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Web building and silk properties functionally covary among species of wolf spider

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Abstract

Although phylogenetic studies have shown covariation between the properties of spider major ampullate (MA) silk and web building, both spider webs and silks are highly plastic so we cannot be sure whether these traits functionally covary or just vary across environments that the spiders occupy. As MaSp2-like proteins provide MA silk with greater extensibility, their presence is considered necessary for spider webs to effectively capture prey. Wolf spiders (Lycosidae) are predominantly non-web building, but a select few species build webs. We accordingly collected MA silk from two web-building and six non-web-building species found in semirural ecosystems in Uruguay to test whether the presence of MaSp2-like proteins (indicated by amino acid composition, silk mechanical properties and silk nanostructures) was associated with web building across the group. The web-building and non-web-building species were from disparate subfamilies so we estimated a genetic phylogeny to perform appropriate comparisons. For all of the properties measured, we found differences between web-building and non-web-building species. A phylogenetic regression model confirmed that web building and not phylogenetic inertia influences silk properties. Our study definitively showed an ecological influence over spider silk properties. We expect that the presence of the MaSp2-like proteins and the subsequent nanostructures improves the mechanical performance of silks within the webs. Our study furthers our understanding of spider web and silk co-evolution and the ecological implications of spider silk properties.

Introduction

Comparing traits between animal species is useful for determining their adaptive value and for assessing whether paired traits are likely to have co-evolved (Harvey & Pagel, 1991; Garland *et al.*, 2005). Nevertheless, making comparisons between species is problematic because the assumption of independence of observations is likely to be violated (Garland *et al.*, 2005; Stone *et al.*, 2011). The phylogenetic comparative methods proposed by Felsenstein (1985) and others (Garland, 1992; Quader *et al.*, 2004; Revell, 2009) can circumvent this issue by measuring and accounting for the phylogenetic branch length differences between species. These methods, nonetheless, are encumbered by other complications (Garland & Adolph, 1994). For instance, they rely heavily on the sampling of large numbers of species with minimal branch length irregularities to minimize the statistical variance across species (Harvey & Pagel, 1991; Garland & Adolph, 1994). They are also designed explicitly for the examination of discrete traits that appear repeatedly throughout a phylogeny (Paradis & Claude, 2002; Rosenberg & Kumar, 2003). Accordingly, phylogenetic comparisons are most commonly used to examine traits, or sets of traits, that

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repeatedly re-emerge across well-resolved phylogenies of mega-diverse taxonomic groups. For this reasons, rare traits and taxa are seldom examined using phylogenetic comparisons (Fisher & Owens, 2004; Violle *et al.*, 2017).

Traits, or sets of traits, that vary within individuals across ecological circumstances (i.e. plastic traits) present additional problems. For these, ecological factors can exert greater influences over many traits than phylogenetic inertia (Dunbar-Co *et al.*, 2009; Pearman *et al.*, 2014). One solution to this conundrum is to sample fewer species over well-defined landscapes or ecosystems and make more detailed trait value measurements that include molecular data where feasible (Rosenberg & Kumar, 2001; Hillis *et al.*, 2003; Garland *et al.*, 2005; Heath *et al.*, 2008). This approach can effectively enhance trait value confidence intervals and thus compensates for the fewer species sampled.

Our understanding of the covariations between spider web building and silk properties is an interesting case in point. Broad phylogenetic studies across the major spider groups have shown that spiders that build webs produce the toughest and most extensible major ampullate (MA) silks (Sensenig *et al.*, 2010; Blackledge *et al.*, 2012; Cranford *et al.*, 2014). However, spider webs and their silks are both highly plastic, that is they vary significantly within individual spiders across environments (Boutry & Blamires, 2013; Blamires *et al.*, 2017a). Hence, we cannot be sure whether these traits functionally covary or happen to vary across the environments over which the spiders examined are found.

Spider MA silk comprises of a lipid-rich layer and a glycoprotein-rich skin covering a protein-based outer and inner core (Sponner *et al.*, 2007; Papadopoulos *et al.*, 2009; Heim *et al.*, 2010; Blamires *et al.*, 2017a). The core consists primarily of two types of proteins, called spidroins; major ampullate spidroin 1-type, or MaSp1-type proteins, and major ampullate spidroin 2-type, or MaSp2-type proteins (Heim *et al.*, 2010; Blamires *et al.*, 2012a,b, 2016; Larracas *et al.*, 2016). The secondary structures of the spidroins have traditionally been thought to be critical for the silk's mechanical properties, with MaSp2-type being generally associated with greater extensibility and toughness and MaSp1-type with greater ultimate strength (Hayashi *et al.*, 1999). Nevertheless, the relative expression of these, and other spidroins, their secondary structures and the consequent mechanical properties of MA silk, differs substantially among and between spider species according to the spider's habitat and/or other ecological needs (Lewis, 2006; Brunetta & Craig, 2010; Garb *et al.*, 2010; Goodacre, 2012; Blamires *et al.*, 2016).

Spider webs function by absorbing the kinetic energy of flying or falling prey as well as holding the weight of the spider (Harmer *et al.*, 2015). We, accordingly, expect that when MA silks are incorporated into webs they have a strength, extensibility and toughness conducive to absorbing the amount of kinetic energy needed to capture the spider's predominant prey. We also expect it to be likely that the larger bodied spiders have stronger and/or tougher MA silks (Piorkowski *et al.*, 2018).

The spider superfamily Araneoidea comprises not only most of the large-bodied web-building spiders, but also many small spiders as well as spiders that do not build webs (Hormiga & Griswold, 2014). Phylogenetic analyses have revealed that the MA silks of web-building Araneoidea have the greatest elasticity and toughness (Swanson *et al.*, 2006a,b; Sensenig *et al.*, 2010). The presence of MaSp2-type proteins has thus far only been reported for the silks of orb-web-building spiders (Orbiculariae clade) (Gatesy *et al.*, 2001; Garb *et al.*, 2006; Bittencourt *et al.*, 2010; Blackledge *et al.*, 2012) and is thought to be important for facilitating MA silk elasticity and toughness within webs (Hayashi *et al.*, 1999; Garb *et al.*, 2006; Blackledge *et al.*, 2012). It may accordingly be expected that enhanced MA silk extensibility and toughness is necessary for spider webs to effectively capture flying or falling prey. We might subsequently predict that web building should correlate with the presence of MaSp2-type proteins among and within different web-building spider clades (Blackledge *et al.*, 2012). The difficulty with testing this prediction within any particular clade is that we need to select a species-rich clade to examine and compare silk protein compositions and properties between web-building and non-web-building spiders from similar regions or ecosystems.

Wolf spiders (family Lycosidae) are a species-rich group largely comprising of cursorial foraging spiders (i.e. they do not build webs) that belong to the diverse retrolateral tibial apophysis (RTA) clade. Molecular evidence suggests that the RTA spiders are derived from the earliest orb-web-building spiders (Blackledge *et al.*, 2009; Bond *et al.*, 2014). A handful of species of wolf spiders from the basal subfamilies Sossipinae and Venoniinae build sheet and funnel webs, whereas the 2000 or so species from the more derived subfamilies do not build webs (Murphy *et al.*, 2006; Spagna & Gillespie, 2008). Unlike those of many web-building spiders, wolf spider webs are devoid of sticky viscous silks and capture prey that falls rather than flies into it (Stefani & Del-Claro, 2015). The web is predicted to be composed primarily of MA silk, which acts in both the capture of prey and, owing its vibratory propagation capacity (Mortimer *et al.*, 2016), an extension of the spider's sensory system, thus enabling it to detect prey or other items that enter the web (see González *et al.*, 2015 and Eberhard & Hazzi, 2017, for examples). Wolf spiders accordingly present as an excellent group for testing the above-mentioned prediction wherever web-building and non-web-building species might co-exist (Blamires *et al.*, 2017b).

In semirural ecosystems of Uruguay, a range of web-building and cursorial foraging wolf spiders are found

across a range of habitat types. We utilized the high regional wolf spider diversity here to test the relationship between MA silk properties (namely the presence of MaSp2-type proteins), protein nanostructures and silk mechanical properties, and the presence or absence of web building, among eight species of Uruguayan wolf spiders. Two of these species (both from the subfamily Sosippinae) build large funnel webs that function to trap falling prey, whereas the other six (from the subfamilies Allocosinae and Lycosinae) do not build webs. To compensate for using just eight species for our phylogenetic comparisons, we made meticulous trait measurements within and among species and included molecular data.

Materials and methods

Collection of spiders

We collected five individuals each from eight species of similar sized wolf spiders: the web-building *Aglaoctenus lagotis* and *A. oblongus* (subfamily Sosippinae), and the non-web-building *Allocosa senex* (subfamily Allocosinae), *Pavocosa gallopavo*, *Lycosa erythrognatha*, *L. inornata*, *L. poliostoma* and *L. thorelli* (subfamily Lycosinae). The two species of *Agloactenus* were the only web-building species found at our sites. As it is included among the Lycosoidea superfamily and is known as a close relative of spiders of the family Lycosidae (Albo *et al.*, 2017), we also collected five individuals of the species *Paratrechalea ornata* (family Trechaleidae) as the outgroup for our phylogenetic comparisons.

All spiders were collected within semirural landscapes near Rivera, San José or Marindia, Uruguay. The two *Agloactenus* species were collected near Rivera. *Aglaoctenus lagotis* was found in dry grasslands, whereas *A. oblongus* was always located close to waterways. *Lycosa inornata* and *L. thorelli* were found in dry grasslands near San José, whereas *L. erythrognatha*, *L. poliostoma* and *P. gallopavo* were more commonly located beneath boulders or within crevices in the soil. *Allocosa senex* was found within sand dunes along riverbanks near Marindia (see Aisenberg *et al.*, 2007). We collected individual *P. ornata* adjacent to water bodies at San José.

To ensure spiders of approximately equal size were used for all ensuing procedures, we weighed all spiders to the nearest 0.001 g using an electronic balance (Ohaus Corp., Pine Brook, NY, USA), and body length measured to the nearest 0.1 mm using digital Vernier calipers (Caliper Technologies Corp., Mountain View, CA, USA), immediately upon capture. The spiders were then taken to the laboratory at either the Centro Universitario de Rivera, Universidad de la República (Rivera) or Laboratorio Ecología del Comportamiento, Instituto de Investigaciones Biológicas 'Clemente Estable' (Montevideo), where the following measurements were made.

Collection of silk

We immobilized the 45 spiders (five each from the nine species) by placing them on ice for ~20 min. We placed each of the spiders' ventral side up on a 150 mm × 100 mm foam platform and immobilized their legs using nonadhesive tape and pins before carefully extruding a single MA silk fibre from the major ampullate spinnerets using tweezers. The fibres were collected using a mechanical spool spun at a constant speed (1 m min^{-1}) under controlled temperature (~25 °C) and humidity (~50% R.H.) in still air (see Blamires *et al.*, 2016; Benamú *et al.*, 2017 for details) for all ensuing analyses.

Amino acid composition analysis

To collect silk for amino acid composition analyses, we spooled, from each spider, a single MA silk fibre around a glass tube. The silks were scraped off the tube using a scalpel blade and weighed to the nearest 0.001 mg on an electronic balance (Pioneer PA214C, Ohaus, Pine Brook, NJ, USA) before being placed into Eppendorf tubes and sent to the Australian Proteomic Analysis Facility, Sydney, Australia.

As MaSp1-type proteins consist primarily of residues of alanine and glycine, whereas MaSp2-type proteins consist of additional residues containing proline and serine, we measured the compositions of the amino acids alanine, glycine, proline and serine using high-performance liquid chromatography to estimate the relative composition of MaSp1- and MaSp2-type proteins in each of the spider's silks.

Mechanical property analysis

We spooled MA silk from five individuals per species by attaching a headframe to the spool. A 240-mm-long cardboard strip with six 10 mm × 10 mm square holes punched at 10 mm intervals was wrapped around the headframe. Double-sided sticky tape was stuck onto the cardboard at the border of the holes. The headframe was rotated once ensuring the silk traversed all of the holes and adhered to the tape. The strip was then removed from the headframe and a drop of Elmer's glue applied at the positions where the silk was fastened to the cardboard. Another frame of equal size with identically positioned holes punched into it was placed on top. The two strips were squeezed together with forceps ensuring that they stuck together. We then cut the strip at the regions between the holes perpendicular to the silk thread, leaving six 10 mm × 10 mm frames holding a single thread of silk for each of the 45 spiders that were silked.

One frame of silk collected for each spider was used to ascertain the width of the thread to account for the cross-sectional area in our tensile tests. We taped the

frame to a microscope slide and examined and photographed the silk under 1000× magnification using a polarized light microscope (CKX41; Olympus, Tokyo, Japan) connected to a SPOT Idea 5 Mp digital camera (Spot Imaging Solutions, Sterling Heights, MI, USA). The images were digitized using the program Spot Basic 4.7 (Spot Imaging Solutions) and the width of each thread determined as a mean of 12 measurements using ImageJ (NIH, Bethesda, MD, USA).

For the remaining 225 frame-mounted silk samples (five frames each from five individuals from each of the nine species), we performed tensile tests as follows. We first placed the 10 mm × 10 mm frames containing a single fibre within the grips of a T150 (Agilent Technologies, Santa Clara, CA, USA) nano-tensile testing machine. We ensured that the grips held the silks firmly at the upper and lower frame edges. The left and right sides of the frames were cut away, and the silks were stretched at a rate of 0.1 mm s^{-1} until the fibre ruptured.

Stress (σ) and strain (ε) were calculated using equations:

$$\sigma = \frac{F}{A}$$

and

$$\varepsilon = \frac{L - L_0}{L_0}$$

where F is the force applied to the specimen measured using the program Nano Suite 1.0 (Agilent Technologies), and A is the cross-sectional area of the thread calculated from the thread diameter assuming a constant thread volume. L is the instantaneous length of the fibre at a given extension value, measured using Nano Suite, and L_0 is the original gage length of the fibre (i.e. 10 mm).

Stress vs. strain curves were plotted for each silk tested, from which we calculated the following mechanical properties: (i) ultimate strength; or the stress at rupture, (ii) extensibility; or the strain at rupture, (iii) toughness; the area under the stress–strain curve, and (iv) Young's modulus (a proxy of stiffness); the slope of the stress–strain curve during its initial, elastic, phase.

X-ray scattering analysis

The alanine/glycine residues of MaSp1-like proteins are expected to promote crystalline β–sheet formations in the MA silk fibres, whereas the addition of proline and serine in MaSp2-like proteins induces the formation of additional type II β-turns and similar nanostructures. To examine these structures in each spider's silk, we performed high energy wide-angle X-ray scattering analyses (WAXS) at the SAXS/WAXS beamline at Australian Synchrotron, Melbourne, Australia.

In the laboratory, we collected silk from each spider on individually constructed 3 mm × 1 mm steel frames with 0.5 mm × 0.5 mm windows. We pulled the silk threads across the frame window and ran the spool for ~2 h, ensuring approximately 2000 rounds of silk were wrapped around each of the frame windows. At Australian Synchrotron, we taped each frame to a sample plate. The plates were mounted onto a plate holder at a distance of 330 mm from the incident X-ray beam. The beam size was confined by a collimator 0.5 mm in diameter. A digital camera was set up enabling us to move the specimens into the line of the beam from outside the hutch. We exposed each sample to the beam for 10–60 s depending on the density. The scattered photons from each silk sample were detected by a Mar 165 imaging plate (Q ≈ 1.45Å). Two-dimensional WAXS images were subsequently developed using the program Scatterbrain (Australian Synchrotron, Melbourne, Australia) and examined as follows.

From the images, we calculated the following: (i) scattering parameter (q), (ii) diffraction angles (2θ), (iii) azimuthal angles, (iv) intensity peaks (I_x) and (v) full-width and half-width maximum intensities (FWHM) of the 2θ and azimuthal angles using Scatterbrain, to estimate crystalline region alignment at the (0 2 0) and (2 1 0) reflection vectors (the vectors associated with scattering from crystalline β–sheets in silks). We then calculated and compared between treatments:

1. crystal size, τ, using Scherrer's equation (Riekel *et al.*, 1999):

$$\tau = K\lambda/\beta\cos\theta$$

   where K is the shape factor, which we assumed to be derived from a sphere hence a value of 0.9 (Glisovic *et al.*, 2008), λ is the incident X-ray wavelength, β is full line widths at half the maximum intensity after subtracting instrumental broadening, and 2θ is the diffraction angles of the (0 2 0) and (2 1 0) reflection vectors.
2. The relative crystalline intensity ratios $I_{020}/I_{\text{amorphous}}$ and $I_{210}/I_{\text{amorphous}}$ with I_{020}, I_{210} and $I_{\text{amorphous}}$, which represent the sum of the intensity peaks at the (0 2 0) and (2 1 0) reflection vectors and the amorphous region, respectively.
3. The crystallinity index, X_c, according to Grubb & Jelinski (1997), and
4. Herman's orientation function, f_c, using the equation:

$$f_c = (3\{\cos^2\varphi\rangle\} - 1)/2$$

where φ is the angle between the c axis and the fibre axis, $\{\cos^2\varphi\}$ is the azimuthal width of the two strongest equatorial reflections, (020) and (210), as determined using the equation:

$$\{\cos 2\varphi\} = 1 - A\{\cos 2\varphi 1\} - B\{\cos 2\varphi 2\}$$

where A = 0.8 and B = 1.2.

Genetic phylogeny construction

As the spiders from the subfamily Sossipinae build webs, whereas those from the subfamilies Allocosinae and Lycosinae and family Trechaleidae do not, it was essential that we discriminate between silk properties associated with web building and those associated with phylogenetic inertia. Nevertheless, a well-resolved wolf spider phylogeny that includes all of the species examined herein does not exist. We therefore estimated a genetic phylogeny for the nine species used prior to performing our analyses.

Upon collection of the silks, we killed and removed the legs of each of the spiders, and whole-genomic DNA was extracted using the commercially available Quick-gDNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA). As explained in the 'Collection of spiders' subsection, we used *P. ornata* (family Trechaleidae) as the outgroup to root the phylogeny. We amplified a fragment of the 28S rRNA gene using the primers 28S 'O' and 28S 'C' as described by Hedin & Maddison (2001). Polymerase chain reactions (PCRs) were performed with Taq DNA polymerase (Tingen Biotech, Beijing, China) in an Arktik thermocycler (Thermo Scientific, Waltham, MA, USA) with the following temperature profile (Murphy *et al.*, 2006): an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, an annealing temperature of 50 °C for 30 s and an extension temperature of 72 °C for 30 s. This was followed by an additional extension of 72 °C for 3 min. We checked PCR amplicons with an electrophoresis in 0.7% agarose gels stained using GoodView (SBS Genetech, Beijing, China) for visualization in UV light. Successful amplicons were purified with the DNA Clean and Concentrator-5 kit (Zymo Research Corp.) and sent to Macrogen, Inc. (www.macrogen.com) for direct Sanger sequencing using the same primers. Chromatograms were checked using Geneious 8.1.6 (Biomatters Ltd., Auckland, New Zealand), and assembled contigs were aligned with ClustalX2 (Larkin *et al.*, 2007). We BLAST-searched all sequences to confirm their taxonomic identity and subsequently deposited the identified sequences in GenBank (www.ncbi.nlm.nih.gov, DNA sequences: accession numbers MF54339–MF543357).

We formulated a phylogenetic tree for our nine species using a Bayesian inference approach in BEAST 1.8.1 (Drummond *et al.*, 2012) based on an alignment of 804 base pairs, the best-fit GTR+G model of nucleotide substitution selected with JMODELTEST2 (Darriba *et al.*, 2012), and estimated base frequencies. We specified a Yule tree with a random, coalescent-based start-up tree. We used an uncorrelated relaxed clock model with a log-normal distribution of the mean rate across branches of the tree. We ran two independent MCMC analyses to sample from the posterior distribution of trees using ten million steps sampled every 1000 steps. After checking for convergence of MCMC runs and high effective sample sizes for all parameters (> 200) in Tracer v1.6 (Rambaut *et al.*, 2014), we summarized 9000 samples (after excluding the first 1000 trees as the 'burn-in') to obtain a maximum clade credibility (MCC) tree using TreeAnnotator from BEAST 1.8.1. Branch support was estimated with posterior clade probabilities.

Statistical analyses

We used redundancy analyses (Van den Wollenberg, 1977) to estimate the variation in (i) amino acid composition (specifically: % alanine, %, glycine, % proline and % serine), (ii) mechanical properties (ultimate strength, extensibility, toughness and Young's modulus) and (iii) nanostructures (crystal size, relative crystalline intensity ratios, crystallinity index and Herman's orientation function) that could be ascribed to species differences. We plotted RDA ordinations, and wherever the properties of the two web-building species (*A. lagotis* and *A. oblongus*) grouped separately from the other species, we interpreted it as web building associating with the properties in question.

We constructed a generalized least squares phylogenetic regression model (Grafen, 1989) to determine whether web building or phylogenetic inertia was associated with the silk amino acid compositions, and/or mechanical properties, and/or nanostructures, across the nine species of spiders examined. The following response parameters were included in the model: (i) amino acid composition, (ii) mechanical properties and (iii) nanostructures. The predictor variable was web building. The values for amino acid composition, mechanical properties and nanostructures were the first RDA scores from our redundancy analyses. Web building was scored as either 0 or 1, where 0 represents web building and 1 represents no web building. We assumed evolutionary divergence among the nine species by Brownian motion and that the regression error term, ε, has variance = σ^2C, where σ^2 is the rate of change and C is the internode branch lengths determined from our phylogeny by a penalized likelihood semiparametric estimator with a smoothing parameter of 0.1 (Pagel, 1998). We constructed a log-likelihood goodness-of-fit model to test among eight alternative hypotheses that explain the association between web building and silk amino acid compositions, and/or mechanical properties, and/or nanostructures (Table 1), with the model best fitting the data identified by a G^2 test. Analyses were performed using a combination of Statistica 13.0 and the glmm and caper packages (Orme *et al.*, 2013) in the program R.

Results

The amino acid compositions, mechanical properties and nanostructure parameters all differed between the

Table 1 Alternative hypotheses explaining the association between web building and silk amino acid compositions and/or mechanical properties and/or nanostructures.

H_1	Web building is associated with amino acid compositions, independent of mechanical properties and nanostructures.
H_2	Web building is associated with amino acid compositions and mechanical properties, independent of nanostructures.
H_3	Web building is associated with amino acid compositions and nanostructures, independent of mechanical properties.
H_4	Web building is associated with mechanical properties, independent of amino acid compositions and nanostructures.
H_5	Web building is associated with mechanical properties and nanostructures, independent of amino acid compositions.
H_6	Web building is associated with nanostructures, independent of amino acid compositions and mechanical properties.
H_7	Web building is associated with amino acid compositions and mechanical properties and nanostructures.
H_0	Web building is associated with are independent of amino acid compositions and mechanical properties and nanostructures.

two web-building *Aglaoctenus* spp. and the other species of wolf spider and *P. ornata* (see Table S1–S3, respectively, for values. Stress–strain curves and raw X-ray diffraction outputs are in Figs S1 and S2). Our redundancy analyses confirmed that the amino acid compositions, mechanical properties and nanostructures of the web-building spiders differed from the non-web-building spiders, with 'species' explaining 82%, 92% and 80% of the variance in each, respectively (Fig. 1).

The genetic phylogeny that we attained had well-resolved and strongly supported relationships between species (Fig. 2). *Paratrechalea ornata* was confirmed as the outgroup. The four *Lycosa* species were clustered in a derived lineage together with *P. gallopavo*, as we expected. The two species of *Aglaoctenus* were grouped together and placed as a sister group to the *Lycosa-Pavacosa* cluster. In addition, *A. senex* appeared as sister to all the other sampled lycosids.

Our regression model tested for the effects of web building while controlling for phylogenetic drift and found a significant relationship between web building and silk amino acid compositions, mechanical properties and nanostructures (Adjusted $R^2 = 0.297$, $F_{1,18} = 17.523$, $P < 0.001$) among the nine species. Of the hypotheses presented in Table 1, the one that best fitted our data (log-likelihood = 0.966, $G^2 = 14.895$, $P < 0.001$) was H_7, that is, that web building effected the silk amino acid compositions, mechanical properties and nanostructures (for all effects see Table S4). Our analyses, accordingly, showed web building to be predominantly associated with silk properties across the spiders examined.

Discussion

Our analyses showed that the silk properties of the web-building spiders differed from those that do not build webs, and that it was web building and not phylogenetic inertia that was associated with the silk properties. The validity of our phylogenetic comparative analyses, however, depends upon the reliability of our genetic phylogeny, which we justify as follows.

Firstly, we used a gene widely employed in spider systematics as a marker of deep and shallow relationships across (Wheeler *et al.*, 2016) and within (Murphy *et al.*, 2006) spider families, as well as within genera (Planas *et al.*, 2013). Secondly, the clusters within our tree (*Lycosa*/*Pavacosa* and *Aglaoctenus*) showed that this marker establishes the same phylogenetic relationships as other analyses (e.g. Murphy *et al.*, 2006). Thirdly, we used a statistically robust Bayesian inference approach based on models of nucleotide substitution and a relaxed clock that appropriately took into account variation in DNA sequence evolution among sites and tree branches. We are thus confident that our tree attained exceptionally good estimates of the phylogenetic relationships among species and their relative branch length differences. Our placement of the nine species is congruent with expected relationships, except for *A. senex*, which we expected to be more closely related to *Lycosa*/*Pavacosa* than to *Aglaoctenus* (Murphy *et al.*, 2006). The affinity of *A. senex* had the lowest support in our tree (see Fig. 2) and was placed at the base of the Lycosidae. The placement of *P. ornata* as the outgroup is consistent with the sister taxa relationship between Lycosidae and Trechaleidae (Wheeler *et al.*, 2016; Albo *et al.*, 2017).

Minor differences between our phylogeny and that of Murphy *et al.* (2006) are likely the result of our sparser taxon sampling rather than any biases in the markers and/or our phylogenetic methods. The reasons for our sparser sampling were that there were only two species of web-building wolf spiders available to us and we only sampled spiders from homogeneous semirural ecosystems to control for any possible environmental influences over trait values (which was critical given the considerable variability in spider silk properties; Boutry & Blamires, 2013). We, however, used meticulous measurements (i.e. a combination of HPLC, tensile testing and synchrotron derived X-ray scattering) to ascertain the amino acid compositions, silk mechanical properties and nanostructures in the MA silks of all nine species, so we are satisfied that we had statistically compensated for a sparse number of species. As the above-mentioned silk property measurements were expensive, operationally complex and time- and resources consuming, a larger scale effort was not feasible. Nonetheless, our measurements and subsequent redundancy and regression analyses found exceptionally strong effects and clearly implicated web building as influential over silk properties among the wolf spiders examined.

Our amino acid composition analyses showed that the silks of the two web-building species, *A. lagotis* and *A. oblongus*, had a greater proline and serine

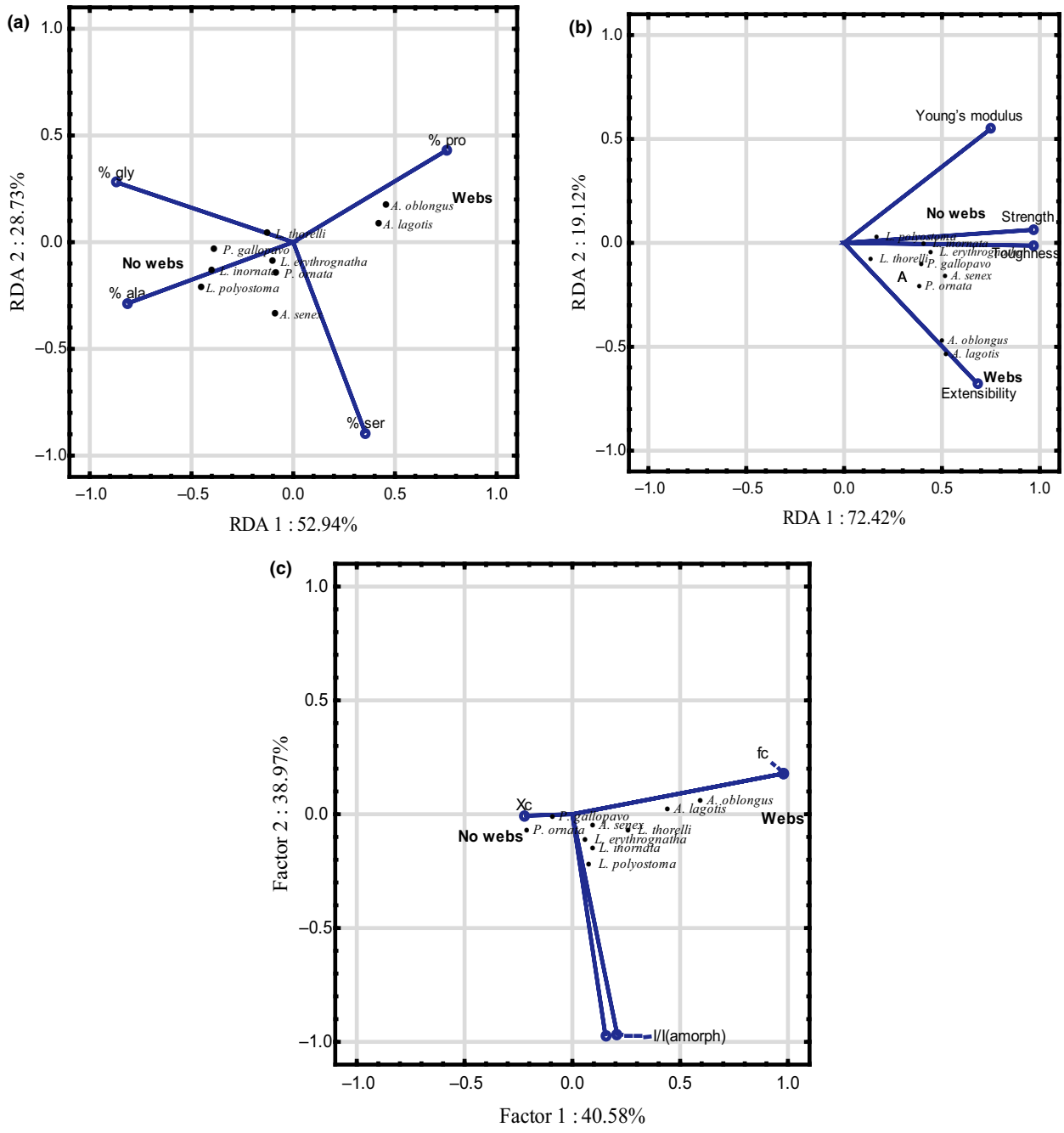

Fig. 1 Redundancy analysis ordinations for (a) amino acid composition (specifically: % alanine, %, glycine, % proline and % serine), (b) mechanical properties (ultimate strength, extensibility, toughness and Young's modulus) and (c) nanostructures (crystal size, relative crystalline intensity ratios, crystallinity index and Herman's orientation function), showing all parameters for the two web-building species (*Aglaoctenus lagotis* and *Aglaoctenus oblongus*) differing from those of the other seven species.

composition but lower alanine and glycine composition compared to the other seven species (Table S1), all of which do not build webs. MaSp2-type proteins contain proline and serine residues in place of the repeated alanine and glycine residues found in MaSp1-type proteins (Lewis, 2006; Blamires *et al.*, 2017a). We thus deduced that the silks of the web-building *A. lagotis* and *A. oblongus* had greater proportions of MaSp2-like proteins than the silks of the seven cursorial foraging spiders. Our subsequent regression modelling showed that web building was the primary factor driving silk protein compositions as well as the silk's mechanical and

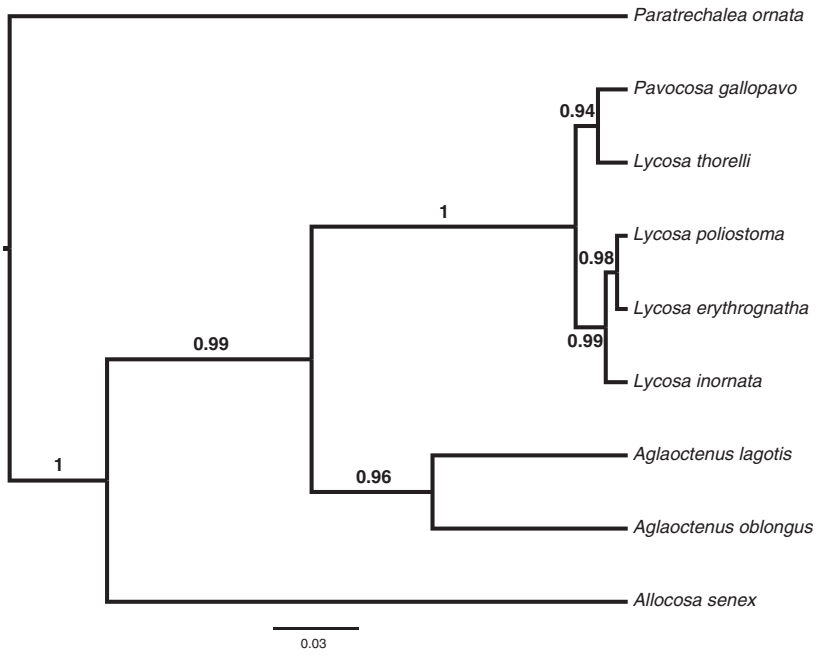

Fig. 2 Bayesian phylogeny of the nine species of spider examined based on the 28S rRNA gene. Posterior clade probability values are shown at the nodes, and the scale bar represents the internode branch lengths in units of number of substitutions per site. [Correction added on 21 May 2018, after first online publication: The terms in Figure 2 was previously erroneous in this article and this has been corrected in this current version.]

structural properties. This conclusion conforms with our *a priori* prediction that web building and the presence of MaSp2-type proteins are correlated within the web-building spider clades, and supports the hypotheses that the silk properties brought about by MaSp2-type proteins, such as increased extensibility and toughness (Hayashi *et al.*, 1999), are required for spider webs to effectively function at capturing moving prey.

According to conventional models (Hayashi *et al.*, 1999; Blamires *et al.*, 2012a, 2017a), the preferential expression of MaSp2-type proteins over MaSp1-type proteins by web-building spiders might bestow their silk with great extensibility and toughness, but it comes at the expense of strength and stiffness (indicated here by Young's modulus values). Nevertheless, we found that the silks of *A. lagotis* and *A. oblongus* had greater extensibility and toughness as well as ultimate strength and stiffness compared to the silks of the other seven species. Our X-ray scattering analysis showed that the silks of *A. lagotis* and *A. oblongus* had greater crystalline densities at the (020) reflection vector than the silks of the other species. In addition, the silks of *A. lagotis* and *A. oblongus* had the greatest crystallinity and crystalline orientations. These results suggest that the silks of these spiders contain stretched and oriented crystalline regions, characteristics that we would expect to be associated with high-performance MaSp1-type-enriched MA silk (Parkhe *et al.*, 1997; Giesa *et al.*, 2016).

It is known that spiders can produce silks high in MaSp2-type spidroins and retain conventionally MaSp1-like nanostructures, or vice versa, as a consequence of the internal environment during spinning (Lewis, 2006; Blamires *et al.*, 2012a, 2016). Variations in pH, salts and shear forces acting within the silk gland during spinning can induce spidroins to undergo structural phase transitions (Dicko *et al.*, 2004; Giesa *et al.*, 2016; dos Santos-Pinto *et al.*, 2016; Blamires *et al.*, 2017a). It thus appears that, in addition to different protein expressions, variations within the glandular environment could have induced the silks of *A. lagotis* and *A. oblongus* to undergo nanostructural changes prior to spinning. The great novelty of our findings is that the silk nanostructures were correlated with the building of webs among the eight wolf spiders species and *P. ornata*, suggesting that the ecological use of MA silk influences a spider's protein expression and the various spinning processes.

We found evidence here that high silk strength, extensibility, toughness and stiffness, and the presence of MaSp2-type proteins correlate with web building among wolf spiders. Whether this trend is true across the entire RTA clade requires further testing within other subgroups that contain web-building species, for instance among spiders from the family Pisauridae (World Spider Catalog, 2017). Our regression model found that web building principally drives the differences in silk properties between web-building and non-web-building spider species. It thus seems reasonable to conclude that MA silk properties brought about by the presence of MaSp2-type proteins, such as exceptional extensibility and toughness, are necessary for spider webs to perform the function of capturing moving prey. Nevertheless, confirmatory tests are required. Our study

definitively showed that closely related spiders can produce silks of vastly different properties to cater for their specific ecological needs. Our work enhances our understanding of the ecological inducers of spider silk property differentiation and the co-evolutionary relationship between web building and silk properties among spiders.

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Ethical statement

The wolf spiders used were not endangered or protected species, although some species are on the list of arachnids of conservation priority for Uruguay. No permits were required for carrying out experiments with any of the species.

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Competing interests

The authors declare they have no competing interests.

Authors' contributions

M.L., A.C., L.F.G., M.B. and S.J.B. designed the research. M.L., A.C., L.F.G., M.B., M.S. and S.J.B. performed the research. A.C., J.F., X.W. and S.J.B. contributed new reagents/analytical tools. M.L., A.C., L.F.G., J.F. and S.J.B. analysed the data. All authors wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1 The % molar compositions (mean ± 1 SE) of the amino acids alanine, glycine, proline and serine in the MA silks of the nine species (the eight wolf spiders and *P. ornata*).

Table S2 Mean ± 1 SE values for the mechanical properties: (i) ultimate strength, (ii) extensibility, (iii) toughness, and (iv) Young's modulus for the MA silks of the nine species.

Table S3 The mean nanostructure parameters the nine species.

Table S4 Generalized least squares regression model to determine whether it was web building and not phylogenetic variation that associated with the silk amino acid compositions, and/or mechanical properties, and/or nanostructures, across the nine species of spider.

Figure S1 Stress–strain curves for the MA silks of the nine species.

Figure S2 X-ray diffraction outputs (two-dimensional scattering images and individual scattering parameter, q, vs. intensity plots) for the MA silks of the nine species.

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