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The stability of hygroscopic compounds in orb-web spider viscous thread

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Abstract. Each of the droplets that form an orb-weaving spider's viscous capture thread are composed of a viscoelastic glycoprotein glue core that is surrounded by an aqueous layer and supported by a pair of protein axial fibers. Low molecular weight organic and inorganic compounds within the aqueous layer confer droplet hygroscopicity, thereby maintaining the glycoprotein's adhesion and extensibility and ensuring that the axial fibers remain supercontracted. These materials also cause droplet volume to change in response to ambient humidity. This study examined the viscous threads of *Argiope aurantia* Lucas 1833, a species found in exposed, low humidity habitats, and *Neoscona crucifera* (Lucas 1838), a nocturnal species found in high humidity forest edge habitats. An earlier study showed the former species' threads to be more hygroscopic than those of the latter. When aged and exposed to chemical fixatives, the hygroscopicity of *A. aurantia* threads decreased, while that of *N. crucifera* threads was unaffected. Fixation eliminated the extensibility of both species' droplets. However, droplet adhesion, as measured by the deflection angle of a thread's axial lines just prior to droplet extension or, in the case of fixed droplets, droplet pull-off, was unaffected. These findings indicate that the compounds that confer greater hygroscopicity to *A. aurantia* viscous threads are more susceptible to chemical fixatives than those in the aqueous layer of *N. crucifera* droplets.

Keywords: Adhesion, *Argiope aurantia*, low molecular weight compounds, *Neoscona crucifera*

High evening and early morning humidity contributes to the formation of the droplets that form an araneoid orb spider's viscous prey capture threads (Fig. 1). These composite threads are spun from three spinning spigots on each of a spider's posterior median spinnerets (Sahni et al. 2013). A flagelliform spigot, which produces a supporting axial line, is flanked by two aggregate glands which coat this line with an aqueous solution as it is drawn out. After the coated lines from the two spinnerets merge, compounds in their aqueous material attract atmospheric moisture, increasing the volume of the viscous cylinder, causing it to become unstable and form into a regular series of droplets though the Rayleigh process (Edmonds & Vollrath 1992). Within each droplet, viscoelastic glycoprotein condenses to form the droplet's adhesive core (Vollrath et al. 1990; Vollrath & Tillinghast 1991; Tillinghast et al. 1993; Vollrath 2006; Choresh et al. 2009). The adhesion and extensibility of this core combine to form a highly integrated and effective adhesive delivery system, which implements a suspension bridge mechanism to sum the adhesion of multiple droplets (Opell & Hendricks 2009; Sahni et al. 2010).

By attracting atmospheric moisture, compounds in the aqueous materials that surround each droplet's glycoprotein core and cover the thread's axial lines (in inner droplet regions) play a crucial role in maintaining thread function (Fig. 2). Ensuring that both the axial lines and the glycoprotein remain hydrated (Opell et al. 2011, 2013) maintains axial line supercontraction (Work 1981, 1982; Shao & Vollrath 1999; Shao et al. 1999) and glycoprotein extensibility (Sahni et al. 2011; Opell et al. 2013).

Inorganic ions ($H_2PO_4^-$, K^+, NO_3^-, Na^+, Cl^-, and Ca_2^+) account for only 10–20% of the dry mass of viscous thread (Townley et al. 2006) whereas low molecular mass compounds (LMM: alanine, betaine, choline, GABamide, glycine, isethionic acid, *N*-acetylputrescine, *N*-acetyltaurine, proline, putrescine, and taurine (Tillinghast et al. 1993; Townley et al. 2006; Townley & Tillinghast 2013)) constitute 40–70% of the dry mass (Fisher & Brander 1960; Anderson & Tillinghast 1980; Tillinghast & Christenson 1984; Townley et al. 1991). As inorganic salts are in low concentrations and LMM do not crystalize as humidity changes, viscous threads continue to function during the often wide daily oscillation in humidity (Opell et al. 2011, 2013). These LMM differ in both their metabolic cost and hygroscopicity. Choline, isethionic acid, and *N*-acetyltaurine are more costly to synthesize and their concentrations decline in threads spun by starved spiders, being replaced by more readily synthesized GABamide and glycine (Townley et al. 2006). However, as choline and N-acetyltaurine are very hygroscopic at low relative humidities (20–30% RH) and glycine absorbs very little water even at moderate humidity (55% RH) (Vollrath et al. 1990), such replacements might be predicted to alter the hygroscopic performance of viscous threads.

Recent evidence indicates that the hygroscopic performance of an orb-weaver's viscous threads is linked to the humidity regime of its habitat (Opell et al. 2013). *Neoscona crucifera* (Lucas 1838), which occupies more humid forest habitats, have less hygroscopic threads than *Argiope aurantia* Lucas 1833 and *Larinoides cornutus* (Clerck 1757), which are found in more exposed habitats where humidity drops during the day. As judged by droplet extensibility and the energy required to extend droplets, performance of *N. crucifera* viscous droplets continues to increase as humidity increases, whereas performance of *A. aurantia* and *L. cornutus* peaks in the 55–60% RH range (Sahni et al. 2011; Opell et al. 2013). Below this range, the glycoprotein cores of the latter two species' droplets are not sufficiently hydrated to adhere and extend as effectively. Above this range, their droplets absorb so much water that their glycoproteins become over-lubricated, extend too readily and release their adhesion to a surface (Sahni et al. 2011; Opell et al. 2013), suggesting that LMM have been selected to perform in the humidity regime of a species' habitat. LMM also contribute more directly to thread

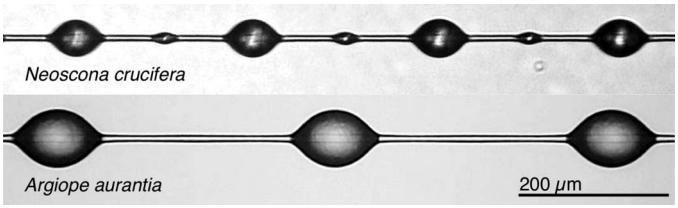

Figure 1.—Viscous threads of *A. aurantia* and *N. crucifera*.

adhesion. By solvating and softening glycoproteins, they facilitate glycoprotein-surface interactions and enhance adhesion at all humidities (Sahni et al. 2014). When combined with the synthetic costs and hygroscopicity of LMM, these differences also suggest that it may be more costly for species that occupy lower humidity habitats to spin viscous threads as more and more costly LMM may be required to facilitate glycoprotein-surface interactions and glycoprotein extension.

A compound's hygroscopicity depends on a number of factors. Because water is a polar molecule, other charged molecules are attracted to water molecules. Sulfates or nitrates can form strong hydrogen bonds with water and nitrate ions can form configurations in which the water bridges the oxygen molecules with hydrogen bonds (Early 2010). The greater interactivity of these more hydrophilic compounds may also cause them to be more interactive with chemical fixatives and, therefore, more easily degraded. In this study, we tested the hypothesis that, due to the greater interactivity of many of their LMM, the hygroscopicity of viscous threads spun by *A. aurantia* is more easily degraded than that of *N. crucifera* threads. We tested this hypothesis by comparing the hygroscopicity of fresh threads of these two species with threads that were aged and threads that were fixed by chemical vapor. The glutaraldehyde and formaldehyde that we used for this purpose are known to crosslink proteins (Fraenkel-Conrat & Olcott 1948; Richards & Knowles 1968; Stratis & Ternynck 1969; Hardy et al. 1976; Marquié, 2001; Sutherland et al. 2008). By crosslinking glycoproteins in the droplet's core, this treatment also permitted us to test the hypothesis that fixation destroys both a droplet's adhesion and its extensibility, two properties crucial for the functioning of viscous threads. Only two glycoproteins, ASG1 and ASG2 (aggregate spider glue 1 and 2), have been identified and, in the two species examined, they are highly conserved (Choresh et al. 2009). However, as glycoproteins have not been characterized for either *A. aurantia* or *N. crucifera*, we make no assumptions about their similarity.

METHODS

Context.—We conducted this study concurrently with an earlier investigation (Opell et al. 2013) with values of fresh (control) threads being taken from that study. Therefore, we summarize procedures that were common to both studies and describe in detail only those methods that were unique to this study. The current study compares droplet volumes at three humidities (20%, 55%, and 90% RH) and droplet extensions at only 90% RH, all at 23 ± 1° C. Humidity values were tightly controlled, as illustrated by the narrow limits (mean ± 1 standard error) of values at which images of fresh droplets were captured: *A. aurantia* (n = 14): 20.5 ± 0.3, 55.1 ± 0.1, 90.2 ± 0.3; *N. crucifera* (n = 16): 20.9 ± 0.3, 55.1 ± 0.1, 90.2 ± 0.1.

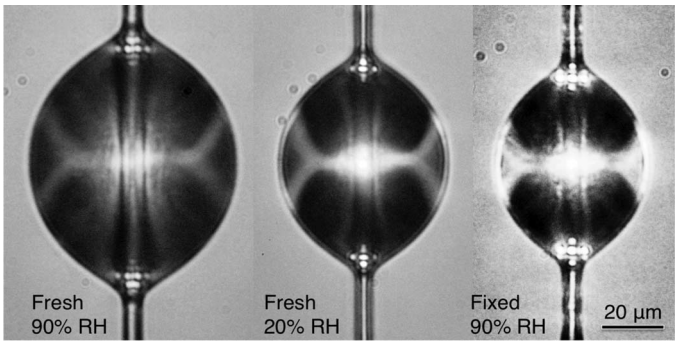

Figure 2.—Viscous thread droplets of *A. aurantia* individual 3, illustrating the effects of relative humidity on fresh and fixed droplet volume.

Collecting threads and extending droplets.—Samples of webs constructed by adult female *N. crucifera* and *A. aurantia* were collected from the vicinity of Blacksburg, Montgomery Co., Virginia during the late summer and early fall of 2011. These web sectors were collected on 15 × 52 cm aluminum frames with Scotch® double-sided tissue tape (Tape 410M; 3M, St Paul, MN, USA) on their 1 cm rims to secure web sectors at their native tensions. In the laboratory, we transferred viscous threads from a frame to the raised supports of a microscope slide sampler and placed this in a humidity-controlled chamber covered by a glass plate. At each test humidity we photographed three of an individual's droplets under a Mitutoyo FS60 inspection microscope equipped with a Canon T1i digital camera and extended, or attempted to extend, one of these droplets. To prepare a droplet for extension, we slid adjacent droplets away using a small needle, moistened with distilled water, leaving a focal droplet in the center of a 4.8 mm long thread strand. We then contacted this droplet with the 413 μm wide tip of a polished steel probe, advanced the probe into the thread for a distance of 500 μm to ensure adhesion, and activated a stepping motor, which was connected to the X axis knob of the stage manipulator and withdrew the probe at 69.5 μm s^{-1}, while a 60 fps video of the droplet's extension was recorded.

Fixing droplets.—All thread images and videos were captured on the same day that a web sample was collected. At the end of each day's droplet testing, we collected two additional thread samples from each individual's web sector. One was placed in a slide box and stored inside a sealed plastic cabinet and the other sample was placed in a closed container with an opened 10 ml glass ampule of 50% glutaraldehyde solution (product 16320, Electron Microscopy Sciences, Fort Washington, PA) and a small beaker containing 10 ml of 37% formaldehyde solution (product 2106-01, J.T. Baker Inc., Phillipsburg, NJ). Threads were fixed at 23 ± 1° C for 41.7 ± SE 2.1 hours in the case of *A. aurantia* and 49.2 ± SE 3.3 hours in the case of *N. crucifera*. After these fixed threads were removed, they were ventilated for at least one hour before being stored as described for aged threads. After cold weather ended our ability to collect web samples, we shifted our attention to photographing aged and fixed droplets and conducting extension trials of fixed threads.

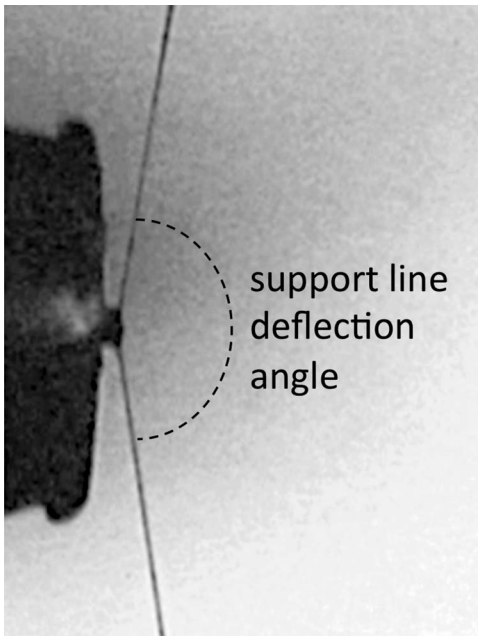

Figure 3.—Angular deflection of the support line of a viscous thread droplet just prior to the initiation of droplet extension or, in the case of fixed droplets, pull-off.

Measuring droplet volume, adhesion, and extension.—An image of a stage micrometer taken at the same magnification as a droplet image served as a scale for measuring suspended droplet length (*DL*, dimension parallel to the thread's axial fibers) and droplet width (*DW*) with Image J (Rasband 1997–2014). Following an earlier study (Opell & Schwend 2007), we computed droplet volume (*DV*) according to the following formula for a parabolic volume:

$$DV = \frac{\left(2\pi \text{ X } DW^2 \text{ X } DL\right)}{15} \quad (1)$$

This formula remains appropriate for droplets as their volumes change with ambient humidity (Liao et al. 2015). The volumes of the three droplets from an individual's thread were averaged to represent that individual in statistical tests.

We assessed droplet adhesion as the angular deflection of the axial line supporting a droplet just prior to the initiation of a droplet extension, as occurred in fresh threads, or release from the probe tip, as occurred in fixed threads (Fig. 3). Smaller angles signify greater droplet adhesive forces. We measured these angles with an OndeSoft screen protractor (Beijing Torrentsoft Technology Co. Ltd., Beijing, China) while viewing movies frame-by-frame with iMove ′11 (vers. 9.0.9. Apple Inc., Cupertino, CA). Droplet extension was measured in a similar manner, using the OndeSoft caliper option, calibrated with the 413-μm width of the probe tip. In the few cases where the droplet's attachment to the thread's axial lines were out of the video image when the filament released, we computed filament length at pull-off by adding to the product of the number of seconds of out-of-frame extension and extension velocity to the measured length of the in-frame portion of the filament.

Because there was the possibility that fixed droplets would not extend, we included an analysis to determine if their pre-extension adhesion could be attributed to the same forces as in fresh droplets. The adhesive force of glycoprotein within droplets is an order of magnitude greater than the capillary force generated by the LMM and water in their aqueous layer (Sahni et al., 2010). Therefore, if the pre-pull-off axial line deflection of a non-extending, fixed droplet is similar to or less than the early extension axial line deflection of a fresh thread, its adhesion can be attributed to the adhesion of its glycoprotein and not to weaker capillary forces. We generated a profile of each species' axial line deflection during droplet extension by dividing the extension duration of each individual's droplet into five intervals (0% = pre-extension, 25%, 50%, 75%, and 99% droplet extension time). A plot of the mean measured axial line deflection at these times provided a profile of force on extending droplets.

Analysis.—We completed some of our *A. aurantia* thread measurements before those of *N. crucifera* and did not begin setting aside aging thread samples until several weeks into our study. Therefore, there were aged *A. aurantia* thread samples

Table 1.—Dimensions and humidity responses of fresh, aged, and fixed threads. (Sample size), mean ± 1 standard error, N = normally distributed, A = anormally distributed.

Droplet dimensions and volumes	*Argiope aurantia*		*Neoscona crucifera*		
	Fresh (14)	Fixed (14)	Fresh (16)	Aged (10)	Fixed (16)
20% RH	20.5 ±0.27	20.09 ±0.06	20.93 ±0.31	19.97 ±0.03	19.96 ±0.03
length μm	60.59 ±3.7	60.3 ± 3.5	42.07 ±2.1	43.6 ±2.4	41.22 ±3.0
width μm	43.89 ± 2.6	42.14 ±2.1	29.91 ±1.4	29.5 ±1.6	28.94 ±1.9
volume $\mu m^3 \times 10^{-2}$	560 ± 96 N	491 ± 71 N	176 ± 23 N	174 ± 37 A	174 ± 37 A
	MP $P = 0.2473$			Wilcoxon $P = 0.8371$	
55% RH	55.07 ±0.12	55 ±0.09	55.16 ±0.15	55 ±0.05	55.04 ±0.04
length μm	63.76 ±4.1	60.32 ±3.8	38.4 ±1.8	43.6 ±2.8	42.41 ±3.0
width μm	47.53 ±3.2	44.4 ±2.1	29.18 ±1.4	31.1 ±1.8	30.25 ±1.8
volume $\mu m^3 \times 10^{-2}$	711 ± 127 N	559 ± 78 N	156 ± 22 N	202 ± 47 A	195 ± 39 A
	MP $P = 0.0925$			Wilcoxon $P = 0.7967$	
90% RH	90.21 ±0.27	89.95 ±0.05	90.19 ±0.11	90 ±0	90 ±0
length	78.89 ±4.5	73.67± 4.2	44.19 ±2.5	52.4 ±3.1	47.88 ±3.2
width μm	59.68 ±3.6	51.57 ±2.5	34.94 ±2.0	37.4 ±2.0	35.79 ±1.9
volume $\mu m^3 \times 10^{-2}$	1335 ± 225 N	848 ± 115 N	267 ± 51 A	329 ± 71 A	284 ± 55 A
	MP $P = 0.0089$			Wilcoxon $P = 0.5766$	

Table 2.—Dimensions of thread droplets and aging time including fixation time. (Sample size), mean ± 1 standard error, N = normally distributed, A = anormally distributed.

	Argiope aurantia				*Neoscona crucifera*			
	Aged 7–21 mean 15±2.2 days		Aged 22–35 mean 28±2.4 days		Aged 14–21 mean 17±1.1 days		Aged 22–38 mean 31±2.2 days	
	Fresh(7)	Fixed(7)	Fresh(7)	Fixed(7)	Fresh(8)	Fixed(8)	Fresh(8)	Fixed(8)
20% volume $\mu m^3 \times 10^{-2}$	551±113 N	632±110 N	569±164 N	349±53 N	204±37 N	229±65 N	147±24 N	117±25 N
	MP $P = 0.48$		MP $P = 0.19$		MP $P = 0.61$		MP $P = 0.19$	
55% volume $\mu m^3 \times 10^{-2}$	776±160 N	714±119 N	646±206 N	404±63 N	177±39 N	253±69 N	135±19 N	137±29 N
	MP $P = 0.81$		MP $P = 0.23$		MP $P = 0.11$		MP $P = 0.93$	
90% volume $\mu m^3 \times 10^{-2}$	1433±304 N	1065±175 N	1237±350 A	630±104 N	349±90 N	368±95 N	184±31 N	199±44 N
	MP $P = 0.15$		Wilcoxon $P = 0.031$		MP $P = 0.74$		MP $P = 0.64$	
Log_N volume $\mu m^3 \times 10^{-2}$	11.67±0.3 N	11.48±0.2 N	11.54±0.2 N	10.95±0.2 N	10.26±0.2 N	9.71±0.2N	10.33±0.2 N	9.69±0.3 N
	MP $P = 0.336$		MP $P = 0.016$		MP $P = 0.77$		MP $P = 0.90$	

for only five individuals; too few for a meaningful analysis and, for this species, our comparisons of droplet volume are between fresh and fixed droplets only. For *N. crucifera*, comparisons include fresh, aged, and fixed droplets (Table 1).

We assembled, summarized and analyzed data using JMP (SAS Institute, Cary, NC). If the mean value of a feature was normally distributed, as shown by Shapiro–Wilk *W*-tests with $P \geq 0.05$, we compared the values using a Matched Pairs T-test (MP). If one or more values were not normally distributed, we used a Wilcoxon/Kruskall–Wallis test to compare means.

RESULTS

The volume of fixed *A. aurantia* droplets appeared less than that of fresh droplets at each humidity, although this difference was significant only at 90% RH (Table 1, Fig. 2). In contrast, aging and fixation had neither an apparent nor a statistically significant effect on the volumes of *N. crucifera* droplets. The duration of droplet fixation did not affect droplet performance as demonstrated by the lack of fit for regressions of droplet volume at 90% (the humidity that provides the most sensitive assay of droplet response) and fixation time in hours (*A. aurantia*: $P = 0.803$, $R^2 = 0.005$; *N. crucifera*: $P = 0.782$, $R^2 = 0.006$).

To isolate the effects of fixation and aging, which we were not able to examine directly for *A. aurantia*, we divided fixed droplets into two groups: those with a shorter time between the start of fixation and droplet image capture and those with longer times (Table 2). Droplets of *N. crucifera* continued to show no apparent or statistical effect of either aging or fixation. For *A. aurantia*, there were greater differences between fresh and fixed droplet volumes for threads that were stored for longer periods although this difference was significant only at 90% RH for threads stored 22 days or longer. This effect of post-fixation droplet aging in *A. aurantia* was supported by a regression of Log_N droplet volume at 90% RH against droplet age (Fig. 4).

The fixed droplets of both *A. aurantia* and *N. crucifera* failed to extend (Table 3). However, as judged by axial line deflection, fixed droplets generated as much adhesion as fresh droplets. The axial line deflection profile of *A. aurantia* shows that the force on extending droplets decreases rapidly during early extension while that of *N. crucifera* shows a smaller decrease in force (Fig. 5). The difference in these profiles reflects the greater hygroscopicity of *A. aurantia* droplets and the lower viscosity of its glycoprotein at 90% RH (Opell et al. 2013; Stellwagen et al. 2014). However, in neither species is there evidence of a switch in adhesive force that would suggest that fixed droplets are relying only on capillary adhesion and fresh droplets on glycoprotein adhesion. Instead, the increasing deflection angle of *A. aurantia* droplets can be attributed to the reduced cross sectional area of its lengthening glycoprotein filament.

DISCUSSION

Our experimental treatments were clearly more extreme than the conditions normally experienced by an orb-web. From this perspective, our study documents the robustness of a viscous thread's hygroscopic system and indicates that the effectiveness of viscous threads even in species like *A. aurantia*, which occupy exposed sites, will decline little over the course of a day.

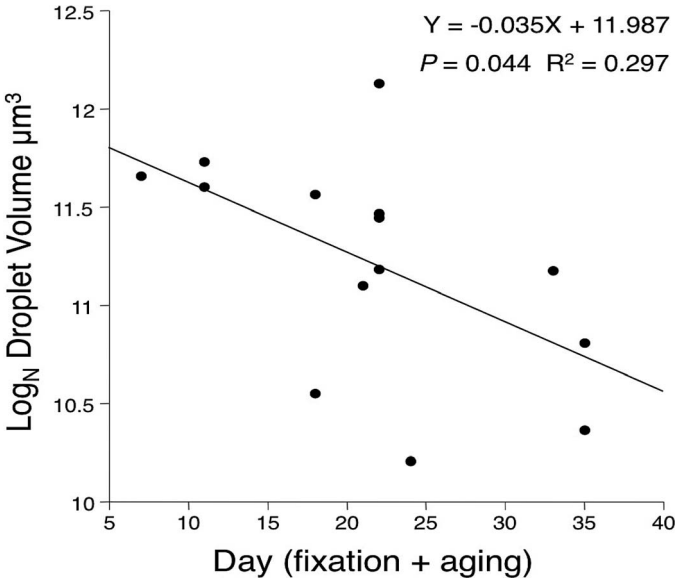

Figure 4.—Regression of the combined effects of fixation and aging on *A. aurantia* viscous thread droplet volume at 90% RH.

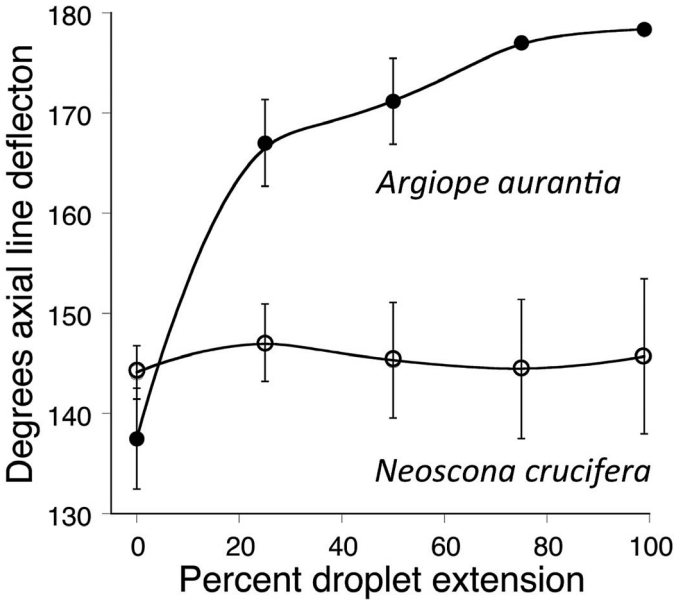

Figure 5.—Axial line deflection profiles for fresh *A. aurantia* and *N. crucifera* droplets. Increasing angle is associated with a decrease in force on a droplet. Error bars are ± 1 standard error. For *A. aurantia*, the points hide the error bars of 75% and 99% extensions.

In contrast to *A. aurantia* viscous droplets, neither fixation nor aging affected the hygroscopicity of *N. crucifera* droplets. This finding supports the hypothesis that hygroscopic viscous threads spun by spiders that occupy low humidity habitats contain more interactive LMM that are more easily degraded. However, in *A. aurantia*, this decrease in droplet hygroscopicity was only statistically significant at 90% RH where greater droplet volume probably makes this difference easier to detect. In this species, the difference between fresh and fixed droplet volume at 90% RH did not become significant until threads were aged for more than 22 days, suggesting aging contributed as much or more to LMM degradation as did fixation. The less stable compounds in *A. aurantia* droplets probably include the more hygroscopic compounds like choline and *N*-acetyltaurine. Unfortunately, compounds in *N. crucifera* threads have not been characterized, so a comparison with *A. aurantia* threads is not possible.

As hypothesized, fixation, which was probably associated with protein crosslinking, caused the glycoprotein cores of both species' threads to lose their extensibility. However, we failed to find support for the hypothesis that fixing droplets affected the adhesion of individual thread droplets, as judged by axial line deflection. Moreover, in neither species is there evidence of a transition from a much weaker pre-extension adhesion (that might be attributed to capillary force) to a greater extension adhesion generated by glycoprotein glue. Instead, glycoprotein adhesion appears to be established at droplet contact and to remain the dominant force as fixed droplets pull-off and fresh droplets extend.

The retention of adhesion in the absence of extensibility observed in fixed droplets has important implications for the adhesion of both viscous thread spans and individual droplets. As the adhesion of a viscous thread span relies on a suspension bridge mechanism that recruits adhesion from multiple droplets (Opell & Hendricks 2009), the loss of droplet extensibility would eliminate the recruitment of adhesion from more than a very few droplets and greatly reduce the adhesion of a thread span. The expression of adhesion by stiff fixed droplets that were incapable of extension indicates that the adhesive regions or side chains of these molecules remain exposed and are not folded inward, only to be exposed upon the contact of highly plasticized glycoprotein. This is consistent with findings that even at 0% RH where glycoprotein was not softened by water, viscous droplets adhered to surfaces, albeit at a much lower level than did fresh threads (Sahni et al. 2014).

ACKNOWLEDGMENTS

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Table 3.—Extension and pre-extension angle of thread support line of fresh and fixed threads at 90% RH. (Sample size), Mean ± 1 standard error, N = normally distributed.

	Argiope aurantia		*Neoscona crucifera*	
	Fresh (14)	Fixed (14)	Fresh (16)	Fixed (16)
Extension µm	4,266 ± 557	No extension	3,941 ± 837	No extension
Pre-extension angle °	139.3 ± 3.9 N	137.5 ± 5.0 N	143.2 ± 2.5 N	137.6 ± 4.8 N
	MP $P = 0.7532$		MP $P = 0.3606$	

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