



Environmentally induced post-spin property changes in spider silks: influences of web type, spidroin composition and ecology

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Many spiders use silk to construct webs that must function for days at a time, whereas many other species renew their webs daily. The mechanical properties of spider silk can change after spinning under environmental stress, which could influence web function. We hypothesize that spiders spinning longer-lasting webs produce silks composed of proteins that are more resistant to environmental stresses. The major ampullate (MA) silks of orb web spiders are principally composed of a combination of two proteins (spidroins) called MaSp1 and MaSp2. We expected spider MA silks dominated by MaSp1 to have the greatest resistance to post-spin property change because they have high concentrations of stable crystalline β -sheets. Some orb web spiders that spin three-dimensional orb webs, such as *Cyrtophora*, have MA silks that are predominantly composed of MaSp1. Hence, we expected that the construction of three-dimensional orb webs might also coincide with MA silk resistance to post-spin property change. Alternatively, the degree of post-spin mechanical property changes in different spider silks may be explained by factors within the spider's ecosystem, such as exposure to solar radiation. We exposed the MA silks of ten spider species from five genera (*Nephila*, *Cyclosa*, *Leucauge*, *Cyrtophora*, and *Argiope*) to ecologically high temperatures and low humidity for 4 weeks, and compared the mechanical properties of these silks with unexposed silks. Using species pairs enabled us to assess the influence of web dimensionality and MaSp composition both with and without phylogenetic influences being accounted for. We found neither the MaSp composition nor the three-dimensionality of the orb web to be associated with the degree of post-spin mechanical property changes in MA silk. The MA silks in *Leucauge* spp. are dominated by MaSp2, which we found to have the least resistance to post-spin property change. The MA silk in *Argiope* spp. is also dominated by MaSp2, but has high resistance to post-spin property change. The ancestry of *Argiope* is unresolved, but it is largely a tropical genus inhabiting hot, open regions that present similar stressors to silk as those of our experiment. Ecological factors thus appear to influence the vulnerability of orb web spider MA silks to post-spin property change. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, 106, 580–588.

ADDITIONAL KEYWORDS: environmental stress – major ampullate silk – mechanical properties – orb web spiders – phylogenetic comparisons – post-spin silk properties.

INTRODUCTION

Spider silks are tough, elastic, and adaptable biomaterials, and consequently industries are interested in developing materials that mimic their properties

(Vollrath, Porter & Holland, 2011; Blamires & Tso, 2012). A disadvantage associated with many potential applications of spider silk, however, is its mechanical instability as a result of post-spin molecular rearrangements, or molecular 'draw-down', of the proteins (Agnarsson, Boutry & Blackledge, 2008; Keten & Buehler, 2010). Molecular models derived from

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synchrotron radiation predict that short-term environmental stresses, such as high temperature or dehydration, interrupt crystalline intermolecular bonds, causing molecular realignment in the silk proteins (Kaplan, 1998; Vollrath *et al.*, 2011). Whereas the physical and chemical bases of post-spin molecular rearrangements in spider silk are partly understood, how they correlate with silk composition or spider ecology is largely unknown.

Phylogenetic analyses show that orb web spiders (Orbiculariae) have modified their web designs over time, probably in adaptation to continuously changing ecological circumstances (Blackledge *et al.*, 2009; Sensenig, Agnarsson & Blackledge, 2010; Dimitrov *et al.*, 2012). Presently, some orb web spiders build webs with a two-dimensional planar architecture that are taken down and replaced daily. This web recycling should limit selection for long-term stability of the threads in orb webs. On the other hand, some species surround their two-dimensional orb webs with a silken barrier or a tangle of MA threads (Lubin, 1973; Blamires *et al.*, 2010). These three-dimensional orb webs usually remain in service for weeks rather than days (Carico, 1986). The non-sticky framework and radial lines of two-dimensional orb webs, and the barrier and tangle threads added to the three-dimensional orb webs, are formed from major ampullate (MA) silk (Blackledge, Kuntner & Agnarsson, 2011). A combination of high strength and extensibility enable MA silk to absorb most of the force when prey strike the web (Gosline *et al.*, 1999; Blackledge *et al.*, 2011).

Depending on temperature, humidity, UV levels, and pollutant concentrations, spider MA silks may naturally undergo significant short-term (i.e. within days) post-spinning mechanical property change as a result of draw-down deformation, causing cross-linking in the amorphous region and the establishment of crystalline β -sheets (Kitagawa & Kitayama, 1997; Perez-Rigueiro *et al.*, 2007; Agnarsson *et al.*, 2008). Different spider silks vary in their chemical composition (Work & Young, 1987), and may change properties at different rates under similar conditions (Vollrath *et al.*, 2011). Sticky spiral (flagelliform and aggregate) and prey-wrapping (aciniform) silks may change in stickiness, strength, and elasticity more readily at room temperature than MA silk (Kitagawa & Kitayama, 1997; Sahni, Blackledge & Dhinojwala, 2011). Ecologically, mechanical property changes in MA silks may serve to increase the strength of orb webs immediately post-construction (Agnarsson, Kuntner & Blackledge, 2010; Blackledge *et al.*, 2011; Vollrath *et al.*, 2011), but it is not known how they influence the long-term functionality of the web.

The MA silks of different orb web spiders are a product of the relative expression of genes encoding

for two proteins (spidroins): MaSp1 and MaSp2 (Savage & Gosline, 2008). These two spidroins differ in amino acid composition, and hence secondary and tertiary molecular structure, with consequent effects on the mechanical properties of the silk (Kaplan, 1998; Ayoub *et al.*, 2007). MaSp1 has a greater concentration of alanine and glycine, but is low in glutamine and free of proline, whereas MaSp2 has prevalent glutamine and proline, but is lower in alanine than MaSp1 (Ayoub *et al.*, 2007; Liu, Shao & Vollrath, 2008; Savage & Gosline, 2008; Blamires, Wu & Tso, 2012). Alanine and glycine harbour small side chains (Nelson & Cox, 2005), and hence form strength-inducing β -sheets, whereas proline disrupts β -sheet formation and promotes the formation of β -coils, endowing fibre extensibility (Liu *et al.*, 2008). As β -sheet formation is associated with enhanced thermal stability in silk (Kaplan, 1998; Keten & Buehler, 2010), silks predominant in MaSp1 should have greater resistance to post-spin mechanical property changes than those predominant in MaSp2. Nonetheless, other features, e.g. flaws in the skin core, crystal size or density, and other associated proteins and inorganic compounds (Kitagawa & Kitayama, 1997; Perez-Rigueiro *et al.*, 2007; Giesa *et al.*, 2011), may influence silk stability. Many orb web spiders with predominately MaSp1 MA silk also build three-dimensional orb webs as adults, e.g. *Cyrtophora* spp., or two-dimensional orb webs surrounded by three-dimensional barrier structures, e.g. some *Nephila* spp. (Liu *et al.*, 2008; Blamires *et al.*, 2012). Accordingly, if MaSp composition influences stability in MA silks, the orb web three-dimensionality may be associated with MA silks with higher resistance to post-spin mechanical property change. Indeed, such an association between orb web dimensionality and silk stability may partially explain why two-dimensional orb webs are rebuilt daily but three-dimensional webs are not (Carico, 1986). Alternatively, the MA silks in three-dimensional orb webs may change properties post-spinning as a means of enhancing web performance, regardless of MaSp composition.

We tested the hypothesis that variations in post-spin mechanical property changes in orb web spider MA silk are associated with MaSp composition and/or orb web dimensionality against the alternative that they are a product of adaptations to the spider's environment or unexplained phylogenetic influences. We determined the amino acid composition, as an estimate of MaSp composition, of the MA silks of 10 species of orb web spider. The silks were incubated at 35 °C and 0% relative humidity over 4 weeks and mechanical tests were performed. The ten spider species comprised two species each from the genera *Nephila* (*Nephila clavata* and *Nephila pilipes*),

Cyclosa (*Cyclosa mulmeinensis* and *Cyclosa confusa*), *Leucauge* (*Leucauge blanda* and *Leucauge tessellata*), *Cyrtophora* (*Cyrtophora unicolor* and *Cyrtophora moluccensis*), and *Argiope* (*Argiope aetherea* and *Argiope aemula*). We used these species because they represent a mixture of two- (*N. pilipes*, *C. mulmeinensis*, *C. confusa*, *L. blanda*, *L. tessellata*, *A. aetherea*, and *A. aemula*) and three-dimensional web builders (*N. clavata*, *C. unicolor*, and *C. moluccensis*). As an adult, *N. clavata* builds a vertical orb web that incorporates sticky threads and is enclosed by a series of barrier threads. *Cyrtophora* spp. builds a horizontal dome web, which lacks aggregate silk, with a dense network of barrier threads above and below the dome (Lubin, 1973; Blamires *et al.*, 2010; see Fig. S1). The barrier threads in both *N. clavata* and *Cyrtophora* spp. are exposed to the environment for weeks at a time, and function similarly: they deter predators and absorb the impact of prey, reducing their kinetic energy and deflecting their flight towards the web (Lubin, 1973; Blamires *et al.*, 2010). We therefore expect the demands on the mechanical properties of the MA silks between these spiders to be similar. All species were found at the forest edge or in understory, with the exception of the two *Argiope* spp., and *Cyclosa mulmeinensis*, which were found in open or grassland ecosystems.

We adapted a generic-level ‘working’ phylogeny from a recent molecular phylogeny (Blackledge *et al.*, 2009) upon which we ‘hung’ web dimensionality (Fig. 1) and proline composition, and calculated the lengths of the branches between generic common ancestors (i.e. ‘internode branch length’). Assigning genus pairs equal internode branch lengths (Grafen, 1989) meant that we could perform inter- and intrageneric comparisons to assess the influence of the MaSp composition and web type on the degree of

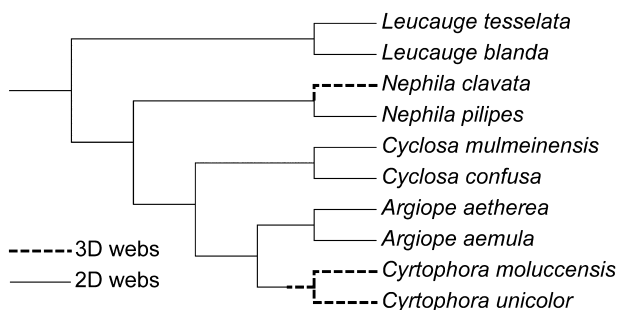


Figure 1. The working phylogeny created to calculate the internode branch lengths used in the comparative analyses of environmentally induced MA silk property changes in orb web spiders. The position of the species that build two-dimensional (2D webs) and three-dimensional (3D webs) orb webs are indicated.

post-spin mechanical property change, with and without the phylogenetic account of Pagel (1998).

MATERIAL AND METHODS

SILK COLLECTION AND PREPARATION

We collected the silks from six adult females per species in temporary field laboratories so that the transportation and housing of the spiders did not impact the ‘as spun’ properties of the silks. In all instances body length and weight were measured upon capture using calipers and an electronic balance, to ensure that all of the individuals that we used for each species were of approximately similar length and mass.

Each spider was anaesthetized using CO₂ before fixing them to a foam platform using non-adhesive tape and pins. We waited 30 min to ensure that there was no influence from the anaesthetic over the properties of the silk (Madsen & Vollrath, 2000), before drawing a length of silk from the MA gland and taping it to a mechanical spool. The MA silk was reeled from the gland at a constant speed (1 m min⁻¹) for 1 h. We used a dissecting microscope to observe the spinnerets to ensure that a single thread of MA silk was consistently drawn and that there was no intervention by other spinnerets.

Fifteen 25-mm sections of MA silk were collected from each individual by mounting taut silk onto cardboard frames (open area = 20 × 20 mm, border = 5 mm) with double-sided adhesive tape around its border (see Kitagawa & Kitayama, 1997). We placed a second cardboard frame with double-sided adhesive tape around its border on top of the original, and stuck the frames together, securing the silk within by squeezing the borders with forceps. The part of the border holding the silk was reinforced by a drop of superglue. The length of the silk thread within each frame was measured by digital calipers before the frames were taped to a microscope slide and examined and photographed using a polarized light microscope (Olympus BX50, Tokyo, Japan) connected to a UC-series Nikon digital camera. The diameter of each thread was determined from the photographs using ImageJ (NIH, Bethesda MD, USA), from which we calculated the cross-sectional area for use in the calculations of mechanical properties.

The remaining silk extracted from each individual was weighed to the nearest 0.01 mg on an electronic balance and placed into 10-μL Eppendorf tubes. We submerged the silk in 6 mol L⁻¹ hexafluoroisopropanol using 500 μL of solvent per mg of silk. The samples were examined under a dissecting microscope to ensure that there were no suspended particles before being dried. The samples were sub-

sequently hydrolysed in 6 mol L⁻¹ HCl for 24 h and the composition of amino acids was determined by high-performance liquid chromatography (Waters Pico-Tag Amino Acid Column, Milford, CA, USA). The proline composition was calculated from the total amino acid compositions after confirming that there were no associations across genera or with web type for any of the other amino acids (MANOVA; Roa's $R = 1.55$; $P > 0.05$).

EXPERIMENT

The 15 lengths of silk collected from each of the 60 spiders (six individuals from ten species) were assigned to one of three treatments ($N = 30$; five silks per individual, per treatment): (1) 'as spun', where thread width measurements and tensile tests were conducted within 48 h of silk collection; (2) 'incubated', where the silk was placed in a vacuum-sealed drying cabinet (DO45; Deng Yng Instruments, Tainan, Taiwan), maintained at 35 °C and 0% relative humidity for 4 weeks, after which thread width measurements and tensile tests were performed within 48 h; and (3) 'laboratory', where silks were placed in the laboratory, maintained at approximately 25 °C and 30% relative humidity, for the same 4-week period as the 'incubated' silks, to control for short-term changes in mechanical properties associated with ageing (Agnarsson *et al.*, 2008), after which thread width measurements and tensile tests were performed within 48 h. We used 35 °C and 0% relative humidity for incubation as these conditions represent an extreme environmental stress that the silks may naturally endure, and is likely to induce rapid property changes. An exposure period of 4 weeks was assigned to approximately represent the maximum time that MA silks might be expected to maintain mechanical function as a component of a web.

TENSILE TESTS

Tensile tests were performed at the Centre for Measurement Standards in Hsinchu, Taiwan. We placed the frames containing single silk threads within the grips of a UTM Micro Bionix tensile tester (MTS Systems Corp., Oakridge, TN, USA), ensuring the position where the frame was held in the grips corresponded to the position where the silk was held between the frames. The silk was subsequently stretched at a rate of 1% of the gage length per second until rupture. The load resolution varied from 2–5 µN, depending on the diameter of the silk tested. Tensile tests were conducted on the silks from all treatments under the same controlled laboratory conditions (~ 25 °C and ~ 30% relative humidity).

True stress and strain were calculated (Blackledge & Hayashi, 2006), and stress–strain curves were plotted using TestWorks 4.0 (MTS Systems Corporation, Eden Prairie, MN, USA). Using the stress–strain curves, we calculated the following mechanical properties: (1) ultimate strength, or stress at rupture; (2) extensibility, or strain at rupture; (3) toughness, or area under the stress–strain curve; and (4) Young's modulus (a measure of stiffness), or the slope of the curve during the elastic phase for each specimen.

STATISTICAL COMPARISONS

We compared the ultimate strength, extensibility, toughness, and Young's modulus of the 'as spun' versus 'incubated' versus 'laboratory' MA silk for each species by way of paired (among individuals), one-factor (the three treatments), MANOVAs. All data were skewed from normality (Kolmogorov–Smirnov tests: $P < 0.05$), so were log₁₀ transformed prior to analyses. Fisher's least-significant difference (LSD) post-hoc analyses were conducted to identify the significant variables, where differences were detected by MANOVA. Significance was identified in accordance with Bonferroni-corrected P values ($0.05/n$), which were applied to account for the multiple testing procedures.

A generalized least squares regression (Pagel, 1998) was carried out using the data from all species combined. The following parameters were included: (1) proline composition, as an estimate of MaSp composition (Liu *et al.*, 2008; Savage & Gosline, 2008); (2) web type, entered as a dummy variable, where 0 represents a two-dimensional orb web and 1 represents a three-dimensional orb web; and (3) mean change in, 'as spun' compared with 'incubated', mechanical properties of the MA silks, ascertained as the first principal component eigenvectors of the properties identified by MANOVA to vary between treatments. We assumed evolutionary divergence among the ten species by Brownian motion (Garland & Ives, 2000), and that the regression error term, ϵ , has variance = $\sigma^2 C$. Where σ^2 is the rate of change and C is the internode branch lengths determined from the working phylogeny as single vectors (Grafen, 1989). We considered the internode branch lengths of all of the genus pairs to be equal to one, and to form the basis for the intergeneric branch-length estimates.

We constructed a log-likelihood goodness-of-fit model to test four alternative hypotheses: (1) MaSp composition is associated with silk post-spin mechanical property change, independent of web type; (2) web type is associated with silk post-spin property change, independent of MaSp composition; (3) MaSp composition and web type are interactively associated with

silk post-spin property change; and (4) silk post-spin property change is independent of MaSp composition and web type. The model best represented by the data was identified by a G^2 test. Support for hypothesis (4) indicates that ecological and/or phylogenetic factors have stronger effects on MA silk post-spin property change than MaSp composition or web type. Differing post-spin property changes in the MA silk of *Argiope* spp., and/or *Cyclosa mulmeinensis*, which inhabit open/grassland, compared with all of the other species measured, were deemed to implicate ecological factors as being influential.

RESULTS

The ultimate strength, toughness, and Young's modulus of the MA silks from all of the species examined changed with treatment, with the exception of *Argiope aemula* (Tables 1 and 2). Extensibility was unaffected by treatment for all of the species (hence was excluded from Table 2). Incubated silk increased in ultimate strength (with the exception of *Cyclosa mulmeinensis* and *Argiope aetherea*, in which the silks were unaffected), toughness (with the exception of *Nephila* spp. and *Argiope aetherea*, in which the silks were unaffected), and Young's modulus (with the exception of *Cyclosa confusa* and *Cyrtophora* spp., in which the silks were unaffected, and *Argiope aetherea*, in which the 'incubation' and 'laboratory' silks were greater than the 'as spun' silks) (Table 2). Much of these changes in silk mechanical properties

Table 1. Results of the multivariate analyses of variance (MANOVAs) comparing the influence of experimental treatment ('as spun', 'laboratory', or 'incubated') on changes in mechanical properties (ultimate strength, extensibility, toughness, and Young's modulus) for major ampullate (MA) silks of the ten species of spider examined (genera are arranged top-to-bottom from longest to shortest branch lengths to common ancestor)

Species	MANOVA		
	Wilk's $\lambda_{10,166}$	Roa's R	P
<i>Leucauge blanda</i>	0.713	3.050	< 0.001
<i>Leucauge tessellata</i>	0.629	4.324	< 0.0001
<i>Nephila clavata</i>	0.722	2.932	0.008
<i>Nephila pilipes</i>	0.769	2.238	0.010
<i>Cyclosa mulmeinensis</i>	0.698	3.227	0.008
<i>Cyclosa confusa</i>	0.759	2.246	0.009
<i>Argiope aetherea</i>	0.760	2.463	0.010
<i>Argiope aemula</i>	0.864	1.256	0.259
<i>Cyrtophora unicolor</i>	0.728	2.853	0.002
<i>Cyrtophora moluccensis</i>	0.703	3.205	0.008

Table 2. The mean mechanical property values of major ampullate (MA) silks of the ten species of spider examined (genera are arranged top-to-bottom from longest to shortest branch lengths to common ancestor), with the results of post-hoc honestly significant difference (HSD) tests comparing the influence of experimental treatment (AS, 'as spun'; Lab, 'laboratory', or Inc, 'incubated') on change in each property: a > b > c, with Bonferroni-corrected P values

Species	Properties										
	Ultimate strength (MPa)			Toughness (MJ m ⁻³)			Young's modulus (GPa)				
	AS	Lab	Inc	AS	Lab	Inc	AS	Lab	Inc	AS	
<i>Leucauge blanda</i>	707 ± 37 ^b	763 ± 41 ^b	929 ± 60 ^a	98 ± 5 ^b	95 ± 8 ^b	143 ± 13 ^a	8.5 ± 0.4 ^b	9.5 ± 0.5 ^b	13.1 ± 1.0 ^a	8.5 ± 0.4 ^b	9.5 ± 0.5 ^b
<i>Leucauge tessellata</i>	565 ± 24 ^b	578 ± 46 ^b	777 ± 38 ^a	79 ± 4 ^b	84 ± 4 ^b	122 ± 18 ^a	6.3 ± 0.3 ^c	8.6 ± 0.4 ^b	10.3 ± 1.1 ^a	6.3 ± 0.3 ^c	8.6 ± 0.4 ^b
<i>Nephila clavata</i>	534 ± 28 ^c	702 ± 36 ^b	791 ± 71 ^a	105 ± 6	116 ± 12	120 ± 12	8.7 ± 0.3 ^b	7.7 ± 2.2 ^b	10.0 ± 0.6 ^a	8.7 ± 0.3 ^b	7.7 ± 2.2 ^b
<i>Nephila pilipes</i>	495 ± 28 ^c	537 ± 39 ^b	596 ± 41 ^a	75 ± 16	79 ± 19	77 ± 14	7.3 ± 0.9 ^b	6.4 ± 0.5 ^c	8.0 ± 0.5 ^a	7.3 ± 0.9 ^b	6.4 ± 0.5 ^c
<i>Cyclosa mulmeinensis</i>	416 ± 18	428 ± 34	434 ± 34	62 ± 3 ^b	57 ± 5 ^b	74 ± 7 ^a	5.2 ± 0.3 ^b	5.5 ± 1.8 ^b	6.7 ± 0.3 ^a	5.2 ± 0.3 ^b	5.5 ± 1.8 ^b
<i>Cyclosa confusa</i>	232 ± 11 ^{ab}	212 ± 22 ^b	254 ± 30 ^a	47 ± 2 ^{ab}	41 ± 3 ^b	54 ± 4 ^a	3.4 ± 0.2	3.2 ± 0.1	4.0 ± 0.3	3.4 ± 0.2	3.2 ± 0.1
<i>Argiope aetherea</i>	651 ± 29 ^b	726 ± 45 ^a	728 ± 31 ^a	202 ± 11 ^b	227 ± 16 ^a	206 ± 20 ^b	8.8 ± 0.4 ^a	7.3 ± 0.5 ^b	6.0 ± 0.9 ^b	8.8 ± 0.4 ^a	7.3 ± 0.5 ^b
<i>Argiope aemula</i>	909 ± 47	970 ± 40	992 ± 51	175 ± 8	160 ± 11	148 ± 10	9.6 ± 0.5	9.8 ± 0.6	10.0 ± 0.7	9.6 ± 0.5	9.8 ± 0.6
<i>Cyrtophora unicolor</i>	486 ± 23 ^c	606 ± 46 ^b	742 ± 61 ^a	121 ± 6 ^b	129 ± 7 ^b	174 ± 17 ^a	7.8 ± 0.4	8.5 ± 0.3	8.6 ± 0.7	7.8 ± 0.4	8.5 ± 0.3
<i>Cyrtophora moluccensis</i>	721 ± 37 ^c	821 ± 46 ^a	929 ± 52 ^a	168 ± 9 ^b	185 ± 16 ^{ab}	204 ± 19 ^a	10.7 ± 0.6	10.2 ± 1.0	9.7 ± 1.1	10.7 ± 0.6	10.2 ± 1.0

were expected if property changes serve to improve the performance of the orb web.

Our regression and subsequent log-likelihood analyses of the MaSp composition and web type data (see Table 3 for a summary) for all species combined found the hypothesis that best fit the results was hypothesis 4, i.e. post-spin property changes in MA silk under environmental stress occurs independent of MaSp composition and web type (Table 4). Hence, ecological and/or phylogenetic factors influence the degree of post-spin mechanical property changes in

the MA silks of orb web spiders. As the post-spin mechanical property changes in *Argiope* spp. MA silks differed to all other species (*Argiope aemula* MA silks were unaffected by incubation and those of *A. aetherea* were affected in a different direction to all of the other species), we considered ecological factors to be most influential.

DISCUSSION

We found that neither MaSp composition nor two- or three-dimensional webs influence the degree of MA silk post-spin mechanical property changes of orb web spider MA silk exposed to 35 °C and 0% relative humidity over 4 weeks. The low proline and high alanine and glycine compositions of MaSp1-predominant silks are associated with the formation of β-sheets, amorphous cross-linking, and the enhanced ultimate strength and molecular stability of the silk (Kaplan, 1998; Keten & Buehler, 2010). Our results suggest that MaSp-mediated β-sheet formation and amorphous cross-linking does not enhance MA silk resistance to environmentally-induced property change. Other mechanisms, e.g. skin-core defects, variations in crystal size or density, and intermolecular bond activation energies must, accordingly, have a more profound influence on the post-spin properties of MA silks (Perez-Rigueiro *et al.*, 2007; Porter *et al.*, 2009; Giesa *et al.*, 2011). Which mechanisms influenced the degree of post-spin mechanical property change detected among the MA silks that we tested, and how they varied across our working phylogeny, however, remains to be elucidated.

The three spiders with MaSp1-predominant MA silks (the two *Cyrtophora* spp. and *N. clavata*) also build three-dimensional orb webs. Our findings imply that the MA silks in the webs of these spiders experience similar changes in mechanical properties under environmental stress as do those of spiders that build

Table 3. Orb web type (classified as two dimensional, 2D, or three dimensional, 3D), percentage (mean ± SE) of proline (PRO) and likely MaSp composition (MaSp1 or MaSp2, according to the likely influence of MaSp1 and MaSp2 on the composition of proline in major ampullate, MA, silk) in the ‘as spun’ MA silk of the ten species of spider examined

Species	Web type	Mean composition (%) ± SE	
		PRO	MaSp1/MaSp2
<i>Leucauge blanda</i>	2D	12.261 ± 0.204	MaSp2
<i>Leucauge tessellata</i>	2D	11.393 ± 1.991	MaSp2
<i>Nephila clavata</i>	3D	0.970 ± 1.501*	MaSp1
<i>Nephila pilipes</i>	2D	8.388 ± 1.313*	both
<i>Cyclosa mulmeinensis</i>	2D	6.688 ± 2.575	both
<i>Cyclosa confusa</i>	2D	8.313 ± 1.552	both
<i>Argiope aetherea</i>	2D	9.425 ± 1.627	MaSp2
<i>Argiope aemula</i>	2D	8.822 ± 0.807	both
<i>Cyrtophora unicolor</i>	3D	3.090 ± 0.443	MaSp1
<i>Cyrtophora moluccensis</i>	3D	4.113 ± 0.554	MaSp1

*Significant intergeneric differences at Bonferroni-corrected P values (MANOVA/HSD post-hoc tests).

Table 4. Goodness-of-fit model of the multiple regression coefficients: web type (β₁; 0, two-dimensional orb web; 1, three-dimensional orb web); silk proline composition (β₂; representing relative proline composition); mechanical properties (β₃; from PCA analysis between ‘as spun’ and ‘incubated’ ultimate strength, toughness, and stiffness values); and a null model (β₀). The model tests four alternative hypotheses (H_A): (1) proline–alanine composition is associated with mechanical property change independent of web type; (2) web type is associated with property change independent of proline–alanine; (3) proline–alanine and web type are interactively associated with property change; and (4) property change is independent of proline–alanine and web type

Models	H ₀	H _A	log-likelihood	G ²	P
β ₀ + β ₂ + β ₃	β ₁ = 0	1	7.654	5.111	0.09
β ₀ + β ₁ + β ₃	β ₂ = 0	2	2.424	-1.368	0.107
β ₁ + β ₂ + β ₃	β ₀ = 0	3	16.696	2.675	0.178
β ₀ + β ₁ + β ₂	β ₃ = 0	4	3.959	-9.930	< 0.001*

*Best-fitting data for the model.

two-dimensional orb webs. MA silk resistance to mechanical property change does not therefore explain why two-dimensional orb web building spiders rebuild their webs more frequently than do three-dimensional orb web builders. The MA silks of both two-dimensional and three-dimensional orb webs seem to undergo significant post-spin property change in order to strengthen the webs post production, so the silks effectively function to capture prey and transmit stimuli (Blackledge *et al.*, 2011; Vollrath *et al.*, 2011). Aggregate and flagelliform silks undergo more rapid environmentally induced property change than MA silks (Sahni *et al.*, 2011; Vollrath *et al.*, 2011). As aggregate and flagelliform silks are absent from orb webs that are not renewed daily, such as those of *Cyrtophora* (Lubin, 1973), regulating the properties of the capture threads presents a better explanation of why two-dimensional orb web spiders renew their web daily but *Cyrtophora* spp. do not, and why *N. clavata* renews its orb web daily but not its barrier web.

We found that MA silks of the intrageneric species pairs undergo similar environmentally induced post-spin mechanical property change, suggesting that resistance or vulnerability to post-spin mechanical property change of the MA silk of a given spider is phylogenetically predestined to some extent. We nonetheless found a contradiction in the genus *Nephila*, where there was a difference in the degree of environmentally induced post-spin property change between *N. clavata* and *N. pilipes*: phylogeny appears to have less of an influence on the degree of environmentally induced post-spin property change in the MA silks of *Nephila* spp. As both *N. clavata* and *N. pilipes* were collected from similar ecosystems at similar times of year, differences in the ecology of the spiders are unlikely to explain the difference in the MA silk property changes found between the two *Nephila* spp. The MA silks of the two spiders differed in proline composition: 0–3% (mean = 0.97%) in *N. clavata* MA silks and 7–10% (mean = 8.39%) in *N. pilipes* MA silks. Hence their MaSp1 and MaSp2 compositions differed considerably. Differences in MaSp composition may, in this instance, explain the intergeneric variation. The relationship between MaSp composition and mechanical property changes in *Nephila* may, on the other hand, be an artifact of the comparative methods. *Nephila* is an ancient genus (Blackledge *et al.*, 2009), so it is plausible that *N. pilipes* and *N. clavata* are more distantly related to each other (see Higgins *et al.*, 2011; Su *et al.*, 2011) than any of the other intrageneric pairs and, as variations in evolutionary divergence time were not accounted for in intrageneric comparisons, variations in relatedness may have gone undetected.

The two species of *Leucauge* had the longest branch lengths from terminus to nearest common ancestor

on our working phylogeny. *Leucauge* spp. also had MA silk with the greatest vulnerability to environmentally induced post-spin mechanical property change. The genus *Argiope* had the shortest, along with *Cyrtophora*, branch length from terminus to nearest common ancestor. *Argiope* spp., incidentally, had MA silks with the greatest resistance to environmentally induced post-spin mechanical property change. Both *Leucauge* spp. and *Argiope* spp., however, expressed MA silks with high proline (~9–12%) and low alanine (~12–23%) compositions. Hence, both MA silks are likely to be composed of predominantly MaSp2. MaSp composition does not therefore explain the contrasting post-spin MA silk mechanical property changes found between these genera. Moreover, the mechanical properties of native MA silks of other *Leucauge* spp. and *Argiope* spp. are not remarkably different from each other or other spiders (Swanson *et al.*, 2006; Perez-Rigueiro *et al.*, 2007; Agnarsson *et al.*, 2010; Sensenig *et al.*, 2010). The silks of the closely related genera *Cyrtophora* and *Argiope* did not exhibit similar vulnerabilities to mechanical property changes under environmental stress, so vulnerability to post-spin property change in spider MA silk does not appear to be driven by phylogenetic lag (Blomberg & Garland, 2002). There thus must be genus-specific molecular mechanisms, e.g. vulnerability to skin-core structural flaws or compounds affecting bond activation energies (Perez-Rigueiro *et al.*, 2007; Porter *et al.*, 2009; Giesa *et al.*, 2011), that influence the vulnerability of spider MA silks to property variation, which may be associated with ecological factors or unascertained evolutionary factors.

Phenotypic plasticity and ecological adaptations influence the extent of mechanical property variation experienced and tolerated in the MA silks of different spiders (Boutry, Rezac & Blackledge, 2011; Blamires *et al.*, 2012). For instance, the MA silks of *Argiope trifasciata* are resistant to UV irradiation, whereas the MA silks of other spiders are more vulnerable (Kitagawa & Kitayama, 1997; Perez-Rigueiro *et al.*, 2007), owing to their different resistances to skin cracking (Perez-Rigueiro *et al.*, 2007). *Argiope* spp. have a greater physiological thermal tolerance compared with other orb web spiders (Kato *et al.*, 2008), thus silk properties and spider physiology may phylogenetically co-vary. A recent *Argiope* phylogeny (Cheng *et al.*, 2010) placed arid African and Eurasian species ancestral to tropical Southeast Asian species. Nevertheless, another phylogeny, albeit using different species, placed the neotropical species as ancestral (Elices *et al.*, 2009). If there is an arid ancestry for *Argiope*, it may to some extent explain the resistance of their MA silk to property changes at environmentally stressful temperatures and humidity, and upon exposure to UV. Regardless of ancestry,

most extant *Argiope* spp., including those used herein, inhabit tropical grasslands, where the spiders and their webs are exposed to solar radiation throughout the day (Miyashita & Shimazaki, 2006). MA silks with resistance to environmentally induced property change may thus be an adaptation to this environment. The ultimate strength of silk of the other species from an exposed ecosystem, *Cyclosa mulmeinensis*, was unaffected by incubation at 35 °C and 0% relative humidity. Nevertheless, all the other mechanical properties changed compared with those of its forest dwelling congener, *C. confusa*. The conditions, especially wind, moisture availability, and sunshine, of *C. mulmeinensis* habitats are variable (Blamires *et al.*, 2010), so it may be necessary for their silks to vary in properties to maintain web function. As both *Leucauge* spp. were found in tropical forests, the high vulnerability of their MA silk to environmentally induced mechanical property change may be explained by it not being necessary for their silk to resist temperature or humidity variations. Ecology of the spider appears to be an important factor influencing the vulnerability of MA silks to environmentally induced mechanical property changes. Nevertheless, more information on the ecosystems used by a range of orb web spiders and the properties of their silks is needed to assess the relationship between ecosystems, habitats, evolutionary history, silk properties, the post-spin environment, and MA silk vulnerability to property changes.

In summary, *Argiope* MA silks had the greatest resistance to environmentally induced post-spin property change, whereas *Leucauge* silks had the least resistance. Our findings were contrary to our a priori prediction that the two *Cyrtophora* spp., or *Nephila clavata*, would have silks with high resistance to post-spin mechanical property change because they express MaSp1-predominant silk, considered to be stronger and more stable than MaSp2-predominant silk, and because they build three-dimensional orb webs and/or barrier webs that are exposed to the environment for days at a time. Our findings suggest that the silks of three-dimensional orb webs are not inherently more resistant to environmentally induced post-spin mechanical property changes than are those of two-dimensional orb webs. Instead, variations in silk property change might be associated with the ecosystems used by the spiders, particularly those exposing webs to solar radiation. We suggest that industries seeking to harness silks with resistance to environmentally induced property change should not only investigate the chemical and physical properties of different spider silks, but also the ecology and evolutionary history of different spiders, as these appear to influence the mechanical stability of the silks.

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REFERENCES

- Agnarsson I, Boutry C, Blackledge TA. 2008.** Spider silk aging: initial improvement in a high performance material followed by slow degradation. *Journal of Experimental Zoology* **309A**: 494–504.
- Agnarsson I, Kuntner M, Blackledge TA. 2010.** Bioprospecting finds the toughest biological material: extraordinary silk from a giant riverine orb spider. *PLoS ONE* **5**: e11234.
- Ayoub NA, Garb E, Tinghitella RM, Collin MA, Hayashi CY. 2007.** Blueprint for a high-performance biomaterial: full-length spider dragline silk genes. *PLoS ONE* **2**: e514.
- Blackledge TA, Hayashi CY. 2006.** Unraveling the mechanical properties of composite silk threads spun by cribellate orb-weaving spiders. *Journal of Experimental Biology* **209**: 3131–3140.
- Blackledge TA, Kuntner M, Agnarsson I. 2011.** The form and function of spider orb webs: evolution from silk to ecosystems. *Advances in Insect Physiology* **41**: 175–262.
- Blackledge TA, Scharff N, Coddington JA, Szuts T, Wenzel JW, Hayashi CY, Agnarsson I. 2009.** Reconstructing web evolution and spider diversification in the molecular era. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 5229–5234.
- Blamires SJ, Lee YH, Chang CM, Lin IT, Chen JA, Lin TY, Tso IM. 2010.** Multiple structures interactively influence prey capture efficiency in spider orb webs. *Animal Behaviour* **80**: 947–953.
- Blamires SJ, Tso IM. 2012.** Ecophysiological influences on spider silk properties and the potential for producing adaptable, degradation resistant biomaterials. In: Aramwit P, ed. *Silks: properties, production and uses*. New York: Nova Science Publishers, 139–154.
- Blamires SJ, Wu CL, Tso IM. 2012.** Variations in protein intake induces variations in spider silk expression. *PLoS ONE* **7**: e31626.
- Blomberg SP, Garland T Jr. 2002.** Tempo and mode in evolution: phylogenetic inertia, adaptation and comparative methods. *Journal of Evolutionary Biology* **15**: 899–910.
- Boutry C, Rezac M, Blackledge TA. 2011.** Plasticity in major ampullate silk production in relation to spider phylogeny and ecology. *PLoS ONE* **6**: e22467.
- Carico JE. 1986.** Web removal patterns in orb weaving

- spiders. In: Shear WA, ed. *Spiders. Webs, behavior, and evolution*. Stanford: Stanford University Press, 306–318.
- Cheng RC, Yang EC, Lin EP, Herberstein ME, Tso IM. 2010.** Insect form vision as one potential shaping force of spider web decoration design. *Journal of Experimental Biology* **213**: 759–768.
- Dimitrov D, Lopardo L, Giribet G, Arnedo MA, Alvarez-Padilla F, Hormiga G. 2012.** Tangled in a sparse spider web: single origin of orb weavers and their spinning work unraveled by denser taxonomic sampling. *Proceedings of the Royal Society B* **279**: 1341–1350.
- Elices M, Plaza GR, Arnedo MA, Perez-Rigueiro J, Torres FG, Guinea GV. 2009.** Mechanical behavior of silk during the evolution of orb-web spinning spiders. *Biomacromolecules* **10**: 1904–1910.
- Garland T Jr, Ives AR. 2000.** Using the past to predict the present: confidence intervals for regression equations in phylogenetic comparative methods. *American Naturalist* **155**: 346–364.
- Giesa T, Arslan M, Pugno NM, Buehler MJ. 2011.** Nanoconfinement of spider silk fibers begets superior strength, extensibility and toughness. *Nano Letters* **11**: 5038–5046.
- Gosline JM, Guerette PA, Ortlepp CS, Savage KN. 1999.** The mechanical design of spider silks: from fibroin sequence to mechanical function. *Journal of Experimental Biology* **202**: 3295–3303.
- Grafen A. 1989.** The phylogenetic regression. *Philosophical Transactions of the Royal Society B* **326**: 119–157.
- Higgins LE, Coddington JA, Goodnight C, Kuntner M. 2011.** Testing ecological and developmental hypotheses of mean and variation in adult size in nephilid orb-weaving spiders. *Evolutionary Ecology* **25**: 1289–1306.
- Kaplan DL. 1998.** Fibrous proteins- silk as a model system. *Polymer Degradation and Stability* **59**: 25–32.
- Kato N, Takasago M, Omasa K, Miyashita T. 2008.** Coadaptive changes in physiological and biophysical traits related to thermal stress in web spiders. *Naturwissenschaften* **95**: 1149–1153.
- Keten S, Buehler MJ. 2010.** Nanostructure and molecular mechanics of spider dragline silk protein assemblies. *Journal of the Royal Society Interface* **7**: 1709–1721.
- Kitagawa M, Kitayama T. 1997.** Mechanical properties of dragline and capture thread for the spider *Nephila clavata*. *Journal of Materials Science* **32**: 2005–2012.
- Liu Y, Shao ZZ, Vollrath F. 2008.** Elasticity of spider silks. *Biomacromolecules* **9**: 1782–1786.
- Lubin Y. 1973.** Web structure and function: the non-adhesive orb web of *Cyrtophora moluccensis*. *Forma et Functio* **6**: 337–358.
- Madsen B, Vollrath F. 2000.** Mechanics and morphology of silk drawn from anesthetized spiders. *Naturwissenschaften* **87**: 148–153.
- Miyashita T, Shimazaki A. 2006.** Insects from the grazing food web favoured the evolutionary habitat shift to bright environments. *Biology Letters* **2**: 565–568.
- Nelson DL, Cox MM. 2005.** *Lehninger principals of biochemistry*. New York: WH Freeman.
- Pagel M. 1998.** Inferring evolutionary processes from phylogenies. *Zoologica Scripta* **26**: 331–348.
- Perez-Rigueiro J, Elices M, Plaza GR, Rueda J, Guinea GV. 2007.** Fracture surfaces and tensile properties of UV-irradiated spider silk fibers. *Journal of Polymer Science* **45**: 786–793.
- Porter D, Vollrath F, Tian K, Chen X, Shao Z. 2009.** A kinetic model for thermal degradation in polymers with specific applications to proteins. *Polymer* **50**: 1814–1818.
- Sahni V, Blackledge TA, Dhinojwala A. 2011.** Changes in the adhesive properties of spider aggregate glue during the evolution of cobwebs. *Scientific Reports* **1**: 41.
- Savage KN, Gosline JM. 2008.** The effect of proline on the network structure on major ampullate silks as inferred from their mechanical and optical properties. *Journal of Experimental Biology* **211**: 1937–1947.
- Sensenig A, Agnarsson I, Blackledge TA. 2010.** Behavioural and biomaterial coevolution in spider orb webs. *Journal of Evolutionary Biology* **23**: 1839–1856.
- Su YC, Chang YH, Smith D, Zhu MS, Kuntner M, Tso IM. 2011.** Biogeography and speciation patterns of the golden orb web genus *Nephila* (Araneae: Nephilidae) in Asia. *Zoological Science* **28**: 47–55.
- Swanson BO, Blackledge TA, Beltran J, Hayashi CY. 2006.** Variation in material properties of spider dragline silk across species. *Applied Physics A: Material Science and Processing* **82**: 213–218.
- Vollrath F, Porter D, Holland C. 2011.** There are many more lessons still to be learned from spider silk. *Soft Matter* **7**: 9595–9600.
- Work RW, Young TC. 1987.** The amino acid composition of major ampullate and minor ampullate silks of certain orb-web building spiders (Araneae, Araneidae). *Journal of Arachnology* **15**: 65–80.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Architecture of orb webs with: (A) two-dimensional planar webs, such as those built by adult *Nephila pilipes*, *Cyclosa mulmeinensis*, *Cyclosa confusa*, *Leucauge blanda*, *Leucauge tessellata*, *Argiope aetherea*, and *Argiope aemula*; (B) horizontal web with three-dimensional barrier structures above and below, as built by adult *Cyrtophora* spp.; and (C) vertical web, with front and back barrier structures, as built by adult *Nephila clavata*.

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