

## Does behavioral isolation prevent interspecific mating within a parallel ecotypic wolf spider radiation from the Galápagos?

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**Abstract.** Behavioral isolation may play an important role in speciation. However, the roles of divergence time and ecological specialization on the evolution of intrinsic barriers to gene flow are poorly understood. On the Galápagos, ecotypic differentiation of *Hogna* Simon 1885 wolf spiders has led to the repeated evolution of morphologically distinct high-elevation and coastal species on Santa Cruz and San Cristóbal. This offers a unique opportunity to investigate the importance of ecological factors and evolutionary history on courtship behavior, but also to explore the opportunity for interspecific gene flow. On San Cristóbal, both high elevation and coastal *Hogna* species clearly showed distinct courtship behavior. This pattern corresponded primarily with variation in male genital organs rather than with ecotypic classification or phylogenetic relationship. Despite low acceptance rates, heterospecific mating was observed, suggesting that potential gene flow within as well as among islands should not be neglected when seeking to understand island radiations.

**Keywords:** Gene flow, parallel evolution, natural selection, sexual selection

The speciation process necessarily involves the reduction of gene flow between actually or potentially interbreeding populations (Coyne & Orr 2004; Futuyma 2005). If populations diverge in allopatry, spatial isolation serves as an initial isolating barrier (Coyne & Orr 2004). This initial barrier might be re-enforced due to the accumulation of differentially selected traits that reduce interspecific attraction and therefore heterospecific mating; i.e., behavioral/sexual isolation (Andersson 1994; Schluter 2000; Panhuis et al. 2001; Masta & Maddison 2002; Rundle & Nosil 2005). In general, mating traits are predicted to diverge between populations due to mechanisms that are not related to the environment, such as genetic drift and sexual selection (Futuyma 2005). In contrast, in the light of ecological speciation, the evolution of mating traits is predicted to correlate with the environment as a byproduct of natural selection (Boughman 2001; McKinnon & Rundle 2002; Rundle & Nosil 2005) and may as such lead to assortative mating of populations that have undergone similar selection pressures. Hence, scenarios wherein allopatric populations each diverged along a similar selection gradient (i.e., parallel divergence) provide a unique opportunity to test the respective roles of sexual and natural selection in the evolution of behavioral isolation (Boughman 2001; Boughman et al. 2005). Although mating traits most frequently evolve independently among species when populations are isolated, they can also be expected to evolve by species interactions when the diverging populations come into secondary contact and suffer reduced hybrid viability (Dobzhansky 1937; Coyne & Orr 1989).

As behavioral isolation is expected to evolve rapidly between incipient species (Del Solar 1966; Gleason & Ritchie 1998) and even faster than intrinsic postzygotic isolation barriers (Coyne & Orr 1989, 1997, 2004; Mendelson 2003), we here focus on the role of behavioral isolation in the radiation of the ground-dwelling wolf spider genus *Hogna* Simon 1885 from the Galápagos (De Busschere et al. 2010, 2012). As male wolf spiders need to persuade females by courting, differences in courting signals are expected to serve as prezygotic

behavioral isolating mechanisms (Andersson 1994; Uetz 2000; Rypstra et al. 2009). Moreover, male wolf spider courtship, which may involve different sensory channels such as visual, vibratory and chemical signals, often leads to elaborate species-specific male courtship displays (Miller et al. 1998; Hebets & Uetz 2000), enabling delineation of species boundaries between morphologically indistinguishable species (Den Hollander & Dijkstra 1974; Uetz & Denterlein 1979; Töpfer-Hofmann et al. 2000; Chiarle et al. 2010).

Within-island habitat specialization was demonstrated to lead to morphologically highly similar *Hogna* species in similar habitats on both San Cristóbal and Santa Cruz (De Busschere et al. 2010, 2012) (Fig. 1). *Hogna galapagoensis* Banks 1902 and *H. junco* Baert & Maelfait 2008 are morphologically difficult to distinguish (with the exception of genital traits) and are referred to as “high elevation species” occurring on the top of Santa Cruz and San Cristóbal in the dense pampa vegetation dominated by ferns and sedges (Fig. 1). Similarly, *H. hendrickxi* Baert & Maelfait 2008 and *H. snodgrassi* Banks 1902 are morphologically difficult to distinguish and are referred to as coastal dry species found in the dry supralittoral and arid zone along the coast in sparsely vegetated dunes and open shrub land on Santa Cruz and San Cristóbal (Baert et al. 2008c) (Fig. 1). High-elevation species are characterized by darker coloration, smaller body size and smaller eyes than coastal dry species (De Busschere et al. 2012). Contemporary gene flow between these species appears absent, based on allozyme allele frequencies (Baert et al. 2008a) and on spatial isolation of high elevation and coastal dry habitats (Fig. 1). Nevertheless, on Santa Cruz, ecological divergence between *H. galapagoensis* and *H. hendrickxi* has been shown to occur in the face of low levels of gene flow (De Busschere et al. 2010), which is in agreement with the very similar shape of their male copulatory organs (De Busschere et al. 2012). Moreover, based upon Loosveldt (2004) and our sampling campaigns, these *Hogna* species seem to have a similar seasonal life cycle, in which activity is concentrated in the warm wet season from January to May, suggesting no potential role for strong

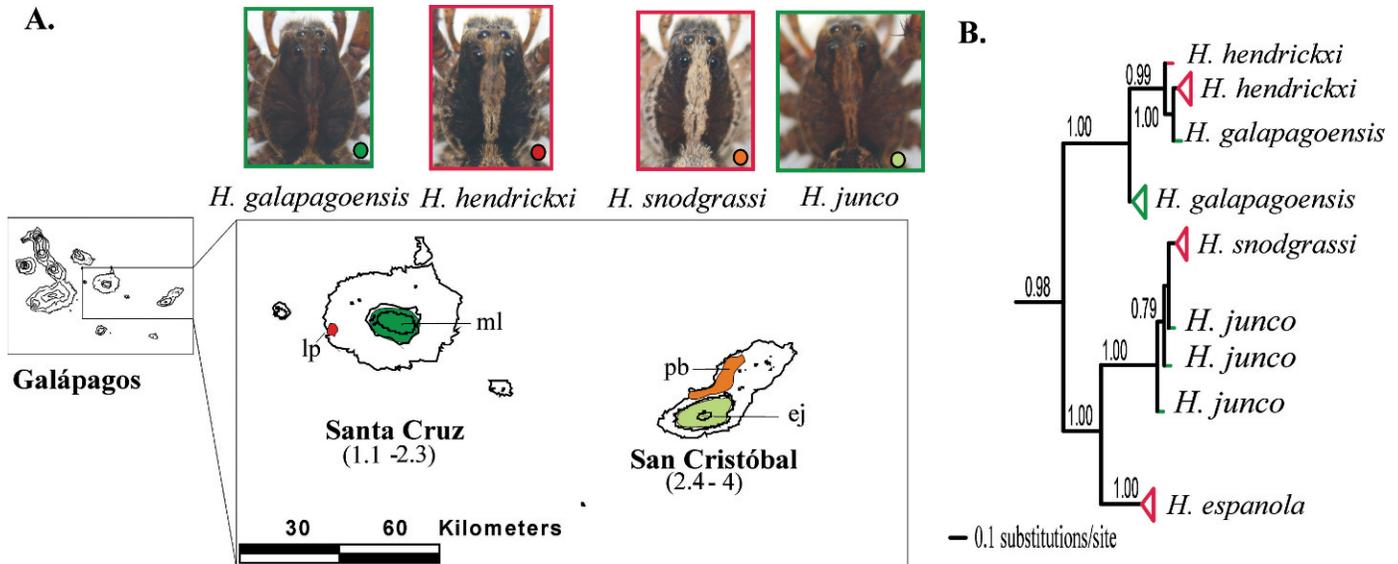


Figure 1.—(A) Geographical distribution (adapted from De Busschere et al. 2010) and (B) COI-28S phylogeny (De Busschere et al., 2010) of high elevation (green) and coastal (red) *Hogna* species on Santa Cruz and San Cristóbal (with exception of *H. espanola*). Node values represent Bayesian posterior probabilities; sampling localities are abbreviated as: Las Palmas (lp), Media luna (ml), Punta Bassa (pb) and Volcán El Junco (ej), and estimated minimum and maximum geological ages (MYA) for each island are in parentheses (D. Geist et al. unpubl. data).

temporal isolation. The latter observation and the signature of historical gene flow demonstrate that gene flow between *H. galapagoensis* and *H. hendrickxi* might still be possible, despite their ancient initial split ( $\sim 0.8$  My) (De Busschere et al. 2010).

This study system allows us to investigate whether strong ecological divergence and/or historical gene flow has led to behavioral isolation by addressing the following research questions: 1) Are there interspecific differences in male courtship behavior, and are these differences related to differences in ecology, or do they correlate with the phylogenetic relationships among the species? 2) Do interspecific differences serve as effective prezygotic isolating barriers? To address these questions, we documented male courtship behavior and performed interspecific mating trials among the four *Hogna* species for which within-island habitat specialization was demonstrated on San Cristóbal and on Santa Cruz.

## METHODS

**Sampling.**—Juvenile and adult *H. galapagoensis* and *H. hendrickxi* from Santa Cruz were sampled at Media Luna (pampa, high elevation) and Las Palmas (coastal), respectively. The species from San Cristóbal, *H. junco* and *H. snodgrassi*, were sampled at Volcán El Junco (pampa, high elevation) and Punta Bassa (coastal) in February 2010, respectively (Fig. 1). Sampling efforts led to a total of 431 specimens with sample sizes ranging from 64 to 187 individuals per species, 52% of which were juveniles. They were housed individually in the quarantine laboratory of the Charles Darwin research station at an average temperature of 20°C and fed ad libitum with two to three wild-caught moths per day (adults) or five fruit flies per day (juveniles). Although many juveniles exuviated in the lab, none of them reached adulthood, suggesting that our laboratory conditions did not adequately mimic the field conditions to induce maturation. Since we could not ensure virginity of the females used in the mating experiments, no

reliable quantitative comparisons can be made concerning the degree of inter- and interspecific acceptance rates (see below).

**Analysis of species-specific male courtship behavior.**—Wild-caught individuals were used in mating trials to describe the species-specific courtship behavior of the four species. The use of wild-caught individuals might confound results due to mating experience, age and mating status of the female. Therefore, we restricted our aim to describing the presence or absence of species-specific male courtship behaviors. Mating trials were performed in a plastic arena (30 × 20 cm) filled with 1 cm of sand. Before each trial, a new filter paper was placed on the sand in order to eliminate signals from previous trials and allowing vibrations. Females were placed in the arena and confronted with 1) a conspecific male, 2) a heterospecific ecotypically similar male (from the other island) or 3) a heterospecific ecotypically dissimilar male (from the same island) after a 5 min period of acclimatization. Trials were observed on average for 20 min. This time frame was chosen based on an initial subset of trials for which we observed that if mating were to occur, it was generally completed within the first 5 min of the experiment. Courtship events were recorded with a HDV camera (SONY HV40 Legria). For each mating trial, the longest recorded complete courtship fragment within those 20 min was used for further analyses. These fragments were chosen if we observed several stages of the mating process starting from the male detecting the female, approaching her and then elaborately courting her until she responded.

A list of five recognizable male courting behaviors was defined and used to score male courtship behavior during the fragment (Table 1; Video 1, online at <http://www.bioone.org/doi/suppl/10.1636/K12-49>). Females' reactions were classified as 1) acceptance of the male (i.e. allowing him to mount), 2) aggressive behavior, or 3) no response. For each mating trial, total male courting time ( $t_{tot}$ ) was assessed, and the absence/presence of male courting behavior was obtained.

Table 1.—Description of male courtship behaviors (see Video 1).

<b>PM</b>	Pedipalp movements: this involves all movements of the male pedipalps and mainly consisted of drumming against the substrate.
<b>FM</b>	Foreleg movements: this involves all movements with the first two pairs of legs and consists of repeatedly raising, waving, tapping, arching and stretching forelegs.
<b>mP</b>	Moderate push-ups: this involves a period of repeated moderate push-ups of the total body invoked by bending the legs.
<b>sP</b>	Strong push-ups: this involves a period of repeated strong push-ups of the total body invoked by strongly bending the legs and leading to jumps.
<b>Po</b>	Poking: repeated poking of the female with the forelegs; forelegs are positioned in front of the male and parallel with the substrate.

First, we tested whether males courted differently; i.e., expressed different courting behaviors to heterospecific females than to conspecific females. This was done for each species by comparing the probability of occurrence of each male courtship behavior toward the females of different species by means of exact Pearson Chi-Square tests (StatXact-5). If no differences were observed in the presence or absence of male courtship behavior with respect to the species of the female, data for heterospecific and conspecific trials were pooled to describe species-specific male courtship. Second, interspecific differences in the probability of expressing a specific courtship behavior among males of the different species were tested with exact Pearson's Chi-Square tests to look for the presence of species-specific courtship behaviors.

**Inter- vs. intraspecific courting.**—Investigating the potential for reproductive isolation should ideally be based upon heterospecific and conspecific choice and no-choice trials using virgin adults. Here, the lack of virgins impedes us from investigating mate preferences directly. However, heterospecific mating trials allowed us to observe whether heterospecific females elicited male courting behavior and whether females could distinguish and reject heterospecific males. Observations of heterospecific acceptances under laboratory conditions might indicate the presence of weak premating barriers. By means of exact Pearson's Chi-Square tests, we tested whether the probability of male courting differed with respect to female species.

## RESULTS

**Interspecific comparison in male courtship behavior.**—Table 2 gives an overview of the total number of trials performed and the number of trials used in the analysis of interspecific comparisons of male courtship behavior. Given that particular courtship traits were consistently expressed irrespective of the species of the female to which the male was exposed to ( $P > 0.17$ ), male courtship data were pooled across female species. Movements of the pedipalps (PM) were observed in males in all four species (Table 3). For the other courtship traits, large differences were observed among species (Table 3). Courtship of males of both species from San Cristobal can be clearly

Table 2.—Sample sizes of total trials and, in parentheses, trials used in courtship analysis.

Males	Females			
	<i>gala</i>	<i>hend</i>	<i>snod</i>	<i>junc</i>
<i>gala</i>	37 (12)	9 (4)	–	13 (5)
<i>hend</i>	13 (5)	13 (6)	3 (1)	–
<i>snod</i>	–	4 (1)	16 (9)	6 (0)
<i>junc</i>	11 (9)	–	5 (4)	16 (10)

distinguished, based on some unique male courting behaviors. *Hogna snodgrassi* males often court for extremely long periods (up to 12 min) by combining palpal drumming with strong push-ups (sP) (Table 3). In comparison, *H. junco* males generally court for much shorter periods and combine pedipalp drumming and quick movements toward the female, and if distance is small, males poke the females repeatedly with their forelegs (Po). Differences in courtship between males of the two Santa Cruz species; i.e., *H. galapagoensis* and *H. hendrickxi*, are much less evident, and both species combine palpal drumming, elaborate movements of the forelegs and moderate push-ups while courting. The latter courtship trait was not observed for the two species from San Cristóbal. Although our quantitative measurements of the courtship of both Santa Cruz species were not significantly different (Table 3), some subtle differences were observed, wherein *H. hendrickxi* males tended to make more use of the second pair of forelegs than *H. galapagoensis* males and often moved their pedipalps sideways while drumming (C. De Busschere pers. observ.). In sum, the species on San Cristóbal, *H. junco* and *H. snodgrassi*, are clearly distinguishable, based upon unique male courtship behaviors.

**Inter and intraspecific copulations.**—Males apparently did not prefer conspecific females, as the number of courtship events a male displayed was not significantly different when exposed to heterospecific females ( $P > 0.45$ ). Although the acceptance rate of courting males was in general very low (10%), few heterospecific mating events were observed, and the acceptance rate among species did not differ from random ( $\chi^2 = 2.6$ ,  $P = 0.46$ ) (Fig. 2). Remarkably, despite clear differences in morphology, *H. galapagoensis* females accepted heterospecific males of *H. hendrickxi*. Moreover, *H. galapagoensis* females also accepted heterospecific males from the distantly related *H. junco*, which has a distinctively different male courtship (Poking) (Fig. 2).

## DISCUSSION

**Interspecific differences in male courtship behavior.**—The mating trials revealed that the high-elevation species *H. junco* and the coastal species *H. snodgrassi*, both from San Cristóbal, show distinct male courtship behaviors. In contrast, our quantitative analysis based on five male courtship traits did not reveal any significant differences between *H. hendrickxi* and *H. galapagoensis* on Santa Cruz. The lack of difference between these species is in strong concordance with earlier studies (Baert et al. 2008b; De Busschere et al. 2012) that noted almost identical male genital traits for *H. hendrickxi* and *H. galapagoensis*, which clearly differ from those of the San Cristóbal species. Furthermore, De Busschere et al. (2012) observed clear interspecific differences in male genital traits between *H. junco* and *H. snodgrassi*. Hence, the variation in male courtship behavior appears congruent with the diver-

Table 3.—Interspecific comparison of male courtship behaviors.

Occurrence	<i>gala</i>	<i>hend</i>	<i>junc</i>	<i>snod</i>	$\chi^2$	<i>P</i>
Total trials	21	12	23	10		
PM	21	12	22	10	1.90	1
FM	21	12	2	10	57.58	<0.0001
mP	19	9	0	0	49.38	<0.0001
sP	1	0	0	10	59.14	<0.0001
Po	0	0	21	0	57.58	<0.0001
Total courting time $t_{tot}$ (s) ( $\pm$ SE)	101 $\pm$ 16	147 $\pm$ 36	50 $\pm$ 10	301 $\pm$ 72		

gence pattern in two male genital structures involved in the copulation process. In contrast, the variation in male courtship behavior contrasts with the ecological divergence into morphologically distinct high elevation and coastal dry species. Therefore, species with a similar habitat preference, which are highly similar in color pattern and in non-genital traits (De Busschere et al. 2012), share no similarities in male courtship behavior, and hence this observation does not suggest that these mating traits evolved as a byproduct of natural selection. Additionally, the variation in male courtship behavior is rather in disagreement with the phylogenetic relationships, as the more recently diverged *H. junco* and *H. snodgrassi* (~0.1 MY ago) are much more different in male courtship behavior than *H. hendrickxi* and *H. galapagoensis*, which diverged approximately 0.8 MY ago, albeit under low levels of gene flow (De Busschere et al. 2010). In sum, on Santa Cruz and San Cristóbal, parallel within-island speciation is only reflected in ecologically relevant traits and not in male courtship behavior. This incongruence indicates that, beside the similar and strong natural selection, different processes influenced the divergence of mating traits.

**Weak prezygotic mating barriers.**—Whether the above-mentioned interspecific differences have the potential to function as premating isolating mechanisms was investigated by performing interspecific mating trials. Beside the differences denoted in male courtship and morphology, we note that hitherto undescribed chemical and vibratory cues might also

influence the outcome of these mating trials (Uetz & Roberts 2002; Roberts & Uetz 2004). Indeed, the latter might be expected, as palpal drumming dominates male courtship, and both drumming and push-up movements might result in vibrations being transmitted through the substrate. In general, female acceptance rate of courting males was low (10%), which was probably due to the use of wild-caught individuals. The latter refers to the possibility that wild-caught females were already fertilized in the field, leading to a possible rejection of courting males in the laboratory (Fernández-Montraveta & Ortega 1990; Rypstra et al 2003). Despite the generally small volume of data, which does not permit us to test for species-specific acceptance rates, females of *H. galapagoensis* accepted heterospecific males. Remarkably, despite clear differences in morphology, females of *H. galapagoensis* accepted heterospecific males of the genetically closely related sister species *H. hendrickxi*. Moreover, *H. galapagoensis* females also accepted heterospecific males from the distantly related but morphologically highly similar *H. junco*, which has a distinct male courtship (Poking). Therefore, despite clear interspecific differences in male courtship behavior and/or morphology, within and between-island acceptances were observed, which suggests that interspecific prezygotic mating barriers are weak for *H. galapagoensis*. This contrasts sharply with other wolf spider studies, in which distinct differences in courtship behavior serve as a strong prezygotic mating barrier (Den Hollander & Dijkstra 1974;

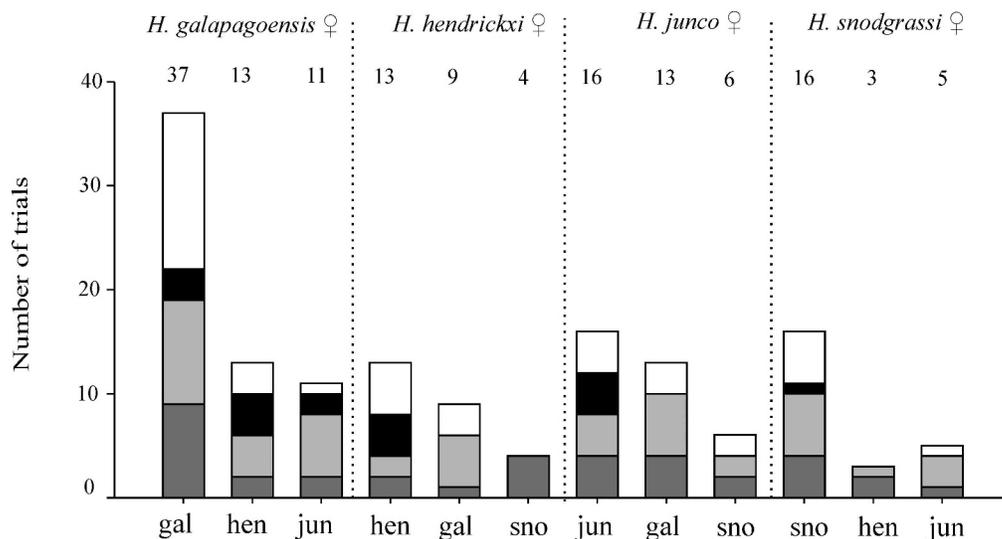


Figure 2.—Number of trials with no males courting (white), acceptance of courting male (black), aggression against courting male (light gray) and no response to courting male (dark gray). Total number of trials are noted above bars.

Uetz & Denterlein 1979; Töpfer-Hofmann et al. 2000; Chiarle et al. 2010). Furthermore, it is remarkable to note potentially weak prezygotic mating barriers, taking into account the deep divergence times [*H. galapagoensis*-*H. hendrickxi*: ~0.8MY and *H. galapagoensis*-*H. junco*: ~1.6MY ago (De Busschere et al., 2010)] and strong ecotypic divergence. The lack of premating barriers has also been found between allopatric lineages of warbler finches on Galápagos, despite differences in song and a long divergence time (1.5–2 MY) (Grant & Grant 2002). Weak prezygotic barriers might be explained by the lack of or weak selection against hybridization due to low levels of gene flow, and hence a predominant divergence in allopatry (Coyne & Orr 1989). Therefore, females of *H. galapagoensis* were not forced to recognize heterospecific males. The lack of frequent interactions has also been suggested for the absence of strong mating isolation between stream and lake sticklebacks (Raeymaekers et al. 2010). Indeed, range overlap between both ecotypes on Santa Cruz and San Cristóbal might have been limited to periods of environmental and climatological change (De Busschere et al. 2010).

Despite the current spatial isolation, the potential for weak prezygotic mating barriers points out that *Hogna* species boundaries, especially of *H. galapagoensis*, could be fragile in the case of future secondary contact. Moreover, the potential for weak prezygotic reproductive barriers for *H. galapagoensis* in combination with the detection of ancient hybridization events between *H. galapagoensis* and *H. hendrickxi* (De Busschere et al. 2010) and of inter-island dispersal of *H. galapagoensis* (Fig. 1), suggest a potential role of within and between-island gene flow in the *Hogna* radiation. Further exploration of the potential role of gene flow should not be neglected in understanding the *Hogna* radiation on Galápagos, as hybridization among diverging populations might enhance the spread of adaptive genetic variation and as such catalyze adaptive divergence (Seehausen 2004; Barrett & Schluter 2008; Schluter & Conte 2009) and facilitate recurrent phenotypic evolution. However, the possibility of other mating barriers such as assortative mating related to habitat preference (Rundle et al. 2000; Boughman 2001), natural selection against migrants and hybrids (Hendry 2004; Nosil & Crespi 2004) and the role of mechanical and postzygotic isolation mechanisms, definitely needs further assessment.

In sum, this study provided an initial view of the role of behavioral isolation among habitat-specialized wolf spiders on the Galápagos and emphasizes the need for further assessment of the degree of reproductive isolation and the potential role of within and between-island gene flow to understand the *Hogna* radiation on the Galápagos.

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