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Reproductive interference between two sibling species of gift-giving spiders

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We investigated the possibility of reproductive interference between two sibling spider species, *Paratrechalea azul* and *Paratrechalea ornata*, which occur syntopically and reproduce synchronously. Males of both species offer a nuptial gift composed of prey wrapped in silk to females. Through laboratory experiments, we evaluated possible asymmetries in the outcome of heterospecific encounters between males and females, and investigated whether chemical signalling could function as a premating barrier between the two species. Males of *P. azul* were unable to discriminate conspecific from heterospecific female draglines, which resulted in wasted time and energy in nuptial gift construction. Males of *P. ornata* incurred a higher cost for discrimination mistakes because most of them were attacked by heterospecific females; 95% lost the nuptial gift upon the attack and 33% were preyed upon. This pattern is probably a consequence of differences in body size between males and females of each species. Both species showed erroneous female choice, but only *P. ornata* females courted heterospecific males, which are considerably larger than conspecific males and may resemble high-quality mating partners. Males of *P. ornata* also made discrimination mistakes, but at a much lower frequency compared to *P. azul* males. The selective pressure for precise recognition of conspecific female signs is probably stronger on *P. ornata* males because misdirected courtship may increase their chances of encountering predatory heterospecific females. This study provides the first detailed evidence of reproductive interference between two reproductively isolated spider species, showing that the costs paid by individuals of different sexes and different species are highly asymmetric.

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Reproductive interference is defined as 'any kind of interspecific interaction during the process of mate acquisition that adversely affects the fitness of at least one of the species involved and that is caused by incomplete species recognition' (Gröning & Hochkirch 2008, page 258). Such fitness losses caused by reproductive interference are not an outcome of competition for mates, but rather of wasting time, energy, nutrients or gametes or even loss of life in sexual interactions with heterospecific individuals (Hochkirch et al. 2007). Given that the mechanisms involved in signal production and reception are evolutionarily constrained by the morphology, physiology or behaviour of the species involved, signal overlap is expected to be more frequent in closely related taxa (de Kort & ten Cate 2001). Indeed, reproductive interference has been reported more frequently among species belonging to the same genus (e.g. Fujimoto et al. 1996; Deering & Scriber 2002; Dame & Petren 2006). Moreover, signal overlap is more likely to be found between allopatric species that have not been exposed to long-term selective

pressures favouring divergence of their species recognition systems (Coyne & Orr 1997). Consequently, most of the empirical evidence of reproductive interference has come from secondary contact zones (e.g. Servedio & Noor 2003) and between invasive and native species (e.g. Rhymer & Simberloff 1996).

Reproductive heterospecific interactions are possible at any stage of mate acquisition, from courtship signalling to fertilization. Therefore, based on the temporal course of mating behaviour, seven types of reproductive interference have been recognized, but the boundaries between them are sometimes elusive, and more than one type has been documented in many species pairs (reviewed in Gröning & Hochkirch 2008). Although there are costs associated with all seven types of reproductive interference, fitness losses are more obvious when species misdirect their courtship and/or mating attempts. These discrimination mistakes are costly because they reduce the breeding performance of the individuals involved and result in wasting time and energy in sexual interactions with heterospecifics (e.g. Wells & Taigen 1989; Collins & Margolies 1991; Ardeh et al. 2004). Additionally, the costs associated with discrimination mistakes are usually asymmetric, with individuals of one species suffering higher costs than individuals of the other (e.g. Fujimoto et al. 1996; Wirtz 1999; Deering & Scriber 2002; Hochkirch et al. 2007).

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The costs associated with reproductive interference are not only species specific and asymmetric but may also vary between the sexes (Pfennig 1998), as it is unlikely that males and females pay similar costs or are indiscriminate to the same degree (Wirtz 1999). Males, for instance, pay reproductive costs when they invest time and energy into courtship and/or mating attempts with heterospecifics, which will not result in successful fertilization (e.g. Kandul et al. 2006). Moreover, males of several arthropod species offer costly nuptial gifts to females (Vahed 1998), which may be wasted in reproductive heterospecific interactions. In the most extreme cases, the price paid by males involved in heterospecific mating attempts may be death if they become prey for an aggressive mimic (e.g. Lloyd 1983). Compared to males, however, females are expected to pay higher costs for heterospecific reproductive interactions (especially those involving mating) because they are usually characterized by a greater reproductive investment and a lower mating frequency (Trivers 1972). Empirical and theoretical evidence has shown that heterospecific mating may reduce female reproductive success by increasing sperm competition, obstructing fertilization, or interrupting embryonic development (Ribeiro & Spielman 1986). Heterospecific mating may benefit females only in rare cases, for example, when mating with a heterospecific male is a prerequisite for embryogenesis (Gumm & Gabor 2005) or when females receive direct benefits, such as valuable nuptial gifts.

In this study, we investigated the possibility of reproductive interference between two semi-aquatic spiders, *Paratrechalea azul* and *Paratrechalea ornata* (Trechaleidae), which are sibling species showing nearly indistinguishable morphology and courtship sequences (Costa-Schmidt et al. 2008). The distribution of these two species is allopatric in most parts of their ranges, but there is a large area of sympatry in southern Brazil, where individuals are found syntopically and synchronously at the margin of forest streams (Carico 2005; Silva et al. 2006). This sympatric zone most likely represents a secondary contact zone between the two species, and recent morphological studies indicate that the shape of the male and female genitalia of both species diverge in the zone of contact (Costa-Schmidt & Araújo 2010). This divergence has been interpreted as a reinforcement mechanism to avoid hybrid formation in syntopic populations (Costa-Schmidt & Araújo 2010).

Males of both *Paratrechalea* species offer a nuptial gift to females, which is composed of prey wrapped in silk and is carried in the males' chelicerae during mate search (Costa-Schmidt et al. 2008). The male's decision to construct a nuptial gift is primarily mediated by chemical cues deposited by conspecific females on their draglines (Albo et al. 2009). However, we do not know whether males are able to discriminate silk from heterospecific females and what costs are associated with possible discrimination mistakes. Given that individuals of *P. azul* are larger than individuals of *P. ornata* (Costa-Schmidt & Araújo 2008), heterospecific encounters are potentially dangerous for *P. ornata*, especially for males carrying a nuptial gift, because they are likely to be more conspicuous and perhaps slower than males without a nuptial gift. Therefore, there should be strong selective pressure on *P. ornata* males to develop mechanisms of species recognition before nuptial gift construction when sharing a habitat with *P. azul*. In contrast, this selective pressure favouring species recognition would be weaker in *P. azul* because males making discrimination mistakes would rarely become prey if they court a heterospecific female.

Although *Paratrechalea* males deposit phagostimulant substances on the silk wrapping around the nuptial gift and these substance elicit female acceptance (Brum et al. 2012), the nuptial gift alone should not play an important role in the recognition of potential conspecific males by females. From the female's perspective, the nuptial gift represents a meal, and regardless the male species presenting the gift, nuptial gifts are expected to be accepted and eaten (Gröning & Hochkirch 2008). Alternatively, heterospecific males resembling high-quality conspecifics may be perceived as especially attractive mates (Pfennig 1998; Ryan 1998). This may be particularly common if heterospecific males are larger than conspecific mates because body size is often an indicator of male quality (Andersson 1994). If females select males based on body size, *P. azul* males, which are larger than *P. ornata* males, would resemble high-quality conspecifics. Trechaleids belong to a clade of spiders with an acute sense of vision (Foelix 2011), and at least for some lycosids (wolf spiders), females show a marked preference for large males with more conspicuous secondary sexual traits (Framenau & Hebets 2007). Therefore, *Paratrechalea* females might evaluate male body size visually during courtship and show some preference for large males.

Despite the fact that some types of reproductive interference are restricted to individuals of the same sex (Gröning & Hochkirch 2008), we focused this study only on interspecific interactions between individuals from different sexes. In the first part of this study, we investigated possible asymmetries in the outcome of heterospecific encounters between males and females of *P. ornata* and *P. azul*. In the second part, we investigated whether chemical signalling could function as a premating barrier between the two species, focusing on both males and females. Regarding males, we investigated whether they are able to discriminate conspecific from heterospecific females based on chemical cues deposited on their draglines. Given that a nuptial gift represents an investment in time and energy, males should invest in nuptial gift construction only in the presence of conspecific females. We also predicted that the higher the risk incurred in discrimination mistakes, the greater the ability of males to recognize cues from heterospecific females and avoid nuptial gift construction. Regarding females, we investigated whether they discriminate conspecific from heterospecific males based on chemical cues deposited on the silk that wraps the nuptial gift. In our first experimental approach, females of both species were exposed exclusively to conspecific and heterospecific nuptial gifts. Even when females are able to recognize conspecific males based on specific cues deposited on the nuptial gift, we expected that they would grab gifts from males of any species because the nuptial gift represents a cost-free meal. In our second experimental approach, we considered the influence of male presence on the acceptance of nuptial gifts by females. If females select males based on body size, *P. ornata* females should accept nuptial gifts from *P. azul* males more frequently than *P. azul* females.

METHODS

Model Organisms

Individuals of *P. azul* and *P. ornata* are mostly found on boulders at the margins of streams and are active mainly at night. Their distributional range includes southern Brazil, Uruguay, and northern Argentina (Carico 2005; Silva et al. 2006). In a sympatric zone in the state of Rio Grande do Sul, Brazil, populations of the two species can be found syntopically and show great overlap in their reproductive seasons (from October to February). Syntopic populations are characterized by a high density of individuals, such that heterospecific encounters are frequent in the field (Costa-Schmidt 2008). Males of both species offer nuptial gifts to females during courtship. Sexual cannibalism under natural conditions appears to be rare, and was not observed during 6 years of intensive field work with the two *Paratrechalea* species (L. E. Costa-Schmidt, personal observations). In the laboratory, sexual cannibalism occurred in only one out of 102 courtship interactions of *P. ornata*, and was not observed to occur in more than 30 courtship interactions of *P. azul* (L. E. Costa-Schmidt, unpublished data).

Animal Collection and Housing

We collected juvenile males and females of both species at Pedra de Amolar River (29°32′20.52″S; 50°14′46.83″W), state of Rio Grande do Sul, Brazil, on two occasions: first, between November and December 2009, and second, in August 2010. In this locality, the two species live together, so it is common to find adults of *P. azul* and *P. ornata* close to each other on the same boulder at night. We captured males and females as subadults to ensure that only virgin individuals would be used in the experiments described below. We transported all individuals to our laboratory at the University of São Paulo, Brazil, where we reared them in individual plastic vials (8 cm high × 6 cm in diameter) covered with a soft textile net. Each plastic vial contained a wood stick as a perch for the spiders and a piece of wetted cotton to maintain humidity. We maintained the spiders at room temperature, which ranged from 21 °C to 25 °C, under a 12:12 h light:dark cycle and fed them three times per week with domestic flies or laboratory-reared cockroach nymphs (∼5 mm). Individuals were used in the experiments 15 days after their last moult to ensure that they were reproductively mature. After the experiments, we kept the individuals alive in the laboratory for other research projects.

Heterospecific Encounters

First, we offered a cockroach nymph (∼5 mm) to the adult males and females 24 h before the trials so that they would be at a similar hunger level. Then, we randomly assigned each individual to one of four experimental groups. In two of the experimental groups, we paired *P. azul* males either carrying ($N = 17$) or not carrying a nuptial gift ($N = 13$) with a haphazardly selected heterospecific female. In the other two experimental groups, we paired *P. ornata* males either carrying ($N = 23$) or not carrying a nuptial gift ($N = 24$) with a haphazardly selected heterospecific female. We conducted the trials between 1500 and 1900 hours under dim light conditions and used each individual (males and females) only once.

Prior to the trials, we placed males from all experimental groups in a receptacle (15 cm length × 10 cm width × 5 cm high) in which they experienced contact with a conspecific female until they showed the 'hyperflexion posture', a stereotypical behaviour that indicates male sexual excitement (Costa-Schmidt et al. 2008). Then, we offered the males of both species a cockroach nymph (∼5 mm) as a prey item for nuptial gift construction. Although males in the experimental groups without a nuptial gift received no cockroach nymph, exposure to a conspecific female served to induce them to perform mate searching in the subsequent trials. Females of both species used in this preliminary phase came from an independent stock and were not used in the trials described below.

We performed the trials in a square glass arena (14 cm sides × 5 cm high). First, we placed one male and one female inside the arena at the same time but prevented them from interacting by keeping one of them (decided by chance) in a translucent cage inside the arena. When both individuals were quiescent, indicating that they were presumably acclimated to the experimental conditions, we removed the cage. We recorded the female reaction during the first contact with the male and classified her reaction into one of three categories: (1) attack, when she suddenly moved towards the male and attempted to grab and bite him; (2) escape, when she touched the male and immediately ran away from him; or (3) hyperflexion, when she raised her two pairs of front legs and folded them above her cephalothorax. For the purpose of this experiment, we considered this last category as 'no attack', but we explore this last category in more detail below (see Species Recognition by Females). For those trials that resulted in female attack, we also recorded the occurrence of male predation and

nuptial gift release for males from experimental groups with a nuptial gift. The trials took no longer than 15 min, and we considered females as nonrespondents when they did not contact the male within this period. We excluded nonrespondent females in the analyses described below. Moreover, in the analysis of attack risk, we used all observations, whereas in the analysis of predation risk, we used only the attacked males.

After the trials, we measured the cephalothorax length, which is the best linear descriptor of body size in *Paratrechalea* species (Costa-Schmidt & Araújo 2008), of all individuals that were not severely injured during the experiment. We performed this measurement by photographing the individuals under a stereomicroscope and analysing the images using the software ImageTool v.3.00 (University of Texas Health Science Center, San Antonio, TX, U.S.A., http://compdent.uthscsa.edu/imagetool.asp). The body size distributions of the individuals used in the experiments were in agreement with the pattern previously recorded in the field, with individuals of *P. azul* (mean ± SD = 4.73 ± 0.32 mm, $N = 77$) being larger than individuals of *P. ornata* (4.06 ± 0.24 mm, $N = 77$; Student's *t* test with variance differences: $t_{141.62} = 14.43$, $P < 0.0001$). More specifically, *P. azul* females were, on average, 20% larger than *P. ornata* males (female size/male size ratio = 1.20 ± 0.10 mm, $N = 47$), and *P. ornata* females were 10% smaller than *P. azul* males on average (0.90 ± 0.07 mm, $N = 30$; Student's *t* test: $t_{74.84} = 15.16$, $P < 0.0001$).

We analysed our data using a model selection procedure employing Bayesian generalized linear models to select which hypothesis best fit the observed data on attack and predation risk. As distribution and linkage functions, we used the binomial family function and the logit function, respectively. For all models, we used either attack or predation on males as the dependent variable, but the predictor variables varied between models, consisting of a combination of body size difference (defined as the difference between female and male cephalothorax length) and nuptial gift presence (categorical, with two levels). Given that individuals of *P. azul* are larger than individuals of *P. ornata* on average, positive body size difference values were mostly related to interactions between *P. azul* females and *P. ornata* males, whereas negative values were mostly related to interactions between *P. ornata* females and *P. azul* males. The combination of predictor variables resulted in five concurrent models for each dependent variable (see Results, Table 1). To infer the maximum likelihood of the concurrent models, we used Akaike's Information Criterion corrected for small sample size (AICc). We ran each model using the function 'bayesglm' from the 'arm' package (Gelman et al. 2012) with its default parameters developed for R software (R Development Core Team 2011).

Species Recognition by Males

We performed this set of experiments from January to February 2010, always conducting the trials between 2000 and 0200 hours. We used 15 *P. azul* males and 16 *P. ornata* males to test the hypothesis that males would invest more in nuptial gift construction when exposed to a stimulus from conspecific females. The female stimulus consisted of draglines obtained from adult virgin females maintained individually for 24 h inside a petri dish (9 cm in diameter) with its bottom covered with filter paper (experimental arena). Albo et al. (2009) demonstrated that female draglines deposited on a substrate induce nuptial gift construction in *P. ornata* males, which is also the case for *P. azul* (L. E. Costa-Schmidt, unpublished data). Approximately 1 h before the trials, we gently removed each female from her experimental arena and placed a male in the arena for acclimation. No visual or physical contact occurred between males and females; thus, the only

intersexual communication occurred strictly via silk deposition on the filter paper. We used a paired design in which we sequentially exposed each male to a stimulus from one conspecific and one heterospecific female. The order of the trials was randomly chosen for each experimental male (*P. azul*: N = 15, *P. ornata*: N = 16), with no effect on the results being detected (data not shown).

The trials began when we offered a live domestic fly to each male inside the experimental arena. We performed videorecording of each trial for 2 h after prey were offered. In cases in which the prey was not captured during this interval, we interpreted the male decision as no investment in nuptial gift construction. We evaluated male investment in nuptial gifts using four proxies. The first was the latency until the first sequence of silk deposition on the prey ('wrapping' sensu Costa-Schmidt et al. 2008), which was taken as a measure of male excitement in response to cues from a virgin female. We expected that males would perform prey wrapping sooner when exposed to draglines of conspecific females. We tested this prediction using a conditional inference tree based on a survival analysis (Kaplan–Meyer estimate of survival). The tested model consisted of two fixed effect variables: the male's specific identity and the female's signal identity. We added a random effect based on males' individual identity to the model to control for data independence because each male was used in both treatment groups. We performed this analysis with R software (R Development Core Team 2011) using the 'party' package (Hothorn et al. 2006).

Our second proxy of male investment in nuptial gift construction consisted of the number of trials in which males performed at least a single wrapping. We expected that males would invest in wrapping more frequently when exposed to draglines of conspecific females. We used a McNemar test for paired frequency samples to compare the frequency of nuptial gift construction by males of each species. The last two proxies were the number of wrappings and the total wrapping time, which are both related to the quality of the nuptial gift. We expected that males would perform more wrapping and spend more time wrapping when exposed to draglines of conspecific females. We tested these two predictions using a paired samples Student's *t* test when the data fitted a normal distribution; otherwise, we applied a Wilcoxon signed-rank test (Zar 1999). For both proxies, we performed the analyses only including males that constructed nuptial gifts, resulting in a sample size of six *P. ornata* and 11 *P. azul* males that were exposed to draglines of both conspecific and heterospecific females. We performed all of these analyses in the R software (R Development Core Team 2011).

Species Recognition by Females

Our first experiment was designed to test the hypothesis that females would show no discrimination when exposed to nuptial gifts from conspecifics and heterospecifics. Again, we used a paired design in which we exposed each female to a nuptial gift constructed by a conspecific and a heterospecific male. The order of the trials was randomly chosen for each experimental female (*P. azul*: N = 15, *P. ornata*: N = 15), with no effect on the results being detected (data not shown). We offered a nuptial gift to each female by placing it in front of her using metal forceps, so that no visual or physical contact between males and females occurred during the experiment. The only source of information available for the females was the nuptial gift. We scored females as respondent if they grasped the nuptial gift with their chelicerae and held it for at least 3 min. This procedure has already been used by our research group and has proved to elicit natural female responses (Brum et al. 2012). We compared the number of females that accepted the nuptial gift from conspecific and heterospecific males using

a McNemar test for paired frequency samples performed in the R software (R Development Core Team 2011).

The second experiment was designed to evaluate the influence of male presence on nuptial gift acceptance by females. The experimental set-up was the same as described in Heterospecific Encounters above, except that here, we focused on cases in which females showed hyperflexion posture towards heterospecific males. This hyperflexion posture is interpreted as a sign of female receptivity or a form of female courtship, and has been recorded for *P. ornata* and *P. azul* females during reproductive interactions with conspecific males (Costa-Schmidt et al. 2008). Given the marked difference in body size between males of these two *Paratrechalea* species, *P. azul* males could resemble high-quality conspecifics to *P. ornata* females. For the same reason, *P. ornata* males would resemble low-quality conspecifics to *P. azul* females. Therefore, we expected that *P. ornata* females would accept nuptial gifts from heterospecifics more frequently than *P. azul* females. We tested this prediction using a Fisher's exact test performed in the R software (R Development Core Team 2011). Additionally, if female hyperflexion is indeed a recognition mistake, *P. ornata* females should show this behaviour even to heterospecific males not carrying a nuptial gift.

RESULTS

Outcome of Heterospecific Encounters

Eleven heterospecific encounters out of 77 resulted in no female reaction (Table 2). However, two-thirds of the heterospecific encounters were followed by a female attack (44/66 = 66.7%). The attack frequency towards heterospecific males was higher for *P. azul* females (39/45 = 86.7%) compared to *P. ornata* females (5/21 = 23.8%; Fig. 1). The attack frequency towards *Paratrechalea* males with a nuptial gift (22/32 = 68.7%) was almost the same as that towards males without a nuptial gift (22/34 = 64.7%). Model selection analysis for attack risk resulted in two selected models (Table 1). The best-fitting model considered only the positive influence of the body size difference between males and females in the probability of attack (Fig. 2). The second best-fitting model

Table 1
Summary of the models fitted to data on attack and predation risks for heterospecific males from *Paratrechalea* females

Model	Predictions	AICc	k	ΔAICc	w_i
Attack risk (complete data set)					
A∼SD	A is influenced only by SD	55.5	2	0.0	0.532
A∼SD*T	A is influenced by the interaction between SD and T	56.7	4	1.2	0.289
A∼SD+T	A is influenced by the additive effect of SD and T	57.7	3	2.2	0.178
A∼1	A is not influenced by SD or T (null model)	86.1	1	30.6	<0.001
A∼T	A is influenced only by T	88.1	2	32.6	<0.001
Predation risk (attacked males only)					
P∼SD+T	P is influenced by the additive effect of SD and T	55.9	3	0.0	0.464
P∼SD	P is influenced by SD	56.5	2	0.6	0.344
P∼SD*T	P is influenced by the interaction between SD and T	58.4	4	2.5	0.131
P∼1	P is not influenced by SD or T (null model)	60.8	1	4.9	0.040
P∼T	P is influenced only by T	62.1	2	6.2	0.020

AICc: Akaike's Information Criterion corrected for small sample size; *k*: number of parameters in the models; ΔAICc: change in AICc for each model in relation to the best-fitted model; w_i: Akaike weight; A: attack; P: predation; SD: size difference between females and males; T: treatment (males with and without a nuptial gift); '+': additive effects.

Table 2
Behavioural responses of *Paratrechalea* females in staged encounters with heterospecific males that carried and did not carry a nuptial gift (NG)

Interaction	Attack	Escape	Hyperflexion	Other	Predation
***P. ornata* female vs *P. azul* male**					
Without NG ($N=13$)	4	8	1	–	–
With NG ($N=17$)	1	8	8	–	–
***P. azul* female vs *P. ornata* male**					
Without NG ($N=24$)	18	4	–	2	10
With NG ($N=23$)	21	2	–	–	7

considered the interaction between the experimental group (males with and without a nuptial gift) and body size difference (Table 1). All but one of the attacked *P. ornata* males released their nuptial gift upon female attack (20/21 = 95.2%; Fig. 1). In contrast, *P. azul* males attacked by *P. ornata* females did not release their nuptial gifts (0/20; Fig. 1).

Only *P. ornata* males were preyed upon after they had been attacked (17/39 = 43.6%; Fig. 1), and the predation frequency was slightly higher among males without a nuptial gift (10/18 = 55.6%) compared to males with a nuptial gift (7/21 = 33.3%). Model selection analysis for predation risk also resulted in two models being selected (Table 1). The best-fitting model considered the positive influence of the body size difference between males and females on predation risk and the additive effect of the experimental group (males with and without nuptial gift) (Fig. 3). The second best-fitted model considered only the positive influence of the body size difference on predation risk (Table 1).

Do Males Discriminate Conspecific from Heterospecific Females?

The latency until the first sequence of silk deposition on the prey ('wrapping') when males were exposed to female dragline signals differed between the two *Paratrechalea* species (conditional inference tree: $P = 0.018$; Fig. 4). Males of *P. ornata* began to wrap the prey sooner after exposure to draglines of conspecific females than after exposure to draglines of heterospecific females (conditional inference tree: $P = 0.042$; Fig. 4). For *P. azul* males, the latency until the first wrapping was similar after exposure to draglines of conspecific and heterospecific females (Fig. 4).

Males of *P. ornata* invested more frequently in nuptial gifts when exposed to draglines of conspecific females (McNemar test: $\chi_1^2 = 5.14$, $P = 0.023$; Fig. 5). All males that constructed nuptial gifts when exposed to draglines of heterospecific females also
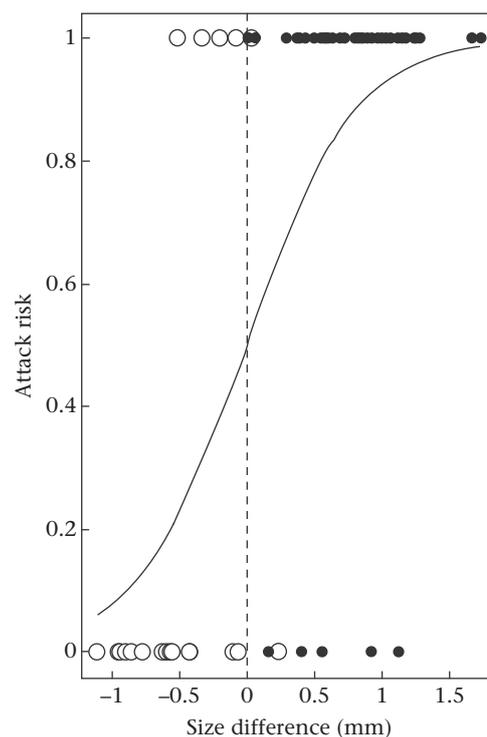

Figure 2. Attack risk for *Paratrechalea azul* and *P. ornata* males in interactions with heterospecific females. Body size difference was defined as female cephalothorax length minus male cephalothorax length. Open circles: interactions between *P. azul* males and *P. ornata* females; solid circles: interactions between *P. ornata* males and *P. azul* females. The line represents the adjustment of the best-fitted model (see Table 1).

constructed nuptial gifts when exposed to draglines of conspecific females, but the opposite was not true (Fig. 5). Three *P. ornata* males did not construct a nuptial gift in either situation (Fig. 5). Males of *P. azul* constructed nuptial gifts at similar frequencies when exposed to draglines of conspecific and heterospecific females (McNemar test: $\chi_1^2 = 0$, $P = 1$; Fig. 5). Most of the males constructed a nuptial gift in both situations, while one male that constructed a nuptial gift when exposed to a conspecific signal did not construct a nuptial gift when exposed to a heterospecific signal (Fig. 5). Two males that constructed a nuptial gift when exposed to a heterospecific signal did not construct a nuptial gift when exposed to conspecific signals and a single male showed no reaction to either signals (Fig. 5). For both species, males showed no difference in the number of wrappings or in the total time of wrapping (Table 3).

Do Females Discriminate Conspecific from Heterospecific Males?

Only *P. ornata* females showed some degree of species recognition when exposed to nuptial gifts from conspecific and heterospecific males (McNemar test: $\chi_1^2 = 5.14$, $P = 0.023$; Fig. 6). Among 15 trials involving conspecific nuptial gifts, the nuptial gift was accepted by *P. ornata* females in all but one case, and among females that accepted conspecific nuptial gifts, seven also accepted the heterospecific nuptial gift (Fig. 6). In contrast, *P. azul* females showed a similar reaction pattern towards conspecific and heterospecific nuptial gifts (McNemar test: $\chi_1^2 = 0.250$, $P = 0.617$). Only one *P. azul* female rejected a conspecific nuptial gift but accepted the heterospecific nuptial gift, whereas three females rejected the heterospecific nuptial
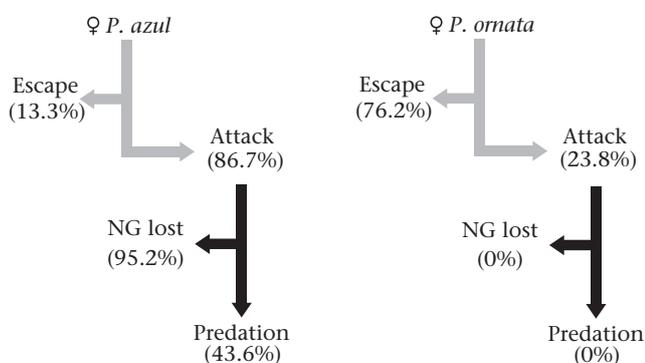

Figure 1. Fluxogram showing the behavioural responses of *Paratrechalea* females (grey arrows) and their consequences for heterospecific males (black arrows) in staged encounters. Percentages in brackets represent the frequency of each event. NG: nuptial gift.
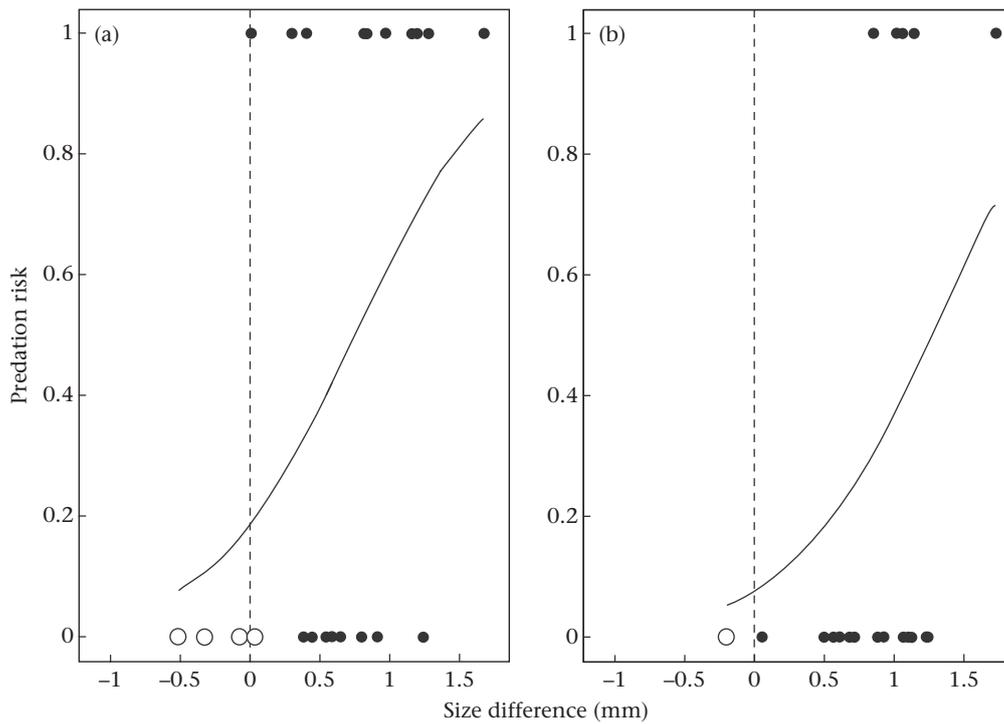
Figure 3. Predation risk for *Paratrechalea azul* and *P. ornata* males (a) with and (b) without a nuptial gift in interactions with heterospecific females. Body size difference was defined as female cephalothorax length minus male cephalothorax length. Open circles: interactions between *P. azul* males and *P. ornata* females; solid circles: interactions between *P. ornata* males and *P. azul* females. The lines represent the adjustment of the best-fitted model (see Table 1).

gift but accepted the conspecific nuptial gift (Fig. 6). The remaining 11 females accepted both conspecific and heterospecific nuptial gifts (Fig. 6).

Furthermore, only *P. ornata* females showed hyperflexion posture towards heterospecific males during the trials (*P. ornata*: 9/30 = 30%; *P. azul*: 0/47; Table 2). Only one of these *P. ornata* females (1/13 = 8%) performed the hyperflexion posture towards a heterospecific male without a nuptial gift, whereas eight of 17 (47%) performed the hyperflexion posture towards heterospecific males with a nuptial gift (Table 2).
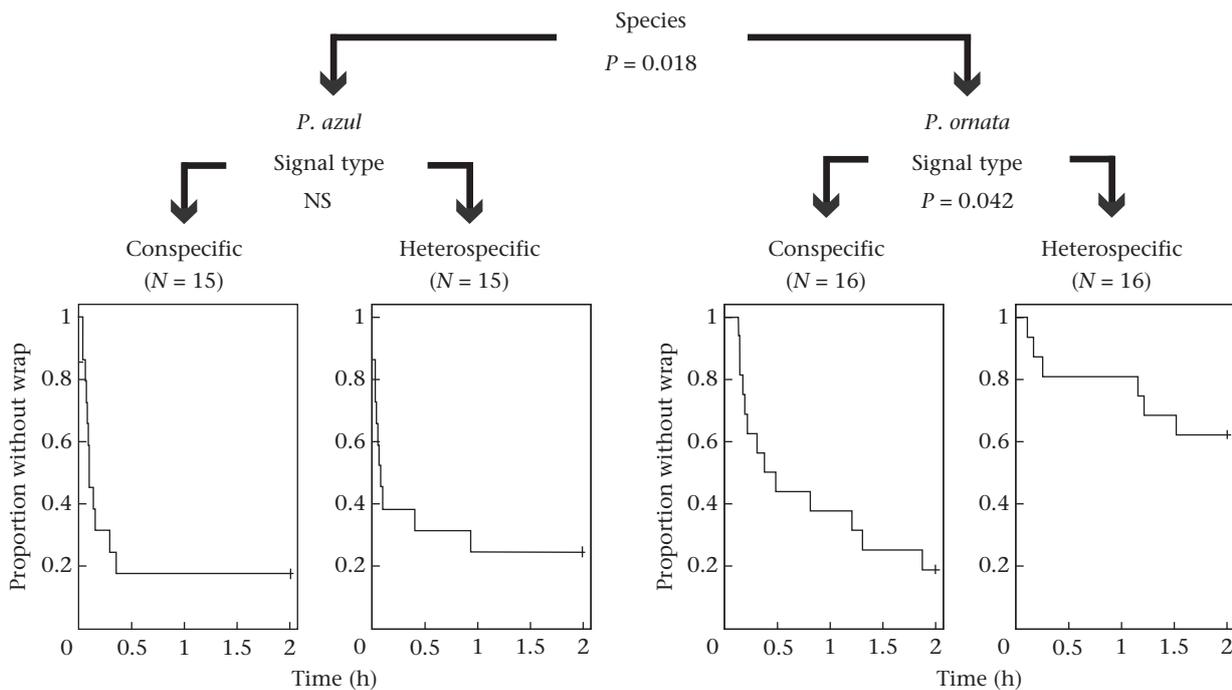

Figure 4. Conditional inference tree for the latency until the first nuptial gift wrapping by *Paratrechalea* males during 2 h trials.
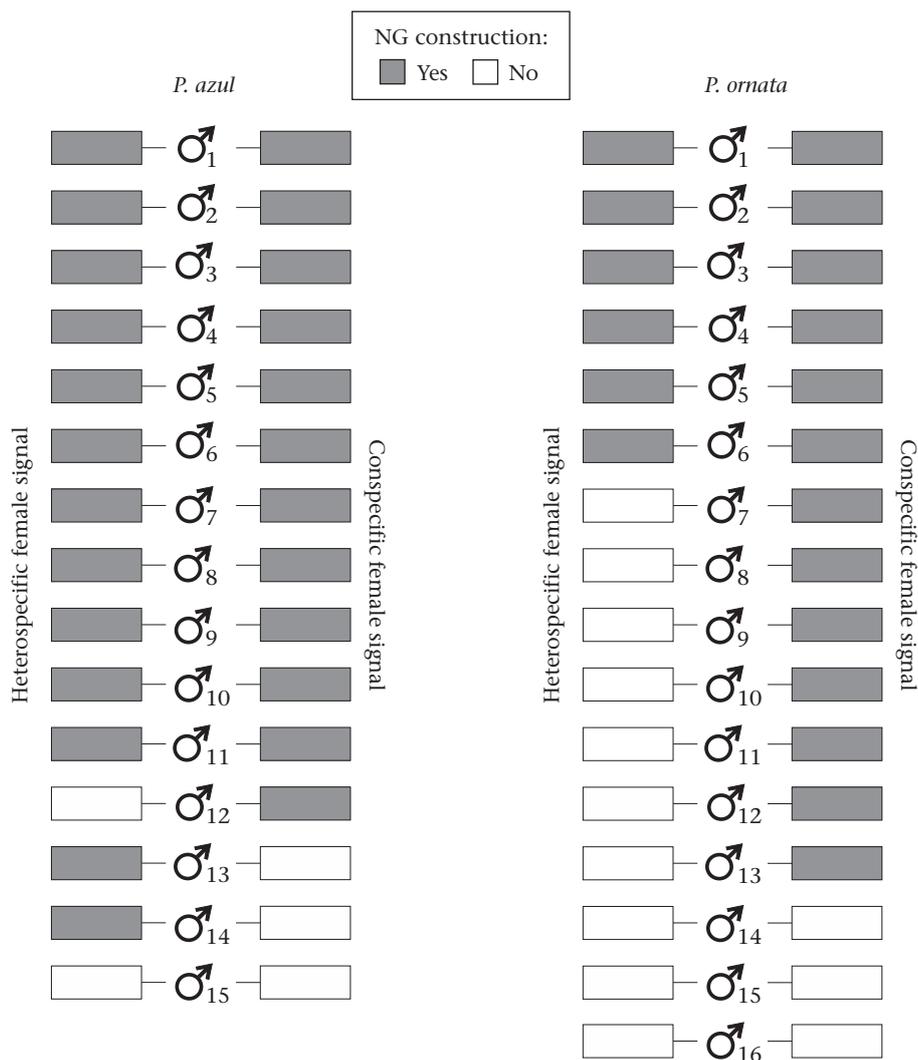
Figure 5. Diagram summarizing the results of the paired experiment designed to investigate whether *Paratrechalea* males are able to discriminate conspecific from heterospecific females based only on chemical cues deposited on their draglines. We evaluated male response as the construction of a nuptial gift (NG) in the presence of conspecific and heterospecific female signals.

DISCUSSION

General Patterns

Here, we present the first evidence of reproductive interference between two sibling spider species in which males offer nuptial gifts

Table 3
Comparisons of the number of prey wrappings and time spent on silk adhesion by *Paratrechalea* males during nuptial gift construction when exposed to signals from conspecific and heterospecific females

Female species signal	Conspecific (mean±SD)	Heterospecific (mean±SD)	Statistics	df	P
P. azul (N=11)					
Prey wrapping (counts)	5.54±2.97	4.54±2.63	Z=12.5	–	0.129
Silk adhesion (s)	255.85±133.93	205.69±82.63	t=−1.163	10	0.134
P. ornata (N=6)					
Prey wrapping (counts)	5.3±1.83	4.5±1.87	t=−1.754	5	0.070
Silk adhesion (s)	235.83±105.35	166.5±69.2	t=−1.752	5	0.070

t: paired Student's t tests; Z: Wilcoxon signed-ranks test.

to females prior to copulation. Males of *P. azul* were unable to discriminate conspecific from heterospecific female signs deposited on a substrate, representing a typical case of misdirected courtship (Fig. 7). This discrimination mistake results in wasted time and energy in nuptial gift construction, because presentation of a nuptial gift is an important component of the male courtship effort in *Paratrechalea* species (Costa-Schmidt et al. 2008; Albo & Costa 2010). However, the costs of reproductive interference for *P. azul* males would most likely be low because they did not court heterospecific females and they consumed the nuptial gift when there was no conspecific female present. Males of *P. ornata* also made discrimination mistakes, but at a much lower frequency than *P. azul* males. The selective pressure for precise recognition of conspecific female signs is most likely stronger on *P. ornata* males because misdirected courtship may increase the chance of encountering predatory heterospecific females. In fact, more than 90% of *P. ornata* males carrying a nuptial gift were attacked by heterospecific females, almost all of which lost the nuptial gift, and nearly 30% of these males were preyed upon. Therefore, the output of reproductive interference is highly asymmetric, with male *P. ornata* paying higher costs than male *P. azul*. This general pattern is most likely a direct consequence of differences in body size between males and females of each species.
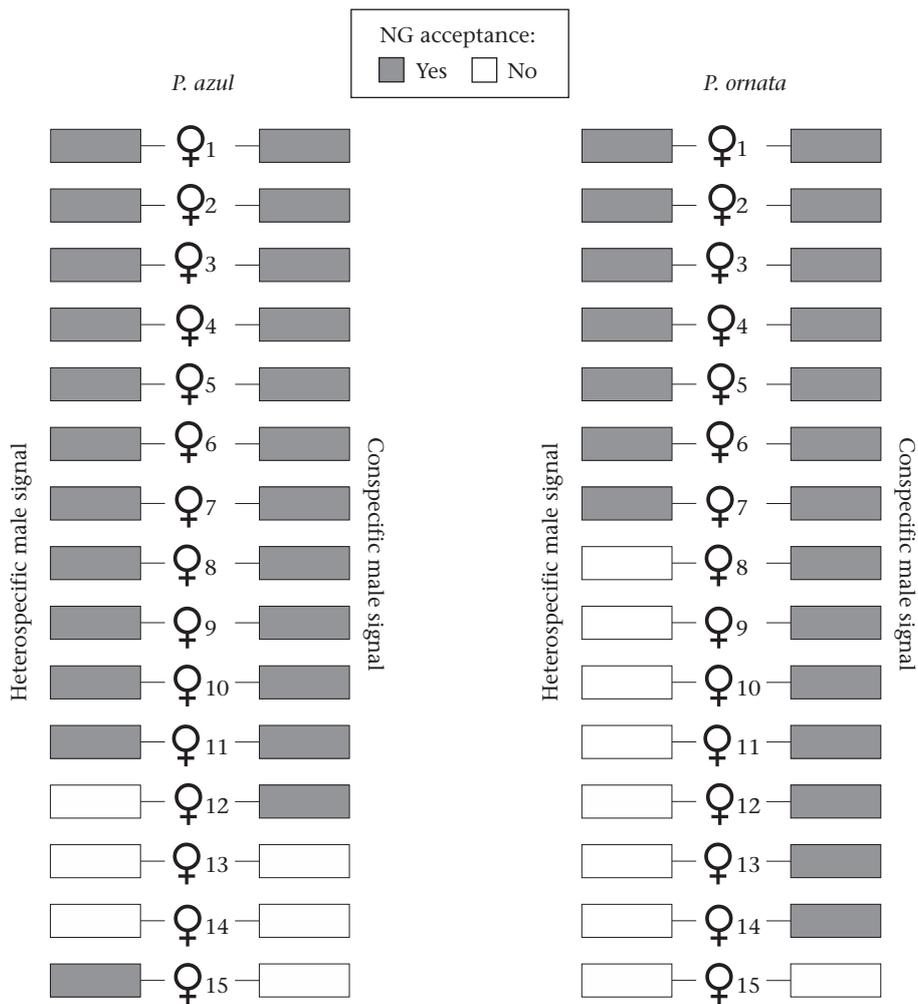
Figure 6. Diagram summarizing the results of the paired experiment designed to investigate whether *Paratrechalea* females discriminate conspecific from heterospecific males based on chemical cues deposited on the silk that wraps the nuptial gift (NG). We evaluated female response as the acceptance of a nuptial gift (NG) from conspecific and heterospecific males.
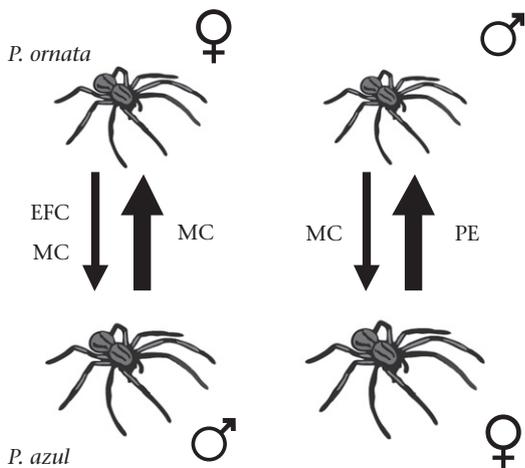

Figure 7. Summary of heterospecific interactions between male and female *Paratrechalea azul* and *P. ornata*. Relative differences in body size between sexes and species are represented. Width of the arrows indicates the frequency of the following interactions: erroneous female choice (EFC), misdirected courtship (MC) and predatory exploitation (PE).

Body size differences may also explain the erroneous female choice of *P. ornata* females towards heterospecific males (Fig. 7). Although *P. ornata* females accepted nuptial gifts from conspecific males more frequently than they did from heterospecific males, some degree of erroneous mate choice occurred because nearly 50% of the females grabbed nuptial gifts from heterospecific males. The interaction between *P. azul* females and *P. ornata* males is more difficult to interpret and classify. Indiscriminate grabbing of both conspecific and heterospecific nuptial gifts could be viewed as erroneous mate choice. However, in the encounters with heterospecific males, *P. azul* females never showed misdirected courtship but, rather, attacked a high proportion of males carrying a nuptial gift. Therefore, it is possible that *P. azul* females are able to recognize chemical signs deposited by heterospecific males on the nuptial gifts and exploit these signs to find and attack potential prey. In this case, the interaction between *P. azul* females and *P. ornata* males would be better described as predatory exploitation of sexual signals (Fig. 7).

Male Perspective

Although sex pheromones are usually attractive only to conspecifics (Wyatt 2003), males of some species may also react to heterospecific sexual cues (e.g. Andrews et al. 1982; Mazor &

Dunkelblum 1992). In *Paratrechalea* species, the decision to construct a nuptial gift is made using pheromonal cues found on silk threads deposited on the substrate by females (Albo et al. 2009). Here, we investigated whether chemical cues deposited on the draglines of heterospecific females could also elicit nuptial gift construction. We found that while *P. ornata* males showed some degree of species recognition, investing in the construction of nuptial gifts more frequently and more promptly when in the presence of conspecific cues, *P. azul* males showed no evidence of species discrimination (Fig. 4). Most adaptive interpretations of species discrimination are related to avoidance of hybridization, which represents an extreme case of reproductive interference (Gröning & Hochkirch 2008). However, in the case of the *Paratrechalea* species studied here, we suggest that predation imposed by heterospecific females is the most important selective force favouring species recognition because males making discrimination mistakes may become prey if they court heterospecific females. Given that only *P. ornata* males suffer from this type of predation, they have most likely developed more accurate mechanisms of species recognition prior to nuptial gift construction when sharing the same habitat with *P. azul*. In the future, it would be interesting to compare the discrimination ability of *P. ornata* males from different populations to test whether males living in allopatry with *P. azul* are more likely to make discrimination mistakes than males living in sympatry.

Our results indicate that possession of a nuptial gift promotes a small increase in the attack risk for *Paratrechalea* males (Fig. 2). At least for *P. ornata*, the nuptial gift was lost in most of the cases in which males were attacked by heterospecific females (Fig. 1). Loss of the nuptial gift can be regarded as a putative expenditure to the males that survive because they invest time, energy and silk in nuptial gift construction (Albo et al. 2009). Interestingly, males without a nuptial gift were consumed slightly more often than males without a nuptial gift (Table 1, Fig. 3). We suggest that the presence of a nuptial gift might protect the males from a predatory heterospecific attack. In fact, one possible role attributed to nuptial gifts is male protection from sexual cannibalism, such that the gift would allow the male to mate without being eaten by the female (Kessel 1955; Bristowe 1958). Although our experiment was not specifically designed to test the protective role of the nuptial gift, behavioural observations show that all *P. ornata* males released the nuptial gift upon female attack (Fig. 1). This behaviour may be a tactic to allow the male increase his speed and/or to distract the potential predator. A similar interpretation has traditionally been used to explain limb autotomy in lizards (Cooper 2003) and harvestmen (Gnaspini & Hara 2007).

Previous studies with the spider *Pisaura mirabilis* have demonstrated that only males in good condition are able to construct a nuptial gift, indicating that this sexual trait is condition dependent (Albo et al. 2011). Similar results have been obtained for *P. ornata* (L. E. Costa-Schmidt & G. Machado, unpublished data), suggesting that the construction of a nuptial gift is costly for spiders. Here, we showed that antagonistic interactions with heterospecific females impose additional costs on *P. ornata* males, represented by the loss of the nuptial gift or even death of the males. If continuous exposure to predation over the course of the reproductive season reduces the fitness of *P. ornata* males carrying a nuptial gift, this sexual trait may act as a reliable indicator of overall male quality. According to this hypothesis, sexual selection favouring nuptial gift expression in *P. ornata* should be stronger in the zone of sympatry with *P. azul* than in the allopatric populations. The available data seem to corroborate this idea: whereas nuptial gift offering is not mandatory for a male to achieve copulation in an allopatric Uruguayan population of *P. ornata* (39% copulation success without a nuptial gift; Albo & Costa 2010), only 16% of males

not carrying a nuptial gift (4/25) achieved copulation at our study site (L. E. Costa-Schmidt & G. Machado, unpublished data), where both *Paratrechalea* species live syntopically.

Female Perspective

Most empirical studies have been devoted to understanding the role of mating signals in the context of female mate choice (Pfennig 1998; Servedio 2007; reviewed in Candolin 2003). However, the importance of mating signals in species recognition becomes evident in situations involving reproductive interference (Gröning & Hochkirch 2008). In *P. ornata*, males deposit phagostimulatory substances on the silk that wraps the nuptial gift, inducing female acceptance during the first phase of the courtship process (Brum et al. 2012). Here, we showed that *P. ornata* females seem to be able to recognize conspecific from heterospecific nuptial gifts, although the frequency of discrimination mistakes was relatively high. This result contradicts our hypothesis that females should accept nuptial gifts from males of any species because this gift represents a cost-free meal. However, if the phagostimulant substances deposited by males on the silk of nuptial gifts act on the female sensory system by exploiting their foraging motivation, as has been suggested for the fishing spider *P. mirabilis* (Bilde et al. 2007), it is possible that the chemical signals include a species-specific component.

Despite the discriminatory ability of *P. ornata* females when exposed to a nuptial gift alone, 47% of these females made discrimination mistakes when exposed to heterospecific males carrying nuptial gifts. In these cases, females showed the stereotyped hyperflexion posture, which is indicative of female receptivity and can be interpreted as a courtship behaviour that induces the male to offer the nuptial gift (Costa-Schmidt et al. 2008). Given that *P. azul* males are 15% larger on average than *P. ornata* males, it is possible that heterospecifics may resemble high-quality conspecifics and may be perceived as especially attractive mates by *P. ornata* females. Mistakes in species and mate quality recognition are expected to be common between closely related species whose geographical ranges have recently overlapped and that have not yet evolved accurate mechanisms of species recognition (Pfennig 2000), which seems to be the case for the two *Paratrechalea* species studied here (Costa-Schmidt & Araújo 2010; present study). However, once the benefits of mate quality recognition become substantial, especially for females, selection should favour individuals that are able to assess both mate quality and species identity simultaneously (Pfennig 1998). To investigate this subject, interpopulation comparisons of the frequency of misdirected courtship in *P. ornata* females should reveal whether recognition mistakes are less common where this species occurs sympatrically with *P. azul*.

The evidence of reproductive interference between *P. azul* females and *P. ornata* males is less clear, and there are at least two possible explanations for our results. When exposed to only a nuptial gift, *P. azul* females showed no indication of species discrimination, as in a typical instance of 'signal jamming' (Gröning & Hochkirch 2008). Thus, these females might be unable to recognize species-specific phagostimulant substances deposited by conspecific males on the silk that wraps the nuptial gift. A second explanation, which is congruent with our hypothesis, is that *P. azul* females are indeed able to recognize conspecific from heterospecific signals on nuptial gifts, but they accept the nuptial gifts indiscriminately because they are an inexpensive food source. In accordance with this second explanation, *P. azul* females never performed the hyperflexion posture towards heterospecific males, regardless of whether the males were carrying a nuptial gift or not. In contrast, in most heterospecific encounters, *P. azul* females attacked and consumed *P. ornata* males. Given that sexual cannibalism has never been observed in *P. azul* (even under limited

laboratory conditions), it appears that females can discriminate heterospecific males using chemical and/or visual cues.

For species in which males rely on conspicuous visual, acoustic or chemical signals to attract females, the mating benefits to be gained from signalling may also result in survival costs because increased conspicuousness may also attract predators or parasitoids that exploit courtship signals (Zuk & Kolluru 1998; Haynes & Yeargan 1999). Additionally, natural enemies can take advantage of the reduced defensive ability of the males when they are expressing conspicuous courtship signals (Magnhagen 1991). Thus, we suggest that *P. azul* females recognize chemical signs deposited by heterospecific males on their nuptial gifts and exploit these signs to find and attack potential prey. Given that the predator and prey in this case are sibling species and most likely share similar sensory channels, *P. ornata* males are particularly vulnerable to predatory exploitation by heterospecific females, which are 20% larger on average. An analogous case of predatory exploitation has been described for the jumping spider *Portia labiata*, in which individuals visually identify and single out egg-carrying females of the spitting spider *Scytodes pallida* as preferred prey (Li & Jackson 2003). Similar to the *Scytodes* eggsac, the *Paratrechalea* nuptial gift is carried with the chelicerae, which may render the carrying male more conspicuous or defenceless against predatory attacks. The hypothesis of predatory exploitation offers interesting possibilities for experimental manipulations, and we predict that *P. azul* males that are forced to carry nuptial gifts constructed by *P. ornata* males will be attacked by conspecific females in staged encounters.

Concluding Remarks

This study provides the first data on reproductive interference between two sibling *Paratrechalea* species that are reproductively isolated. Several important questions remain to be answered, and field studies will be required to obtain a thorough understanding of the costs of reproductive interference for individuals of these two species living in sympatry and allopatry. This study system therefore offers a good opportunity for investigating the ecological and evolutionary role of reproductive interference in two closely related species that diverged recently but show broad secondary overlap in their geographical ranges.

Acknowledgments

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