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COMMENTARY

Connecting materials, performance and evolution: a case study of the glue of moth-catching spiders (Cyrtarachninae)

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ABSTRACT

Morphological structures and extended phenotypes are made possible by materials that are encoded by the genome. Nearly all biomaterials are viscoelastic, which means that to understand performance, one must understand the strain rate-dependent properties of these materials in relevant ecological interactions, as the behavior of a material can vary dramatically and rapidly. Spider silks are an example of materials whose properties vary substantially intra- and inter-specifically. Here, we focus on aggregate silk, which functions as a biological adhesive. As a case study to understand how a material manifests from genome through organism to ecology, we highlight moth-specialist spiders, the Cyrtarachninae, and their glues as an ideal experimental system to investigate the relationship between genomics and ecologically variable performance of a biological material. There is a clear eco-evolutionary innovation that *Cyrtarachne akirai* and related species have evolved, a unique trait not found in other spiders, a glue which overcomes the scales of moths. By examining traditional orb-weavers, *C. akirai* and other subfamily members using biomechanical testing and genomic analysis, we argue that we can track the evolution of this novel bioadhesive and comment on the selection pressures influencing prey specialization. The importance of the ecological context of materials testing is exemplified by the poor performance of *C. akirai* glue on glass and the exceptional spreading ability and adhesive strength on moths. The genetic basis for these performance properties is experimentally tractable because spider silk genes are minimally pleiotropic and advances in genomic technologies now make possible the discovery of complete silk gene sequences.

KEY WORDS: Adhesion, Aggregate silk, Arachnology, Biomechanics, Long-read sequencing

Introduction

For the ecological physiologist or comparative biomechanist, the study of function often begins with the parsing of an organism's integrated phenotype into separate structures. This reductionist approach facilitates analysis, and often uses morphometric geometry to quantify, characterize and differentiate size and shape (Bookstein, 1997). What analytic geometry misses, by design, are the non-geometrical features of structure: material properties. Material properties add to our understanding of function by characterizing how forces applied to and by the structure, via Newton's third law, cause the part to change shape dynamically. Flowing, sticking, stretching, shortening, twisting, bending or compound reconfigurations require mechanical work that the structure may store and release as elastic energy, dissipate as heat or both. This dynamic mechanical behavior, viscoelasticity, depends on material properties – such as storage and loss moduli or stiffness and toughness – that characterize size- and shape-free contributions to material behavior. Adding to their functional importance, material properties may change automatically as an organism moves, shifting the function of various parts from brake to spring, or vice versa, depending on the exact pattern and magnitude of the forces and the structural reconfigurations that they generate. Finally, material properties are derived from the genome, which expresses the proteins and the biochemical machinery that assembles proteins and other chemicals into intracellular, cellular, multi-cellular and extracellular structures. Thus, if one seeks a broad understanding of the causal mechanisms involved in functions governing an organism's behavior – and hence performance and evolution – then it is imperative to study the material properties of the structures of that organism at multiple levels.

In this Commentary, we argue that materials play a causal role in the performance of individuals and the evolution of their populations (Fig. 1), an extension of the morphology–performance–fitness (MPF) framework (Arnold, 1983) that has been supported by experiments such as evolving digital robots (Corucci et al., 2018) and physical biorobots (Long et al., 2006). That materials and their properties play a causal role in MPF may seem self-evident, yet one can find many recent publications that measure morphology as structural geometry devoid of material properties, correlate it with performance, and make evolutionary arguments based on material-free shape alone. Rather than critiquing those works directly, our goal is to illuminate what may be lost by showing what additional explanatory power is gained when materials and their properties are an explicit part of the MPF approach. Our discussion has two parts: (1) the conditional performance of materials in general, and how that constrains any explanation of mechanical function or evolution; and (2) a special case of the important role of materials in understanding differences in behavior and evolution: the glues that allow a subfamily of spiders to do something other web weavers cannot; namely, catch moths.

Conditional performance of structural materials

Material properties are rarely fixed and are usually in flux, even in the laboratory. For example, the dynamic storage modulus, E' – a property that measures a material's ability to store and return elastic energy – may change, often in non-linear ways, depending on how quickly (rate) and to what degree (magnitude) the structure is reconfigured by an external force in a testing apparatus (Lakes, 1998). Just as important, E' of the material may change as we change other environmental factors: temperature and humidity being two with large effects. For biological materials, the resulting variability in E' makes it impossible to have a single value that represents its conditional performance (Porter et al., 2016). Thus, as we consider the material's genomic, organismal and ecological

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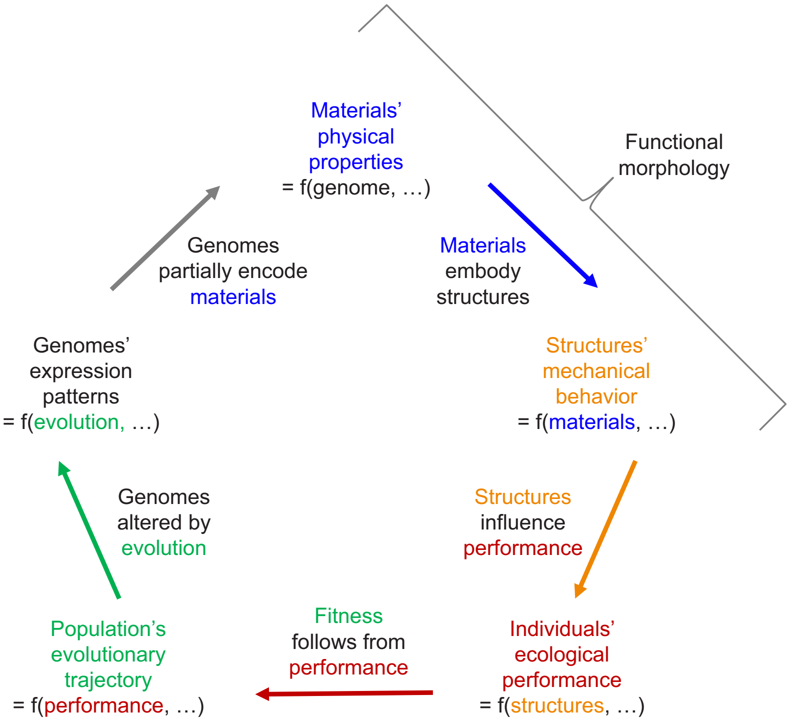

Fig. 1. Materials play a causal role in the performance of individuals and the evolution of populations. When considered as a phenotype in and of themselves, materials are more closely tied to the genome than are the structures that they embody. The materials determine, in part, structural behavior, measured as the intrinsic mechanical behavior of a cell, tissue or organ, under physiological strain and force dynamics. The structural behaviors that support the integrated operation of the whole organism influence the performance of the organism in the ecological context that determines its lifetime evolutionary fitness. Relative fitness determines, in part, the composition of the genomes of individuals in the next generation. This causal loop, inspired by Arnold's (1983) morphology–performance–fitness (MPF) framework for studying adaptation, makes explicit the role of materials in the complete set of an organism's morphology (measurable aspects of 'structure, physiology, or behavior', Arnold, 1983); in this framework, a material, such as the silk of spiders, can be a direct target of selection and/or an indirect target through the structures that it constitutes. The notation f() is a shorthand for 'function of' as in *y*=f(*x*) or '*y* is a function of *x*'.

context – its chemical constituents created directly and indirectly by the genome, its structural contribution to the organism, and its place in the larger environmental setting – the complexity of the problem increases. A conceptual and methodological challenge for biologists is this: how do we close the explanatory gap between genomics and morphology, performance, and fitness?

Our model is inspired by Arnold's (1983) MPF model, expanding it as a method of biomechanical–evolutionary hypothesis testing, where each step informs the test conditions and hypotheses of the one after it. Mechanical behavior of a structure – combining material and structural features – determines function in the immediate ecological and behavioral context. It is here that our process begins. Materials are tested under the simplest laboratory conditions, determining overall material properties in a context that is directly relatable to previous studies. Secondarily, the material properties are characterized and put into the context of behavior of both the material and the organisms it is coming into contact with, which we term ecological relevance. While we stress the need for ecologically relevant tests, we are not ignorant to the wealth of knowledge already in existence. It is important that tests continue to be comparable with past scientific studies. Our claim is that limiting these studies to only simple laboratory conditions leads to an evolutionary fallacy of material evolution as adaptation towards ecologically naive optima instead of materials adapting to solve particular problems in an ecologically dynamic world.

Even the interactions of seemingly generalist organisms, such as orb-weaving spiders, and their prey will happen under vastly different ecological conditions, and thus the exact nature of that relationship and pressures exerted on that material will be different. For example, the interaction between diurnal spiders and lepidopteran prey will be under different temperature and humidity conditions than the interaction between nocturnal species of the same order. The relationship between ecological conditions and foraging strategies has been shown to lead to differences in salt concentrations and material properties of the aggregate glue droplets in spider webs (Higgins et al., 2001; Amarpuri et al., 2015). A key note is that when analyzing these materials, proper interspecies comparison of their true properties cannot be considered outside of ecologically relevant scenarios; comparison of these properties otherwise can lead to misleading understanding of their evolution and their genomic basis. Evolution of novel and improved materials occurs under new and changing conditions and thus recently evolved traits are not innately superior to more ancestral ones. One such example is the variable adhesive properties of capture silk spun by spiders to retain prey. A recent study showed that the fluffy capture threads produced by cribellate orb-weavers, once believed to rely entirely on Van der Waals forces and be adhesively inferior to more recently evolved liquid adhesives, actually have a vital interaction with prey cuticle (Bott et al., 2017). These seemingly simple threads help to draw in the waxy cuticle of the insect, fusing with them, leading to drastically increased strength: a behavior which could not be seen on traditional testing substrates such as glass. The inability to observe such intricate interactions is also why some biomaterials are largely ignored, ultimately leading to gaps in our understanding and missed opportunities for biomimicry.

Materials often have a very clear connection to the genome; the genetic basis of materials and their functions generally exhibit less pleiotropy and complexity than other genotype–phenotype relationships. But even in the simplest systems, complications in the genome-to-protein and material-to-performance relationships present difficulties when attempting to draw correlations between genome and material properties. One complicating factor is that many proteins undergo post-translational modifications which can substantially alter the protein structure relative to the initial gene sequence (Mann and Jensen, 2003; Ramazi and Zahiri, 2021). For instance, adhesive properties in spider glue are thought to be impacted by post-transcriptional modification of glycoproteins and a recent study in cobweb weavers demonstrated that glue proteins exhibit greater glycosylation and phosphorylation than proteins in silk fibers (Ayoub et al., 2021). Thus, there may be aspects of expressed proteins not captured by the genome-predicted sequence and in such systems it would be informative to conduct quantitative proteomic studies to identify post-translational modifications associated with the evolution of novel protein function (Ayoub et al., 2021). Additionally, advances in techniques such as cryo-electron microscopy (cryo-EM) can be used to generate high-resolution images of protein structures (Cressey and Callaway, 2017; Shen, 2018).

Biomaterials, proteinaceous ones in particular, are more closely tied to the genome than are the structures that they embody. For example, point mutations alter the primary structure of collagen, a change that alters its microstructural fiber architecture and impacts the mechanical properties at the tissue level of the bony structure it partially creates (for review, see Cranford et al., 2013). This understanding of the tight mapping of genome to material has been exploited by genetic fusion to create a recombinant protein from spider and mussel genomes that self-assembles into an adhesive fiber; those fibers, in turn, have mechanical properties that are explained by the contributions of the different genetic elements expressing domains that work together in the supramolecular protein structure to enhance adhesion energy per protein molecule (Aich et al., 2018). Thus, mechanical properties at the molecular level determine structural behavior measured as the intrinsic behavior of an organ, tissue or silk under dynamic strain and force. The structural behaviors that support the organism influence the performance of the organism in the ecological context that determines its lifetime fecundity and evolutionary fitness. Relative fitness determines, in part, the composition of the genomes of individuals in the next generation (Fig. 1). This causal loop, inspired by Arnold's (1983) MPF framework for studying adaptation, makes explicit the role of materials in an organism's morphology; in this framework, a material can be a direct target of selection and/or an indirect target through the structures that it constitutes. As an example of this process, we outline our present research on the aggregate glue of a moth-specialist subfamily of spiders, Cyrtarachninae: their glue has been shown to be unimpressive on glass but has exceptionally strong silk on prey substrates (Diaz et al., 2018b).

Case study: ecological relevance of moth-catching glue

Spider silks are an example of a class of materials whose properties vary substantially within a single individual and across species. We highlight one type of silk, aggregate glue, which functions as a biological adhesive (Foelix, 2011; Townley and Tillinghast, 2013). As a case study to understand how a performance-enhancing material functions from genome through organism to ecology, we provide our framework for the work we are currently conducting on the conditional performance and evolution of the aggregate glue produced by the moth-specialist spider *Cyrtarachne akirai* (Tanikawa et al., 2014; Araneidae). In this system, there is a clearly defined eco-evolutionary innovation: *C. akirai* and related species have evolved a trait that is not found in any other spiders; namely, a glue that can overcome the scales of moths.

Though moths are plentiful within environments, they are more difficult to catch than other flying insects because of their sacrificial layer of scales (Foelix, 2011; Stowe, 1986; Yeargan, 1988). These amazing structures provide the moth with many evolutionary advantages including excretion of pheromones, camouflage and the ability to escape spider webs. The connection between these scales and the moth's body is relatively weak and when they come into contact with a web, the scales stick to the glue on the capture threads and flake off; this system is akin to dirt contamination on a wall when attempting to put up a poster. The combination of this weakened adhesion force and moth thrashing generally leads to escape (Stowe, 1986; Diaz et al., 2020).

There are two clades of spiders, both in the Araneidae, that have evolved techniques for specializing on moth prey (Stowe, 1978, 1986; Harmer and Herberstein, 2010). The first clade encompasses the ladder web builders, *Scoloderus*. These spiders build an elongated orb-web, that allows the moth to slowly tumble down it. As the prey tumbles down the web, the moth is essentially cleaned of its scales, leading to its eventual adhesion to the web somewhere below its initial contact point. While these webs are elongated, their other silk properties – namely, investment in capture threads and aggregate glue – appear to be the same as those of other orb-weavers (Stowe, 1978, 1986; Harmer and Herberstein, 2010). The second clade, subfamily Cyrtarachninae, have altered their web structure, creating horizontal webs that utilize significantly larger glue droplets than other spider species (Fig. 2). This group consists of the horizontal web builders, *Cyrtarachne*, triangle web builders, *Pasilobus*, and bolas spiders, *Mastophora*. These webs are also known as minimized orb webs as they utilize fewer capture threads but more aggregate glue per thread than traditional orb-webs. Here, we describe the properties of the silk of one species, *C. akirai.*

Initially, it was suggested that *C. akirai* capture threads would have higher tensile strength relative to those of other species, as they rely on fewer threads interacting with prey to retain them, often catching them on a single strand. This hypothesis was found to be false and thus the next step was to characterize the aggregate glue, which we expected to be exceptionally strong to compensate for these average capture threads. However, this supposition was also not supported, as *C. akirai* glue was found to be proportionally weaker, when normalized for glue volume, on glass. The measured adhesive strength and tensile strength would imply that the web of *C. akirai* should be incapable of retaining moth prey (Cartan and Miyashita, 2000; Diaz et al., 2018a, 2020).

To address this dilemma, we coupled adhesion tests with spreading videos, looking to characterize the impedance of aggregate glue by the microstructure of the scales, and correlate the ability of glue to spread with adhesion strength: two properties that are often at odds with one another. We also conducted ecologically relevant biomechanics tests. When non-moth-specialist orb-weaver capture threads were brought into contact with moth substrates, the adhesion strength dropped relative to glass, measuring nearly zero (Diaz et al., 2018b, 2020). This decrease was independent of glue volume or size: the adhesive toughness was the same, limited by the number of prey scales contacted (Diaz et al., 2018b, 2020). However, when *C. akirai* strands were tested on moth cuticles, a dynamic and drastic behavior was instead observed (Fig. 3). On glass, *C. akirai* strands behaved like others, spreading to 2 times their initial diameter, but when brought into contact with moth scales, *C. akirai* glue spread exceptionally far, between 6 and 10 times its initial radius (Diaz et al., 2020). This led to substantially increased adhesion strength, now proportionally stronger than for other traditional orb-weaver species (Diaz et al., 2018b, 2020). This 'hyper-spreading' occurs beneath the scales and serves to increase the surface area of glue contact (Diaz et al., 2020). This allows the glue to attach the scales to the underlying prey cuticle and also causes adjacent scales to be glued to one another, creating a larger 'metascale' which needs to be peeled as a unit, increasing overall adhesion strength (Diaz et al., 2020).

Because moth scales are often between 40 and 100 µm in diameter, they appear as flat dust, but with magnification it can be seen that the surface of the scales is highly variable (Fig. 4). Scales vary in both size and shape, and their layout appears to be randomly distributed across the insect's body. Individual scales have ridges across them, creating a microstructure, as well as a complicated macrostructure as

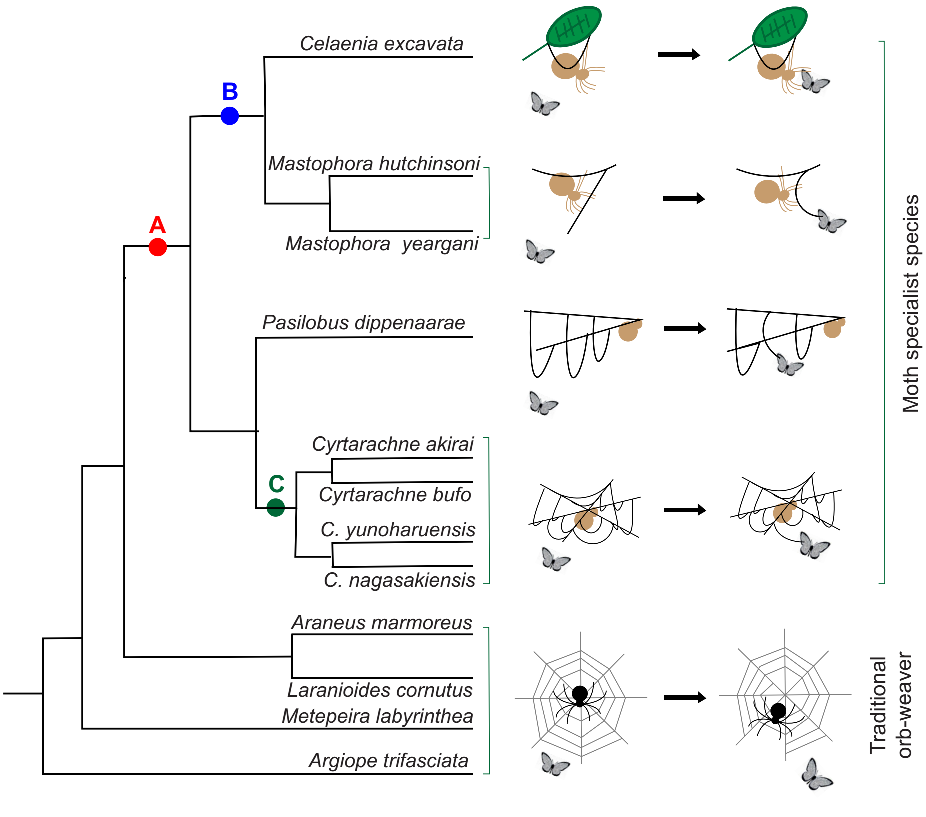

Fig. 2. Evolutionary origin and diversification of moth-catching spiders. The subfamily Cyrtarachninae (A) contains moth-specialist species which have altered the classic orb-web structure. This group contains many variations on the orb-web, including the horizontal dangling webs produced by our study species Cyrtarachne (C) and the genus *Pasilobus* that creates similar but triangle-shaped webs. The most extreme of these web minimizers include *Mastophora*, bolas spiders (B), which utilize pheromones to lure in male moths, then strike their prey with a single sticky glue droplet. All of these species utilize substantially larger glue droplets than traditional webs and hunt in high humidity conditions.

scales are not merely arranged next to one another but overlap and create a vast system of channels throughout the surface of the moth wing. Based on SEM and confocal microscopy, 3D models of the scale layout and of individual scales have been constructed to test how these features vary between moth species and impact fluid flow (Fig. 4). The spreading of *C. akirai* glue through the moth scale matrix seems to draw water away from the center of the glue droplet, separating the water from glycoproteins, causing a relative drying event, crosslinking the glycoproteins and hardening the glue. This contrasts with traditional orb-weaving spiders whose glue droplets exhibit homogeneity in their distribution of salts and proteins. We hypothesize that this complicated moth scale topography creates capillary action, interacting with the glue and pulling it further than possible on glass.

Capillary action is when a liquid's adhesive attraction to a substrate is higher than its internal cohesive forces. This attraction leads to spreading on the substrate, drawing the liquid bulk mass forward through inertia, a behavior similar to the formation of a meniscus. The droplet fluctuates between these two forces, pulling the liquid forward until it spreads out thin enough to reach equilibrium. The complex topography of the moth scales leads to a plethora of conjoining capillary tubes that allow the glue droplets to flow forward at a faster rate than on glass, and side channels which allow them to change direction as they come into contact with adjacent scales (Diaz et al., 2020; Figs 3 and 4). This behavior is limited by the material properties of the glue and hints at why *Cyrtarachne* only makes webs in high humidity, maximizing spreading and thus adhesive strength. In this way, the adaptations of *C. akirai* are both behavioral, limiting web behavior, and chemical, lowering glue viscosity, such that the glue is able to separate while spreading. This leads us to conclude that the behavior of this system is an interaction among the *C. akirai* glue, the topography of the moth scales and humidity, leading to a micro-drying event, hardening and strengthening the aggregate glue; ultimately, a behavior easily missed in ideal laboratory conditions. Glue of species with similar viscosity and material properties, such as the model organism *Larinioides cornutus*, could be made to spread similar to *C. akirai* glue at extremely high humidity (>90% relative humidity), but for these species it did not lead to an increase in adhesive strength: instead, failure now occurred as a result of weak cohesive forces (Diaz et al., 2020). This observation leads us to believe that the glue of *C. akirai* is of a unique composition compared with that of others, instead of resulting from a different level of gene expression, as it is able to harden in a way not shown by traditional orb-weavers.

Glue properties are dependent on spidroins produced in aggregate silk glands

Although we have not been able to test the properties of all Cyrtarachninae species from ecological observations of web-building behavior, the secret to catching moths seems to be twofold: (1) use a lot of glue and (2) spread and harden the glue quickly. The first adaptation can merely be a behavioral change, similar to the altered structure of the minimized webs, but *C. akirai*'s increased adhesion strength on moth scales shows it is not losing cohesive ability as do other glues. Thus, this second adaptation seems to be largely molecular in origin as changes in the genetic and protein structure would be necessary to cause the phase change and permanent loss of water seen in these species over time. Glue properties are dependent, in part, on silk genes produced in aggregate silk glands. Primary among these are the spidroins, the central protein constituents of silk fibers. Orb-web weaving spiders utilize a diversity of silks produced specifically in seven different types of specialized glands (Blackledge and Hayashi, 2006; Foelix, 2011). Each gland largely expresses spidroins of one type that serve a specific function (e.g. dragline silk from the major ampullate gland).

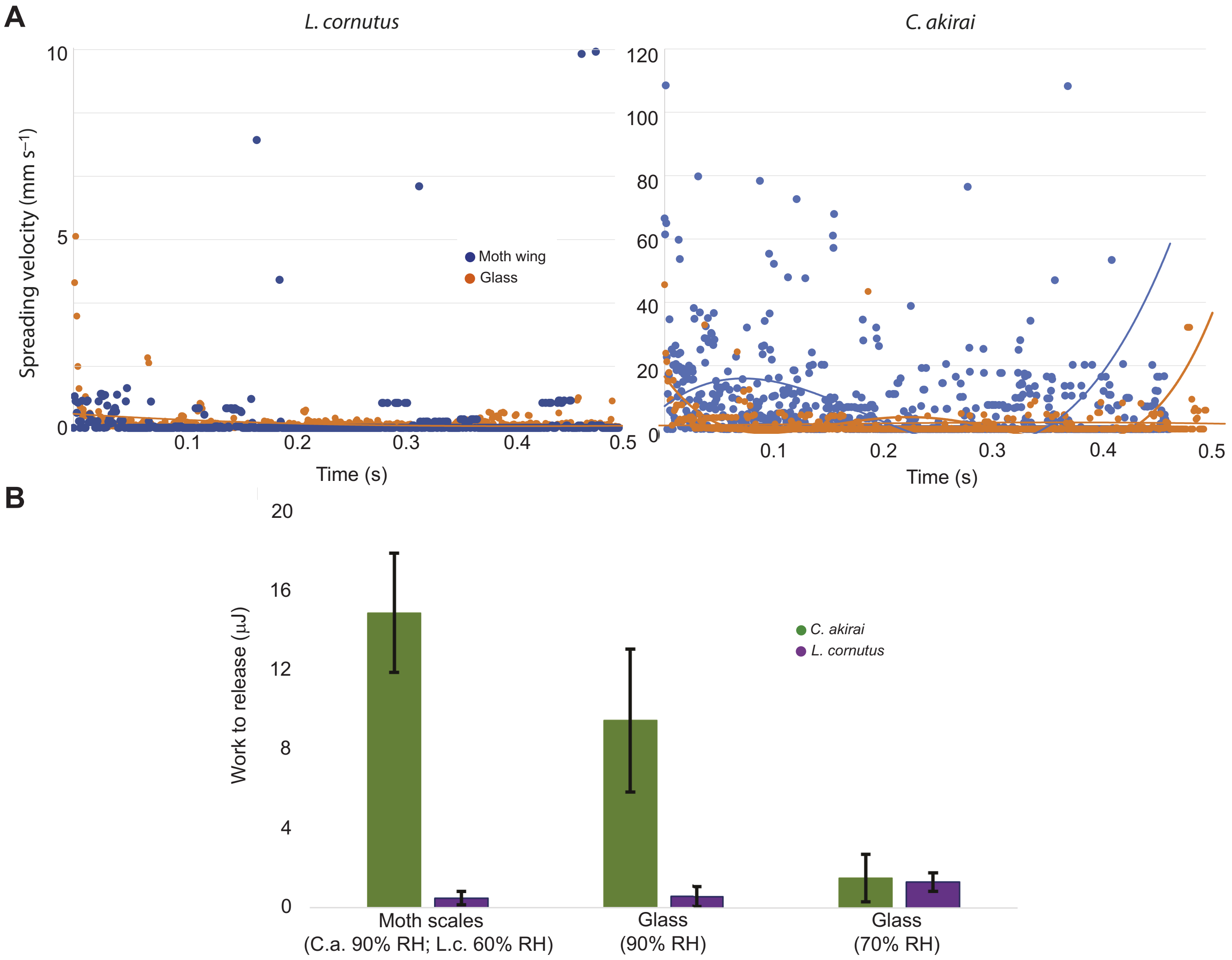

Fig. 3. Velocity of aggregate glue spreading at >90% relative humidity (RH) and associated adhesion forces. For raw data, see Tables S1 and S2. (A) Glue droplets were filmed coming into contact with each substrate at 2000 frames s^{-1} and the furthest edge was measured as total spreading distance. Tests were conducted on moth wings (blue) and glass (orange). Velocity was calculated between each frame for the leading edge. Each set of points consists of one trial example over 0.5 s and trendlines are third order polynomials. *Cyrtarachne akirai* glue droplets spread substantially faster than *Laranioides cornutus* glue droplets on both substrates. *Cyrtarachne akirai* glue droplets spread faster on moth scales than on glass, being enhanced by the scales. *Laranioides cornutus* glue droplets failed to adequately spread on moth scales, with near-zero velocity at all times. (B) When capture threads are brought into contact with a substrate adhesion strength, total work done before detachment varies based on both substrate chemistry and the ambient humidity at which tests are conducted. *Cyrtarachne akirai* capture threads are weaker on glass than on moth scales. They also lose most adhesion strength below 90% RH. Inversely, *L. cornutus* aggregate glue shows increased adhesion strength as humidity falls from 90% RH to 70% RH. Its strength is weakest on moth scales with almost no measurable work done before detachment (Diaz et al., 2018b, 2020).

The aggregate gland is thought to have the most recent evolutionary origin among the different gland types as it arose with the use of wet sticky adhesives on orb-web catching fibers (Garb, 2013). More basal orb-web weavers use a dry adhesive catching system involving cribellar silk whose gland and spidroins do not appear homologous to those of aggregate silk. All spidroin genes belong to a single gene family that has diversified throughout spider evolutionary history to produce the array of functionally divergent silk types (Garb, 2013; Collin et al., 2018). These genes all show a common protein organization characterized by short conserved terminal regions flanking a large repetitive region composed of stereotypical motifs that confer specific mechanical properties (e.g. high proline content increases extensibility) (Brooks et al., 2005; Hayashi et al., 1999; Liu et al., 2008; Malay et al., 2017). It is noteworthy that these similarly structured genes from the same gene family can produce proteinaceous products as diverse as strong fibers and liquid glues.

Examination of the genetic basis of the performance properties of aggregate glue is experimentally tractable for two primary reasons. First, spider silks are a minimally pleiotropic system, with most silk genes highly expressed in silk glands and negligibly expressed in non-silk tissues. Several recombinant studies have demonstrated that functional silk fibers, albeit with diminished mechanical properties, can be produced from the transgenic expression of constructs containing only the terminal and limited repetitive sequence from a single spidroin (Adrianos et al., 2013; Brooks et al., 2008; Heidebrecht et al., 2015; Saric et al., 2021; Xia et al., 2010; You et al., 2018). Second, recent advances in genomic technologies have made possible the discovery of complete silk gene sequences, which were previously inaccessible (Babb et al., 2017; Kono et al., 2019; Sheffer et al., 2021; Stellwagen and Burns, 2021; Stellwagen and Renberg, 2019; Zhou et al., 2021). Genomic comparisons coupled with our biomechanical tests will allow us to understand

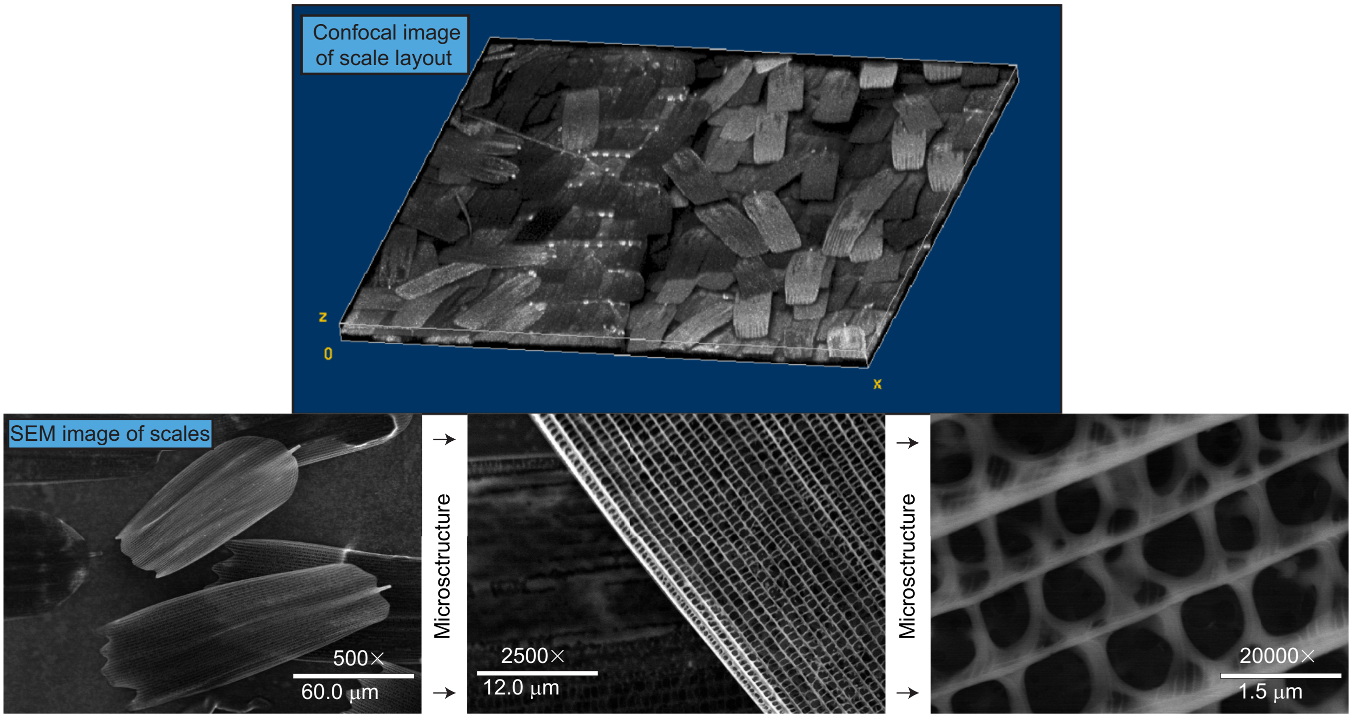

Fig. 4. Three-dimensional structure of moth scales. Top: moths are covered with microscopic scales of varying shapes and sizes, growing in patches, overlaying one another, creating a complex topography as shown here with confocal microscopy. The combination of these microstructures and their chemical composition render them superhydrophobic, resisting water flow. Bottom: using SEM, we can photograph these microstructures to model them with both 3D printing and for computational analysis. Confocal microscopy enables us to determine the scale layout and analyze the channels and gaps created by the overlay of scales which allow glue to flow through. The moth shown is from the family Tortricidae (unpublished data).

changes in amino acid composition and how they affect glue behavior. Recent studies using long-read Oxford Nanopore (ON) data, one of which was focused on the bolas spider *Mastophora phrynosoma*, has revealed the full sequence and structure of aggregate spidroins (Stellwagen and Burns, 2021; Stellwagen and Renberg, 2019). This research has verified the presence of two primary copies, *AgSp1* and *AgSp2*, and elucidated key aspects of intraspecific and interspecific variation. These genes have the largest coding regions recorded to date among spidroins and, like fiber-forming spidroins, are composed primarily of highly homogenized repetitive sequences. The amino acid translation of these repeat regions in both species is characterized by abundant threonine residues that are likely targets of O-linked glycosylation, a post-translational modification expected to enhance glue adhesive properties (Singla et al., 2018; Stellwagen and Renberg, 2019; Tillinghast et al., 1992). There is a sizable difference, however, in the repeat structure of these genes between *M. phrynosoma* and the orb-web weaving garden spider *Argiope trifasciata*. In *AgSp2*, the largest repeat unit (hereafter referred to as an ensemble repeat, ER) in *M. phrynosoma* is roughly 4 times larger than in *A. trifasciata* but maintains homology with *A. trifasciata* across the entire unit in an alternating pattern, indicating that this larger unit arose from several duplications of the smaller unit (Stellwagen and Burns, 2021).

We have also recently conducted ON long-read sequencing and assembly of *Mastophora* (*M. hutchinsoni*) and *Argiope* (*A. argentata*) taxa, providing an opportunity to conduct additional comparisons between bolas spiders and conventional orb-web weaving spiders. Fig. 5A provides the full-length gene organization for both *AgSp1* and *AgSp2* in both species and is consistent with what was described by Stellwagen and colleagues (Stellwagen and Burns, 2021; Stellwagen and Renberg, 2019) (although a full-length *AgSp1* was not provided for *M. phrynosoma*). For analysis of the repeat structure, we have defined the core repeat units slightly differently from Stellwagen and Burns (2021) and Stellwagen and Renberg (2019), using a highly conserved motif, PGTTPG, as the basis for unit identification. This approach essentially combines two of the four Stellwagen subgroup (SG) units into a single unit and places the 'tail' region in the middle of one of these combined units. Ultimately, comparison of *AgSp* genes from several more species will be needed to identify the most appropriate repeat unit delineation. Regardless of unit definition, the regions of homogenized repetitive motif structure (termed RM regions) are virtually identical between congeneric species and similar between *Mastophora* and *Argiope*. One difference between the studies is that we have identified a third RM zone in *AgSp1* of both *M. hutchinsoni* and *A. argentata* that maintains distinct sequence differences with repeats in RM2 (Fig. 5A).

The use of the PGTTPG repeat unit allows for a more comprehensive homology assessment within and between genes. As such, all RM regions in the *AgSp* genes of *Mastophora* and *Argiope* contain a paired unit structure defined by alternating homologous units (units 1 and 2 in Fig. 5B). Unit 1 exhibits less variation (and is invariant in size, always encoding 45 amino acids) than unit 2, which contains the hypervariable 'tail' region. Ensemble repeats may be composed of a single paired unit (as in all *Argiope* genes, ER=2) or a combination of slightly variant paired units (as in *Mastophora AgSp2* RM, ER=8; and *AgSp1* RM2, ER=4; Fig. 5A,B). The paired unit homology extends across both *AgSp* paralogs and species as a phylogenetic analysis of the consensus sequence of the PGTTPG units from each distinct RM region in all genes places all unit 1 sequences and unit 2 sequences as separate monophyletic groups (Fig. 5C). The presence of a conserved core structure (the

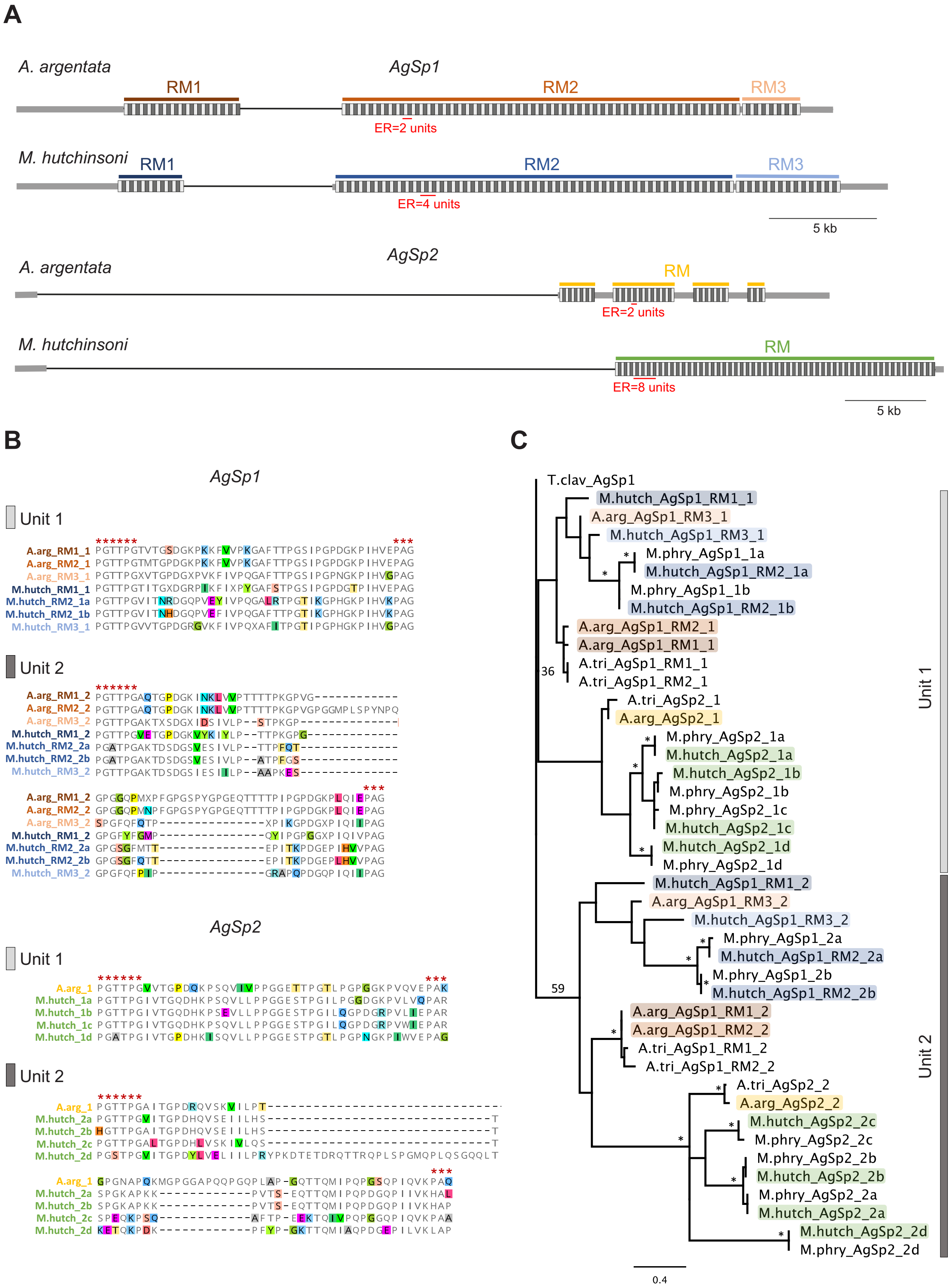

Fig. 5. See next page for legend.

Fig. 5. Sequence and structure of aggregate spidroins. (A) Gene organization of *AgSp1* and *AgSp2* in *Argiope argentata* and *Mastophora hutchinsoni* (NCBI accession no. OK615814–OK615817). Thin black lines represent intron sequences, hatched regions indicate coding sequences with stereotypical repeat organization and wide gray lines depict other coding regions. Repeat motif (RM) regions with sequence variants are classified as different numbered zones. Red bars indicate the size of a standard ensemble repeat (ER) within that region. RM1 and RM3 regions in both species have an ER composed of two units. (B) Alignment of consensus sequences for the corresponding repeat unit type from each RM zone in the *AgSp* gene products. The unit 2 sequence is arranged in two blocks. Colored sequence names correspond to the RM colors in the gene representations. Conserved motifs PGTTPG and PAX that delineate the repeat units are identified with asterisks. The ensemble repeat units comprise the concatenation of all sequences (unit 1 and 2) for a given RM zone. (C) Phylogenetic relationships among the consensus sequences of PGTTPG units from all distinct RM regions in the four *AgSp* genes plus the repeat units from *A. trifasciata* and *M. phrynosoma* (Stellwagen and Burns, 2021; Stellwagen and Renberg, 2019). The ML tree was generated with PhyML (Guindon and Gascuel, 2003) (LG+G4+I) with 1000 bootstraps. Sequence from *Trichonephila clavipes* was used to root the tree. Colors on taxa names correspond to those used in A and B. Asterisks indicate nodes with bootstrap values >90% and the bootstrap values for the two nodes defining the unit 1 and unit 2 monophyletic groups are provided as numbers. The sequence alignment for the tree is provided in Fig. S1.

paired repeat unit) that evolves into different configurations suggests this is a central genetic attribute responsible for variation in the mechanical properties of glue behavior. Most studies examining the structure–function relationship associated with spider silk focus on fine-scale molecular characteristics such as amino acid composition and simple motif frequencies (e.g. GPGXX motifs in MaSp2) (Adrianos et al., 2013; Blamires et al., 2015; Brooks et al., 2008; Craig et al., 2020; Gatesy et al., 2001; Malay et al., 2017). We argue that higher-level sequence organization, such as ensemble repeats, may be of greater ecological and evolutionary significance. Overall, our exploration of glue mechanical behavior with the Cyrtarachninae provides an ideal opportunity to test this assertion and identify the fundamental genetic units driving the evolution of a novel material.

Summary – a general framework

What this work on spiders shows is that comparisons of glue properties and behavior among species must be done not at a standard temperature and humidity but at the ecologically relevant conditions for each species. Materials are at the heart of this problem of conditional performance: the physical properties of glue, the structural behavior as a droplet, and its ecological performance catching moths are variable from moment to moment in the life of a single spider, changing with changes in temperature, humidity or the surface of the contact medium. This same type of conditional performance is manifested in different ways in different organisms. For example, some shark species swim in cold water and some in hot; thus, comparisons of the elastic energy storage capabilities of their vertebral columns should be done not under standard laboratory conditions but at ecologically relevant temperatures and strain rates. The challenge presented by material properties is a general one that must be recognized in order to close the explanatory gap between genomics and morphology, performance and fitness (Fig. 1).

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Competing interests

The authors declare no competing or financial interests.

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Data availability

AgSp sequence data for *M. hutchinsoni* and *A. argentata* are available in the National Center for Biotechnology Information (NCBI), accession number OK615814–OK615817.

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