# Reproductive isolation between two populations of *Aglaoctenus lagotis*, a funnel-web wolf spider

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*Aglaoctenus lagotis* (Lycosidae: Sosippinae) is a spider that, in contrast to the predominant wandering habit of the family, constructs funnel webs. The species is widely distributed throughout the Neotropics and is credited with high levels of intraspecific variation. Here, we evaluate whether reproductive isolating barriers operate between some populations of *A. lagotis*. We used heterotypic encounters between individuals from two distant localities: southern Uruguay (SU) and Central Argentina (CA). Additionally, we used spiders from an intermediate locality, western Uruguay (WU), where both forms of the species overlap (SU.WU was used to describe individuals from WU reminiscent of those from SU; and CA.WU was used to describe individuals from WU reminiscent of those from CA). No copulations occurred between SU and CA individuals, whereas a single and atypical copulation occurred between SU.WU and CA.WU individuals. Attacks (only by females on males) were rare. In tests of choice based on silk cues, SU males did not prefer homotypic cues but almost did not court CA females, whereas CA males preferred homotypic cues but usually courted heterotypic females. These findings, with a previously reported temporal asynchrony between populations, suggest the occurrence of reproductive isolation between both spider forms and a speciation process favoured by the wide distribution and plasticity of the species.   © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **114**, 646–658.

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## INTRODUCTION

According to the biological species concept (Mayr, 1942), speciation occurs with the evolution of reproductive isolation between populations (Coyne & Orr, 1998; Tregenza, 2002). The process may occur in allopatry (when populations become geographically separated, resulting in little or no gene flow between them) or in sympatry (when geographical barriers do not exist and the separation occurs based on intrinsic reproductive isolating barriers) (Futuyma, 2005). In any scenario, speciation is considered as complete when reproductive isolation is observed in sympatry (Coyne & Orr, 1998). Sometimes, reinforcement can occur in areas of coexistence, where sibling species with unfit hybrid offspring increase reproductive isolation barriers (The Marie Curie SPECIATION Network, 2012). Mating occurrence does not always ensure the absence of speciation but can signal a process of incomplete divergence or one finished in which hybrids are unable to leave fertile offspring (Butlin & Tregenza, 1997).

Studies focusing on speciation processes and the putative mechanisms that promote them usually compare morphological, chemical, and behavioural characteristics between sibling species in sympatric

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areas (Stratton, 1997; Gabirot et al., 2010; Oxford & Croucher, 2014; Santos et al., 2014), or between members from the same species that show differences in any of the characteristics mentioned (Clark & Biesiadecki, 2002; Roberts, 2006; Gabirot, López & Martín, 2012; Marchiori, Bartholomei-Santos & Santos, 2014). Many publications on species origin can be found in amphibians (Verrell, 2003; Pröhl et al., 2006), fishes (Barluenga et al., 2006; González-Zuarth & Macías-García, 2006), snails (Kuppernagel & Baur, 2011), insects (Claridge, den Hollander & Morgan, 1985; Saarikettu, Limatainen & Hoikkala, 2005; Veen et al., 2011), and spiders (Gillespie, 1999; Ayoub, Riechert & Small, 2005; Crews & Hedin, 2006). Particularly in wolf spiders, studies have been carried out in the wandering species (Uetz & Denterlein, 1979; Barthel & von Helversen, 1990; Kronestedt, 1990; Miller et al., 1998; Aisenberg & Costa, 2008; De Busschere & Hendrickx, 2013; Puzin, Leroy & Pétillon, 2014), the majority in the Lycosidae family (Foelix, 2011), but almost no data exist on the atypical web-living species, except for the morphological study of the genus Sosippus by Brady (1972).

Behaviour evolves quickly in response to environmental changes, which is why it may be the most easily detected feature in speciation events (Barlow, 2002). Indeed, several studies suggest the emergence of new species as a result of differences in behaviour between populations during sexual interactions (Pillay & Rymer, 2012; Oh & Shaw, 2013), although not all cases with intraspecific differences of this kind could reflect individual variation or personalities (Claridge & Morgan, 1993). As sexual interactions usually involve a rich communicatory exchange (Coyne & Orr, 2004), in some scenarios multiple signals are used to recognize conspecifics, whereas in others a single signal can give information about more than one individual trait, such as sexual identity and mate quality (Candolin, 2003; Hebets & Papaj, 2005; Rypstra et al., 2009). Sometimes, only some communication channels are involved in species recognition and they can be in conflict with those channels concerning mate choice (Leonard & Hedrick, 2009; Velásquez et al., 2013) or lead to potential misleading recognition, especially between closely related species (Wyman et al., 2014). Spiders with complex courtship behaviours (involving more than one communication channel), such as salticids, agelenids, and lycosids, have been considered good candidates for studying the function of the different communication signals (Uetz & Denterlein, 1979; Costa & Capocasale, 1984; Hebets & Uetz, 1999; Elias, Hebets & Hoy, 2006; Galasso, 2012; Chiarle & Isaia, 2013).

In species with wide geographical distribution, variation (in behaviour, morphology, and phenology, among others) tends to increase as a result of the diverse conditions to which their members are exposed (De Witt & Scheiner, 2004; Moraiti et al., 2012). In some scenarios, this intraspecific variation has a strong genetic basis (Begon, Townsend & Harper, 2006; Velásquez et al., 2013), whereas in others it results from expression of the same genotype in different environments (phenotypic plasticity; Pigliucci, 2001). Both sources of variation can lead to speciation (West-Eberhard, 2003, 2005; Ghalambor et al., 2007; The Marie Curie SPECIATION Network, 2012) but can also persist without the occurrence of reproductive isolation (Nossil, 2008; Michel, 2012). However, when the variation is related to sexual behaviour, with the special role it plays in lack of sexual incompatibility (Verrell, 1999), the probability of reproduction isolation is high. Many examples have been reported in spiders, even of species that only appear to differ from others in mating behaviour (Costa & Capocasale, 1984; Barthel & von Helversen, 1990; Töpfer-Hofmann, Cordes & von Helversen, 2000). According to Galasso (2012), divergence in mating behaviour, at least originated by assortative matings, would be improbable in web-building spiders because of the occurrence of all sexual encounters in the web, a predicable arena compared with those of the wandering spiders.

Aglaoctenus lagotis (Holmberg 1876) is a Sosippinae lycosid spider that, unlike most members of the family, is sedentary, inhabiting funnel webs (Capocasale, 1982; Foelix, 2011). Its taxonomic status has received several changes (Santos & Brescovit, 2001), but currently it is the species with the widest geographical distribution of the small South American genus Aglaoctenus (from Uruguay to Venezuela) (Piacentini, 2011). Previous publications on A. lagotis have described sexual behaviour (Stefani et al., 2011), oviposition and postembryonic development (Stefani & Del-Claro, 2011), and the effects of habitat fragmentation (Stefani & Del-Claro, 2014) in Brazilian populations. Additionally, Santos & Brescovit (2001) characterized A. lagotis as a highly variable species, although no interpopulation studies were reported prior to González et al. (2013).

González et al. (2013) described different sexual repertoires in two distant populations of A. lagotis (south Uruguay and central Argentina), both of which were dissimilar to the description published by Stefani et al. (2011) for populations from Brazil. The most representative courtship acts in southern Uruguay were web stretching, striding forward, and foreleg elevation, whereas in central Argentina they were alternate waving, web stretching, and leg tapping. Females from both localities performed leg tapping, commonly during the first stages of courtship interactions (González et al., 2013). Copulation occurs

with the male mounted on the female dorsum and opposite to her (the typical copulating position for all lycosid spiders), and males performed several ejaculations per insertion of their palps, which differed in duration and frequency between southern Uruguay and central Argentina individuals (González *et al*., 2013). Additionally, a later study showed that those distant populations present marked differences in their phenological patterns, so much so that sexual periods occur in different seasons (González, Costa & Peretti, 2014). Individuals also seemed to differ in body-pigmentation patterns (M. González, unpubl. data). To date, it is unknown whether these differences are effectively generating a reproductive barrier between these populations.

In this study, we evaluate whether mechanisms of mating isolation occur between populations of *A. lagotis*. With this purpose we investigate heterotypic sexual behaviours during direct sexual encounters (courtship and copulation tests) and by male choice based on female silk cues. According to Tregenza (2002), the observation of sexual behaviours between populations of a single species and between hybrid zones from closely related species are important approaches for detecting speciation events. Therefore, here we work with individuals from two distant localities (southern Uruguay and central Argentina) (allopatric) and with members from a third, geographically intermediate, locality (western Uruguay), where both forms of the species co-occur (one of them resembling those from southern Uruguay and the other those from central Argentina) (sympatric). We test how much the different sexual repertoires reported for *A. lagotis* influence the sexual isolation between populations.

When we began interpopulation studies with *A. lagotis*, we expected to find that the intraspecific variation attributed to the species by Santos & Brescovit (2001) was caused by phenotypic plasticity. This assumption was based on the wide distribution given to this lycosid and the diversity of habitats involved, as is characteristic in the Neotropics (Morrone, 2001) region within which this species is distributed. Additionally, the revision of the genus *Aglaoctenus* (Santos & Brescovit, 2001) unifies previously described species of the genus in only two (even after considering specimens from the localities studied here). Under this hypothesis we predict that greater variation will be found in morphological and behavioural reproductive traits of species from the Neotropics than in the species from the comparatively homogeneous Palaeotropics (Macías-Ordóñez, Machado & Macedo, 2014), but distributed within populations as between populations. This is why we expect that our experimental encounters under laboratory conditions (as in nature, sexual periods of the forms do not overlap) will lead to heterotypic sexual displays and copulations, but in lower proportions than in intrapopulation encounters reported by González *et al*. (2013), whereas no preferences based on silk cues are expected (as shown for other spiders by Barth & Schmitt, 1991). However, bearing in mind the differences encountered in previous studies (González *et al*., 2013, 2014), the intraspecific variation could not be homogeneously distributed, putting in doubt a single-species hypothesis for *A. lagotis*. This study sheds light on the controversial taxonomic history of this subfamily of spiders and the speciation processes involved in these sedentary web-living members of a family of wandering spiders.

## MATERIAL AND METHODS

### COLLECTING AND HOUSING

We chose three localities (environmental characteristics given in González *et al*., 2014) for collecting adult and subadult individuals of *A. lagotis*: Piedras de Afilar, Canelones, southern Uruguay (SU: 34°43′44″S, 55°30′46″W) and Sierras Chicas, western Córdoba, central Argentina (CA: 30°57′10″S, 64°15′28″W) as the two distant localities, and Fray Bentos, Rio Negro, western Uruguay bordering with Argentina (WU: 33°06′45″S, 58°17′11″W) as the third locality, geographically intermediate to the previous locations, where the distribution of the two populations overlap (Fig. 1). Collection periods were consistent with the sexual period of each population (González *et al*., 2014): early autumn (during March and April of 2011 and 2012) for SU, late winter/early spring (from August to October of 2011 and 2012) for CA, and during March of 2011 and 2012 in WU. During that last period and in that locality we found individuals resembling those from SU (thereafter termed SU.WU) and others resembling those from CA (termed CA.WU). We distinguished them based on external body-pigmentation patterns and phenological patterns (we collected during March, encountering small juveniles from CA.WU and larger, almost penultimate, individuals from SU.WU) (González *et al*., 2013, 2014). We captured the spiders during daylight, by manually blocking their silk tubes. We obtained 68 subadult individuals and eight adult males from Piedras de Afilar (SU), 72 subadult individuals and six adult males from Sierras Chicas (CA), and 60 subadults from Fray Bentos (WU). Males from the field were considered as virgins because we captured them in their own webs, before abandonment to search for females (M. González, pers. observ.; reported for other funnel-web spiders by Singer & Riechert, 1995).

Individuals belonging to SU (and SU.WU) had an orange cephalothorax during the subadult stages
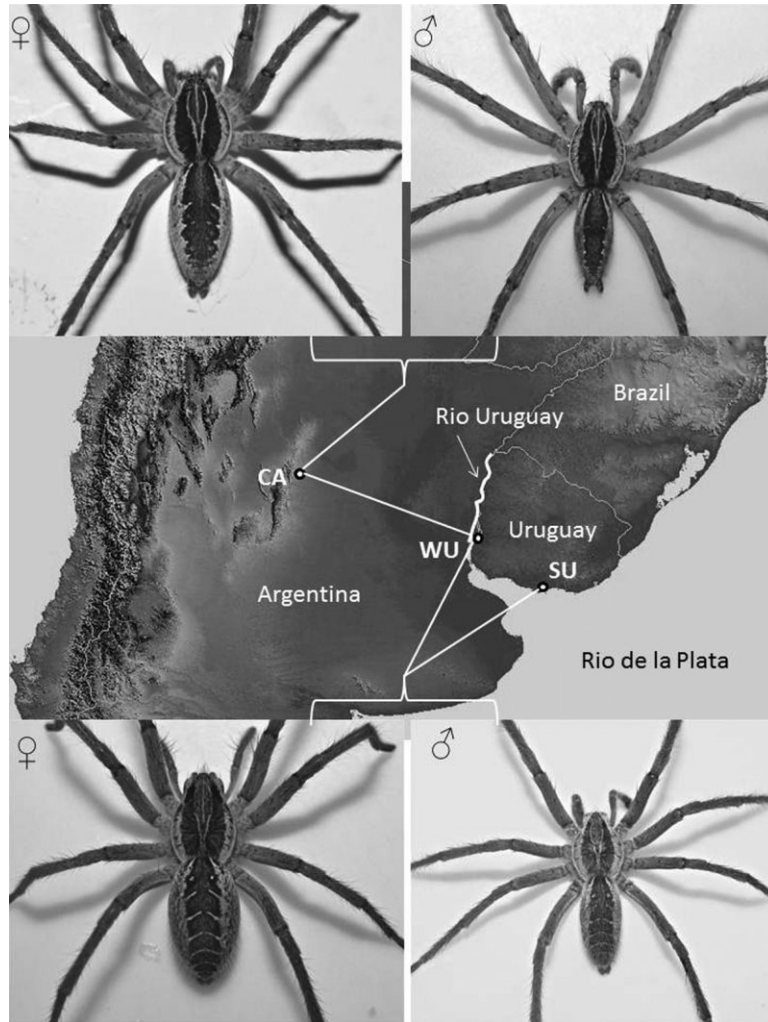
**Figure 1.** Geographical location of the localities studied (southern Uruguay (SU), central Argentina (CA), and western Uruguay (WU) and body-pigmentation patterns of *Aglaoctenus lagotis* individuals inhabiting each locality; females and males from SU (and SU.WU) are at the bottom and females and males from CA (and CA.WU) are at the top.

(Supporting Information Fig. S1), which turned brown on reaching maturity. There are two dorsolateral white bands on the abdomen, from which several pairs of white lines, originating at an angle of 45°, converge in the midline to form forward-pointing 'chevrons' (Fig. 1). The sexual period occurred during autumn, and the maternal period occurred during spring and summer (González *et al*., 2014). Individuals assigned to CA (and CA.WU) had a brown cephalothorax during all developmental stages. The pattern on the abdomen was the same as above except that the 45° white lines did not meet in the midline (Supporting Information Fig. S1 and Fig. 1). The reproductive period (sexual plus maternal) takes place during spring and summer (González *et al*., 2014).

We performed the trials at the Laboratorio de Etología, Ecología y Evolución, IIBCE, Montevideo, Uruguay, and the Laboratorio de Biología Reproductiva y Evolución, IDEA-UNC, Córdoba, Argentina. Because of the different phenological patterns of the individuals involved in the trials, they were raised in laboratory conditions to achieve temporal overlapping of sexual periods, which are separated under natural conditions. We accelerated the growth of CA and CA.WU by keeping them at an average temperature of 23 °C, and slowed the growth of SU and SU.WU by keeping them at 20 °C. Adult and subadult spiders were individually housed in Petri dishes (of diameter 9.5 cm and height 1.5 cm) with a piece of cotton soaked in water. We fed individuals twice a week with a mixed diet of mealworm

larvae (*Tenebrio* sp.; Coleoptera: Tenebrionidae) and small crickets (*Acheta domestica*; Orthoptera: Gryllidae). We monitored individuals daily and recorded the occurrence of moulting in subadults to determine the exact date that they reached adulthood. We deposited voucher specimens (from all three localities) in the arachnological collections of the Facultad de Ciencias, UdelaR, Montevideo, Uruguay, and the Cátedra de Diversidad Animal I, Facultad de Ciencias Exactas, Físicas y Naturales, UNC, Argentina.

### EXPERIMENTAL DESIGN

*Sexual interactions*

We exposed SU males to CA females occupying their webs (15 couples), as well as CA males to SU females in their webs (15 couples) (trials between distant localities). We also exposed males of SU.WU to females of CA.WU (10 couples) and males of CA.WU to females of SU.WU (10 couples) (trials within overlapping locality, see Table 1). We used virgin females 10 days after they had reached adulthood (ensuring chemical sexual attractiveness, according to Baruffaldi & Costa, 2010, for another wolf spider) and males 7 days after reaching adulthood (to ensure cuticle hardiness), or at least 7 days after their capture in the field, to allow acclimatization to captivity conditions. All males were exposed to homotypic females (not included in the experimental groups) a week before experimental trials to ensure that they were capable of courting. We performed the trials during daylight, according to our observations of matings at the field (González *et al.*, 2013). We carried out the experimental trials in terrariums (length 30 cm, width 16 cm, height 20 cm), with a 2-cm layer of sand and 2 cm of wood-chips as substrate, simulating leaf litter. We also added small branches to provide refuge and web support, and a water source in small lids. To allow funnel-web construction we placed each virgin female in the arena 5 days before the trial.

The trial started when we placed one male (randomly selected) on the margin of the female web. We registered the occurrence and duration of the behavioural acts of both individuals according to González *et al.* (2013). We recorded the trials using a Sony DCR-SR85 HD digital video camera and analyzed the videos using JWATCHER software (Blumstein, Evans & Daniel, 2000). After each trial, we weighed the adult individuals and measured their carapace width, a representative measure of body size in spiders (Eberhard *et al.*, 1998; Foelix, 2011). Courtship and mating frequencies obtained were compared with data from homotypic encounters in SU and CA published by González *et al.* (2013), observed under the same laboratory conditions used here. Crosses between SU-SU.WU or CA-CA.WU individuals were not attempted because of differences in the timing of the experiments in this study and the seasons of maturity in the field in SU and CA, respectively.

*Male choice on silk cues*

We tested whether searching males are able to discriminate between females from their own locality and those from another locality based exclusively on silk cues. Trials were performed only with individuals from the distant localities (SU and CA) as there was an insufficient number of individuals from the overlapping locality (WU). We used only virgin individuals. We registered the choice of a male (N = 20, from each locality) when exposed simultaneously to silk threads deposited by two females: one female from the same locality and the other from the distant locality. We used a T-shaped device (see Fig. 2) with two arms, separated by a removable cardboard barrier (modified from Baruffaldi & Costa, 2014). The floor of the device was covered with black corrugated cardboard (replaced with a new one for each trial) to facilitate attachment of the silk threads and its visualization by the observer. Additionally, we prevented deposition of silk thread on the walls of the device, and the concomitant loss of silk threads during

**Table 1.** Percentages of courtship, copulation, and attack occurrence during the experimental trials

| | *N* | ♀ Courtship (%) | ♂ Courtship (%) | Copulation (%) | Attack (%) |
|---|---|---|---|---|---|
| Distant localities | | | | | |
| ♀CA × ♂SU | 15 | 27 | 47 | 0 | 0 |
| ♀SU × ♂CA | 15 | 27 | 60 | 0 | 7 |
| Overlapping localities | | | | | |
| ♀CA.WU × ♂SU.WU | 10 | 30 | 0 | 0 | 0 |
| ♀SU.WU × ♂CA.WU | 10 | 70 | 80 | 10 | 0 |

CA, central Argentina population; CA.WU, western Uruguay reminiscent of CA; SU, southern Uruguay population; SU.WU, western Uruguay reminiscent of SU.
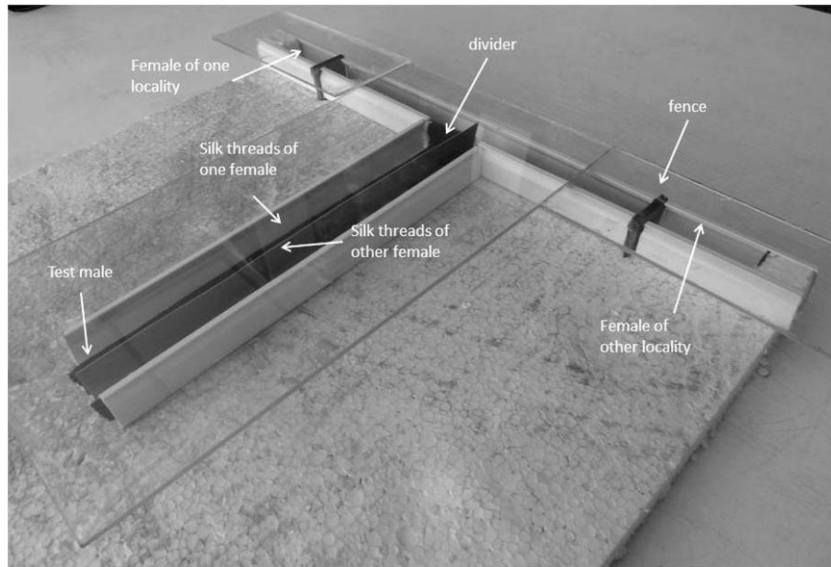
**Figure 2.** T-Shaped device used to evaluate male choice when exposed, simultaneously, to silk threads from females of the two distant localities (southern Uruguay and central Argentina).

barrier removal, by covering the walls with liquid petrolatum (Hronsky, 2006). Each female was placed in one of the arms 48 h before the trial, allowing her silk to be deposited. Before the beginning of the trial, each female was confined behind a fence at the ends of the branches of the 'T' (to facilitate their removal from the experimental device) and the central barrier in the stem of the T was removed. Thus, each male was placed in the basal end of the 'T', exposing him to the silk threads of both females at the same time. Each trial lasted for 1 h, a time period considered sufficient in similar studies of lycosids (Aisenberg, Baruffaldi & González, 2010; Stefani *et al*., 2011). We registered first male choice (when the male turned at one corner of the T-shape device), time spent in the chosen arm (permanence), and time spent by the male to make the decision (choice latency).

## STATISTICAL ANALYSIS

We analysed the results using the statistical packages PAST v.1.18 (Hammer, Harper & Ryan, 2001) and NCSS 2007 (Hintze, 2007). We checked data for conformity to a normal distribution using the Shapiro–Wilk test and homogeneity of variances with the Levene test. We used the Kruskall–Wallis one-way analysis of variance and the Mann–Whitney $U$-test for nonparametric data with adjustment made to the $P$ value using Bonferroni correction. Frequencies were compared using the chi-square test for two independent samples and the exact Fisher probability test (for small samples). We performed multiple linear regressions to establish whether the size and the weight of individuals were related to the duration or intensity of the courtship they performed.

## RESULTS

### SEXUAL INTERACTIONS

Courtship, copulation, and attack occurrences in all experimental groups are shown in Table 1. We did not observe copulations between SU and CA individuals. Although males of both localities courted females from the other, courtship occurrence was more frequently performed by CA males (Fisher's exact test: $P = 0.012$). The percentages of females that showed leg tapping to males from their corresponding locality were the same in SU and CA. When females performed leg tapping, males always courted. Females attacked males only in the SU (female) × CA (male) group (Table 1). In a total of 20 trials between SU.WU × CA.WU, only one copulation occurred in the SU.WU (female) × CA.WU (male) group. All courting males from CA performed the complete pattern of courtship (web stretching and alternate waving). Fourteen per cent of SU males displayed the complete courtship pattern (i.e. web stretching, forelegs elevation, and striding forwards), whereas the remaining males only performed web stretching. CA.WU males showed the courtship pattern reported for CA but SU.WU males only performed web stretching.

In the trials [SU (female) × CA (male)] in which females did not attack the males, three males tried to mount the females (that had previously performed leg tapping), but the females did not lie on the web (as is necessary in lycosids for the mount occurrence)

**Table 2.** Frequencies (number of occurrences min$^{-1}$) of the different behaviours registered during courtship and copulation

| | ♀ Courtship | ♂ Courtship | | | | Copulation | | |
| | Leg tapping | Alternate waving | Web stretching | Foreleg elevating | Striding forward | Insertion | Ejaculation | Body-shaking |
|---|---|---|---|---|---|---|---|---|
| Distant localities | | | | | | | | |
| ♀CA × ♂SU | 0.04 | – | 0.76 | 0.53 | 0.58 | – | – | – |
| ♀SU × ♂CA | 0.57 ± 0.68 | 2.53 ± 1.31 | 1.93 ± 1.40 | – | – | – | – | – |
| Overlapping localities | | | | | | | | |
| ♀CA.WU × ♂SU.WU | – | – | – | – | – | – | – | – |
| ♀SU.WU × ♂CA.WU | 0.37 ± 0.33 | 2.86 ± 1.72 | 0.95 ± 0.36 | – | – | 0.77 | 2.71 | 0.21 |

Data are shown as mean ± standard deviation (SD).

(Foelix, 2011). In six sexual encounters, individuals remained motionless during all the trial and in the others males courted, but never touched, the females. In the trials CA (female) × SU (male), seven males courted (rarely the complete behavioural pattern) and four females performed leg tapping, but they never touched each other. The other sexual pairs stayed motionless. In the SU.WU (female) × CA.WU (male) trial, in which copulation did not occur, the majority of the females and males courted and three of them tried to mount repeatedly, but the females did not lie on the web. In the other trials, males courted but never touched the female, except for one in which both individuals stayed motionless. In the CA.WU (female) × SU.WU (male) trial, males did not court but three females performed leg tapping. However, they never touched each other and stayed motionless for most of the experimental time.

The only SU.WU (female) × CA.WU (male) couple that copulated had the longest courtship duration and an intermediate copulation duration, compared either with other experimental groups or with homotypical trials reported by González *et al*. (2013). Courtship duration (mean ± SD) was 15.4 min (SU × SU, 2.91 ± 3.33 min; CA × CA, 9.47 ± 6.10 min) including 0.78 leg tapping min$^{-1}$, 3.70 alternate waving min$^{-1}$, and 1.30 web stretching min$^{-1}$. Leg-tapping and web-stretching frequencies were highest in the group SU.WU (female) × CA.WU (male), and alternate waving was the third highest (out of 10). Copulation duration was 19.3 min (SU × SU, 60.9 ± 55.5 min; CA × CA, 7.84 ± 3.54 min), which included a rate of 0.77 insertions min$^{-1}$, 2.71 ejaculations min$^{-1}$, and 0.21 female shakings min$^{-1}$ (Table 2). The female died 1 month after copulation, without ovipositing. Her carapace width was 4.2 mm and she weighed 0.24 g, a small female in her group. The male carapace width was 4.7 mm and he weighed 0.22 g, a large male in his group (Table 3).

**Table 3.** Measurements of selected body characteristics of females and males from each experimental group

| Gender | N | Carapace width (mm) | Body weight (g) |
|---|---|---|---|
| ♀ | | | |
| SU | 15 | 5.29 ± 0.52 | 0.33 ± 0.09 |
| SU.WU | 10 | 5.04 ± 0.72 | 0.31 ± 0.09 |
| CA | 15 | 4.61 ± 0.56 | 0.28 ± 0.06 |
| CA.WU | 10 | 4.65 ± 0.49 | 0.31 ± 0.07 |
| ♂ | | | |
| SU | 15 | 4.99 ± 0.75 | 0.24 ± 0.05 |
| SU.WU | 10 | 4.87 ± 0.29 | 0.25 ± 0.05 |
| CA | 15 | 5.16 ± 0.49 | 0.23 ± 0.04 |
| CA.WU | 10 | 4.38 ± 0.58 | 0.19 ± 0.03 |

Values are mean ± standard deviation (SD).

Body measurements of individuals of different groups (carapace width and weight) are shown in Table 3 and the statistical comparisons between them are included in Table 4. Females from SU were larger than those from CA and CA.WU. Also, males from SU and from SU.WU were heavier than those from CA.WU. Comparisons (multiple linear regressions) analysing whether body measurements of individuals (females or males) affected their courtship duration and intensity (number of behavioural acts/time spent performing them) were nonsignificant in all groups. Neither body size nor weight of the individuals was related to courtship duration, either in distant localities [SU (female) × CA (male): females, $R^2 = 0.99$, d.f. = 2, $F = 27.67$, $P = 0.14$; males, $R^2 = 0.67$, d.f. = 2, $F = 2.66$, $P = 0.18$; SU (male) × CA (female): females, $R^2 = 0.38$, d.f. = 2, $F = 0.82$, $P = 0.54$; males, $R^2 = 0.08$, d.f. = 2, $F = 0.11$, $P = 0.95$] or in the locality of sympatry [SU.WU (female) × CA.WU (male): females, $R^2 = 0.33$, d.f. = 2, $F = 0.81$, $P = 0.13$; males, $R^2 = 0.39$,

**Table 4.** Statistical comparisons between body measurements of individuals among the experimental groups

| Experimental group | Carapace width (mm) | | Body weight (g) | |
| --- | --- | --- | --- | --- |
| ♀SU vs. ♀CA | $H = 8.646$, | $U = 13.5, P = 0.01$* | $H = 1.846$, | – |
| ♀SU vs. ♀SU.WU | $P = 0.023$* | $U = 37.5, P = 0.23$ | $P = 0.605$ | – |
| ♀SU vs. ♀CA.WU | | $U = 19.5, P = 0.01$* | | – |
| ♀CA vs. ♀SU.WU | | $U = 28.5, P = 0.32$ | | – |
| ♀CA vs. ♀CA.WU | | $U = 39.0, P = 0.95$ | | – |
| ♀SU.WU vs. ♀CA.WU | | $U = 34.5, P = 0.32$ | | – |
| ♂SU vs. ♂CA | $H = 8.714$, | $U = 76.0, P = 0.32$ | $H = 9.471$, | $U = 44.0, P = 0.43$ |
| ♂SU vs. ♂SU.WU | $P = 0.032$* | $U = 69.5, P = 0.98$ | $P = 0.023$* | $U = 39.0, P = 0.96$ |
| ♂SU vs. ♂CA.WU | | $U = 37.5, P = 0.06$ | | $U = 14.0, P = 0.02$* |
| ♂CA vs. ♂SU.WU | | $U = 46.0, P = 0.16$ | | $U = 46.5, P = 0.17$ |
| ♂CA vs. ♂CA.WU | | $U = 23.0, P = 0.01$** | | $U = 34.5, P = 0.04$* |
| ♂ SU.WU vs. ♂CA.WU | | $U = 25.5, P = 0.07$ | | $U = 16.0, P = 0.010$** |

We used the global non-parametric Kruskal–Wallis test (first column) and the pairwise non-parametric Mann–Whitney $U$-test (second column) with Bonferroni correction. $H$ and $U$ are the statistics of the Kruskal-Wallis and Man-Whitney $U$-test, respectively. *Significant $P$ values without Bonferroni correction; **significant $P$ values post-Bonferroni correction.

d.f. = 2, $F = 1.05$, $P = 0.16$]. No relationship was found between body measurements and courtship intensity, either in distant localities [SU (female) × CA (male): females, $R^2 = 0.93$, d.f. = 2, $F = 4.31$, $P = 0.34$; males, $R^2 = 0.23$, d.f. = 2, $F = 0.40$, $P = 0.76$; SU (male) × CA (female): females, $R^2 = 0.38$, d.f. = 2, $F = 0.82$, $P = 0.54$; males, $R^2 = 0.09$, d.f. = 2, $F = 0.07$, $P = 0.98$] or in the locality of sympatry [SU.WU (female) × CA.WU (male): females, $R^2 = 0.36$, d.f. = 2, $F = 0.56$, $P = 0.68$; males, $R^2 = 0.51$, d.f. = 2, $F = 1.05$, $P = 0.49$]. We excluded data of the CA.WU (female) × SU.WU (male) group because no males and only three females courted, and at an extremely low frequency.

All homotypic encounters in SU and CA ($N = 15$, for each population) triggered copulations (González *et al.*, 2013) differing significantly, being completely absent in the heterotypic scenario (Fisher's exact test: $P = 1.28 \times 10^{-8}$). Regarding courtship, González *et al.* (2013) reported that in SU × SU sexual encounters the most representative acts were web stretching (8.00 ± 3.2 times/individual), striding forward (6.57 ± 3.61 times), and forelegs elevation (3.25 ± 2.0 times/individual), which were all performed by 100% of the males at least once during each trial, whilst seven females performed leg tapping (2.00 ± 0.89 times). In CA × CA trials, the most representative behavioural acts were alternate-waving (21.12 ± 14.77 times) and web-stretching (10.90 ± 7.10 times), performed by all males, and leg tapping (20 ± 11.53 times) performed by all females. When females from SU were exposed to males from CA, the number of courting individuals, as well as the frequencies of behavioural acts, appeared to be considerably lower than in homotypic trials; eight males (out of 15) performed alternate waving (2.53 ± 1.31 times) and nine performed web stretching (1.93 ± 1.40 times), whereas four females performed leg tapping (0.57 ± 0.68 times). This sharp decline was also observed in trials between females from CA and males from SU compared with homotypic encounters; four males (out of 15) performed web stretching (0.38 ± 0.53 times/individual) and only one showed striding forward (0.53 times) and forelegs elevation (0.58 times), whereas four females performed leg tapping (0.044 ± 0.011 times). Therefore, in a heterotypic scenario, fewer males courted than when exposed to females from their own populations (Fisher's exact test: $P = 0.012$). There were fewer courting females in the heterotypic scenario than in the homotypic scenario (Fisher's exact test, $P = 0.047$).

## MALE CHOICE ON SILK CUES

Ten (of 20) males from SU chose first the arm with silk threads deposited by females from their own locality and eight males chose first the arm of females from the other locality ($\chi^2 = 0.22$, $P = 0.64$). The remaining two males did not make a choice in the stipulated time. Only two males changed to the other female arm after the first choice; one changed to SU and the other to CA. Permanence (37.69 ± 21.34 min) and choice latency (17.89 ± 18.96 min) were highly variable and did not differ between males that chose silk threads deposited by females from their own locality and those that chose females from the other locality (permanence: $U = 21.0$, $P = 0.19$; choice latency: $U = 16.0$, $P = 0.07$). Fifteen males from CA chose arms of females from their own locality and two

males chose those from the other locality, showing significant differences ($\chi^2 = 9.94$, $P = 0.002$). The remaining three males did not make a choice on time, remaining at the basal end of the experimental device. No male changed his choice during the trial. Permanence ($34.90 \pm 21.94$ min) and choice latency ($19.39 \pm 21.05$ min) were highly variable and we did not find significant differences between males who chose female silk threads from their locality and those who chose threads from females belonging to the other locality (permanence: $U = 8.0$, $P = 0.37$; choice latency: $U = 11.0$, $P = 0.62$).

## DISCUSSION

Individuals of *A. lagotis* from the two distant populations – southern Uruguay and central Argentina – did not mate with each other and males performed a courtship repertoire that was strongly population-specific. Additionally, the locality with the two coexisting forms (those resembling southern Uruguay and those resembling central Argentina) in western Uruguay, showed a similar scenario of isolation between them with the occurrence of only one heterotypic copulation, with atypical characteristics and that did not generate offspring. The occurrence of courtship was asymmetric, as has been observed in heterotypic crossing experiments with other species (Miller *et al*., 1998; Kitano, Mori & Peichel, 2007): SU males rarely showed all the normal behavioural displays of courtship, whereas CA males courted females from their own and from the other population displaying the complete courtship repertoire. However, the number of courting males and courtship intensity were always lower in heterotypic than in homotypic scenarios described by González *et al*. (2013), but attacks between heterotypic individuals were rare. Additionally, male choices based only on female silk cues were not as conclusive, and SU males did not show a clear preference for tacto-chemical signals from homotypic females. Conversely, CA males clearly preferred cues from females of their own population.

More recently, we have performed preliminary observations of the co-occurrence of both forms in three widely separated localities of Uruguay (Lavalleja, Paysandú, and Rivera). Preliminary heterotypic studies between spiders from these localities showed a similar situation to those observed in WU: individuals behaved according to their similarity to the reference population (i.e. SU or CA) and mating occurred only between individuals of the same form. Additionally, new observations on local environmental conditions suggest differences in microhabitat preferences between the forms: SU individuals appear to be associated with grasslands; and CA individuals appear to be associated with shrubs and trees, and also with meadows. To date, we have not observed individuals with intermediate shape, behaviour, or phenology. In summary, the results strongly suggest the existence of reproductive isolation between these two forms and that a speciation process is taking place. Moreover, individuals of Misiones province (Northern Argentina) have yet another sexual repertoire (M. González, unpubl. data), different from those reported for CA (González *et al*., 2013) and Brazilian (Stefani *et al*., 2011) populations, suggesting a mosaic of divergences within *A. lagotis* that needs further investigation. This does not preclude the existence of pronounced intraspecific variation – suggested by Santos & Brescovit (2001) for *A. lagotis* – within each form.

Most studies of spiders report the existence of new species based on sexual behaviour and genital morphology in sympatry or in hybridization zones of closely related species (Stratton, 1997; Töpfer-Hofmann *et al*., 2000; Ayoub *et al*., 2005; Costa-Schmidt & Machado, 2012; Galasso, 2012; Puzin *et al*., 2014), although few find total sexual isolation (Costa & Capocasale, 1984; Barth & Schmitt, 1991; Aisenberg & Costa, 2008). In lycosids, Barthel & von Helversen (1990) reported speciation based on data from crossing experiments as also did Reiskind & Cushing (1996), who even found intermediate individuals and mating in hybridization zones. Meanwhile, Kronestedt (1990) defined new species based on differences in courtship and genitalia, and Miller *et al*. (1998) did so from interpopulation variations in sexual behaviour in *Schizocosa crassipes*, while mentioning phenological differences. In *A. lagotis*, almost all appointed characteristics have been tested. González *et al*. (2013) showed the existence of at least two sexual repertoires, González *et al*. (2014) observed different phonologies, and, in the present study, we confirmed that those differences agree with the existence of two forms sexually isolated. Indeed, they do not interbreed, even though they are in sympatry (as well as also showing distinctive body-coloration patterns). Putative genital and molecular differences await confirmation.

The discordance found between the communication channels employed by different populations of *A. lagotis* for recognition (in the experiments based on silk cues) suggests another divergence between them. The SU males showed no preference for females' silk cues of their own population, but at the time of the sexual interactions they did not court CA females. Meanwhile, CA males showed preferences for females' silk cues of their own population, but usually courted SU females, whereas females showed a clear rejection, although they did not attack. Both SU and CA would be giving different relative importance to visual/seismic and chemical signals, as has been analysed in

other groups of animals (Verzijden *et al.*, 2010; Taylor *et al.*, 2011; Oh & Shaw, 2013), but is also common in spiders (Hebets & Uetz, 1999; Galasso, 2012), including several (wandering) lycosids. In *Schizocosa duplex* and *Schizocosa uetzi* the seismic channel, but not the visual channel, is crucial for recognition of conspecifics, whereas in *Schizocosa stridulans* and *S. crassipes* the visual cues are crucial (Hebets & Uetz, 1999). Costa & Capocasale (1984), Uetz & Roberts (2002), and Roberts & Uetz (2004) described spider species in which males were unable to recognize conspecifics based on chemical cues in female silk but succeed in heterospecific sexual encounters, when the female was also present. Another possible explanation for our findings in *A. lagotis* would be that there are no qualitative differences in the silk cues of the two populations but in central Argentina they are more intense (i.e. more pheromone deposition). Future studies focused on communicational channels involved in sexual behaviour in each form would be useful for clarifying these observations.

The aim of this study was to establish the degree of inconsistency at the level of sexual behaviour among individuals from distant localities (SU and CA) and in WU, an area where both forms of *A. lagotis* overlap (SU.WU and CA.WU). We observed that differences in the sexual repertoire of these forms of *A. lagotis* (González *et al.*, 2013) reflect a sexual communication barrier, which isolates them, in addition to the asynchrony in their respective phenologies (González *et al.*, 2014). The high intraspecific variation suggested for the species (Santos & Brescovit, 2001) – based on phenotypic plasticity or genetic differences – and the heterogeneous environments in the Neotropics (Morrone, 2001) could be involved in the divergence. Beyond that, differences between forms are strongly distinctive to be explained only by any type of intraspecific variation. We do not know whether this divergence occurred in sympatry or allopatry, but allopatry *per se* does not seem to explain reproductive isolation among the forms, as it persists in sympatry. At the same time we did not find greater divergence in coexistence localities (elements of reinforcement), as could be expected more under a sympatric divergence. The role played by sexual selection in the differentiations (given the different sexual repertoires found for the forms), or those of the ecological factors (given the asynchrony in phenology and possible differences in microhabitats) and web-living habit are unresolved. The hypothesis postulated by Gillespie (1999) – that webs are a favouring factor of divergence (as a result of their association with habitat vegetal structure) – do not appear as a complete explanation because webs of both forms of *A. lagotis* could be found at the same vegetal strata. Alternatively, the prediction of Galasso (2012), about

less speciation by sexual selection in web spiders than in nonweb spiders (based on the idea that assortative matings are less expected in the primers as the interactions always occur in a predictable arena, i.e. the web in this study), could offer a better explanation. However, this would also not be a decisive factor in *A. lagotis* as different sexual repertoires exist, even though both occur in web. If *A. lagotis* presents the low dispersion rate shown for other funnel-web spiders (Singer & Riechert, 1995), local differentiations might have favoured divergence.

Finally, perhaps the synonomysing of several species of this genus by Santos & Brescovit (2001) should be revised in the light of the new data. Information about genital morphology, which appears to vary between populations (M. González, unpubl. data), as well as about population genetics, will be critical in determining the evolutionary robustness of the differences already encountered in *A. lagotis*. So far, we could say that the differences reported above seem to affect intraspecific recognition and suggest a not very recent divergence. This study is the first to shed light on speciation processes occurring in a scarcely studied Neotropical wolf spider, atypical for its sedentary web-living habits.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Body pigmentation patterns from both forms of subadults *A. lagotis; a*: SU (and SU.WU); b: CA (and CA.WU).