

Research



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Meta-analysis reveals materiomorphic relationships in major ampullate silk across the spider phylogeny

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Spider major ampullate (MA) silk, with its combination of strength and extensibility, outperforms any synthetic equivalents. There is thus much interest in understanding its underlying materiomorph. While the expression of the different silk proteins (spidroins) appears an integral component of silk performance, our understanding of the nature of the relationship between the spidroins, their constituent amino acids and MA silk mechanics is ambiguous. To provide clarity on these relationships across spider species, we performed a meta-analysis using phylogenetic comparative methods. These showed that glycine and proline, both of which are indicators of differential spidroin expression, had effects on MA silk mechanics across the phylogeny. We also found serine to correlate with silk mechanics, probably via its presence within the carboxyl and amino-terminal domains of the spidroins. From our analyses, we concluded that the spidroin expression shifts across the phylogeny from predominantly MaSp1 in the MA silks of ancestral spiders to predominantly MaSp2 in the more derived spiders' silks. This trend was accompanied by an enhanced ultimate strain and decreased Young's modulus in the silks. Our meta-analysis enabled us to decipher between real and apparent influences on MA silk properties, providing significant insights into spider silk and web coevolution and enhancing our capacity to create spider silk-like materials.

1. Introduction

Biomimetics, the study of natural phenomena to inspire the development of synthetic materials and processes, is an exciting frontier field of research spanning the biological, chemical and material sciences sub-disciplines [1]. Effectively merging these sub-disciplines within a single research programme is required to gain an understanding of the materiomorph (the interplay of factors from the underlying genetic expression through to macro-scale physical properties) of the subject of investigation [2]. Spider major ampullate (MA) silk, with its unique combination of extensibility and strength, is perhaps the world's toughest natural material [3]. It is accordingly an excellent candidate for materiomorph-inspired biomimetics [1,4].

MA silk is a protein-based nanocomposite spun from a liquid precursor [3,5]. It comprises a fibrillar core coated by a thin skin that is easily removed by chemical treatment. High-resolution imaging techniques such as atomic force microscopy (AFM) reveal that the 1–5 μm MA silk fibres are made up of smaller nano-scale fibrils oriented along the fibre's axis [6,7]. The fibrillar substructure comprises hard nanocrystalline regions, which are thought to impart the silk's strength, suspended within rubbery amorphous matrices that are thought to be responsible for its extensibility [8–11].

Two proteins are considered to be present in MA silk, Major Ampullate Spidroin 1 and 2 (MaSp1 and MaSp2), although there is some evidence of variants of these proteins among some spiders [12]. The proteins comprise

highly conserved amino (N-) and carboxyl (C-) terminal domains, between which are the repetitive regions that make up the bulk (approx. 95%) of the protein [13,14]. The majority of the repetitive region is made up of the short repetitive amino acid motifs GGX, (A)_n and (AG)_n within MaSp1 with an addition of a GPGXX and loss of (AG)_n within MaSp2 (where A = alanine, G = glycine, P = proline, and X may be either Q = glutamine or Y = tyrosine or L = leucine; [15]). The GGX and GPGXX motifs form helical and nano-spring structures within the amorphous region and are responsible for the silk's elasticity [15,16]. (A)_n and (AG)_n motifs form tightly packed β -pleated sheets that form nanocrystallites. A network of dense hydrogen bonds within the nanocrystallites influence silk strength [17,18].

From the abovementioned models of MA silk structure and function, it has been deduced that the amino acid composition of MA silk is, for any given spider species, a strong predictor of its likely mechanical performance [3]. These deductions have led researchers to make further inferences about the functional role of MA silk in spider webs and the influence of the expression of the two spidroins on the evolutionary trajectories of spiders and webs. It has been deduced, for instance, that MaSp1 appeared in the MA silks of the earliest web-building spiders, while MaSp2 appeared relatively recently in the silks of the more derived orb-weaving spiders [19,20]. The high extensibility bestowed by a high proline composition within MaSp2 thereupon has been considered integral to the capacity of two-dimensional orb webs to withstand the impacts of fast flying and/or large prey [19,21,22].

Studies examining variability in MA silk properties within single spider species across environments have, nevertheless, brought the nature of the relationship between spidroin expression and silk mechanics into question [23–25]. These have shown that factors such as the speed at which the fibre is spun, exposure of the spider to the wind, or the nutritional state of the spider, can affect MA silk mechanical properties irrespective of spidroin composition [26–28]. Moreover, MA silk properties can additionally vary across physical environments, as spider/silk age, diets and behaviours vary, and as a result of the incorporation of additional spidroins [3,28–32]. Accordingly, our understanding of whether the evolutionary emergence, or differential expression, of specific spidroins is integral to the mechanical performance of MA silk remains somewhat inconclusive.

Differentiating between real and apparent coevolutionary relationships between traits across species is notoriously difficult. Phylogenetic comparative methods can be a useful tool if sufficient sample sizes can be attained [33], otherwise they suffer from biases due to the underlying assumptions and the limitations of the statistical methodologies [34]. In the aforesaid case of spider MA silk, the logistical complexities required to attain large enough numbers of spiders, across a broad enough phylogeny, and perform the necessary spidroin expressional and mechanical property measurements (each requiring specialized machinery and technical expertise) is beyond most laboratories. Classical phylogenetic comparative methods are thus rendered impractical as a means for uncovering the nature of the relationship between spidroin expression and MA silk mechanics.

Meta-analyses of published datasets are another powerful way of deciphering between real and apparent effects and/or causal relationships [35,36]. Since the underlying models, datasets and computing power has increased exponentially of late, so too has the effectiveness of meta-analyses [35].

Accordingly, meta-analyses have increasingly been used to understand the nature of the relationships among covarying biological traits across species [35,36]. Hence, they present as an ideal tool for ascertaining the effects of spidroin expression on MA silk mechanical properties.

We thus herein scanned the literature reporting spider MA silk spidroin expressions (most commonly as amino acid compositions) and mechanical properties and built a database. We thereupon used a meta-analysis that incorporated phylogenetic comparative methods to ascertain whether the emergence of MaSp2 was integral to the development of MA silk's mechanical properties.

2. Methods

2.1. Data collection

We searched the Web of Knowledge and Scopus databases for articles using the search terms: (i) spider silk, (ii) amino acid, (iii) protein, (iv) composition, (v) mechanical and (vi) properties and compiled an across species database. To further refine the search, 'NOT' terms were used to exclude publications deemed irrelevant. The full list of articles used can be found in the electronic supplementary material. All articles were searched and screened using the platform PRISMA [37].

We obtained the full text of any articles that reported the tensile mechanical properties and/or the amino acid composition of spider MA silk. Inclusion of an article into the database was limited to primary publications that included tabulated data on at least three tensile properties, and/or four amino acids, or any combination including both amino acid and tensile properties. The tensile properties that we searched for and recorded were Young's modulus (or modulus), ultimate strength (or stress), ultimate strain (or strain, or extensibility) and toughness. We also searched and recorded the silk amino acid profile of each spider species. However, the final analysis was limited to the amino acids alanine, glutamine, proline, glycine and serine, due to limited data available on other amino acids since they were commonly found at low percentages. We made sure that all of the articles included documented the properties of silks that had been forcibly collected in the laboratory under controlled conditions. This was to ensure that the properties reported were not affected by the spider's habitat, diet, behaviour or the incorporation of other spidroins.

2.2. Phylogenetic analysis

To control for the possible non-independence of sampling as a consequence of the likely phylogenetic relatedness among the species covered [38], we performed a phylogenetic comparative analysis using an up-to-date spider molecular phylogeny [39]. From this phylogeny, a fresh tree was constructed for the spiders covered by the database using the ape package in R, with the branch lengths computed using the Grafen method with a power of 1, with the compute.brln function applied within ape [40]. Once constructed, any missing data points (approx. 33.5% of the data) were imputed using an ancestral state reconstruction method in the rPhylopars package within R, which takes into account phylogenetic relatedness, to append missing values [41].

To take account for any error associated with imputed values, we used the variance of the interpolated data points to simulate the dataset 1000 times to form an array [42]. The statistical analysis was then run and pooled from this array to ensure that the regressions were robust to the interpolated data points. The amino acid percentage of alanine, glycine, proline, glutamine and serine were each correlated with four material properties: Young's modulus, ultimate strength, ultimate strain and toughness,

using a weighted phylogenetic generalized least-squares regression. The assigned weights corresponded to the number of individual data points for each species. This process is mathematically analogous to a meta-analysis where the data points are weighted according to their relative inverse variance [43].

3. Results

Our literature search uncovered 1672 publications, 514 of which were duplicates between the two search engines. Of the 1158 studies remaining, 1114 were excluded due to lack of relevance, available data, or methods involving additional treatments or alterations to the silk (see electronic supplementary material). After an additional 22 papers were included from a back search, data were retrieved on 85 species from 66 studies for inclusion in the ensuing analysis. The 85 species represented 44 genera across 21 spider families. *Nephila* and *Argiope* were the most represented genera with 129 mentions, making up approximately 41% of the data. The full list of references used in this study is in the electronic supplementary material.

We found that glycine composition (electronic supplementary material, table S1) had the greatest effect on MA silk mechanical properties and was positively correlated with Young's modulus and ultimate strength, and negatively correlated with the ultimate strain (figure 1 and electronic supplementary material, table S1). Proline composition was substantially greater among the more derived spiders than the ancestral spiders (figure 1), which we interpreted as indicative of the emergence of MaSp2 in these spiders. We found that proline composition was negatively correlated with Young's modulus and positively correlated with ultimate strain. Accordingly, we could confidently conclude that MaSp2 expression does have an influence on the mechanical properties of the MA silks of different spiders. Unexpectedly, serine composition was negatively correlated with silk toughness (figure 2 and electronic supplementary material, table S1). Moreover, alanine and glutamine were not significantly correlated with any of the mechanical properties included in our analyses across the phylogeny.

4. Discussion

While it has been a subject of intensive research for decades, conflicting information across studies that differed in focus (e.g. between versus within species comparisons) have meant we have not had, until now, a clear picture whether or not the expression of certain spidroins is integral to enhancing the mechanical performance of spider MA silk. We performed a meta-analysis using phylogenetic comparative methods and found that the expression of MaSp2 does appear to be integral to the development of MA silk's exceptional mechanical properties. The specific effects our analyses uncovered and their implications are detailed below.

We found that the Young's modulus, or the silk's ability to resist permanent deformation, was positively associated with glycine composition but negatively associated with proline composition. Since a greater proline composition, concomitant with a reduced glycine composition, can be ascribed to an upregulation of MaSp2 expression at the expense of MaSp1 expression [3], we are confident in ascribing this effect

as a consequence of MaSp2 expression among derived orb-weaving spiders.

The reason that Young's modulus decreases with an increasing proline composition is because proline is a branched amino acid that can form zwitterions (i.e. molecular species that contain both a positively and negatively charged group) [21]. Accordingly, it readily bonds across and within the protein chain and alters protein structure, transforming beta-sheets into beta-coils, spirals and turns. Moreover, it reduces protein alignment in MA silk when present in higher abundance [21]. Additionally, proline may become hydroxylated post-translation and form hydroxyproline within MA silk [41]. Indeed, hydroxyproline is thought to provide flagelliform silk with its extreme extensibility [44]. We therefore surmised that it is serving a similar function in increasing extensibility in MA silk. The changes in silk structure and alignment as a result of the presence of proline or hydroxyproline within MaSp2 induce slip-stick stretching within the protein chains that may become manifested as a decrease in the fibre's modulus when under tension [45].

Young's modulus and glycine composition were both greater among basal spider species, whose MA silk is traditionally thought to comprise predominantly MaSp1. MaSp1 is high in glycine because it contains multiple GGX and GA motifs [20]. The GGX motif within the amorphous region of MaSp1 is associated with 3_{10} -helical and turn structures containing few inter-molecular hydrogen bonding sites resulting in enhanced fibre stiffness [16].

The ultimate strain is a measure of the amount of deformation the silk can undergo as a proportion of its original size before breaking. The ultimate strain was influenced by both proline and glycine compositions. Again, these results suggest a role for MaSp2 in facilitating high strains within certain MA silks. Indeed, the GPGXX motif within MaSp2 induces silk proteins to form type 1 and type 2 β -turns within the amorphous region of MA silk, which are thought to enhance ultimate strain while increasing fibre stability [23,46,47].

Ultimate stress is the maximum capacity of the silk fibre to resist tension while being stretched. We found it to be positively correlated with MA silk glycine compositions across our spider phylogeny. This result is intriguing since alanine is the major component of nanocrystalites, which are thought to be the major contributor to strength in MA silk fibres [3,23]. Our finding, nevertheless, may be explained as being a consequence of a high prevalence of the $(GA)_n$ motif inducing the formation of β -sheets within the crystalline region resulting in stiffer silks [23,45]. MaSp1 predominant silks are generally stiffer, which was explained as a consequence of the $(GA)_n$ motif forming close-fitting hydrogen bonds around the nanocrystalites [48].

We found that toughness, a measure of a silk fibre's ability to absorb energy during deformation without fracturing, was negatively correlated with serine composition. This surprised us because serine is not thought to form any major structural motifs within the repetitive regions. It is nonetheless commonly found throughout MaSp2, which may explain its partial negative association with Young's modulus [17]. Curiously, serine did not correlate with ultimate strain, which we would expect if serine had influenced the structural alignments within the amorphous region in a similar way to proline. Serine is abundant within the C- and N-terminal domains of both MaSp1 and MaSp2. These domains have a regulatory function over dimerization and folding in both

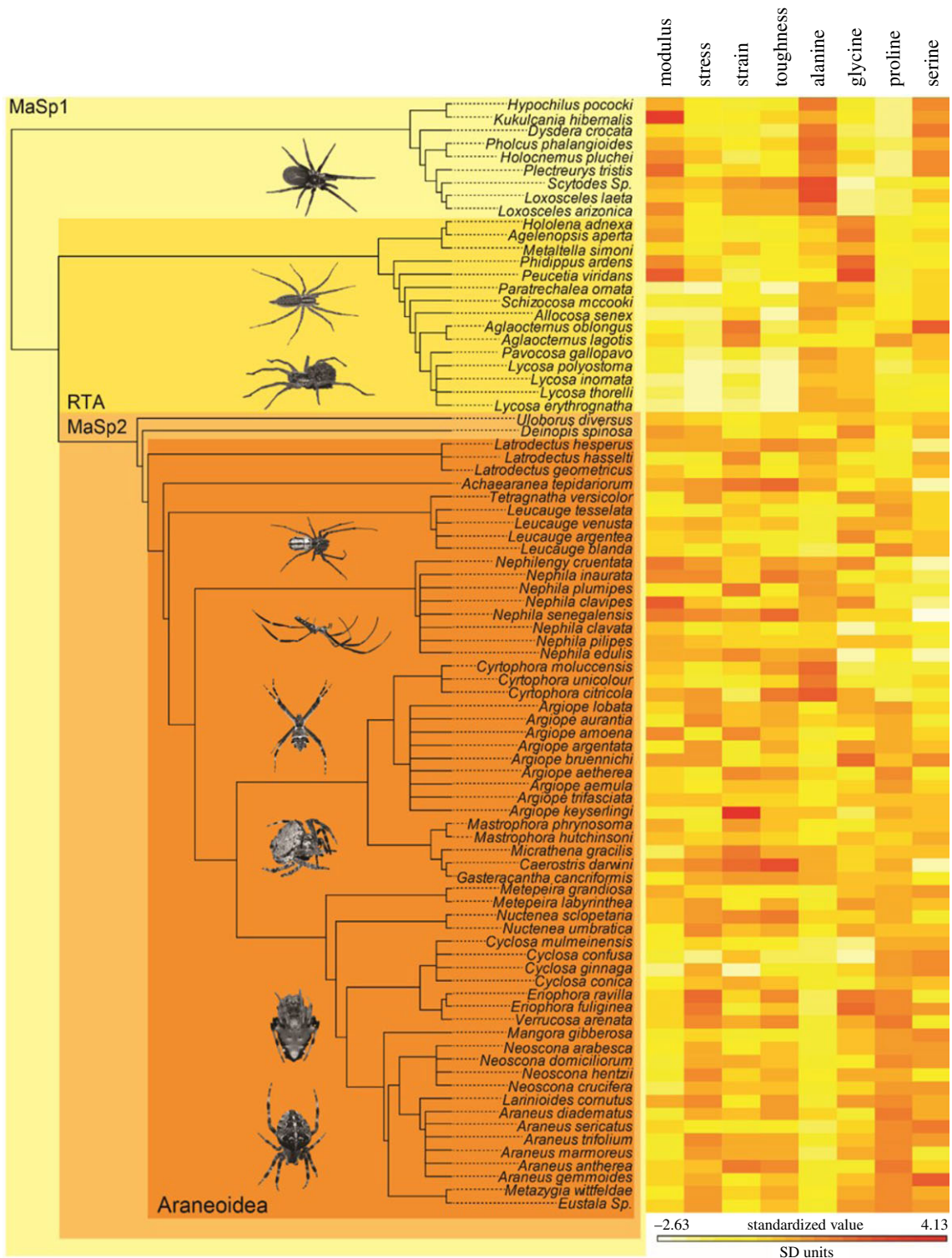


Figure 1. Phylogenetic tree and standardized trait values generated from the literature search showing each species within this study resolved to genus. The tree depicts the major clades involved within this study and development/potential utilization of the two MaSp proteins. MaSps are displayed at the top left and major clades on the bottom left of the highlighted areas. The colour of the box corresponds to the relative value of each trait lower values in white and higher in red.

spidroins during spinning [17,49]. The sequence and size of the terminal domains is highly conserved so an increase in serine composition may be a consequence of the silk containing shorter repetitive regions, i.e. the higher serine results from the C- and N-terminal domains making up a proportionally larger component of the constituent proteins. Given the length of the repetitive region is known to affect MA silk mechanics via its influence on protein folding, any changes in the proportional component of the C- and N-terminal domains might be manifested as changes in both the composition of serine and aspects of silk mechanical performance [14,23].

A close examination of the MA silk amino acid compositions across our phylogeny (figure 1) reveals a truncated shift in proline composition across the ancestral to modern spiders from quite low (1–4%) to high (10–14%). Other studies reporting MA silk amino acid compositions in different spider species have found similar results, albeit across smaller phylogenies [21,25,29,30]. Those studies have generally attributed the truncated shift in proline composition to differential expression of MaSp2, driven largely by ecological factors such as building webs of different type or spider diet. Our meta-analysis supports these findings across a broader

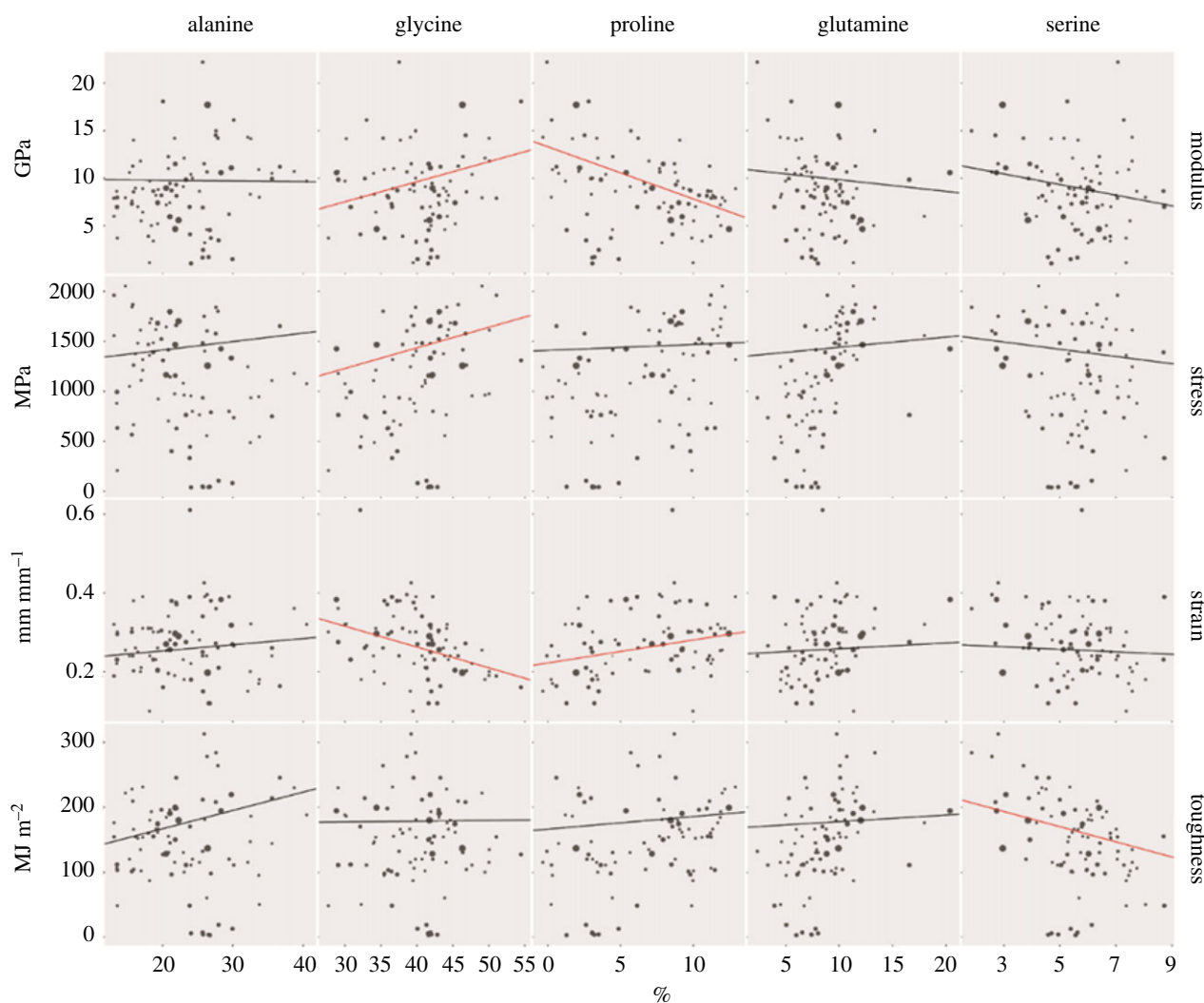


Figure 2. Pooled phylogenetic generalized least-squares regressions of each major amino acid (alanine, glycine, proline, glutamine and serine) against each material property; where modulus is Young's modulus, stress is ultimate stress and strain is ultimate strain. Significant correlations are displayed with red regression lines and data points are scaled and weighted in size by number of individuals.

spider phylogeny, suggesting that an ecologically driven advent of MaSp2 expression might be integral to the emergence and functionality of different spider web forms [19–22].

5. Conclusion

We performed a meta-analysis that used phylogenetic comparative methods to shed light on the influence of spidroins, and the amino acids they are comprised, on the properties of spider MA silk. We found that spidroin expression does indeed appear to be integral to the development of MA silk's mechanical properties. Some of the amino acids that we found to be integral to promoting certain mechanical properties, e.g. proline's influence on Young's modulus and ultimate strain, we had predicted at the outset, whereas others, e.g. serine's influence on toughness, we had not.

We nevertheless consider our study to have achieved more than consolidating previous findings and/or unearthing some

unexpected ones. By performing a multi-species comparative meta-analysis, we were able to decipher among real and apparent influences on MA silk properties. Such insights have potential to improve our capacity to test hypotheses about spider silk and web evolutionary radiations [50] and the effects of other covarying factors, such as changes in the spinning apparatus across spider phylogenies [51]. They also provide valuable information about the silk materiomie, which better informs the development of synthetic proteins and other, e.g. recombinant, forms of spider silk biomimetics.

Data accessibility. Data are accessible from Dryad.

Authors' contributions. H.C.C., D.P., S.K., S.J.B. and M.M.K designed the study. H.C.C. and D.P. compiled the database; H.C.C. and S.K. carried out data analysis, H.C.C. drafted the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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