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CANNIBALISTIC SOCIETY: STUDYING SOCIAL BEHAVIOR AND LEARNING UNDER
HIGH SOCIAL RISK

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CANNIBALISTIC SOCIETY: STUDYING SOCIAL BEHAVIOR AND LEARNING UNDER
HIGH SOCIAL RISK

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Abstract

Risks of sociality, including competition and conspecific aggression, are particularly pronounced in venomous invertebrates such as arachnids. Spiders show a wide range of sociality, with differing levels of cannibalism and other types of social aggression. To have the greatest chance of surviving interactions with conspecifics, spiders must learn to assess and respond to risk. One of the major ways risk assessment is studied in spiders is via venom metering, in which spiders choose how much venom to use based on prey and predator characteristics. While venom metering in response to prey acquisition and predator defense is well-studied, less is known about its use in conspecific interactions. In Chapter 1, I discuss that due to the wide range of both sociality and venom found in spiders, they are poised to be an excellent system for testing questions regarding whether and how venom use relates to the evolution of social behavior and, in return, whether social behavior influences venom use and evolution. I focus primarily on the widow spiders, *Latrodectus*, as a strong model for testing these hypotheses, and my hypotheses in Chapter 2. In Chapter 2, I test for the presence of aversion learning, a risk-management tool, in *Latrodectus mactans* juveniles. Although no aversion learning was evident from the results, there were potential signs of other, nonassociative learning types. The results indicate that aversion learning is not utilized by juvenile *L. mactans* to reduce the risk of cannibalism in the social environment, leaving the question of how *L. mactans* maintains sociality while mitigating risk open. Given that successful responses to risk are vital for maintaining sociality, comparative analysis of spider taxa in which venom metering and sociality vary can provide valuable insights into the evolution and maintenance of social behavior under risk.

Chapter 1: Venom and social behavior: the potential of using spiders to evaluate the evolution of sociality under high risk

1. Introduction

Many social species benefit from cooperation, though there are an assortment of risks associated with social behaviors, such as disease spread ¹, predation threats¹, and conspecific competition ^{2,3}. The definition of sociality fluctuates, but Wilson's definition of sociality as the tendency of conspecifics to live cooperatively in groups is currently widely accepted ⁴. This paper will consider social behavior as any type of interaction between or among conspecifics. Conspecific competition over resources such as food and mates can result in anything from mild inconvenience to the death of the individual ^{5,6}.

Many species deal with social risk through engaging in multiple social interactions and adjusting their responses accordingly. There are many different mechanisms involved in this behavioral modification. When an individual "loses" an agonistic social interaction, hormonal changes in the brain may cause changes in behavior that make the individual more likely to lose in a future interaction [4]. Likewise, the "winning" individual experiences physiological changes that may result in this individual more likely to continue to win in future interactions, a phenomenon known as the "winner/loser effect"⁷. These mechanisms can help to establish social hierarchy and/or to create a cohesive social environment.

Sociality's delicate balance of risk and reward is present in invertebrates as well as vertebrates, and some of the most highly studied cases of invertebrate sociality include the eusocial invertebrate systems such as order Hymenoptera (bees, wasps, ants, etc.). These eusocial organisms live in a highly specialized caste system, with only one colony member reproducing and others raising the offspring ⁸. Eusocial individuals can recognize members of

their colony⁹, communicate with members of their colony¹⁰, and can learn from observing other colony members^{11–13}. Eusocial species also engage in collective defense of the colony against external threats. It has been hypothesized that the key to the evolution of eusociality is partially in this defense, through the evolution of venomous stingers¹⁴. This hypothesis has received some support via the scarcity of non-venomous eusocial hymenopterans and presence of non-venomous solitary hymenopterans¹⁴, and termites are both non-venomous and eusocial. Another intriguing piece of evidence for the correlation between venom and social evolution in invertebrates is the evolution of immune-attacking secretions in eusocial aphids, which have many venom-like properties and could be a precursor to venom¹⁵.

While Hymenopterans are widely lauded as model systems for the study of the evolution of sociality, one difficulty in using this group for comparative studies on the evolution of social behavior lies in the few independent origins of sociality, with only 7-8 instances across all social hymenopterans¹⁶. Spiders, like social insects, show the nearly the full range of social categories, and sociality has been estimated to have evolved independently 15-16 times¹⁷. Recent comparative genomic work suggests intriguing convergence of molecular evolution in the development of sociality¹⁸. As the majority of spider groups are venomous, this group provides a promising foundation for examining potential links between the evolution of venom and sociality in concert with Hymenopteran studies. Yet, while highly social spiders are well-studied, we know little about the properties of their venom, whether/how its molecular components and function differ from less social spiders, and whether spider venom components and/or function change over evolutionary transitions.

Researchers have proposed an index of sociality across four broad categorical levels: solitary, subsocial, social, and eusocial¹⁹. This way of categorizing sociality qualitatively

categorizes species based on various behaviors and aspects of natural history¹⁹. Although this categorization is based on a linear continuum or stair-step model, this is not necessarily the case in nature, as some of the different “levels” could actually occur across separate evolutionary directions. For example, subsociality could be an evolutionary end and not just a step on the way to eusociality²⁰. While this categorization accurately describes many species, many others fall between these categories, or show indications of sociality that may not match specifically the descriptors that make up the category definitions¹⁹. These in-between species are often less researched than their counterparts, but may represent important transitions in the evolution of social systems.

One group of organisms that lies between categories is the widow spiders (*Latrodectus* spp.). Members of the genus *Latrodectus* are considered facultatively social, live in large juvenile groups from hatching through two weeks following emergence from the egg sac, and adult females aggregate outside of the breeding season [19, 32]. Group living in these systems is dependent on nutritional state, prey availability, and population density [32]. Communally living juveniles and females frequently cannibalize their conspecifics, including kin^{21,22}. To survive in this high-risk social environment, *Latrodectus* individuals must recognize social risks and alter their behavior in response. While individuals in many taxa adjust their responses based on outcomes of social interactions²³, we understand little about the social ecology of venomous, cannibalistic species such as *Latrodectus*, where negative interactions could result in death. These extreme consequences, together with *Latrodectus*’s facultative sociality, make *Latrodectus* an excellent system to study responses to social risk in comparison with genera having more highly social spider systems (see section 2 for examples). By exploring how individuals respond to social risk in a high-risk environment where consequences are amplified, we can reach a

greater understanding of causes and consequences of social behavior. Additionally, members of this genus are highly venomous and lie between traditional stair-steps of social categories. This system, along with comparisons to other spiders, provides an excellent opportunity to test hypotheses relating the evolution of social behavior and venom. This review will focus primarily on non-eusocial arachnids and discuss the literature examining how cannibalistic, venomous taxa such as *Latrodectus* adapt to the inherent risks of social interaction, and how evolution of venom may be intricately intertwined with social behaviors.

2. Sociality in Arachnids

Arachnids are typically known for being solitary, but this assessment has key exceptions ²⁴. Juveniles of certain amblypygid species have been observed aggregating and associating with their mothers ²⁴, and some burrowing scorpions (*Heteromepstrus fulvipes*) have been witnessed dividing labor and sharing food ²⁵. Burrowing scorpion sociality possibly evolved due to the necessity of burrows for predator avoidance and protection from dehydration. Juvenile scorpions struggle with burrow construction, and sharing may increase survival ²⁶, suggesting an important role for juvenile social environment. In contrast to expected cannibalism among juveniles in the scorpions (See section 4.1.2 for more information on sibling cannibalism), no cannibalization was observed in the lab ²⁵.

2.1. Differences Between Solitary and Social Spiders

Sociality in spiders is divided into two main classifications²⁷: “cooperative” species, also referred to as “quasi-social”²⁸, which share duties such as care of young and foraging, and “communal” species, which reside in aggregations but undergo all duties alone and do not engage in maternal care ²⁹. More recent terminology classifies cooperative species as “non-

territorial permanent-social”, and communal species as “communal-territorial” or “territorial permanent-social”²⁹. While most spiders are considered solitary, there are at least 53 known species of communal-territorial spiders³⁰ and 25 of non-territorial permanent-social¹⁷. No social spiders have been identified as eusocial³¹. Sociality in spiders has numerous independent evolutionary origins¹⁷, likely associated with collective defense and hunting abilities³², but any concurrent changes in venom properties or use along with these evolutionary transitions remain understudied. Recent genomic comparisons among social, subsocial, and solitary spiders found mainly differences in genes associated with immunity and metabolism, supporting hypotheses relating social evolution in spiders to hunting and defense, but genomic and behavioral studies and data in spiders across varying social structures remain scarce¹⁸.

Although only certain species are labeled as “social spiders,” spiders, like other arachnids and other taxa, follow a spectrum of sociality not limited to just social spiders and solitary spiders. *Latrodectus*, for example, is not classified as a social spider genus by the aforementioned papers, but exhibits several known social behaviors. *Latrodectus* adult females are known to aggregate with juveniles and other females during the fall and winter, dispersing only in the spring and summer in time for mating season^{21,33}. Additionally, *Latrodectus* juveniles remain in groups for at least two weeks prior to dispersal and have been observed sharing food³⁴.

Both social and solitary spider taxa show evidence of kin recognition. Juvenile cannibalistic social spiders preferentially attack non-related juveniles over related juveniles in the lab, only cannibalizing the latter when the alternative is starvation^{35–37}. Even fully solitary wolf spiders³⁸ and amblypygids³⁹ are less likely to prey on kin than on other unrelated individuals. Evidence for kin recognition is seen in facultatively group living *Latrodectus* as well. Juvenile *Latrodectus*

are more likely to cannibalize nonrelated individuals in studies conducted in the lab, and sometimes will completely decline to cannibalize kin^{40,41}. Pheromones associated with silk have been found to play an important role in providing kin recognition cues in both social and solitary spiders^{42–47}. Males use silk pheromones to find females in many *Latrodectus* species^{48,49}, and therefore chemical cues seem like a promising mechanism for kinship recognition in this group, although this has yet to be formally tested. Alternatively, *Latrodectus* may not use chemical cues: developmental synchrony prevents cannibalism in *L. hesperus*, suggesting a pathway for familiarity over chemical kin recognition in forming cannibalism decisions⁵⁰. Untangling these non-mutually exclusive mechanisms can help shed light on cannibalistic decision-making and, ultimately, group dynamics in subsocial species.

Given the prevalence of kin recognition among cannibalistic social arachnids and some non-social arachnids, kin likely recognition contributes to the evolution of sociality in cannibalistic species. Interestingly, many colonies of social spiders are highly inbred, despite the evolutionary cost of inbreeding^{51,52}. Whether this is because of proximity to other colonies or a consequence of kin selection remains under discussion. However, interaction of any type between colonies is rare, increasing support for the role of kin selection in cannibalistic sociality⁵². Further research into the transition from outbreeding to inbreeding in social spiders could provide valuable insight into the mechanisms behind the evolution of sociality in cannibalistic and/or venomous species.

One potential differentiation between solitary and social spiders lies in venom properties. In Hymenoptera, the venom of solitary and social wasps differ both in content and use^{53,54}. Solitary wasps mainly use venomous stings for prey capture, stinging to paralyze. In social wasps, stings are used primarily for defense of the colony, providing evidence for the theory of venom evolution as a function of defense to facilitate the evolution of sociality⁵⁴. Unfortunately, there

are no comparative analyses of venom contents in social and solitary spiders. However, one intriguing example exists of a communal spider taxon that is entirely non-venomous⁵⁵, suggesting that venom may potentially have been lost in conjunction with the evolution of communal living. Whether venom in spiders is a pathway for evolution of sociality, or the lack of venom is an adaptation to sociality, the possibility of a connection between venom and social evolution remains an unknown but intriguing area of study.

3. Risk Assessment

Individuals must assess risk from predators, prey, and conspecifics, and then make decisions based on that assessment. Web-spinning and hunting spiders both learn to identify and react to potentially dangerous venomous prey, such as ants or wasps⁵⁶. Spitting spiders, for example, use venom both in hunting and for defense. They are risky to attack from the front due to their spitting behavior, in which they rapidly eject a mixture of venom, silk, and glue at the predator, prey⁵⁷, or even occasionally kin⁵⁸. Additionally, spitting spiders regulate their spit expenditure in response to more difficult prey or more risky situations, similar to how many other spiders regulate their venom expulsion (section 3.1)⁵⁸. However, contrary to the similarity of spitting expenditure to venom expenditure, and despite the spit containing venom components, the spit does not appear to have any toxic effects on invertebrates, despite previous thought⁵⁸. Some peptide components of the venom have been described, but no receptors have been identified as of yet, and the function of the known peptides remains unclear⁵⁹.

Spiders are known to behaviorally modify attacks based on risk assessment of prey. *Portia* jumping spiders, for example, will only attack spitting spiders from the back, with one key exception. If the spitting spider is carrying an egg sac, *Portia* spiders will attack it from the front, since it cannot spit⁶⁰, suggesting that *Portia* avoid the spitting defense despite venom from the

spit not having toxic effects on invertebrates [44]. Web spiders similarly adapt to risky prey, learning through experience to identify which vibrations on their web signify a high-risk prey, item such as an ant, and avoiding those vibrations ⁶¹. Although web-spinning spiders will occasionally catch risky (typically venomous) prey, in a field count only about 10% of prey caught by web-spinning spiders was classified as “risky”, e.g., orders Araneae (spiders) and Hymenoptera (wasps) ⁶². This low percentage is potentially due to learned avoidance behavior. While many risks that spiders face come from either predators or their prey, in social situations spiders face cannibalism from their conspecifics, placing them in the interesting position of being at once predator preying on risky prey *and* potential risky prey themselves. For sociality to be feasible in these cannibalistic taxa, spiders must be able to assess and respond to social risk. As risk assessment in both predator and defense contexts has been demonstrated across multiple spider taxa [50-52], it is possible that spiders engage in similar risk assessment in social interactions, providing the foundations needed to evaluate whether and how venom influences social behavior and vice versa.

3.1. *Venom Metering*

When faced with risks, spiders have a series of choices, typically to play dead, run, or attack [42]. Of these, attacking is potentially the most costly. A spider can attack with webbing or venom. Both of these are presumably costly to produce, and venom in particular is very complex in composition^{63,64}. In part because of these costs, venomous organisms typically meter their venom use, injecting only a minimal or optimal amount for each defensive or offensive encounter^{65,66}. For example, in lab-based studies, rattlesnakes of all ages injected more venom into larger prey than into smaller prey, after being exposed to prey of various sizes ^{67,68}. Spiders are no exception, also capable of venom metering. Juvenile *Latrodectus hesperus* spiders relied

primarily on evasive behaviors in response to predator cues in the lab, switching to more aggressive behaviors such as biting and presumed venom expulsion as they grew older and bigger, though it is not certain whether this difference was caused by age or size ⁶⁹. In the aforementioned *L. hesperus* study, spiders tested repeatedly as adults acclimatized to the predator cues, with more experienced spiders preferring more evasive behaviors than aggressive behaviors ⁶⁹. This indicates that both age/size and experience play a role in *L. hesperus* venom metering, with larger and less experienced spiders relying more heavily on combative behaviors than smaller or more experienced spiders. When *Latrodectus* spiders do bite, they often administer “dry bites” (bites without venom) when defending themselves against humans or other potential threats, and the amount of venom that they do inject is directly correlated with the threat level ⁷⁰. Some spiders such as hunting spiders (*Cupiennius salei*) adjust their venom based on olfactory cues and the behavior or perceived threat level of their prey, not simply the prey size ⁶³. Additionally, ontogenetic shifts in venom profiles and toxin abundance from juvenile to adulthood and after have been observed in tarantulas, directly relating to size ⁷¹, and some differences in venom composition have been shown to exist in juvenile and adult *Latrodectus* [69], although these differences have yet to be formally described. Despite these examples, few ontogenetic studies have been conducted in *Latrodectus* or closely related spiders ^{71,72}, representing a fruitful area of study for understanding the evolution and function of spider venom. Changes in venom composition during ontogeny may relate to behavioral changes, including venom metering. Taken together, these findings suggest that age, experience, and cue type may all influence venom metering in spiders across multiple contexts, such as prey capture, predator defense, and perhaps social interactions such as cannibalism.

4. Social risk

4.1. Cannibalism

Cannibalism is often associated with spiders, especially *Latrodectus*, and is one of the major risks that individual spiders face in social interactions. Cannibalism can occur between individuals of either sex and any age ^{73,74}. Adults and juveniles of some invertebrates gain nutrients by eating unhatched eggs, though adult females sometimes show preference for eggs laid by other females, possibly indicating kin recognition ⁷⁵. Conversely, mother spiders sometimes offer themselves up as a meal for their offspring, providing nutrients for the next generation in a process called “matrphagy” ^{76,77}. Old or weak spiders will also often end up as a meal for their younger, healthier relatives ^{76,78}.

Current research on arachnid venom metering focuses on responses to predator or prey stimuli, however, little is known about venom metering in cannibalistic interactions ⁶⁶. Cannibalism could easily be interpreted as interactions with prey or interactions with a predator, so it is difficult to draw inferences from current research. However, certain defensive or aggressive tactics such as increased venom or bites based on aggression level of the other spider likely still apply. For example, many cannibalizations occur with a larger spider attacking a smaller spider ⁵⁰. In this situation, the smaller spider may display defensive rather than aggressive strategies, resulting in less risk for the cannibalizing spider. The cannibalizing spider’s reaction to more aggressive strategies from the smaller spider is unknown, as there is little research on the details of how such cannibalizations outside of mating contexts occur. Understanding behaviors occurring during cannibalism can help shed light on the use and evolution of venom in response to conspecific behaviors, and whether and how they differ from standard foraging or defensive behaviors.

Research on cannibalism in spiders focuses nearly entirely on the cannibalistic behavior, and seldom addresses the use of venom, assuming that females (and other cannibalizing spiders) use venom during cannibalism as they would against prey. Conversely, in one species of spider, the male bites the female during courtship, paralyzing her to prevent any resistance ⁷⁹. The effect of this male spider's venom on the female indicates that spiders are susceptible to venom of their own species. However, venom is known to differ between male and female scorpions, and female paralysis in courtship might be simply a sexual adaptation ⁸⁰. More research is needed to determine the use of venom during cannibalism, and the effectiveness of venom on spider conspecifics, to allow testing of hypotheses regarding venom and evolution of social behavior.

4.1.1. Courtship and Mating

Risk assessment research in *Latrodectus* typically focuses on male mate choice, since sexual cannibalism occurs frequently. All species in *Latrodectus* engage in sexual cannibalism, but males invite cannibalism in only three documented species, *L. mirabilis* (Argentinian black widow) (L. Barrufaldi, *personal communication*), *L. hasselti* (redback spiders) and *L. geometricus* (brown widow spiders) ⁸¹. Males of the latter two species engage in a "somersault" behavior in which the males present their abdomen to the females, leading to the males' death and consumption ^{82,83}. Although this might seem to be the opposite of risk mitigation, self-sacrifice increases the time the female spends copulating ⁸⁴, which is directly related to paternity ⁸⁵. Despite the male's willingness, however, the female will not always consume the male, likely due to her hunger state ⁸⁶. It can be difficult for males to find mates, and so it is less risky for a male to give the ultimate investment to one female than to search for a second mating opportunity ⁸⁷. Because females disperse from their colonies and live solitarily during mating season ³³, males may or may not be able to find other females. The male can either take the risk

of cannibalism with the benefit of a guaranteed mate, and invest himself fully in the offspring from that mating, or he can try to find another mate, with the risk of dying before he can do so. In addition, the journey itself to find another female can be particularly dangerous. Field studies on both *L. pallidus* and *L. hasselti* indicate high mortality rates for journeying males ^{87,88}.

While males of self-sacrificial species do not appear to take action to reduce the risk of sexual cannibalism, many males in other species, such as *L. hesperus*, that are able to mate multiple times, will ⁸⁹. The first male to mate with a female is likely to get sperm precedence over males who mate with the female later ⁹⁰. To be the first male to mate with a female, males will sometimes mate with subadult females that are about to molt to adulthood, puncturing the exoskeleton above the newly-formed genitalia. Subadult females are less likely than adult females to engage in sexual cannibalism, for reasons that are currently unknown. Despite this reduced cannibalism risk, males do not appear to prefer subadult females over adult females, perhaps because of a lower production of pheromones by the subadults that could make them harder for males to find ^{91,92}. *L. hesperus* males are also capable of detecting female hunger, which may prevent cannibalization. Information about female hunger is transmitted through pheromones in the web ⁹³, and well-fed females tend to be more robust and larger than hungry females. In a laboratory study, *L. hesperus* males showed a clear preference for well-fed females ⁹⁴, potentially decreasing cannibalism risk as female hunger is correlated with cannibalism rates ⁸⁶. Moreover, among females and juveniles, larger individuals tend to cannibalize smaller individuals ⁵⁰, indicating different assessments used in different cannibalistic contexts. We were unable to find any studies discussing whether the females of any species use venom during these interactions, or if the use of venom differs between species where males invite cannibalism versus others. However, in *L. hasselti*, one of the species in which males self-sacrifice, males

will die within two days of copulation, even if they survive the female's initial attack⁸², either to envenomation or injury. Comparing venom use during sexual cannibalism across these species could provide important insights into how spiders use social context in venom metering, a potential first step for the evolution of more complex social interactions and structures.

4.1.2. Sibling Cannibalism

Another common type of cannibalism is sibling cannibalism. Even in many solitary species of spider, juveniles remain clustered on their mother's web, dispersing in later instars. If food is scarce, they will begin either matrophagy (feeding off their mother) or cannibalizing their siblings for nutrients. Cannibalism rate varies between different clutches and maternal lineages, but follows a few common guidelines^{40,41}. Cannibalization is typically size dependent, with larger spiders cannibalizing smaller, lower risk spiders. Accordingly, a low variance in spider size within a clutch will often decrease cannibalism rates^{50,95}. Additionally, juveniles in the field often only aggregate with their siblings, but in the lab juveniles preferentially cannibalize unrelated juveniles, providing evidence for kin recognition (section 2.1)³⁶. As we do not currently know how venom develops over ontogeny, it is unclear if smaller spiders are "lower risk" due to low production of venom, capability of venom metering, or if their smaller body hinders them in an aggressive encounter.

4.1.3. Avoiding Cannibalization

One way to mitigate the risk of cannibalism is communication. Invertebrates communicate using a mixture of several signal modes, including visual, seismic (vibratory), and chemical stimuli⁹⁶. Web-spinning spiders use their webs to transmit vibrational signals^{97,98}. These signals

can be used to establish territory, indicate defensiveness or aggression (preventing the need to use costly venom)⁹⁹, and to find mates. In addition to transmitting vibrational signals, *Latrodectus* webs contain sex pheromones. Volatile pheromones act as attractants for male *Latrodectus*, indicating the position of webs as well as information about the female¹⁰⁰. Once the male arrives on the web, he gains information from contact pheromones on the silk¹⁰¹. Contact sex pheromones also allow *Latrodectus* males to discriminate between webs of well-fed and hungry females, reducing their risk of cannibalism⁹³ (for more detail on sexual cannibalism, see Section 4..1). The transmission of both vibratory and pheromonal signals allow spiders to avoid risk, staying out of another individual's territory, or away from a hungry female. The presence of these communication methods suggest potential ways for spiders to mitigate costly venom use if they can communicate risk prior to social interactions.

4.2. Social Learning

Spiders learn and modify their behavior through social interactions. An example of social learning can be seen in *Schizocosa ocreata* and *S. royneri* (wolf spiders). Females of these species learn mating preference from exposure to males, with inexperienced females having no preference and experienced females preferring males with larger brushes¹⁰². Unfortunately, relatively few studies focus on learning in social spiders or other spiders like *Latrodectus*, although there are examples of early social environment influencing physiological, morphological, and behavioral traits later in life (developmental plasticity) [47]. For example, adult male *L. hasselti* resting metabolic rates differed when reared as juveniles with or without females and on different diets, resulting in differences in morphology and response to females¹⁰³. The presence of developmental plasticity encourages further theory and research regarding this and other mechanisms potentially related to learning in spiders. Given the high-risk social

system in *Latrodectus*, study of learning-related mechanisms could reveal new insights into how sociality is maintained in cannibalistic, venomous organisms and potentially shed light on the evolution of sociality in spiders.

One aspect of social learning in *Latrodectus* that remains unknown is the relationship between venom, cannibalization, and social learning. *Latrodectus* are known to cannibalize as juveniles, and it is often assumed that such cannibalization is venomous, though studies that address this specifically are scarce (section 4.1). However, there are many interesting observations that have never been formally studied. For example, in the laboratory, *Latrodectus* spiderlings have a much lower mortality rate if they are separated from each other after two weeks, instead of immediately after hatch (L. Gatch, N. Singh, pers. obs.). This could be merely because they are fragile before this time (though this seems unlikely as they are observed to move and build webs properly after transfer), or there could be a potential benefit of being around their siblings, even with cannibalization occurring. This enhanced survival rate may be related to the advantage of being able to share prey. However, there is another intriguing possibility. While there is some evidence that snakes may learn how to properly meter venom for attacking prey by gaining experience hunting as juveniles ⁶⁷, little is known about how spiders learn to meter their venom ⁶⁶. It is possible that the cannibalization of siblings is not simply a way of acquiring resources, but may allow juveniles to learn how to meter their venom on a more stationary target that does not require web use.

5. Conclusions

The relationship between venom and the evolution of complex social structures in invertebrates is an intriguing topic of study, yet has been primarily addressed in systems with complex, eusocial structures. Spiders, provide an excellent system to compare venom use and

evolution across taxa of varying social levels and social structures. Venom's widespread use among invertebrates and vertebrates, and its effects on defense and social behavior, make its presence and evolution a potentially valuable tool for studying both social and venom evolution in a broader context.

Chapter 2. No evidence for aversion learning in juvenile widow spiders (*Latrodectus mactans*)

Introduction

Across taxa, the ability to alter behavior based on past experience (learning) benefits individuals by allowing them to quickly and persistently modify their behavior in response to environmental changes and conditions^{104,105}. Learning can improve survival and fitness outcomes; for example, starlings learn to avoid sick individuals to decrease chances of infection¹⁰⁶, and problem-solving skills are connected with improved parental care and offspring survival in house sparrows¹⁰⁷. Therefore, learning across multiple contexts can have significant impacts on individual success, and different contexts may require different mechanisms of learning.

One type of learning that individuals use to adapt to variable situations is aversion learning, where a novel cue is paired with an aversive stimulus, resulting in changes in behavior. Many species learn to identify cues that could signal predation or other threats through this process. For example, chicks (*Gallus gallus domesticus*) learn to associate certain colors with unpalatable prey, which allows them to avoid prey that are potentially poisonous and therefore dangerous¹⁰⁸. In the other direction, lemon damselfish (*Pomacentrus moluccensis*) learn to identify predatory fish versus non-predators via chemical odor signals in the water¹⁰⁹. Through these mechanisms, organisms may identify different types of predators and prey that require different behavioral responses. This can have important effects on foraging efficiency and safety: for example, the orb weaving spider *Nephila clavipes* learns to identify risky prey based on vibrations made on the web and adjusts behavior accordingly³. However, while there has been significant interest in

understanding how aversion learning impacts foraging behavior and efficiency, less is known about aversion learning in other contexts – for example, in a social environment where conspecifics may either represent a cooperative opportunity, such as taking down larger prey than can be done individually, or a predation risk.

For aversion learning to occur, cues must vary enough that it is not practical for individuals to have innate responses to each cue, such as with generalist species adapting to many different prey species. While aversion learning is often studied in relation to predation, it may also play an important role in social behaviors. Social interactions can result in either positive or negative outcomes, and individuals will have different levels of risk associated with their interactions. Aversion learning can help individuals assess this risk and respond accordingly. When a social cue is associated with an aversive stimulus, this can lead to individuals adjusting their behavior to avoid future interactions, known as social aversion. For example, in Mongolian gerbils, social aversion can be experimentally induced when gerbils are paired with a conspecific and simultaneously injected with a mild toxin¹¹⁰. Consequences in social contexts can range from having food or territory stolen, to death from fighting or cannibalism. These risks and associated behaviors can also differ in species with different levels and approaches to social living. Because of this, social species offer unique insights into aversion learning.

One taxon that includes a wide range of sociality and social risk is class Arachnida, with species ranging from fully solitary to sharing food and labor in social contexts^{17,30,111}. In many arachnid species, a communal juvenile phase with juveniles dispersing in later instars is followed by a solitary adult phase. Here, living socially brings unique risks such as cannibalism that can make it difficult to differentiate between friend and foe. While we know that vertebrates such as birds

and mammals can navigate tricky social environments by learning to associate aversive cues with certain conspecifics^{106,110}, and that arachnids as well as vertebrates have mechanisms for kin recognition that can assist with social living¹¹², less is understood about how invertebrates navigate similar social contexts involving conspecifics and aversion. In this experiment, we examine aversion learning in a spider known for both high levels of cannibalism and social life stages, *Latrodectus mactans* (widow spiders). *Latrodectus* are one of several arachnids with high-risk social interactions, in which negative outcomes may include cannibalism¹¹³, but positive outcomes may include food sharing¹¹⁴. After hatching, juvenile *Latrodectus* stay clustered in their mother's web for about ten to fourteen days, fitting the definition of communal subsociality. During this time, the spiderlings share space and prey^{114,115}, and sometimes cannibalize each other as well¹¹³. Different egg sacs have differing rates of cannibalism¹¹³, leading to variation of social risk among familial groups. After the juvenile social period, juveniles disperse into the surrounding area, shifting from social to solitary. Here, we tested whether juvenile *Latrodectus* learn to associate an aversive stimulus (simulated attack) with a neutral vibrational cue during this social period. Although lack of short term plasticity in web structure is reported for *Latrodectus* adults¹¹⁶, there is potential for cryptic female choice⁴⁹ and venom metering decisions¹¹⁷, hinting at potential cognitive capabilities. Additionally, evidence of behavioral syndromes¹¹⁸ and memory¹¹⁹ support the higher cognitive ability that would facilitate learning. Initial lab testing showed potentials of behavioral plasticity and learning in adult widow spiders (pers.obs), but this would require higher sample size for more certainty. These experiments have been conducted in adult spiders, with no current research on learning in juveniles. We predicted that juveniles would respond differently to the presented cue after

training than before training, and that colonies (egg sacs) would differ in their behavioral response.

Methods

Spider collection and maintenance

Adult *Latrodectus mactans* females and egg sacs were collected from May-July, 2021 from local community sites in the Oklahoma City, Oklahoma area using 20-dram vials. After collection, spiders were transported to the University of Oklahoma.

Adult females were kept in clear circular 16 oz deli cups with sticks to provide structure for web-building. Females were fed one mealworm every 4 weeks, with an additional mealworm whenever they laid a new egg sac¹²⁰. All egg sacs collected in the field were labeled “field” and any egg sacs laid in the lab were labeled with the date they were laid. Females were checked Mondays, Wednesdays, and Fridays for new egg sacs. Egg sacs were kept in 20-dram vials, gently separated from the mother with forceps. After hatching, a subset of juveniles from each egg sac were separated into individual transparent 9-dram vials using a paintbrush within 48 hours and entered experimental assays 24 hours after separation. Spiders were kept solitarily, without social interaction, as this experiment focuses on the learning and cognitive ability of *Latrodectus* at this life stage, and not the specific responses to potentially hostile social interactions. Juvenile vials contained a single cotton swab for web-building structure. Due to natural mortality and differences in numbers of offspring produced from each egg sac, the final experimental sample size was 596 juveniles from 6 different egg sacs (Table 1). Of these egg sacs, 4 were collected from the field, 2 were laid in the lab.

Experimental assays

We conducted experimental assays over 11 consecutive days, separated into three phases: pre-test, training, and post-test (Figure 1). Spiders were randomly assigned to the control group or the aversive group upon separation. The neutral cue was a vibration administered via tapping the vial wall with the hard end of a paintbrush, and the aversive stimulus was a gentle poke with the soft end of a paintbrush, as spiderlings were too small to use vibration or shock stimuli^{121,122}.

The goal of the pre-test was to determine the spiders' base reaction to the paintbrush tap cue without any trained association. During the pre-test phase (days 1-3), once daily we opened the vial, tapped the outside of the vial wall ~2 cm from the spider with the hard end of a paintbrush, and recorded responses after the opening of the vial and after the paintbrush tap (neutral cue).

We recorded two binary response behaviors, 'web,' or whether the spider spun web, characterized by movement of legs without movement of body, and 'flight,' any movement of the spider away from the location of the paintbrush tap, two anti-threat behaviors commonly recorded in spiders¹²³. We initially recorded several other behaviors, but none were performed enough to analyze. We also recorded overall presence/absence of any response. After we recorded behavior, we closed the lid and replaced the vial. No aversive stimulus was used during the pre-test or post-test.

During the training phase (days 4-8), we opened the spider vial, tapped the outside of the vial wall ~2 cm from the spider with the hard end of a paintbrush (neutral cue), and then immediately either closed the top (control) or poked the spiderling with the soft end of the paintbrush (aversive stimulus), followed by closing the top. We chose a poke stimulus because, although it has been shown that a poke is less effective than a pinch stimulus for provoking response in

Latrodectus individuals, there is still a precedent for a poke stimulus provoking a response, and the size of the juveniles makes a pinch unfeasible¹¹⁷. We recorded the same response characters as in the pre-test phase both after the vial opening and the paintbrush tap (neutral cue).

During the post-test phase (days 9-11), we opened the spider vial, tapped the outside of the vial wall ~2 cm from the spider with the hard end of a paintbrush (neutral cue). No aversive stimulus was administered during this phase. We recorded the spider response after the opening of the vial (first response; supplemental Table S1) and after the paintbrush tap (second response; Table 1). We recorded first response to control for behavior caused by any vibrations caused to the vial or web by removing the lid of the vial. We report only second response here because the tapping of the vial is a novel cue to the spiderlings, unlike removal of the lid, since lids were removed each time the spiderling was fed. All spiderlings were fed twice per week (3-4 times during the trial) with one small *Drosophila*. A total of n=4 observers recorded behavior data.

Data analysis

We recorded data as binary for each day, describing whether or not an individual performed one of the recorded behaviors. For each individual, we calculated the proportion of days they performed each behavior in the pre-testing period (across days 1 - 3) and then the post-testing period (across days 9 – 11). We subtracted the resulting pre-test proportion from the post-test proportion, using these values for the response variables in our linear mixed models. The values range from -1 to +1, with negative values representing a decrease in the behavior from the pre-test to the post-test, and positive values representing an increase in the behavior from pre-test to post-test. We ran linear mixed models including treatment (control, aversive) as our fixed effect, and observer and egg sac as our random effects. We analyzed the fixed effect with a type III

ANOVA with Satterthwaite's method and analyzed the random effects with a RANOVA. Fit was determined based on a Shapiro-wilks test of normality. Due to natural mortality, not all individuals had recorded behaviors for all three pre- and post-trial days. Therefore, we could not include trial part (pre- vs. post-trial) as a fixed effect in our linear mixed model. Instead, difference between pre and post trial behavior proportions were analyzed via T-tests to measure whether and how behaviors differed between pre- and post-test. We analyzed treatments both separately and together; there was no difference and so we report combined results only.

All data were analyzed and visualized in R version 4.3.1, using packages lme4¹²⁴ and lmerTest¹²⁵. If juvenile widow spiders learn to associate the aversive cue with the neutral stimulus in this experiment, we expect to see a significant effect of treatment, in that juveniles that were exposed to aversive stimuli will differ in their behavior post-training from juveniles not exposed to aversive stimuli.

Results

We did not find a difference between treatments for any of our recorded behaviors (web: $F_{1,240.75} = 1.36$, $p = 0.24$, flee: $F_{1,259.51} = 1.77$, $p = 0.18$, overall response rate: $F_{1,200.32} = 0.44$, $p = 0.51$)(Fig 2; Table 2). We did not find a significant change in behavior between pre- and post-test for overall response rate ($t_{511} = -0.17$, $p = 0.90$)(Fig 2A). However, there was an effect of trial part (pre- vs. post-trial) for web spinning ($t_{511} = 10.70$, $p = 2.2e-16$) and fleeing behaviors ($t_{511} = -8.62$, $p = 2.2e-16$) (Table 3), such that spiderlings increased web-spinning and decreased fleeing in the post-test period (Fig 2B,C).

We found a significant effect of egg sac on all behaviors (overall response rate: LRT = 4.80, $p = 0.03$; web: LRT = 6.92, $p = 0.009$; flee: LRT = 7.35, $p = 0.007$) (Table 2).

Discussion

Aversion learning is widespread across animal taxa and may be important during repeated social interactions. However, when interaction between conspecifics may result in either positive or negative outcomes, it is unclear whether aversive learning will be expected to evolve as a result. Our results did not show support for general aversion learning in *Latrodectus mactans* juveniles but did support the presence of habituation and sensitization to cues, such that juveniles increased web spinning, a behavior associated with aggression, and decreased fleeing, a behavior associated aggression avoidance, in response to the neutral cue over time¹²³. We also found a significant effect of egg sac, and juveniles of the same egg sac showed similar behaviors regardless of treatment, supporting observations from previous studies relating to correlations between egg sac and behavior¹¹³. Altogether, our results suggest that maternal identity plays a larger role in behavioral change over time than aversive learning in *L. mactans* juveniles.

We did not find evidence for differences in behavioral change across treatments, suggesting no aversive learning occurred in our study. However, we did find evidence that behaviors changed over time. There are several, non-mutually exclusive explanations for this behavioral change: ontogenetic shifts in behavior, and nonassociative learning (habituation and sensitization).

Various behaviors, including aggression, change with age in both social and solitary spiders, correlated with a growth in size¹²⁶. In the social spider *Stegodyphus*, aggression increased over tests conducted as spiderlings passed through the first few instars, though it is not clear if this

increase was due to ontogenetic shift in behavior, or to experience¹²⁷. Our trials on *Latrodectus* juveniles were conducted within the first 15 days after hatch from egg sac, meaning that the juveniles were primarily in the 2nd instar during trials, with a few molting to third instar¹²⁸. We did not record behavioral differences between instars as there was not a big enough sample size of third instars to conduct a statistical analysis. Current evidence suggests that *Latrodectus* juveniles begin to shift in aggression starting in the 4th instar, and changing again in the 6th, with no significant difference in behavior between the second and third instars, and the fourth and fifth.¹²³ While we were not able to explicitly test between instars, our results do suggest changes over time in aggression even in these early stages, perhaps due to older, larger spiders having more capacity to use web as a defense, while younger, smaller spiderlings may have fewer protein resources and/or their silk too thin to be used in defense or offense. Future work detailing behavior within instars would shed light on how and why behavior changes over time in this species.

Nonassociative learning is often thought of as less complex and thus more common than associative learning¹²⁹, and both have been observed in invertebrates^{129–132}. Habituation, a decline in response to a repeated stimulus over time, and sensitization, an increase in response to a repeated stimulus over time, are two common forms of nonassociative learning. Our results show evidence of both sensitization and habituation, with spiders spinning web more as the experiment progressed, but fleeing from the cue less as the experiment progressed, showing a general shift from less aggressive behavior to more aggressive behavior. Overall behaviors performed did not change from the beginning to the end of the experiment, indicating that spiders, instead of increasing or decreasing reactions, instead switched from one reaction (running away) to another (spinning web, perhaps in preparation for either prey or predator

defense). These results suggest that over time, juveniles do change their response to a formerly neutral cue.

Egg sac played a large role in offspring response. There was significant variation among egg sacs, and egg sacs showed consistency in behavior regardless of treatment. This suggests that maternal effects (whether genetic or not) influenced offspring behavior and the extent to which offspring habituated or were sensitized to the stimulus. Previous research on *Latrodectus* juveniles has found that cannibalism rates varied significantly based on egg sac¹¹³. Taken together, it is possible that this variation in rates of cannibalism is tied to nonassociative learning and/or differences in timing of behavior shifts, and future studies identifying correlations between cannibalism rate and learning ability can begin to disentangle these effects. Altogether, our results suggest that maternal effects, and not aversive learning, have a strong influence on behavioral response of juvenile *L. mactans* over time. Future studies examining variation in learning and behavior shifts among familial groups could be a fruitful avenue for investigating genetic and environmental components of learning in *Latrodectus* and potentially other arachnids.

There are several potential reasons for the lack of aversion learning observed in this study. In *Latrodectus*, a negative encounter with a conspecific may result in cannibalism, and with few individuals surviving this interaction, aversion learning might not occur. The environment that the juveniles are in during the social period might also be stable and predictable, with cannibalism a common threat. If this is the case, any risk response might be canalized, with a specific behavior set for all individuals from hatch instead of learned. In addition, we cannot rule out that our aversive stimulus was not sufficient to elicit an aversive response. Previous research with adult *Latrodectus* shows a poke eliciting avoidance responses, but only rarely provoking a

specific aggressive response, release of venom. The release of venom was much more commonly witnessed after a pinch to body or leg¹¹⁷. Here, due to the size of the juveniles, a pinch was not possible. However, juveniles did respond to the paintbrush poke, suggesting it did elicit a negative response. Finally, we did not test aversion learning in a specific social context, as we kept individuals separated to both isolate this specific life stage and prevent cannibalism. Future work evaluating whether social context influences aversion learning in *Latrodectus* may provide insight into context specificity of learning across life stages. Whether juveniles have different thresholds for aggressive or aversive responses than adults remains unknown, but may play a role in changes in types of learning over development.

Invertebrates often use multimodal cues, taking information from vibrations, chemical cues, vision, and other sensory modalities^{133–138}. In organisms that use multimodal cues, conflicting cues can disrupt associative learning, as one cue may overshadow another and prevent response to the overshadowed cue¹³¹. *Latrodectus* is known to use primarily vibratory cues when communicating with conspecifics, in mating context and in situations such as in territorial rivalry contests between adult females, with the addition of some pheromonal chemical communication primarily seen in sexual courtship settings and mate choice^{139–141}. In this experiment, we used vibratory cues to mimic this, attempting to imitate spider interactions via association between a negative experience and a specific vibrational cue.

Although we did not find evidence of associative learning in this experiment, we did see potential evidence of nonassociative learning, specifically habituation and sensitization, although other causes of behavioral change are still a possibility. It is possible that either the cue we chose to use as our aversive cue was overshadowed by other cues, or the juvenile *L. mactans* social period does not have the factors needed for aversion learning to evolve. Aversion learning might

not be useful in cannibalistic contexts, because interactions that would produce an aversive response might not be repeated enough, as they might too often lead to death on the first interaction. High-risk interactions like this in vertebrates as well as invertebrates could instead favor evolution of canalized responses or other mechanisms to prevent cannibalization. As social environment and context changes over development in *Latrodectus* and many similar arachnids, future work examining different types of learning over the lifetime of individuals may help elucidate the importance of learning across taxa.

Figures and Tables

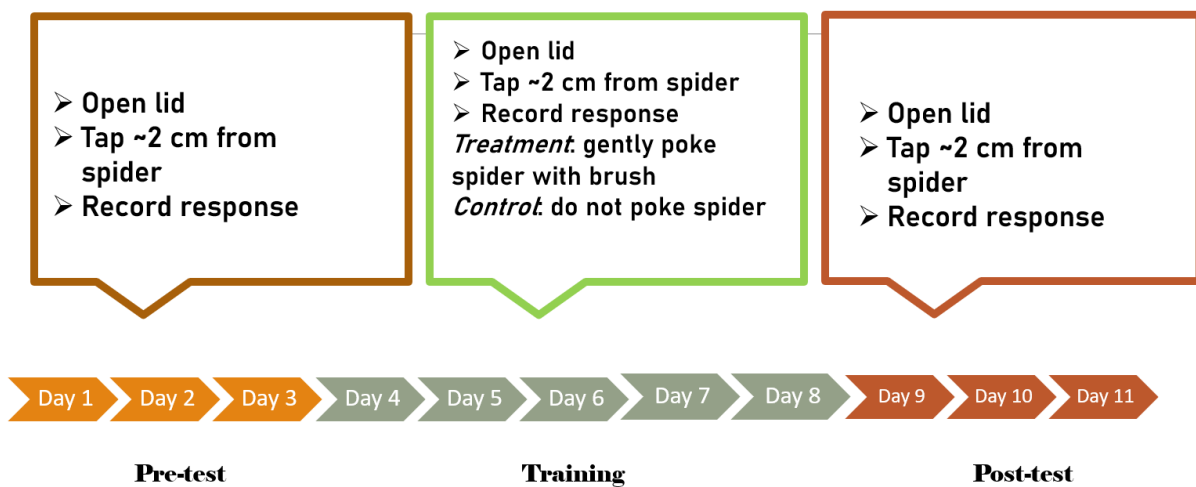


Figure 1: Experiment schedule. Individual juveniles were tested with a neutral cue once per day for three days prior to training (pre-test), were either trained with an aversive stimulus paired with the neutral cue (treatment) or received no cue (control) once per day for eight days (training) and tested with the neutral cue again once per day for three days (post-test).

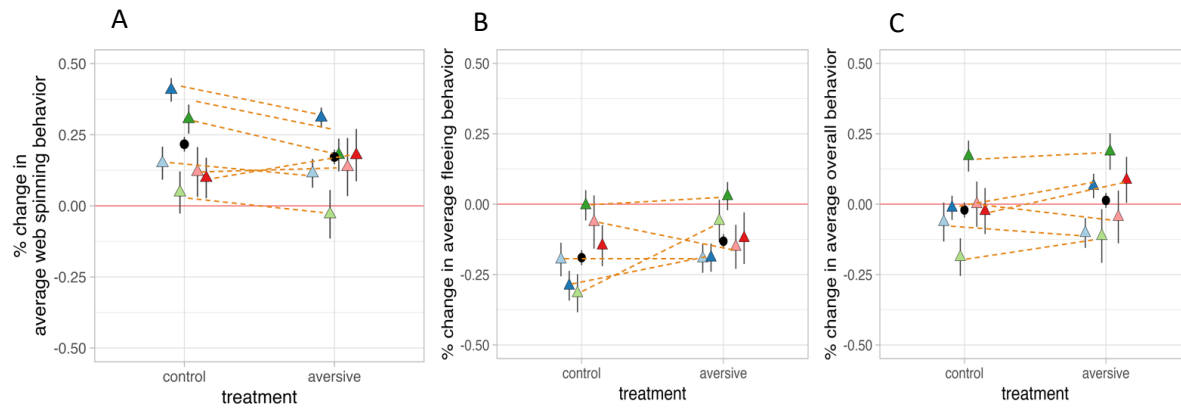


Figure 2. Change in behavior pre- and post-training across treatments. Solid black circles and lines indicate mean and standard error, triangular colored dots indicate individual egg sacs. Solid red line indicates no change, above the line indicates an increase, below the line indicates a decrease. Dotted lines connect treatment groups of the same egg sac. A) Both control and aversive treatments showed an increase in web spinning post-training. B) Both control and aversive treatments showed a decrease in fleeing post-training. C) Both control and aversive treatments showed no change in total behaviors performed pre- and post-training.

Eggsac	Count	Origin
F0004-21-1	120	field
F0004-21-2	137	lab
F0011-21-1	67	field
F0023-21-1	82	field
F0029-21-1	43	lab
F0031-21-1	63	field

Table 1: Final sample size per egg sac, with the lab or field origin of each eggsac.

	% Change in Overall Response Rate					
ANOVA	<i>Sum Sq</i>	<i>Mean Sq</i>	<i>Num DF</i>	<i>DenDF</i>	<i>F value</i>	<i>P-value</i>
Treatment	0.08	0.08	1	200.32	0.44	0.51
Random Effects (RANOVA)	<i>Npar</i>	<i>Loglik</i>	<i>AIC</i>	<i>LRT</i>	<i>p</i>	<i>Npar</i>
Egg Sac	4	-290.35	588.69	4.80	0.03	4
Observer	4	-288.48	584.96	1.07	0.30	4

	% Change in Web Spinning					
ANOVA	<i>Sum Sq</i>	<i>Mean Sq</i>	<i>Num DF</i>	<i>DenDF</i>	<i>F value</i>	<i>P-value</i>
Treatment	0.21	0.21	1	240.75	1.36	0.24
Random Effects (RANOVA)	<i>Npar</i>	<i>Loglik</i>	<i>AIC</i>	<i>LRT</i>	<i>p</i>	<i>Npar</i>
Egg Sac	4	-261.65	531.31	6.92	0.009	4
Observer	4	-259.61	527.21	2.83	0.09	4

	% Change in Fleeing					
ANOVA	<i>Sum Sq</i>	<i>Mean Sq</i>	<i>Num DF</i>	<i>DenDF</i>	<i>F value</i>	<i>P-value</i>
Treatment	0.30	0.30	1	259.51	1.77	0.18
Random Effects (RANOVA)	<i>Npar</i>	<i>Loglik</i>	<i>AIC</i>	<i>LRT</i>	<i>p</i>	<i>Npar</i>
Egg Sac	4	-283.54	575.07	7.35	0.007	4
Observer	4	-281.42	570.84	3.11	0.08	4

Table 2. Linear mixed model results for percent change in overall responses, percent change in fleeing, and percent change in web spinning, restricted to the response of the individual after the neutral tap cue (second response). All models included treatment as a fixed effect and egg sac and observer as random effects.

% Change in Overall Response Rate			
T-test	<i>t</i>	<i>df</i>	<i>p</i>
Change in behavior (pre-post)	-0.17	511	0.86
% Change in Web Spinning			
T-test	<i>t</i>	<i>df</i>	<i>p</i>
Change in behavior (pre-post)	10.70	511	2.2e-16
% Change in Fleeing			
T-test	<i>t</i>	<i>df</i>	<i>p</i>
Change in behavior (pre-post)	-8.62	511	2.2e-16

Table 3. T-tests evaluating whether percent change in behavior differed from zero in overall responses, percent change in fleeing, and percent change in web spinning,

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