretti, 2010), so this genital structure could be found under mosaic evolution. This offers great possibilities for the morpho-functional study of diverse characters and contexts and allows different outcomes in a scenario of RCD.

There are several records of interspecific mating in scorpions (Auber, 1963; Matthiesen, 1968; Probst, 1972; Le Pape & Goyffon, 1975; Peretti, 1993; Peretti et al., 2000). Although many scorpions use pheromones for sex encounter, males are vagrant in scenarios of indirect competition for females, and there are records of overlapping species distributions and coexistence of species, phenomena such as RI or RCD between closely related species have not yet been extensively assessed. Here, we explored the occurrence of RCD in two closely related scorpion species of the genus *Urophonius* Pocock, 1893 (*U. brachycentrus* and *U. achalensis*, Bothriuridae) (Ojanguren-Affilastro et al., 2020) that have partially sympatric ranges with overlapping reproductive seasons and share the same habitat requirements and life-history traits (Maury, 1969; Acosta, 1985; Ojanguren-Affilastro et al., 2020). These scorpions have winter habits and adaptations for this lifestyle, which is rather peculiar among scorpions (Ojanguren-Affilastro et al., 2020; Garcia et al., 2021). These species do not possess specific recognition through chemical signals, which, together with a promiscuous mating system with scramble competition, leads to an asymmetric RI scenario with heterospecific mating (Oviedo-Diego et al., 2020, 2021). The coexistence of these species raises the question of whether there are morphological, reproductive barriers, that may hinder or prevent the culmination of heterospecific mating, given the costs they may entail in terms of gamete loss, female plugging (Oviedo-Diego et al., 2019, 2020; Romero-Lebrón et al., 2019) or potential hybridization. For these reasons, we evaluated the existence of RCD in the shape and size of somatic characters used

in courtship (non-genital contact characters) and genital characters of hemispermatothores to observe whether these metrics responded concordantly or follow a mosaic pattern under specific recognition and sexual selection pressures. Additionally, we determined the phenotypic variation by analyzing the coefficient of variation of these characters in contexts of sympatry and allopatry of both species to complement the analysis of the selective regimes that could explain the morphological variability in these species. Complementarily, we consider the influence of environmental and geographic factors on the morphological patterns found. The results from multiple lines of evidence account for the inherent complexity of sexual characters in scorpions and provide clues about the possible selective pressures behind their evolution.

## 2. Material Amd Methods

### 2.1.1 Study Species and Sampling

*Urophonius brachycentrus* has a wide geographic range distributed throughout central Argentina, while *U. achalensis* is endemic to the mountainous regions of Córdoba in central Argentina (Acosta, 1985; Ojanguren-Affilastro, 2020). The two species share partially sympatric distribution areas in the Sierras Grandes that are part of the Sierras Pampeanas Centrales (Acosta, 1985), part of a fundamental orographic system of extra-Andean mountain formations in Argentina, were formed in the Lower Paleozoic (about 300 and 350 million years ago). Adult scorpions of *U. achalensis* and *U. brachycentrus* were collected during the day during the mating season (May-August) (Acosta, 1985; Maury, 1969; Ojanguren-Affilastro et al., 2020) for three consecutive years (2018, 2019, 2020) by turning over rocks. We collected individuals in two allopatric populations of *U. brachycentrus* (31°22'42.4"S 64°35'34.0"W, 876 m.a.s.l.; 31°31'46.3"S 64°51'52.7"W, 996 m.a.s.l.), two allopatric populations of *U. achalensis* (31°35'49.1"S 64°44'49.3"W, 2030 m.a.s.l., 31°21'17.3"S 64°48'21.3"W, 1927 m.a.s.l.), and in two sympatric populations (31°23'13.5"S 64°46'10.2"W, 1796 m.a.s.l.; 31°34'07.6"S 64°42'43.8"W, 1610 m.a.s.l.).

### 2.1.2 Processing of individuals and selected characters

Individuals from field collections were identified and sexed (Ojanguren-Affilastro, 2005) with a Nikon SMZ 1500 stereo zoom microscope and preserved in 80% EtOH in glass containers for morphological studies. Classical and geometric morphometric studies were carried out, and measurements of characters were compared between sexes and study species in different contexts (sympatry vs. allopatry) (n = 25 per population context and per sex of each species) (Table 1, Fig. 1). We selected characters under both natural and sexual selection pressures, used during feeding, defense, and courtship traits such as pedipalps, chelicerae and telson vesicle (Table 1, Fig. 1). Also, we analyzed characters used only in a sexual context, such as characters for female stimulation (caudal gland) or characters for grasping the female pedipalps during courtship (pedipalp apophyses) (Table 1, Fig. 1). Finally, we measured genital characters involved in sperm transfer that has also been shown to be under sexual selection pressures (Olivero et al., 2015; Peretti, 2010) (Table 1, Fig. 1). To analyze the selected characters, individuals were dissected, and internal structures were extracted with fine tweezers for photographic treatment. The individuals were measured using images taken under the stereo zoom microscope with a digital coupled camera (Nikon Digital Sight DS-F11-U2). Because the internal female genitalia consist of flexible structures that vary in size and shape according to the female reproductive status (Peretti, 2010), morphometric analysis was not performed. In subsequent analyses, individuals and characters with damaged or incomplete portions were not considered.

Table 1

Morphological characters selected in *Urophonius* species analyzed. The type of character (somatic or genital), the corresponding sex, the functional role, and the measurement technique used are indicated. Abbreviations: AL, absolute length; RL, relative length; NS, Natural selection; SS, Sexual selection. See landmark positions in Fig. 1 and descriptions in Table S1.

Morphological character	Sex and type of character		n	Methodology	Functional role
Prosome	Somatic in both sexes		n = 122	Geometric morphometry (Landmarks = 8)	Body size indicator (Polis & Sissom 1990; McLean et al., 2018).
			n = 112		
Chelicera			n = 113	Classic morphometry (AL, RL)	Character used during feeding and courtship where the pair touch and rub chelicerae during 'chelicera massage' or 'kiss' (under NS and SS pressures) (Carrera et al., 2009).
			n = 114		
Pectine			n = 126	Classic morphometry (AL, RL)	Character used for mechano-chemical-sensory recognition, foraging, mate searching and spermatophore deposition site in courtship (under NS and SS -slight- pressures) (Polis & Sissom, 1990; Peretti et al., 2001)
			n = 100		
Pedipalp	Grasping characters		n = 128	Geometric morphometry (Landmarks = 5 + Semilandmarks = 21)	Character used during defense, feeding and grasping of the other sex during courtship (under NS and SS pressures) (Polis & Sissom 1990; Peretti et al., 2001; Olivero et al., 2014).
			n = 121	Geometric morphometry (Landmarks = 4 + Semilandmarks = 21)	
Pedipalp apophysis	Somatic in males		n = 122	Geometric morphometry (Landmarks = 5 + Semilandmarks = 16)	Character used for the correct grasping and locking of pedipalps during courtship (only under SS pressure) (Peretti et al., 2001).
Telson vesicle	Somatic in both sexes		n = 122	Geometric morphometry (EFA = 8 harmonic)	Character used during feeding and agonistic interactions, during courtship in sexual stinging of the female and gland rubbing (under NS and SS pressures) (Polis & Sissom, 1990; Peretti, 1993; Fox et al., 2015; Sentenská et al., 2017; Olivero et al., 2017, 2019).
			n = 122		Character used during feeding and agonistic interactions, sometimes during courtship movements indicative of receptivity (under NS and SS pressures) (Polis & Sissom 1990; Fox et al., 2015).
Caudal gland	Somatic in males	Stimulation character	n = 122	Geometric morphometry (EFA = 7 harmonic)	External secretory gland on the dorsal side of the telson used during courtship where the male rubs the female to increase female receptivity (under SS pressures) (De la Serna de Esteban, 1978; Peretti, 1997; Olivero et al., 2017, 2019).
Hemispermatothore lamella	Genital in male	Genital character	n = 117	Geometric morphometry (Landmarks = 4 + Semilandmarks = 24)	Genital character that will form the spermatophore involved in the copulatory mechanics for indirect sperm transfer, acting as a lever for sperm release (under NS and SS pressures) (Peretti et al., 2001).

Morphological character	Sex and type of character	n	Methodology	Functional role
Hemispermatoophore capsular lobe		n = 108	Geometric morphometry (EFA = 6 harmonic)	Genital character that will form the copulatory cone of the spermatophore that enters and evert inside the female genitalia, guiding the sperm during sperm transfer (under NS and SS pressures) (Peretti et al., 2001; Olivero et al., 2014).

Table 2

Coefficients of variation (CVs) of multiple somatic and genital characters of male and female *Urophonius achalensis* and *U. brachycentrus* scorpions from sympatric and allopatric areas. Morphological character, sex, CVs value and statistical significance value (between species and contexts) are indicated (p-values < 0.05 indicated in bold). : males, : females. Letters indicate significant differences between character CVs (p-values < 0.05)

Morphological character	Species	<i>U. achalensis</i>		<i>U. brachycentrus</i>		Differences between spp.	Differences between contexts		
		Sex/Context	sympatry	allopatry	sympatry		allopatry	<i>U. achalensis</i>	<i>U. brachycentrus</i>
Chelicerae length	♂		5.525 <sup>b</sup>	5.098 <sup>b</sup>	6.266 <sup>b</sup>	5.037 <sup>b</sup>	0.605	0.735	0.379
	♀		4.220 <sup>b</sup>	5.605 <sup>b</sup>	5.09 <sup>b</sup>	5.831 <sup>b</sup>	0.134	0.169	0.354
Pecten length	♂		7.263 <sup>b</sup>	6.141 <sup>b</sup>	6.824 <sup>b</sup>	6.955 <sup>b</sup>	0.150	0.195	0.251
	♀		5.363 <sup>b</sup>	6.962 <sup>b</sup>	6.984 <sup>b</sup>	6.872 <sup>b</sup>	0.323	0.241	0.092
Pedipalp length	♂		4.591 <sup>b</sup>	3.248 <sup>b</sup>	5.092 <sup>b</sup>	6.155 <sup>b</sup>	0.025	0.119	0.324
	♀		3.581 <sup>b</sup>	4.439 <sup>b</sup>	5.416 <sup>b</sup>	5.643 <sup>b</sup>	0.010	0.274	0.506
Pedipalp apophysis length	♂		10.741 <sup>a</sup>	11.267 <sup>a</sup>	12.329 <sup>a</sup>	16.227 <sup>a</sup>	0.018	0.301	0.191
Telson vesicle length	♂		3.472 <sup>b</sup>	4.832 <sup>b</sup>	4.223 <sup>b</sup>	4.785 <sup>b</sup>	0.444	0.145	0.585
	♀		4.471 <sup>b</sup>	5.315 <sup>b</sup>	5.413 <sup>b</sup>	6.331 <sup>b</sup>	0.215	0.371	0.169
Caudal gland length	♂		10.359 <sup>a</sup>	12.653 <sup>a</sup>	16.111 <sup>a</sup>	13.020 <sup>a</sup>	0.249	0.320	0.307
Hemispermatoophore lamella length	♂		3.982 <sup>b</sup>	5.111 <sup>b</sup>	4.756 <sup>b</sup>	5.245 <sup>b</sup>	0.484	0.306	0.692
Hemispermatoophore capsular lobe length	♂		4.447 <sup>b</sup>	5.925 <sup>b</sup>	4.951 <sup>b</sup>	5.451 <sup>b</sup>	0.677	0.285	0.730
Hemispermatoophore frontal crest length	♂		8.466 <sup>ab</sup>	8.539 <sup>ab</sup>	7.749 <sup>ab</sup>	7.842 <sup>ab</sup>	0.786	0.970	0.547

## 2.1.3 Morphometric studies

### 2.1.3.1 Classic morphometric and coefficient of variation analysis

The chelicerae and the pectines were analyzed by linear measurements (due to methodological difficulties in applying geometrical morphometry) by analyzing absolute and relative lengths (prosome length as body size index - McLean et al., 2018) (Table 1). These measurements were taken from photographs obtained for each character with ImageJ software tools (Schneider et al., 2012). Measurements were taken three times by the same person, and the measurement error was calculated (Sokal & Rohlf, 1995).

The coefficient of variation (CV) is widely used as indirect evidence to know the selective pressures that might be operating on morphological characters (Pomiankowski & Møller, 1995; Eberhard et al., 1998; Peretti et al., 2001). We compared this parameter across different types of characters in males and females from both contexts (sympatry *versus* allopatry). We used the modified formula:  $CV' = [(sd_y/mean_y) * (1 - r^2)^{1/2} * 100]$ , where  $sd_y$  is the standard deviation of the character,  $mean_y$  is the arithmetic mean of the character,  $r^2$  is the determination coefficient between the character and a measure of body size (prosome length) and \* indicates multiplication symbol

(Eberhard et al., 1998; Calbacho-Rosa et al., 2019). CVs were statistically compared using the 'asymptotic\_test' function of the *cvequality* package (Marwick & Krishnamoorthy, 2019).

### 2.1.3.2 Geometric morphometric analysis

We took digital images of selected characters in male and female scorpions with a scale close to the character, and the images were assembled with TPSutil software (Rohlf, 2015). Sets of anatomical Landmarks (Bookstein, 1991) and semilandmarks were established using TPSDig2 (Rohlf, 2004; Bookstein, 1997; Gunz & Mitteroecker, 2013). We used landmarks in the prosome, the hemispermatophore lamella, the pedipalp, and the apophysis of this structure (Table 1, Fig. 1, Table S1). Sliding landmarks or semilandmarks were used to enhance geometric information about curvatures between adjacent landmarks in the pedipalp, the pedipalp apophysis, and the hemispermatophore lamella (Fig. 1). In other characters (hemispermatophore capsular lobe, telson vesicle and caudal gland) we quantified shape using an elliptic Fourier analysis (EFA) (following Santibáñez-López et al., 2017, 2021) that allowed us to explore small differences in defined shapes from contour characterization (Kuhl & Giardina, 1982; Ferson et al., 1985; Hammer & Harper, 2006) (Fig. 1).

The shape coordinates of each character were subjected to a Generalized Procrustes Analysis (Gower, 1975) with the 'gpagen' function of the *geomorph* package (Schlager, 2017; Adams et al., 2017) in R software (R Core Team, 2021) to remove non-shape variables (translation, rotation, size) from the dataset to compare shape by contrasting with a mean generated from a consensus matrix (Rohlf & Slice, 1990; Adams et al., 2017). The size proxy of each character was retained from the GPA analysis (i.e., Centroid size) for subsequent analyses (Bookstein, 1991; Zelditch et al., 2004). To account for semilandmarks in the GPA calculation, we used the 'slider2d' function of the *Morpho* package (Schlager et al., 2021). EFA was performed using the *momocs* package (Iwata & Ukai, 2002; Bonhomme et al., 2014).

A Principal Component Analysis (PCA) was performed to visualize and explore the general trends of the distribution of total morphological variation in morphospace from both the data yielded by the GPA as well as the data obtained from the EFA using the 'plotTangentSpace' function of the *geomorph* package. Principal components can be considered as reorganized and uncorrelated morphological features representing different aspects of the total shape variation. Additionally, vectors that reflected shape variation along x/y axes were used to visualize magnitudes and overall shape changes with the *geomorph* package (Bookstein, 1991). Multivariate analysis of variance (MANOVA) was performed with the function 'procD.lm' of the *geomorph* package with resampling permutations procedure to calculate the significance of shape variables. The variation in shape of the first two principal components (since they captured more than 70% of the morphological variation) was analyzed in detail. First, we checked the allometric component (influence of size on shape) of the characters with the functions 'procD.lm' and 'plotAllometry' of the *geomorph* package. If we found allometry in the sample, we calculated residual values of the shape variables for subsequent analyses (Outomuro & Johansson, 2017).

### 2.1.3.3 Statistical analysis

Measurements obtained by classical and geometric morphometry were compared between species and contexts (sympatry *versus* allopatry) with linear mixed models (LMMs) in R. Separate models were performed for each character and sex (because in some characters the number of Landmarks was not equal for males and females) where we set as response variables the linear measurements, size variables (centroid size) or shape variables (PCs scores) and the fixed effects were species (levels: *U. achalensis* / *U. brachycentrus*) and contexts (levels: sympatry / allopatry). The interaction between these explanatory variables was evaluated to corroborate RCD patterns. We added populations of origin as random effects to account for the variability contributed to this factor. Due to the influence of altitude on morphological variability, we added the altitude where individuals were collected as another random effect. Analyses were performed with the package *lme4* (Bates et al., 2011) and *lsmmeans* (Lenth, 2016) for a posteriori test (with Bonferroni correction) if necessary. Model validation was assessed graphically and by residual analysis.

### 2.1.4 Influence of environmental factors on morphological characters

Complementarily, in a subset of data, we explored whether environmental factors might correlate with any of the phenotypic characters measured; because, for example, the clinal or geographic variation present in our study system may be influencing the patterns found (Goldberg & Lande, 2006). As altitude may be strongly associated with temperature and humidity, we considered the variation of these environmental factors in our analysis by obtaining the mean annual temperature and mean annual rainfall rasters from Geoportal IDESA (<http://geoportal.idesa.gob.ar/>, data from last year available: 2017). With the QGIS program 3.26 (QGIS Development Team, 2020), we mapped the distribution of the collected individuals (using the geo-referenced latitude and longitude data for each individual). We used the 'extractRandomClim' function of the *raster* package (Hijmans et al., 2015) in R to extract the mean annual temperature and mean annual rainfall values for each collection point. Subsequently, we explored the relationships between these environmental factors with size (centroid size, absolute length) and shape (PCs scores) previously calculated (see 2.1.3.2) with linear mixed models (LMMs). We



acknowledge that other environmental factors (e.g., soil characteristics, atmospheric pressure, food availability) may sustain some of the phenotypic variation among species and populations. Still, the scoring of these factors was beyond the scope of this study, so our estimates of environmental effects on phenotype are prospective.

### 3. Results

#### 3.1.1 Morphological variation across contexts

We compared multiple sexual characters involved in courtship and sperm transfer in males' and females' scorpions from sympatric and allopatric contexts. We observed different patterns of phenotypic variation in different directions (convergences and divergences) in each species (Fig. 2), and the shape and size appear to respond independently to different selective pressures. The morphometric results for each character analyzed in both sexes are detailed below, first evaluating the size and then the variation in shape.

##### 3.1.1.1 Chelicerae and pecten: asymmetric convergence in size only in females

We observed an asymmetric convergence in the absolute length of both chelicerae ( $\chi^2 = 34.180$ ,  $p < 0.001$ ) and pectines ( $\chi^2 = 45.894$ ,  $p < 0.001$ ) in females (*U. brachycentrus* more similar to *U. achalensis* in sympatry) (Fig. 2). Neither contexts nor species showed differences in the relative lengths of chelicerae or pectines. We only found interspecific differences in the relative cheliceral length in males, with *U. brachycentrus* males having larger chelicerae ( $\chi^2 = 64.348$ ,  $p < 0.001$ ). However, all the other variables did not differ between species or contexts.

##### 3.1.1.2 Prosome and telson vesicle: size convergence

Centroid size of the prosome showed symmetric convergence in females of both scorpion species, with species becoming more similar in sympatry than in allopatry ( $\chi^2 = 26.907$ ,  $p < 0.001$ ) and asymmetric convergence in males (*U. brachycentrus* more similar in sympatry than in allopatry) ( $\chi^2 = 8.507$ ,  $p = 0.004$ ) (Fig. 2). In terms of shape, the Procrustes MANOVA showed no significant variation according to species and context. PC1 comprised almost half of the morphological variation (Females: 46.49%, Males: 45.85%), showing interspecific differences (*U. brachycentrus* more compressed prosome than *U. achalensis*) (Females:  $\chi^2 = 31.992$ ,  $p < 0.001$ ; Males:  $\chi^2 = 19.895$ ,  $p < 0.001$ ) (Fig. 2). PC2 explained an 18.44% of the variation in females and 13.82% in males and showed no differences between species or contexts in either sex. PC3 accounted for the 13.37% of the variability in females without differences between species or contexts. In contrast, PC3 in males representing the 12.52% of morphological variability was different between species ( $\chi^2 = 9.783$ ,  $p = 0.002$ ) and contexts ( $\chi^2 = 6.827$ ,  $p = 0.006$ ) but we found no significant interaction between these factors.

Regarding the telson vesicle, in females, we found a pattern of symmetric convergence in the centroid size with both species becoming more similar in sympatry than in allopatry ( $\chi^2 = 32.176$ ,  $p < 0.001$ ) (Fig. 2). In males the convergence was asymmetric, as only males of *U. brachycentrus* presented a shift in the size of this character towards sympatry ( $\chi^2 = 6.118$ ,  $p = 0.013$ ). The Procrustes MANOVA showed significant shape variation according to species in both sexes (Females:  $F = 4.269$ ,  $p = 0.001$ ; Males:  $F = 4.404$ ,  $p = 0.001$ ), but the interaction between species and context was not significant (Fig. 2). In females, we found significant differences between species in telson vesicle shape reflected in PC1 (54%) (Females:  $\chi^2 = 22.441$ ,  $p < 0.001$ ) and PC2 (19.57%) (Females:  $\chi^2 = 21.034$ ,  $p < 0.001$ ). Also, in males, PC1 (67.48%) showed differences between species ( $\chi^2 = 36.965$ ,  $p < 0.001$ ) (Fig. 2), while in PC2 (12.21%) there were no significant differences between species or contexts.

##### 3.1.1.3 Pedipalp in females: asymmetric convergence in size and divergence in shape

We found asymmetric convergence in pedipalp centroid size, with species more similar in sympatry than in allopatry due to a shift of *U. brachycentrus* ( $\chi^2 = 19.812$ ,  $p < 0.001$ ) (Fig. 2, 3A). In terms of shape, the Procrustes MANOVA showed significant variation according to species and context ( $F = 7.788$ ,  $p = 0.001$ ). PC1 explained 38.10% of morphological variability, and we found asymmetric divergence in PC1, with *U. brachycentrus* females showing a shift relative to sympatric *U. achalensis* females and allopatric females ( $\chi^2 = 8.294$ ,  $p = 0.004$ ) (Fig. 3B). PC2 explained 26.95% and PC3 10.60% of morphological variation although these shape variables showed no significant differences between species or contexts.

##### 3.1.1.4 Pedipalp and apophysis in males: asymmetric divergence in shape

Male pedipalp size showed only interspecific differences, with larger pedipalp and apophysis in *U. achalensis* than *U. brachycentrus* ( $\chi^2 = 84.839$ ,  $p < 0.001$ ) (Fig. 2, 3A). The Procrustes MANOVA showed significant variation by species and context ( $F = 3.321$ ,  $p = 0.006$ ). Regarding the pedipalp, the PC1 explained 45.25% of the morphological variability, and we found a pattern of asymmetric divergence in PC1 (*U. brachycentrus* males with higher pedipalp and shorter fixed fingers than allopatric males and sympatric *U. achalensis* males) ( $\chi^2 = 10.069$ ,  $p = 0.002$ ) (Fig. 3B, D-E). PC2 accounted for 20.21% and PC3 a 9.99% of the variability, and this component showed no differences between species or contexts (Fig. 3D).

For the pedipalp apophysis size, we found interspecific differences ( $\chi^2 = 38.651$ ,  $p < 0.001$ ), with apophysis of *U. achalensis* being larger than those of *U. brachycentrus* (Fig. 2, 3C). The Procrustes MANOVA showed significant variation in the interaction between species and context ( $F = 3.419$ ,  $p = 0.014$ ). PC1 (accounting for 31.11% of the variation) showed no significant differences between species or contexts. In contrast, PC2 explaining 21.07% of the morphological variation, showed significant differences between species in sympatry, and not in allopatry ( $\chi^2 = 10.221$ ,  $p = 0.002$ ) (Fig. 3C, E). Moreover, the shape of the apophysis was different between sympatric and allopatric populations of *U. brachycentrus* so that this displacement pattern would be an asymmetric divergence. Morphological variability was also distributed between PC3 (9.34%) and PC4 (8.56%), although these morphological variables did not vary between contexts and only between species in PC4 ( $\chi^2 = 8.685$ ,  $p = 0.003$ ).

### **3.1.1.5 Caudal gland: asymmetrical convergence in size**

Caudal gland size showed a pattern of asymmetric convergence, with *U. brachycentrus* males more similar to *U. achalensis* males in sympatry and differing significantly from allopatric population males (with smaller gland) ( $\chi^2 = 10.087$ ,  $p = 0.002$ ) (Fig. 2). The Procrustes MANOVA showed significant variation only according to species ( $F = 155.064$ ,  $p < 0.001$ ), but the interaction between species and context was not significant. Regarding shape, PC1 almost completely comprised all morphological variability (92.81%), and we only found significant interspecific differences (*U. brachycentrus* showing a more compressed and wider caudal gland than *U. achalensis*) ( $\chi^2 = 155.774$ ,  $p < 0.001$ ). PC2, with an explanation of only 2.86% of the morphological variation, did not differ between species or contexts.

### **3.1.1.6 Hemispermatothore lamella: asymmetrical divergence in shape**

Hemispermatothore lamella size varied only at the interspecific level ( $\chi^2 = 86.714$ ,  $p < 0.001$ ), with lamella of *U. achalensis* males always being larger than those of *U. brachycentrus* (Fig. 2, 4A). In terms of shape, the Procrustes MANOVA showed significant variation according to species and context ( $F = 3.223$ ,  $p = 0.006$ ). Almost half of the lamella morphological variation was represented by PC1 (43.41%) (Fig. 4B-C). This shape showed asymmetric divergence, as *U. brachycentrus* males differed from their allopatric conspecifics with a wider lamella, also differing from sympatric *U. achalensis* males ( $\chi^2 = 6.791$ ,  $p = 0.009$ ) (Fig. 4C-D). PC2 comprised 15.33% and the PC3 14.02% of the morphological variation but these shape variables showed no differences between species or contexts (Fig. 4C).

### **3.1.1.7 Hemispermatothore capsular lobes: asymmetrical divergence in size**

We found a pattern of asymmetric divergence in the hemispermatothore capsular lobe size, with males of *U. brachycentrus* in sympatry having larger lobes than the rest of the male groups ( $\chi^2 = 12.784$ ,  $p < 0.001$ ) (Fig. 2). We found no significant interaction between species and context in the Procrustes MANOVA, but there was variation in shape according to species ( $F = 4.847$ ,  $p = 0.001$ ). PC1 explained 31.96% and PC3 16.19% of the morphological variance, and none of the shape variables resulted in different between species or contexts. PC2 accounted for the 25.52% and differed between contexts ( $\chi^2 = 3.926$ ,  $p = 0.048$ ) and marginally between species ( $\chi^2 = 3.319$ ,  $p = 0.068$ ), but the interaction between context and species was not significant.

## **3.1.2 Coefficients of variation of morphological characters**

We found different values of CVs according to the type of character analyzed and sex (Table 3). The chelicerae, the pecten, the pedipalp, and the telson vesicle showed relatively low CVs values in both sexes and species, with no statistical differences in CVs between these characters, between species or between contexts. Only the pedipalp' CVs differ between species, higher in *U. brachycentrus* than in *U. achalensis* in both sexes. In contrast, other male characters used exclusively during sexual interactions, such as the caudal gland and the pedipalp apophysis, showed high CVs, significantly different from the previously mentioned characters. In the case of the pedipalp apophysis for grasping during courtship, we found higher variation values in *U. brachycentrus* than in *U. achalensis*. Genital characters such as the length of the hemispermatothore lamella or the hemispermatothore capsular lobe showed low values of CVs with no differences between species or contexts. The only exception was the frontal crest of the hemispermatothore, which showed high CVs values compared to other genital characters in both species.

Table 3

Influence of environmental factors on multiple somatic and genital characters of male and female *Urophonius achalensis* and *U. brachycentrus* scorpions from sympatric and allopatric areas. Character and compared parameter, sex, statistic value and statistical significance value are indicated (values < 0.05 indicated in bold). Abbreviations: AL, absolute length; cs, centroid size; hum, humidity (rainfall); hum:sp, interaction term between humidity and species fixed effect; PC, principal component 1–2; temp, temperature fixed effect; temp:sp, interaction between temperature and species fixed effects, ♂ : males, ♀ : females

Morphological character	Sex	Fixed effect	F	p-value	Sex	Fixed effect	F	p-value
Prosome	cs	♀	temp:sp	12.102	♂	temp:sp	68.449	<b>&lt; 0.005</b>
	PC1	♀	temp	0.053	♂	temp	3.324	0.072
	PC2	♀	temp	0.123	♂	temp	0.589	0.445
	PC3	♀	temp	0.826	♂	temp	0.165	0.686
	cs	♀	hum	0.207	♂	hum	5.424	<b>0.022</b>
	PC1	♀	hum	0.002	♂	hum	0.021	0.885
	PC2	♀	hum	0.977	♂	hum	3.929	0.051
	PC3	♀	hum	2.437	♂	hum	0.231	0.632
Pedipalp	cs	♀	temp:sp	5.129	♂	temp:sp	8.876	<b>0.004</b>
	PC1	♀	temp	1.58	♂	temp	2.715	0.103
	PC2	♀	temp	1.885	♂	temp	0.205	0.652
	PC3	♀	temp	0.004	♂	temp	0.015	0.904
	cs	♀	hum	0.416	♂	hum	1.505	0.223
	PC1	♀	hum	0.081	♂	hum	0.069	0.793
	PC2	♀	hum	2.802	♂	hum	0.987	0.323
	PC3	♀	hum	3.629	♂	hum	0.818	0.365
Chelicerae	AL	♀	temp:sp	12.904	♂	temp:sp	15.457	<b>0.0002</b>
	AL	♀	hum	0.001	♂	hum	0.001	0.996
Pecten	AL	♀	temp:sp	7.361	♂	temp:sp	21.884	<b>&lt; 0.005</b>
	AL	♀	hum	0.421	♂	hum	0.037	0.848
Telson vesicle	cs	♀	temp:sp	4.957	♂	temp:sp	8.371	<b>0.005</b>
	PC1	♀	temp	0.134	♂	temp	1.783	0.185
	PC2	♀	temp	2.787	♂	temp	0.897	0.348
	cs	♀	hum	0.264	♂	hum	2.614	0.109
	PC1	♀	hum	0.017	♂	hum	0.476	0.492
	PC2	♀	hum	2.159	♂	hum	0.753	0.389
Pedipalp apophysis	cs	♀	temp	0.197				0.659
	PC1	♀	temp	0.325				0.570
	PC2	♀	temp	1.026				0.314
	PC3	♀	temp	0.136				0.713
	PC4	♀	temp	0.812				0.373
	cs	♀	hum	0.019				0.888
	PC1	♀	hum	2.748				0.101

Morphological character		Sex	Fixed effect	F	p-value	Sex	Fixed effect	F	p-value
	PC2	♀	hum	1.796	0.184				
	PC3	♀	hum	0.188	0.666				
	PC4	♀	hum	1.107	0.298				
Caudal gland	cs	♀	temp:sp	8.485	<b>0.003</b>				
	PC1	♀	temp	0.329	0.569				
	PC2	♀	temp	2.068	0.154				
	cs	♀	hum	0.447	0.504				
	PC1	♀	hum:sp	5.400	<b>0.023</b>				
	PC2	♀	temp	0.764	0.385				
Hemispermatothore Lamella	cs	♀	temp:sp	13.602	<b>0.0004</b>				
	PC1	♀	temp	2.648	0.108				
	PC2	♀	temp	3.392	0.073				
	PC3	♀	temp	2.144	0.147				
	cs	♀	hum	1.934	0.168				
	PC1	♀	hum	0.015	0.902				
	PC2	♀	hum	0.929	0.341				
	PC3	♀	hum	0.159	0.691				
Hemispermatothore capsular lobe	cs	♀	temp:sp	4.152	<b>0.046</b>				
	PC1	♀	temp	2.526	0.117				
	PC2	♀	temp	0.005	0.945				
	PC3	♀	temp	1.642	0.205				
	cs	♀	hum	0.725	0.398				
	PC1	♀	hum	0.112	0.739				
	PC2	♀	hum	3.025	0.087				
	PC3	♀	hum	0.087	0.769				

### 3.1.3 Influence of environmental factors on morphological characters

We found that the size (centroid size and absolute length) of almost all the characters analyzed varied with temperature (Table 3). We found a significant statistical interaction between temperature and species in all cases, so temperature-dependent morphological variations were observed only in *U. brachycentrus*, with no relationship in *U. achalensis*. Generally, both sexes of this species had larger characters in colder areas (at higher altitudes) and smaller characters in warmer areas (at lower altitudes). This was observed for both sexes' prosome, pedipalp, chelicerae, pecten, and telson vesicle. In males, we also found this same pattern of variation in *U. brachycentrus* for the caudal gland and genital characters, although we did not observe it in the pedipalp apophysis. The pattern of variation found in the size of many characters coincides with the convergence asymmetrical in *U. brachycentrus*. The shape of none of the analyzed structures showed variation with temperature.

As for humidity (rainfall), we found patterns of morphological variation of some characters regarding this environmental factor (Table 3). We observed that females of both species presented a larger prosoma in more humid areas. In addition, we found an interaction between humidity and species for caudal gland shape (PC1). That is, in *U. brachycentrus*, males presented a gland with negative PC1 values in more humid areas. This morphological change is associated with more slender and less rounded gland. The shape of no other character was affected by humidity.

## 4. Discussion

We found great morphological variability between sympatric and allopatric contexts in the studied model species of scorpions. Our study revealed main novel insights about the evolution of shape and size of somatic and genital characters in an animal model so far understudied but with great potential for further research. We were able to observe complex patterns of phenotypic variation in different directions (convergences and divergences) in size and shape, which allows us to suggest a possible mosaic evolution in certain sexual characters in these scorpion species. The integration of the results allows us to infer an asymmetric RCD in the shape of certain sexual characters of both sexes key for courtship success (i.e., grasping characters) and sperm transfer (i.e., genital characters of the hemispermatophore). Intriguingly, although we found low phenotypic variation in some genital characters, others showed high variation which could reflect that some characters are under antagonistic selective pressures. The convergence patterns found in the size of many characters were due to environmental fluctuations linked to the altitude cline of the geographic system. In the following discussion, we analyze in depth the remarkable patterns of phenotypic variation, the possible selection pressures underlying this variability, and the consequences of the RCD in the mating system and coexistence for these scorpion species.

### 4.1 Reproductive character displacement in pedipalps

We obtained evidence of phenotypic divergence in shape and size of multiple somatic characters used in courtship in *U. brachycentrus*, while *U. achalensis* showed no divergence in any character between sympatric and allopatric populations. *Urophonius brachycentrus* males of the sympatric zone differed from their conspecifics and *U. achalensis* males by having more globose pedipalps and apophyses with a lower crest deeper. *U. brachycentrus* females showed an RCD pattern also in the shape of their pedipalps, with the pedipalps being more globose in sympatric zones. Therefore, the pattern of divergence in pedipalp shape was complementary in males and females. RCD results in mechanical incompatibilities (due to mechanisms under natural selection such as the "lock-and-key" hypothesis) that can hinder the culmination of heterospecific matings, promoting reproductive isolation (Eberhard, 2004). We know that there is incompatibility at the behavioral level since, in heterospecific mating, females show more resistance events (Oviedo-Diego, M. pers. obs.), which sometimes causes the pedipalps of both sexes to be released, interrupting the mating. Multiple biomechanical variables may be involved in these events, such as the pedipalp muscles and grip strength, as well as probably the optimal fit given by the morphology of the apophysis. Analyzing these variables together could help better understand the determinants of "pedipalp grasping" success in scorpions (van der Meijden et al., 2012) and its relationship to species-specificity in heterospecific matings. Peretti et al. (2000) report that some of the intercrosses between *Bothriurus flavidus* and *B. prospicus* from areas of sympatry could progress to courtship but not to complete matings, but it is unclear which factors lead to mating interruption. Interestingly, it was noted that in intercrosses between *B. cordubensis* and *B. noa*, some matings were interrupted by female resistance events (Peretti et al., 2000). Although the latter species are allopatric, likely, mechanical incompatibilities in pedipalp grasping are also occurring in this pair of species.

The pedipalp apophysis is a key character for the correct attachment of the pedipalps during the mating dance (Ábalos & Hominal, 1974; Maury, 1968; Peretti, 1993), although little is known about the mechanics of the coupling and adjustment with the female pedipalps (Peretti, 1993). The morphospace of this character was complex, and although its summary into a few dimensions allowed us to simplify this complexity, we believe that future studies should be carried out to complete the understanding of the selective forces underlying the evolution of this character. According to the hypotheses of morphological evolution, RCD could be expected under the hypotheses of structural or sensory "lock-and-key" natural selection. Under these hypotheses, species-specific morphological complementarity could exist with similar divergence in both sexes (Eberhard, 1985; Arnqvist, 1997), so it is necessary to include the morphological variability of female characters in future studies. At the same time, the male pedipalp apophysis showed a high coefficient of variation indicative of sexual selection pressures (directional selection) and not consistent with characters that are under stabilizing selection according to the "lock-and-key" hypothesis (Peretti et al., 2001). This could suggest that the pedipalp apophysis in *U. brachycentrus* is under different and maybe opposite selective pressures. On the one hand, the shape of the apophysis (which could be summarized as apophysis depth) showed divergence in sympatry to ensure mechanical isolation. Still, on the other hand, the size of the apophysis seems to be influenced by sexual selection pressures.

### 4.2 Reproductive character displacement in male genital characters

As we observed in some somatic characters, we found evidence of RCD in characteristics of the hemispermatophores of *U. brachycentrus*. In addition, we found that these characters had low CVs, which would support some type of stabilizing selection on these characters (Peretti et al., 2001). Males of this species showed hemispermatophores with a more compressed lamella and larger capsular lobes than allopatric males and sympatric *U. achalensis* males. The size of larger capsular lobules in *U. brachycentrus* could be partly explained by the increase in size of females of this species towards the sympatric zone, as morphological complementarity is expected

for mechanical isolation by the "lock-and-key". Although there are examples of these hypothesis in arthropods (Mikkola, 1992, 2008; Sota & Kubota, 1998; Usami et al., 2006; Nagata et al., 2007; Takami et al., 2007; Tanabe & Sota, 2008; Sota & Tanabe, 2010; Wojcieszek, & Simmons, 2012; Kubota et al., 2013; Nishimura et al., 2022), it is a hypothesis that has been discarded in several species as in general, genitalia diverge much more in males than in females, and it is not so common to find morphological complementarity (Eberhard, 1985; Shapiro & Porter, 1989; Masly, 2012). Like to our conclusion for the pedipalp apophysis, it would be necessary to evaluate the female component to confirm this hypothesis in these species. However, although there may be rather cryptic differences, the female genital atrium is flexible and has a relatively "uniform" structure (Peretti, 2003, 2010). Therefore, the female genitalia in these species does not mechanistically prevent the entry of heterospecific male genitalia, which would also not support the "lock-and-key" hypothesis.

Some particular zones of the hemispermatophore (i.e., frontal crest area) had a very high phenotypic variation, suggesting that their variability is not so much restricted, which is not consistent with a stabilizing selection (Eberhard et al., 1998; Peretti et al., 2001). These results would indicate that the morphological variation of at least some areas of the genitalia of these species would be explained rather by sexual selection hypotheses (Peretti, 2003, 2010; Monod et al., 2017). The frontal crest of the lamella fits into the inter-coxal space of the female, and there could be a 'passive' choice by 'mechanical adjustment' (Eberhard, 1985; Huber & Eberhard, 1997). Also, the capsular lobes possess micro-ornamentations contacting the female genital atrium wall that could have a stimulatory role, which could be contemplated in a female cryptic choice hypothesis (Peretti, 2003, 2010). Larger capsular lobes could be related to a larger contact surface of ornamentations with the female genital atrium and, consequently, a greater stimulation that could be linked to cryptic female choice.

An inevitable question at this point is: if some portions of the genitalia are under sexual selection pressures, why does RCD exist in others? An interesting option could be that the female may bias, by cryptic choice, the use of sperm or other variables (e.g., hardening of the genital plug) according to characteristics evaluated in the interaction of the genitalia, such as (a) greater stimulation by larger capsular lobes (would explain the RCD in the hemispermatophores capsule lobe), (b) by mechanical adjustment of coxae of the first pair of legs with hemispermatophore frontal crest (would explain the RCD in the lamella of hemispermatophore) or fit between male capsular lobe and female genital atrium. We could say that there would be a "combination" of the sensory/mechanical "lock-and-key" hypothesis, where females can recognize the species-specificity of the male genitalia (and thus RCD would be promoted) but where physiological changes would not occur immediately but at the post-copulatory level mediated by female cryptic choice processes.

This interaction between sexual and natural selection hypotheses could be expected to explain the evolution of genitalia in these species, where there is intense competition between males at the intra and interspecific level and promiscuity in their mating and where females must not only exercise mate choice at the pre-copulatory level, but copulatory and post-copulatory mechanisms seem to be necessary to avoid hybridization. A similar example seems to occur in hybridizing *Drosophila* species, where the male genitalia differ in size and shape, and the external female genitalia shows no interspecific differences (Coyne, 1983). In interspecific mating, the intrusion of the male genitalia differentially contacts the female genitalia so that females can store and use sperm according to the specific identity of the male (Price et al., 2001). This is called "cryptic reproductive isolation" and maybe a by-product of multiple evolutionary forces acting at the intra- and interspecific level (Price et al., 2001). As we have emphasized above, it is now recognized that mate choice and specific recognition are part of a continuum and that the forces of sexual and natural selection may interact in multiple ways explaining patterns of sexual diversification across species (Ryan & Rand, 1993; Liou & Price, 1994; Boake et al., 1997; Mendelson & Shaw, 2012). Keeping these interactions in mind is critical for analyzing possible hypotheses of genital evolution (Simmons, 2014).

The reinforcement model postulates the emergence of successive reproductive isolation barriers if these become ineffective (Howard, 1993; Coyne & Orr, 2004; Butlin & Smadja, 2018). If mechanical isolation existed in the past leading to an RCD pattern but subsequently the effectiveness of this barrier, weakened pre-copulatory barriers may have been generated (such as RCD in pedipalps and behavioral incompatibilities), and the RCD in the genitalia may have persisted rather than reverted to the previous morphological scenario. This, in turn, could have resulted in the genitalia (or some of its parts) being able to diversify under other pressures more "freely." This argument is supported by the fact that in this system, there is a pre-copulatory filter in heterospecific matings, and only 10 to 20% of these reach sperm transfer (Oviedo-Diego, M. per obs). Perhaps the existence of a percentage of matings that reach this point is sufficient for the maintenance of the RCD observed in genital characters.

## 4.3 Environmental variations promote size convergence of multiple characters

The overall size, but no shape, of individuals converged in sympatry, i.e., individuals were more similar in size when the species were together, and this pattern was particularly strong for *U. brachycentrus*. This could be observed in males and females for the prosome and

the telson vesicle. Convergence was also observed in females for cheliceral size and in males for caudal gland size and hemispermatophore lamella. The patterns of convergence found in size could follow the rule of Atkinson (1994, 1995) that predicts larger body sizes at lower temperatures (Horne et al., 2015). Most ectotherms grow more slowly and mature with larger body sizes in colder environments (Angilletta et al., 2004). This increase in size may be adaptive when it allows for increased fecundity or higher survival or reproductive rates (Stearns, 1992). In scorpions, it is known that different numbers of molts or the period between molts can affect the final body size of individuals (Sarmiento et al., 2008; Seiter et al., 2020), so through this mechanism, they could reach different sizes depending on environmental characteristics such as temperature or humidity, as well as variations in diet (Sarmiento et al., 2008).

The effect of altitude and temperature change was probably more drastic in *U. brachycentrus* due to the large altitudinal and temperature difference between the allopatric and sympatric populations compared. Temperature is predicted to affect the body size of individuals of both sexes similarly (Hirst et al., 2015), and in *U. brachycentrus*, we found that males and females increase in size. However, this increase could be seen reflected in different characters in each sex, which is perhaps related to sexual dimorphism due to different life habits or phenotypic plasticity in thermal gradients (Fairbairn, 2005; Blanckenhorn et al., 2006; Stillwell & Fox, 2007). Females had a general increase in size, including their chelicerae, a key character for digging and gestation chambers (Maury, 1968, 1969, 1977). Males increased in the body and caudal gland size, a character for sexual interactions (Peretti, 1997). The shape changes in the caudal gland related to humidity are intriguing, considering that this gland produces complex chemistry secretions with numerous compounds where geographic variation among different populations has been demonstrated previously (Olivero et al., 2015). Future studies will aim to determine whether the dynamics of secretion production or effectiveness of secretion rubbing may depend on these shape variations and whether this correlates with behavioral differences between species and allopatry and sympatry contexts.

In scorpions, it is known that geographic variability may exist (Harington, 1983; Abdel-Nabi et al., 2004; Olivero et al., 2012, 2015; Yamashita & Rhoads, 2013) and that the size of individuals may be affected by environmental gradients (Jochim et al., 2020; Lira et al., 2021). For example, Jochim et al. (2020), studying the morphology of a species complex of the family Vaejovidae, found a pattern of morphological convergence very similar to our results. In mountainous areas of Arizona, individuals at higher elevations were larger, resulting in individuals of different species being more similar in the middle areas of the gradient (Jochim et al., 2020). These authors argue that RCD does not occur in these species and that these scorpions probably follow Bergmann's rule, although they do not discuss these aspects further (Jochim et al., 2020). Because of this type of geographic variation, RCD studies must contemplate ecological factors as promoters of morphological variation (Goldberg & Lande, 2006; Kosuda et al., 2016).

## 4.4 Species asymmetry in morphological variability

Asymmetric RI and RCD have been reported multiple times (Bordenstein et al., 2000; Pfennig & Simovich, 2002; Smadja & Ganem, 2005; Cooley et al., 2006; Cooley, 2007; Hochkirch et al., 2007; Costa-Schmidt & Machado, 2012) and it generally occurs when there are interspecific differences in the intensity of selective pressures to avoid heterospecific interactions because species suffer different costs from RI (Pfennig & Simovich, 2002; Cooley, 2007). Also, asymmetric outcomes in morphological variability between species may indicate interspecific differences in morphological plasticity. Divergent characters can also be plastic or can be expressed facultatively when individuals face competition with heterospecifics, so plasticity has been a proposed mechanism to explain character displacement (Robinson & Wilson, 1994; Pfennig & Murphy, 2002; Rice & Pfennig, 2007; Pfennig & Pfennig, 2010; Stuart et al., 2017). Species with broad distribution, exposed to a wide range of environmental conditions, and with ample genetic variation may exhibit more remarkable phenotypic plasticity (DeWitt & Scheiner, 2004; Lavergne et al., 2004; Pigliucci et al., 2006). For example, Crowder et al. (2010) found that the globally distributed whitefly *Bemisia tabaco* biotype exhibited greater plasticity in reproductive behavior, which could result in greater success in avoiding the costs of RI than other biotypes. Here, *Urophonius* species present asymmetries in the RI degree they may be undergoing since males of *U. brachycentrus* are more indiscriminate in their mating decisions than males of *U. achalensis* (Oviedo-Diego et al., 2021). Moreover, *U. brachycentrus* presented higher male-biased operational sex ratios than *U. achalensis* in the sympatric zone (Oviedo-Diego, M. pers. obs.), which could mean males under greater scramble competition to find females and that this species could suffer higher costs due to RI (Oviedo-Diego et al., 2020; 2021). In turn, *U. brachycentrus* showed the most remarkable morphological variations, being the most widely distributed species compared to *U. achalensis*, endemic to the highland area under analysis (Acosta 1985, 1993; Ojanguren-Affilastro, 2005; Ojanguren-Affilastro et al., 2020). This complex social and geographic scenario could translate into strong selective pressures for interspecific recognition during mating or sperm transfer and the existence of RCD patterns in an asymmetric manner, being *U. brachycentrus* the species that suffers more RI costs and the most morphologically plastic to manifest changes under these pressures.

## 4.5 Mixed selective pressures on multiple characters in scorpions

Our results reveal a strong variation in the size and shape of somatic and genital characters, which supports the notion that morphological traits are the result of multiple selective pressures and that different dimensions of the same character (e.g., shape, size) may be reflecting different evolutionary responses (mosaic evolution). This is most noticeable in characters used in multiple activities in the organism's life. We found evidence of the existence of RCD for the pedipalps shape of both sexes in sympatric populations, an evolutionary response to avoid crossbreeding and strengthen reproductive isolation among these species. In turn, other characters showed high geographic variability in size reflected in patterns of convergence towards the sympatry zone, which could affect the mating system of these species, promoting RI and explaining the high values of phenotypic variation found in characters used in sexual interactions (e.g., caudal gland, pedipalp apophysis). It is noteworthy the different selective pressures under which the genitalia would be, also under natural selection pressures showing an RCD pattern in shape, although manifesting in other portions of the hemispermaphore very high phenotypic variation which would indicate possible sexual selection pressures acting mainly in the crest zone.

Peretti (2010) highlights the existence of mixed patterns in the genitalia of scorpions, where morphological complexity results from different selective regimes. This has also been observed in other arachnids (Huber, 1996, 2004) and insects (Song & Wenzel, 2008; Simmons et al., 2009; Song & Bucheli, 2010; Rowe & Arnqvist, 2012; House et al., 2013; Frazee & Masly, 2015) where characters are under multiple, often contradictory or inconsistent pressures. Studies in water striders suggest that the non-intromittent genitalia have differing degrees of selection acting upon them (Danielsson & Askenmo, 1999; Bertin & Fairbairn, 2005; Rowe & Arnqvist, 2012). Another example was reported in the dung beetle *Onthophagus taurus* which has shown that different sections of male genital morphology may be under different selective regimes (Song & Wenzel, 2008; Simmons et al., 2009) as the shape of the aedeagus is subject to directional sexual selection, but genital sclerites that penetrate the female genitalia are subject to stabilizing and disruptive nonlinear selection (Simmons et al., 2009). In addition, in *O. taurus*, the genitalia shape diverges rapidly due to directional sexual selection, whereas size remains unaffected in the process (Simmons et al., 2009). Similarly, it has been reported for the millipede *Antichiropus variabilis* that genitalia shape responded to stabilizing pressures (supporting the occurrence of lock-and-key), although genitalia size did not follow this pattern and responded to environmental gradients (Wojcieszek & Simmons, 2012). This is like to our results, where the shape of certain structures responds to specific recognition variations with low phenotypic variation, and size shows patterns of variation linked to geographic and environmental differences. The size and shape of the same structure may respond in this mosaic manner, independently to different selective pressures, perhaps due to genetic or developmental decoupling (Macagno et al., 2011; Rowe & Arnqvist, 2012; Wojcieszek & Simmons, 2012; Richmond, 2014). Future studies will aim to assess the consistency of these results with allometric patterns between populations, and coevolution between female and male characters, as well as explore the morphological complexity of the traits by assessing the modularity of the subunits of the characters (e.g., Kuntner et al. 2009; Tatarski & Cassis, 2010; Rowe & Arnqvist, 2012; Genevicius & Schwertner, 2017; Genevicius et al., 2020)

## Conclusions

We found a remarkable morphological variability in both scorpion species that was determined in part by geographic and environmental variations, in part by sexual selection pressures at the intra- and interspecific level, and in part by natural selection pressures during species recognition. We report a pattern of asymmetric morphological variation where one of the scorpion species (*U. brachycentrus*) suffered an increase in size in several characters to the sympatric zone due to environmental factors (showing a pattern of morphological convergence). This increase in size and a scenario of promiscuity probably led to certain characters undergoing intense sexual selection pressures, which is reflected in the high phenotypic variation found. However, key characters for mating success, such as grasping or genital characters, experienced morphological divergence in males and females, implying a mechanical incompatibility that could function as a barrier promoting reproductive isolation. However, some characters that showed variation by RCD were also found to be under sexual selection pressures, suggesting a complex scenario of mixed selective regimes acting on these characters. On the other hand, the non-concordant results on the pressures on the size and shape of characters enlighten us on the complexity inherent in the evolution of multi-functional traits in scorpions. This comprehensive study presents novel results in an ancestral group that has not been studied from this perspective and provides interesting insights for evaluating characters under multiple selective pressures in animal systems with RI.

## Declarations

Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Please refer to "Competing Interests and Funding" below for more information on how to complete this section.



*Competing interests:* The authors declare no competing interests with regard to this manuscript and the material implicated.

## Ethical standards

We declare that the experiments comply with the current laws of Argentina. This investigation adheres to the ASAB/ABS Guidelines for the Use of Animals in Research (Buchanan et al., 2012), and the use of animals was reviewed and approved by the animal care review committee at the Instituto de Diversidad y Ecología Animal (IDEA), CONICET-UNC, Argentina, where we performed the experiment.

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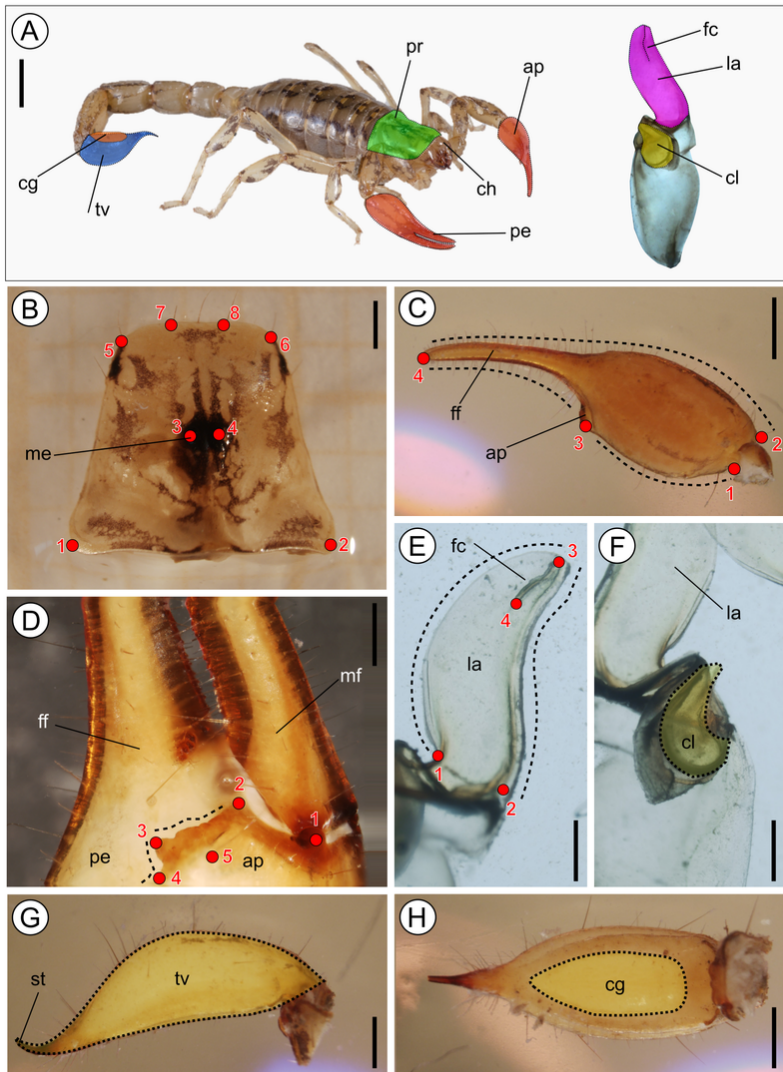


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## Figures



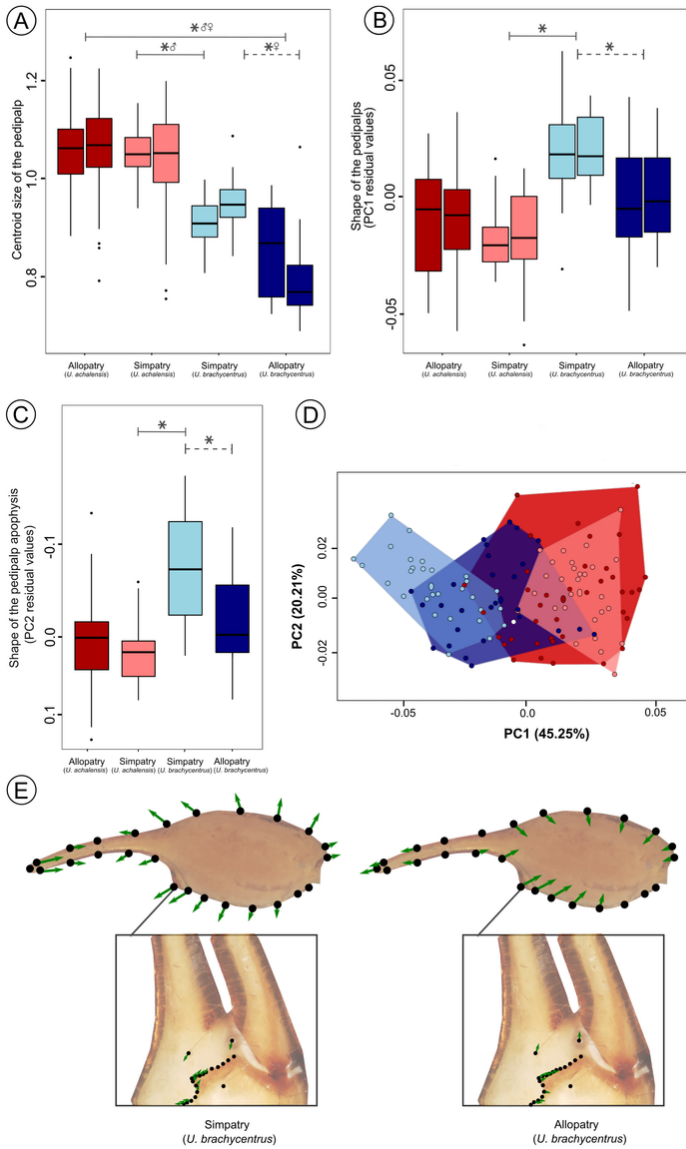
**Figure 1**

Selected characters for morphological study in two scorpion species of *Urophonius achalensis* and *U. brachycentrus*. A. General diagrams of measured somatic and genital characters. B. Prosoma. C. Lateral view of the male pedipalp. D. Apophysis of the male pedipalp. E. Lamella of the hemispermatophore with frontal crest. F. Capsular lobe of the hemispermatophore. G. Dorsal view of male telson. H. Ventral view of male telson with caudal gland. Abbreviations: ap, pedipalp apophysis; cg, caudal gland; ch, chelicerae; cl, hemispermatophore capsular lobe; fc, hemispermatophore frontal crest; ff, pedipalp fixed finger; la, hemispermatophore lamella; me, median eye; mf, pedipalp mobile finger; pe, pedipalp; pr, prosoma; tv, telson vesicle; st, sting. References: Red dots and numbers, Landmarks (descriptions in Table S1); dotted line with more separated stroke, positioning of semilandmarks; dotted line with a narrower stroke, character analyzed by elliptical Fourier analysis (EFA). Scales: A= 5 mm in scorpion, 0.5 mm in hemispermatophore, B-C, G-H=1 mm; D-F=0.5 mm

Morphological character	Sex	Allopatry		Sympatry		Allopatry	
		<i>U. brachycentrus</i>		<i>U. brachycentrus</i>		<i>U. achalensis</i>	
Prosoma Telson vesicle	♀		Size →		← Size		
	♂		Size →				
Chelicerae Pecten	♀		Size →				
	♂						
Pecipalp	♀		Size →		≠		
	♂		Shape →		≠		
Pecipalp apophysis	♀		Shape →		≠		
	♂				≠		
Caudal gland	♀		Size →		≠		
	♂				≠		
Hemispermaphore lamella and capsular lobe	♀		Shape →		≠		
	♂		Size →		≠		

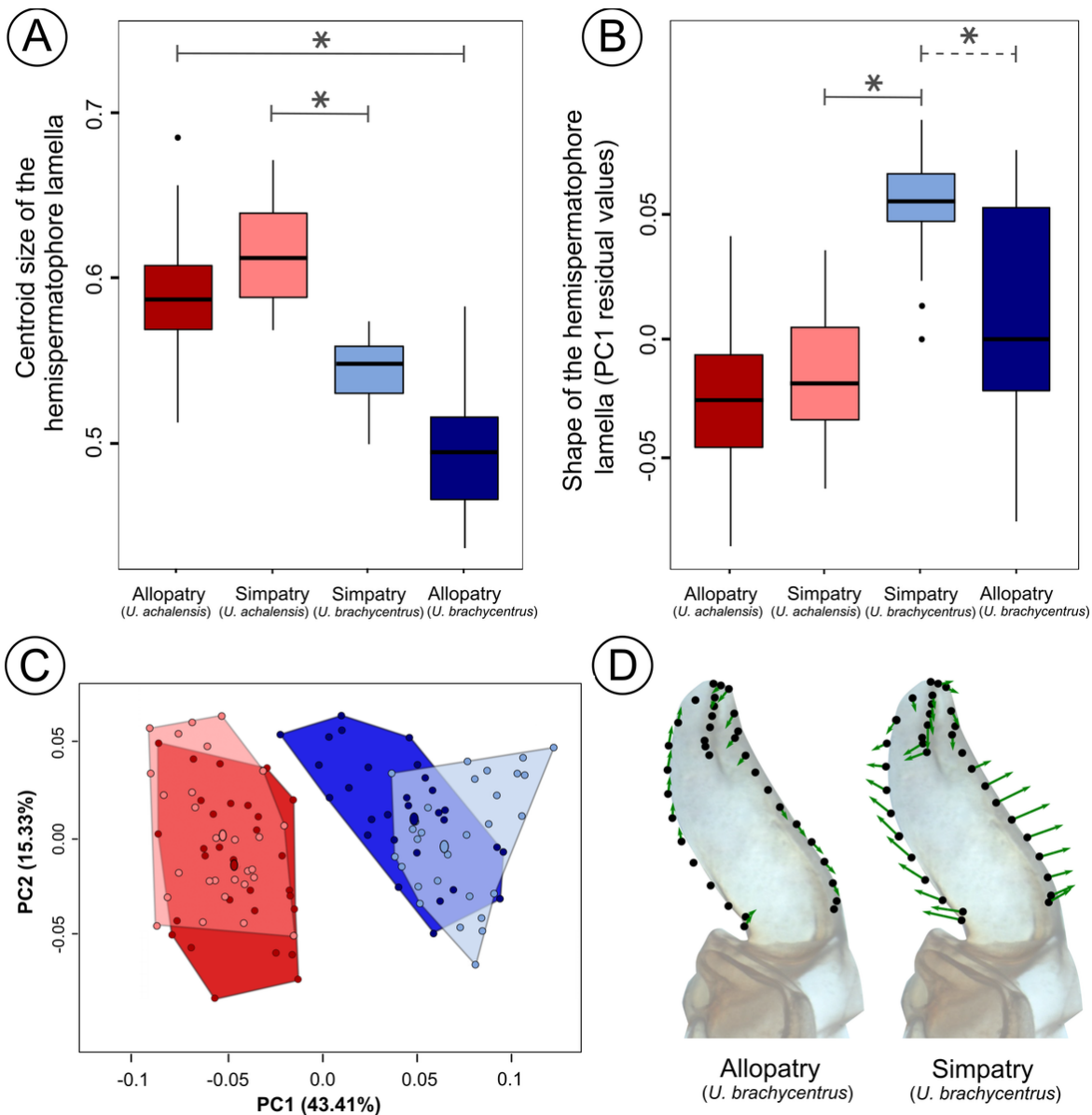
**Figure 2**

Diagrams showing the summary of morphological variation in size and shape of somatic and genitalia characters in scorpions in different contexts of sympatry and allopatry. Each character is scaled at the intrasexual level. Gray area in the middle of the plate indicates sympatric zone. Gray arrows, characters undergoing convergence; black arrows, characters undergoing divergence (RCD). : males, : females, ≠: Statistical differences between species in sympatry



**Figure 3**

Interspecific and intraspecific morphological variation in pedipalp and male pedipalp apophysis in *Urophonius achalensis* and *U. brachycentrus* from sympatric and allopatric zones. A. Pedipalp size of males (first box) and females (second box) indicated by centroid size. B. Pedipalp shape (PC1) of males (first box) and females (second box) and differences between species and contexts C. Shape of pedipalp apophysis of males (PC2) and differences between species and contexts. Statistical differences indicated in each graph: continued line showed interspecific differences, dashed line: intraspecific differences (between allopatric and sympatric contexts), : males, : females. D. Male pedipalp morphospace indicating the morphological distribution of individuals along two principal components of variation. Numbers in parentheses on each axis showing percentage of variance explained by each principal component. Color reference following A-C. E. Summary of morphological changes in PC scores of extremes individuals (minimum in sympatric population and maximum in allopatric population) of *U. brachycentrus*, Top: shape of male pedipalp (PC1 scores); Below: shape of male pedipalp' apophysis (PC2 scores); black dots showing landmarks and semilandmarks showing consensus conformation, orientation of arrows (vectors) indicating direction of morphological change and arrow longitude indicating magnitude of change



**Figure 4**

Interspecific and intraspecific morphological variation in the hemispermatophore lamella of *Urophonius achalensis* and *U. brachycentrus* males from sympatric and allopatric zones. A. Size of hemispermatophore lamella indicated by centroid size. B. Hemispermatophore lamella shape (PC1) and differences between species and contexts. Statistical differences indicated in each graph: continued line showed interspecific differences, dashed line: intraspecific differences (between allopatric and sympatric contexts). C. Morphospace indicating the morphological distribution of individuals along two principal components of variation. Numbers in parentheses on each axis showing percentage of variance explained by each principal component. Color reference following A-B. D. Summary of morphological changes in PC1 scores of extremes individuals (maximum in sympatric population and minimum in allopatric population) of *U. brachycentrus*, black dots showing landmarks and semilandmarks showing consensus conformation, orientation of arrows (vectors) indicating direction of morphological change and arrow longitude indicating magnitude of change

## Supplementary Files

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- [TableS1.xlsx](#)