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SHORT COMMUNICATION

Proteomics suggest pyriform silk attaches orb web capture spiral junctions

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Abstract. Orb web weaving spiders, like *Argiope trifasciata* (Forsskål, 1775), rely on sticky aggregate glue droplets on the capture spiral of their web to retain prey. Here, we compared the protein composition of the glue droplets placed at the bottom of the web early in capture spiral construction, to those placed at the inside of the web late in web construction. We found a lower abundance of aggregate proteins in the inner web, where the droplets are smaller, are less extensible, and contain a higher proportion of water. Interestingly, we also found that pyriform spidroin, which anchors bridging lines of the web to the substrate, was more abundant in the inner region of the web, which has a higher density of capture spiral-radial line junctions. We propose that pyriform spidroin has a previously unidentified role in orb webs: anchoring the capture spiral to radial lines.

Keywords: *Argiope trifasciata*, aggregate glue, silk gland-specific expression
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Orb web weaving spiders in the family Araneidae rely on the web's sticky capture spiral to intercept and retain prey (Eberhard 2020). Like other large, vertical orb webs, the webs of *Argiope trifasciata* (Forsskål, 1775) are typically asymmetrical: after putting down strong frame and radial lines made of stiff major ampullate silk (Foelix 2011), the spider constructs capture spiral switchbacks at the bottom of the web (Rhisiart & Vollrath 1994; Zschokke 2011; Zschokke & Nakata 2015), resulting in a larger lower web region. Once the bottom switchback region is complete, orb weavers start constructing the capture spirals inward until reaching the center hub (Peters 1955; Peters 1970; Venner et al. 2000). Capture spiral thread consists of flagelliform fibers covered by aqueous glue, which flows from aggregate gland spigots and is configured as a series of regularly spaced droplets (Townley & Tillinghast 2013). Aggregate glands and their chemically sticky glue are unique to the Araneoidea, a superfamily comprised of at least 17 families and about 25% of described spider species, including orb web, cobweb, and related spiders (Coddington 1989; Bond & Opell 1998; Dimitrov et al. 2017; Wheeler et al. 2017; World Spider Catalog 2023). Araneoid spiders also use pyriform silk to attach the web's structural fibers to surrounding substrates (Foelix 2011). Pyriform silk forms cement-like discs after extrusion (Römer & Scheibel 2008; Wirth et al. 2019; Greco et al. 2020). In contrast, the aggregate silk adhesive retains the aqueous nature found in the silk gland (Römer & Scheibel 2008; Townley & Tillinghast 2013).

The aggregate aqueous glue droplets have impressive physical properties that vary considerably among species, including hygroscopicity, humidity responsiveness, toughness, and elasticity (Kelly et al. 2019; Opell & Stellwagen 2019; Opell et al. 2021, 2022). They are composed of low molecular mass compounds, salts, and proteins (Townley & Tillinghast 2013). Low molecular mass compounds (LMMCs) and salts confer hygroscopicity which is essential in determining the material properties of the glue droplet (Jain et al. 2018; Opell et al. 2018). The absorption of atmospheric moisture by LMMCs and salts results in the solvation of proteins within the glue droplet (Sahni et al. 2011). These proteins, including aggregate spidroins AgSp1 and AgSp2 (Stellwagen & Renberg 2019), are highly glycosylated and phosphorylated (Ayoub et al. 2021, 2023), which are both common post-translational modifications seen in other biological adhesives (Wagner et al. 1992; Stewart & Wang 2010; Stewart et al. 2011; Tarakhovskaya 2014; Waite 2017). The solvation of these highly modified proteins makes the glue droplets sticky, allowing for prey capture (Sahni et al. 2014; Amarpuri et al. 2015, 2022; Singla et al. 2018).

The adhesive properties of aggregate glue droplets have been shown to differ depending on where they are located on the capture spiral for the orb weaver *A. trifasciata* (Opell & Stellwagen 2019). Glue droplets at the bottom of the web are significantly larger, tougher, and more extensible. They also have a lower percentage of water than the droplets in the inner region (Opell & Stellwagen 2019). Proteins are responsible for a glue droplet's material properties, including cohesion and adhesion. Because material properties of glue droplets change between the early-constructed bottom region of the web and the later-constructed inner region of the web, we hypothesized that there would also be differences in the protein composition of the glue droplets. Opell & Stellwagen (2019) proposed that the spider runs out of aggregate material during web construction. If true, we predict a lower abundance of aggregate expressed proteins in the inner portion of the web than the bottom.

To test this hypothesis, we collected 10 *A. trifasciata* spiders from Blacksburg, Virginia on September 18, 2021, and maintained them in the lab until October 20, 2021, in Lexington, VA. Spiders were kept in large ($81 \times 81 \times 18$ cm, $43 \times 61 \times 6$ cm, or $43 \times 46 \times 9$ cm) wood-framed boxes with plexiglass fronts and backs that allowed spiders to make vertical orb webs. We lined the bottom of the box with wet sponges to maintain humidity levels. The room lights were set on a 24-hour timer (12 hours on, 12 hours off), and there were windows allowing dim natural light into the lab. We attempted to hand-feed the spiders one small (1–2 cm long) cricket daily, after web collection. If the spider did not feed, we left the cricket in the remaining parts of the web and the cricket was consumed before the next web collection. A previous study demonstrated that *A. trifasciata* glue droplets from spider webs spun in the field and webs spun in these housing conditions have the same features (Ayoub et al. 2023), justifying the use of lab built webs for protein comparisons. While it is possible that long-term rearing could change the protein composition of the capture spirals, we would expect that change to be consistent across the entire web, allowing for comparison between the bottom and inner region of the webs on any given day. We did see some deterioration in web building over time; however, we only collected samples from webs that had a full capture spiral, complete with clearly distinguishable switchbacks at the bottom of the web and spirals at the inner region.

Each day before collecting threads, we misted a web with deionized water to make it easier to see. Each individual spider had a designated petri dish containing two pairs of forceps. One pair was labeled "bottom" and was used to collect switchback threads. The other pair was labeled "inner" and was used to collect threads just beneath the web's hub (See Supplementary Fig. S1, online at https://doi.org/10.1636/JoA-S-23-020.1).

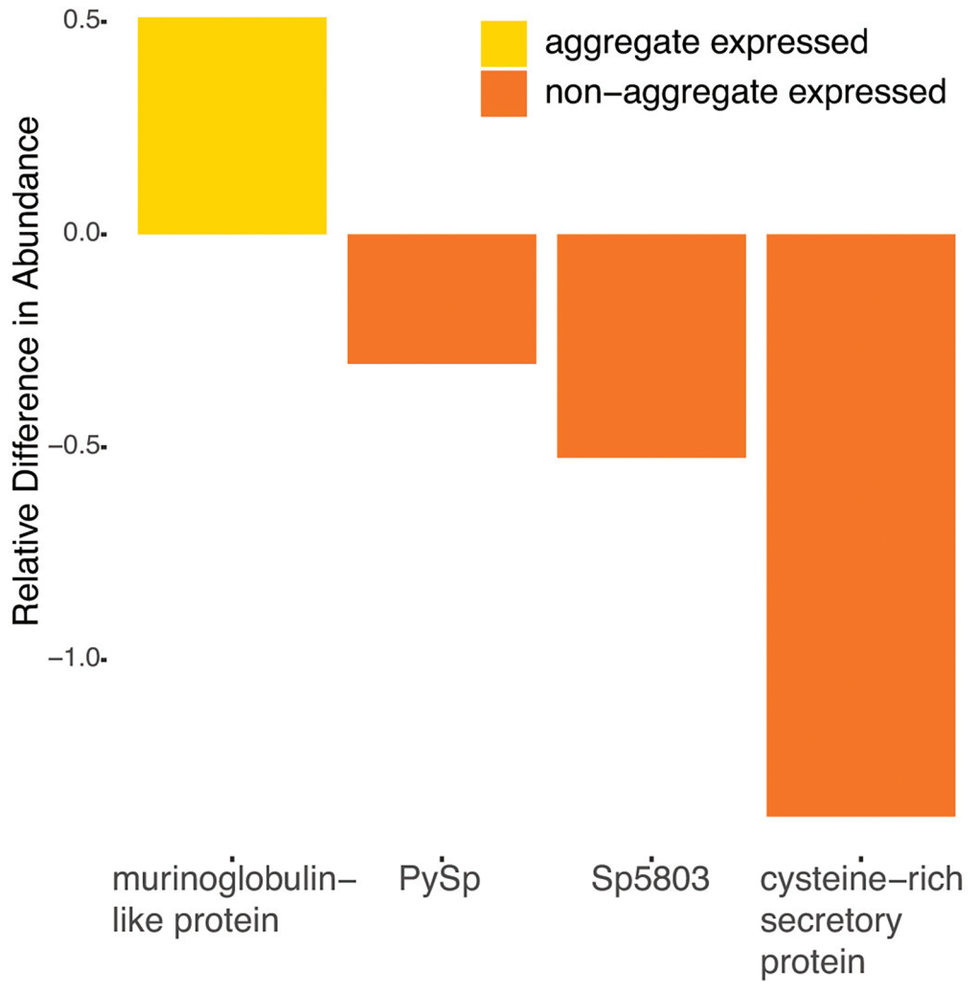

Figure 1.—Relative difference in protein abundance, *d(i)*, of the four proteins that differed significantly between capture spiral samples taken from the bottom region of the web compared to the inner region of the web calculated according to Tusher et al. (2001). Positive *d(i)* indicates higher abundance in the bottom region. See also Supplementary Table S1. Location of expression (aggregate silk glands versus non-aggregate silk glands) was determined using high-throughput sequencing of silk gland RNA (Ayoub et al. 2023).

To the best of our ability, we targeted lines with visible glue droplets and avoided the dry hub. We took pictures of each spider web prior to and post capture spiral collection to verify the locations of collection and that radial lines were still present after collection (e.g., Supplementary Fig. S1). While we cannot confirm with complete certainty that our samples only consisted of sticky capture spiral threads, our mass spectrometry failed to detect any major ampullate spidroins (MaSp-s) and only detected a small amount of minor ampullate spidroin (MiSp) in two samples, one bottom and one inner. Due to the inconsistent presence of MiSp, we removed it from further analysis (See Supplemental Table S1, online at https://doi.org/10.1636/JoA-S-23-020.s2). The absence of MaSp and MiSp in our proteomics despite the ability of our methods to detect these proteins (see Ayoub et al. 2021, 2023) suggests we effectively avoided major ampullate silk from the hub and radii, and that most of our spiders had effectively removed the temporary spiral, which is composed of minor ampullate silk.

We collected enough capture spirals from two individual spiders for protein purification and mass spectrometry. To generate a third replicate of each region, we combined switchback threads from eight other spiders and the inner capture spirals of these eight individuals, resulting in three paired bottom and inner samples (6 total). To compare protein components of the two regions, we followed the silk purification, tryptic digest, and mass spectrometry procedures outlined in Ayoub et al. (2021, 2023). In brief, we solubilized capture spirals in hexafluoroisopropanol and then dried the samples. We resuspended the dried proteins in guanidine hydrochloride, denatured the proteins with boiling in the presence of dithiothreitol, alkylated with iodoacetamide, and diluted with ammonium bicarbonate. These proteins were subjected to overnight digestion with trypsin at a 1:20 ratio of trypsin to silk protein. We cleaned the resulting peptides with C18 ZipTips and sent them to the University of Arizona Proteomics core for LC-MS/MS on a Q Exactive Plus mass spectrometer with chromatography, flow rates, and data collection mirroring methods described in Ayoub et al. (2021). Initial searches were completed with Thermo Proteome Discoverer against a *de novo* assembled transcriptome generated from three aggregate gland and three non-aggregate gland RNA-seq libraries (Ayoub et al. 2023, SRR21590965-8). The Proteome Discoverer-identified proteins were used in subsequent searches with MaxQuant (Cox & Mann 2008) with the addition of spidroins based on genome sequencing (Stellwagen & Renberg 2019; Diaz et al. 2022). We manually shortened these spidroins to remove repetitive regions to decrease the computational time and to not bias the false discovery rate (FDR) (see Ayoub et al. 2023 for more details). We completed label free quantitation (LFQ) on the resulting database using default parameters in MaxQuant.

There were 46 proteins with an LFQ value > 0 in at least three of the six samples (ProteomeXchange PXD044266, Supplementary Table

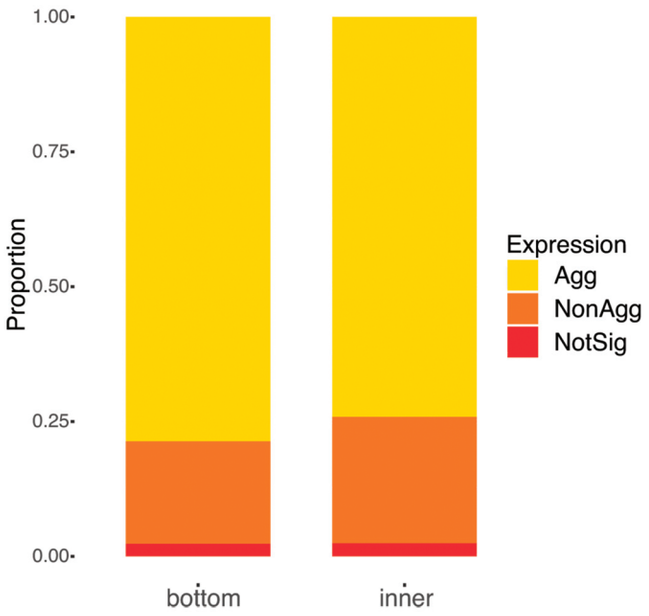

Figure 2.—Relative proportion of capture spiral protein composition that can be attributed to aggregate (Agg), versus non-aggregate (NonAgg) expressed proteins in the bottom compared to the inner region of *A. trifasciata* orb webs. Location of expression (aggregate glands versus other non-aggregate silk glands) was determined from high-throughput sequencing of silk gland RNA (see text and (Ayoub et al. 2023)). NotSig proteins are encoded by transcripts that did not significantly differ in expression levels between aggregate and non-aggregate glands. Using summed LFQ values, we determined that there is a significantly higher abundance of aggregate proteins in the bottom region of the web compared to the inner region of the web (one-tailed t-test, $P = 0.0274$).

S1). We examined the proteins found in at least three of the silk samples, in case there were proteins found only in the bottom samples or only in the inner samples. We found four proteins to be in different abundance between the bottom samples and the inner samples (two-tailed paired t-test, p-value < 0.05, Figure 1). To account for performing 46 t-tests, we estimated the FDR (Benjamini & Hochberg 1995) and found all proteins had an FDR > 0.05, indicating that these differences were not significant. Nevertheless, the patterns uncovered by the t-test are consistent with the hypothesis that the spider is running out of aggregate material towards the end of capture spiral production. Of the four proteins identified as potentially different between the bottom and inner regions of the web, one (TRINITY_DN2280_c0_g1, homologous to a murinoglobulin-like protein) had significantly higher expression in aggregate glands ((Ayoub et al. 2023) and see next paragraph), and was more abundant in the bottom of the web ($P = 0.0435$, Fig. 1). The other three had significantly higher expression in the other types of silk glands (Ayoub et al. 2023), and were more abundant in the inner region of the web. These were Sp5803, a spidroin expressed in flagelliform glands (Ayoub et al. 2023) ($P = 0.0220$); TRINITY_DN1211_c0_g1, which is homologous to a cysteine-rich secretory protein ($P = 0.0236$); and the pyriform spidroin, PySp ($P = 0.0322$). These patterns could be due to the loss of aggregate material at the inner part increasing the relative contribution of non-aggregate (e.g., flagelliform and pyriform) expressed proteins in the innermost part of the web.

To further evaluate the hypothesis that aggregate material is depleted during web construction, we estimated the proportion of the total capture spiral protein composition that could be attributed to aggregate-expressed proteins. We considered a protein aggregate expressed if its encoding transcript was significantly more abundant in the three aggregate gland RNAseq libraries than the three non-aggregate gland libraries with a FDR < 0.01 according to DESeq2 (Love et al. 2014, see also Ayoub et al. 2023). We then summed the LFQ of all these aggregate-expressed proteins and divided by the total LFQ of the 46 proteins consistently found in capture spirals (Supplementary Table S1) to obtain relative abundance of aggregate-expressed proteins (Fig. 2). We additionally found that the total LFQ of aggregate expressed proteins in the inner region of the web was significantly less than the total LFQ of these proteins in the bottom region (one-tailed t-test, $P = 0.0274$). This, paired with the data on individual proteins, provides evidence to support our original hypothesis that there is a difference in protein composition of glue droplets between the inner and bottom region of the web, due to the spider running out of aggregate material near the end of web construction.

The presence of PySp in capture spirals, as well as the higher abundance of PySp in the inner region of the orb web, is especially intriguing. Although admittedly a small proportion of the total capture spiral, ~0.16 percent of the total LFQ of the bottom region and ~0.25 percent of the inner region, the presence of PySp is somewhat surprising, as pyriform silk is placed on the outermost structural fibers, working as a cement to adhere the spider web to the surrounding environment (Blasingame et al. 2009). Microscopy also suggests that aggregate glue holds the capture spiral to the radial lines, and that capture spiral-radial line junctions differ in structure compared to other thread junctions (Work 1981; Greco et al. 2019) (Supplementary Fig. S1c), so we did not expect to find PySp in capture spirals. However, the higher abundance of PySp in the inner region strongly suggests that pyriform silk may also play a role in keeping the capture spiral attached to the radial lines. The innermost part of the orb web has the highest concentration of capture spiral-radial line junctions, which would increase the abundance of any proteins that contribute to these junctions. Furthermore, video recordings show that some orb weavers briefly touch the pyriform spigots to the capture spiral and radial line during web construction (Eberhard 2010). PySp at the capture spiral-radial line junctions may confer some of the cement-like properties of pyriform silk to these junctions, which could allow the force of prey hitting the web to transfer to the sturdier radial lines, keeping the web intact after impact (Sensenig et al. 2012; Greco et al. 2019). Morphologically, the glue at the capture spiral-radial line junctions may appear to be more aqueous and aggregate-like because the interactions between PySp and proteins or hygroscopic molecules in aggregate silk secretions could prevent hardening of the pyriform silk. One way to test this hypothesis would be to make recombinant PySp and compare its morphology in the presence versus absence of LMMCs and salts. Additional proteomics work from other species would also determine if PySp is generally present in capture spirals of orb webs or if the finding is unique to *Argiope* species. Coupled with biomechanical analysis of capture spiral-radial line junctions, cross-species comparisons could evaluate the contribution of pyriform versus aggregate expressed proteins in web performance.

Our proteomic analysis of different regions of the capture spiral highlights a pattern of less aggregate material in the inner region of the capture spiral compared to the bottom region, consistent with our hypothesis that the spider runs out of aggregate material as web construction progresses. Finding PySp at a higher abundance in the inner region compared to the bottom region could also provide evidence that PySp contributes to capture spiral–radial line junctions. Cross-species comparisons of protein composition of different regions of the capture spiral could provide evidence in support of the patterns described in this communication. In addition, proteomic work on the interactions between PySp and hygroscopic molecules or other proteins found in the capture spiral may explain the significance of PySp in the capture spiral.

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SUPPLEMENTAL MATERIALS

Supplementary Figure S1.— *A. trifasciata* rearing and collection box; pyriform spidroin attachment disc and capture spiral–radial line junction (Greco et al. 2019). Available online at https://doi.org/10.1636/JoA-S-23-020.1

Supplementary Table S1.— Proteins identified in this study and their characteristics. Available online at https://doi.org/10.1636/JoA-S-23-020.2

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