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# Protein Composition Correlates with the Mechanical Properties of Spider (*Argiope trifasciata*) Dragline Silk

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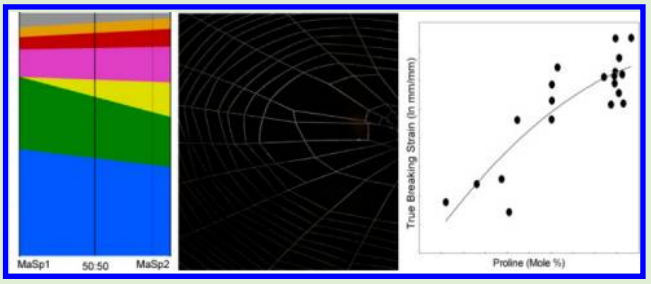
**ABSTRACT:** We investigated the natural variation in silk composition and mechanical performance of the orb-weaving spider *Argiope trifasciata* at multiple spatial and temporal scales in order to assess how protein composition contributes to the remarkable material properties of spider dragline silk. Major ampullate silk in orb-weaving spiders consists predominantly of two proteins (MaSp1 and MaSp2) with divergent amino acid compositions and functionally different microstructures. Adjusting the expression of these two proteins therefore provides spiders with a simple mechanism to alter the material properties of their silk. We first assessed the reliability and precision of the Waters AccQ-Tag amino acid composition analysis kit for determining the amino acid composition of small quantities of spider silk. We then tested how protein composition varied within single draglines, across draglines spun by the same spider on different days, and finally between spiders. Then, we correlated chemical composition with the material properties of dragline silk. Overall, we found that the chemical composition of major ampullate silk was in general homogeneous among individuals of the same population. Variation in chemical composition was not detectable within silk spun by a single spider on a single day. However, we found that variation within a single spider's silk across different days could, in rare instances, be greater than variation among individual spiders. Most of the variation in silk composition in our investigation resulted from a small number of outliers (three out of sixteen individuals) with a recent history of stress, suggesting stress affects silk production process in orb web spiders. Based on reported sequences for MaSp genes, we developed a gene expression model showing the covariation of the most abundant amino acids in major ampullate silk. Our gene expression model supports that dragline silk composition was mostly determined by the relative abundance of MaSp1 and MaSp2. Finally, we showed that silk composition (especially proline content) strongly correlated with some measures of mechanical performance, particularly how much fibers shrunk during supercontraction as well as their breaking strains. Our findings suggest that spiders are able to change the relative expression rates of different MaSp genes to produce silk fibers with different chemical compositions, and hence, different material properties.




## INTRODUCTION

Spider dragline silks are an exceptional class of natural proteins whose material properties, especially toughness, exceed all other known natural materials.[1] This performance makes spider dragline silk an attractive model for industrial and biomedical applications ranging from high-performance fabrics to tendon repair to tissue scaffolds.[2] Known as major ampullate silk due to the glands from which it is spun, dragline silk also plays a key role in how spider webs dissipate prey energy.[3] Understanding the relationship between the protein composition of major ampullate silk and its functional properties is therefore critical both to understanding the diversification of spiders as dominant predators of flying insects[4] and to the development of silk-based biomimetic materials.[2]

In orb-weaving spiders, major ampullate silk consists of two large proteins (250−350 kDa), known as major ampullate spidroins, which have different amino acid compositions and are identified as MaSp1 and MaSp2.[5,6] Major ampullate silk's structure is a composite of rigid nanocrystals that provide strength and stiffness embedded in an amorphous network that provides extensibility to the fibers.[5] cDNA analyses of major ampullate silk glands of orb-weaving spiders (Orbiculariae) reveal that alanine and glycine are more abundant in MaSp1, while proline occurs almost exclusively in MaSp2.[7] Alanine and glycine promote $\beta$-sheets that stack together to form the nanocrystals, while proline disrupts the formation of $\beta$-sheet crystals and instead promotes $\beta$-spirals in the amorphous region of the silk.[5] Hence, MaSp1, with high glycine and alanine content, is particularly important for formation of the $\beta$-sheet crystalline region of silk fibers and gives strength and stiffness to the fibers. On the other hand, MaSp2, with its high proline content, mostly occupies the amorphous regions connecting the $\beta$-sheet crystals to each other and hence increases the extensibility of the fibers.[5,8−11] The combination of these two proteins with notably different compositional elements helps to explain spider silks exceptional material properties and makes

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spider silk a good model to study fibrous protein structure−function relationships.

Orb web spiders depend upon their webs to feed.[12,13] Therefore it might be beneficial for spiders to change their silk composition to tune their web performance for different environments. Blamires, et al.[14] and Tso, et al.[15] reported the effect of diet on the composition of major ampullate silk, demonstrating that spiders can exert some control over the chemical composition of their silk. However, these studies only gave a rough estimate of intraspecific variation in silk composition over several days so that we still do not know over how small of a temporal or spatial scale silk composition can vary.

Supercontraction provides a convenient tool to relate the chemical composition of silks to their material properties. Supercontraction is a phenomenon where spider silk shrinks up to 50% of its length after exposure to water and is an important property from the structure−function standpoint.[16,17] During supercontraction, water interrupts the intermolecular bonds between amorphous regions of silk proteins allowing the microstructure to move to a more disorganized level. In other words, the interaction of water with the silk increases the entropy of the silk's microstructure, providing the energy for the contraction to occur.[17−19] Thus, the ability of silk to supercontract is an important measure of both the alignment of molecules and the intermolecular bonding among silk proteins. The proline content of major ampullate silk correlates significantly with the amount that spider silks contract (shrink capacity),[9] and proline is hypothesized to enhance silk mobility in the presence of water by providing more sites for water molecules to make hydrogen bonds.[18] Therefore, proline facilitates the movement of $\beta$-sheet crystals and increases the shrink capacity of silk.[8,9,16,20,21] Proline content correlates positively with shrink capacity of silk at the interspecific level.[9,16] However, the relationship between proline content and silk mechanics is largely understudied at the intraspecific level, even though such investigation better controls for alternative influences on silk mechanics and could provide crucial evidence for plasticity in the expression of silk genes. The best studied system is *Nephila pilipes*, where proline content and material properties of major ampullate silk seem to vary among populations[15] and in response to changes in diet.[22] However, these relationships are not always found.[23,24]

To understand structure−function relationships, we need reliable methods to measure mechanical properties and chemical composition of silk. However, spinning conditions (e.g., silking speed, shear forces, hemolymph pH, etc.) interact with the chemical composition of silk to affect silk microstructure and ultimately its mechanical properties.[5,20,25,26] Elices et al.[20] reported that supercontraction acts as a "reset switch" that relaxes the arrangement of silk microstructure to a defined mechanical ground state by removing most of the effects of silking speed and shear forces to help consistently measure silk mechanical properties. Therefore, the mechanical properties of supercontracted fibers are more closely derived from their chemical compositions and can reveal structure−function relationships not readily apparent within the variation of naturally spun silk.[4]

MaSp1 and MaSp2 are difficult to distinguish using common gel electrophoresis methods because large quantities of silk are required so that amino acid composition analysis is used in most studies investigating spider silk composition.[9,15,22,23] Based upon translated cDNA sequence, proline is abundant in MaSp2, while MaSp1 is almost proline-free.[7,27,28] Thus, many recent studies focus on proline in major ampullate silk as an indicator of silk MaSp1 content relative to MaSp2, when comparing mechanical performance of dragline silk among spiders.[9,14,15,22−24,29] Studies investigating variation in proline content across different spider species report a significant correlation between proline content and the mechanical performance of major ampullate silk.[9,29] However, spinning morphology and physiology likely varies among species in ways that may correlate with or enhance the effects of protein composition on silk properties. Our study therefore focuses exclusively on variation within a single species, so that differences in amino acid composition more likely reflect variation in gene expression than details of gland morphology or gene sequence, both of which vary among species.[28,30,31] The degree to which individual spiders can control the protein composition of their silk is ultimately critical to understand the ecology and evolution of these high-performance fibers.[32]

While several studies show consistent chemical composition of major ampullate silk among individuals of the same species,[7,26,27,33] other studies suggest that amino acid composition of major ampullate silk varies at the intraspecific level,[22−24,34,35] especially across large spatial or temporal scales. Most of these studies use mixtures of several meters of major ampullate fiber to get the required quantity of silk for amino acid composition analysis, and none of them assess the potential variation in silk composition over a short length of a silk fiber. Many of them also neither optimize their analyses for spider silk fiber nor assess the precision of their methods. Here, by using the Waters AccQ-Tag amino acid composition analysis kit, we propose a reliable and more sensitive method to analyze the composition of major ampullate silk with very low quantity of material (as low as 150 ng or 3 cm of silk). More importantly, we assess the reliability and precision of amino acid composition analysis for spider silk. We then investigate variation in the chemical composition of major ampullate silk within and among individual spiders of the same species at multiple spatial and temporal scales. Finally, we relate variation in chemical composition of silk to mechanical performance.

## EXPERIMENTAL SECTION

**Spiders and Silk Collection.** We collected nine adult female *Argiope trifasciata* from the University of Akron Field Station at the Bath Nature Preserve (Bath, OH) and purchased seven more adult female *A. trifasciata* from a distributor (tarantulaspiders.com) in Florida. We kept all spiders inside cages (40 × 40 × 10 cm) at the University of Akron's greenhouse with a natural light dark cycle (∼12 h) and fed all of them with house crickets, *Acheta domestica*.

We forcibly silked the spiders to collect pure samples of major ampullate silk under controlled conditions. To silk a spider, we anesthetized it with $CO_2$ for 30−50 s and fixed it on a glass platform using Scotch tape. We allowed the spider to recover for 3−5 min and then, under a stereomicroscope, carefully collected major ampullate silk, using procedures detailed below. We collected samples only from a single major ampullate gland (either the right or left) for each individual.

For mechanical testing, we collected three to five samples of silk from a 60 cm length of dragline for each of six individual spiders (in total 46 samples). Each spider was sampled for up to 4 days within a 15-day period. We mounted the silk samples across 15.7 mm diameter holes in cardboard using cyanoacrylate adhesive (Superglue). Blackledge, et al.[36] showed that measuring the diameter of each testing sample directly by using polarized light microscopy at 1000X can provide a better estimate of the diameter of silk threads when the diameter varies across their lengths or among individuals compared to

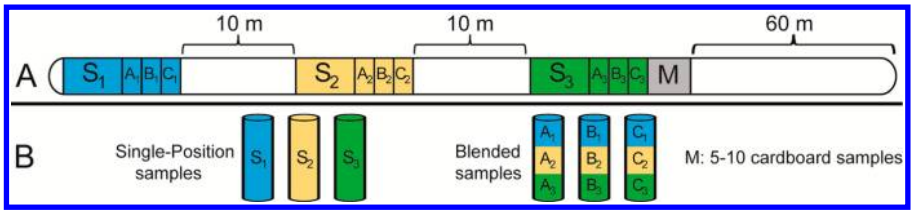


**Figure 1.** Sampling scheme for assessing the measurement precision and the variation in amino acid composition within a single silk fiber. Panel A shows the relative position of the samples on a silk fiber. Panel B shows the final six samples for amino acid composition analysis. Variation within single draglines was assessed by taking samples at the beginning, middle, and end of 20 m of a single dragline ($S_1$, $S_2$ and $S_3$). Measurement precision was assessed by combining silk from each of those three locations into three blended cocktails (A, B, and C), each of which was independently hydrolyzed and analyzed. The blending should have assured homogeneous composition among the samples. We collected 5−10 cardboard-mounted samples from the M region for mechanical testing and then continued the silking process for an additional 60 m to ensure that the next sampling day's collections were at a significantly greater spatial distance than any comparisons within a single day.

destructively measuring diameter from an exemplar sample using SEM and extrapolating to tensile tested samples. Therefore, we estimated the diameter of each sample based on the average of three measurements taken along its length using polarized light microscopy at 1000X.[36] We then measured the material properties (true breaking stress, true breaking strain, Young's modulus, toughness, and shrink capacity) of the samples using a Nano Bionix UTM tensile tester (Agilent Technologies, Phoenix, AZ, USA) as described previously.[37,38] We normalized the mechanical properties of each sample based on its diameter, assuming constant volume as the silk stretched.[39] The Nano Bionix tensile tester was equipped with a humidity chamber and had a load resolution of ∼1 uN and an extension resolution of ∼1 um. Briefly, we mounted a sample at room temperature and humidity. We pulled the silk until it generated 20 uN load to ensure the sample was taut. We then increased the humidity to more than 75% Rh within 60 s to supercontract the sample. Then, we relaxed the sample until slacked (i.e., until load decreased to 0) and dried the chamber to less than 5% Rh within 90 s. Then, we performed a tensile test on the dried silk at a strain rate of 0.1 $s^{-1}$ based upon the current length of the contracted sample. We measured the shrink capacity (Sc) as:

$$\mathrm{Sc} = \frac{l_o - l_s}{l_o}$$

where $l_o$ is the original length of the fiber, and $l_s$ is the length of the just taut, supercontracted, fiber.

For amino acid composition analysis, we collected $4 \times 10^5$ $\mu m^3$ (9−15 cm in length) samples of major ampullate silk. To collect consistent volumes of material, we first measured the diameter of major ampullate silk from the samples described above. Then, based on the diameter and the required volume, we calculated and collected the corresponding length (9−15 cm) of the dragline. As the dragline came out of the major ampullate silk gland, we wrapped it around a clean thin glass rod (0.2−0.3 mm in diameter) attached to a rotating axel. The silk was wrapped around the terminal end (1−2 cm) of the rods. We then cut the terminal end and transferred it to the bottom of a 6 × 50 mm glass test tube. We used powder free vinyl gloves during the entire procedure to minimize contamination.

**Amino Acid Composition Analysis.** We used the established protocol described by Smith[40] to perform vapor phase hydrolyzation and amino acid composition analysis. We used a hydrolysis vial with a PTFE cap (Eldex Laboratories, Napa, CA, USA), which holds fourteen 6 × 50 mm glass test tubes, each containing an individual silk sample. However, to minimize the possibility of cross contamination, we placed up to eight prepared test tubes containing silk into the hydrolysis vial and filled the vacant spaces between them with blank tubes. We used the blank samples to estimate the cross contamination among samples in a single hydrolysis vial. We added 400 $\mu$L of 6N HCl to the bottom of the hydrolysis vial and vacuum-sealed it using a vacuum pump with an ultimate vacuum of 5 Pa. We hydrolyzed samples under 6N HCl vapor at 115 °C for 21 h. Afterward, we removed the inner sample tubes, dried them under a vacuum, and derivatized amine containing compounds with an aminoquinolyl-NHS compound (AQC) using a Waters AccQ-Tag kit (Waters, Milford, MA, USA). We separated the derivatized amino-acids with a 3.9 × 150 mm C18 column from the AccQ-Tag kit using an ÄKTAPurifier high-performance liquid chromatography (HPLC) system with UV detection. We analyzed chromatographs and integrated peak areas using Unicorn 5.20 software (GE Healthcare, Pittsburgh, PA, USA). We calibrated the instrument with five amino acid standard concentrations (50−1000 pmol) and used the calibration file to calculate the relative content of each amino acid as mole percent per 100 residues. Because asparagine and glutamine are deaminated to their respective acids under HCl hydrolyzation, we reported them as mixtures of asparagine/aspartic acid and glutamine/glutamic acid. We performed HPLC at room temperature, thus, we were not able to distinguish between arginine and threonine peaks, and we reported them as a mixture of arginine/threonine. Much of our analysis focuses on the percentage of proline in silk because this amino acid occurs almost exclusively in MaSp2 so that it is often used as an indicator of silk MaSp1 content relative to MaSp2.[15,22]

**Precision and Spatial Variation.** Little is known about the potential for variation in amino acid composition of spider silk over small spatial scales (e.g., within cm along the same fiber). Prior work often either used longer lengths of threads, thereby averaging out any potential variation, or too few samples to detect potential variation in composition.[15,35,41] Moreover, the precision with which the amino acid composition of spider silk can be determined has never been quantified. Thus, it is impossible to know the degree to which minor variation in measurements of amino acid compositions among silk fibers might be biologically meaningful versus simple error. To check the reliability of our amino acid composition analysis method, and whether or not major ampullate silk composition varied across a single dragline, we silked three spiders (identified as #25, 30, and 37 hereafter) on three separate days within a 7 day period. On each sampling day, we collected three single-position samples ($S_1$, $S_2$ and $S_3$ in Figure 1) at 10 m intervals along with three additional subsamples from each of those locations (Figure 1). One subsample from each location would later be combined into a single blended sample according to its relative position along the silk fiber, to make a total of three blended samples (A, B and C in Figure 1). These blended samples should have averaged out any real chemical variation in the silk fiber so that their chemical compositions would be identical to one another. Therefore, any measured variation in chemical composition could most likely be attributed to error inherent in the amino acid composition analysis itself. The length of each individual subsample was one-third of the length of each single-position sample. Thus, the volumes of all six final samples were equal. At the end of the third location, we collected 5−10 cardboard-mounted samples (region M in Figure 1) for mechanical testing so that their chemical composition could be inferred from the last single-position sample ($S_3$ in Figure 1).

Knowing the statistical precision of any measurement is critical for understanding how much of the variation among samples might be due to real biological differences rather than random error. Therefore, we calculated the statistical precision of our method by taking the average of the coefficients of variation of the blended samples for the

major amino acids in spider dragline (glycine, alanine, proline, serine and glutamine/glutamic acid). These amino acids comprised more than 90% of a dragline silk, thus they better represent method precision compared to less abundant amino acids. To examine whether or not the variation in silk composition within 20 m of a single major ampullate silk fiber was significantly different from the measurement precision, we ran F-tests comparing the variances of the three single-position samples versus the three blended samples for each individual spider (five F-tests in total). Then, we calculated the overall p-value using a Fisher's combined probability test.

**Variation through Time.** To compare variation in silk composition among days, we used the data from all the above spiders (#25, 30 and 37) and two more individual spiders (#22 and 62) whose silk was collected for three consecutive days, as well as one more spider (#52) whose silk was collected on three separate days spread over a 10 day period. In addition, we silked spider 25 for one more day (a fourth sampling day), which was a total of 15 calendar days after the spider was first sampled. On each day, we collected two samples for amino acid composition analysis and 5−10 cardboard-mounted samples for mechanical testing. We took care to minimize the space between samples, so the chemical composition of cardboard samples could be inferred from the amino acid samples. Finally, we continued the silking process for 60 m at the end of each day. Spiders have a reservoir of liquid silk dope of unknown volume waiting to be spun into silk.[13] We had no way to assess how much silk any spider might have spun outside of our sampling, but this protocol did ensure that the next sampling day's collections were at a significantly greater spatial distance than any comparisons within a single day. Because of the poor understanding of how the silk dope may or may not be regulated physiologically by the spider, we also considered how silk fibers might vary both as a function of the distance from prior samples (a measure of material removed from the reservoir) and as time since last collection (presumably a measure of potential for physiological manipulation of the reservoir).

**Variation among Individuals.** To compare variation in chemical composition among individual spiders, we used the data from all six spiders above and collected one to four amino acid samples from 10 more *A. trifasciata*. Six of those spiders were from a different population (designated here as Florida spiders). We purchased them from a distributor and shipped them to the laboratory. Therefore they were not only a geographically distinct population, but they also likely were under greater physiological stress prior to the experiment, compared to the locally captured "Ohio" spiders.

**MaSp1−MaSp2 Expression Model.** Several studies suggest that variation in major ampullate silk composition is potentially determined by the relative abundance of MaSp1 and MaSp2.[22,24,42] However, other factors can also affect silk composition, such as the expression of other silk genes in the major ampullate gland,[43] potential post-translational modifications (currently uninvestigated), and the existence of multiple alleles for some MaSp proteins.[44]

If the composition of major ampullate silk is mostly determined by the relative abundance of MaSp1 versus MaSp2 proteins, then the amino acid composition of the silk should vary proportionally across a defined set of values determined by the MaSp1/MaSp2 ratio. At one extreme, the amino acid profile is pure MaSp1 protein, which can be inferred from MaSp1 cDNA, and at the other extreme the silk is pure MaSp2 protein. Thus, by knowing the ratio of MaSp1/MaSp2, we can predict the whole amino acid profile of a silk fiber. Proline is virtually unique to MaSp2 so that knowing the proline ratio alone should be enough to predict the silk amino acid profile and expression levels of the two proteins.

With this assumption, and based on reported sequences for the repetitive regions of MaSp1 (14 repetitive units in total 549bp) and MaSp2 (18 repetitive units in total 851bp) in *A. trifasciata* (accession #: AAZ15371, AAK30596 and AAK30595), we designed a gene expression model to estimate the amino acid composition of a pure MaSp1 and a pure MaSp2 dragline silk (Table 1). We used the model to evaluate the expected linear changes between different pairs of amino acids as gene expression level changes (Figure 2). We then used the gene expression model to estimate the relationship between pairs of amino acids in the major ampullate silk and calculated the slope and intercept of the line as the average of data points for each axis. We plotted our real data and compared them to the best fit of the gene expression model to assess how well a simple variation in MaSp1/MaSp2 expression explained the variation in our data set, compared to alternative explanations, such as expression of other silk genes in the major ampullate gland, contamination, or experimental error.

**Table 1. Estimated Amino Acid Composition of MaSp1 and MaSp2 Based on the Repetitive Regions of Reported Sequences for MaSp Proteins in *Argiope trifasciata***

| amino acids | MaSp1 | MaSp2 |
|---|---|---|
| glycine | 43.90 | 36.55 |
| alanine | 29.69 | 20.56 |
| proline | - | 14.22 |
| serine | 5.10 | 7.40 |
| glutamine | 11.29 | 14.69 |
| tyrosine | 4.01 | 4.35 |
| arginine | 0.91 | 1.06 |
| threonine | 0.36 | 0.47 |
| valine | 0.55 | 0.59 |
| leucine | 3.10 | - |
| aspartic acid | 0.36 | 0.12 |
| phenylalanine | 0.18 | - |
| glutamic acid | 0.55 | - |

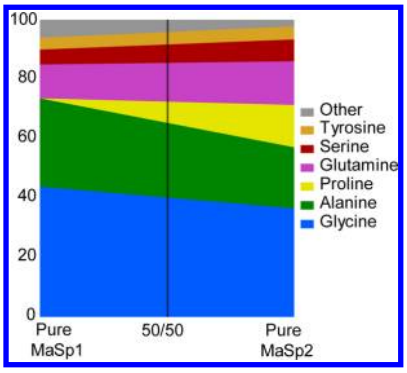


**Figure 2.** Gene expression model for estimating the amino acid profile of dragline silk in *Argiope trifasciata*. Amino acid composition of dragline silk varies proportionally across a defined set of values determined by the MaSp1/MaSp2 ratio. The amino acid composition of MaSp proteins are estimated based on the repetitive regions of reported sequences for MaSp proteins in *Argiope trifasciata*. There is no proline in the repetitive regions of MaSp1 cDNA so that it can be used as indicator of the relative expression of the two genes.

**Structure−Function Relationships.** We used polynomial regressions to assess the relationship between proline content and material properties of silk. We applied a Bonferroni correction to achieve a global $\alpha = 0.05$ because individual measures of material properties are intrinsically correlated with one another.

## RESULTS

**Precision and Spatial Variation.** We calculated the precision of our method by taking the average of the coefficients of variation calculated from the three blended samples collected from a single spider on a single day (coefficient of variation: glycine = 0.012, alanine = 0.013, proline = 0.014, serine = 0.019, and glutamine/glutamic acid = 0.026). The total amino acid content of each sample was between 0.5 and 6 nmol, and the cross contamination among samples in a single hydrolysis vial was less than 1% of the total amino acid content of each sample. Thus, cross contamination

did not affect the results significantly. Figure 3 compares the proline content of single-position and blended samples in three

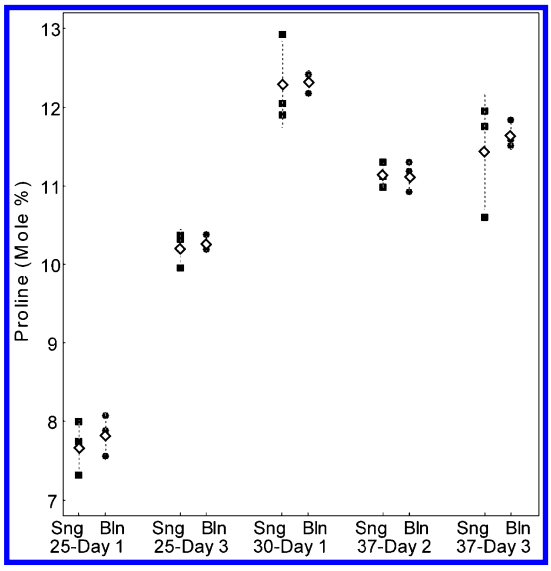


**Figure 3.** Natural variation in proline content of major ampullate silk compared with measurement precision. The open diamonds correspond to the mean proline content of the dragline, and the dashed lines show standard deviation. Fisher's combined probability test showed that variation in proline content within 20 m of single draglines was no greater than measurement precision ($p$ = 0.23). Single (Sng) and blended (Bln) samples are described in Figure 1. The spider identities and day numbers correspond to other figures.

spiders collected across multiple days. Although two outlier data points (spider 37-day 3 and spider 30-day 1) suggest variation in chemical composition across samples taken within 20 m of major ampullate silk, the coefficients of variation of single-position and blended samples were not statistically different ($p$ = 0.23, Figure 3) for the whole data set, supporting that dragline silk does not vary chemically over small spatial scales (i.e., meter length). Moreover, the two outlier data points showed alanine and glycine values that were lower than suggested by cDNA for major ampullate silk in *A. trifasciata* (Table 1 shows the acceptable ranges), and likely resulted from partial hydrolyzation as analyzed in the Discussion section. Therefore, any potential variation in chemical composition within 20 m of major ampullate silk fiber was below the detection level of our method.

**Variation through Time.** We found that the proline content of silk from individual spiders could vary substantially over multiple days, but was usually remarkably consistent for most spiders. Proline increased by 80% (from 7.3% to 13.1% of all amino acids) after 15 days in spider 25 and decreased by 62% (from 8.5% to 5.3%) after 3 consecutive days in spider 62 (Figure 4). However, silk proline content was constant over 20 m of silk collected continuously in a single day for most spiders and was constant across 100 m of silk for spider 56 (Figure 5). Silk composition was remarkably homogeneous across time and typically varied by less than ±2% proline across 3 days for most individuals (Figure 4). However, the average coefficient of variation within a single day (c.v. = 0.02) was significantly lower than the average coefficient of variation over multiple days (c.v. = 0.08, Figure 5).

**Variation among Individuals.** The proline content of major ampullate silk varied from 5.2−13.6% among individual

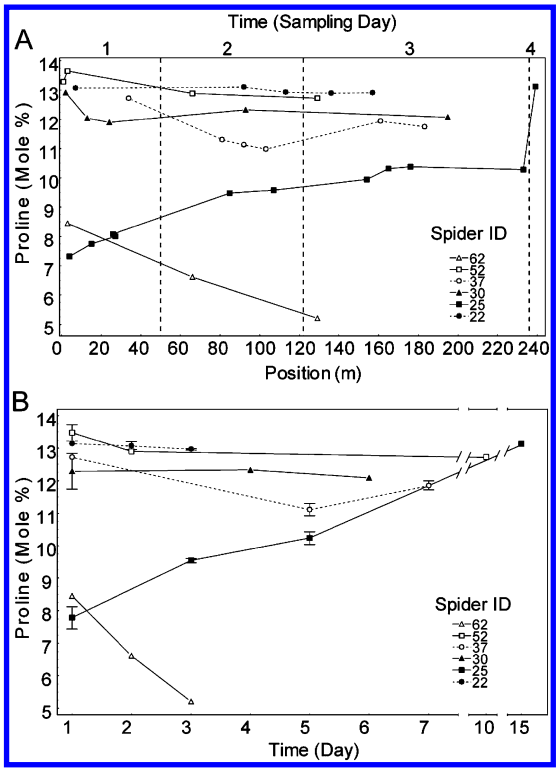


**Figure 4.** Natural variation in proline content of major ampullate silk as a function of distance or time. Panel A shows the variation in proline content of the silk collected on different days (not necessarily contiguous) from six spiders against the position of the samples along the reeled silk. Position is the relative distance of the sample to the total length of the reeled silk. Spiders might produce and use more silk than we collected between each day. Proline variation was minimal both within draglines and across spiders, with the exception of two individuals that were noteworthy for the unusually low proline content of their silk. Panel B shows the same data expressed as day of collection (e.g., number of real calendar days since day 0) instead of distance of dragline reeled from the spider. The spider identities correspond to other figures.

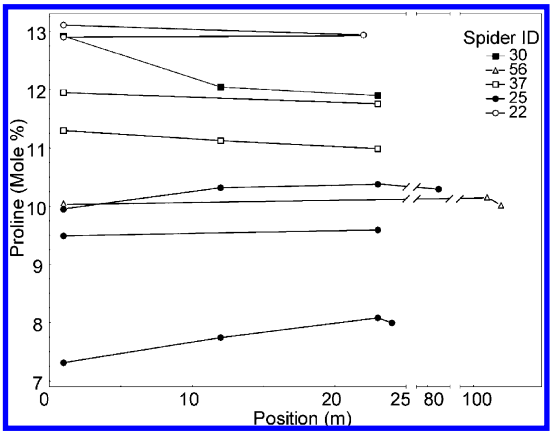


**Figure 5.** Variation in proline content of major ampullate silk within and among days. Each line connects samples collected in the same day. Five spiders are compared, most for multiple days. The lines connect silk samples taken from different positions on an individual spider's dragline on a single day. The spider identities correspond to other figures. Silk proline content is consistent within a single day, while it varies among days for some individual spiders (e.g., spider 25).


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*A. trifasciata*. Despite this broad range, proline content was remarkably homogeneous among most individuals, varying by less than ±2% among 13 of 16 individuals (Figure 6). However, the variation within a single spiders' silk across days can be larger than variation among individuals (e.g., spider 25 in Figure 6).

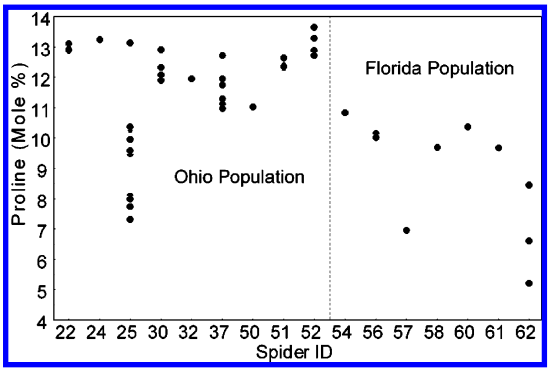


**Figure 6.** Variation in proline content of major ampullate silk among individual spiders. Sixteen spiders from two populations were compared. There was a wide range of proline content (5.2% - 13.6%) among the silk of individual *Argiope trifasciata*. However, most of the variation in silk composition resulted from samples collected from just three spiders (25, 57 and 62), two of which had a recent history of "shipping" stress. Moreover, silk proline content was consistent among most individuals within a single population. Each dot represents a sample of dragline. The spider identities correspond to other figures.

**Variation among Populations.** Overall, the amino acid compositions of major ampullate silks differed significantly between the Ohio and Florida spiders ($p < 0.001$). Silk proline content was more consistent within populations, varying by less than ±1.5% among most individuals of the same population. The shipped spiders (Florida population) on average produced major ampullate silk with significantly lower proline content ($p < 0.001$, Figure 6). Major ampullate silk from Florida spiders had more glycine, alanine, arginine/threonine, and phenylalanine, but less serine, glutamine/glutamic acid, and proline (Figure 7). These correlations are consistent with the MaSp1/MaSp2 gene expression model (Figure 8) and suggest that Ohio spiders express more MaSp2 in their major ampullate silk. We emphasize that this variation correlates with major differences in how the two groups of spiders were handled, in addition to their geographic origins, as the Florida spiders were shipped through the mail enduring a period of prolonged stress where they lacked food and water and were subjected to unknown temperature changes.

**Does MaSp1/MaSp2 Expression Explain Variation in Major Ampullate Silk Composition?** We compared our amino acid data (45 samples from 16 spider individuals) to our MaSp1-MaSp2 expression model to assess how well variation in MaSp1 versus MaSp2 expression explains the variation in silk composition (Figure 8). The gene expression model explained the majority of the variation in alanine (75%) and much of glycine (44%) and glutamine/glutamic acid (51%). In addition, there was no meaningful correlation between tyrosine and proline (Figure 8), as predicted by the gene expression model because tyrosine is nearly equally abundant in MaSp1 and MaSp2 cDNA (4.01% and 4.35%, respectively). The absolute values measured for tyrosine were less than expected because tyrosine is recovered in low yield under HCl hydrolyzation.

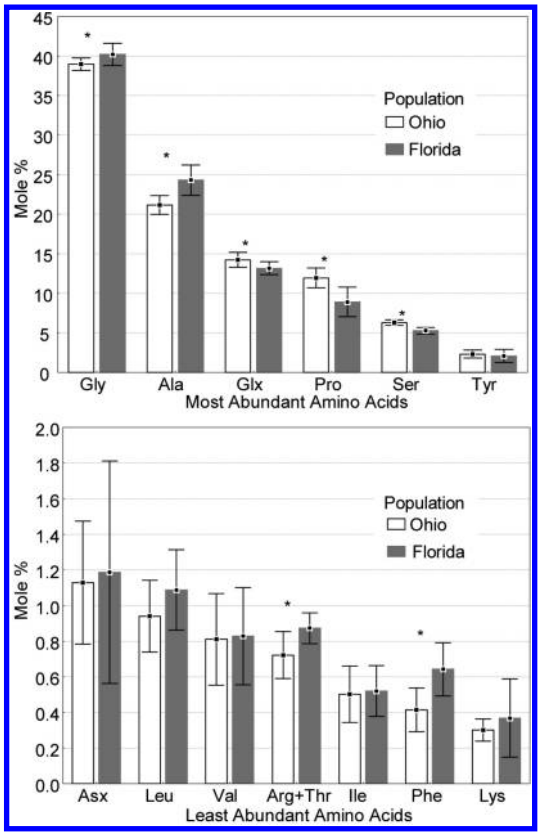


**Figure 7.** Comparison of mean ± SD amino acid compositions of major ampullate silk fibers for spiders from Ohio (34 samples from 9 individuals) and Florida (11 samples from 7 individuals). Three letter amino acid abbreviations are used. Asterisks show statistically significant pairwise comparisons from 13 *t* tests with a Bonferroni correction ($p < 0.05$). The amino acid percentages were all normally distributed enough for parametric statistics with the exception of Ile. Because asparagine and glutamine will be deaminated to their respective acids under HCl hydrolyzation, we report them as mixtures of asparagine/aspartic acid (Asx) and glutamine/glutamic acid (Glx). Cystine and methionine are nonquantifiable in the HCl hydrolyzation process, thus, they are excluded from this analysis. Amino acids associated with MaSp2 (Glx, Pro, and Ser) are higher in Ohio spiders, and amino acids associated with MaSp1 (Gly and Ala) are higher in Florida spiders. There is no difference in tyrosine which is expected, because tyrosine is nearly equally abundant in both MaSp1 and MaSp2 cDNA.

**Structure–Function Relationships.** Amino acid composition correlated strongly with some aspects of the mechanical performance of the silk (Figures 9 and 10). In particular, we found a strong correlation between major ampullate silk proline content and its shrink capacity after supercontraction ($R^2$ = 0.75, $p < 0.001$). The correlation is positive and plateaus after reaching 13% proline. Proline content also strongly correlated with true breaking strain ($R^2$ = 0.75, $p < 0.001$), but not with true breaking stress ($R^2$ = 0.13, $p$ = 0.32). There was a weak correlation between proline content and toughness ($R^2$ = 0.24, $p$ = 0.09), whereas Young's modulus did not correlate with silk proline content ($R^2$ = 0.17, $p$ = 0.20).

## DISCUSSION

Our study showed that the chemical composition of major ampullate silk was homogeneous over 20 m of a single spider silk fiber and across 100 m for at least one spider (#56, Figure 5). Silk chemical composition was also generally consistent

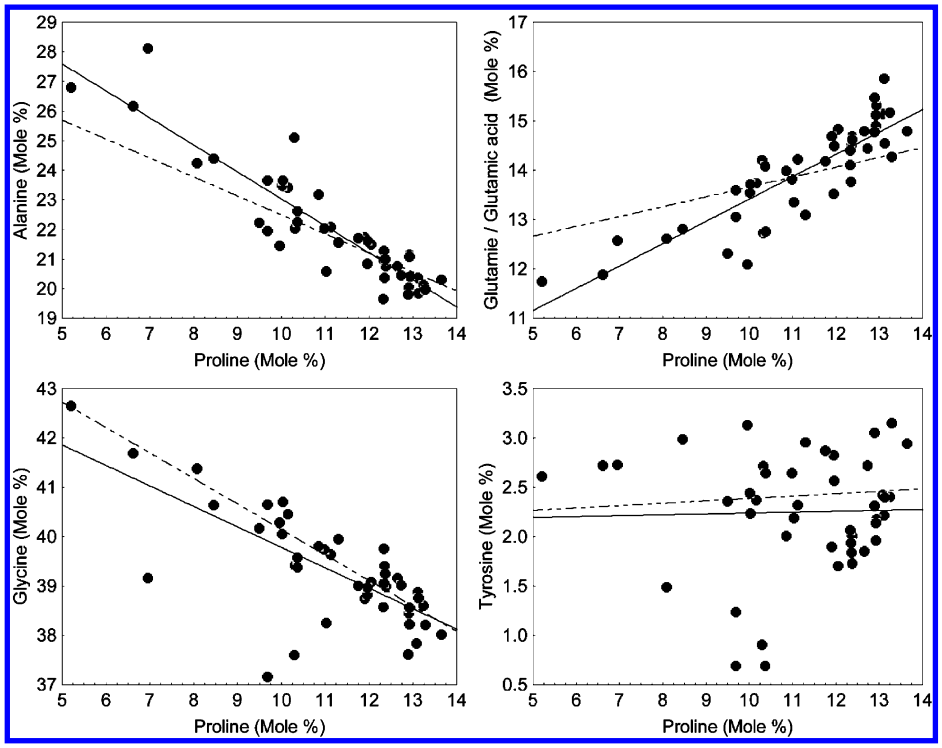


**Figure 8.** Covariation in amino acid composition as predicted by relative expression of MaSp1 versus MaSp2. Proline is used to estimate the relative MaSp2 content of the silk because it is largely absent from MaSp1. In each plot, the sold line shows the best fit of the data, and the dashed line shows the best fit from the gene expression model, based on reported sequences for the repetitive regions of MaSp1 and MaSp2 in *Argiope trifasciata*. The gene expression model explains most of the variation in alanine and much of glycine and glutamine/glutamic acid. There is no meaningful correlation between tyrosine and proline, which is predicted by the gene expression model as well.

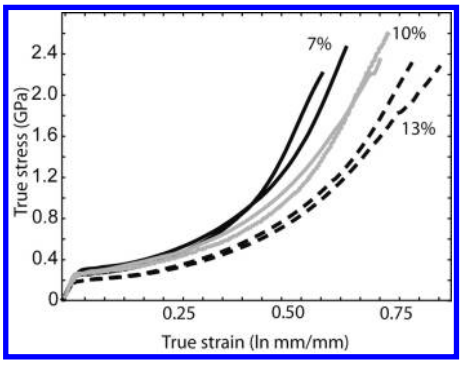


**Figure 9.** Tensile behavior of supercontracted silk with different proline content. Two exemplar stress–strain curves, each from different spiders, are shown for major ampullate silk with ~7, 10 and 13% proline content (black, gray and dashed lines respectively). Note the increase in extensibility as proline content increases.

across longer lengths of fibers produced over multiple days (Figure 4). Amino acid composition was similar within individual spiders (0.02 coefficient of variation), with the exception of two individuals whose silk was both more variable and generally substantially lower in proline. We found consistent variation in silk composition among individuals from two populations ("Ohio" versus "Florida"), although geography was confounded with differences in how the spiders were handled. Finally, we demonstrated that variation in the chemical composition of major ampullate silk correlates with mechanical properties. In particular, proline-rich silk was more extensible and shrank more after supercontraction, further validating the "structure-function" model of MaSp1/MaSp2 previously supported at the interspecific level.[8−10,18,29]

Hydrolyzation is a key step in amino acid composition analysis. The goal of hydrolyzation is to uniformly and nonspecifically break down all peptide bonds without losing free amino acids, but this is imperfect for some proteins. The classical HCl hydrolyzation is conducted under a vacuum at 110 °C for 18−24 h.[45] However, the stability of peptide bonds between amino acids varies. The peptide bonds between hydrophobic amino acids (e.g., alanine, valine, leucine and isoleucine) are relatively stable under HCl hydrolyzation. If the target protein is rich in these hydrophobic amino acids, a partial hydrolyzation will lead to a significant underestimation of the hydrophobic amino acids and overestimation of the other amino acids. Since poly alanine is a common domain in major ampullate silk, the alanine recovery is a very good indicator of the quality of hydrolyzation. When the alanine content of a sample is radically lower than the expected value, the sample should therefore be evaluated with caution.

Based on the general guideline for HCl hydrolyzation,[40] we hydrolyzed our samples at 115 °C for 21 h and we found few partially hydrolyzed silk samples (3 out of 68). Although the recovery of free amino acids may decrease by increasing the hydrolyzation time, the relative abundance of most amino acids does not change significantly, while alanine recovery increases dramatically (unpublished data). Under HCl hydrolyzation, tryptophan will be destroyed, cystine will be recovered as cysteine, and methionine and cysteine will be recovered in low, nonquantifiable yields. However, these amino acids comprise less than 0.5% of the total amino acid composition of major ampullate silk in orb-weaving spiders.[33,34,46,47]

The complete sequences of MaSp1 and MaSp2 of *Argiope trifasciata* are still unknown. Therefore, to assess how well the variation in MaSp1/MaSp2 expression explains the variation in

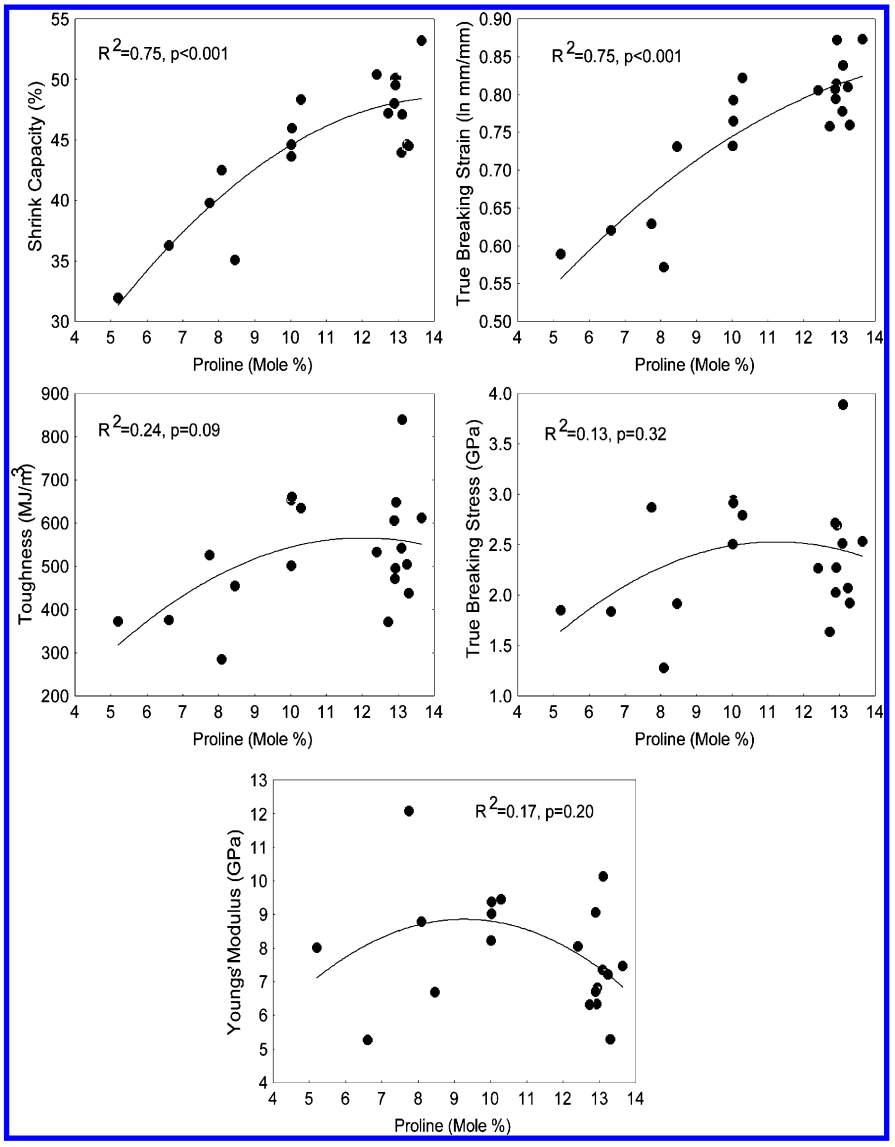


**Figure 10.** Correlations between proline content and mechanical properties of major ampullate silk. Each point is the average of as many as four mechanical test replicates within 50 cm of a single sample for amino acid composition analysis. Data are from multiple locations along draglines from six spider individuals. In total, 46 mechanical tests and 20 amino acid samples were examined. Polynomial (quadratic) regression is used to obtain the best fit. There is a strong correlation between major ampullate silk proline content and its shrink capacity after supercontraction. Proline is also strongly correlated to strain and weakly correlated to toughness. Neither stress nor modulus correlates with proline content.

our data set, we developed a gene expression model based on the internally repetitive regions of the proteins based upon reported cDNA sequences. We used the repetitive regions because they comprise the bulk of the protein (more than 90% in *Latrodectus hesperus* where full gene sequences are available[7]). Despite using incomplete sequences, the estimated gene expression model explained the majority of the variation in alanine and much of glycine and glutamine/glutamic acid. The pattern of covariation among amino acids (Figure 2) also supports that major ampullate silk composition in *A. trifasciata* is mostly determined by the relative abundance of MaSp1 and MaSp2. Thus, proline can be used as a reliable indicator of the whole amino acid profile of major ampullate silk, which is why much of our analysis focuses on the percentage of proline.

The amino acid composition of major ampullate silk varied significantly ($p < 0.001$) over multiple days in spiders 62 and 25 (Figure 4), both of which initially showed the lowest proline level reported in *A. trifasciata*. Their silk also differed mechanically compared to other spiders (e.g., Figure 10). Notably, spider 25 showed a trend toward recovery to the normal state in both proline level (Figure 4) and mechanical performance of the silk (Figure 10). Therefore, we suspect that these two spiders produced unusual silk due to the stresses that they encountered during the collecting, storing, and shipping processes. Because MaSp2 production is energetically more expensive than MaSp1, spiders may down regulate MaSp2 expression to save energy when stressed.[22] However, this is speculative, and finding the ultimate cause of this pattern needs more investigation.

The variation in proline content of a single spider's silk within a day is significantly lower than the variation among days (Figure 5). This suggests *A. trifasciata* produces nearly homogeneous silk fibers within a single day, but the composition can vary among days as well as individuals. We suggest change in relative expression rates of MaSp1 and MaSp2 to be the main pathway for the production of silk fibers


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with different chemical compositions within an individual of *A. trifasciata*.

Silk proteins are stored in silk glands waiting to be spun into silk fiber. This causes a time lag between secreting and spinning a silk fiber with new chemical composition. Therefore, not only the fiber's length but also the sampling time could be an important factor in assessing natural variation. We found that both silk composition and mechanical performance were consistent among days for spiders that produced silk with more than 44% shrink capacity (e.g., at least 10% proline) at the start of the experiment. Therefore, we suggest checking the mechanical behavior of major ampullate silk before starting an experiment to avoid including abnormal spiders in the study.

Several studies suggest that the amino acid composition of silk varies among populations of spiders.[15,24,48] We did not explicitly aim to test this hypothesis, but Ohio spiders showed higher proline content in major ampullate silk compared to Florida spiders (Figure 7). While this could be an evidence of geographic differences in silk gene expression, we think that it is equally likely that the Florida spiders were stressed due to collecting, storing, and/or shipping processes. This stress hypothesis is supported by our observations in which some spiders from Florida produced normal silk from the first day of arrival, while others produced silk with notably unusual mechanical properties (e.g., low shrink capacity) within two days of arrival in the laboratory, but then produced silk with normal amino acid compositions and typical mechanical properties after being kept for a week in the greenhouse (e.g., unpublished data for spider 25 and others). In contrast, locally collected Ohio spiders were more consistent in amino acid composition.

**Does Amino Acid Composition Correlate with Silk Mechanics?** We found that the proline content of major ampullate silk correlates positively with shrink capacity and true breaking strain (Figures 9 and 10). Both correlations are positive and plateau after 13% proline. Other studies show this correlation between proline content and elasticity across silk for different spider species[8,20] and for biological fibers in general.[49] These correlations are evidence for the hypothesis that proline increases the mobility of fibrous protein microstructure.[8,18,49,50] Thus, a silk fiber with more proline has more mobile microstructure so that it is more extensible and also shrinks more during supercontraction. Contrary to the other studies, proline does not correlate with breaking stress and Young's modulus in our study. Liu et al.[9] showed that spider silk proline content correlates with breaking stress and Young's modulus. However, the relationship was not linear and both of these correlations were strongest in the range of 0−6% proline, with the strength of the relationship declining after 7% proline. The proline contents of our samples were in the range of 5−13% (and mostly 10−13%). Therefore, it is possible that we did not see these correlations simply because the proline content of the silk was already high enough to put the material well beyond the amyloid-elastomer transition boundary hypothesized by Rauscher et al.[49] Most of these prior studies also investigate variation across different spider species.[9,29] However, these species vary not only in the relative expression of MaSp1 and MaSp2, but also in the amino acid sequences of each MaSp protein (e.g., *Argiope trifasciata* versus *Latrodectus hesperus*). We therefore conclude that the actual MaSp genes' sequences (the number of repetitive regions, the length of each unit, and the amino acid identity of the units) interact with their relative abundances in determining silk mechanical properties.

## CONCLUSIONS

Reliable analysis of the chemical composition of small quantities of spider silk is critical for investigating the structure−function relationships that make spider dragline silk such an incredibly tough material. Understanding these relationships is also a crucial step in the development of biomimetic applications for silks. We showed that the chemical composition of major ampullate silk is in general remarkably homogeneous within a single population of spiders across multiple spatial and temporal scales. However, chemical composition can also vary substantially across individual spiders or from day to day in ways that correlate with the material properties of spider silk. We showed that variation in chemical composition is not detectable within silk spun by a single spider on a single day. Variation in chemical composition of silk spun by a single spider across different days can be as much as the variation among individuals. However, most variation in silk composition in our investigation resulted from a small number of outlier spiders with a recent history of stress. Thus, we conclude that the chemical composition of spider dragline silk should be mostly homogeneous within a population of spiders experiencing similar, and generally benign, conditions, even across large spatial and temporal scales. We also showed that variation in the chemical composition of spider silk helps to explain mechanical performance. The strong correlation between major ampullate silk proline content and its shrink capacity after supercontraction, as well as its breaking strain, suggests that spiders can change the relative expression of different MaSp genes to produce silk fibers with different mechanical properties, thereby providing spiders with a mechanism to potentially tailor their silk to function in different environments.

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. M.M., T.C.L., and T.A.B. designed the experiment; M.M. performed the experiment and analyzed the data. M.M., T.C.L., and T.A.B. wrote the manuscript.

### Notes

The authors declare no competing financial interest.

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## REFERENCES


(1) Agnarsson, I.; Kuntner, M.; Blackledge, T. A. *PLoS One* **2010**, *5*, e11234.
(2) Vepari, C.; Kaplan, D. L. *Prog. Polym. Sci.* **2007**, *32* (8−9), 991−1007.
(3) Sensenig, A. T.; Kelly, S. P.; Lorentz, K. A.; Lesher, B.; Blackledge, T. A. *J. Exp. Biol.* **2013**, *216* (18), 3388−3394.
(4) Blackledge, T. A.; Pérez-Rigueiro, J.; Plaza, G. R.; Perea, B.; Navarro, A.; Guinea, G. V.; Elices, M. *Sci. Rep* **2012**, *2*, 782.
(5) Eisoldt, L.; Smith, A.; Scheibel, T. *Mater. Today* **2011**, *14* (3), 80−86.
(6) Lewis, R. V. *Acc. Chem. Res.* **1992**, *25* (9), 392−398.
(7) Ayoub, N. A.; Garb, J. E.; Tinghitella, R. M.; Collin, M. A.; Hayashi, C. Y. *PLoS One* **2007**, *2* (6), e514.
(8) Liu, Y.; Shao, Z. Z.; Vollrath, F. *Biomacromolecules* **2008**, *9* (7), 1782−1786.
(9) Liu, Y.; Sponner, A.; Porter, D.; Vollrath, F. *Biomacromolecules* **2008**, *9* (1), 116−121.
(10) Savage, K. N.; Gosline, J. M. *J. Exp. Biol.* **2008**, *211*, 1937−1947.
(11) Hayashi, C. Y.; Shipley, N. H.; Lewis, R. V. *Int. J. Biol. Macromol.* **1999**, *24* (2−3), 271−275.
(12) Blackledge, T. A. *J. Arachnol.* **2012**, *40* (1), 1−12.
(13) Foelix, R. F. *Biology of Spiders*, 3rd ed.; Oxford University Press: Oxford, 2011.
(14) Blamires, S. J.; Chao, I. C.; Tso, I. M. *J. Exp. Biol.* **2010**, *213* (22), 3906−3910.
(15) Tso, I. M.; Wu, H. C.; Hwang, I. R. *J. Exp. Biol.* **2005**, *208* (6), 1053−1061.
(16) Boutry, C.; Blackledge, T. A. *J. Exp. Biol.* **2010**, *213*, 3505−3514.
(17) Work, R. W. *Text. Res. J.* **1977**, *47* (10), 650−662.
(18) Savage, K. N.; Gosline, J. M. *J. Exp. Biol.* **2008**, *211*, 1948−1957.
(19) Blackledge, T. A.; Boutry, C.; Wong, S. C.; Baji, A.; Dhinojwala, A.; Sahni, V.; Agnarsson, I. *J. Exp. Biol.* **2009**, *212*, 1981−1989.
(20) Elices, M.; Plaza, G. R.; Pérez-Rigueiro, J.; Guinea, G. V. *J. Mech. Behav. Biomed. Mater.* **2011**, *4* (5), 658−669.
(21) Work, R. W. *J. Arachnol.* **1981**, *9* (3), 299−308.
(22) Blamires, S. J.; Wu, C. L.; Tso, I. M. *PLoS One* **2012**, *7* (2), e31626.
(23) Blamires, S. J.; Wu, C. L.; Blackledge, T. A.; Tso, I. M. *Biol. J. Linn. Soc.* **2012**, *106*, 580−588.
(24) Tso, I. M.; Jiang, S. Y.; Blackledge, T. A. *Ethology* **2007**, *113*, 324−333.
(25) Vollrath, F.; Madsen, B.; Shao, Z. Z. *Proc. R. Soc. Lond. B* **2001**, *268* (1483), 2339−2346.
(26) Madsen, B.; Shao, Z. Z.; Vollrath, F. *Int. J. Biol. Macromol.* **1999**, *24* (2−3), 301−306.
(27) Garb, J. E.; Ayoub, N. A.; Hayashi, C. Y. *BMC Evol. Biol.* **2010**, *10*, 243.
(28) Gatesy, J.; Hayashi, C.; Motriuk, D.; Woods, J.; Lewis, R. *Science* **2001**, *291* (5513), 2603−2605.
(29) Elices, M.; Plaza, G. R.; Arnedo, M. A.; Pérez-Rigueiro, J.; Torres, F. G.; Guinea, G. V. *Biomacromolecules* **2009**, *10* (7), 1904−1910.
(30) Davies, G. J. G.; Knight, D. P.; Vollrath, F. *Tissue Cell* **2013**, *45* (5), 306−311.
(31) Townley, M. A.; Tillinghast, E. K.; Cherim, N. A. *Philos. Trans. R. Soc. London B* **1993**, *340* (1291), 25−38.
(32) Harmer, A. M. T.; Blackledge, T. A.; Madin, J. S.; Herberstein, M. E. *J. R. Soc. Interface* **2011**, *8*, 457−471.
(33) Lombardi, S. J.; Kaplan, D. L. *J. Arachnol.* **1990**, *18* (3), 297−306.
(34) Work, R. W.; Young, C. T. *J. Arachnol.* **1987**, *15* (1), 65−80.
(35) Craig, C. L.; Riekel, C.; Herberstein, M. E.; Weber, R. S.; Kaplan, D.; Pierce, N. E. *Mol. Biol. Evol.* **2000**, *17* (12), 1904−1913.
(36) Blackledge, T. A.; Cardullo, R. A.; Hayashi, C. Y. *Invertebr. Biol.* **2005**, *124* (2), 165−173.
(37) Blackledge, T. A.; Hayashi, C. Y. *J. Exp. Biol.* **2006**, *209* (13), 2452−2461.
(38) Blackledge, T. A.; Swindeman, J. E.; Hayashi, C. Y. *J. Exp. Biol.* **2005**, *208* (10), 1937−1949.
(39) Guinea, G. V.; Pérez-Rigueiro, J.; Plaza, G. R.; Elices, M. *Biomacromolecules* **2006**, *7* (7), 2173−2177.
(40) Smith, B. J. *Protein Sequencing Protocols*, 2nd ed.; Human Press: Totowa, NJ, 2003.
(41) Casem, M. L.; Turner, D.; Houchin, K. *Int. J. Biol. Macromol.* **1999**, *24* (2−3), 103−108.
(42) Boutry, C.; Blackledge, T. A. *J. Exp. Zool.* **2008**, *309A*, 542−552.
(43) Garb, J. E.; DiMauro, T.; Vo, V.; Hayashi, C. Y. *Science* **2006**, *312* (5781), 1762−1762.
(44) Ayoub, N. A.; Hayashi, C. Y. *Mol. Biol. Evol.* **2008**, *25* (2), 277−286.
(45) Stein, W. H.; Moore, S. *Cold Spring Harb. Symp. Quant. Biol.* **1950**, 179−190.
(46) Anderson, S. O. *Comp. Biochem. Physiol* **1970**, *35*, 705−711.
(47) Xu, M.; Lewis, R. V. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87* (18), 7120−7124.
(48) Higgins, L. E.; Townley, M. A.; Tillinghast, E. K.; Rankin, M. A. *J. Arachnol.* **2001**, *29* (1), 82−94.
(49) Rauscher, S.; Baud, S.; Miao, M.; Keeley, F. W.; Pomes, R. *Structure* **2006**, *14* (11), 1667−1676.
(50) Hayashi, C. Y.; Lewis, R. V. *J. Mol. Biol.* **1998**, *275* (5), 773−784.