

Putative microbial defenses in a social spider: immune variation and antibacterial properties of colony silk

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Abstract. The accumulation of microbes in and around the large, perennial nests of social arthropods can increase the potential for interactions between individuals and harmful pathogens. Accordingly, many social insects utilize multiple organizational lines of individual and collective defenses against microbes. The interaction between microbes and social spiders, however, has been almost entirely unexplored. Here, we use the social spider *Stegodyphus dumicola* Pocock 1898 (Araneae: Eresidae) to (1) probe how innate immunity varies among individuals and (2) determine if two types of silk extracted from their colonies can inhibit the growth of the entomopathogenic bacteria *Bacillus thuringiensis*. Individual spiders' innate immunity against lyophilized cells of *Micrococcus luteus* varied negatively with their boldness, a behavioral metric important for individual foraging and the organization of collective behaviors. Further, silk from both the capture webs and retreats of uncontaminated colonies inhibited the growth of *B. thuringiensis* to a small degree. Thus, web construction might represent a form of collective anti-microbial defense in these social spiders. This preliminary evidence suggests that social spider societies may exhibit antimicrobial defenses on multiple levels of organization, including both individual- and group-level defenses.

Keywords: Antibacterial defense, *Bacillus thuringiensis*, immunity, silk, *Stegodyphus dumicola*

Associations with microorganisms (mutualistic, benign, or harmful) far exceed in frequency any of the other species interactions with which a social animal may be challenged (Ezenwa et al. 2012; McFall-Ngai et al. 2013). Individuals can reduce their likelihood of infection via behavioral (Meyling & Pell 2006), dietary (Singer et al. 2009), or physiological defenses. However, defenses of the arthropod innate immune system can be extremely metabolically costly and social arthropods often exhibit decreased individual-level defenses relative to solitary species (e.g., Freitak et al. 2003; Gwynn et al. 2005; Jacot et al. 2005; Evans et al. 2006). Social insects compensate for these costs, along with their increased risk of disease outbreaks, with a collective “social immunity” via a network of cooperative social interactions (Wilson-Rich et al. 2009; Cremer et al. 2007; Cremer & Sixt 2009). In addition to external sources of pathogens, the physical nests that social arthropods construct can themselves harbor large microbial loads (Hart & Ratnieks 2001; Rosengaus et al. 2003; Chapuisat et al. 2007; but see Wagner et al. 1997). For example, the webs of social spider colonies are often littered with old and fresh prey carcasses (Ward & Enders 1985; Tietjen 1986; Rypstra & Tiley 1991), potentially producing a hotbed for microbial growth (Okafor 1966; Tietjen 1980; Tietjen et al. 1987). How, then, would these seemingly less organized, non-eusocial arthropod societies cope with the increased microbial biota in their nests?

Much of the history documenting the behavioral interactions between spiders and microbes concerns hygienic and sanitary behavior. Hygienic prey carcass-removal behavior has been observed in two African social spiders, *Agelena consociata* Denis 1965 (Furey & Riechert 1989) and *Stegodyphus dumicola* Pocock 1898 (Soydaner 2013). Additionally, the Central American communal mesh-weaver, *Mallos gregalis* (Simon 1909) (Tietjen 1980) and the solitary crab spider *Misumena vatia* (Clerck 1757) (Morse 2008) exhibits sanitary excretory behavior away from their silk, which has been suggested as a means to avoid fouling. These examples illustrate a considerable parallel between the sanitary behavior

of social spiders and that of most social insect societies (López-Riquelme & Fanjul-Moles 2013). Conversely, it also appears that *M. gregalis* actually captures new prey that are attracted to the odor produced by yeasts growing on old prey carcasses in the nest (Tietjen et al. 1987), suggesting a positive interaction between some colony-associated microbes and the host spiders. Most important to our understanding of the survivorship of social spider colonies are the descriptions of colony-wide fungal growth in extinct colonies of both old-world and new-world social spiders (Henschel 1998; J.N. Pruitt, pers. comm.). Although it is yet unknown whether fungal epizootics actually cause these colony collapse events, or whether the fungal growth occurs post-mortem, our more detailed understanding of fungal pathogens in non-social spiders suggests these microbes could similarly be important enemies of social spiders (reviewed in Evans 2013). Together, these varied cases demonstrate that more studies are needed to better characterize the nature of social spider/microbe interactions and the mechanisms by which these interactions are carried out.

The general spider immune system has been described in detail (Kuhn-Nentwig & Nentwig 2013). It is similar to the innate immune systems of insects, characterized by a broad defense with limited specificity (Kuhn-Nentwig & Nentwig 2013). Its function is centralized around hemocytes that combat invading cells via phagocytosis, encapsulation, melanization, and the constitutive production of antimicrobial peptides (Fukuzawa et al. 2008; Kuhn-Nentwig & Nentwig 2013). Further, general measures of individual immunocompetence can vary predictably with behavioral traits, as has been described both within and among wolf spiders populations (Ahtiainen et al. 2004, 2005). Recently, González-Tokman et al. (2014) described cuticular antifungal substances in the subsocial crab spider *Diaea ergandros* Evans 1995. Additionally, given that spiders' association with silk encompasses nearly all aspects of their life history (Brunetta & Craig 2010), arachnologists have long speculated that silk may itself contain antimicrobial properties. Recently, Wright &

Goodacre (2012) discovered that silk produced by the common funnel-weaver, *Tegenaria domestica* (Clerck 1757), inhibits the growth of the gram-positive *Bacillus subtilis* in a bacteriostatic fashion. It follows, then, that the webs of social spiders could also exhibit antimicrobial properties, if their silken colonies are indeed at a high risk of accumulating microbes. Here, in a purely exploratory fashion, we probe individual immunity variation and silk-based antibacterial defenses in *S. dumicola*.

METHODS

Animal and bacteria collection.—We collected *S. dumicola* colonies in the southern Kalahari Basin, South Africa, along roadside fences and in *Acacia* bushes. This social spider lives in highly female-biased, inbred social groups of a few dozen up to several hundred individuals (Henschel et al. 1995). Their colonies contain two main functional units: a dense three-dimensional webbed retreat where spiders reside and a cribellate-silk capture web where spiders encounter, capture, and consume prey items (Peters 1992). We collected bacteria from spider cuticles *in situ* by wiping a sterile cotton swab across the entire carapace of one haphazardly chosen adult female spider in each of 20 different colonies in the field. We then plated these samples directly onto lysogeny broth (LB) agar. We did not collect from more than five colonies within 10 km. These plates were incubated at 35°C for 24 h and then stored at 4°C. After bacteria were collected from this subset of spiders, spider colonies and bacterial samples were transported back to the University of Pittsburgh. Individual bacterial colonies were isolated and re-plated several times. After spiders were extracted from their colonies, we measured their body mass (g) using a digital scale immediately before experimentation. Spiders were isolated in 30 ml plastic cups with a piece of chicken wire to facilitate web-building, maintained at ambient temperature and natural light:dark cycles, and fed a diet of one 2-week old domestic cricket weekly. Two spiders from each source colony were used in the immunocompetence assay.

Identification of cuticular bacteria.—Bacterial identification was performed with 300bp 16S ribosomal DNA sequencing and MicroSeq® BLAST Software (SeqWright Genomic Services, Houston, TX 77054). We identified the common gram-positive soil bacterium *Bacillus thuringiensis* on spiders from two colonies approximately 50 km apart. Many common strains of *B. thuringiensis* are generalist entomopathogens (Aronson et al. 1986). We grew *B. thuringiensis* continuously on LB agar plates and maintained them at 4°C. For full BLAST report see Supplemental Material S1, online at <http://dx.doi.org/10.1636/M15-12.s1>.

Behavioral assays.—To determine if immunocompetence in *S. dumicola* varies with the spiders' behavioral tendencies, we first tested each individual's "boldness" (Sloan Wilson et al. 1994), defined as their latency to resume activity after an aversive stimulus, by simulating the approach of an avian predator (Riechert & Hedrick 1993; Lohrey et al. 2009; Pruitt & Riechert 2012). This behavioral metric is highly repeatable in *S. dumicola* (repeatability ~ 0.63; Keiser et al. 2014a, b) and is positively associated with an individual's propensity to initiate prey capture events (Pruitt & Keiser 2014). We placed spiders ($n = 42$) in clear plastic arenas (diameter = 12 cm),

allowed them a 60 s acclimation period, and administered two rapid puffs of air to the spider's anterior prosoma using an infant nose-cleaning bulb. We then measured the latency for spiders to resume activity (i.e., to move at least one full body length). Trials were terminated after 600 s, and we used the inverse of their latency to move as a metric of their "boldness" (600 minus latency to move, in sec). That is, individuals with long latencies to resume movement had a lower boldness score while individuals that resume movement rapidly have a higher boldness score. Boldness assays were performed between 0900 and 1100 h.

Individual immunocompetence.—We conducted a lytic response assay to estimate the concentration of antimicrobial peptides in the spiders' hemolymph (following the protocol of Ahtiainen et al., 2004, 2005, 2006). We anaesthetized 42 adult female *S. dumicola* with CO₂ and extracted a 0.5 µl sample of hemolymph from a puncture directly posterior to the epigastric furrow using a sterilized Hamilton syringe. Hemolymph samples ($n = 42$) were mixed with 20 µl of 0.01M phosphate buffered saline (PBS), vortexed at 1500 rpm for 3 s, and frozen at -80°C. Thawed samples were vortexed at 1500 rpm for 3 s and pipetted into a 96-well flat-bottom microplate. Then, all samples were mixed with 80 µl of a 0.20 mg/ml PBS solution of lyophilized *Micrococcus luteus* cells (Sigma Chemical Co.; St. Louis, MO) and vortexed at 1500 rpm for 3 s. We then measured the samples' optical density at 492 nm immediately after mixing and again after 10 minutes using a BioTek Microplate reader (BioTek US, Winooski, VT). The strength of the immune response is determined by the magnitude by which the optical density of the solution decreases over this time. Frozen and thawed hemolymph was used as a control ($n = 10$). Due to an increase in optical density via sedimentation of the hemolymph suspension, the mean amount by which control samples increased in optical density (change in optical density = 0.0043) was subtracted from the observed values of experimental samples.

Antibacterial properties of silk.—We placed 10 adult female spiders originating from the same source colony into a clean 500 ml plastic cup with a piece of sterilized chicken wire to facilitate web construction ($n = 12$ experimental colonies from 12 different source colonies). These spiders were not fed in these cups, and they remained sealed for one week to allow spiders to produce new silk in an environment uncontaminated by prey or prey remains. We tested this silk for antibacterial properties against a strain of *B. thuringiensis* collected from the cuticle of an adult female *S. dumicola in situ* (described above). We created a lawn of bacteria on a petri dish containing LB agar by applying 20 µl of a liquid bacterial culture of *B. thuringiensis* grown in LB broth at 35°C for 24 hours and spread the solution evenly using a sterile inoculating loop (Thermo Fisher Scientific Inc., Waltham, MA). This was performed immediately prior to applying the spider silk.

We used sterilized forceps to wrap a thin layer of silk around a sterile filter paper disk (diameter = 6 mm), dipped it in ethyl acetate, an organic solvent commonly used for extractions of antibiotics (Dutia 2004), and placed it directly onto the surface of the agar, leaving the silk strands intact. Preliminary experiments suggest that ethyl acetate itself does not have antibacterial activity against *B. thuringiensis* (unpubl.

data). However, to test whether any antibacterial activity observed was due to the structural properties of the silk, or one of its chemical constituents, we also mixed separate silk samples with 0.5 ml of ethyl acetate and vortexed them for 15 s. We then dipped filter paper disks in this solution and applied the disks to the agar surface as before. Control disks were dipped only in ethyl acetate. We performed this assay with both silk types that are used in the construction of *S. dumicola* colonies: (1) non-sticky retreat silk and (2) capture web cribellate silk ($n = 15$ disks per treatment allocated across 15 petri dishes). Each petri dish contained 6 different disks: (1) vortexed capture silk, (2) vortexed retreat silk, (3) intact capture silk, (4) intact retreat silk, (5) ethyl acetate control, and (6) an untreated filter paper disk control. The petri dishes were incubated at 35°C for 24 h, after which we then measured the annular radius of the zones of inhibition around the disks.

Statistical analyses.—We used non-parametric Spearman's rank correlation to test the relationships between individual boldness, body mass, and immunocompetence (i.e., hemolymph lytic activity). To test the effects of spider silk on bacterial growth, we used a general linear mixed model with treatment as an independent variable and the radius of the zone of inhibition as the response variable. Source colony ID and treatment nested in agar plate ID were included as random effects. All statistical analyses were performed in JMP version 10 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Individual immunocompetence.—Hemolymph collected from spiders with a greater measurement of boldness exhibited a weaker immune response when compromised with the bacteria *M. luteus* (mean change in optical density = -0.01 , SE = 0.005; Spearman's $\rho = 0.33$, df = 40, $P = 0.04$; Fig. 1). Although boldness was negatively correlated with body mass (Spearman's $\rho = -0.44$, df = 40, $P = 0.009$), our measure of innate immunity only showed a non-significant positive trend with body mass (Spearman's $\rho = -0.30$, df = 40, $P = 0.08$).

Antibacterial properties of silk.—Filter paper disks wrapped with intact silk inhibited the growth of the bacteria *B. thuringiensis* while disks dipped in a silk-ethyl acetate solution were not significantly different from the control treatment ($F_{4, 89} = 7.23$, $P < 0.0001$; Fig. 2). Intact spider silk produced zones of inhibition, though relatively small on average, over four times larger than any other treatment type. There was no difference in the antimicrobial activity of silk originating from two different parts of the colony, the capture web vs. the retreat (Fig. 2).

DISCUSSION

Interactions between microbes and hosts by and large represent the most common ecological interaction in which any animal or plant participates. Large, stable animal societies must therefore exhibit antimicrobial defenses across multiple levels of organization (e.g., individual and collective defenses). Here, we demonstrated that individuals' innate immune responses to lyophilized bacterial cells varied negatively with their boldness in the social spider *S. dumicola*. We also demonstrated that two functionally different silks used to construct *S. dumicola* colonies can weakly inhibit the growth of the gram-positive entomopathogen *B. thuringiensis*.

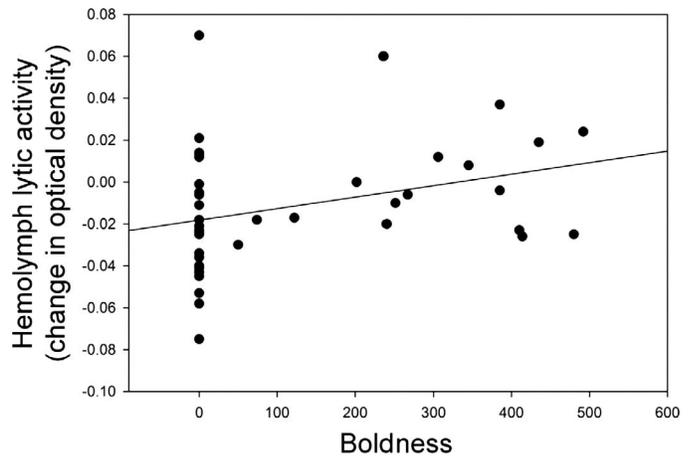


Figure 1.—Individuals with greater boldness values (i.e., those that resumed activity faster during our boldness assay) were associated with decreased lytic activity relative to shy spiders, suggesting a weaker immune response (Spearman's $\rho = 0.33$, df = 40, $P = 0.04$). Lytic activity values below zero indicate a decrease in optical density over the sampling interval, suggesting a stronger lytic activity.

We identified a negative relationship between individual boldness and innate immune defense. Many studies have demonstrated that individuals with higher boldness or more pronounced behavioral traits (e.g., sexual advertisement, anti-predator behavior) have a weaker investment in immune defenses (Rigby & Jokela 2000; McKean & Nunney 2001; Roberts et al. 2004; Ahtiainen et al. 2005; Kortet et al. 2007; Niemelä et al. 2012). This trade-off between individual immunity and other traits like anti-predator behavior should be expected based on traditional life history theory (Stearns 1992; Norris & Evans 2000; Zuk & Stoehr 2002). However, the magnitude by which individual immunocompetence and other behavioral traits are negatively related should be contingent

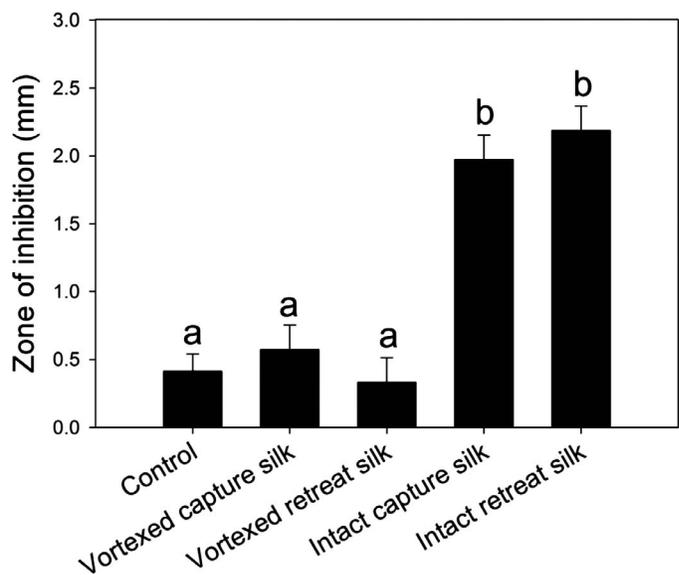


Figure 2.—Intact spider silk from both the capture web and retreat significantly inhibited the growth of *B. thuringiensis* ($F_{4, 89} = 7.23$, $P < 0.0001$). Bars significantly different from one another are denoted with different letters (Tukey's HSD post-hoc test).

on whether or not the behavior of interest is indeed metabolically costly (Norris & Evans 2000).

Alternatively, *positive* relationships between measurements of immunity and behavioral traits like social dominance (Zuk & Johnsen 2000; Ahtiainen et al. 2006) and resource holding potential (Koskimäki et al. 2004) have also been documented. The mechanistic underpinnings of the boldness/immunity relationship in this system are entirely unknown. Although we found no relationship between body mass and immunity, the relationship between immunocompetence and nutritional gain/feeding rate should be explored further (Chandra 1983; Saino et al. 1997). Bold individuals initiate more foraging events (Pruitt & Keiser 2014) and are the first to begin consuming the subdued prey item, thus gaining the greatest nutritional benefit from the foraging bout (Amir et al. 2000). Perhaps, though, this investment in extra-oral digestion is metabolically costly and is related to other systems of innate immunity (Haerberli et al. 2000). Further, we observed broad variation in immunity among individuals that received a boldness value of 0, potentially because the 600 s cutoff for our boldness assay truncates the data set and does not account for variation among individuals beyond that point. Future studies on the relationship between behavioral traits and immunity should allow for a larger window of measurements.

It should be particularly profitable to investigate both social and solitary species of *Stegodyphus* in unison to test the hypothesis that individual investment in immune defenses increases with sociality or perhaps that among-individual variance in immunity is greater in social species (Wilson et al. 2003). *Stegodyphus* spp. represent a promising case to test within-colony or within-family pathogen transmission, as adult females are consumed by their offspring at the end of their lives (Seibt & Wickler 1987; Schneider 2002; Salomon et al. 2005; Ruch et al. 2012). Given that pathogen transmission is commonly regarded as a selective force against cannibalism, especially among kin, the role of this pivotal life-history event in colony health is particularly intriguing (Pfennig 1997; Pfennig et al. 1998).

Just like the way many social insects collect and phylogenetically treat their colonies with antimicrobial substances (Christe et al. 2003; Chapuisat et al. 2007), social spiders may similarly be able to reduce microbial growth via the antibacterial properties of their silk. Our results indicate that intact silk limits bacterial growth while ethyl acetate impregnated with silk had no antibacterial properties. Wright & Goodacre (2012) demonstrated that silk treated with Proteinase K had a reduced antimicrobial ability, suggesting that one or more protein elements are involved as active agents. Here, the lack of antibacterial activity in the vortexed silk may have been a result of a dilution in the concentration of products exhibiting the antibacterial activity. Sanggaard et al. (2014) recently discovered ~132 proteins in the silk of the social spider *S. mimosarum* Pavesi 1883, which is a key step to identifying the causative agents of antimicrobial activity.

The zones of inhibition produced around the silk-treated disks were relatively minor compared to the inhibition of other *Bacillus* spp. by commercial antibiotic extractions (Coonrod et al. 1971). We are unsure if this is because of the actual inhibitory nature of spider silk or a low concentration of

antibacterial properties due to our extraction methods. Regardless, this research provides an important step towards understanding collective antimicrobial defenses in social spiders, a group wholly ignored in the ecoimmunology literature. Future studies should employ dilution techniques to determine the minimum inhibitory concentration (i.e., mass of silk) necessary to inhibit bacterial growth, and attempt to identify the causative antimicrobial agents via proteomic methodologies.

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