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Uncovering Spider Silk Nanocrystalline Variations That Facilitate Wind-Induced Mechanical Property Changes

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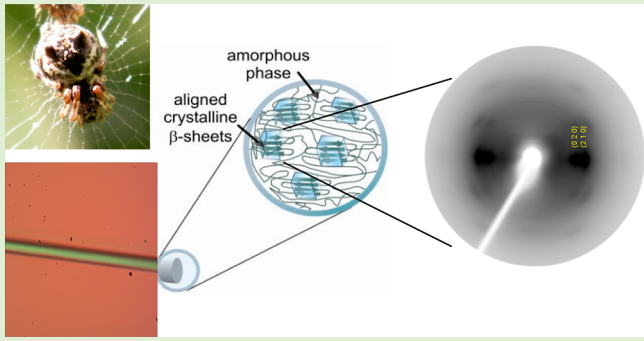
ABSTRACT: Spider major ampullate (MA) silk varies in mechanical properties when spun in different environments. Amino acid compositional changes induced by variations in MaSp1 and MaSp2 expression, and various biochemical and physiological glandular processes induce silk property variability. Quantifying the contributions of these mechanisms on silk variability may facilitate the development of silk biomimetics. Wind is a medium that induces variations in MA silk mechanics. We exposed the spider *Cyclosa mulmeinensis* to wind and measured the amino acid composition, tensile mechanics, and crystalline structure of its MA silk using HPLC, tensile tests, and X-ray diffraction. We found the mechanical properties of MA silks from spiders exposed to wind to differ from unexposed spiders. The amino acid compositions did not differ, but X-ray diffraction found a lower crystal density and greater β-sheet alignment relative to the fiber axis in the silks of spiders exposed to wind. We found no evidence that the mechanical property variations were a product of profound changes to the alignment of the protein within the amorphous region. We conclude that variations in the density and alignment of the crystalline β-sheets, probably accompanied by some alignment change in the amorphous region as a result of "stretching" during spinning of the silk, probably explains the mechanical property variations that we found across treatment subgroups. As *C. mulmeinensis* MA silk increases both in strength and elasticity when the spiders are exposed to wind, bioengineers may consider it as a model for the development of high-performance silk biomimetics.



INTRODUCTION

Biomimetics, the design and production of materials and devises based on natural materials, organisms, or structures,[1,2] is forging forward due to developments in many exciting new fields. Biomateriomics, the detailed study of biological materials from nano- to macro-scopic hierarchical levels,[3] is one such field. Spider major ampullate (MA) silk is a remarkable biological material because of its combined extreme extensibility and tensile strength. It, therefore, serves as a good model for biomateriomics investigations and is potentially useful as a biomimetic.[3,4] Spider silk-inspired materials earmarked for development include bullet proof clothing, high performance ropes, sensors, biodegradable sutures, and nanoscale material scaffolds.[5] Understanding the mechanisms by which spider MA silk varies in response to environmental variation is of particular interest to bioengineers as it may provide inspiration for the development of "smart materials", i.e., materials that adjust their properties when environmental stimuli changes,[5−9] for medical or other applications.

High-performance synthetic materials are usually exceptionally strong or extensible. Few materials yield both properties. Spider MA silk is an exception, yielding both high strength and extensibility.[4,10] The principal method used to produce synthetic silk analogues has been to clone and amplify the silk gene products and mechanically or electrically spin the secretions into a solidifying solution[10−12] (for alternative processes, see the work of Teulé et al.[13]). Such procedures have, however, thus far failed to produce silk analogues whose strength and/or extensibility resembles spider silk.[4] It is, therefore, imperative to understand the physiological and biochemical mechanisms by which silk is naturally spun across multiple hierarchical levels.[3,5,14−16]

Spider MA silk tensile properties vary in response to a multitude of environmental stimuli. The precise mechanisms that induce MA silk tensile property variations are unclear but may include a combination of changes in silk amino acid composition and post secretion biochemical and physiological processes.[17] Notwithstanding, the influence of these mechanisms has not been experimentally tested simultaneously within individual spiders. Amino acid composition is predicted to influence tensile mechanics because certain amino acids facilitate the production of silk proteins with determinable secondary and

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tertiary structures and properties.[18,19] Nevertheless, MA silk tensile properties may vary without variations in amino acid composition and, conversely, MA silk amino acid compositions may vary without necessarily being accompanied by variations in tensile properties.[20−22] Post secretion processes, accordingly, appear to be highly influential over MA silk tensile properties.

Spider MA silk is predicted to comprise a combination of two proteins (spidroins). Spidroin 1, or MaSp1, consists of multiple $(GA)_n$, $(GGX)_n$ and $(A)_n$ repeated amino acid sequences.[23] Nuclear magnetic resonance (NMR) analyes have revealed that these sequences promote the formation of crystalline β-sheets in the assembled silk fibers.[24,25] Spidroin 2, or MaSp2, on the other hand consists of additional multiple $(GPGXX)_n$ repeated sequences.[26] NMR has revealed that the proline in MaSp2 inhibits β-sheet formation and stacking and promotes the formation of crystalline β-spirals and type-II β-turns in the assembled fibers.[10,27] The crystalline β-sheet formations promoted by MaSp1 give the silk strength, while the β-spirals and β-turn formations promoted by MaSp2 give the silk extensibility.[4,10,28] According to the MaSp model, the mechanical properties of spider silk are principally a product of the ratio of their MaSp1 and MaSp2 expression. Indeed, spiders feeding on different types of prey vary the spidroins that are expressed in their MA silk, and this manifests as variations in the chemical and physical properties of their silk.[22,29−31] While the MaSp model suggests that silk strength is likely to be traded-off against extensibility, the MA silk of some spiders, such as that of *Cyclosa mulmeinensis*,[20,21] may simultaneously increase in strength and extensibility in certain circumstances.

Silk travels through the silk gland as liquefied dope before being drawn from the spinneret as a solid. The gland consists of a tail, sac, duct, and valve. The dope proteins are secreted by the epithelial cells into the sac and stored as a concentrated aqueous solution before flowing through the duct and drawn at the valve.[10] The duct is divided into three limbs: limb 1, 2, and 3.[10,32] Prior to the dope reaching the limit of limb 3, the crystalline β-sheets, β-spirals, β-turns, 3_1-helices or other structures are fully formed, but may change in alignment relative to the fiber axis further along the gland. Techniques using synchrotron radiation, such as X-ray diffraction techniques, have found that physiological and biochemical processes at different sites in the duct affect the proportion and orientation of crystalline β-sheets, β-spirals, β-turns, 3_1-helices, or other structures.[4,10,14,32] Shear stresses at the valve immediately prior to drawing also align the proteins relative to the fiber axis in the crystalline and noncrystalline amorphous regions.[4,24,33] Amorphous region alignment is extenuated by the spider eliciting drag friction on the fiber via contraction of the valve spigot as the silk is spun.[15,32] Studies exploiting varying degrees of supercontraction, i.e., exposing solid silk fibers to different humidities to sequentially misalign the proteins in the amorphous region, have been used to ascertain the degree to which alignment of proteins in the amorphous region induces variations in MA silk mechanical properties.[21,34−36]

Spiders whose webs are regularly exposed to extreme environments, such as strong wind or high temperatures, might be expected to have exceptionally variable silk properties.[20,37] Variations in wind speed is particularly challenging for spiders and their webs.[20,37−41] Webs exposed to strong wind must be composed of MA silk of particularly high strength to withstand wind drag and high extensibility to optimally capture prey without tearing.[20,40] The dust spider, *Cyclosa mulmeinensis*, is an orb web spider that builds webs exposed to strong winds in its natural habitat.[20,38,41] Moreover, *C. mulmeinensis* MA silk increases in both strength and extensibility without changing amino acid composition upon exposure to wind. The ground state properties of *C. mulmeinensis* have been measured,[21] thus, the influence of protein alignment in the amorphous region may be determinable in this species by comparing the degree of variation of wind exposed silks with the ground state properties.

To test the relative influence of environmentally induced changes in MaSp expression and glandular spinning processes on MA silk mechanical properties within individual spiders, we exposed *C. mulmeinensis* to wind and measured the changes in MA silk amino acid composition, tensile properties, and crystalline structures using HPLC, tensile tests, and synchrotron-derived X-ray diffraction, respectively. We also compared the variations in mechanical properties of MA silks of wind exposed and unexposed spiders with published *C. mulmeinensis* MA silk properties in their native and ground states to ascertain whether the alignment of proteins in the amorphous region during spinning influences the mechanical property variation.

MATERIALS AND METHODS

Spider Collection and Acclimation. We collected adult female *C. mulmeinensis* from shrubs beside a peanut plantation in Huwei, Yunlin County, Taiwan (120°22′31.47″ E, 23°38′57.54″ N) all year round. Spiders were collected on their webs by placing two circular wooden frames (diameter = 200 mm) with superglue around their rims on either side of a web, moving them toward each other carefully until they touched. We pressed the touching frames firmly together in order to stick them to each other and burnt away any web lying outside the frames using burning incense. We temporarily removed each spider from their web to measure its mass (±0.1 mg), using an electronic balance (PJ300; Mettler Toledo, Greifensee, Switzerland). The spiders were returned to their frame-mounted webs within which they were taken back to the laboratory. Before performing the following experiments, we acclimated the spiders in the laboratory under a 12:12 h light−dark cycle for 3 days; feeding them one fruit fly and lightly spraying the webs with tap water each day.

Manipulative Experiment. As *C. mulmeinensis* is small (adult body length <6 mm), its body condition and silk production may be affected by multiply silking individuals.[41] Accordingly, we did not obtain silk from any individual spider twice. Rather, we randomly divided 120 spiders into two groups: a pretreatment and a treatment group (n = 60 in each). The individuals in the pretreatment group were further randomly divided into two subgroups (n = 30 in each), designated P1 and P2. Spiders in the treatment group were divided into a wind exposed subgroup (W subgroup) and an unexposed subgroup (N subgroup) (n = 30 in each subgroup). In a separate laboratory, individuals in the P1 and P2 subgroups had their silks collected prior to the commencement of the experiment.

We subjected the W subgroup to constant wind (speed = 1.1 ms^{-1}) over 7 days, while the N subgroup were placed in the same laboratory as the W subgroup for the same 7 days but not subjected to wind. The wind was generated by 120 × 120 mm electric fans (Cooler Master; AREC Peripherals, Inc., Taipei, Taiwan) placed 400 mm from the spiders' dorsum. Relative humidity and temperature data loggers (Hobo U23, Pro v2, Onset, USA) were set up in the laboratory beside six representative webs from each treatment to make sure that relative humidity and temperature did not differ significantly between the locations where spiders in the W and N subgroups were placed. After 7 days, we terminated the experiment and performed the following procedures.

Amino Acid Composition and Mechanical Property Measurement. We anaesthetized, using CO_2, each spider used in the experiments before placing them ventral side up between two metal grids that were glued together with foam rubber. We waited 30 min to ensure that there was no influence of anesthesia over silk properties before drawing MA silk from each individual using forceps.

The extracted silk was taped to a mechanical spool, which was reeled at a constant speed (5 mm s^{-1}) for 20 min. We viewed the spinnerets under a dissecting microscope during silking to ensure only a single fiber of MA silk was consistently taken.

From each spider, 10 25-mm sections of taut MA silk fiber were individually 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 and photographed under 1000× magnification using a polarized light microscope (BX 50, Olympus, Tokyo, Japan) 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 (C.C.W.) under controlled temperature (~25 °C) and humidity (~30% R.H.) in still air, so reeling speed or postspin handling had no influence on variations in the mechanical properties of the silks.

The remaining silk from each individual was weighed to the nearest 0.01 mg on an electronic balance and placed into 10 μL tubes (Eppendorf, Hamburg, Germany), where they were submerged in 99% hexaflouro-isopropanol (500 μL mg^{-1} silk). The solutions were examined to ensure there were not any suspended particles before being dried and hydrolyzed in 6 mol l^{-1} HCl for 24 h. Their amino acid compositions (as percentages) were determined by reverse-phase high-performance liquid chromatography (Waters Pico-Tag Amino Acid Column, Milford CA, USA) at the Instrument Center, National Tsing-Hwa University, Taiwan.

Tensile tests were performed under controlled temperature and humidity on the frame-mounted silks from each individual at the Industrial Technology Research Institute, Hsinchu, Taiwan, approximately 10 days after their collection. We first placed the frames containing single silk fibers within the grips of a UTM Nano Bionix tensile testing machine (MTS Systems Corporation, Oakridge, TN, USA), ensuring that the grips held the silk firmly at the edge of the frame.[42,43] The silks were then stretched at a rate of 0.1 mm s^{-1} until rupture. The load resolution varied from 2 to 5 μN depending on the diameter of the silk.

True stress (σ) and strain (ε) were calculated by[44]

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

where F is the force applied to the specimen and A is the cross-sectional area of the thread calculated from diameter, assuming a constant thread volume,[45] and

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

where L is the instantaneous length of the fiber at a given extension value and L_0 is the original gage length of the fiber. Stress−strain and load−extension curves were plotted for each silk using TestWorks 4.0 (MTS Systems Corporation, Eden Prairie MN, USA), from which we calculated the following mechanical performance parameters: (1) ultimate strength, or the stress at rupture; (2) extensibility, or the strain at rupture; (3) toughness, the total work of extension, calculated as the area under the stress strain curve; (4) Young's modulus (stiffness), calculated as the slope of the curve during the initial elastic phase for each specimen; (5) ultimate tension, or the force applied at rupture; and (6) breaking energy, or the area under the load-extension curve.

X-ray Diffraction. We exposed an additional 30 spiders each to conditions similar to those described above for the W and N treatment subgroups. MA silk from all of the spiders was reeled onto 3 mm × 1 mm steel frames with a 0.5 mm × 0.5 mm window at the same constant speed as that used to collect silk for the mechanical property measurements (5 mm s^{-1}) for ~60 min per individual. The frames were attached to a mechanical spool until 2000 rounds (the amount of MA silk needed to effectively perform X-ray diffraction) were collected. Due to the small silk reserves of *C. mulmeinensis*, one frame was used to collect the combined silks of all 30 spiders in each subgroup. We exposed each sample to X-rays generated by the BL01C2 beamline at the National Synchrotron Radiation Research Center, Taiwan. Samples were aligned parallel to a detector at a distance of 300 mm. The incident X-ray wavelength was 1.033 Å. The beam size was confined by a collimator 0.5 mm in diameter. Two-dimensional diffraction patterns were recorded for each silk sample with a Mar 345 imaging plate with the typical exposure period lasting 120 min. One-dimensional diffraction profiles were developed from the two-dimensional diffraction images using Fit2D software so the crystalline density and structure could be compared between samples. We calculated the size of crystals from the full widths at half-maximum diffraction peaks using Scherrer's equation.[46,47] The degree of alignment of β-sheets along the silk thread was estimated by examining azimuthal angles.

Statistical Analysis. We assessed whether the tensile properties of the MA silks of spiders from the P1 and P2 differed using an analysis of variance (ANOVA). We also assessed whether spider weight and spiral features differed between the pretreatment subgroups, and the W and N treatment subgroups using ANOVA (n = 300 threads were used for between subgroup analyses: 10 threads x 30 individuals). We used multivariate analyses of variance (MANOVA) to determine whether amino acid compositions and MA silk tensile properties differed between the pretreatment and W and N treatment subgroups. When MANOVA showed a significant difference, we performed individual ANOVAs on each of the variables to ascertain the significantly differing variables across treatments. We performed Kolmogorov−Smirnov tests to assess the normality of the data ($P < 0.05$) prior to all analyses, $\log_{10}$ transforming data that failed the test. Amino acid composition and MA silk extensibility were measured as percentages, so these data were arcsine transformed prior to analyses. All statistics were performed using SAS (SAS Foundation for Statistical Computing, North Carolina, USA).

RESULTS

Spider mass did not differ significantly between the P1 and P2 subgroups (P1 = 9.70 ± 0.44 mg; P2 = 9.58 ± 0.53 mg; $F_{1,59}$ = 0.03, P = 0.85), or between W and N subgroups (W = 9.73 ± 0.44 mg; N = 9.49 ± 0.33 mg; $F_{1,100}$ = 0.18, P = 0.67), thus spider mass had no influence over silk property variations.

The compositions of amino acids of *C. mulmeinensis* MA silk did not differ significantly between the pretreatment subgroups (MANOVA: λ = 0.80, $F_{5,23}$ = 1.15, P = 0.36; Table 1) or between the W and N treatment subgroups (MANOVA: λ = 0.95, $F_{5,39}$ = 0.37, P = 0.86, Table 1). These compositions were similar to other MA silk amino acid compositions reported for *C. mulmeinensis*.[20,21,42] MA silk tensile properties, likewise, did

Table 1. Mean (± SE) Amino Acid Composition (%) of *C. mulmeinensis* MA Silk: P1 and P2 Are the Pretreatment Subgroups and W and N Are the Wind and No Wind Treatment Subgroups, Respectively

treatment	alanine	glycine	glutamine	proline	serine
P1	11.76 ± 0.55	27.22 ± 1.13	5.94 ± 0.24	8.17 ± 0.34	5.76 ± 0.19
P2	12.44 ± 0.82	28.03 ± 1.75	6.63 ± 0.37	8.35 ± 0.46	6.06 ± 0.24
N	12.64 ± 0.41	28.40 ± 0.75	6.08 ± 0.21	8.40 ± 0.25	5.95 ± 0.13
W	12.45 ± 0.55	28.47 ± 1.00	6.03 ± 0.20	8.56 ± 0.31	5.99 ± 0.16

Table 2. Tensile Properties (Mean ±1 SE) of *C. mulmeinensis* MA Silk: (A) P1 and P2 Represent the Pretreamtent Subgroups, and (B) W and N Represent the Wind and No Wind Treatment Subgroups, Respectively[a]

	treatment	Young's modulus (Gpa)	ultimate strength (Mpa)	extensibility (mm mm^{-1})	ultimate tension (μN)	toughness (Mpa)	breaking energy (μJ)	thread diameter (μm)
(A)	P1	6.10 ± 0.47	977.22 ± 97.05	0.24 ± 0.10	0.98 ± 0.11	116.19 ± 14.57	2.96 ± 0.43	1.27 ± 0.03
	P2	6.33 ± 0.61	947.60 ± 97.67	0.23 ± 0.01	1.25 ± 0.24	108.65 ± 13.10	3.63 ± 0.76	1.32 ± 0.06
	$F_{1, 41}$	0.09	0.05	0.56	0.05	0.15	0.61	0.80
	P	0.77	0.83	0.46	0.83	0.70	0.44	0.37
(B)	N	7.32 ± 0.5	1127.86 ± 109.6	0.22 ± 0.01	1.13 ± 0.12	131.08 ± 15.46	3.34 ± 0.46	1.28 ± 0.04
	W	9.75 ± 0.98	1498.99 ± 143.9	0.25 ± 0.01	1.65 ± 0.13	185.73 ± 19.21	5.29 ± 0.49	1.37 ± 0.04
	$F_{1, 58}$	4.90	4.21	6.56	4.21	4.91	8.39	2.54
	P	0.03	0.04	0.01	0.04	0.03	<0.001	0.11

[a] F and P values are for ANOVAs between subgroups.

not significantly differ between the pretreatment subgroups (MANOVA: λ = 0.82, $F_{7,35}$ = 1.07, P = 0.40, Table 2A). However, the tensile properties of silks from the W and N subgroups differed significantly (MANOVA test, λ = 0.77, $F_{7,52}$ = 3.21, P = 0.04). ANOVAs comparing the tensile properties between the W and N subgroup found that the ultimate strength, extensibility, toughness, Young's modulus, ultimate tension and breaking energy of the MA silks of spiders in the W subgroup were all significantly greater than those from the N subgroup (Table 2B). Thus, *C. mulmeinensis* that had been exposed to wind produced MA silk with greater ultimate strength and extensibility than spider that had not been exposed to wind.

All of the pretreatment tensile property values were between those reported for *C. mulmeinensis* native MA silk and those reported for *C. mulmeinensis* MA silk in a ground state.[21] Nevertheless, post-treatment property changes did not conform to the property changes found when *C. mulmeinensis* MA silks are supercontracted from a native to a ground state.[21] Thus, disruption of protein alignment within the amorphous region alone cannot explain the changes in property that occur when *C. mulmeinensis* MA silk are exposed to wind.

Since *C. mulmeinensis* MA silk fibers are exceptionally thin (mean = 1.3 μm, range = 0.5−1.5 μm), the diffraction quality was not as good as that found for other spider silks, such as for *Nephila* spp. and *Argiope* spp.[46−49] This issue was largely overcome by using a substantially longer (~120 min rather than ~30 min) X-ray exposure time. The two-dimensional X-ray diffraction pattern (Figure 1) showed a textured structure of β-sheets aligned relative to the fiber axis, similar to that of the MA silks of *Nephila pilipes*.[48] Figure 2 shows the one-dimensional X-ray diffraction profiles of the MA silk samples of the wind disturbance group (W) and no wind disturbance group (N) integrated from the two-dimensional patterns.

The diffraction intensity of N was stronger than that of W, as indicated on the two-dimensional diffraction image at the (0 2 0) and (2 1 0) Bragg reflections of Figure 1. These results indicate that N had a higher β-sheet density than W. This was verified by the crystalline size estimates, which were 4.00 and 3.54 nm for N and W, respectively. These are approximately 3 times smaller than the crystal size estimates for *Nephila* spiders.[47,48] We expect this discrepancy to be a product of *C. mulmeinensis*' MA silk threads being ~3 times thinner than those of *Nephila* spp. We found azimuthal angle peaks for (0 2 0) and (2 1 0) of 16.4(3)° and 18.1(4)°, respectively, for N, and 30.7(18)° and 33.5(15)° for W (Figure 3), hence there were variations in the β-sheet alignment; with the β-sheets of W being more aligned relative to the fiber axis than those of N.

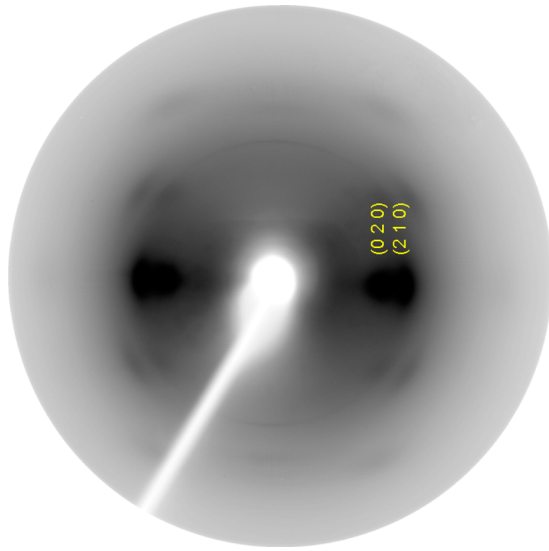

Figure 1. A two-dimensional X-ray diffraction pattern of *C. mulmeinensis* MA silk obtained from spiders from the no wind (N) treatment group.

Due to the low resolution of the amorphous halo and significant amounts of background scattering, we could not ascertain whether there were similar variations in amorphous alignment patterns. Nevertheless, we expected, based on the results of X-ray diffraction studies of *Nephila* spp.,[47,48] that crystalline alignment variations are accompanied by variations in alignment in the amorphous region. The crystalline size and alignment variations, and likely accompanying amorphous alignment variations, between the treatment subgroups most likely explains the change in *C. mulmeinensis* MA silk mechanical properties that occurred when the spiders were exposed to wind.

DISCUSSION

Here we empirically showed that variations in crystalline β-sheet density and structure (in this case, the relative alignment of the crystalline β-sheets) and variations in protein alignment in the amorphous region in the MA silk of *C. mulmeinensis* exposed to wind differed from the MA silk of *C. mulmeinensis* that had not been exposed to wind. Moreover, these changes are induced in the absence of any variation in the ratio of MaSp1 or MaSp2 expression. Such variations may be responsible for the tensile property variations, including the simultaneous increase in strength and extensibility that occur in *C. mulmeinensis* MA silk when the spiders were exposed to wind.[20] Protein alignment in the amorphous region of the silk alone does not seem to be

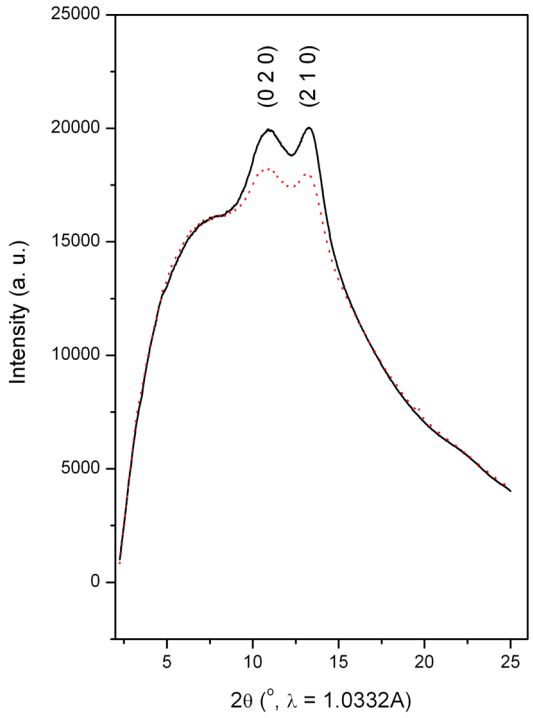

Figure 2. One-dimensional X-ray diffraction profiles perpendicular to silk direction of MA silks of *C. mulmeinensis* receiving the two experimental treatments. The solid black line represents the MA silks of the no wind treatment group (N) and the dotted red line represents that of the wind disturbance group (W).

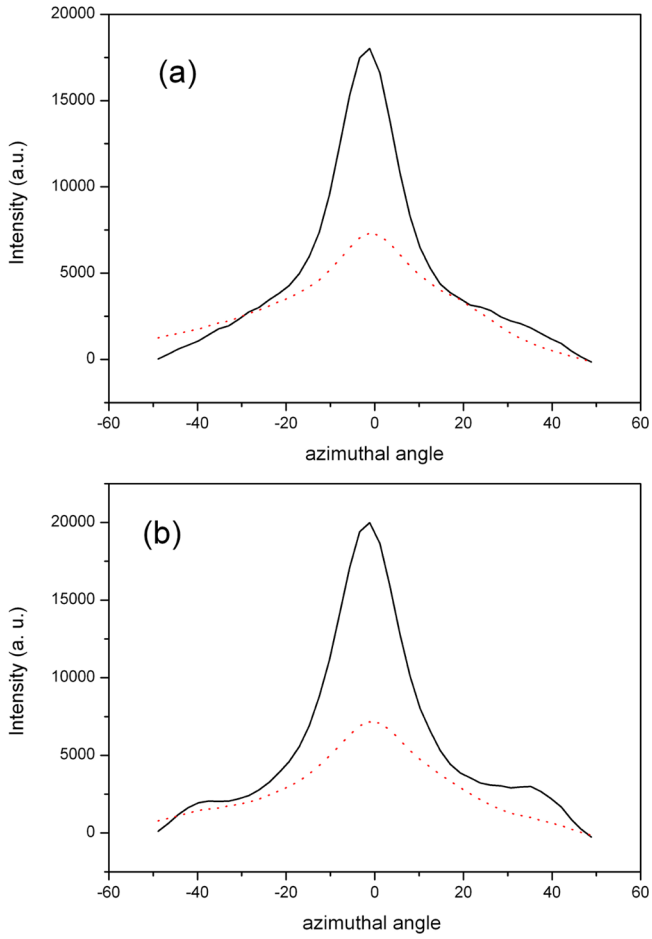

Figure 3. Azimuthal angle of the (a) (0 2 0) and (b) (2 1 0) reflection angles of *C. mulmeinensis* MA silk. The solid black line represents the MA silk sampled from spiders from the no wind (N) treatment. The dotted red line represents the MA silk sampled from spiders from the wind (W) treatment.

responsible for the property variations that we found, since: (1) the MA silk property changes induced by exposing *C. mulmeinensis* to wind differed to the property changes induced when *C. mulmeinensis*' MA silks are supercontracted in water, where the influence of amorphous protein alignment is eliminated,[21,50] and (2) a concomitant increase in strength and extensibility was found while, in contrast, supercontracted silks generally decrease in strength while simultaneously increasing in extensibility.[7,34,35,51,52] Our findings suggest that strength and extensibility can be simultaneously enhanced in spider MA silks by changes in crystalline β-sheet density, secondary structure, and alignment without the need for variations in MaSp expression. An understanding of the postsecretion physiological and biochemical means by which these changes are generated within the silk gland would provide invaluable insights for developing high-performance biomimetic silk analogues and smart materials.

We found the β-sheet nanocrystal sizes for silks from the N and W treatments to differ. Recently it was reported[53] that β-sheet nanocrystal size and density impacts on the larger-scale mechanical properties of silk. Significantly, the toughness increases when β-sheet nanocrystal size or density decreases. We confirmed these findings by finding an increase in toughness with decreasing β-sheet nanocrystal density in silks from spiders that had been exposed to wind. The X-ray diffraction patterns and azimuthal angles that we found suggest that there were also variations in the degree of alignment of the crystalline β-sheets in the MA silk of the wind exposed spiders. These patterns suggest that the silks of wind exposed spiders had become "stretched" in the crystalline regions and these may bring about increases in ultimate strength in the silks via slip-stick and other similar molecular-level mechanisms.[14,16] Similar crystalline stretching has been shown to occur when silks are drawn at high speed.[33,54,55] We, however, drew all of the silks in still air at constant speeds; 5 mm s^{-1}, which we had predetermined as the fastest reeling speed possible without damaging the fibers, so we were confident that variations in reeling speeds could not have induced stretching in the crystalline region in MA silks from wind exposed spiders.

We expected that protein alignment in the amorphous region did not have a profound influence on the tensile properties of the silks of spiders exposed to wind because the variations were not in accord with those found in *C. mulmeinensis* MA silk superconstraction tests.[21] Nevertheless, since strength and extensibility are products of the elastic and plastic components of the silk and not of the crystalline structure alone, there would have been a degree of variation in protein alignment in the amorphous region in the silks of spiders across the treatment subgroups. This variation probably contributed in some way to the variations in silk mechanical properties.[16,32,33,56−58] Further quantitaive analyses of the relative role of amorphous alignment using supercontraction coupled with detailed X-ray diffraction, NMR, and infrared (e.g., FTIR) analyses are, nonetheless, needed to determine the precise extent to which any variations in protein alignment influences the tensile properties in the wind exposed spiders.

Although the diffraction pattern shown in Figure 2 exhibits a clear signal recognizable from background noise, to calculate

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the percent crystallinity we needed to deconvolute this profile into (020), (210) Bragg reflections and amorphous, broad peak and background (air) diffraction patterns. In attempting to do this we found that, because of the overt thinness of the fibers, the air scattering signal dominated the background and inhibited accurate calculations of percent crystallinity. Nevertheless, from Figure 2, we can deduce that the diffraction peak intensity for (020) and (210) of N is higher than that of W, so we surmize that the percent crystallinity for N is likely to be higher than that of W. Confirmatory analyses nonetheless are required to verify our deduction.

In summary, we showed that postsecretion processes enable the spider *C. mulmeinensis* to vary the mechanical properties of its MA silk when exposed to wind. Moreover, we showed that these changes include simultaneous increases in strength and extensibility and are induced by processes acting on the formation and alignment of the β-sheets, e.g., shear stresses, without concomitant changes in MaSp1: MaSp2 expression. X-ray diffraction measurements showed that the crystalline regions of the MA silks of wind exposed spiders had a lower β-sheet density than those of spiders not exposed to wind. Moreover, alignment of the β-sheets also varied between the treatment subgroups; with those in the silks from spiders in the W subgroup being more aligned relative to the fiber axis than those from spiders in the N subgroup. A change in protein alignment within the amorphous region may have also exerted some influence on the mechanics of the MA silks of wind-exposed spiders.[21,32,56−58] Our measurements, nonetheless, were unable to ascertain to what extent this influence was exerted. Ecologically, the variations in MA silk mechanical properties probably serve to reduce wind-induced tearing of the silk when webs are exposed to strong wind. We predict that if the threads are spun in strong wind, a greater frictional stress is applied at the spigot valve and would result in an increased shear force on the silk during extrusion, promoting greater alignment in the amorphous region and further strengthening of the fibers. We are currently undertaking studies to test this prediction.

CONCLUSION

C. mulmeinensis MA silk crystalline β-sheet density and structure but not amino acid composition differed between spiders that had been exposed to wind and spiders that had not been exposed to wind, which culminated in differences in silk mechanics across the treatment subgroups. Protein alignment in the amorphous region seemed not to substantially influence tensile properties, but this inference remains to be confirmed. The mechanical properties of the MA silks of *C. mulmeinensis* exposed to wind appeared to be modified by changes in crystalline β-sheet density and alignment. Shear stresses at the valve and variations in the physiology and biochemistry within the sac or duct may have induced these variations. The mechanical properties, particularly strength and extensibility, of *C. mulmeinensis* silks are highly variable, perhaps more so than those of other orb web spiders.[20,21,41,42] Moreover, they are highly responsive to variations in the wind speed that the spiders experience. Accordingly, the MA silk of this spider may be of interest to researchers and engineers seeking synthesizable and adaptable biomimetics.[2,3,6] We suggest researchers look at the molecular structure and mechanics of *C. mulmeinensis* MA silk at increasingly finer scales to understand the precise molecular mechanisms inducing its variability.

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Notes

The authors declare no competing financial interest.

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