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### Supplementary data

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# Post-secretion processing influences spider silk performance

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Phenotypic variation facilitates adaptations to novel environments. Silk is an example of a highly variable biomaterial. The two-spidroin (MaSp) model suggests that spider major ampullate (MA) silk is composed of two proteins—MaSp1 predominately contains alanine and glycine and forms strength enhancing  $\beta$ -sheet crystals, while MaSp2 contains proline and forms elastic spirals. Nonetheless, mechanical properties can vary in spider silks without congruent amino acid compositional changes. We predicted that post-secretion processing causes variation in the mechanical performance of wild MA silk independent of protein composition or spinning speed across 10 species of spider. We used supercontraction to remove post-secretion effects and compared the mechanics of silk in this ‘ground state’ with wild native silks. Native silk mechanics varied less among species compared with ‘ground state’ silks. Variability in the mechanics of ‘ground state’ silks was associated with proline composition. However, variability in native silks did not. We attribute interspecific similarities in the mechanical properties of native silks, regardless of amino acid compositions, to glandular processes altering molecular alignment of the proteins prior to extrusion. Such post-secretion processing may enable MA silk to maintain functionality across environments, facilitating its function as a component of an insect-catching web.

**Keywords:** biomaterial plasticity; MaSp expression model; mechanical properties; orb web; spider silk; supercontraction

## 1. INTRODUCTION

Differential expression of phenotypes across environments within or between individuals, termed phenotypic variation, flexibility or plasticity depending on the authority, promotes adaptations to novel environments in animals [1–4]. The influences of morphological, biochemical, physiological and behavioural trait variations on animal fitness accordingly are well described [5–7]. Secreted biomaterials, such as silk, slime, nacre and byssus, exert functions external to the animal and, as a consequence, they may be exposed to spatial and temporal environmental variations. Variability in their physical and chemical properties across environments thus appears imperative for these materials to sustain their functions [8–10].

Spider dragline, or major ampullate (MA), silk is a secreted biomaterial with high tensile strength coupled with high extensibility, rendering it desirable for

industries to mimic the production process for the synthesis of analogous materials for specific purposes [11,12]. However, attempts to clone or to spin regenerated silk have, to date, produced fibres with inferior physical properties compared with those naturally secreted by spiders [11]. A multitude of factors influence the physical and chemical properties of spider silk, including genetic effects, phylogenetic signal, post-secretion physiological and biochemical processes and factors acting during spinning and post-spinning [12–14]. Researchers currently do not fully understand the relative influence of each of these processes on silk properties and whether their influences vary in heterogeneous environments [12]. Indeed, failure to quantitatively account for these factors may be a reason for why attempts to synthetically produce analogous materials have thus far not succeeded [11–13].

The glycine, glutamine, alanine and proline composition from spun MA silk is considered an indicator of its physical properties in accordance with a two-spidroin (MaSp) model developed for the model spider, *Nephila clavipes* [15,16]. The model suggests that MA

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silk is a product of the relative expression of two-spidroins, MaSp1 and MaSp2, which differ in amino acid composition and mechanical properties. MaSp1 has greater concentrations of alanine but is low in glutamine and virtually free of proline, while MaSp2 has prevalent glutamine and proline but is lower in alanine than MaSp1 [15,17–19]. Alanine and glycine form motifs that promote the formation of strength-inducing  $\beta$ -sheets and cross-linking in the amorphous region while proline disrupts  $\beta$ -sheet formation instead promoting the formation of  $\beta$ -coils endowing the fibre greater extensibility. MaSp1-predominant silk therefore is predicted to have low proline, high alanine and remarkable ultimate strength, while MaSp2-predominant silk is predicted to have high proline and greater extensibility [15,16,20,21]. Whether the model is applicable to other taxa of spiders is unknown, but examination of the chemical and mechanical properties of different spiders indicates that the spidroin compositions in the MA silks of many spiders may differ substantially to those of *Nephila clavipes* [21–23]. Nonetheless, the influence of specific amino acids, e.g. proline, on the mechanical properties of spider silk is considered consistent across species [17,18].

Under certain circumstances, e.g. when silk are spun when dropping compared with horizontal walking, the physical properties of freshly spun MA silk can vary within individual spiders, even without concomitant variations in amino acid composition [24–26]. Processes additional to protein expression thus influence the mechanical performance of MA silk. The molecular alignment and flow dynamics of the liquid spinning dope can be altered along the glandular duct by the actions of various physiological and biochemical post-secretion processes, e.g. ion exchange and variations in pH and hydration levels [27–29]. Moreover, other variations in gland morphology, physiology and/or biochemistry appear largely responsible for many of the variations in MA silk properties found between individuals and species [30,31]. Variations in reeling speed, friction at the spinning valve and the thermal and hydric environment into which the silk is spun can induce further variations in MA silk properties [25,26,32]. The spider therefore controls and seemingly ‘freezes into place’ the alignment of the molecules during spinning [24,26,30]. The effectiveness of this process is limited, nonetheless, by the amount of amorphous cross-linking, which may be inhibited by the presence of proline [16]. Of the post-spinning environmental processes that influence MA silk properties, its tendency to shrink up to 50 per cent of its original length and become rubbery when exposed to water, a phenomena called ‘supercontraction’ [33], is the most marked [33–36]. Supercontraction involves saturation of the intermolecular bonds and the subsequent formation of hydrogen bonds inducing the protein backbone to lose its alignment [37]. The supercontracted state may be considered a ‘ground state’ for MA silk because amino acid composition variation influences the physical properties without the influence of post-secretion processing [36,37,39].

A recent comparative study [13] found wide variation in MA silk mechanical properties across spiders but

there was little or no phylogenetic signal affecting silk properties. Since reeling methods and the post-spinning environment were accounted for in the analyses, post-secretion processing was assumed to exert the most substantial influence on MA silk mechanical properties in all spiders. Nonetheless, without any estimate of MaSp expression or reference to silk properties in a ‘ground state’ conjecture pertaining to the degree of influence exerted by post-secretion processing is speculative as genetic inputs [20,40] could not be entirely ruled out.

Here, we performed a comparative study of the natural variations in native spider silk properties relative to that of silk in the ‘ground state’ for 10 species of orb web spiders from five genera (two species each from *Argiope*, *Cyclosa*, *Cyrtophora*, *Leucauge* and *Nephila*) to determine the extent that post-secretion processes influence the performance of the MA silk of wild spiders. For each species, we collected individuals from different regions of Taiwan to maximize variation in MA silk amino acid compositions. We determined: (i) the MA silk amino acid composition, (ii) the mechanical properties of the MA silk in the supercontracted ‘ground state’, and (iii) the mechanical properties of native MA silks from individuals collected in the wild. Comparisons of (ii) with (iii) allowed us to determine the degree of mechanical property variation within and between genera to assess the degree to which amino acid composition variations influence the mechanical properties of native silk. By making inter- and intra-generic comparisons, we could assess the influence of amino acid composition on silk mechanical properties with and without the influences of phylogenetic signal taken into account. We interpreted any within or between species differences in the mechanical properties of native and ‘ground state’ silk not accompanied by amino acid compositional differences as a result of physiological and/or biochemical processes within the gland acting on the silk.

## 2. MATERIAL AND METHODS

### 2.1. Spider collection

We collected adult females of 10 species of orb web spider, two species each from the genera: *Argiope* (*Argiope aetherea* and *Argiope aemula*, Araneidae), *Cyclosa* (*Cyclosa mulmeinensis* and *Cyclosa confusa*, Araneidae), *Cyrtophora* (*Cyrtophora unicolor* and *Cyrtophora moluccensis*, Araneidae), *Leucauge* (*Leucauge blanda* and *Leucauge tessellata*, Tetragnathidae) and *Nephila* (*Nephila clavata* and *Nephila pilipes*, Nephilidae). For each species, a total of 12 individuals were collected. All individuals of any one species were collected at the same time of year to exclude season as an influence in the comparisons within species. We accounted for non-random error effects encountered when making interspecific and inter-generic comparisons by making independent contrasts of the phylogenetic branch lengths [41], calculated by single vector analyses (see Blamires *et al.* [42] for details), derived from a recently published molecular phylogeny that includes these genera [43].

## 2.2. Spider and web measurements

There is covariation between MA silk properties, body condition and web type and architecture among species of orb web spiders [42,44,45]; so accounting for phylogeny alone does not eliminate all of the extraneous bias that may influence silk properties in different spiders. We therefore measured carapace length and width of each spider collected using digital calipers in the field prior to collecting their MA silk. For each species at each of its collection sites, we randomly selected five webs of adult female spiders of approximately similar size as those used for silk collection and counted the number of radii and measured, using a tape measure, the orb and hub radius in four cardinal directions (up, down, left and right). We calculated the web capture area and mesh height (i.e. the spacing between spiral threads) using a published formula [46]. Because they build three-dimensional webs with horizontally orientated orbs, it was not possible to make the earlier-mentioned measurements on the webs of *Cyrtophora* spp. Accordingly, we estimated web volume (in litres) exclusively for this genus using measurements of vertical web length and horizontal width in the four cardinal directions input into a published formula [47], and counted the number of vertical supporting frame threads.

## 2.3. Silk collection, amino acid determination and tensile testing

We extracted MA silk from each individual immediately upon collection in the field to avoid any effects of transportation or housing on silk properties. We anaesthetized all spiders using CO<sub>2</sub> before fixing them to a foam platform using non-adhesive tape and pins. We waited 30 min to ensure that there was no influence of anaesthesia over silk properties before drawing MA silk from each individual using a mechanical spool. MA silk was reeled from each spider at a constant speed (1 m min<sup>-1</sup>) for 1 h. The spinnerets were observed under a dissecting microscope to ensure that a single fibre was consistently drawn. Ten 25 mm sections of taut MA silk fibre were mounted onto cardboard frames (open area = 20 × 20 mm, border = 5 mm) with double-sided adhesive tape around its border. A second cardboard frame with double-sided adhesive tape around its border was placed on top of the original and the frames were stuck together securing the silk within by adding one drop of superglue at the position where the silk was secured between frames and squeezing the borders with forceps. The frames containing silk were taped to a microscope slide and examined, at 1000× magnification, and photographed using a polarized light microscope (BX 50, Olympus, Tokyo) connected to a UC-series Nikon digital camera. The width of each thread was determined from the photographs using the program IMAGE J (NIH, Bethesda MD, USA). All silks were extracted by the same method by the same researcher (S.J.B.) under controlled temperature (approx. 25°C) and humidity (approx. 30% R.H.) so spinning speed or post-spin handling had no influence on variations in the mechanical properties of the silks.

The remaining extracted silk from each individual was weighed to the nearest 0.01 mg on an electronic balance, placed into 10 µl tubes (Eppendorf, Hamburg, Germany) and submerged in 6 mol l<sup>-1</sup> hexafluoro-isopropanol (500 µl mg<sup>-1</sup> silk). The samples were examined to ensure there were no suspended particles before being dried and hydrolysed in 6 mol l<sup>-1</sup> HCl for 24 h, whereupon the amino acid composition (percentages) was determined by high-performance liquid chromatography (Waters Pico-Tag Amino Acid Column, Milford CA, USA) at the Instrument Center, National Ching-Hwa University, Taiwan. Proline composition was calculated as a percentage of total amino acids.

Native silk tensile tests were performed under controlled temperature (approx. 25°C) and humidity (approx. 30% R.H.) on five silk samples from each individual at the Industrial Technology Research Institute, Hsinchu, Taiwan, within 14 days of collecting as follows. Firstly, we placed the frames containing single silk fibres within the grips of a UTM Nano Bionix tensile tester (MTS Systems Corporation, Oakridge TN, USA), so that the grips held the silk firmly at the edge of the frame. The silks were stretched at a rate of 1 per cent of the gauge length per second until rupture. The load resolution varied from 2 to 5 µN, depending on the diameter of the silk being tested.

True stress ( $\sigma$ ) and strain ( $\epsilon$ ) were calculated by [48]

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

where  $F$  is the force applied to the specimen and  $A$  is the cross-sectional area of the thread calculated from diameter assuming constant thread volume [26,49], and

$$\epsilon = \log e \frac{L}{L_0},$$

where  $L$  is the instantaneous length of the fibre at a given extension value, and  $L_0$  is the original gauge length of the fibre. Stress–strain curves were plotted for each silk using TestWorks v. 4.0 (MTS Systems Corporation, Eden Prairie MN, USA). From which we calculated the following mechanical performance parameters: (i) ultimate strength (or the stress at rupture); (ii) extensibility (or the strain at rupture); (iii) toughness (the total work of extension), calculated as the area under the stress–strain curve; and (iv) Young's modulus (stiffness), calculated as the slope of the curve during the initial elastic phase for each specimen.

Tensile tests of supercontracted silks were done on a further five silk samples from each individual at the University of Akron, USA. The rationale for these tests was twofold. Firstly, the supercontraction behaviour of silk is particularly responsive to variation in proline and, secondly, the effects of spinning conditions are largely removed as an influence over mechanical behaviour [13,14,36,37]. The test was done by: (i) supercontracting the fibres at 100 per cent relative humidity within a stainless steel chamber (see Agnarsson *et al.* [35] for details) while held within the grips of a UTM Nano Bionix tensile tester without tension applied so as to ascertain how much stress was generated when restrained, (ii) relaxing the fibres while wet



to determine the supercontraction percentage shrink (PS), calculated as the absolute difference between the pre-shrink ( $\ell_0$ ) and post-shrink ( $\ell_1$ ) fibre length (see Boutry & Blackledge [36] for details), (iii) drying the fibres at maximum relaxation, and then (iv) subjecting them to tensile testing as outlined for native silks. The measurements corresponded to the ‘ground state’ outlined by Elices *et al.* [38,39], which removes many of the post-secretion effects on silk performance. All tensile testing was conducted within 14 days of silks being collected in the field.

#### 2.4. Statistical comparisons

We used analysis of covariance (ANCOVA) to ascertain whether proline composition varied between intra-generic pairs and/or within species from different regions. We used a series of multivariate analyses of covariances (MANCOVA) to ascertain whether: (i) the mechanical properties of the native silks collected from different locations differed within species and (ii) the mechanical properties (ultimate strength, extensibility, toughness and Young’s modulus) of the native silks differed within those of the supercontracted silk for each species. For each ANCOVA/MANCOVA, we used the multiple covariates: (i) body condition, calculated as the residuals between carapace length and width and (ii) principal component eigenvectors of the web architectural parameters measured for each species (table 1). Using eigenvectors accounted for the different web types, the different architectural parameters measured, and the wide range of parameter values, across the 10 species. All analyses were conducted using independent contrasts of the inter-generic branch lengths [41]. We used Levene’s tests, Kolomogrov–Smirnov tests and homogeneity of slopes tests (all  $p > 0.05$ ) to check the data for heterogeneous variances and conformity to normality, and that the covariate slopes were homogeneous and parallel. Where data failed these tests,  $\log_{10}$  or fourth root transformations were performed. Proline compositional data were arcsine-transformed for normalization.

We additionally performed a series of multiple regressions. Firstly, to ascertain the ‘ground state’ mechanical properties that vary with proline in each species, we performed individual multiple regressions between proline and the mechanical properties of the supercontracted silks for each species. We then used the data for all of the species combined to ascertain whether there were congruence between proline compositional variations and variations in PS, the mechanical properties of the supercontracted silks and the mechanical properties of the native silks, and whether these variations depended on inter-generic branch lengths. We did this using two approaches: (i) standard forward and backward linear regression and (ii) regression using independent phylogenetic contrasts [41]. We tested the data for normality, linearity, homoscedasticity, co-linearity and singularity by a combination of Q–Q plots and scatter-plots,  $\log_{10}$  or fourth-root transforming the data where necessary. Bonferroni-corrections to  $p$ -values were applied, where appropriate, to account for multiple testing.

Table 1. First, second and third principal component eigenvectors (PC1, PC2 and PC3) of the web architectural parameters (number of radii, web capture area and mesh height) measured for each species.

species	eigenvalues			% variation explained
	PC1	PC2	PC3	
<i>Argiope aemula</i>	1.339	1.125	0.536	44.62
<i>Argiope aetherea</i>	1.484	1.175	0.431	49.97
<i>Cyclosa confusa</i>	1.557	0.778	0.665	51.90
<i>Cyclosa mulmeinensis</i>	1.462	0.938	0.602	48.73
<i>Cyrtophora moluccensis</i> <sup>a</sup>	1.110	0.890	—	55.49
<i>Cyrtophora unicolor</i> <sup>a</sup>	1.213	0.787	—	60.23
<i>Leucauge blanda</i>	1.438	0.999	0.563	52.98
<i>Leucauge tessellata</i>	1.783	0.992	0.226	59.42
<i>Nephila clavata</i>	1.527	0.852	0.621	50.81
<i>Nephila pilipes</i>	1.715	0.723	0.625	57.17

<sup>a</sup>Two web parameters were measured in *Cyrtophora* spp.; web volume and number of vertically supporting frame threads.

### 3. RESULTS

Since we found none of the 10 species to have significant differences in their MA silk proline compositions or mechanical properties between individuals from different locations (per cent proline: ANCOVA; all  $p > 0.10$ , mechanics: MANCOVA; all Bonferroni-corrected  $p > 0.01$ ), we pooled data from the two locations for each species to perform within genus analyses. The only intra-generic species pairs for which we found a difference in MA silk proline composition was the two *Nephila* species (table 2).

Examination of typical stress–strain curves across species showed that there were two patterns of mechanical performance (figure 1). The stiffer silks, labelled ‘pattern A’, comprised silks from spiders of the genera *Nephila*, *Cyrtophora* and *Argiope aetherea*, while the more stretchy silks of ‘pattern B’ comprised silks from spiders of the genera *Cyclosa*, *Leucauge* and *Argiope aemula* (figure 1). For nine of the 10 species, the exception being *Argiope aemula* for whom differences were found but they were statistically insignificant ( $p = 0.08$ ), each of the mechanical properties of the ‘ground state’ silks differed significantly from those of native silks (table 3).

Proline composition influenced the mechanics of silks in the ‘ground state’ in all species with the exception of the two species of *Argiope* (electronic supplementary material, table S1). In *Cyclosa* spp., *Leucauge* spp. and *N. clavata* proline was negatively correlated with ultimate strength. In *Cyrtophora* spp., proline was positively correlated with extensibility, while in *N. pilipes* it was both positively correlated with extensibility and negatively correlated with ultimate strength (see electronic supplementary material, table S1). Of the mechanical properties examined across species, PS (table 3 and figure 2) and extensibility (table 4 and figure 3a) were positively correlated with proline composition in the ‘ground state’, whereas ultimate strength and Young’s modulus were negatively correlated

Table 2. Composition (% of total amino acids) of proline (PRO) for 10 species of spider.  $F$ - and  $p$ -values are for intra-generic analyses of covariance of proline composition with body condition and the eigenvectors of a principal component analysis of web architecture parameters as covariates.

genus	species	PRO	$F_{1,10}$	$p$
<i>Argiope</i>	<i>A. aemula</i>	$9.712 \pm 1.201$	2.606	0.174
	<i>A. aetherea</i>	$10.451 \pm 1.284$		
<i>Cyclosa</i>	<i>C. confusa</i>	$8.313 \pm 1.552$	0.273	0.778
	<i>C. mulmeinensis</i>	$7.798 \pm 2.385$		
<i>Cyrtophora</i>	<i>C. moluccensis</i>	$3.913 \pm 1.535$	2.193	0.182
	<i>C. unicolor</i>	$2.990 \pm 1.063$		
<i>Leucauge</i>	<i>L. blanda</i>	$12.775 \pm 0.204$	0.872	0.330
	<i>L. tessellata</i>	$11.373 \pm 1.113$		
<i>Nephila</i>	<i>N. clavata</i>	$0.778 \pm 1.501$	31.094	<0.0001
	<i>N. pilipes</i>	$8.890 \pm 1.365$		

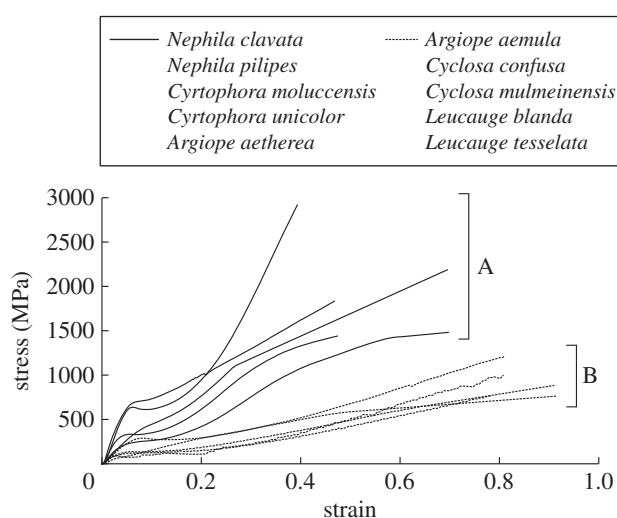


Figure 1. Comparison of typical stress–strain curves for each of the 10 species, showing that the mechanical properties of the silks of each species conform to one of two patterns: pattern A (area A) represents stiffer fibres and pattern B (area B) represents more extensible fibres.

(table 4 and figure 3*b,c*). Nonetheless, only PS and extensibility were significantly correlated with proline composition when the influence of phylogenetic signal was accounted for (table 4). Extensibility was positively correlated with proline composition in native MA silks using a standard regression (table 4 and figure 3*b*) but not when phylogenetic signal was accounted for (table 4). Toughness was not correlated with proline composition variations in ‘ground state’ MA silks (figure 3*d*). Ultimate strength, toughness and Young’s modulus were not correlated with variations in proline composition in native silks (table 4). There was, therefore, a contrast between the native and ‘ground state’ mechanical properties indicating that post-secretion processes substantially affect the mechanical properties of wild orb web spider silks.

#### 4. DISCUSSION

We tested whether amino acid variations influenced silk properties, as predicted by the two-spidroin model, using

MA silks from spiders in their natural habitat and found that variation in native MA silks differed from that of silk supercontracted to its ‘ground state’. The properties of the ‘ground state’ MA silks conformed to the predictions of the model but the properties of native silks did not. Hence the model alone does not explain the degree of natural variation in native MA silk properties among different spider species because native silk mechanical properties are largely influenced by post-secretion processes. Our findings may explain why: (i) no phylogenetic signal influence on MA silk properties was found by Swanson *et al.* [13], (ii) attempts to clone and spin recombinant silk have failed to produce fibres with properties analogous to those naturally secreted by spiders [11], and (iii) MA silk mechanical property changes induced by different prey types, levels of nutrient acquisition and ambient conditions are not necessarily accompanied by congruent changes in amino acid composition [21,22,50]. Moreover, the correlations between ultimate strength and extensibility and proline show that a trade-off between ultimate strength and extensibility exists among orb web spider MA silks in the ‘ground state’, in contrast to Swanson *et al.* [13], who used only native silk, and this trade-off is associated with silk proline composition (see also [17,18]).

Amino acid compositions have been shown to influence mechanical properties in supercontracted MA silks for *N. clavipes*, *Latrodectus hesperus* and *Araneus diadematus* [8,13,40,51] and all of the species examined herein. Although we found an association between per cent proline and ultimate strength, extensibility and Young’s modulus of the ‘ground state’ MA silks across 10 species, no relationship was found in wild native MA silks (other than for extensibility when phylogenetic signal was not accounted for). Despite a range of proline, alanine and glycine compositions being found among the different MA silks, the mechanical properties of the native silks were relatively similar between and within species compared with the ‘ground state’ after the influences of phylogeny, spider body condition and web architecture were accounted for. Such contrasts between the variations in native MA silk mechanical properties and variations in amino acid compositions means post-secretion processes acting in the gland [24–27] influence the molecular

Table 3. Mechanical properties (mean  $\pm$  s.e.) of native state (NS) and ground state (GS) MA silks of 10 species of spider. Results of individual MANCOVAs show that all properties differed between native and supercontracted silks at Bonferroni-corrected  $p \leq 0.01$ .

species	properties										MANOVA
	ultimate strength (MPa)		extensibility (mm mm <sup>-1</sup> )		toughness (MJ m <sup>-3</sup> )		Young's modulus (GPa)		Wilk's $\lambda_{10,166}$	$p$	
	NS	GS	NS	GS	NS	GS	NS	GS			
<i>A. aemula</i>	921 $\pm$ 46	1108 $\pm$ 55	0.3 $\pm$ 0.1	0.8 $\pm$ 0.1	166 $\pm$ 12	323 $\pm$ 20	8.8 $\pm$ 0.6	3.8 $\pm$ 0.6	0.864	0.08	
<i>A. aetherea</i>	688 $\pm$ 24	1499 $\pm$ 50	0.4 $\pm$ 0.2	0.7 $\pm$ 0.1	205 $\pm$ 18	426 $\pm$ 20	7.4 $\pm$ 0.5	2.1 $\pm$ 0.5	0.760	0.001	
<i>C. confusa</i>	207 $\pm$ 11	832 $\pm$ 22	0.3 $\pm$ 0.1	0.8 $\pm$ 0.2	48 $\pm$ 6	222 $\pm$ 24	3.7 $\pm$ 0.3	1.3 $\pm$ 0.1	0.759	<0.0001	
<i>C. mulmeinensis</i>	422 $\pm$ 18	1152 $\pm$ 88	0.3 $\pm$ 0.3	0.9 $\pm$ 0.1	59 $\pm$ 5	302 $\pm$ 32	5.3 $\pm$ 0.5	1.4 $\pm$ 0.1	0.698	<0.0001	
<i>C. moluccensis</i>	721 $\pm$ 36	1446 $\pm$ 95	0.3 $\pm$ 0.2	0.4 $\pm$ 0.2	176 $\pm$ 8	219 $\pm$ 24	10.4 $\pm$ 0.3	6.2 $\pm$ 1.0	0.703	0.001	
<i>C. unicolor</i>	486 $\pm$ 22	1717 $\pm$ 81	0.2 $\pm$ 0.1	0.4 $\pm$ 0.7	147 $\pm$ 5	292 $\pm$ 28	8.3 $\pm$ 0.4	6.9 $\pm$ 0.3	0.728	0.001	
<i>L. blanda</i>	554 $\pm$ 37	751 $\pm$ 51	0.5 $\pm$ 0.2	0.9 $\pm$ 0.1	97 $\pm$ 10	253 $\pm$ 32	5.9 $\pm$ 0.8	2.4 $\pm$ 0.5	0.713	<0.0001	
<i>L. tessellata</i>	565 $\pm$ 23	976 $\pm$ 45	0.3 $\pm$ 0.1	0.9 $\pm$ 0.1	101 $\pm$ 7	209 $\pm$ 36	7.3 $\pm$ 0.1	1.8 $\pm$ 0.4	0.629	<0.0001	
<i>N. clavata</i>	665 $\pm$ 43	2143 $\pm$ 127	0.2 $\pm$ 0.1	0.4 $\pm$ 0	115 $\pm$ 15	488 $\pm$ 50	8.2 $\pm$ 0.5	6.9 $\pm$ 2.2	0.722	<0.0001	
<i>N. pilipes</i>	516 $\pm$ 32	993 $\pm$ 66	0.3 $\pm$ 0.2	0.7 $\pm$ 0.2	77 $\pm$ 13	550 $\pm$ 59	7.6 $\pm$ 0.7	6.2 $\pm$ 0.2	0.769	<0.0001	

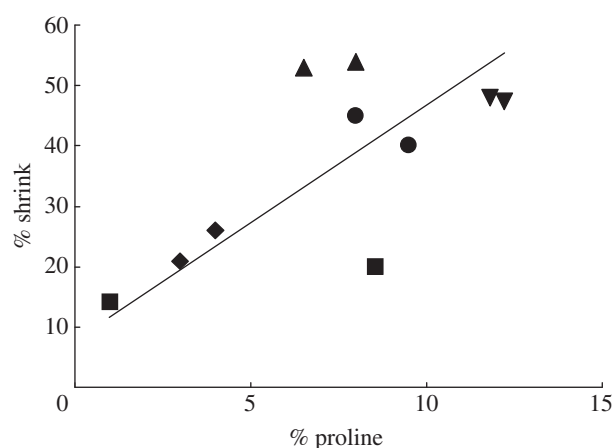


Figure 2. Relationship, showing standard regression curve, between the % shrink and % proline (used to represent MaSp2 compositions in the regression model) of 'ground state' (supercontracted) silks using data for all 10 species examined combined. Each point represents a mean value obtained for six individuals of one species. Triangles, *Cyclosa* spp.; squares, *Nephila* spp.; diamonds, *Cyrtophora* spp.; circles, *Argiope* spp.; inverted triangles, *Leucauge* spp.

alignments and crystal structures of the silk proteins. Another potential determining factor is the effect of spinning action over MA silk mechanical properties. Spiders may vary the reeling speed of the MA silk or differentially apply friction at the spinning valve under different ecological circumstances and consequently alter the natural silk properties [24,25]. We, however, expect this effect was minimal here as the rate of pull during spinning was standardized across individuals.

All of the genera studied herein conformed to the generalization that amino acid composition determines their 'ground state' mechanical properties. The one exception was the two species of *Argiope*. The stress-strain curves of the two *Argiope* also differed more than any other intra-generic pairs, despite having similar amino acid compositions. The result was surprising as we would have expected the two species of *Nephila* to differ more owing to their differences in proline composition. It, nonetheless, highlights that although proline composition generally predicts MA silk mechanical properties in the 'ground state', the specific mechanical responses across a range of stresses may be species specific. Other studies have found similar deviations in the mechanical properties of both supercontracted and native MA silks between different species of *Argiope* [42,52]. It is beyond the scope of this paper to speculate about why the mechanical properties of MA silks vary considerably between *Argiope* spp., but we consider it an intriguing question for further research.

Most of the genera we used construct relatively typical two-dimensional vertically aligned orb webs. However, *Cyrtophora* spp. builds three-dimensional webs that consist of fine-meshed silken barrier threads that form a three-dimensional barrier structures extending above and beneath a non-sticky horizontal orb structure. The MA silks of the two *Cyrtophora* species had greater ultimate strength in the 'ground state' than those of any other species except *Nephila clavata*, which, incidentally, builds three-dimensional barrier

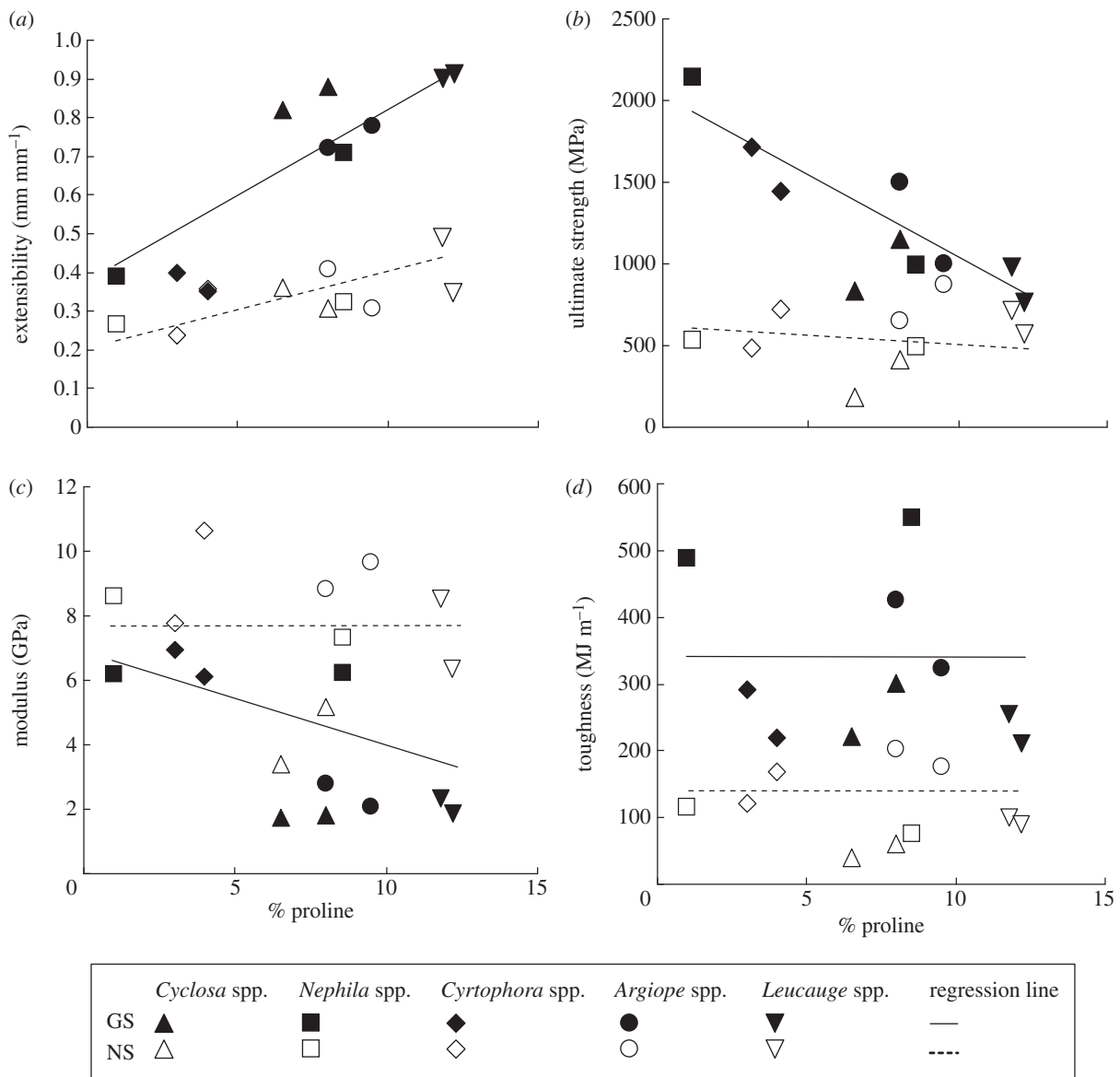


Figure 3. Relationship between the mechanical properties: (a) ultimate strength, (b) extensibility, (c) toughness and (d) Young's modulus and % proline (used to represent MaSp2 compositions) of 'ground state' (GS) and native state silks (NS) for each species. Each point represents a mean value obtained for six individuals of one species.

Table 4. Multiple regression models (standard and independent contrasts of phylogenetic branch lengths) show the association between proline composition in spider MA silks and the mechanical properties: percentage shrink (PS), ultimate strength, extensibility, toughness and Young's modulus for silks in the ground state (supercontracted) silk (standard regression: adjusted  $R^2 = 0.627$ ;  $p = 0.001$ , independent contrasts: adjusted  $R^2 = 0.412$ ;  $p = 0.013$ ) and native state silks (standard regression: adjusted  $R^2 = 0.036$ ;  $p = 0.715$ ; independent contrasts: adjusted  $R^2 = 0.032$ ;  $p = 0.750$ ). Data are from all species combined.

property	ground state silk						native state silk					
	standard regression			independent contrasts			standard regression			independent contrasts		
	$\beta$	$t_{14}$	$p$	$\beta$	$t_{14}$	$p$	$\beta$	$t_{14}$	$p$	$\beta$	$t_{14}$	$p$
percentage shrink	0.473	3.098	0.007	-0.549	-3.433	0.004	—	—	—	—	—	—
ultimate strength	-0.805	-2.246	0.040	-0.315	-1.398	0.184	0.390	1.487	0.203	0.023	0.550	0.583
extensibility	0.889	3.283	0.005	0.522	2.706	0.009	0.063	0.650	0.039	0.246	1.111	0.285
toughness	0.601	0.164	0.124	-0.079	-0.298	0.679	0.584	1.405	0.160	0.623	1.551	0.143
Young's modulus	-0.690	-2.711	0.016	0.164	0.650	0.562	0.005	0.154	0.877	0.189	0.412	0.686



webs either side of its two-dimensional orb web [42]. The two species of *Cyrtophora* and *N. clavata* also had MA silks with the lowest proline compositions. However, the closest relatives to *N. clavata* and *Cyrtophora*, such as *N. pilipes* and the two *Argiope* spp., had MA silks with higher proline and lower alanine compositions. Such results indicate that web dimensionality may be indirectly related to MA silk spidroin expression and/or mechanical properties. We suggest MA silks of more two-dimensional and three-dimensional orb web building spiders be compared to elucidate the relationship between web dimensionality, silk performance requirements and spidroin expression patterns.

To recapitulate, while the two-spidroin, MaSp, model is explanative of the variations found in the mechanical properties of 'ground state' MA silks, post-secretion processes appear to influence native silk mechanical properties in wild spiders. While the properties of MA silk may vary within individuals across environments [21,25,26,32,50], we found that the properties of native MA silk from the 10 species of orb web spider used herein were perceptibly homogeneous. The question remaining to be resolved is: why is a genetic mechanism allowing for plasticity (amino acid composition, presumably as a product of spidroin expression) nullified in nature by post-secretion processing? An answer probably lies in there being different selective pressures acting on spidroin expression and MA silk mechanical properties. Spidroin expression seems to have diversified to provide the means for supercontraction shrink to accommodate the tension requirements of different web types [36]. Because MaSp2 and its orthologues are more expensive to metabolically synthesize than MaSp1, selection has facilitated plasticity in spidroin expression to respond to unpredictability in the spider's diet and environment [21,50]. Mechanical property constancy, on the other hand, is required to maintain MA silk functionality as an integral component of an energy absorbing prey capture web and spider life line [13,44] independent of the environment. Our study suggests that the latter is facilitated by post-secretion processes.

The implication of our finding that post-secretion processes profoundly affect spider MA silk performance independent of factors, such as spidroin expression and reeling speed, is that it suggests that post-secretion processes be examined in different spiders and it should be accounted for in any attempts to create analogous materials. Moreover, MA silk is an externally secreted biomaterial that is often exposed to heterogeneous environments and we have showed that its properties can be significantly altered by post-secretion processes. Nevertheless, whether post-secretion processes also influence the physical properties of other externally secreted biomaterials remains to be resolved.

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