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Ontogeny of Venom Use and Venom Composition in the Western Widow Spider Latrodectus Hesperus

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LOMA LINDA UNIVERSITY
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Ontogeny of Venom Use and Venom Composition in the Western Widow Spider *Latrodectus hesperus*

By

David Roger Nelsen

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I would like to dedicate my dissertation to my wife and parents.

Marie, you are amazing. Thank you for loving me.

&

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CONTENTS

TABLES

Tables Page

FIGURES

Figures Page

ABBREVIATIONS

RP-FPLC	Reverse Phase Fast Protein Liquid Chromatography
ACN	Acetonitrile
TFA	Trifluoroacetic Acid
FA	Formic Acid
LC-MS/MS	Liquid Chromatography Mass Spectrometry
MALDI	Matrix-Assisted Laser Desorption/Ionization
PPV	Positive Predictive Value
NPV	Negative Predictive Value
α-LTX	Alpha-Latrotoxin
α-LCT	Alpha-Latrocrustotoxin
α-LIT	Alpha-Latroinsectotoxin
β-LIT	Beta-Latroinsectotoxin
γ-LIT	Gamma-Latroinsectotoxin
δ-LIT	Delta-Latroinsectotoxin
ε-LIT	Epsilon-Latroinsectotoxin

ABSTRACT OF THE DISSERTATION

Ontogeny of Venom Use and Venom Composition in the Western Widow Spider *Latrodectus hesperus*

by

David R. Nelsen

Doctor of Philosophy, Graduate Program in Biology
Loma Linda University, June 2013
Dr. William K. Hayes, Chairperson

I investigated the behavioral ecology of venom and venom use by the western widow spider (*Latrodectus hesperus*), emphasizing the role of ontogeny. In an introductory paper, I reviewed existing definitions of venom and poison, and refined these by adding a third category of toxic biological secretions: toxungen. These three can be distinguished by mode of toxin delivery and presence of a wound. In the first of four empirical studies, I investigated venom use by adult females in the context of threat assessment. A single brief poke at the lowest threat level elicited primarily avoidance responses ("move" and "retract"), repeated prodding at medium threat incited increased silk-flicking, and gentle pinching at highest threat provoked increased biting. Spiders modulated venom expenditure by delivering 2.2–fold more venom per bite when pinched on the body compared to a leg, and 2.3–fold more venom when target presentations were separated by a long (5–min) rather than a short (5–sec) interval. The second study investigated the ontogenetic development of defensive behaviors. Spiders relied largely on non–combative behaviors early in life and switched to more combative behaviors, including silk flicking and biting, as they increased in size. Sex differences in behavior were comparatively negligible. Spiders habituated to the repeated testing by exhibiting

fewer combative behaviors than naïve spiders upon reaching adult size. In the third study, I developed an ethogram of the prey capture sequence of adult females feeding on crickets (*Acheta domesticus*) approximately 1.5 times their size. I identified 21 behaviors exhibited during three major phases: detection, immobilization, and prey manipulation. Spiders delivered an average of 15.2 (range 0–31) brief bites, with initial bites primarily to a leg. In the fourth study, I investigated ontogenetic and sexual variation in venom composition. Initial results requiring validation by improved methodology suggested that female venom becomes increasingly complex with age, whereas male demonstrates a more complex pattern. This dissertation represents the first major study of defensive venom metering in spiders. My findings support a growing body of literature suggesting that spiders are capable of cognition, and evaluate information from their body and environment when making decisions.

CHAPTER ONE

INTRODUCTION

Spiders comprise a diverse group of predators belonging to the order Araneae, consisting of 43,678 described species within 3898 genera and 112 families (Platnick 2013). Belonging to the arthropod class Arachnida, most easily recognized by the presence of eight legs, spiders are one of the most frequently encountered arachnids. However, among the general public there can still be considerable confusion. In the strict sense, spiders can be distinguished from other arachnids by the presence of opisthosomal silk–producing spinnerets, naked cheliceral fangs, cheliceral venom glands, a copulatory device on the male pedipalp, and absence of the trochanterofemoral depressor muscle in the walking legs (Selden and Dunlop 1998). Of course, spiders are generally thought of as anything possessing two body segments and eight legs, and are well known for their use of silk and venom. Spiders can be found on almost every continent and in almost every ecological environment, including some that spend much of their time under water. Spiders range in size from <1 to 90 mm body length, with the majority being small, in the 2–10 mm range (Foelix 1996). Spiders are an integral part of many ecosystems and are often the dominant entomophagous (insect–eating) predators, capturing prey using many different strategies: webs, silk nets, sit–and–wait ambushing, active hunting, lassoing, and more. Because of the aforementioned diversity in size, habitat, and diet, spiders show a wide range of behavioral adaptations that facilitate their particular lifestyle.

Organisms must make decisions on a daily basis that affect resource acquisition and energy expenditure (Bednekoff 2007; Caro 2005; Ferrari et al. 2009; Lima and Bednekoff 1999; Lima and Steury 2005; Wisenden 2000). Every action an organism does or does not undertake has an impact on its survival and fitness. Thus, selection acts continually to refine behavioral and physiological traits in order to minimize risks to the organism while maximizing energy and fitness gains. However, making predictions and testing decision–making processes is not straightforward, as an understanding of how the animal gains information from its environment (i.e., the sensory organs it possesses and uses) and a myriad of possible interactions must be considered. Types and sensitivity of sensory organs vary across species, and within a species different senses may be more important during certain tasks than others (Uetz and Roberts 2002).

Venom, which will be defined and its attributes reviewed in Chapter 2, has originated independently in many animal groups. Venom is often primarily used for predation and defense, but may also be used for other purposes (see Chapter 2). Although venom confers many benefits, there are also costs. A growing body of work has found that venom is metabolically expensive to make and maintain (McCue 2006; Nisani et al. 2007; Nisani et al. 2012; Pintor 2010). Haphazard venom use could also result in biological costs; for example, if during prey capture or defense an organism uses all of its venom, the animal will be at greater risk of predation or losing out on subsequent meals until the venom has been regenerated. It is therefore reasonable to assume that animals judiciously utilize their venom to balance the tradeoffs between venom's different functions. Indeed, numerous studies suggest that this is the case; recent reviews of the

venom metering (also referred to as venom optimization) hypothesis have been published by Hayes (2008) and Morgenstern and King (2012).

The overriding objective of this dissertation was to investigate the behavioral ecology of venom use in both predatory and defensive contexts, and the roles of ontogeny and sex differences in defensive behaviors and venom (toxin) development within the western widow spider *Latrodectus hesperus*.

Specific Objectives

In this dissertation, I begin with a review of toxic biological secretions in Chapter 2. My co–authors and I started out by reviewing the myriad definitions of venom. We then discussed the features common to all definitions, and proposed three new functionally–based definitions of venom, poison, and toxungen. The latter category represents a new term that helps to resolve ambiguities in distinguishing between venom and poison. We finished our discussion by proposing a classification scheme of the organisms that employ these toxic biological secretions.

To better understand the behavioral ecology of venom in the spider *L. hesperus*, and the role of ontogeny in shaping it, I conducted several experiments on the defensive use of venom, the ontogenetic development of defensive behaviors, the predatory use of venom within an ethogram constructed for prey capture, and the identification of ontogenetic and sexual variation in the venom. *Latrodectus hesperus* belongs to the spider family Theridiidae. Members of the genus *Latrodectus* comprise some of the most well–known and feared spiders worldwide because of their medically relevant venom. The group includes widow spiders of the Americas, the Australian red back spider, and

Africa's button spiders. The venom possessed by members of the genus is functionally very similar, and unique among other spiders in that its main toxins are large molecular weight proteins in the 110–140 kDa range (Kuhn-Nentwig et al. 2011; Rohou et al. 2007). *Latrodectus* venom consist of seven major taxon-specific proteins in three categories: (1) alpha-latrotoxin (α-LTX), which is vertebrate specific with a molecular mass of 130 kDa; (2) five latroinsectotoxins (α, β, γ, δ, ε-LIT), which are insect specific at 110–140 kDa; and (3) alpha-latrocrustotoxin (α-LCT), which is crustacean specific at120 kDa. The venom also includes many low molecular weight proteins (Gasparini et al. 1994; Volkova et al. 1995).

Latrodectus hesperus is the largest widow spider found in North America, averaging 3.5 cm in length. The species ranges from southwestern Canada into northern Mexico. These spiders are polyphagous predators, but generally feed on ground-active arthropod prey (Salomon 2011).

In Chapter 3, I examined the defensive behaviors of *L. hesperus* using two separate experiments. The first experiment tested for risk assessment by subjecting the spiders to three threat levels in a repeated measures design. I hypothesized that the spiders would show differential use of defensive behaviors across threat levels with behaviors like bite, accompanied with potential venom use, preferentially being used at the highest threat level. The second experiment investigated the spider's ability to actively control the amount of venom delivered in a defensive bite. I subjected spiders to a series of treatments and allowed the spiders to interact with three separate targets per observation period. I hypothesized that the spiders would use more venom in the high threat condition.

In Chapter 4, I examined the ontogenetic development of defensive behaviors from the second instar through maturity. I also included a naïve control group to test for possible habituation to the treatment. I hypothesized that young (small) spiders would rely largely on non-combative behaviors, but transition with growth to use more combative behaviors, such as silk flicking and biting. I also hypothesized that repeated exposure to the treatment would result in habituation—a decrease in defensiveness (characterized by the use of metabolically and biologically expensive behaviors)—compared to naïve controls at the same age.

In Chapter 5, I developed an ethogram of the predatory sequence, defining 21 discrete behaviors and quantifying the duration and frequency of each. Although entirely descriptive, this ethogram will provide a basis from which future studies can be designed and evaluated.

In Chapter 6, I collected preliminary data to evaluate ontogenetic and sexual variation in the venom of *L. hesperus*. Based on known characteristics of *Latrodectus* venom (see Kuhn-Nentwig et al. 2011), I hypothesized that certain toxins, particularly α-latrotoxin, would not be present in the earliest instars, and become expressed later in older and larger spiders. I also hypothesized that the venom of male spiders would differ from female venom, especially between mature instars. We evaluated differences in venom using a variety of techniques. Current results are suggestive but inconclusive, as steps are being taken to improve procedures and obtain more definitive data.

In Chapter 7, I summarize and discuss the results from my research. Collectively, the findings from my work should provide a clearer picture of how a medically important synanthropic spider produces its venom and relies on it for survival.

References

Bednekoff, P. A. (2007). Foraging in the face of danger. In: *Foraging Behavior and Ecology.* (eds. D.W. Stephens, J.S. Brown, & R.C. Ydenberg), University of Chicago Press, Chicago, Illinois, USA, 305–329.

Caro, T. (2005). *Antipredator defenses in birds and mammals*. University of Chicago Press.

Ferrari, M. C., Sih, A., & Chivers, D. P. (2009). The paradox of risk allocation: a review and prospectus. *Animal Behaviour*, *78*(3), 579–585.

Foelix, R. F. (1996). *Biology of Spiders*. Oxford University Press, USA.

Gasparini, S., Kiyatkin, N., Drevet, P., Boulain, J. C., Tacnet, F., Ripoche, P., Forest, E., Grishin, E., & Menez, A. (1994). The low molecular weight protein which co-purifies with alpha-latrotoxin is structurally related to crustacean hyperglycemic hormones. *Journal of Biological Chemistry*, *269*(31), 19803–19809.

Hayes, W. K., Herbert, S. S., Harrison, J. R., & Wiley, K. L. (2008). Spitting versus biting: differential venom gland contraction regulates venom expenditure in the Black-necked Spitting Cobra, Naja nigricollis nigricollis. *Journal of Herpetology*, *42*(3), 453–460.

Kuhn-Nentwig, L., Stöcklin, R., & Nentwig, W. (2011). Venom composition and strategies in spiders: is everything possible?. *Advances in Insect Physiology*, *40*, 1.

Lima, S. L., & Bednekoff, P. A. (1999). Temporal variation in danger drives antipredator behavior: the predation risk allocation hypothesis. *The American Naturalist*, *153*(6), 649–659.

Lima, S. L., & Steury, T. D. (2005). Perception of predation risk: the foundation of nonlethal predator–prey interactions. In: *Ecology of Predator–Prey Interactions.* Oxford University Press, Oxford, 166–188.

McCue, M. D. (2006). Cost of producing venom in three North American pitviper species. *Copeia*, *2006*(4), 818–825.

Morgenstern, D., & King, G. F. (2012). The venom optimization hypothesis revisited. *Toxicon 63*, 120–128.

Nisani, Z., Dunbar, S. G., & Hayes, W. K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *147*(2), 509–513.

Nisani, Z., Boskovic, D. S., Dunbar, S. G., Kelln, W., & Hayes, W. K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon*, *60*(3), 315–323.

Pintor, A. F., Krockenberger, A. K., & Seymour, J. E. (2010). Costs of venom production in the common death adder (*Acanthophis antarcticus*). *Toxicon*, *56*(6), 1035–1042.

Platnick, N. I. 2013. The world spider catalog, version 13.5. American Museum of Natural History, online at http://research.amnh.org/iz/spiders/catalog. DOI: 10.5531/db.iz.0001.

Rohou, A., Nield, J., & Ushkaryov, Y. A. (2007). Insecticidal toxins from black widow spider venom. *Toxicon*, *49*(4), 531–549.

Selden, P. A., & Dunlop, J. A. (1998). Fossil taxa and relationships of chelicerates. In: *Arthropod fossils and phylogeny*, (ed. G.D. Edgecombe) Springer, Netherlands, 303–331.

Salomon, M. (2011). The natural diet of a polyphagous predator, *Latrodectus hesperus* (Araneae: Theridiidae), over one year. *Journal of Arachnology*, *39*(1), 154–160.

Uetz, G. W., & Roberts, J. A. (2002). Multisensory cues and multimodal communication in spiders: insights from video/audio playback studies. *Brain, Behavior and Evolution*, *59*(4), 222–230.

Volkova, T. M., Pluzhnikov, K. A., Woll, P. G., & Grishin, E. V. (1995). Low molecular weight components from black widow spider venom. *Toxicon*, *33*(4), 483–489.

Wisenden, B. D. (2000). Olfactory assessment of predation risk in the aquatic environment. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *355*(1401), 1205–1208.

CHAPTER TWO

POISONS, TOXUNGENS, AND VENOMS: REDEFINING AND CLASSIFYING TOXIC BIOLOGICAL SECRETIONS AND THE ORGANISMS THAT EMPLOY THEM

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Abstract

Despite extensive study of poisonous and venomous organisms and the toxins they produce, a review of the literature reveals inconsistency and ambiguity in the definitions of "poison" and "venom." These two terms are frequently conflated with one another, and with the more general term, "toxin." We therefore clarify distinctions among three major classes of toxins (biological, environmental, and anthropogenic or man-made), evaluate prior definitions of venom which differentiate it from poison, and propose more rigorous definitions for poison and venom based on differences in mechanism of delivery. We also introduce a new term, "toxungen," thereby partitioning toxic biological secretions into three categories: poisons lacking a delivery mechanism, i.e., ingested, inhaled, or absorbed across body surface; toxungens delivered to the body surface without an accompanying wound; and venoms delivered to internal tissues via creation of a wound. We further propose a system to classify toxic organisms with respect to delivery mechanism (absent versus present), source (autogenous versus heterogenous), and storage of toxins (aglandular versus glandular). As examples, a frog that acquires toxins from its diet, stores the secretion within cutaneous glands, and transfers the secretion upon contact or ingestion would be heteroglandular-poisonous; an ant that produces its own toxins, stores the secretion in a gland, and sprays it for defense would be autoglandular-toxungenous; and an anemone that produces its own toxins within specialized cells that deliver the secretion via a penetrating wound would be autoaglandular-venomous. Adoption of our scheme should benefit our understanding of both proximate and ultimate causes in the evolution of these toxins.

Introduction

Poisonous and venomous organisms have generated both fascination and loathing since the beginning of recorded history. They have also inspired considerable research across a broad range of disciplines. Despite the extraordinary attention given to these animals and the toxins they produce, substantial confusion remains regarding the distinction between "poison" and "venom." Even a cursory review of the scientific literature reveals inconsistencies and ambiguities in definitions of these terms, as well as frequent conflation with the more general term, "toxin." Furthermore, the definition for venom, which has the most precise meaning, is often excessively narrow and excludes many toxic secretions classically thought of as venoms.

Despite this long and continuing history of conflation (e.g., Osterhoudt, 2006; Gibbs, 2009), biologists and toxicologists alike have gradually forged an important distinction, primarily in mechanism of delivery: poisons are typically ingested or passively encountered, whereas venoms are typically injected by means of a specialized device (Mebs, 2002). This distinction, though based on proximate causation, can help to clarify the evolution of these toxins in terms of ultimate causation (*sensu* Ayala, 1999). The mechanisms by which organisms deliver toxins relate to how the toxins function and their evolution. Toxins delivered by passive contact or ingestion function best for defense, whereas those delivered via a penetrating wound are especially well suited for predation, and therefore are often under different selective pressures (Mebs, 2002; Brodie 2009). Understanding such distinctions can inform our efforts to develop applications for biotechnology and pharmaceutical purposes.

In this paper, we first clarify the distinctions among three major classes of toxins (biological, environmental, and anthropogenic), but limit further consideration to a single group—the biological toxins. Second, we review the literature to critically evaluate prior definitions of venom which set it apart from poison, and assess which components of the definitions work better than others. Third, we propose more rigorous definitions for poison and venom based on readily defined differences in mechanism of delivery, and introduce a new term, "toxungen" (pronunciation: tox-unj'-en), to further reduce ambiguity. Accordingly, we partition toxic biological secretions into three categories: poisons, toxungens, and venoms. Fourth, we develop a classification system for toxic biological secretions that specifies not only mechanism of delivery (absent versus present), but also source of toxins (autogenous versus heterogenous) and storage (aglandular versus glandular).

As a result of our effort, we seek: (1) to develop a more rigorous and comprehensive terminology and classification of toxic biological secretions, thereby facilitating consistency in usage and discussion; (2) to unify and place in better context a diverse and fractured body of literature; and (3) to develop an improved framework for studying the evolution of these toxins, including their biochemical structure, associated structures (for synthesis, storage, and application), mechanism of delivery, functional roles in nature, and biodiversity.

Toxins

To clarify the definitions of venom and poison, we first discuss a common feature of both: they are comprised of one or more toxins. Toxins are substances that, when

present in biologically relevant quantities, cause dose-dependent pathophysiological injury to a living organism, thereby reducing functionality or viability of the organism. Onset of effects may be immediate or delayed, and impairment may be slight or severe. Relative quantity, or dose, is important because many ordinarily innocuous substances, including water, can become toxic to organisms at abnormally high levels, and many highly toxic substances can be harmless in minute quantities. As Theophrastus of Hohenheim (Paracelsus), the Swiss–German physician and "Father of Toxicology," put it, "All things are poison and nothing [is] without poison. Only the dose makes a thing not to be poison" (Poerksen, 2003). This axiom of toxicology posits that the effects of substances can vary depending on dose, which is a shared property of the substance and the target organism, including its receptors (Stumpf, 2006).

Little agreement exists on how toxins are classified (Hodgson, Mailman & Chambers, 1988; Schiefer, Irvine & Buzik, 1997; Eaton and Klaassen, 2001; Hayes, 2001). Based on perusal of the literature and on internet sources, which reflect common usage, we categorize toxins into three general classes:

● ***Biological toxin*** – A substance produced by a living organism that is capable of causing dose-dependent pathophysiological injury to itself or another living organism; sometimes called a “biotoxin.”

● ***Environmental toxin*** – A naturally occurring substance in the environment that is not produced by an organism but is capable of causing dose-dependent pathophysiological injury to a living organism. Examples include arsenic, mercury, and lead.

● ***Anthropogenic toxin*** – A substance produced by humans that does not otherwise occur in the environment which is capable of causing dose-dependent pathophysiological injury

to a living organism; often called a “man-made toxin” and sometimes called a “toxicant.” Examples include DDT, dioxin, and polychlorinated biphenyls (PCBs).

Toxins are not in themselves living, replicating organisms, nor are they contagious, as in certain biological or chemical “agents” used in biological warfare (e.g., bacteria, viruses, prions, or fungi). The term toxin is most appropriately applied to a single chemical substance (Mebs, 2002; Menez, Servent & Gasparini, 2002). Thus, complex mixtures of toxins, such as the venoms of snakes, should not be labeled a toxin in the singular sense. The term poison is often used to describe toxins of all three classes, whereas venom normally encompasses only biological toxins. However, humans may be uniquely capable of employing all three toxin types as venoms—via deliberate injection into tissues—for research and development purposes (e.g., biotechnology and medical applications), or for more nefarious objectives (e.g., harming other organisms, including humans). Other animals can accumulate environmental or anthropogenic toxins, and could conceivably use them for venom.

Hereafter, we restrict consideration largely to biological toxins, and within this context we show that poison and venom can and should be readily distinguished.

Existing Definitions of Venom

To better understand the distinction between poison and venom, we reviewed the multiple definitions of venom found in the primary and secondary literature. Definitions were found by reading through numerous venom-related articles, toxicology or toxinology textbooks, scientific dictionaries, and books dedicated to venom or venomous animals. This review allowed us to consolidate the most essential components into a

single, more concise definition of venom. In the process, however, we have better defined the term poison as well, because many definitions of venom relate it to poison. Moreover, our review convinced us that, for added clarity, a new class of toxins should be recognized that is distinct from poisons and venoms.

Our review of the literature revealed a handful of shared components, or properties, among existing definitions of venom (Table 1). These included: 1) hierarchy and exclusiveness; 2) source of secretion; 3) mode of transmission, often including a specialized delivery structure or delivery system; 4) purpose (i.e., biological role or function); and 5) method of delivery being either active or passive. We examine each of these in turn.

Table 1. Six frequent components of definitions of venom from various literature sources, illustrating the remarkable lack of consensus.

Source of definition	Hierarchy and exclusiveness		Source of secretion		Delivery structure/system	Mode of transmission			Purpose		Active application
	Toxin	Poison	Gland	Sub-gland		Injection	Wound	Contact	Predation	Defense	
Primary Literature											
(Roth and Eisner, 1962)					×	×			×	×	
(Beard, 1963)		×			×	×	×				
(Welsh, 1964)			×	×	×	×	×	×	×	×	
(Halstead, 1965)		×			×	×	×				
(Russell, 1965)		×	×	×	×						
(Freyvogel, 1972)	×		×		×	×	×	×	×	×	
(Oehme et al., 1975)			×			×			×		×
(Bettini and Brignoli, 1978)						×	×				
(Mebs, 1978)			×		×	×			×	×	
(Schmidt, 1982)			×		×				×	×	
(Sharma and Taylor, 1987)	×		×								
(Auerbach, 1988)	×		×		?	×		?			
(Meier and White, 1995)			×	×	×	×			×	×	
(Russell, 2001)		×	×	×	×						
(Mebs, 2002)			×	×	×	×			×	×	×
(Kuhn-Nentwig, 2003)			×			×					×
(Eisner et al., 2005)			×			×					
(Brodie, 2009)			×		×	×					×
(Fry et al., 2009b)			×		×		×		×	×	
(Mackessy, 2009)			×		×	×	×				
(Wuster, 2010)						×			×	×	
Secondary literature											
Academic Pr Dic (1992)	×					×					
Mosby Dict (1998)		×			×						
Macmillan Dict (1999)	×	×			×	×					
Collins Dict Med (2005)		×									
Dorland Dict (2007)		×									
Mcgraw-Hill (2003)		×									
Taber Dict (2009)		×			×						

Hierarchy and Exclusiveness

Hierarchy and exclusiveness should be expected in definitions of venom. By hierarchy, toxins are properly understood to be singular substances, toxic secretions deployed against other organisms are often comprised of multiple toxins (and often include non-toxic constituents as well), and organisms can possess multiple toxic secretions. As alluded to above, exclusivity, particularly between a poison and a venom, has also been deemed desirable in classifying toxins.

Of the 28 venom definitions gleaned from the literature, five classified venom as a toxin, ten as a poison, and one as both a toxin and a poison. Fourteen did not specify a hierarchical classification in their definition. Lack of hierarchy is evident in statements such as, "venoms are most commonly produced by the organisms that possess them, while toxins are often sequestered from an outside source or modified from external building blocks" (Brodie, 2009). Lack of exclusiveness is evident in, "all venoms are poisons, but not all poisons are venoms" (Halstead, 1965). Clearly, toxin, poison, and venom are frequently conflated even by knowledgeable sources.

The Oxford Dictionary of English Etymology (1966) describes the origin of the word venom as being derived from the Latin word *venenum*, meaning "poison," "drug," or "potion." The origin of poison derives from the Latin *potionem* (nom. potio), meaning "potion," or a "poisonous drink" (*Oxford Dictionnary of English Etymology*1966). Venom and poison are clearly related to each other in that they are both comprised of one or more biological toxins, as generally defined. However, the terms venom and poison, although linked in origin, have now taken on different connotations within the context of biological secretions, which 16 of 24 definitions attempted to make clear (i.e., the

consensus position) and which we support. Accordingly, authors often and appropriately refer to a puffer fish (Tetraodontidae) as poisonous because of the toxic tissues which cause pathophysiological problems for predators upon consumption, and rattlesnakes (Viperidae) as venomous because they inject toxins into their prey via hollow fangs.

If toxicologists persist in an effort to create mutually exclusive categories for poisons and venoms, then both hierarchy and exclusiveness are appropriate for defining venom. Thus, poisons and venoms should be formally recognized as substances comprised of one or more toxins, and they should be defined so as to maintain their distinctiveness. However, two caveats merit mention: 1) a poison or venom can be composed of a single toxin, in which case the toxin would be equivalent to a poison or venom; and 2) because poison and venom will ultimately be defined by how they are deployed, a single substance can be used as both a poison and as a venom, even by the same organism.

Source of Secretion

Our use of the term "secretion" is predicated on recognition that tissues, glands, cells, and even subcellular structures can produce secretions. Venoms typically consist of a secretion containing one or more toxins. Many existing venom definitions specified whether the secretion is glandular (produced in a gland) or glandular/sub-glandular (produced within either a gland, a collection of specialized cells, or a single cell) in origin. Indeed, 10 definitions specified that venoms are glandular, five allowed venoms to be glandular or sub-glandular, and 12 did not specify the origin (most of these were from secondary sources). All biological toxins must be made and/or stored somewhere in the

organism; therefore, it is redundant to specify in the definition that the secretion is glandular or sub-glandular. Moreover, if the definition of venom includes the stipulation that it must be glandular in origin, then cnidarians would not be considered venomous, as the toxins are produced by and stored within a single specialized cell called a cnidocyte or nematocyte (Lotan *et al*., 1995; Ozbek, Balasubramanian & Holstein, 2009). Yet cnidarians, which do not possess a true gland for venom production or storage, are universally regarded to be venomous—a point surprisingly overlooked by many authorities on venom. Thus, we agree with the consensus position (if secondary sources are included) that specifying the source or storage site for the secretion need not be included in the definition of venom, and the same is true for poison. The term secretion should also be avoided in the definition of venom because humans, at least, are capable of deploying toxins that would not be secretions of biological origin (e.g., injecting refined toxic chemicals into other organisms; Mebs, 2002).

Mode of Transmission, Including a Delivery Structure or Delivery System

The mode of transmission refers to how a biological toxin is delivered to the recipient. Venom was most often defined as being delivered specifically via injection (12 definitions), with other definitions specifying more generally injection or delivery via a wound (six definitions). One definition included delivery via mere external contact. Eight definitions did not specify mode of transmission (the majority of these were secondary references).

The word "injection" has the connotation of introducing a substance relatively deep into the tissues of the target through an often highly specialized structure, such as a medical syringe, rattlesnake fang, or scorpion stinger. This is, indeed, the most common method that venomous animals use to deliver their toxic secretions. However, there are many animals that deliver toxins through less specialized methods. Gila monsters (*Heloderma suspectum*) and many colubrid snakes possess teeth that are grooved rather than hollow (in contrast to viperid and elapid snake fangs), and their toxic secretion must be chewed rather than injected into the target organism, with the toxins penetrating the wound via surface tension and diffusion (Fry *et al*., 2006; Young *et al*., 2011). Members of the Formicidae ant family deliver piercing bites with their mandibles, and spray venom from their abdominal storage glands into the wound (McGain and Winkel, 2002; Eisner, Eisner & Siegler, 2005). Similarly, the soldier castes of some termite species inflict damage with their mandibles while simultaneously secreting toxins from their frontal glands onto their victims (Prestwich, 1979, 1984; Quennedey, 1984; Schmidt, 1990). Larvae of the beetle *Phengodes lateicollis* subdue millipedes by puncturing the prey's body with its mandibles, and then injecting fluids from its gut that paralyze the millipede (Eisner *et al*., 2005). Thus, delivery of venom via a wound comprises a more general and applicable description of envenomation, and we therefore reject the consensus criterion of delivery by injection.

We propose that any definition of venom should stipulate that the biological toxin is delivered via mechanical trauma produced by some kind of structure that results in a wound. Because a structure, whether specialized (e.g., fang) or general (e.g., unmodified tooth), is necessary to create the wound, we find it sufficient for the definition to require

toxin delivery via a wound and redundant to specify how the wound is created other than by an assumed mechanism.

Two definitions (Welsh, 1964; Freyvogel, 1972) allowed for the topical application of venom. There are a host of biological toxins that are applied externally by means of a sometimes elaborate mechanism, but the inclusion of these would require serious changes to the current understanding and usage of the term venom. Nevertheless, it is understandable why Freyvogel (1972) and Welsh (1964) included the topical application of biological toxins as venoms. Spitting cobras (genera *Naja* and *Hemachatus*), for example, can introduce their biological toxins to an enemy via injection by fangs, or by spraying it, aiming at the recipient's face and eyes. Both delivery mechanisms result in pathophysiological injury, so why would we refer to the secretion as a venom in one usage and not in the other? Inclusion of topical application of a biological toxins in the classification of venoms would necessitate inclusion of a host of other organisms as venomous that are not commonly thought to be so, thus defeating the purpose of this paper: greater clarity in a definition. We will discuss the special case of topically applied biological toxins shortly, but for now we return to the features of a classically defined venom.

Biological Role(s)

Numerous definitions of venom focused on its biological role(s), or purpose(s), with one stipulating that venom is only used for predation, and nine stating that venom is used for either predation or defense. In many cases (17 definitions), however, no

distinction regarding the role of venom was made. Is it important to specify within a definition the purpose of venom?

Most venomous animals, such as viperid and elapid snakes, employ their toxins for predation and defense. However, venomous animals may use their venoms for a range of other purposes. Male duck-billed platypuses (*Ornithorhyncus anatinus*), for example, use their toxins and delivery apparatus primarily in the context of mate competition, using it against male conspecifics during mating and territorial disputes (Torres *et al*., 2000). This use should qualify as a venom regardless of whether it can also be used for defense. Scleractinian coral colonies and many actiniarian (anemones) use venom for predation and defense, but also possess specialized tentacles to attack other nearby colonies, thereby protecting and expanding their own territory in the context of intraspecific and interspecific competition for space (Williams, 1991). Again, use of toxins for competition should qualify as a venom regardless of whether it is also used for predation and defense. In addition to the use of venom for self and/or colony defense (generally by injection), some hymenopterans also spray their "venom" to keep their broods free of parasites in the context of hygiene (Oi and Pereira, 1993), and some ants spray the same secretion that is used as a venom for trail marking in the context of communication (Blum, 1966; Mashaly, Ali & Ali, 2010). Clearly, venoms can be co-opted or exapted for other purposes, just as secretions serving other purposes can be co-opted or exapted to become a venom.

Because venom can be used for more than predation and defense, the stipulation that venom must serve a defensive or predatory role seems excessive and unnecessary. Thus, we agree with the consensus position in omitting a biological role from the

definition. Further, the fact that a single secretion may be delivered in multiple ways (e.g., biting and spraying) and serve multiple functions (e.g., defense, predation, competition, communication) means that individual secretions may be categorized in multiple ways simultaneously. We will revisit this notion.

Active Application

Four authors specified that venom is "actively applied," whereas the remainder made no such specification. Although the behavioral act of delivering venom was not common among the definitions surveyed, we should consider its merits. As Mebs (2002) stated, "venoms are actively applied for both prey acquisition, which may include predigestion, and as a defense against predators." This language implies a deliberate or reflexive act on the part of the venomous animal in response to an external stimulus. But is this true for organisms that are commonly considered venomous, and what level of "activity" is necessary to be considered active application?

Numerous widely accepted examples of envenomation obfuscate the meaning of active application. Snakes, of course, deliver their venom by biting, and scorpions and bees deliberately sting their victim. Many fish (e.g., stone fish, genus *Synanceia*, and lionfish, genus *Pterois*), however, have venomous spines that deliver toxins only defensively when the recipient (victim) initiates contact. Likewise, the toxin-bearing, harpoon-like cnidocytes of cnidarians and ctenophores are often fired due to incidental contact by recipient organisms. Do these involve "active" participation by the venomous animal? One could argue that venomous fish must erect their toxin-laden spines, or that the cnidocytes have cnidocil triggers, and these qualify as active application. However,

caterpillars of the genus *Lonomia* have stiff, permanently erect, urticating hairs that penetrate tissue and deliver venom upon contact initiated by the recipient. In this latter case, the caterpillar requires no active participation to defend itself via injection of toxins. A freshly deceased caterpillar could also do this every bit as effectively as a live specimen.

Thus, we agree with the consensus position that active application of toxins involving a specific behavior or intention should not be a part of the definition of venom, as its inclusion would not result in further clarity.

Three Classes of Toxic Biological Secretions: Poisons, Toxungens, and Venoms

From our critical assessment of existing definitions of venom, we propose the following mutually exclusive definitions for three major classes of toxic biological secretions, with distinctions delineated in Table 2:

● ***Poison*** – A toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that results in self-induced toxicity or is passively transferred without a delivery mechanism from one organism to the internal milieu of another organism without mechanical injury, usually through ingestion, inhalation, or absorption across the body surface.

● ***Toxungen*** – A toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that is actively transferred via a delivery mechanism from one organism to the external surface of another organism without mechanical injury.

● ***Venom*** – A toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that is passively or actively transferred from one organism to the internal milieu of another organism via a delivery mechanism and mechanical injury.

Table 2. Critical components and features that distinguish the three major categories of biological toxins.

Biological toxin	Delivery mechanism	Penetration wound	Mechanism of transfer or deployment
Poison	No	No	Ingestion, inhalation, or absorption across body surface
Toxungen	Yes	No	Delivered to body surface without accompanying wound
Venom	Yes	Yes	Delivered to internal tissues via wound

Although our interest here is in toxic biological secretions (i.e., what animals normally possess), which are ordinarily comprised of one or more biological toxins, we render our definitions more general by simply including the essence of a toxin: "a toxic substance causing dose-dependent physiological injury." Poison has a widely accepted usage that encompasses environmental and anthropogenic toxins in addition to biological toxins. Self-induced toxicity is included in the definition of poison because a dysfunction of metabolism can result in poisoning of the individual. Moreover, environmental and anthropogenic toxins can be diffusely distributed among the tissues of an organism, rendering it toxic, and therefore comprising a poison. Thus, our definitions are general enough to include environmental and anthropogenic toxins as poisons, toxungens, and venoms.

We propose with these definitions a new class of toxins, the toxungens, to provide greater clarity to the distinction between poisons and venoms. Numerous animals deliver

their toxins by spraying, spitting, or smearing, including representatives among flat worms, insects, arachnids, cephalopods, amphibians, and reptiles (Sutherland and Lane, 1969; Koopowitz, 1970; Brodie and Smatresk, 1990; Deml and Dettner, 1994; Eisner *et al.*, 2005). These modes of delivery do not fit well within the traditional meaning of either a poison or a venom. We therefore propose the term "toxungen" (pronunciation: tox-unj'-en), a new word derived by combining two Latin nouns: *toxicum*, meaning toxic, and *unguentum*, meaning balm or ointment. Thus, this word has the connotation of a toxic ointment, or a toxin that is applied to the outside of the victim's body. We realize that this combination of toxicum and unguentum does not follow proper Latin grammar, but we feel that the combination adequately refers to the original roots while being combined in a way to produce a meaningful word with semblance to venom and poison.

Although toxungens could be classified with poisons, there are reasons to consider them distinct. In addition to the difference in delivery, selection has often acted uniquely on the secretions of animals that spray, spit, or smear their toxins. Spitting cobras, for example, lack a subunit in their venom that in other cobras binds the cardiotoxin, rendering the unbound cardiotoxin more injurious to the eye membranes (Ismail *et al*., 1993). Several arthropods that spray or smear their toxin incorporate a spreading agent with their secretion that increases penetration through the target animal's cuticle and enhances toxicity (e.g. whip scorpions: Eisner *et al*., 1961; termites: Prestwich, 1984; earwigs: Eisner, Rossini & Eisner, 2000). By lumping toxungens and poisons together, important details regarding evolution of the toxins and their deployment may be overlooked. The term "contact poison" exists in the literature, particularly for insecticides of anthropogenic use, but also for arthropod smearing of toxins (Prestwich,

1984; Heredia, de Biseau & Quinet, 2005). With our terminology, toxungens would be a subclass of contact toxins. Toxins passively transferred to surfaces represent contact poisons, whereas those actively delivered to surfaces comprise toxungens.

Our definitions for three distinct biological secretions incorporate just two of the five components, or properties, that we identified as common among prior definitions of venom: 1) hierarchy and exclusiveness (each secretion type is comprised of one or more toxins, but defined to maintain exclusiveness); and 2) mode of transmission (the primary means of distinction among the three toxic secretion classes). We argue that mode of transmission alone is both critical and sufficient for distinguishing these toxic secretions, depending on whether a delivery structure or delivery system exists (satisfied by toxungen and venom, but not by poison), and whether a penetration wound is created (satisfied only by venom). Further, our definitions explicitly reject the following components, or properties, that many authors have used to define a venom: 1) type of secretion (glandular synthesis and/or storage is irrelevant); 2) biological role (restriction to defensive or predatory function is irrelevant); and 3) active delivery (whether the organism employs a specific behavior or action to deliver the secretion is irrelevant). Interestingly, our definition of venom matches the consensus position for four of the five components among the 27 published definitions we considered, but rejects the consensus view that venom must be injected (a wound is necessary, but the toxins may be delivered into the wound without injection). We believe our definitions are both robust and succinct.

As we will elucidate further, organisms that employ these three major classes of toxic secretions can be recognized as “poisonous,” “toxungenous,” or “venomous,”

respectively. Some organisms exhibit more than one of these characteristics. We emphasize that while our definitions are mutually exclusive, individual secretions and the animals that rely on these toxins should not necessarily be constrained within one of these three toxic secretion classes.

To illustrate the adequacy and utility of our definitions, we offer three examples within a single vertebrate class: Amphibia. Toxins in the skin secretion of the golden dart frog (*Phyllobates terribilis*) can be transferred through recipient-initiated ingestion and possibly direct skin absorption resulting from contact (Myers, Daly & Malkin, 1978). Because the frog lacks a distinct mechanism for delivering the toxins to the surface of the recipient, or through a wound created in the recipient, we consider the secretion to be a poison and the frog to be poisonous. The toxins of the fire salamander (*Salamandra salamandra*) can be sprayed at potential predators up to 2 m away, and can be aimed in the direction of the attacker, which presumably can be deterred by the secretion (Brodie and Smatresk, 1990). Because the salamander has a distinct delivery mechanism which does not involve production of a wound, we consider the secretion to be a toxungen and the salamander to be toxungenous. The Brazilian casque-headed tree frog (*Corythomantis greeningi*) possesses specialized ossified spicules on the top of its skull, with toxin-containing glands in the overlying skin. When disturbed, the frog thrashes the top of its head toward the recipient. The spicules can puncture the frog's skin and associated glands, and cause mechanical damage to the recipient as well, thereby delivering the toxins to the recipient's internal tissues (Jared *et al*., 2005). In this case, the secretion is a venom and the frog is venomous because it delivers the toxins by means of tissue injury. These examples also illustrate how a secretion and the animal that produces it may be

classified in at least two categories. If the toxin delivery mechanisms of the fire salamander (deployed as a toxungen) and casque-headed tree frog (deployed as a venom) fail to foil a predator, these and other skin toxins may still function as a poison against a predator that licks or consumes the amphibian. Thus, the fire salamander would be both toxungenous and poisonous, and the casque-headed tree frog would be both venomous and poisonous.

Our definition of venom, taken to its logical conclusion, recognizes that organisms other than animals can be venomous. Venoms, as generally recognized, have evolved across a diverse range of animals, varying in complexity from single-celled cnidarians to multicellular mammals (Mebs, 2002). Must we arbitrarily restrict the term "venom" to a single clade or kingdom, Animalia? If so, then why? Is such an argument based on complexity? Organisms in other kingdoms—including many that rival or exceed the complexity of cnidarians—solve problems in remarkably analogous or even identical ways using biological toxins delivered via the creation of wounds. Phage viruses, for example, employ sophisticated injection systems that deliver lytic proteins and DNA into their victims, resulting in unambiguous pathogenesis (Rossmann *et al*., 2004). Bacteria similarly use sophisticated injection systems to introduce toxic proteins into their victims with devastating consequences (Kenny and Valdivia, 2009; Beeckman and Vanrompay, 2010). Among protists, the ciliate *Dileptus gigas* discharges harpoon-like, toxin-filled projectiles called toxicysts when pursuing prey, which rupture the victim's cell membrane and deliver the toxins, resulting in paralysis or death of the target (Visscher, 1923; Miller, 1968). Fungi produce a dizzying assortment of penetration structures to penetrate host cells and deliver toxins that can incapacitate their victims

(Luo *et al*., 2007; Liu, Xiang & Che, 2009). Among plants, many members of the genus *Urtica* (nettles) possess specialized trichomes that penetrate the tissues of other organisms and deliver toxic substances such as oxalic acid, tartaric acid, acetylcholine, serotonin, and histamine (Fu *et al*., 2006). Without a cogent argument for restricting venom to a single kingdom, these examples of convergent evolution could rightfully be considered venomous organisms that deliver venom by means of venom delivery systems.

Returning to humans, we emphasize that they can be facultatively poisonous, toxungenous, and venomous. Humans can become poisonous, potentially, by accumulating toxic substances in their tissues. They can apply toxins by spraying or smearing them on other organisms. And they can inject toxic substances into other organisms. Some may object to any consideration of humans being toxic, but a simple example illustrates how profound their use of toxins can be. Humans have acquired the technology to spray toxins across vast swaths of the planet (Pimentel, 2009; Brookes and Barfoot, 2010), largely directed toward plants (herbicides) and insects (insecticides). In so doing, humans may now be the most ecologically relevant toxungenous organism on the planet.

Classifying Organisms That Use Poisons, Toxungens, and Venoms

Apart from the general (and frequently botched) distinction between poisons and venoms, biological toxins have been categorized by previous workers in a variety of ways (Bonventre, Lincoln & Lamanna, 1967; Army, 1998; Ogata and Ohishi, 2002; Hewlett and Hughes, 2005; Pimenta and De Lima, 2005; Vetter and Schmidt, 2006; Calvete,

Juarez & Sanz, 2007). These include, at the organismal level, the 1) organisms that produce them; 2) the anatomical source; and 3) organisms susceptible to them. They also include, at the suborganismal level, their 4) chemical structures; 5) major biological effects; 6) primary cellular or tissue targets; 7) molecular mechanisms of action; 8) sub-molecular binding sites; and even 9) levels of toxicity. In contrast to the toxins, classifying the organisms that produce these toxins has lacked a formal structure. In general, many toxic organisms are referred to as poisonous or venomous, but there has been disagreement and confusion here as well (Brodie, 1989; Rodríguez-Robles, 1994; Kardong, 1996)

We argue that organisms which use biological toxins should be classified to highlight the evolutionary and proximate source of their chemical armament. Different selective pressures have influenced whether an organism sequesters toxins from its diet, co-opts its own proteins for use as toxins, or appropriates the toxins synthesized by another species. Since poisons, toxungens, and venoms all exhibit a high degree of variability with respect to source, storage, and delivery, we propose a binomial nomenclature to identify each of these attributes for any given organism. Given recent interest in the diversification and biological roles of these toxins (Fry *et al*., 2008; Fry *et al*., 2009b; Vonk *et al*., 2011), and the acute need for detailed toxin databases driven by recent technological advances and bioprospecting interests (He *et al*., 2008; Jungo *et al*., 2010; Herzig *et al*., 2011; Kaas *et al*., 2012), a classification scheme at the organismal level that combines the origin, storage, and deployment of such toxins becomes pragmatic. Further, the classification scheme we propose distinguishes whether the organism uses its toxins as a poison, toxungen, or venom (or in multiple ways).

Table 3 summarizes our binomial classification scheme based on delivery mechanism, source of acquisition, and storage of toxins. Our scheme yields 12 categories, including four within each group of poisonous, toxungenous, and venomous organisms. The first term in the binomial is a contraction that combines the distinction between intrinsic (autogenous) versus extrinsic (heterogenous) acquisition of venom, and whether the organism stores its toxins within a specialized structure (glandular or aglandular). The second term in the binomial indicates whether the organism is poisonous, toxungenus, or venomous, depending on use of a delivery mechanism and generation of a wound. Table 3 also includes examples of organisms in each of the 12 groups, and these are discussed in the sections that follow.

Table 3. Classification of toxic organisms based on delivery (presence of delivery mechanism, wound), source of acquisition (synthesis), and storage (gland) of toxin.

Classification	Delivery mechanism	Wound	Synthesis	Storage gland	Representative example[a]
Autoaglandular-poisonous	Absent	Absent	Autogenous	Absent	Meloidae beetles
Autoglandular-poisonous	Absent	Absent	Autogenous	Present	Rhinocricidae millipedes
Heteroaglandular-poisonous	Absent	Absent	Heterogenous	Absent	*Pitohui* birds
Heteroglandular-poisonous	Absent	Absent	Heterogenous	Present	Dendrobatidae frogs
Autoaglandular-toxungenous	Present	Absent	Autogenous	Absent	None known
Autoglandular-toxungenous	Present	Absent	Autogenous	Present	*Myrmicaria* ants
Heteroaglandular-toxungenous	Present	Absent	Heterogenous	Absent	*Phrynosoma* horned lizards
Heteroglandular-toxungenous	Present	Absent	Heterogenous	Present	*Hapalochlaena* octopuses
Autoaglandular-venomous	Present	Present	Autogenous	Absent	*Lonomia* caterpillars
Autoglandular-venomous	Present	Present	Autogenous	Present	Viperidae snakes
Heteroaglandular-venomous	Present	Present	Heterogenous	Absent	Erinaceidae hedgehogs
Heteroglandular-venomous	Present	Present	Heterogenous	Present	Chaetognath worms

[a]Citations for representative examples are supplied in the text.

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Poisonous Organisms

Poisonous organisms lack a specialized structure for delivery of their toxins. Thus, delivery of toxic secretion is normally a passive strategy. Although transfer of poison relies on ingestion or contact, poisonous organisms may still employ adaptive tactics to deploy or otherwise enhance the anti-predator efficacy of their toxins. These tactics include enhanced skin secretion in the presence of a predator (Saito *et al*., 1985) and specific postures used to present toxin-dense regions of the body toward would-be molesters (Toledo and Jared, 1995; Lenzi-Mattos *et al*., 2005; Mori and Burghardt, 2008;

Kingdon *et al*., 2011; Toledo, Sazima & Haddad, 2011). Unfortunately, deciphering whether the source of toxin is autogenous or heterogenous can sometimes be difficult. Further, some organisms fall into several classes because a portion of their toxins are stored in glands while the remainder are more widely distributed in other tissues.

Autoaglandular-poisonous organisms produce their own toxins but lack a storage gland and a delivery apparatus. The toxins are often widely distributed among their tissues. Numerous organisms can be identified within this group, including examples among bacteria (Bonventre *et al*., 1967; Amano, Takeuchi & Furuta, 2010; Linhartova *et al*., 2010; Aktories, 2011), protists (Sykes and Huntley, 1987; Turner *et al*., 1998; Wolfe, 2000; Ianora *et al*., 2006), fungi (Buck, 1961; Vetter, 1998; Bennett and Klich, 2003; Rohlfs *et al*., 2007; Reverberi *et al*., 2010), and plants (Harborne, 1999a; Acamovic, Stewart & Pennycott, 2004; Winde and Wittstock, 2011). Examples among animals appear to be scarce. Blister beetles (family Meloidae), as a potential example, accumulate highly toxic cantharidin in their hemolymph and bleed reflexively from their leg joints when disturbed, thereby facilitating contact with the toxin (Carrel and Eisner, 1974; Dettner, 1987). Although the cantharidin is produced in the accessory glands, and is likely employed as a mate attractant (Carrel *et al.*, 1993; Nikbakhtzadeh *et al.*, 2012), its use for defensive purposes clearly functions within an autoaglandular context.

Autoglandular-poisonous organisms produce their own toxins and store them within a gland, but lack a delivery apparatus. Unicellular organisms lack glands, and therefore are excluded from this category. Examples abound, however, among plants and animals. Many plants, such as those in the nightshade family (Solanaceae), secrete and store toxins within glandular trichomes on their surface for protection against insects

(Eigenbrode, Trumble & White, 1996; Maffei, 2010). Among animals, the tropical millipede *Rhinocricus padbergi* possesses a pair of repugnatorial glands that secrete toxic benzoquinones directly to the surface of its body when threatened (Valderrama *et al.*, 2000; Arab *et al.*, 2003). Amphibians, having an abundance of toxin-laden cutaneous glands, may be the best studied group in this category (Daly, 1995; Toledo and Jared, 1995; Brizzi and Corti, 2007).

Heteroaglandular-poisonous organisms cannot produce their own toxic secretion, so they must acquire their toxins from other organisms. Lacking glands for storage, the toxins are often widely dispersed among the tissues. Exogenous toxins can be acquired in at least four ways: via ingestion (bioaccumulation), symbiotic bacteria, copulation, and maternal transfer to gametes and young. Several marine invertebrates and fishes appear to sequester toxins from their diet (Kvitek, 1991; Becerro, Starmer & Paul, 2006; Derby and Aggio, 2011), as do some insects (Nishida, 2002; Opitz and Muller, 2009) and several birds *(*Dumbacher, Spande & Daly, 2000; Dumbacher *et al*., 2004; Dumbacher, Menon & Daly*, 2009*). Human-released toxins can also accumulate in animals, rendering them toxic (Mebs, 2002). Symbiotic bacteria can synthesize toxins for their metazoan host, as documented in some marine invertebrates and fishes (Chau, Kalaitzis & Neilan, 2011). Perhaps most remarkable, males of several beetle species transfer toxins to females via copulation, whereupon the toxins disperse in hemolymph (Holz *et al*., 1994; Nikbakhtzadeh *et al*., 2007; Nikbakhtzadeh *et al*., 2012). Maternal transfer of toxins to eggs, presumably conferring protection to the eggs and/or larvae, has been documented in marine invertebrates and fishes (Lindquist, Hay & Fenical, 1992; Noguch and Arakawa, 2008; Pawlik *et al*., 1988), terrestrial invertebrates including insects (Schroeder *et al.*,

1999; Bezzerides *et al*., 2004; Nikbakhtzadeh *et a*l., 2012), and amphibians (Akizawa *et al*., 1994).

Heteroglandular-poisonous organisms similarly acquire their toxins from other organisms, but store the toxins within glands. Examples involving acquisition by food exist among marine invertebrates (West *et al*., 1996) and abound in insects (Blum, 1981; Pugalenthi and Livingstone, 1995; Morgan, 2010). Several amphibians also fall into this category. Frogs of the family Dendrobatidae, for example, acquire batrachotoxins from their arthropod food source (Saporito *et al*., 2011; Saporito *et al*., 2009), and secrete the toxins through skin glands to the surface of their body (Daly *et al*., 1994; Daly, 1995; Saporito *et al.*, 2010). Although most snakes possessing toxins are venomous, several species sequester diet-derived toxins within their nuchal glands, and maternally transfer the toxins to offspring (Williams and Brodie, 2004; Hutchinson *et al*., 2008; Mori *et al*., 2011). We are unaware of toxin production by symbiotic bacteria within this group, though examples can be anticipated.

Toxungenous Organisms

Toxungenous organisms possess the capacity to deliver their toxic secretion by means other than mere contact, but do not inflict a wound to introduce the toxins. Whereas poison delivery is essentially passive and relies primarily on the actions of the victim to introduce the toxins, toxungen delivery depends on actions taken by the toxic organism. Toxungen delivery often involves a specialized delivery apparatus, though this is not always required.

Autoaglandular-toxungenous organisms produce their own toxins, but do not store them within glands. This combination of features, apparently, is exceptionally rare, as we were unable to find any examples. Nevertheless, there may be organisms that satisfy the characteristics of this category.

Autoglandular-toxungenous organisms synthesize their own toxins and sequester them within glands. Many examples can be identified within this group. *Parabuthus* scorpions, the fire salamander (*Salamandra salamandra*), and spitting cobras (*Naja* spp. and *Hemachatus haemachatus*), for example, can spray their glandular secretions, which are toxic when contacting the eyes of mammalian predators (Newlands, 1974; Brodie and Smatresk, 1990; Chu et al., 2010). Most toxungenous organisms use their secretion for defense. However, whereas numerous ant and wasp species spray their glandular secretions for defensive purposes (Kenne *et al*., 2000), some ant species cooperatively seize, spread-eagle, and then smear toxins onto their prey to subdue them (e.g., Richard, Fabre & Dejean, 2001; Dejean and Lachaud, 2011). In these examples, the fire salamander is both poisonous (toxic via consumption) and toxungenous, and the cobras, scorpions, ants, and wasps are both toxungenous and venomous because they not only spray but also inject their toxic secretions. Insects that spray benzoquinones, such as bombardier beetles (family Carabidae), may represent additional examples. These beetles store hydroquinones and hydrogen peroxide in a two-chambered gland. When threatened, the beetle combines these two chemicals in a mixing chamber along with water, catalases, and peroxidases, and the exothermic reaction results in production of a scalding vapor, containing 1,4-benzoquinones, that is used to deter predators (Eisner *et al*., 1977; Eisner *et al*., 2000a). Some evidence suggests that benzoquinones can exert toxic effects

on predators (Eisner 1958, 1960; Eisner, Rossini & Eisner, 2000b; Paysse, Holder & Coats, 2001; Eisner *et al.*, 2005; also see Souza and Willemart, 2011).

Heteroaglandular-toxungenous organisms acquire their toxins from other organisms but do not store them in a gland. Finding examples proved to be difficult, but the Texas horned lizard (*Phrynosoma cornutum*) may fit this category. Several studies reported that the blood squirting response of *P. cornutum*, directed primarily toward canids, elicits a strong aversion response, particularly when blood is directed at the oral cavity; however, the chemical that acts as the deterrent has not been isolated, and whether it causes a pathophysiological response remains unclear (Sherbrooke and Middendorf, 2004; Sherbrooke and Mason, 2005). Therefore, more experimentation is needed to determine if the blood of the Texas horned lizards has a toxic effect, and thus truly represents a heteroaglandular-toxungenous organism. Humans, however, make abundant use of exogenously acquired toxins, especially for weed and insect control (Pimentel, 2009; Brookes and Barfoot, 2010). By dramatically altering the environment through toxin application, humans have become the most influential toxungenous organism on the planet.

Heteroglandular-toxungenous organisms also acquire their toxins from other organisms, but sequester them within glands. Tetrodotoxin, for example, is produced by bacteria in the Vibrionaceae family and acts by selectively blocking the activity of certain subtypes of voltage-gated sodium channels in nerves and cardiac and skeletal muscle (Watters, 2005). Some animals possess channels that are resistant to these toxins, which allows them to accumulate tetrodotoxin either in their tissues or within specialized glands. The ringed octopus (*Hapalochlaena maculosa*) harbors these bacteria in its

salivary gland, and possesses a venom comprised largely, but not exclusively, of tetrodotoxin. In addition to introducing tetrodotoxin during a bite, it can eject saliva into the water around a crab, move a distance away, and wait for the toxin to take effect (Sutherland and Lane, 1969). Thus, this species is both toxungenous and venomous. The tiger keelback (*Rhabdophis tigrinus*), a colubrid snake found in eastern Asia, sequesters toxins (bufadienolides) from toads (like *Bufo bankorensis*) in its nuchal glands (Chen *et al.*, 2012). Under pressure during physical contact, these glands can spray the toxic secretions up to a meter, whereupon contact with the eye causes acute burning pain and tissue injury (Chen *et al*., 2012). Tiger keelbacks also possess venom glands associated with enlarged maxillary teeth (Ferlan *et al.*, 1983), making them both venomous and toxungeous.

Venomous Organisms

Venomous organisms deploy their toxins by introducing them via mechanical trauma to the internal milieu of other organisms. The scope of venomous organisms is vast, not just among animals, but also among bacteria, protists, fungi, and plants, as mentioned previously. Delivery structures or delivery systems are nearly as diverse as the organisms possessing them, ranging from the intricate design of hypodermic viper fangs to the hollow spines employed by certain caterpillars (Mebs, 2002). In this section, we provide examples only from animals.

Autoaglandular-venomous organisms synthesize their own venom but do not store it within glands. Numerous examples exist, including the aforementioned cnidarians and ctenophores, which produce and store their toxins within individual cells. The caterpillar

Lonomia oblique comprises a good metazoan example. These caterpillars possess no gland that produces the venom; instead, secretory epithelium that underlies the tegument and spines secretes the toxins, which are concentrated at the tips of the spines. When contact is made with the spine, the tip containing the venom breaks off and causes a cutaneous reaction in the victim (Veiga, Blochtein & Guimar, 2001).

Autoglandular-venomous organisms possess the most sophisticated toxin delivery systems, including venom glands and usually an elaborate delivery apparatus. This group has garnered more attention from researchers than any other. Representatives include numerous marine and terrestrial invertebrates, many fishes, several amphibians, several lizards, numerous snakes, and several mammals (Mebs, 2002). Some authorities consider hematophagous (blood-sucking) organisms (e.g., mosquitoes, tsetse flies, fleas, leeches), which secrete injurious enzymes, to be in this group (Fry *et al.*, 2009b). Slow Lorises (genus *Nycticebus*) represent an unusual case in which the secretion from the brachial gland, when combined with saliva from licking of the gland, becomes toxic, and can be used defensively when biting conspecifics or potential predators (Alterman, 1995; Hagey et al., 2007).

Heteroaglandular-venomous organisms procure their toxins from other organisms and lack glands for storage. In the prior section on poisonous animals, we described four sources of exogenous toxins: ingestion (bioaccumulation), symbiotic bacteria, copulation, and maternal transfer to gametes and young. In this group, we find a fifth source: deliberately co-opting the toxins or venom apparatus of another organism. Several examples illustrate this group. The hedgehog (*Erinaceus europaeus*) preys upon poisonous toads (*Bufo* sp), and anoints its spines with the toxic secretion of its prey by

rubbing or licking the toxins onto its spines (Brodie, 1989). The spines may then puncture a would-be attacker, delivering the toxins through a wound. Nudibranchs feed on hydrozoans and then store the undischarged hydrozoan nematocysts on their external surface for protection (Greenwood and Garrity, 1991; Mebs, 2001). Certain crabs similarly co-opt the nematocysts of anemones by situating the entire anemone on their carapace or claws (Chintiroglou, Doumenc & Guinot, 1996; Karplus, Fiedler & Ramcharan, 1998). Even humans are facultatively heteroaglandular-venomous organisms. The indigenous Embera Indians of Western Columbia, for example, used darts coated with poison (batrachotoxins) from a poison dart frog (*Phyllobates* sp.) for hunting (Myers *et al.*, 1978). The Indians would collect the poison by impaling or restraining a poison dart frog with a stick, rub their darts on the frog's back, and then dry the toxins on the dart over a fire (Myers *et al.*, 1978). How is this different than a hedgehog spreading toxins on its spines, or a crab using an anemone for protection? Indeed, as Mebs (2002) observed, *Homo sapiens* has become one of the most dangerous venomous animals, utilizing natural toxins (e.g., batrachotoxins) and manufactured "toxicants" (e.g., chemical warfare) for both defense and predation. We have also co-opted toxins for more benevolent purposes, such as use in human and veterinary medicine (Reisner, 2004; Chaddock and Acharya, 2011; King, 2011).

Heteroglandular-venomous organisms store the toxins acquired from other organisms in one or more glands. Accumulating evidence suggests that a number of marine worms, including chaetognath (Thuesen and Kogure, 1989), nemertean (Ali *et al*., 1990; McEvoy, Rogers & Gibson, 1998), and platyhelminth (Planoceridae) (Ritson-Williams, Yotsu-Yamashita & Paul, 2006) representatives, sequester tetrodotoxin

produced by symbiotic bacteria within their glands, and deliver it through a wound for predation and possibly defense (Williams, 2010). Several species of blue-ringed octopus (*Hapalochlaena lunulata*) represent another example, having a highly toxic secretion containing tetrodotoxin, apparently produced by *Vibrio* bacteria within its posterior salivary glands (Hwang *et al*., 1989), which can be injected into prey and predators, though other autogenous toxins appear to be present (Fry *et al*., 2009a). These octopuses can also transfer the toxin maternally to their offspring (Williams *et al*., 2011).

Toxin Evolution: the Influence of Delivery Mechanism

In the ongoing co-evolutionary arms races between organisms that employ toxins and those affected by them, continual toxin variation is often important for keeping the toxic organism one step ahead of its competitors (Kordis and Gubenek, 2000). Toxic organisms employ a wide range of different toxins, which vary from small secondary metabolites to larger peptides and proteins (Mebs, 2001, 2002). Different taxonomic groups of toxic organisms generally employ different classes of toxins. Poisonous animals generally possess toxins that are small secondary metabolites, whereas venomous organisms generally produce toxic secretions that contain peptide or protein toxins (Mebs, 2002). These differences may result largely from the interaction of the evolutionary drive toward increased toxin variation with the functional constraints of toxin delivery mechanism.

The major constraint on poisons stems from their passive route of delivery. These toxins must be resistant to digestion if delivered via ingestion, or must have properties that enable them to penetrate the external surface of the organism they come in contact

with. Protein toxins will generally not work for this kind of application, since most proteins are readily broken down by digestive action (Mebs, 2002b) and are generally too large to be absorbed across a body surface (Bos and Meinardi, 2000). The use of secondary metabolites overcomes these constraints; however, because secondary metabolites are produced via complex metabolic pathways employing many different chemical reactions catalyzed by multiple enzymes (Mebs, 2001; Wright, 2002), they may be less able to undergo rapid evolution.

Venom toxins bypass these constraints because they are delivered directly to the tissues. This may mean that the major factor that governs venom effectiveness over time, considering the evolution of venom resistance, is its ability to generate significant variation. Having direct genetic control over toxin production, rather than indirect control via modification of one or more enzymes (as secondary metabolites require), allows for the creation of a significantly more diverse array of toxins (Mebs, 2001). Indeed, most genes coding for venom protein toxins are a part of large multigene families, suggesting significant gene duplication and subsequent modification, thereby promoting rapid evolution (Kordis and Gubenek, 2000; Fry *et al.*, 2009b).

The difficulty in evolving new secondary metabolite toxins may also influence whether an organism acquires its toxins autogenously or heterogenously. Despite the fact that evolving resistance to a toxin involves significant costs (Brodie and Brodie, 1999; Mebs, 2001), it may be, in some circumstances, easier to evolve resistance to a toxin and then sequester that toxin than it is to evolve a new secondary metabolite *de novo*. This may be one reason why most examples of heterogenous acquisition of toxins come from poisonous animals that sequester secondary metabolite toxins, whereas nearly all

venomous animals employ autogenous toxins. Toxin availability may also be an issue, as primary consumers generally have greater access to the protective toxins synthesized by producers (cyanobacteria, autotrophic protists, algae, plants) than do predators. Thus, although bioaccumulation can occur up the trophic ladder (Wang, 2008; Miller *et al.*, 2010), heterogenous acquisition of toxins is more frequent among invertebrates than vertebrates.

Whether toxin delivery is active or passive can impact how selection acts. Poisonous organisms primarily use their toxins for defense (Meier and White, 1995; Mebs, 2002). In animals, effective use of the poison for defense is often closely linked to the animal's aposematic adaptations (Sherratt, 2002; Blount *et al.*, 2009), which can take the form of coloration, behavior, or olfactory cues (Eisner and Grant, 1981). Potential predators must acquire the capacity, through innate recognition or learning, to avoid these toxic animals in order for the toxins to be employed as part of an effective defense. This need may set up a situation where selection acts to prevent the development of overly toxic poisons, since a potential predator cannot learn anything if the poison results in its death (Mebs, 1994).

Another consideration is that poisons, by virtue of their passive transfer, may not necessarily act to preserve the life of the individual. In poisonous plants, this isn't much of a problem, since these plants can afford to lose many leaves and branches to consumption without risk of the whole plant dying. Animals, in contrast, generally can't survive when a significant portion of their body is consumed; however, this is often what must happen if an attacker is to consume a significant dose of the animal's poison. This means the selective pressures pushing animals towards being poisonous sometimes act at

a level above that of the individual. This may be why many poisonous arthropods tend to be found in aggregations of closely related individuals, suggesting that their toxicity evolved through kin selection (Pasteels, Gregoire & Rowell-Rahier, 1983).

Organisms that more actively or more precisely control their toxins may begin to shift the level of selection back to the individual. Control of toxin deployment can evolve in multiple ways. First, some organisms concentrate their toxins in strategic places and utilize behavior to place higher concentrations of their toxins in the path of their attacker (Toledo and Jared, 1995; Lenzi-Mattos *et al*., 2005; Mori and Burghardt, 2008; Kingdon *et al.*, 2011; Toledo *et al*., 2011). Second, some organisms employ toxins that are inducible rather than constitutive, enabling them to increase their secretion of toxins when an attacker is present (Harborne, 1999b). Plants, for example, often increase toxin production following browsing, and more so in tissues subject to the highest rates of browsing (Zangerl and Rutledge, 1996). Pufferfish can increase toxin release when a predator approaches (Saito *et al*., 1985). Third, because toxin production and storage entails both energetic and ecological costs, selection has favored judicious use of toxin in a number of venomous animals, ensuring optimal venom expenditure during defensive or predatory contexts (i.e., venom metering or venom optimization; (Wigger, Kuhn-Nentwig & Nentwig, 2002; Hayes, 2008; Herbert and Hayes, 2008). Judicious toxin use occurs in toxungenous delivery as well (Obin and Vander Meer, 1985). Finally, selection has further refined the delivery systems of some animals so that toxins can be deployed as toxungens via spitting, spraying, or squirting, thereby avoiding the risk of physical contact with a potentially dangerous enemy.

Active delivery of toxins to the attacker, rather than the attacker coming to get the toxins, allows the toxins to be used not only for defense, but also for predation. Thus, in contrast to poisons (and most toxungens), venoms often serve a predatory function. For species that rely on venom for subduing and procuring prey, the toxins are under intense selection to quickly paralyze, kill, and even digest the victim. Although defensive use of toxungens and venoms benefits from aposematism and predator recognition, active delivery of toxins for predation, by contrast, is generally more effective with crypsis rather than aposematism.

Conclusions

Toxins are substances that, when present in relatively minute physiological concentrations, cause dose-dependent pathophysiological injury to a living organism, thereby reducing functionality or viability. Toxins may be categorized into three general classes: biological, environmental, and anthropogenic.

Venom and poison are functionally distinct, and should not be conflated. A detailed literature review of the definitions of venom reveals several features in common: hierarchy and exclusiveness, source of secretion, mode of transmission, purpose, and active/passive delivery. Our revised definition includes hierarchy and exclusiveness and mode of transmission, but excludes source of secretion, purpose, and active delivery.

Poison – A toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that results in self-induced toxicity or is passively transferred without a delivery mechanism from one organism to the internal milieu of

another organism without mechanical injury, usually through ingestion, inhalation, or absorption across the body surface.

Venom - A toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that is passively or actively transferred from one organism to the internal milieu of another organism via a delivery mechanism and mechanical injury.

We argue for the creation of a new category of toxic biological secretions, toxungen. A toxungen is defined as a toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that is actively transferred via a delivery mechanism from one organism to the external surface of another organism without mechanical injury.

We argue that organisms which use biological toxins should be classified to highlight the evolutionary and proximate source of their chemical armament. We propose a classification scheme that distinguishes organisms based on three attributes of the toxin: its production or acquisition (autogenous, heterogenous), storage (glandular or aglandular), and nature (venomous, poisonous, toxungenous).

The themes argued in this paper may be novel, and some readers may counter that they are unwarranted, but we believe they will better organize and unify a fractured body of literature. The improved definitions and classification scheme should make these terms more accessible to and better understood by both researchers and the general public.

References

Academic Press Dictionary of Science and Technology., (1992). Venom, in: Morris, C.G. (Ed.), Academic Pr, San Diego.

Acamovic, T., Stewart, C.S., Pennycott, T., (2004). Poisonous plants and related toxins. CABI, Wallingford, Oxon, UK.

Akizawa, T., Mukai, T., Matsukawa, M., Yoshioka, M., Morris, J., Butler Jr, V., (1994). Structures of novel bufadienolides in the eggs of a toad, *Bufo marinus*. *Chemical and Pharmaceutical Bulletin (Tokyo)* 42, 754.

Aktories, K., (2011). Bacterial protein toxins that modify host regulatory GTPases. *Nature Reviews: Microbiology* 9, 487–498.

Ali, A.E., Arakawa, O., Noguchi, T., Miyazawa, K., Shida, Y., Hashimoto, K., (1990). Tetrodotoxin and related substances in a ribbon worm *Cephalothrix linearis* (Nemertean). *Toxicon* 28, 1083–1093.

Alterman, L., (1995). Toxins and toothcombs: potential allospecific chemical defenses in *Nycticebus* and *Perodicticus*, In *Creatures of the Dark: The Nocturnal Prosimians* (eds L. Alterman, G.A. Doyle, M.K. Izard), pp. 413–424. Plenum Press, New York.

Amano, A., Takeuchi, H., Furuta, N., (2010). Outer membrane vesicles function as offensive weapons in host-parasite interactions. *Microbes and Infection* 12, 791–798.

Arab, A., Zacarin, G., Fontanetti, C., Camargo-Mathias, M., Dos Santos, M., Cabrera, A., (2003). Composition of the defensive secretion of the Neotropical millipede *Rhinocricus padbergi* Verhoeff 1938 (Diplopoda: Spirobolida: Rhinocricidae). *Entomotropica* 18, 79–82.

Army, U., (1998). Medical response to chemical warfare and terrorism. Aberdeen Proving Ground, MD: Chemical Casualty Care Division, USAMRIID.

Auerbach, P., (1988). Clinical therapy of marine envenomation and poisoning, In *Handbook of Natural Toxins vol. 3: Marine Toxins and Venoms*. (ed A.T. Tu), Marcel Dekker, New York.

Ayala, F.J., (1999). Adaptation and novelty: teleological explanations in evolutionary biology. *History and Philosophy of the Life Sciences* 21, 3.

Beard, R.L., (1963). Insect toxins and venoms. *Annuual Review of Entomology* 8, 1–18.

Becerro, M.A., Starmer, J.A., Paul, V.J., (2006). Chemical defenses of cryptic and aposematic gastropterid molluscs feeding on their host sponge *Dysidea granulosa*. *Journal of Chemical Ecology* 32, 1491–1500.

Beeckman, D., Vanrompay, D., (2010). Bacterial secretion systems with an emphasis on the chlamydial Type III secretion system. *Current Issues in Molecular Biolology* 12, 17–41.

Bennett, J.W., Klich, M., (2003). Mycotoxins. *Clinical Microbiology Review* 16, 497–516.

Bettini, S., Brignoli, P.M., 1978. Review of the spider families, with notes on the lesser-known poisonous forms, In *Arthropod Venoms: Handbook of Experimental Pharmacology*, vol. 48 (ed S. Bettini), pp. 101–120. Springer-Verlag, Berlin.

Bezzerides, A., Yong, T.H., Bezzerides, J., Husseini, J., Ladau, J., Eisner, M., Eisner, T., (2004). Plant-derived pyrrolizidine alkaloid protects eggs of a moth (*Utetheisa ornatrix*) against a parasitoid wasp (*Trichogramma ostriniae*). *Proceedings of the National Academy of Sciences* 101, 9029.

Blount, J.D., Speed, M.P., Ruxton, G.D., Stephens, P.A., (2009). Warning displays may function as honest signals of toxicity. *Proceedings of the Royal Society of London B Biological Sciences* 276, 871–877.

Blum, M.S., (1966). Source and specificity of trail pheromones in *Termitopone monomorium* and *Huberia* and their relation to those of some other ants. *Proceedings of the Royal Entomological Society of London Series A General Entomology* 41, 155–160.

Blum, M.S., (1981). *Chemical Defenses of Arthropods*. Academic Press, New York.

Bonventre, P.F., Lincoln, R.E., Lamanna, C., (1967). Status of bacterial toxins and their nomenclature: need for discipline and clarity of expression. *Bacteriological Reviews* 31, 95–109.

Bos, J.D., Meinardi, M.M.H.M., (2000). The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Expimental Dermatology* 9, 165–169.

Brizzi, R., Corti, C., (2007). Cutaneous antipredatory secretions and pheromones in anurans and urodeles. *Marine and Freshwater Behaviour and Physiology* 40, 225–231.

Brodie, E., (1989). *Venomous Animals*. St. Martin's Press, New York.

Brodie, E.D., III, (2009). Toxins and venoms. *Current Biology* 19, R931–935.

Brodie, E.D., Smatresk, N.J., (1990). The antipredator arsenal of fire salamanders: spraying of secretions from highly pressurized dorsal skin glands. *Herpetologica* 46, 1–7.

Brodie, E.D., III, Brodie, E.D., Jr. (1999). Costs of exploiting poisonous prey: evolutionary trade-offs in a predator-prey arms race. *Evolution* 626–631.

Brookes, G., Barfoot, P., (2010). Global impact of biotech crops: Environmental Effects, 1996–2008. *AgBioForum* 13, 76–94.

Buck, R.W., (1961). Mushroom toxins: a brief review of literature. *New England Journal of Medicine* 265, 681–686.

Calvete, J.J., Juárez, P., Sanz, L., (2007). Snake venomics. Strategy and applications. *Journal of Mass Spectrometry* 42, 1405–1414.

Carrel, J., McCairel, M., Slagle, A., Doom, J., Brill, J., McCormick, J., (1993). Cantharidin production in a blister beetle. *Cellular and Molecular Life Science* 49, 171–174.

Carrel, J.E., Eisner, T., (1974). Cantharidin: potent feeding deterrent to insects. *Science* 183, 755–757.

Chaddock, J.A., Acharya, K.R., 2011. Engineering toxins for 21st century therapies. *The FEBS Journal* 278, 899–904.

Chau, R., Kalaitzis, J.A., Neilan, B.A., (2011) On the origins and biosynthesis of tetrodotoxin. *Aquatic Toxicology* 104, 61-72.

Chen, Y.C., Yen, D.H.T., Chen, Y.W., Huang, M.S., Huang, C.I., Chen, M.H., (2012). Toxin ophthalmia caused by nuchal gland secretion of the Taiwan tiger keelback (*Rhabdophis tigrinus formosanus*). *Journal Formosan Medical Association* 1–4.

Chintiroglou, C.C., Doumenc, D., Guinot, D., (1996). Anemone-carrying behaviour in a deep-water homolid crab (Brachyura, Podotremata). *Crustaceana* 69, 19–25.

Chu, E.R., Weinstein, S.A., White, J., Warrell, D.A., (2010). Venom ophthalmia caused by venoms of spitting elapid and other snakes: Report of ten cases with review of epidemiology, clinical features, pathophysiology and management. *Toxicon* 56, 259–272.

Collins Dictionary of Medicine., (2005). venom, Collins, New York.

Daly, J.W., (1995). The chemistry of poisons in amphibian skin. *Proceedings of the National Academy of Science* 92, 9–13.

Daly, J.W., Garraffo, H.M., Spande, T.F., Jaramillo, C., Rand, A.S., (1994). Dietary sources for skin alkaloids of poison frogs (Dendrobatidae). *Journal of Chemical Ecology* 20, 943–955.

Dejean, A., Lachaud, J.P., (2011). The hunting behavior of the African ponerine ant (*Pachycondyla pachyderma)*. *Behavioural Processes* 86, 169–173.

Deml, R., Dettner, K., (1994). *Attacus atlas* caterpillars (Lep., Saturniidae) spray an irritant secretion from defensive glands. *Journal of Chemical Ecology* 20, 2127–2138.

Derby, C.D., Aggio, J.F., (2011). The neuroecology of chemical defenses. *Integrative and Comparative Biology* 51, 771–780.

Dettner, K., 1987. Chemosystematics and evolution of beetle chemical defenses. *Annual Review of Entomology* 32, 17–48.

Dorland's Illustrated Medical Dictionary., (2007). Venom, Elsevier Health Sciences, New York.

Dumbacher, J.P., Menon, G.K., Daly, J.W., (2009). Skin as a toxin storage organ in the endemic New Guinean genus *Pitohui*. *Auk* 126, 520–530.

Dumbacher, J.P., Spande, T.F., Daly, J.W., (2000). Batrachotoxin alkaloids from passerine birds: a second toxic bird genus (*Ifrita kowaldi*) from New Guinea. *Proceedings of the National Academy of Science* 97, 12970–12975.

Dumbacher, J.P., Wako, A., Derrickson, S.R., Samuelson, A., Spande, T.F., Daly, J.W., (2004). Melyrid beetles (Choresine): a putative source for the batrachotoxin alkaloids found in poison-dart frogs and toxic passerine birds. *Proceedings of the National Academy.of Science* 101, 15857–15860.

Eaton, D.L., Klaassen, C.D., (2001). *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th ed. McGraw-Hill, New York.

Eigenbrode, S., Trumble, J., White, K., (1996). Trichome exudates and resistance to beet armyworm (Lepidoptera: Noctuidae) in *Lycopersicon hirsutum f. typicum* accessions. *Environmental Entomology* 25, 90–95.

Eisner, T., (1958). The protective role of the spray mechanism of the bombardier beetle, *Brachynus ballistarius* Lec. *Jounal of Insect Physiology* 2, 215–216, IN217–IN218, 217–220.

Eisner, T., (1960). Defense mechanisms of arthropods. II. The chemical and mechanical weapons of an earwig. *Psyche* 67, 62–70.

Eisner, T., Aneshansley, D.J., Eisner, M., Attygalle, A.B., Alsop, D.W., Meinwald, J., (2000a). Spray mechanism of the most primitive bombardier beetle (*Metrius contractus*). Journal of Experimental Biology 203, 1265–1275.

Eisner, T., Eisner, M., Siegler, M., (2005). *Secret Weapons: Defenses of Insects, Spiders, Scorpions, and Other Many-legged Creatures.* Belknap Press of Harvard University Press, Cambridge, Mass.

Eisner, T., Grant, R.P., (1981). Toxicity, odor aversion, and olfactory aposematism. *Science* 213, 476–476.

Eisner, T., Jones, T., Aneshansley, D., Tschinkel, W., Silberglied, R., Meinwald, J., (1977). Chemistry of defensive secretions of bombardier beetles (Brachinini, Metriini, Ozaenini, Paussini). *Journal of Insect Physiology* 23, 1383–1386.

Eisner, T., Meinwald, J., Monro, A., Ghent, R., (1961). Defence mechanisms of Arthropods. I. The composition and function of the spray of the whipscorpion, *Mastigoproctus giganteus* (Lucas)(Arachnida, Pedipalpida). *Journal of Insect Physiology*, 6, 272-298.

Eisner, T., Rossini, C., Eisner, M., (2000b). Chemical defense of an earwig (*Doru taeniatum*). *Chemoecology* 10, 81–87.

Ferlan, I., Ferlan, A., King, T., Russell, F.E., (1983) Preliminary study on the venom of the colubrid snake *Rhabdophis subminatus* (red-necked keelback). *Toxicon* 21, 570-574.

Freyvogel, T.A., 1972. Poisonous and venomous animals in East Africa. *Acta Tropica* 29, 401.

Fry, B., Roelants, K., Norman, J., (2009a). Tentacles of venom: toxic protein convergence in the kingdom animalia. *Journal of Molecular Evolution* 68, 311–321.

Fry, B.G., Roelants, K., Champagne, D.E., Scheib, H., Tyndall, J.D.A., King, G.F., Nevalainen, T.J., Norman, J.A., Lewis, R.J., Norton, R.S., (2009b). The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. *Annual Review of Genomics and Human Genetics* 10, 483–511.

Fry, B.G., Scheib, H., van der Weerd, L., Young, B., McNaughtan, J., Ramjan, S.F.R., Vidal, N., Poelmann, R.E., Norman, J.A., (2008). Evolution of an arsenal. *Molecular and Cellular Proteomics* 7, 215–246.

Fry, B.G., Vidal, N., Norman, J.A., Vonk, F.J., Scheib, H., Ramjan, S.F.R., Kuruppu, S., Fung, K., Hedges, S.B., Richardson, M.K., Hodgson, W.C., Ignjatovic, V.,

Summerhayes, R., Kochva, E., (2006). Early evolution of the venom system in lizards and snakes. *Nature* 439, 584–588.

Fu, H.Y., Chen, S.J., Chen, R.F., Ding, W.H., Kuo-Huang, L.L., Huang, R.N., (2006). Identification of oxalic acid and tartaric acid as major persistent pain-inducing toxins in the stinging hairs of the nettle, *Urtica thunbergiana*. *Annals of Botany* 98, 57–65.

Gibbs, F.W., (2009). Medical understandings of poison circa 1250–1600. Unpublished Ph.D. Dissertation, University of Wisconsin, Madison.

Greenwood, P.G., Garrity, L.K., (1991). Discharge of nematocysts isolated from aeolid nudibranchs. *Hydrobiologia* 216, 671–677.

Hagey, L.R., Fry, B.G., Fitch-Snyder, H., (2007). Talking defensively, a dual use for the brachial gland exudate of slow and pygmy lorises, In *Primate Anti-Predator Strategies* (eds S.L. Gursky, K.A.I. Nekaris), pp. 253–272. Springer, New York.

Halstead, B.W., (1965). *Poisonous and venomous marine animals of the world. Vol. I. Invertebrates*. U.S. Govt. printing office, Washington, D.C.

Harborne, J.B., (1999a). Plant chemical ecology, In *Comprehensive Natural Products Chemistry* (ed K. Mori), pp. 137–196. Pergamon Press Inc, Oxford. Elsevier, Amsterdam.

Harborne, J.B., (1999b). Recent advances in chemical ecology. *Natural Product Report* 16, 509–523.

Hayes, A.W. (ed), (2001). *Principles and Methods of Toxicology*, 4th ed., Taylor & Francis, Ann Arbor, MI.

Hayes, W.K., (2008). The snake venom-metering controversy: levels of analysis, assumptions, and evidence, In *The Biology of Rattlesnakes* (eds W.K. Hayes, K.R. Beaman, M.D. Cardwell, S.P. Bush), pp. 191–220. Loma Linda University Press, Loma Linda, California.

He, Q.-Y., He, Q.-Z., Deng, X.-C., Yao, L., Meng, E., Liu, Z.-H., Liang, S.-P., (2008). ATDB: a uni-database platform for animal toxins. *Nucleic Acids Research* 36, D293–D297.

Herbert, S.S., Hayes, W.K., (2008). Venom expenditure by rattlesnakes and killing effectiveness in rodent prey: do rattlesnakes expend optimal amounts of venom? In *The Biology of the Rattlesnakes* (eds W.K. Hayes, K.R. Beaman, M.D. Cardwell, S.P. Bush), pp. 221–228. Loma Linda University Press, Loma Linda, California.

Heredia, A., de Biseau, J.C., Quinet, Y., (2005). Toxicity of the venom in three neotropical *Cromatogaster* ants (Formicidae: Myricinae). *Chemoecology* 15, 235-242.

Herzig, V., Wood, D.L.A., Newell, F., Chaumeil, P.A., Kaas, Q., Binford, G.J., Nicholson, G.M. Grose, D., King, G.F., (2011). ArachnoServer 2.0, an updated online resource for spider toxin sequences and structures. *Nucleic Acids Research* 39, D653-D657.

Hewlett, E.L., Hughes, M.A., (2005). Toxins, in: *Principles and Practice of Infectious Diseases*, 6th ed. (eds G.L. Mendell, J.E. Bennett, R. Dolin), pp. 24–33. Churchill Livingstone, New York.

Hodgson, E., Mailman, R.B., Chambers, J.E., (1988). *Dictionary of Toxicology*. McMillan Reference, London.

Holz, C., Streil, G., Dettner, K., Dutemeyer, J., Boland, W., (1994). Intersexual transfer of a toxic terpenoid during copulation and its paternal allocation to developmental stages: quantification of cantharidin in cantharidin-producing oedemerids (Coleoptera: Oedemeridae) and *Cantharipbilous pyrochroids* (Coleoptera: Pyrochroidae). *Zeitschrift Fur Naturforschung C: Journal of Biosciences* 49, 856–864.

Hutchinson, D.A., Savitzky, A.H., Mori, A., Meinwald, J., Schroeder, F.C., (2008). Maternal provisioning of sequestered defensive steroids by the Asian snake *Rhabdophis tigrinus*. *Chemoecology* 18, 181–190.

Hwang, D., Arakawa, O., Saito, T., Noguchi, T., Simidu, U., Tsukamoto, K., Shida, Y., Hashimoto, K., (1989). Tetrodotoxin-producing bacteria from the blue-ringed octopus *Octopus maculosus*. *Marine Biology* 100, 327–332.

Ianora, A., Boersma, M., Casotti, R., Fontana, A., Harder, J., Hoffmann, F., Pavia, H., Potin, P., Poulet, S.A., Toth, G., (2006). New trends in marine chemical ecology. *Estuaries and Coasts* 29, 531–551.

Ismail, M., Al-Bekairi, A.M., El-Bedaiwy, A.M., Abd-El Salam, M.A., (1993). The ocular effect of spitting cobras: II. Evidence that cardiotoxins are responsible for the corneal opacification syndrome. *Clinical Toxicology*, 31, 45-62.

Jared, C., Antoniazzi, M., Navas, C., Katchburian, E., Freym¸ller, E., Tambourgi, D., Rodrigues, M., (2005). Head co-ossification, phragmosis and defence in the casque-headed tree frog (*Corythomantis greeningi*). *Journal of Zoology* 265, 1–8.

Jungo, F., Estreicher, A., Bairoch, A., Bougueleret, L., Xenarios, I., (2010). Animal toxins: how is complexity represented in databases? *Toxins* 2, 262–282.

Kaas, Q., Yu, R., Jin, A.H., Dutertre, S., Craik, D.J., (2012). ConoServer: updated content, kowledge, and discovery tools in the conopeptide database. *Nucleic Acids Research* 40, D325-D330.

Kardong, K.V., (1996). Snake toxins and venoms: an evolutionary perspective. *Herpetologica* 36–46.

Karplus, I., Fiedler, G., Ramcharan, P., (1998). The intraspecific fighting behavior of the Hawaiian boxer crab, *Lybia edmondsoni*: fighting with dangerous weapons? *Symbiosis* 24, 287–302.

Kenne, M., Schatz, B., Durand, J.L., Dejean, A., (2000). Hunting strategy of a generalist ant species proposed as a biological control agent against termites. *Entomologia Experimentalis et Applicata* 94, 31–40.

Kenny, B., Valdivia, R., 2009. Host-microbe interactions: bacteria. *Current Opinion in Microbiology* 12, 1.

King, G.F., (2011). Venoms as a platform for human drugs: translating toxins into therapeutics. *Expert Opinion on Biological Therapy* 11, 1469–1484.

Kingdon, J., Agwanda, B., Kinnaird, M., O'Brien, T., Holland, C., Gheysens, T., Boulet-Audet, M., Vollrath, F., (2011). A poisonous surprise under the coat of the African crested rat. *Proceedings of the Royal Society of London B Biological Sciences* doi: 10.1098/rspb.2011.1169.

Koopowitz, H., (1970). Feeding behaviour and the role of the brain in the polyclad flatworm, *Planocera gilchristi*. *Animal Behavior* 18, 31–35.

Kordis, D., Gubenek, F., (2000). Adaptive evolution of animal toxin multigene families. *Gene* 261, 43–52.

Kuhn-Nentwig, L., (2003). Antimicrobial and cytolytic peptides of venomous arthropods. *Cellular and Molecular Life Sciences* 60, 2651–2668.

Kvitek, R.G., (1991). Paralytic shellfish toxins sequestered by bivalves as a defense against siphon-nipping fish. *Marine Biology* 111, 369–374.

Lenzi-Mattos, R., Antoniazzi, M.M., Haddad, C.F.B., Tambourgi, D.V., Rodrigues, M.T., Jared, C., (2005). The inguinal macroglands of the frog *Physalaemus nattereri* (Leptodactylidae): structure, toxic secretion and relationship with deimatic behaviour. *Journal of Zoology* 266, 385–394.

Lindquist, N., Hay, M.E., Fenical, W., (1992). Defense of ascidians and their conspicuous larvae: adult vs. larval chemical defenses. *Ecological Monographs*, 547–568.

Linhartova, I., Bumba, L., Masin, J., Basler, M., Osicka, R., Kamanova, J., Prochazkova, K., Adkins, I., Hejnova-Holubova, J., Sadilkova, L., Morova, J., Sebo, P., (2010). RTX proteins: a highly diverse family secreted by a common mechanism. *FEMS Microbiology Reviews* 34, 1076–1112.

Liu, X.Z., Xiang, M.C., Che, Y.S., (2009). The living strategy of nematophagous fungi. *Mycoscience* 50, 20–25.

Lotan, A., Fishman, L., Loya, Y., Zlotkin, E., (1995). Delivery of a nematocyst toxin. *Nature* 375, 456–456.

Luo, H., Liu, Y.J., Fang, L., Li, X., Tang, N.H., Zhang, K.Q., (2007). *Coprinus comatus* damages nematode cuticles mechanically with spiny balls and produces potent toxins to immobilize nematodes. *Applied and Environmental Microbiology* 73, 3916–3923.

Mackessy, S.P., (2009). The field of reptile toxinology: snakes, lizards, and their venoms. In *Handbook of venom and toxins of reptiles* (ed S.P. Mackessy), p. 3–23. CRC, Boca Raton.

MacMillan Dictionary of Toxicology., (1999). venom, Macmillan Publishers Ltd, New York.

Maffei, M.E., (2010). Sites of synthesis, biochemistry and functional role of plant volatiles. *South African Journal of Botany* 76, 612–631.

Mashaly, A.M.A., Ali, A.S., Ali, M.F., (2010) Source, optimal dose concentration and longevity of trial pheromone in two *Monomorium* ants (Formicidae: Hymenoptera). *Journal of King Saud University Science* 22, 57–60._

McEvoy, E.G., Rogers, A., Gibson, R., (1998). Preliminary investigation of *Vibrio alginolyticus*-like bacteria associated with marine nemerteans. *Hydrobiologia* 365, 287–290.

McGain, F., Winkel, K.D., (2002). Ant sting mortality in Australia. *Toxicon* 40, 1095–1100.

McGraw-Hill Dictionary of Scientific and Technical Terms., (2003). venom, McGraw-Hill, New York.

Mebs, D., (1978). Pharmacology of reptilian venoms, In *Biology of the Reptilia* (eds C. Gans, K. Gans), pp. 437–560. Academic Press, New York.

Mebs, D., (1994). The strategic use of venoms and toxins by animals. *Universitas* 3, 213–222.

Mebs, D., (2001). Toxicity in animals. Trends in evolution? *Toxicon* 39, 87–96.

Mebs, D., (2002). *Venomous and poisonous animals: a handbook for biologists, toxicologists and toxinologists, physicians and pharmacists*. CRC Press, Boca Raton.

Meier, J., White, J., (1995). *Handbook of clinical toxicology of animal venoms and poisons*. CRC Press, Boca Raton, FL.

Menez, A., Servent, D., Gasparini, S., (2002). *The Binding Sites of Animals Toxins Involved Two Components: A Clue for Selectivity, Evolution and Design of Proteins?* John Wiley & Sons, West Sussex, England.

Miller, M.A., Kudela, R.M., Mekebri, A., Crane, D., Oates, S.C., Tinker, M.T., Staedler, M., Miller, W.A., Toy-Choutka, S., Dominik, C., (2010). Evidence for a novel marine harmful algal bloom: cyanotoxin (microcystin) transfer from land to sea otters. *PloS One* 5, e12576.

Miller, S., (1968). The predatory behavior of *Dileptus anser*. *Journal of Eukaryotic Microbiology* 15, 313–319.

Morgan, E.D., (2010). *Biosynthesis in Insects: Advanced Edition*. Royal Society of Chemistry, Cambridge.

Mori, A., Burghardt, G.M., (2008). Comparative experimental tests of natricine antipredator displays, with special reference to the apparently unique displays in the Asian genus, *Rhabdophis*. *Journal of Ethology* 26, 61–68.

Mori, A., Burghardt, G.M., Savitzky, A.H., Roberts, K.A., Hutchinson, D.A., Goris, R.C., (2011). Nuchal glands: a novel defensive system in snakes. *Chemoecology*, 1–12.

Mosby's Emergency Dictionary., (1998). venom, Elsevier Health Sciences, New York.

Myers, C.W., Daly, J.W., Malkin, B., (1978). A dangerously toxic new frog (*Phyllobates*) used by Embera indians of western Colombia, with discussion of blowgun fabrication and dart poisoning. *Bulletin of the American Museum of Natural History* 161, 307–366.

Newlands, G., (1974). The venom-squirting ability of *Parabuthus* scorpions (Arachnida: Buthidae). *South African Journal of Medical Science* 39, 175–178.

Nikbakhtzadeh, M., Vahedi, M., Vatandoost, H., Mehdinia, A., (2012). Origin, transfer and distribution of cantharidin-related compounds in the blister beetle *Hycleus scabiosae*. *Journal of Venomous Animals and Toxins including Tropical Diseases* 18, 88–96.

Nikbakhtzadeh, M.R., Dettner, K., Boland, W., Gade, G., Dotterl, S., (2007). Intraspecific transfer of cantharidin within selected members of the family Meloidae (Insecta: Coleoptera). *Journal of Insect Physiology* 53, 890–899.

Nishida, R., (2002). Sequestration of defensive substances from plants by Lepidoptera. *Annual Review of Entomology* 47, 57–92.

Noguch, T., Arakawa, O., (2008). Tetrodotoxin–distribution and accumulation in aquatic organisms, and cases of human intoxication. *Marine Drugs* 6, 220–242.

Obin, M.S., Vander Meer, R.K., (1985). Gaster flagging by fire ants (*Solenopsis* spp.): functional significance of venom dispersal behavior. *Journal of Chemical Ecology* 11, 1757–1768.

Oehme, F., Brown, J., Fowler, M., (1975). *Toxins of animal origin*. Macmillan Publishing, New York.

Ogata, N., Ohishi, Y., (2002). Molecular diversity of structure and function of the voltage-gated Na+ channels. *Japanese Journal of Pharmacology* 88, 365–377.

Oi, D.H., Pereira, R.M., (1993). Ant behavior and microbial pathogens (Hymenoptera, Formicidae). *Florida Entomologist* 76, 63–74.

Opitz, S.E.W., Muller, C., (2009). Plant chemistry and insect sequestration. *Chemoecology* 19, 117–154.

Osterhoudt, K.C., (2006). The lexiconography of toxicology. *Journal of Medical Toxicology* 2, 1–3.

Oxford Dictionary of English Etymology., (1966). Oxford University Press, London.

Ozbek, S., Balasubramanian, P.G., Holstein, T.W., (2009). Cnidocyst structure and the biomechanics of discharge. *Toxicon* 54, 1038–1045.

Pasteels, J.M., Gregoire, J.C., Rowell-Rahier, M., (1983). The chemical ecology of defense in arthropods. *Annual Review of Entomology* 28, 263–289.

Pawlik, J.R., Kernan, M.R., Molinski, T.F., Harper, M.K., Faulkner, D.J., (1988). Defensive chemicals of the Spanish dancer nudibranch *Hexabranchus sanguineus* and its egg ribbons: macrolides derived from a sponge diet. *Journal of Experimental Marine Biology and Ecology* 119, 99–109.

Paysse, E.A., Holder, S., Coats, D.K., (2001). Ocular injury from the venom of the southern walkingstick. *Ophthalmology* 108, 190–191.

Pimenta, A.M.C., De Lima, M.E., (2005). Small peptides, big world: biotechnological potential in neglected bioactive peptides from arthropod venoms. *Journal of Peptide Science*11, 670–676.

Pimentel, D., (2009). Environmental and economic costs of the application of pesticides primarily in the United States. In *Integrated Pest Management: Innovation-development Process* (eds R. Peshin, A.K. Dhawan), pp. 89–111. Springer, New York.

Poerksen, G., (2003) Paracelsus. Septum Defensiones. *Die Selbstverteidigung eines Aussenseiters*. Schwabe AG Verlag, Basel.

Prestwich, G.D., (1979). Chemical defense by termite soldiers. *Journal of Chemical Ecology* 5, 459–480.

Prestwich, G.D., (1984). Defense-mechanisms of termites. *Annual Review of Entomology* 29, 201–232.

Pugalenthi, P., Livingstone, D., (1995). Cardenolides (heart poisons) in the painted grasshopper *Poecilocerus pictus* F. (Orthoptera: Pyrgomorphidae) feeding on the milkweed *Calotropis gigantea* L. (Asclepiadaceae). *Journal of the New York Entomology Society* 103, 191–196.

Quennedey, A., (1984). Morphology and ultrastructure of termite defense glands, In *Defensive Mechanisms in Social Insects* (ed H.R. Hermann), pp. 151–200. Praeger, New York.

Reisner, L., (2004). Biologic poisons for pain. *Current Pain and Headache Report* 8, 427–434.

Reverberi, M., Ricelli, A., Zjalic, S., Fabbri, A.A., Fanelli, C., (2010). Natural functions of mycotoxins and control of their biosynthesis in fungi. *Applied Microbiology and Biotechnology* 87, 899–911.

Richard, F.J., Fabre, A., Dejean, A., (2001). Predatory behavior in dominant arboreal ant species: the case of *Crematogaster* sp. (Hymenoptera: Formicidae). *Journal of Insect Behavior* 14, 271–282.

Ritson-Williams, R., Yotsu-Yamashita, M., Paul, V.J., (2006). Ecological functions of tetrodotoxin in a deadly polyclad flatworm. *Proceedings of the National Academy of Science* 103, 3176–3179.

Rodríguez-Robles, J.A., (1994). Are the Duvernoy's gland secretions of colubrid snakes venoms? *Journal of Herpetology* 28, 388–390.

Rohlfs, M., Albert, M., Keller, N.P., Kempken, F., (2007). Secondary chemicals protect mould from fungivory. *Biology Letters* 3, 523–525.

Rossmann, M.G., Mesyanzhinov, V.V., Arisaka, F., Leiman, P.G., (2004). The bacteriophage T4 DNA injection machine. *Current Opinions in Structural Biology* 14, 171–180.

Roth, L.M., Eisner, T., (1962). Chemical defenses of arthropods. *Annual Review of Entomology* 7, 107–136.

Russell, F.E., (1965). Marine toxins and venomous and poisonous marine animals. *Advances in Marine Biology* 3, 255–384.

Russell, F.E., (2001). Toxic effects of terrestrial animal venoms and poisons, In *Casarett and Doullís Toxicology: The Basic Science of Poisons* (ed C.D. Klaassen), pp. 945–964. McGraw Hill, New York.

Saito, T., Noguchi, T., Hashimoto, K., Harada, T., Murata, O., (1985). Tetrodotoxin as a biological defense agent for puffers (*Fugu niphobles*, *F. vermicularis vermicularis* and *F. pardalis*). *Bulletin of the Japanese Society for the Science of Fish* 51.

Saporito, R.A., Isola, M., Maccachero, V.C., Condon, K., Donnelly, M.A., (2010). Ontogenetic scaling of poison glands in a dendrobatid poison frog. *Journal of Zoology* 282, 238–245.

Saporito, R.A., Norton, R.A., Andriamaharavo, N.R., Garraffo, H.M., Spande, T.F., (2011). Alkaloids in the mite *Scheloribates laevigatus*: further alkaloids common to oribatid mites and poison frogs. *Journal of Chemical Ecology* 37, 213–218.

Saporito, R.A., Spande, T.F., Garraffo, H.M., Donnelly, M.A., (2009). Arthropod alkaloids in poison frogs: a review of the "dietary hypothesis." *Heterocycles* 79, 277–297.

Schiefer, H.B., Irvine, D., Buzik, S.C., (1997). *Understanding Toxicology: Chemicals, Their Benefits and Risks*. CRC Press, Boca Raton, FL.

Schmidt, J.O., (1982). Biochemistry of insect venoms. *Annual Review of Entomology* 27, 339–368.

Schmidt, J.O., (1990). *Insect defenses: adaptive mechanisms and strategies of prey and predators*. State Univ of New York Press, New York.

Schroeder, F.C., Gonzàlez, A., Eisner, T., Meinwald, J., (1999). Miriamin, a defensive diterpene from the eggs of a land slug (*Arion* sp.). *Proceedings of the National Academy of Science* 96, 13620.

Sharma, R.P., Taylor, M.J., (1987). Animal toxins, In *Handbook of Toxicology* (eds T.J. Haley, W.O. Berndt), pp. 439–470. Hemisphere Publishing, New York.

Sherbrooke, W.C., Mason, J.R., (2005). Sensory modality used by coyotes in responding to antipredator compounds in the blood of Texas horned lizards. *Southwestern Naturalist* 50, 216–222.

Sherbrooke, W.C., Middendorf, G.A., (2004). Responses of kit foxes (*Vulpes macrotis*) to antipredator blood-squirting and blood of Texas horned lizards (*Phrynosoma cornutum*). *Copeia*, 652–658.

Sherratt, T.N., (2002). The coevolution of warning signals. *Proceedings of the Royal Society of London B Biological Sciences* 269, 741–746.

Souza, E.S., Willemart, R.H., (2011). Harvest-ironman: heavy armature, and not its defensive secretions, protects a harvestman against a spider. *Animal Behavior* 81, 127–133.

Stumpf, W.E., (2006) The dose makes the medicine. *Drug Discovery Today* 11, 551-555.

Sutherland, S., Lane, W., (1969). Toxins and mode of envenomation of the common ringed or blue-banded octopus. *Medical Journal of Australia* 1, 893.

Sykes, P.F., Huntley, M.E., (1987). Acute physiological reactions of *Calanus pacificus* to selected dinoflagellates: direct observations. *Marine Biology* 94, 19–24.

Taber's Cyclopedic Medical Dictionary., (2009). venom, F.A. Davis Company, Philadelphia.

Thuesen, E.V., Kogure, K., (1989). Bacterial production of tetrodotoxin in four species of Chaetognatha. *Biological Bulletin* 176, 191–194.

Toledo, L.F., Sazima, I., Haddad, C.F.B., (2011). Behavioural defences of anurans: an overview. *Ethology Ecology and Evolution* 23, 1–25.

Toledo, R.C., Jared, C., (1995). Cutaneous granular glands and amphibian venoms. *Comparative Biochemistry and Physiology Part A Physiology* 111, 1–29.

Torres, A., De Plater, G., Doverskog, M., Birinyi-Strachan, L., Nicholson, G., Gallagher, C., Kuchel, P., (2000). Defensin-like peptide-2 from platypus venom: member of a class of peptides with a distinct structural fold. *Biochemical Journal* 348, 649.

Turner, J.T., Tester, P.A., Hansen, P.J., (1998). Interactions between toxic marine phytoplankton and metazoan and protestan grazers, In *Physiological Ecology of*

Harmful Algal Blooms (eds D.M. Anderson, A.D. Cembella, G.M. Hallegraeff), pp. 452–474. Springer-Verlag, Heidelberg.

Valderrama, X., Robinson, J.G., Attygalle, A.B., Eisner, T., (2000). Seasonal anointment with millipedes in a wild primate: a chemical defense against insects? *Journal of Chemical Ecology* 26, 2781–2790.

Veiga, A., Blochtein, B., Guimar,,es, J., (2001). Structures involved in production, secretion and injection of the venom produced by the caterpillar *Lonomia obliqua* (Lepidoptera, Saturniidae). *Toxicon* 39, 1343–1351.

Vetter, J., (1998). Toxins of *Amanita phalloides*. *Toxicon* 36, 13–24.

Vetter, R.S., Schmidt, J.O., (2006). Semantics of toxinology. *Toxicon* 48, 1–3.

Visscher, J.P., (1923). Feeding reactions in the ciliate, *Dileptus gigas*, with special reference to the function of trichocysts. *Biological Bulletin* 45, 113–143.

Vonk, F.J., Jackson, K., Doley, R., Madaras, F., Mirtschin, P.J., Vidal, N., (2011). Snake venom: from fieldwork to the clinic. *Bioessays* 33, 269–279.

Wang, D.Z., (2008). Neurotoxins from marine dinoflagellates: a brief review. *Marine Drugs* 6, 349–371.

Watters, M.R., (2005). Tropical marine neurotoxins: venoms to drugs. *Seminars in Neurology* 25, 278–289.

Welsh, J.H., (1964). Composition and mode of action of some invertebrate venoms. *Annual Review of Pharmacology* 4, 293–304.

West, D.J., Andrews, E.B., Bowman, D., McVean, A.R., Thorndyke, M.C., (1996). Toxins from some poisonous and venomous marine snails. *Comparative Biochemistry and Physiology Part C Toxicology and Pharmacology* 113, 1–10.

Wigger, E., Kuhn-Nentwig, L., Nentwig, W., (2002). The venom optimisation hypothesis: a spider injects large venom quantities only into difficult prey types. *Toxicon* 40, 749–752.

Williams, B.L., (2010). Behavioral and chemical ecology of marine organisms with respect to tetrodotoxin. *Marine Drugs* 8, 381–398.

Williams, B.L., Brodie, E.D., (2004). A resistant predator and its toxic prey: persistence of newt toxin leads to poisonous (not venomous) snakes. *Journal of Chemical Ecology* 30, 1901–1919.

Williams, B.L., Hanifin, C.T., Brodie, E.D., Caldwell, R.L., (2011). Ontogeny of tetrodotoxin levels in blue-ringed octopuses: maternal investment and apparent independent production in offspring of *Hapalochlaena lunulata*. *Journal of Chemical Ecology* 37, 10–17.

Williams, R., (1991). Acrorhagi, catch tentacles and sweeper tentacles: a synopsis of ëaggressioníof actiniarian and scleractinian Cnidaria. *Hydrobiologia* 216, 539–545.

Winde, I., Wittstock, U., (2011). Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system. *Phytochemistry* 72, 1566–1575.

Wolfe, G.V., (2000). The chemical defense ecology of marine unicellular plankton: constraints, mechanisms, and impacts. *Biological Bulletin* 198, 225–244.

Wright, J.L.C., (2002). Attack and Defend: The Function and Evolution of Bioactive or Toxic Metabolites, In *Proceedings of the Xth International Conference on Harmful Algae. Florida Fish and Wildlife Conservation Commission* (eds K.A. Steidinger, J.H. Landsberg, C.R. Tomas, G.A. Vargo) St. Petersburg, FL.

Wuster, W., (2010). What's your poison? *Heredity* 104, 519.

Young, B.A., Herzog, F., Friedel, P., Rammensee, S., Bausch, A., van Hemmen, J.L., (2011). Tears of venom: hydrodynamics of reptilian envenomation. *Physical Review Letters*106, 198103.

Zangerl, A.R., Rutledge, C.E., (1996). The probability of attack and patterns of constitutive and induced defense: a test of optimal defense theory. *American Naturalist* 599–608.

CHAPTER THREE

POKE BUT DON'T PINCH:

RISK ASSESSMENT AND VENOM METERING IN THE

WESTERN WIDOW SPIDER (*Latrodectus hesperus*)

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Abstract

The capacity to assess threat and modulate defensive behavior accordingly is widespread among animals. Although numerous studies have demonstrated that venomous animals possess cognitive control of venom expenditure in predatory contexts, few have examined venom metering in defensive contexts. In this study, we investigated venom use in the context of threat assessment in the western widow spider (*Latrodectus hesperus*), a synanthropic, medically relevant species found throughout western North America. To elicit defensive behaviors, we subjected wild-caught adult females ($N = 43$) to single (low threat) and repeated (medium threat) prods with a gelatin "finger," and repeated pinches between two gelatin fingers (high threat) within a repeated-measures design. In experiment 1, poking at the lowest threat level elicited primarily avoidance responses ("move" and "retract"), but silk-flicking increased at moderate threat with repeated prodding. Pinching at the highest level of threat provoked significantly more biting. In Experiment 2, spiders modulated venom expenditure by delivering 2.2-fold more venom per bite when pinched on the body compared to having a leg pinched. Spiders also expended 2.3-fold more venom when successive target presentations were separated by a long (5 min) compared to a short (5 sec) interval. Because silk and venom require a metabolic cost to replace, they can be viewed as limited commodities that should be used judiciously. Collectively, these findings support the growing body of literature showing that animals have the ability to cognitively meter their venom. This is also the first study to demonstrate that spiders actively control venom expulsion during defensive interactions.

Introduction

The survival of animals represents a continual trade-off between acquisition of resources, reproduction, and avoidance of predation (Bednekoff 2007; Caro 2005; Ferrari et al. 2009; Lima and Bednekoff 1999; Lima and Steury 2005; Wisenden 2000). While an unsuccessful attempt at resource acquisition or reproduction may be overcome by subsequent successes, failing to avoid predation is the end. Thus, an organism's ability to perceive and respond to a threat is under high selective pressure (Kats and Dill 1998; Lima and Dill 1990). Accordingly, risk assessment, also known as threat sensitivity, offers an excellent opportunity to understand the complex relationships between environmental cues and an organism's ability to modulate their behaviors in response. Although many studies have tested risk assessment in vertebrates and invertebrates, comparatively few studies have focused on venomous organisms, such as snakes (Glaudas and Gibbons 2005; Glaudas et al. 2006), spiders (Jackson et al. 1990, 1992, 1993; Lohrey et al. 2009; Riechert and Hedrick 1990; Taylor et al. 2005), and other arachnids (Grostal and Dicke 2000; Nisani and Hayes 2011).

Venom deployment is a ubiquitous strategy among organisms, and has evolved independently in multiple groups (Lewis and Garcia 2003; Morgenstern and King 2013). Venoms generally comprise a complex mixture of proteins and/or non-proteinaceous compounds. Individual venoms may be composed of hundreds, to thousands, of peptides and proteins (Escoubas et al. 2006; Nascimento et al. 2006). Venoms may even have redundancies in toxin activity, with several different toxins all affecting the same receptor subtype (Morgenstern and King 2013). The complexity and redundancy all result in a measurable metabolic cost to synthesize, store, and maintain venom (Pintor 2010; Nisani

et al. 2007; Nisani et al. 2012; McCue and Mason 2006). The cost of venom can also be inferred by examples of its loss as a result of shifts in diet (Li et al. 2005) or changes in prey capture behavior (Hayes 2008; King 2004; Wigger et al. 2002).

Animals make decisions about venom use on at least two levels: whether to use venom, and how much to use. Dry bites and stings have been reported in numerous species, with evidence suggesting a cognitive component (Alves De Rezende et al. 1998; Hayes et al. 2002; Minton 1990; Nisani and Hayes 2011). Venom metering (or venom optimization), the ability to actively control the amount of venom delivered during a bite or sting, has been studied across many species and in both defensive and predatory contexts. Predatory venom metering has been observed in several species of snakes (Hayes 1992, 1993; Hayes et al. 1995, 2002), and the spider *Cupiennius salei* (Boevé et al. 1995; Malli et al. 1998; Malli et al. 1999; Wigger et al. 2002; Wullschleger and Nentwig 2002), and is related to factors such as prey size (Boeve 1994; Hayes et al. 1995; Hayes et al. 2002; Malli et al. 1998; Malli et al. 1999), prey type (Boeve et al. 1995; Hayes 1992; Rodríguez-Robles and Leal 1993), struggle intensity (Malli et al. 1999; Rodríguez-Robles and Leal 1993; Wigger et al. 2002), satiety (Hayes 1993), and venom availability (Hostettler and Nentwig 2006; Wullschleger and Nentwig 2002). Defensive venom metering has been comparatively less studied, with experiments limited to scorpions (Nisani and Hayes 2011) and snakes (Hayes et al. 2002; Hayes et al. 2008). Heretofore, risk assessment and defensive venom metering have not been examined in spiders.

The western widow spider (*Latrodectus hesperus*), is a medically relevant, synanthropic species found in western North America, ranging from southern British

Columbia into Mexico. We chose to study *Latrodectus hesperus* because of its well-known reliance on venom for defense, frequent human contact, and local abundance. Defensive behaviors against rodent predators were described by Vetter (1980), though not in relation to risk assessment, biting, and venom expenditure. Vetter (1980) concluded that silk flicking occurs only at highest levels of threat. Based on anecdotal evidence, d'Amour et al. (1936) suggested that venom use similarly occurs only at highest levels of threat, and was under the volition of the spiders, but no study to date has investigated venom metering within *Latrodectus*.

The goal of this study was to investigate how the western widow modulates its defensive behavior during different levels of threat, emphasizing the decisions it makes in using its metabolically expensive venom. We sought to experimentally answer two key questions: 1) Does *L. hesperus* modulate its defensive behavior at different levels of threat? And 2) does *L. hesperus* exhibit cognitive control over venom expulsion (i.e., venom metering)? Affirmative answers would suggest that *L. hesperus* is capable of risk assessment. The first of two experiments examined defensive behaviors exhibited at three levels of threat. The second experiment considered specific factors that influence biting and venom expenditure, including location of contact on the spider's body by a simulated predator, interval between successive predator attacks, and sequence of successive attacks.

Methods

Spider Collection, Housing, and Care

We collected spiders in the spring and summer months (generally May–

September) from Redlands, Loma Linda, and Colton, California (San Bernardino County). Spiders were housed in 540-mL plastic deli cups at 22.2° C on a 12 hr light-dark cycle. We provided spiders with a small stick that facilitated web construction, and offered house crickets (*Acheta domestica*), once every 2 weeks. No water was provided, as it was deemed unnecessary.

Experiment 1: Risk Assessment

Forty-five adult female spiders were initially collected for this experiment, but two individuals died before completion of the experiment, yielding a final sample of $N = 43$. We subjected each spider to three threat conditions (low, medium, and high) in a repeated-measures design. Each threat involved a simulated attack by gelatin "fingers." The fingers were made by adding 4 packets Knox gelatin (28.8 g; Associated Brands, Medina, NY, USA) to 0.240 L of water. Once all granules were moistened, the mixture was refrigerated for 2 hr. The mixture was then melted using a double boiler, with temperature never exceeding 54.4 °C. Gelatin was poured into plastic containers and refrigerated for 24 hr. Gelatin was removed from the containers and then sliced into 6 x 3 x 2 cm (L x W x H) rectangles. The gelatin fingers were mounted on 19.5-cm steel micro spatulas (model #702700, Carolina Biological Supply Company, Burlington, NC, USA) before being used in experiments (Fig 1). New gelatin fingers were used for each trial. Gelatin fingers enabled spiders to bite and pierce the “offender;” however, we were unable to recover and measure venom injected into the gelatin.

Figure 1: Gelatin fingers mounted on steel micro spatulas to pinch western widow (*Latrodectus hesperus*) spiders within high threat condition of experiment 1

For low threat, spiders were tested in their home containers while remaining within their web. The treatment consisted of a single 1-sec prod using a single gelatin finger randomly directed at the cephalothorax, abdomen, or leg of a spider; we targeted different body parts to maximize variation in behavioral responses, but pooled the data since many presentations contacted more than one body part. The medium threat, representing a more persistent predator, also took place within the home container. This condition followed the same procedure as the low threat, but consisted of 60 brief prods at one prod/sec for a total observation time of 60 sec. For high threat, we coaxed the spider to climb onto a gelatin finger, and then subjected the spider to a series of three gentle whole-body pinches between two gelatin fingers, thereby modeling a predator's grasp. Each individual pinch lasted 10 sec, with a minimum of 10 sec between successive

pinches. The steel micro spatula helped to keep the spider on the gelatin finger, as the spider could not readily climb the spatula itself.

We subjected each spider to one threat per day over three successive days in a randomized sequence. We identified and recorded five primary defensive behaviors of *L. hesperus*, as defined in Table 4. The experimenter (DRN), who for consistency conducted all trials, made an audio recording while narrating each trial, and subsequently analyzed the recordings for presence/absence, frequency, and duration of each behavior observed. Observations were aided by a desk-mounted magnifying lamp (L745BK, Luxo Corp, Elmsford, NY, USA), facilitating the observation of fine behaviors, such as biting.

Table 4: Definitions and contexts of western widow (*Latrodectus hesperus*) defensive behaviors.

Behavior	Structure used	Definition	Threat
Move	Whole body	Spider moves entire body in response to stimulus, usually away from it	Low
Silk-flick	Legs IV and spinnerets	Legs IV first move toward spinnerets, then extended simultaneously or asynchronously toward stimulus; leg contact is made with stimulus and visibly viscous silk attached	Low and High
Bite	Chelicerae and fangs	Spider moves chelicerae close to gelatin "finger" or parafilm target, distal ends of chelicerae open laterally, and fangs are inserted as chelicerae close medially	Low and High
Retract leg(s)	One or more leg(s)	Spider moves (adducts) one or more legs medially from initial position	Low
Play dead	Whole body	Spider drops from web, retracts (adducts) all legs medially against body, and remains motionless ≥1 sec	Low and High

Experiment 2: Venom Metering

To measure venom expenditure during biting, we prepared targets to be bitten from which we could readily collect the venom. The target comprised a 1.5-mL snap-cap plastic tube with a single sheet of Parafilm® (Bemis Company Inc., Neenah, WI, USA) stretched over the opening via gloved hands to avoid protein contamination. We tested spiders ($N = 20$) by presenting three successive targets to bite within four randomly ordered conditions combining presentation interval (2 levels) and pinch location (2 levels) in a 3 x 2 x 2 repeated-measures design. For each of the four trials, spaced 2 weeks apart, we removed the spider from its home container, either coaxing the spider to the top of its container using 114-mm long forceps (model 4527, BioQuip Products, Rancho Dominguez, CA, USA) and then pinching the leg with the same forceps, or by grasping the spider by the abdomen with a gloved hand (model 304362073, Handgards, El Paso, TX, USA). Once removed and while still grasped by a leg or by the abdomen, the Parafilm® surface of the target was positioned so that the spider's chelicerae and fangs were in contact. We repeated the target presentation two more times, either 5 sec apart (short interval, modeling a single persistent encounter) or 5 min apart (long interval, presumably modeling three separate encounters). Spiders were allowed to interact with each target for 5 sec. When target presentations were 5 min apart, we returned spiders to their home container between each presentation. The spider's interaction with the target was observed with aid of a dissection microscope (Nikon SMZ-10A, Nikon Instruments Inc., Melville, NY, USA) set to 7.5 x magnification. Number of bites delivered during a given pinch was determined by counting the number of puncture marks on the Parafilm®. A bite consisted of two punctures from the two fangs spaced closely together. If only one

puncture was observed, then the bite was scored as 0.5. After observing all spiders on a given day, each spider was fed and not offered food again until the next trial 2 weeks later.

Immediately after each trial, the Parafilm® stretched over each tube was cut by razor along the internal diameter of the tube to collect venom deposited on the surface. We retained only the small portion of Parafilm® to reduce contamination from extraneous proteins, especially silk. We placed the cut portion of the Parafilm® within the snap-cap tube, and added 150 μL of carbonate buffer (50 mM). We then briefly agitated the Parafilm® and carbonate buffer mixture with a VWR Vortexer 2 (set to 8; VWR International LLC., Radnor, PA, USA), and then immediately placed the sample on ice till samples were permanently frozen at -80 °C. Independent of interval, spiders were pinched three separate times for each observation. Within the short interval the three pinches occurred when spiders were repositioned before the presentation of the next target.

We measured venom expenditure using an indirect enzyme-linked immunosorbent assay (ELISA) on hydrophobic 96-well plates (Immulux cat # 1000, Dynex, Chantilly, VA, USA). Antigen was bound to the plate by adding 100 μL of the bite samples (unknown quantity of antigen in carbonate buffer) and standard samples (0, 0.03, 0.06, 0.13, 0.25, 0.50, and 1.0 μg/mL of venom) to separate wells. Two to three additional wells served as reagent controls. We could not test samples in duplicate or triplicate because of the minute venom amounts. We then covered the plates with scotch tape and incubated them overnight at 4^{o} C. The following day, plates were warmed to room temperature and then washed 3x with 300 μL of phosphate-buffered saline (PBS;

pH 7.4). Wells were then blocked with 200 μL SuperBlock (#37515, Pierce, Rockford, IL, USA) for 3 min, flicked to remove liquid, and repeated two more times. The plates were again washed 3x with 300 μL PBS-Tween (PBS + 0.05% Tween-20, Sigma P6585 low peroxide/carbonyl, St. Louis, MO, USA). Next, we added 100 μL of primary antibody (Aracmyn Plus, Bioclon, Mexico City, Mexico), diluted 1:25,000 in Diluent (10% blocking protein in PBS-Tween) to each well, covered the plate with scotch tape, and incubated it on a shaker for 1 hr at room temperature. After incubation, the plates were washed 3x with 300 μL PBS-Tween. We then added 100 μL of secondary antibody (ab6921, Rabbit anti-horse IgG, Abcam, Cambridge, MA, USA, diluted 1:1,000 in Dilutent) to each well, covered the plate with scotch tape, and incubated it on a shaker for 1 hr at room temperature. Dilutions of the primary and secondary antibodies were determined using a checkerboard assay. After incubation, the plates were again washed 3x with 300 μL of PBS-Tween. Finally, we added 100 μL of substrate (Bio-Rad 172-1067, Hercules, CA, USA) to each well and incubated the plate uncovered for 20–30 min. Plates were read on a Dynex MRX II (Chantilly, VA, USA) with absorbance at 630 nm and reference at 490 nm. Venom quantities from spider bites were calculated based on regression of the five control samples plotted on a quadratic curve. All estimates <0.06 μg, the low limit of detectability, were assigned a value of 0 μg. The coefficients of variation (r^2) for standard curves ranged from 0.97–0.99, indicating high reliability of venom measurement. Each plate had its own standards made from the same stock on the same day.

For this experiment, we obtained three dependent variables: quantity of venom delivered, number of bites, and quantity of venom per bite.

Analyses

For experiment 1, we relied mostly on non-parametric tests (Zar, 1996) to evaluate the dependent variables because assumptions of normality and homoscedasticity were not met. We used Cochran's *Q* test, followed by McNemar's test for pairwise comparisons, to compare the proportion of spiders that engaged in a behavior across threat levels and across the series of pinches within the high threat level. We employed Friedman's ANOVA, followed by Wilcoxon tests for pairwise comparisons, to compare the frequency of each behavior across all threat levels and across pinches within the high threat. We used Spearman's rank correlation to test the effect of handling time on frequency of bites within the high threat. Effect sizes for Cochran's *Q* and Friedman's ANOVA were computed as Kendall's *W,* indicating strength of association (Green and Salkind 2005), with values of ~0.1, ~0.3, and ≥0.5 roughly corresponding to small, medium, and large effects, respectively (Cohen 1988). Following Nakagawa (2004), we chose not to adjust alpha for multiple tests.

For experiment 2, we conducted 3 x 2 x 2 (sequence of successive pinches x pinch location x interval duration) repeated-measures analyses of variance (ANOVAs) for each dependent variable. Assumptions of normality and homoscedasticity were often not met. However, because the multivariate assumption of sphericity was usually met, and results of non-parametric tests (Wilcoxon tests and Friedman's ANOVAs) applied to the same data yielded identical conclusions for main effects, we report only the parametric results. We computed partial η^2 as a measure of effect size, indicating approximate percent of variance explained by main effects and interactions, with values of ~0.01, ~0.06 and ≥0.14 loosely considered small, medium, and large, respectively (Cohen 1988). Although

partial η^2 values tend to be upward-biased (Pierce et al. 2004), they never summed to >1.0 in our models. We also used a Spearman's rank correlation to test the association between number of bites delivered to a target and quantity of venom expended. We further calculated coefficients of variation (σ / μ) for venom per bite (for all targets pooled, $N = 240$; 20 spiders x 12 targets per animal), total venom expended across all bites per individual ($N = 20$), and venom expended for all targets bitten a single time ($N = 67$).

We conducted all analyses using SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA) and alpha set at 0.05. All measures of central tendency are reported as mean ± 1 S.E.

Results

Experiment 1: Risk Assessment

All five defensive behaviors recorded differed significantly among the three threat conditions (Table 5).

Table 5: Comparison of spider behaviors across threat levels in experiment 1 ($N = 43$)

Dependent Variable	Low Threat	Medium Threat	High Threat	*P*-value	Kendall's *W*
Retract (%)	16[a]	16[a]	0[b]	0.016[c]	0.16
Move (%)	84[a]	100[b]	100[b]	0.001[c]	0.16
Play dead (%)	0[a]	16[b]	9[ab]	0.016[c]	0.10
Silk-flick (%)	5[a]	56[b]	44[b]	< 0.001[c]	0.31
Silk-flicks (#)	0.05 ± 0.03[a]	3.7 ± 0.84[b]	1.8 ± 0.43[b]	< 0.001[d]	0.32
Bite (%)	0[a]	2[a]	60[b]	< 0.001[c]	0.58
Bites (#)	0[a]	0.02 ± 0.02[a]	2.7 ± 0.54[b]	< 0.001[d]	0.60

[a,b] Different superscipts indicate significant pairwise differences based on McNemer test
[c] Cochran's *Q*
[d] Friedman's ANOVA
mean ± SE

Three variables were significantly greater for medium and high threat compared to low threat, with higher proportions of spiders exhibiting "move" and "silk-flick," and spiders delivering more silk-flicks (all *P*s < 0.001). Move usually involved retreat, but we did not record direction of movement. The proportion of spiders exhibiting "bite" and number of bites was similar for low and medium threat, but substantially greater at high threat, when spiders were pinched rather than poked (both $P < 0.001$). Spiders seldom retracted legs during the trials, but while "retract" did not differ between the low and medium threat conditions, we never observed this behavior high threat trials. Similarly,

few spiders exhibited "play dead," but the proportion of spiders doing so was significantly greater for medium threat than low threat ($P = 0.016$). During the high threat trials, we observed no significant decline or increase in silk-flicks or bites among the three successive pinches (Table 6).

Table 6: Comparison of spider behavior among pinches within high threat condition of experiment 1 ($N = 43$)

Dependent Variable	Pinch One	Pinch Two	Pinch Three	*P*-value	Kendall's *W*
% that bite	36	34	39	0.854[a]	<0.01
# of bites/pinch (mean ± SE)	0.59 ± 0.14	0.64 ± 0.16	0.84 ± 0.19	0.520[b]	0.02
% that silk-flick	11	27	16	0.128[a]	0.05
# of silk-flicks/pinch (mean ± SE)	0.32 ± 0.16	0.89 ± 0.18	0.59 ± 0.19	0.147[b]	0.04

[a] Cochran's *Q*
[b] Friedman's ANOVA

Spiders were removed from the home web during the high threat trials and manipulated between two gelatin fingers. Under these conditions, the majority of spiders (68%) rapidly dropped off the gelatin fingers onto the floor, a distance of ca. 1 m. The drop was rapid but controlled, as spiders attached a dragline to the gelatin fingers prior to dropping. Drops nevertheless were rapid enough that spiders sometimes bounced off the ground upon impact. Two spiders were injured and died from this manipulation, but no other injuries were noted. Due to difficulty in handling, high threat observations lasted an

average of 2.53 min (range 1.45–3.97 min). We found no correlation of handling time with silk-flicks (r_s = -0.19, P = 0.21) or number of bites (r_s = -0.09, P = 0.55).

Experiment 2: Defensive Venom Metering

We examined three dependent variables in this experiment: quantity of venom delivered, number of bites, and quantity of venom per bite (Table 7).

Table 7: Venom expenditure, number of bites, and venom per bite by spiders in Experiment 2.

Independent variable	Total Venom (µg)	Total Bites	Venom/Bite (µg)
Location			
Leg	60.7 ± 10.1	12.9 ± 1.1	5.6 ± 1.4
Body	78.7 ± 14.5	7.7 ± 0.8	12.4 ± 2.7
	$F_{1,19}$ = 1.15, P = 0.30 Partial η^2 = 0.06	$F_{1,19}$ = 14.49, P < 0.001 Partial η^2 = 0.43	$F_{1,19}$ = 4.86, P = 0.040 Partial η^2 = 0.20
Interval			
Short	42.0 ± 7.4	10.1 ± 0.8	5.7± 1.6
Long	97.4 ± 16.5	10.5 ± 0.9	10.2 ± 2.1
	$F_{1,19}$ = 9.84, P = 0.005 Partial η^2 = 0.34	$F_{1,19}$ = 0.19, P = 0.67 Partial η^2 = 0.01	$F_{1,19}$ = 3.79, P = 0.067 Partial η^2 = 0.17
Sequence			
Tube 1	41.1 ± 10.2	6.5 ± 0.6	7.3 ± 1.9
Tube 2	41.1 ± 9.7	6.8 ± 0.6	6.6 ± 1.8
Tube 3	57.2 ± 11.1	7.2 ± 0.5	8.4 ± 1.9
	$F_{2,38}$ = 0.84, P = 0.44 Partial η^2 = 0.04	$F_{2,38}$ = 1.13, P = 0.33 Partial η^2 = 0.06	$F_{2,38}$ = 0.66, P = 0.52 Partial η^2 = 0.03

Spiders were presented three parafilm-covered snap-cap tubes to bite after being pinched in two locations (leg, body) and with two different intervals between successive presentations (5 sec, 5 min). Main effects of 2 x 2 x 3 (location x interval x sequence) repeated-measures ANOVA are indicated; see text for details on the one significant interaction (location x venom for number of bites).

Quantity of venom delivered was similar for the two pinch locations and for the sequence of three successive bites. However, the main effect of interval was significant,

as spiders delivered 2.3-fold more venom when biting during the long intervals between successive bites compared to the short intervals ($P = 0.005$). A two-way interaction also existed between location and sequence ($F_{2,38} = 4.31$, $P = 0.021$, partial $\eta^2 = 0.19$). When a leg was pinched, more venom was expended in the first pinch compared to subsequent pinches, but when the body was pinched, venom expenditure increased with each successive pinch (Fig. 2). No other interactions were significant (data not provided here).

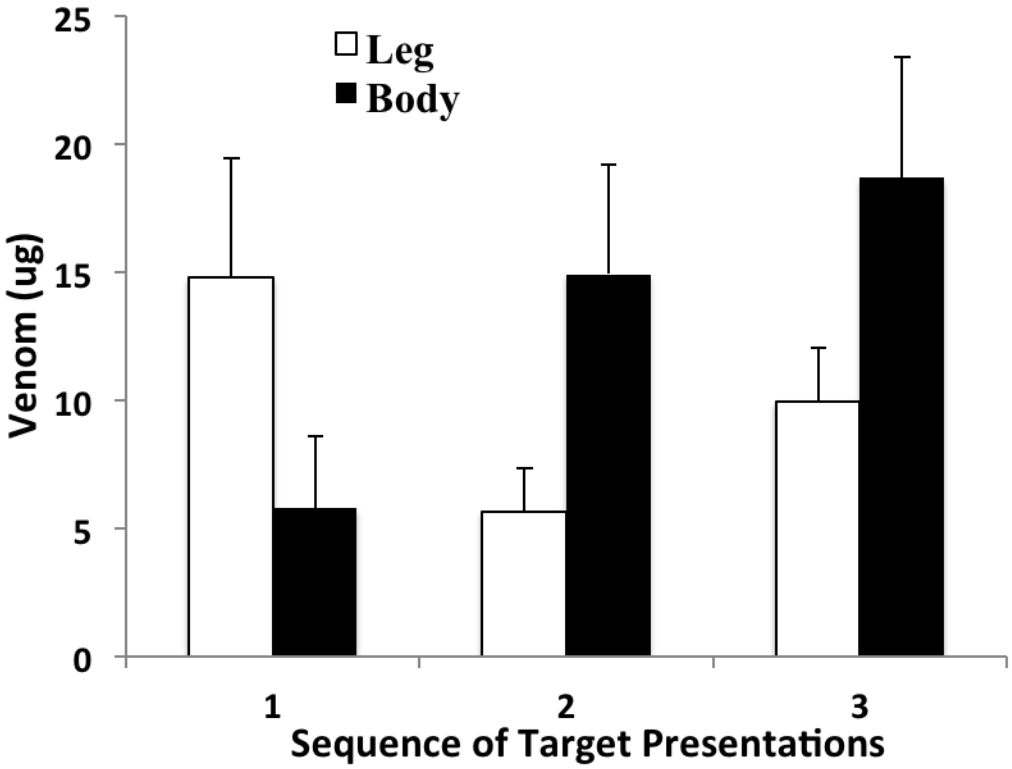

Figure 2: Mean (± 1 S.E.) venom expended by western widow (*Latrodectus hesperus*) spiders illustrating location x sequence interaction in experiment 2 ($N = 20$ for each mean).

Number of bites was similar for the two intervals and the three bites. However, the main effect of location was significant, as spiders delivered 1.7-fold more bites when

a leg was pinched than when the body was pinched ($P < 0.001$). Although total venom expended was similar when a leg or the body was pinched, the amount of venom expended per bite averaged 2.2-fold greater when the body was pinched ($P = 0.040$). The amount of venom per bite also approached significance for interval ($P = 0.067$, partial $\eta^2 = 0.20$; note large effect size), with 1.8-fold more venom injected per bite for long intervals between successive bites compared to short intervals. No interactions were significant for number of bites or venom per bite.

Spiders ($N = 240$) bit individual targets a mean of 1.7 ± 0.1 times (range 0–5), depositing 11.0 ± 1.4 μg venom per bite (range 0–142.7 μg). Of these, 65.4% were below the threshold for venom detectability with our assay. Those bitten a single time ($N = 67$) received a mean of 16.0 ± 3.2 μg venom (range 0–110.0 μg). Of these, 53.7% were below the threshold for venom detectability and were potentially "dry" bites. Some targets bitten up to five times contained no measurable venom. When dry bites were removed, targets bitten a single time ($N = 31$) received 34.6 ± 5.3 μg venom (range 12.3–97.7 μg). When all targets were pooled and treated as independent ($N = 240$), there was a weak but significant positive correlation between number of bites and total venom expended ($r_s = 0.18$, $P = 0.006$). Coefficients of variation were 2.13 for venom per bite per target ($N = 203$), 0.72 for total venom expended across all bites per individual ($N = 20$), and 1.65 for venom per bite for targets bitten a single time ($N = 67$).

Discussion

In this study, we sought to demonstrate that *L. hesperus* modulates defensive behavior based on different levels of threat, and makes corresponding decisions about

venom use. Experiment 1, which compared defensive behaviors at three levels of threat, revealed that poking elicits primarily avoidance responses, whereas pinching provokes more defensive responses including silk-flicking and biting. Experiment 2, which examined the factors that influence biting and venom expenditure, indicated that spiders modulate venom expenditure depending on which body part is pinched and the interval between successive simulated attacks.

Results from experiment 1 confirmed that *L. hesperus* exhibits a range of defensive behaviors, and uses them differentially depending on level of threat. Under low threat, spiders exhibited primarily non-confrontational behaviors, including move (84%) and retract (16%). Only a few responded by flicking silk (5%), and none responded by biting. Changes in movement patterns, including arrested or diminished movement, is a common antipredator response observed in other spider species (Barnes et al 2002; Johnson and Sih 2007; Lohrey et al 2009; Persons et al. 2002). When prodded repeatedly under medium threat, a higher proportion of *L. hesperus* exhibited move (100%), played dead (16%), and flicked silk (56%), but only one attempted to bite (2%). The increase in silk-flicking indicated an escalation in defensiveness, but nearly half the spiders remained non-confrontational. When pinched under high threat, defensiveness escalated further, with the majority of spiders biting (60%). Spiders could have retaliated more often with bites at lower threat levels (one actually did so), as some snakes and tarantulas are prone to do (pers. obs.), but the spiders exhibited biting only as a measure of last resort when physically grasped. Although escalated defensiveness in the high threat condition could have resulted from the longer duration, neither silk-flicking nor biting were associated with duration of trial in the high threat condition. Retract was not observed during high

threat, probably due to the forceful nature by which spiders were removed from their containers.

Vetter (1980) similarly reported that mature females of *L. hesperus* use silk-flicking for defense only at higher levels of perturbance. In the lab, females which silk-flicked were better protected against rodent predators (*Peromyscus* spp.) than those whose spinnerets were experimentally blocked. Mature females, which likely suffer greater predation than other age groups, were most likely to flick silk. Males, in contrast, lose the capacity to produce the silk upon maturation. The silk provided a mechanical rather than a chemical deterrent to the rodents. Defensive use of silk has been observed in other families of spiders (Blackledge and Wenzel 2001; McAlister 1960), but the act of throwing or flicking silk may be unique to family Theridiidae.

Silk-flicking may be preferable to biting because the spider maintains distance from the attacker. During a bite, the cephalothorax is brought into direct contact with the threat, resulting in sensitive organs being exposed to potential injury. The spider's short fangs and relatively weak jaws also impose constraints on envenomation. When dealing with rodent predators, *L. hesperus* never attempted to bite (Vetter, 1980). However, our findings indicate willingness to bite when physically pinched for an extended period of time.

Experiment 2 supported our hypothesis that *L. hesperus* possesses cognitive control over venom expenditure. The first level of venom control is whether to use venom during a bite or sting. Dry bites or stings have been reported during defensive encounters in snakes (Hayes et al. 2002) and scorpions (Nisani and Hayes 2011), and our results suggest they exist in *L. hesperus*. Although our ELISA could not reliably measure

venom samples below 0.06 μg, this value was less than 1% of the mean mass of venom measured on targets bitten a single time (16 μg), suggesting that many bites were functionally dry. Some targets lacking measurable venom were bitten up to five times, which suggests individual proclivities toward dry bites.

The second level of venom control is how much venom to inject during a bite. Clearly, the amounts of venom delivered by *L. hesperus* varied substantially. Spiders ejected anywhere from 0–110.0 μg venom on targets bitten once, averaging 34.6 μg when apparent dry bites were excluded. Coefficients of variation were similar to those reported for snakes (Hayes 2008) and the scorpion *P. transvaalicus* (Nisani and Hayes 2011). Thus, we can reject any notion that *L. hesperus* always ejects a consistent bolus of venom (i.e., the "bullet hypothesis;" Hayes et al., 1995). d'Amour et al. (1936) reported that an adult female *L. hesperus* possesses 128 μg venom in the gland, of which 27% is on average released in the envenomating bites we measured.

The ultimate question, however, is whether the spiders possess cognitive control over venom expulsion, or whether variable venom release is beyond their control. We found that location of the pinch (to leg or body) influenced the number of bites delivered and the amount of venom per bite. Spiders bit more frequently during a leg pinch, but delivered 2.2-fold more venom per bite during a body pinch. This might be expected because a body pinch presents a greater threat to the spider. If a leg is lost, the spider can survive and fitness is not greatly reduced (Brueseke et al 2001), but pinches or bites to the body can be lethal (Vetter, 1980). Furthermore, the significant interaction between venom and sequence of target suggests that spiders responded differently to the persistent threat depending on where they were pinched. Spiders pinched by the leg expulsed the

most venom with the first of three targets, whereas those pinched by the body ejected the most venom with subsequent targets. Some snakes (Hayes et al. 2002) and scorpions (Nisani and Hayes 2011) either increase or decrease the amounts of venom expended in successive bites or stings. Considering the large effect size, spiders also likely delivered more venom during the long intervals (5 min) between successive pinches than during the short intervals (5 sec). We suggest that the spiders treated the short intervals as a single predatory encounter and the long intervals as separate events requiring additional venom for each new attacker.

The use of venom and silk in defense, although potentially effective strategies, are also costly. The metabolic cost of venom has been demonstrated in snakes (McCue and Mason 2006; Morgenstern and King 2013; Pintor et al. 2010) and the scorpion *Parabuthus transvaalicus* (Nisani et al. 2007; Nisani et al. 2012). Studies also suggest there is a metabolic cost associated with silk production (Craig et al. 1999; Tanaka 1989). Because these two products can be considered limited commodities (Hayes 2008), spiders should be judicious in dispensing them. Our results support the suggestions of Vetter (1980) and d'Amour et al. (1936) that widow spiders make decisions about use of silk and venom, respectively, and deploy them only at highest levels of threat.

One caveat regarding venom measurement bears mention. Scorpion venom has well documented heterogeneity; that is, the first venom expelled from the gland has a different chemical composition than venom expelled during later stings (Inceaglu et al. 2003; Nisani and Hayes 2011). Venom heterogineity may also exist in spiders, but only one published abstract has explored this possibility (Morgenstern et al. 2012). If venom heterogeneity exists in *L. hesperus*, venom differences across successive stings could

result in differential recognition by the antibodies of our ELISA measurement, resulting in bias in the quantities of venom measured. The independent variable most likely affected by venom heterogeneity would be comparisons among the three successive targets.

In conclusion, our findings support the growing body of literature showing that animals have the ability to cognitively meter their venom (Boeve 1994; Boevé et al. 1995; Hayes 1992, 1993; Hayes et al. 1995; Hayes et al. 2002; Hayes et al. 2008; Hostettler and Nentwig 2006; Malli et al. 1998; Malli et al. 1999; Nisani and Hayes 2011; Rodríguez-Robles and Leal 1993; Wigger et al. 2002; Wullschleger and Nentwig 2002). This is the first study to demonstrate that spiders actively control venom expulsion during defensive interactions.

References

Alves De Rezende, N., Maia Torres, F., Borges Dias, M., Campolina, D., Chavez-Olortegui, C., & Faria Santos Amaral, C. (1998). South American rattlesnake bite (*Crotalus durissus* sp) without envenoming: insights on diagnosis and treatment. *Toxicon*, *36*(12), 2029–2032.

Barnes, M. C., Persons, M. H., & Rypstra, A. L. (2002). The effect of predator chemical cue age on antipredator behavior in the wolf spider *Pardosa milvina* (Araneae: Lycosidae). *Journal of Insect Behavior*, *15*(2), 269–281.

Bednekoff, P. A. (2007). Foraging in the face of danger. In: *Foraging behavior and ecology.* (eds. D.W. Stephens, J.S. Brown, & R.C. Ydenberg), University of Chicago Press, Chicago, Illinois, USA, 305–329.

Blackledge, T. A., & Wenzel, J. W. (2001). Silk mediated defense by an orb web spider against predatory mud-dauber wasps. *Behaviour*, *138*(2), 155–171.

Boevé, J. L. (1994). Injection of venom into an insect prey by the free hunting spider *Cupiennius salei* (Araneae, Ctenidae). *Journal of Zoology*, *234*(1), 165–175.

Boevé, J. L., Kuhn-Nentwig, L., Keller, S., & Nentwig, W. (1995). Quantity and quality of venom released by a spider (*Cupiennius salei*, Ctenidae). *Toxicon*, *33*(10), 1347–1357.

Brueseke, M. A., Rypstra, A. L., Walker, S. E., & Persons, M. H. (2001). Leg autotomy in the wolf spider *Pardosa milvina*: a common phenomenon with few apparent costs. *The American Midland Naturalist*, *146*(1), 153–160.

Caro, T. (2005). *Antipredator defenses in birds and mammals*. University of Chicago Press, Chicago, Illinois, USA

Cartwright, P., Halgedahl, S. L., Hendricks, J. R., Jarrard, R. D., Marques, A. C., Collins, A. G., & Lieberman, B. S. (2007). Exceptionally preserved jellyfishes from the Middle Cambrian. *PLoS One*, *2*(10), e1121.

Craig, C. L., Hsu, M., Kaplan, D., & Pierce, N. E. (1999). A comparison of the composition of silk proteins produced by spiders and insects. *International Journal of Biological Macromolecules*, *24*(2), 109–118.

Cohen, J. (1988). *Statistical power analysis for the behavioralsSciences*. 2nd ed. Lawrence Erlbaum Associates, Inc., Hillsdale, NJ, USA.

d'Amour, F. E., Becker, F. E., & Van Riper, W. (1936). The black widow spider. *The Quarterly Review of Biology, 11*(2), 123–160.

Escoubas, P., Sollod, B., & King, G. F. (2006). Venom landscapes: mining the complexity of spider venoms via a combined cDNA and mass spectrometric approach. *Toxicon*, *47*(6), 650–663.

Ferrari, M. C., Sih, A., & Chivers, D. P. (2009). The paradox of risk allocation: a review and prospectus. *Animal Behaviour*, *78*(3), 579–585.

Fu, D., Zhang, X., & Shu, D. (2011). A venomous arthropod in the Early Cambrian Sea. *Chinese Science Bulletin*, *56*(15), 1532–1534.

Glaudas, X., & Gibbons, J. W. (2005). Do thermal cues influence the defensive strike of cottonmouths (*Agkistrodon piscivorus*)? *Amphibia Reptilia*, *26*(2), 264.

Glaudas, X., Winne, C. T., & Fedewa, L. A. (2006). Ontogeny of Anti-Predator Behavioral Habituation in Cottonmouths (*Agkistrodon piscivorus*). *Ethology*, *112*(6), 608–615.

Green, S. B., and N. J. Salkind. (2005). *Using SPSS for windows and macintosh: analyzing and understanding data*. 4th ed. Pearson Prentice Hall, Upper Saddle River, NJ, USA.

Grostal, P., & Dicke, M. (2000). Recognising one's enemies: a functional approach to risk assessment by prey. *Behavioral Ecology and Sociobiology*, *47*(4), 258–264.

Hayes, W. K. (1992). Prey-handling and envenomation strategies of prairie rattlesnakes (*Crotalus viridis viridis*) feeding on mice and sparrows. *Journal of Herpetology*, *26*(4), 496–499.

Hayes, W. K. (1993). Effects of hunger on striking, prey-handling, and venom expenditure of prairie rattlesnakes (*Crotalus viridis viridis*). *Herpetologica*, *49*(3), 305–310.

Hayes, W. K., Herbert, S. S., Harrison, J. R., & Wiley, K. L. (2008). Spitting versus biting: differential venom gland contraction regulates venom expenditure in the black-necked spitting cobra, *Naja nigricollis nigricollis*. *Journal of Herpetology*, *42*(3), 453–460.

Hayes, W. K., Herbert, S. S., Rehling, G. C., & Gennaro, J. F. (2002). Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. *Biology of the vipers. Eagle Mountain Publishing, Eagle Mountain, UT*, 207–233.

Hayes, W. K., Lavín-Murcio, P., & Kardong, K. V. (1995). Northern Pacific rattlesnakes (*Crotalus viridis oreganus*) meter venom when feeding on prey of different sizes. *Copeia*, *1995*(2), 337–343.

Hostettler, S., & Nentwig, W. (2006). Olfactory information saves venom during prey‐capture of the hunting spider *Cupiennius salei* (Araneae: Ctenidae). *Functional Ecology*, *20*(2), 369–375.

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., Pessah, I. N., & Hammock, B. D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proceedings of the National Academy of Sciences*, *100*(3), 922–927.

Jackson, R. R., Brassington, R. J., & Rowe, R. J. (1990). Anti‐predator defences of *Pholcus phalangioides* (Araneae, Pholcidae), a web‐building and web‐invading spider. *Journal of Zoology*, *220*(4), 543–552.

Jackson, R. R., Rowe, R. J., & Campbell, G. E. (1992). Anti‐predator defences of Psilochorus sphaeroides and *Smeringopus pallidus* (Araneae, Pholcidae), tropical web‐building spiders. *Journal of Zoology*, *228*(2), 227–232.

Jackson, R. R., Rowe, R. J., & Wilcox, R. S. (1993). Anti‐predator defences of *Argiope appensa* (Araneae, Araneidae), a tropical orb‐weaving spider. *Journal of Zoology*, *229*(1), 121–132.

Johnson, J. C., & Sih, A. (2007). Fear, food, sex and parental care: a syndrome of boldness in the fishing spider, *Dolomedes triton*. *Animal Behaviour*, *74*(5), 1131–1138.

Kats, L. B., & Dill, L. M. (1998). The scent of death: chemosensory assessment of predation risk by prey animals. *Ecoscience*, *5*(3), 361–394.

King, G. F. (2004). The wonderful world of spiders: preface to the special Toxicon issue on spider venoms. *Toxicon*, *43*(5), 471–475.

Lewis, R. J., & Garcia, M. L. (2003). Therapeutic potential of venom peptides. *Nature Reviews Drug Discovery*, *2*(10), 790–802.

Li, M., Fry, B. G., & Kini, R. M. (2005). Eggs-only diet: its implications for the toxin profile changes and ecology of the marbled sea snake (*Aipysurus eydouxii*). *Journal of Molecular Evolution*, *60*(1), 81–89.

Lima, S. L., & Bednekoff, P. A. (1999). Temporal variation in danger drives antipredator behavior: the predation risk allocation hypothesis. *The American Naturalist*, *153*(6), 649–659.

Lima, S. L., & Dill, L. M. (1990). Behavioral decisions made under the risk of predation: a review and prospectus. *Canadian Journal of Zoology*, *68*(4), 619–640.

Lima, S. L., & Steury, T. D. (2005). Perception of predation risk: the foundation of nonlethal predator–prey interactions. In: *Ecology of predator–prey interactions. Oxford University Press, Oxford*, 166–188.

Lohrey, A. K., Clark, D. L., Gordon, S. D., & Uetz, G. W. (2009). Antipredator responses of wolf spiders (Araneae: Lycosidae) to sensory cues representing an avian predator. *Animal Behaviour*, *77*(4), 813–821.

Malli, H., Imboden, H., Kuhn-Nentwig, L., (1998). Quantifying the venom dose of the spider *Cupiennius salei* using monoclonal antibodies. *Toxicon, 36*, 1959–1969.

Malli, H., Kuhn-Nentwig, L., Imboden, H., Nentwig, W., (1999). Effects of size, motility and paralysation time of prey on the quantity of venom injected by the hunting spider *Cupiennius salei*. *Journal of Experimental Biology, 202*, 2083–2089.

McAlister, W. (1960). The spitting habit in the spider *Scytodes intricata* Banks (Family Scytodidae). *Texas Journal of Science*, *12*, 17–20.

McCue, M. D. (2006). Cost of producing venom in three North American pitviper species. *Copeia*, *2006*(4), 818–825.

Minton, S.A., 1990. Venomous bites by nonvenomous snakes: an annotated bibliography of colubrid envenomation. *Journal of Wilderness Medicine, 1*, 119–127.

Morgenstern, D., Hamilton, B., Sher, D., Jones, A., Mattius, G., Zlotkin, E., Venter, D., & King, G. F. (2012). The bio-logic of venom complexity. In *Toxicon* (Vol. 60, No. 2, pp. 241–242). Pergamon, Philadelphia, Pennsylvania, USA.

Morgenstern, D., & King, G. F. (2012). The venom optimization hypothesis revisited. *Toxicon 63*, 120–128.

Nakagawa, S. (2004). A farewell to Bonferroni: the problems of low statistical power and publication bias. *Behavioral Ecology*, *15*(6), 1044–1045.

Nascimento, D. G., Rates, B., Santos, D. M., Verano-Braga, T., Barbosa-Silva, A., Dutra, A. A., Biondi, I., Martin-Eauclaire, M.F., de Lima, M.E., & Pimenta, A. (2006). Moving pieces in a taxonomic puzzle: venom 2D-LC/MS and data clustering analyses to infer phylogenetic relationships in some scorpions from the Buthidae family (Scorpiones). *Toxicon*, *47*(6), 628–639.

Nisani, Z., Boskovic, D. S., Dunbar, S. G., Kelln, W., & Hayes, W. K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon*, *60*, 315–323

Nisani, Z., Dunbar, S. G., & Hayes, W. K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *147*(2), 509–513.

Nisani, Z., & Hayes, W. K. (2011). Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering. *Animal Behaviour*, *81*(3), 627–633.

Oron, U., & Bdolah, A. (1973). Regulation of protein synthesis in the venom gland of viperid snakes. *Journal of Cell Biology*, *56*(1), 177–190.

Perret, B. A. (1977). Venom regeneration in tarantula spiders—I. Analysis of venom produced at different time intervals. *Comparative Biochemistry and Physiology Part A: Physiology*, *56*(4), 607–613.

Persons, M. H., Walker, S. E., & Rypstra, A. L. (2002). Fitness costs and benefits of antipredator behavior mediated by chemotactile cues in the wolf spider *Pardosa milvina* (Araneae: Lycosidae). *Behavioral Ecology*, *13*(3), 386–392.

Pierce, C. A., Block, R. A., & Aguinis, H. (2004). Cautionary note on reporting eta-squared values from multifactor ANOVA designs. *Educational and psychological measurement*, *64*(6), 916–924.

Pintor, A. F., Krockenberger, A. K., & Seymour, J. E. (2010). Costs of venom production in the common death adder (*Acanthophis antarcticus*). *Toxicon*, *56*(6), 1035–1042.

Riechert, S. E., & Hedrick, A. V. (1990). Levels of predation and genetically based anti-predator behaviour in the spider, *Agelenopsis aperta*. *Animal Behaviour*, *40*(4), 679–687.

Rodríguez-Robles, J. A., & Leal, M. (1993). Effects of prey type on the feeding behavior of *Alsophis portoricensis* (Serpentes: Colubridae). *Journal of Herpetology*, 163–168.

Rotenberg, D., Bamberger, E. S., & Kochva, E. (1971). Studies on ribonucleic acid synthesis in the venom glands of *Vipera palaestinae* (Ophidia, Reptilia).*Biochemical Journal*, *121*(4), 609.

Tanaka, K. (1989). Energetic cost of web construction and its effect on web relocation in the web-building spider *Agelena limbata*. *Oecologia*, *81*(4), 459–464.

Taylor, A. R., Persons, M. H., & Rypstra, A. L. (2005). The effect of perceived predation risk on male courtship and copulatory behavior in the wolf spider *Pardosa milvina* (Araneae, Lycosidae). *Journal of Arachnology*, *33*(1), 76–81.

Vetter, R. S. (1980). Defensive behavior of the black widow spider *Latrodectus hesperus* (Araneae: Theridiidae). *Behavioral Ecology and Sociobiology*, *7*(3), 187–193.

Wigger, E., Kuhn-Nentwig, L., Nentwig,W., 2002. The venom optimisation hypothesis: a spider injects large venom quantities only into difficult prey types. *Toxicon, 40*, 749–752.

Wisenden, B. D. (2000). Olfactory assessment of predation risk in the aquatic environment. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *355*(1401), 1205–1208.

Wullschleger, B. & Nentwig, W. (2002). Influence of venom availability on a spider's prey-choice behaviour. *Functional Ecology*, *16*(6), 802–807.

Zar, J. H. (1996). Biostatistical analysis, 3rd Edition. Prentice Hall, Englewood Cliffs, New Jersey, USA.

CHAPTER FOUR

ONTOGENETIC DEVELOPMENT OF DEFENSIVE BEHAVIORS IN THE WESTERN WIDOW SPIDER (*Latrodectus hesperus*)

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Abstract

The threats an animal must deal with changes during ontogeny, especially in relation to changes in body size, experience, defensive repertoire, and vulnerability to predators. Spiders offer a unique opportunity to test the ontogenetic development of antipredatory strategies, as they display a suite of defensive behaviors including the use of both silk and venom, which are metabolically costly and, as limited commodities, should be used judiciously. We repeatedly subjected western widow spiders (*Latrodectus hesperus*) to low and high threat conditions over successive instar stages (sheds) to experimentally test three hypotheses. Spiders were poked by a taxidermy mouse specimen in the low-threat condition, and pinched by the same predator model against the wall of its home container in the high-threat condition. Consistent with our first hypothesis, spiders relied largely on non-combative behaviors early in life and switched to more combative behaviors, including silk flicking and biting, as they increased in size. Consistent with our second hypothesis, age exerted a much greater influence than sex differences for males and females within the range of equivalent body sizes. Consistent with our third hypothesis, spiders habituated to the repeated testing by exhibiting fewer combative behaviors than naïve spiders upon reaching adult size. Collectively, these findings suggest that selection has favored age-specific antipredator strategies that can be modified by experience.

Introduction

As animals age and increase in body size, they face changing threats from predators. Individual body size influences vulnerability to predation, with neonates and

juveniles often at higher risk of predation than adults (Brown et al. 2004; Peters 1983). As individuals grow, they become better able to avoid predators, and the suite of potential predators decreases as prey exceed gape limitations (McCoy et al. 2011; Miller et al. 1988; Werner and Gilliam 1984). Through risk assessment, or threat sensitivity, animals glean risk-specific information from their environment to select appropriate behavioral responses (Bednekoff 2007; Caro 2005; Ferrari et al. 2009; Lima and Bednekoff 1999; Lima and Steury 2005; Wisenden 2000). During ontogeny, risk assessment and the behaviors employed in response to risk need to change, and these should be under strong selection.

Most venomous animals have at their disposal chemical weaponry that can be relied upon for defense. However, several factors can constrain defense via venom, including size of the venom delivery apparatus, capacity to deliver venom into a predator's tissues, amount of venom that can be injected, and relative toxicity of the venom (Haight 2010; Hayes et al. 2002; Hayes and Mackessy 2010; Kardong and Lavin-Murcio 1993). Venom deployment also entails costs related to venom regeneration (e.g., ecological and metabolic: Hayes, 2008; McCue 2006; Nisani et al. 2007, 2012; Pintor et al. 2010) and risk of injury (e.g., damage to venom apparatus and predator retaliation: Hayes et al. 2002). These constraints can be expected to vary during ontogeny in ways that might influence the appropriate responses to risk assessment (Haight 2008, 2010; Mooney and Haloin 2006; Rowe and Owings 1992; Troupe 2009; Uma and Weiss 2012). Prior experience can also shape the antipredator use of venom (Glaudas et al. 2006).

Spiders are unique among venomous animals in producing yet another secretion to defend themselves: silk. Most spiders construct webs from silk to provide a home that

also ensnares prey items for food consumption. However, a number of spiders can deploy silk as an antipredator tactic, which can serve as a mechanical deterrent (McAlister 1960; Vetter 1980; Suter and Stratton 2013) or function as a decoy to facilitate escape (Blackledge and Wenzel 2001). Adult female widow spiders (genus *Latrodectus*, family Theridiidae), for example, defend themselves by vigorously flicking silk at potential predators (Vetter 1980; see also Chapter 3). Numerous spider species also incorporate stabilimenta into their webs, which significantly enhance escape from certain hymenopteran predators (Blackledge and Wenzel 2001). Spider silks are complex, proteinaceous secretions, and silk production, like venom synthesis, entails an energetic cost (Craig et al. 1999; Tanaka 1989). Thus, both venom and silk should be regarded as limited commodities (sensu Hayes 2008) that should be used judiciously.

Among venomous animals, spiders comprise an excellent model organism to test the ontogenetic development of defensive behaviors. Spiders are often precocial and develop through sequential molts that can be readily identified. They usually reside within silk webs, relying on venom only as a last resort. They can be acquired in large numbers and easily maintained in the lab. We chose to study the Western Widow (*Latrodectus hesperus*) for several reasons: its synanthropic nature and local abundance, medically relevant venom, and ease of rearing in captivity. Two studies have previously examined ontogenetic shifts in the antipredator behaviors of genus *Latrodectus*. Vetter (1980) showed that silk use of *L. hesperus* against live rodent predators (*Peromyscus* spp.) increased with age, but disappeared from adult males. Troupe (2009), based on mean scores from a ranking of 22 behaviors, reported a general increase with age in defensiveness of *L. mactans* when poked by a probe.

The purpose of this study was to investigate ontogenetic shifts in defensive behaviors of *L. hesperus* by repeatedly subjecting individual spiders to the same threat stimulus during their progression through sequential molt stages. By using two threat stimuli representing low- and high-threat conditions, we were able to experimentally test three hypotheses. First, within each threat level, we hypothesized that spiders would exhibit primarily non-combative behaviors when smaller during the early instars, and switch to more combative strategies as they aged and increased in body size. Second, although females attain a larger body size than males (Baerg 1923), we hypothesized that age would exert a stronger influence than sex within the range of body sizes where males and females are equivalent. Third, we hypothesized that spiders subjected to repeated testing would exhibit habituation upon completing their final shed (as adults), and therefore exhibit less responsiveness and combativeness than naïve spiders at the same age.

Methods

Spider Husbandry, Sexing, and Aging

We collected spiders in the spring and summer months (generally May–September) from Redlands, Loma Linda, and Colton, California (San Bernardino County). Spiders were housed in 540-mL plastic deli cups within a small room in the laboratory at 22^{o} C on a 12-hr light-dark cycle. We provided spiders with a small stick that facilitated web construction, and offered house crickets (*Acheta domestica*) biweekly. No water was provided, as it was deemed unnecessary.

Some wild caught females produced egg sacs. We removed the hatched spiderlings from their natal container and housed them individually in 350-mL plastic deli cups under conditions identical to the adult females. Instars 2–3 were fed fruit flies (*Drosophila melanogaster* or *Drosophila* sp.) twice a week, and instars 4–6 were fed larger fruit flies once per week. Instars 7+ were treated the same as adult female spiders. Spiders of all ages were housed within the same room.

We sexed spiders using pedipalp morphology once the spiders had reached a sufficient size, typically around the 6th or 7th instar (Bhatnagar and Rempel 1962). We determined the age of spiders based on number of sheds. We called the first shed observed outside the egg sac shed 1; however, this corresponds to the spider becoming a 3rd instar, as the first molt occurs within the egg sac before emergence (Deevey 1949). The number of sheds to reach maturity (following the last shed) varied from 4–6 sheds for males and 7–9 sheds for females. When the experiments were conducted, we were unaware of a method to sex the smallest spiderlings (instar 3), but one has since been proposed (Mahmoudi et al. 2008) and confirmed (Chapter 6). Thus, in the experiments that follow, we were blind to sex as we conducted behavioral observations on the younger spiders.

Experimental Procedures

We conducted two experiments in the laboratory. The first took place during mid-June to mid-November, and the second from mid-February to late July; we assumed there would be no seasonal effect since the spiders bred year-round in the lab. For both experiments, spiderlings were tested at each instar stage through maturity in a repeated-

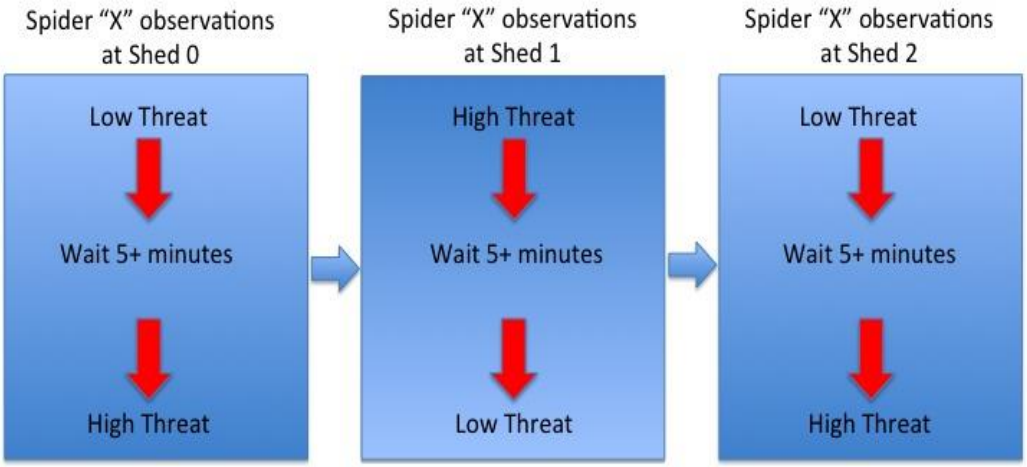
measures design. Because the number of sheds varied between and within sexes, we maximized our sample size by limiting most comparisons to sheds 0–4 (instars 2–6) for males, and sheds 0–7 (instars 2–9) for females (i.e., there were smaller numbers of instar 7+ males and instar 10+ females). Tests were always conducted 3–4 d after ecdysis, at which time spiders appeared to have hardened their exoskeleton. Prior to the start of each experiment, spiderlings placed within individual containers were left undisturbed for 72 hr to construct their web. Figure 3 illustrates the study design.

Experiment 1

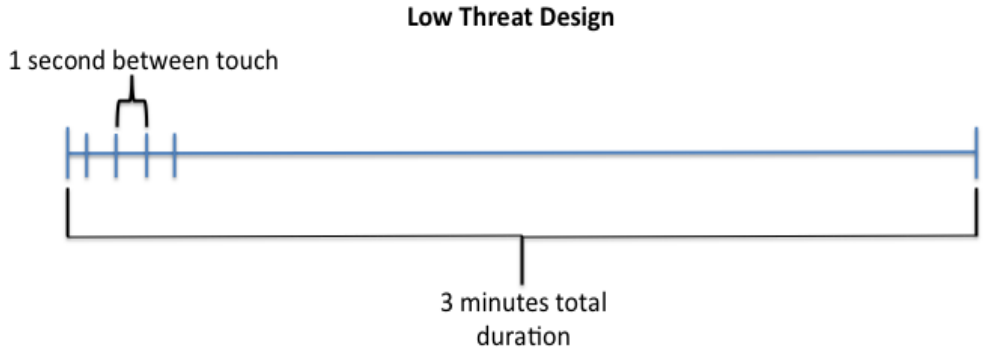
Individual spiders (N = 11 males, 7 females), repeatedly tested at each age class, were subjected to both a low-threat and high-threat stimulus with an intervening 5-min rest period. Order of treatment was randomly assigned at shed 0 and alternated thereafter over successive sheds. Observations were aided by a Nikon SMZ-10A dissection scope (Nikon Instruments Inc., Melville, NY, USA), which facilitated observation of fine behaviors, such as biting.

For the low-threat stimulus, we prodded a subject in its home container once per second for 180 sec with a taxidermy specimen of a laboratory mouse (*Mus musculus*). The mouse was stuffed with cotton and wire to maintain rigidity. We chose this stimulus to mimic a natural rodent predator (Vetter, 1980) in size, odor, and texture, but interactions could be readily standardized across all observations. Mice were wiped with a dry Kimwipe® (34120, Kimberly–Clark Professional, Roswell, GA, USA) after every use. We used an online metronome (http://www.metronomeonline.com/) to standardize the frequency of prods.

A



B



C

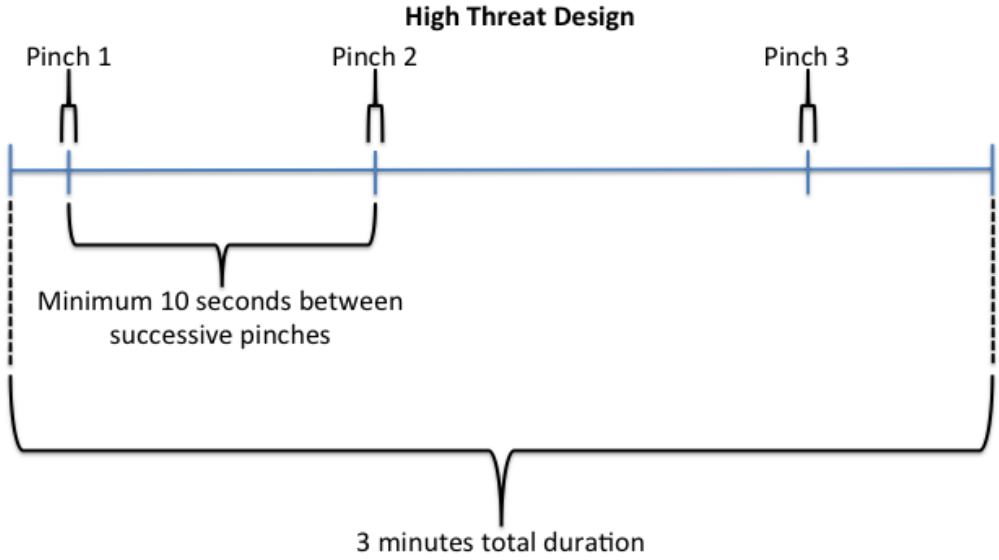


Figure 3: Study design. A) Illustrates observation of a spider across two sheds; alternating order of treatment repeated across all sheds. B) Design of the low threat. C) Design of the high threat.

The high-threat stimulus consisted of three separate pinches of the taxidermy mouse's tail gently pressing the spider against the container wall. Pinches were randomly directed to the leg(s) or prosoma (dorsal or ventral side). All pinches occurred within a 3-min observation period (Fig. 3). Each pinch lasted 10 sec (standardized with use of metronome), with successive pinches separated by a minimum of 10 sec, but often longer due to the difficulty of pinching spiders adequately without causing injury. The entire trial was always completed within 180 sec (mean duration 134 sec; range 80–180).

During observations, the onset and termination of each behavior was spoken aloud and recorded digitally. Audio recordings were subsequently analyzed to transcribe the frequency and duration of each behavior identified (Table 8). Audio recordings under low threat contained data on frequency and duration of each behavior performed. Due to slight gaps in the audio record between successive behaviors and rounding errors due to multiple events, some trials summed to <180 sec, which necessitated standardization of all behavioral durations to sum to 180 sec. Under high threat, manipulation of the spider and interruptions of natural behavior during pinches rendered the frequency and duration of behaviors problematic; therefore, we analyzed only presence/absence of behaviors.

Experiment 2

We conducted this experiment to examine the possible effect of habituation on behavioral responses. We randomly assigned spiders to one of two cohorts: the "experienced" group was tested at each shed as in experiment 1, whereas the "naive" group, was tested only once after the final shed (sheds 4, 5, or 6 for males, sheds 7, 8, or 9 for females).

Table 8: Definitions of behaviors exhibited by western widow (*Latrodectus hesperus*) spiders, including threat levels that most often elicited them.

Behavior	Structure used	Definition	Threat
Barrier	Entire body	Spider maneuvers entire body so that an object, usually the stick, is placed between spider and last direction of stimulus.	Low
Move	Entire body	Spider moves entire body in response to stimulus, usually away from it.	Low
Play dead	Entire body	Spider drops from web, retracts (adducts) all legs medially against body, and remains motionless ≥1 sec.	Low & High
Slight move	Entire body	Similar to Move, except movements are very short distances from origin, followed by a full stop.	Low
Pull free	Legs and/or entire body	Spider attempts to pull trapped body part from being pinned by stimulus, often using free legs to gain leverage for pull.	High
Retract leg(s)	One or more leg(s)	Spider moves (adducts) one or more legs medially from initial position.	Low
On mouse	Entire body	Spider moves entire body onto mouse target.	Low
Silk-flick	Legs IV and spinnerets	Legs IV first move toward spinnerets, then extended simultaneously or asynchronously toward stimulus; leg contact is made with stimulus and visibly viscous silk attached.	Low & High
Bite	Chelicerae and fangs	Spider moves chelicerae close to "predator," distal ends of chelicerae open laterally, and fangs are inserted as chelicerae close medially	Low & High
Bite attempt	Chelicerae and fangs	Movements similar in character to Bite, but penetration or completion of Bite not confirmed.	Low & High
Fang flare	Chelicerae and fangs	Spider opens chelicerae laterally but does not immediately close them, or closes them but not on or near any stimulus structure.	Low & High
Fang use	Chelicerae and fangs	A sum of the behaviors Bite, Bite attempt, and Fang flare.	Low & High
Autotomize	Legs and/or entire body	Spider pulls entrapped leg away from stimulus with sufficient force that entrapped limb is ripped off. Spider then moves away from stimulus.	High
No reaction	None	Spider makes no discernible movements.	Low & High

Thus, we compared the behavioral responses of adults only for habituation. Spiders of both groups were tested on the same day, with order of spiders and threat presentations randomly assigned. Because the experienced group was tested in an identical manner to spiders in experiment 1, the two groups were pooled for certain analyses.

Analyses

The first experiment started with 30 individuals, but due to deaths and exclusion from final analysis because of sex uncertainty, ended with $N = 18$ (11 males, 7 females). Similarly, the second experiment started with 36 individuals and ended with 27, with 11 males and 2 females in the experienced group, and 12 males and 2 females in the naive group. Due to the lack of sex certainty when spiderlings were originally assigned to treatment groups, we had too few females in experiment 2; however, due to negligible sex differences in behavior determined from subsequent analyses, we pooled the sexes for select comparisons. Because of design differences between experiments and treatment groups, four major analyses were necessary. We conducted all analyses using SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA), with alpha set at 0.05.

Low Threat

The low-threat data included frequency and duration of each behavior observed, and allowed us to test hypotheses about age and sex differences within 5 x 2 mixed analysis of variance models (ANOVAs; Zar, 1996), treating age (sheds 0–4) as a within-subjects factor, and sex as a between-subjects factor. We limited age comparisons to the

sheds that males and females shared in common and for which there were adequate data. Most data were positively skewed and heteroscedastic, with $\log_{10}$ transformations improving just three of the 10 dependent variables ("*barrier,*" "*silk flick,*" and "*play dead*"). Although parametric tests are robust to departures from assumptions, we additionally ran several non-parametric tests (Zar, 1996) to confirm parametric results for the main effects of age (Friedman's ANOVAs, within sexes and for pooled sexes) and sex (Mann–Whitney *U*-tests, within each age group and averaged across all age groups). In nearly all cases the parametric and non-parametric interpretations were identical, so we report both sets of analyses only when they differed. For significant age effects, we report the trend (i.e., linear or quadratic). We computed partial η^2 as a measure of effect size for the parametric ANOVAs, indicating approximate percent of variance explained by main effects and interactions, with values of ~0.01, ~0.06 and ≥0.14 loosely considered small, medium, and large, respectively (Cohen 1988). Effect sizes for Cochran's *Q* and Friedman's ANOVA were computed as Kendall's *W,* indicating strength of association (Green and Salkind 2005), with values of ~0.1, ~0.3, and ≥0.5 roughly corresponding to small, medium, and large effects, respectively (Cohen 1988). We computed the correlation coefficient *r* as a measure of effect size for the Mann–Whitney *U*-test, with values of ~0.1, ~0.3, and ≥0.5 roughly corresponding to small, medium, and large effects, respectively (Cohen 1988). Following Nakagawa (2004), we chose not to adjust alpha for multiple tests, and comment on experimentwise error in the Discussion.

High Threat

The high-threat data consisted of the presence/absence of various behaviors. A

previous study (Chapter 3) determined that no significant interaction existed between the presence of a behavior and the order of pinch, so we pooled data across all three pinches. Assumptions of normality and homoscedasticity were poorly met. We tested these dichotomous dependent variables with both semi-parametric (generalized estimating equation, GEE; Zeger and Liang 1986) and non-parametric (Cochran's *Q* and Fisher's exact Chi-square; Zar, 1996) tests. We chose not to report the preferred omnibus GEE results because they frequently disagreed with non-parametric results and some variables could not be tested because of empty or sparsely-populated cells. Thus, we had to examine the effects of age and sex separately using the non-parametric tests, which also meant we could not test for interactions. We evaluated the effect of age on behaviors using Cochran's *Q* test. Sheds 0–4 were tested within males, within females, and for pooled sexes. We also analyzed sheds 0–7 within females. We tested the effect of sex on behaviors with Fisher's exact Chi-square at each of sheds 0–4. Effect size for Fisher's test was computed as Phi (Φ), with values of ~0.1, ~0.3, and ≥0.5 roughly corresponding to small, medium, and large effects, respectively (Cohen 1988). We computed a Spearman's rank correlation to test the effect of age (Females shed 0–7) on the proportion of individuals that performed *bit*e.

Low Threat Versus High Threat

We used nonparametric McNemar's tests (Zar, 1996) to evaluate the effect of threat level on those behaviors that were measured in both conditions (i.e., presence/absence). We ran tests separately for the experimental and control groups.

Males and females were pooled when running these tests since their behaviors differed little. We reported Φ as a measure of effect size.

Naïve Versus Experienced

We tested the effect of habituation within each threat level for experienced (N = 31) and naïve (N = 14) spiders that attained the ultimate shed, with sexes pooled since their behaviors differed little. We used a Mann–Whitney U test within the low threat for frequency and duration of behaviors, and Fisher's exact Chi-square tests within the high threat presence/absence of behaviors.

Results

Low Threat

Figure 4 and Table 9 summarize the results of the 5 x 2 (age x sex) ANOVAs. A number of non-confrontational behaviors differed significantly among groups. We found a shed-by-sex interaction for *barrier* (time: P = 0.035; frequency: P = 0.039). Females maneuvered their body to position a barrier between themselves and the stimulus more frequently and for longer durations than males at sheds 0–1, but used this tactic less frequently by sheds 3 and 4. The decline in *barrier* use by females appeared to persist through shed 7 (Fig. 4). The frequency of *move* (P = 0.020) increased across sheds in a linear fashion ($F_{1,29}$ = 26.49, P = 0.032, partial η^2 = 0.15), whereas the duration showed a significant shed-by-sex interaction (P = 0.006), with females spending less time than males performing *move* during sheds 0–1 and more time in sheds 3–4 (Fig. 4). *Play dead* increased across sheds for both frequency (P = 0.016; quadratic effect: $F_{1,29}$ = 4.26, P =

0.048, partial $\eta^2 = 0.13$) and duration ($P = 0.040$; quadratic effect: $F_{1,29} = 4.46$, $P = 0.043$, partial $\eta^2 = 0.13$). The trend for *play dead* persisted among females through shed 7 (Fig. 4). Frequency of *retract* increased significantly among age groups, but this was detected only with the non-parametric Friedman's ANOVA (pooled across sexes due to non-significant sex differences; df = 4, X^2=15.12, $P = 0.004$). *Slight move* changed across sheds in both frequency ($P = 0.019$) and duration ($P = 0.031$); however, whereas frequency of *slight move* increased and then decreased during ontogeny (quadratic effect: $F_{1,29} = 6.20$ $P = 0.019$, partial $\eta^2 = 0.176$), duration of slight move was more complex, approaching a significant interaction ($P = 0.082$) with males showing an increase and females a decrease across sheds (Fig. 4). While *slight move* among females decreased through shed 4, it appeared to increase again in stages 5–7 (Fig. 4).

Several confrontational behaviors also differed among groups. Spiders occasionally climbed onto the mouse stimulus, and *on mouse* had a significant shed-by-sex interaction for both frequency ($P = 0.006$) and duration ($P = 0.017$). Females climbed *on mouse* less frequently and for shorter durations than males during sheds 0–1, but did so more often and for longer durations during sheds 2–4. Spiders used *silk flick* more frequently across sheds ($P = 0.001$; quadratic effect: $F_{1,29} = 8.40$, $P = 0.007$, partial $\eta^2 = 0.23$), and duration of *silk flick* approached significance (0.051; linear effect: $F_{1,29} = 8.11$, $P = 0.008$, partial $\eta^2 = 0.22$). The behaviors *bite* and *fang use (bite + bite attempt + fang flare)* showed negligible changes during ontogeny and no differences between sexes.

Although the interactions suggested that sex differences exist in the ontogeny of behavioral changes, the relative lack of a sex effect can be better appreciated from the non-parametric results. Only seven (7%) of the 100 non-parametric (Mann–Whitney *U*)

tests comparing the sexes among the 20 dependent measures within the five age groups were significant (data not provided here), which is close to the number of tests expected to be significant by chance (5% with alpha = 0.05).

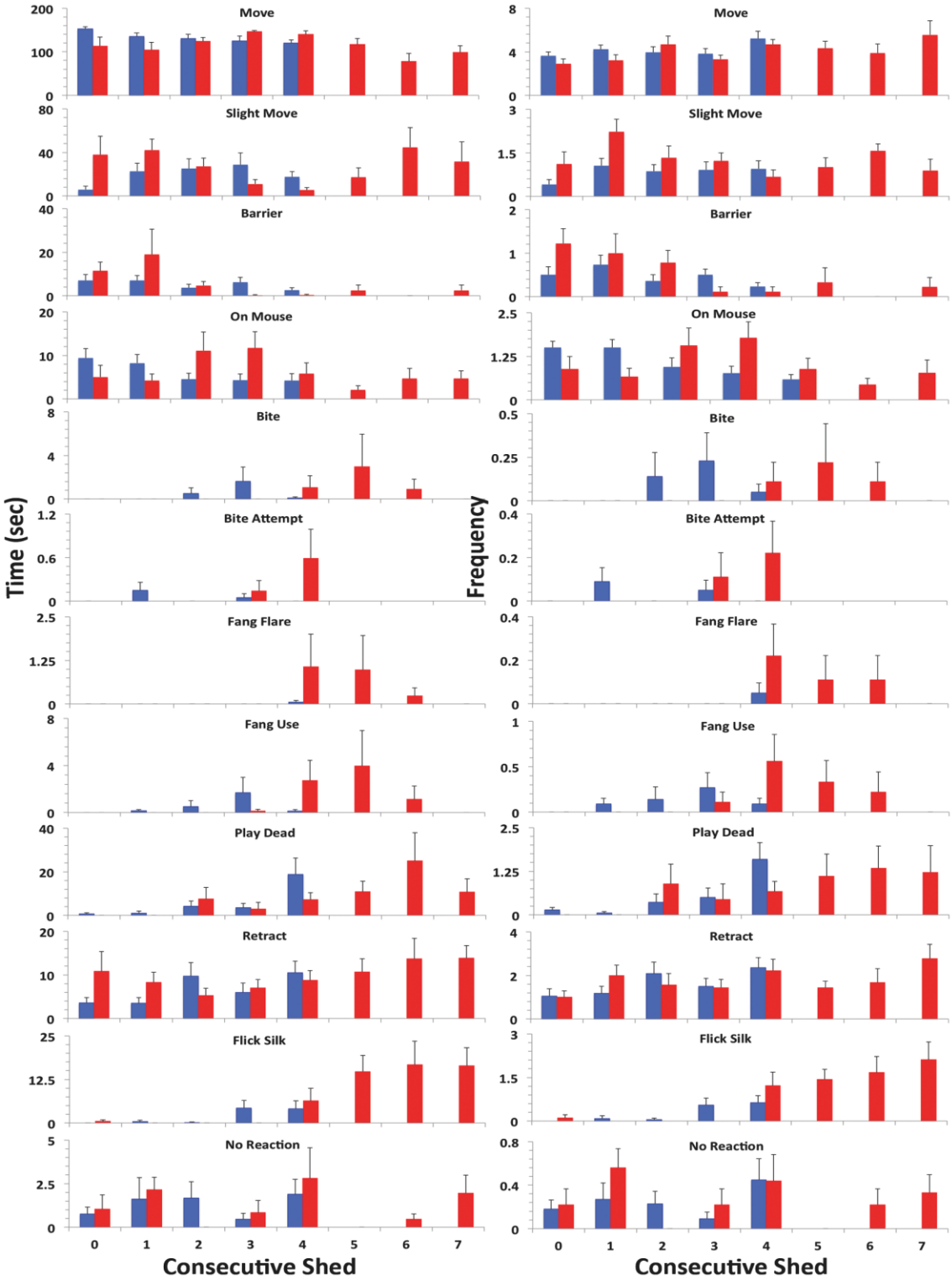


Figure 4: Mean (± 1 S.E.) values for frequency and duration of behaviors exhibited by *Latrodectus hepsperus* during ontogeny (males, sheds 0–4 in blue, $N = 22$; females, sheds 0–7 in red, $N = 9$) when prodded by the low-threat stimulus (taxidermy mount of mouse).

Table 9: Tests of significance for each behavior within the low threat ($N = 45$).

Behaviors		Shed			Sex			Interaction		
		$F_{4,116}$	P	Partial η^2	$F_{1,29}$	P	Partial η^2	$F_{4,116}$	P	Partial η^2
Barrier	Frequency	3.00	0.021	0.09	0.40	0.535	0.01	2.62	0.039	0.08
	Duration	3.23	0.015	0.100	0.10	0.755	0.00	2.69	0.035	0.09
Move	Frequency	3.05	0.020	0.10	0.75	0.392	0.03	0.75	0.560	0.03
	Duration	0.72	0.578	0.02	0.59	0.451	0.02	3.87	0.006	0.12
Play dead	Frequency	4.29	0.016[a]	0.13	0.21	0.649	0.01	1.40[a]	0.253	0.05
	Duration	3.95	0.040[a]	0.12	0.29	0.592	0.01	1.14[a]	0.314	0.04
Slight move	Frequency	3.08	0.019	0.10	4.13	0.051	0.13	1.54	0.195	0.05
	Duration	2.76	0.031	0.09	2.96	0.096	0.09	2.13	0.082	0.07
Retract	Frequency	2.45	0.071[a]	0.08	0.00	0.986	0.00	0.69[a]	0.557	0.02
	Duration	0.69	0.589[a]	0.02	0.33	0.572	0.01	1.91[a]	0.118	0.06
On mouse	Frequency	1.17	0.327	0.04	0.14	0.707	0.01	3.77	0.006	0.12
	Duration	0.62	0.646	0.02	0.65	0.427	0.02	3.16	0.017	0.10
Silk flick	Frequency	7.89	0.001[a]	0.21	0.00	0.983	0.00	2.21[a]	0.120	0.07
	Duration	3.28	0.051[a]	0.10	0.16	0.689	0.01	0.96[a]	0.381	0.03
Bite	Frequency	0.51	0.551[a]	0.02	0.43	0.517	0.02	0.72[a]	0.456	0.02
	Duration	0.57	0.487[a]	0.02	0.16	0.691	0.01	0.99[a]	0.344	0.03
Fang use	Frequency	2.34	0.104[a]	0.08	0.02	0.884	0.00	2.27[a]	0.110	0.07
	Duration	1.45	0.244[a]	0.05	0.02	0.901	0.00	2.21[a]	0.134	0.07
No reaction	Frequency	1.88	0.119	0.06	1.17	0.289	0.04	1.33	0.264	0.04
	Duration	1.75	0.144	0.06	1.28	0.267	0.04	1.37	0.250	0.05

5 x 2 (age x sex) analyses of variance (ANOVAs) and partial η^2 for effect size

[a] P-value based on Greenhouse-Geisser adjustment to degrees-of-freedom

High Threat

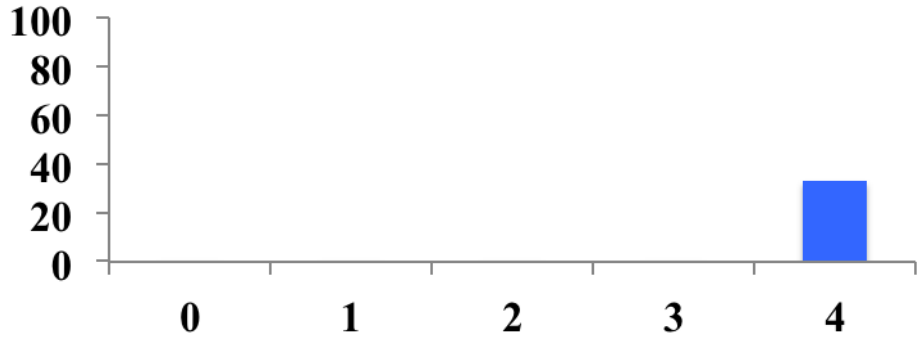
Due to statistical constraints, we separately tested the effects of sex on the presence/absence of behaviors within each of sheds 0–4, and the effects of age within each sex (males, sheds 0–4; females, sheds 0–4 and sheds 0–7) and for both sexes pooled (sheds 0–4). Relevant results (statistical outcomes only) are summarized in Table 10. None of the seven behaviors subjected to analysis differed between sexes at any specific shed, and this despite 35 individual tests (seven behaviors x five age groups). Thus, specific results are not presented. Within males, none of the behaviors differed among age groups. Within females, however, *bite* differed significantly among sheds 0–4 (P = 0.017) and sheds 0–7 (P = 0.007), with an increase in proportion of spiders relying on *bite* as spiders aged (sheds 0–7: Spearman's r = 0.77, P = 0.042; Fig. 5). *Bite* propensity also varied with age for both sexes pooled (P = 0.027), but not in a clear linear fashion (Fig. 5). *Fang use* similarly increased with age, but only within females among sheds 0–4 (P = 0.019; Fig. 6). Finally, *silk flick* increased with age as well, but only for both sexes pooled (P = 0.037; Fig. 6).

Table 10: Comparisons of presence/absence of behavior among age groups within the high threat.

Behavior	Males, sheds 0–4 (N = 22)			Females, sheds 0–4 (N = 9)			Females, sheds 0–7 (N = 9)			Pooled sexes, sheds 0–4 (N = 31)		
	$X^2_{(df=4)}$	P	Kendall's W	$X^2_{(df=4)}$	P	Kendall's W	$X^2_{(df=4)}$	P	Kendall's W	$X^2_{(df=4)}$	P	Kendall's W
Play dead	5.24	0.263	0.06	4.73	0.316	0.13	5.83	0.559	0.09	4.70	0.319	0.04
Pull free	3.00	0.558	0.03	—	—	—	7.00	0.429	0.11	3.00	0.558	0.02
Silk flick	8.50	0.075	0.10	4.00	0.406	0.11	10.43	0.165	0.17	10.18	0.037	0.08
Bite	6.63	0.157	0.08	12.00	0.017	0.33	19.43	0.007	0.31	10.93	0.027	0.09
Fang use	5.14	0.273	0.06	4.33	0.363	0.12	16.79	0.019	0.27	6.56	0.161	0.05
Autotomize	8.00	0.092	0.09	—	—	—	7.00	0.429	0.11	8.00	0.092	0.07
No reaction	8.49	0.075	0.10	1.60	0.809	0.04	3.21	0.865	0.05	5.71	0.222	0.05

Cochran's Q tests and Kendall's W for effect size; pull free and autotomize absent in females during sheds 0–4

A



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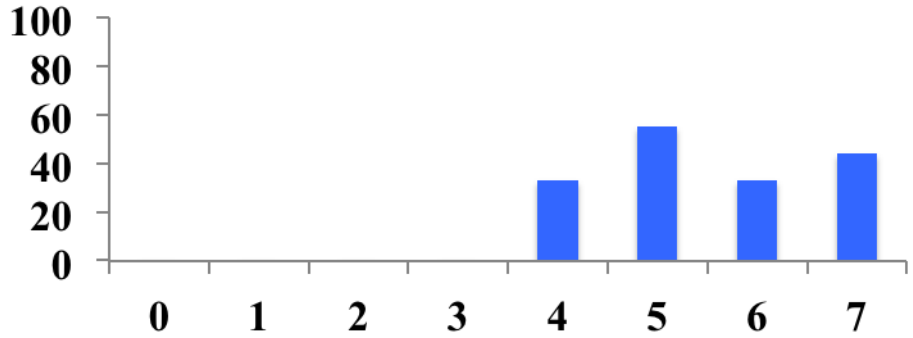

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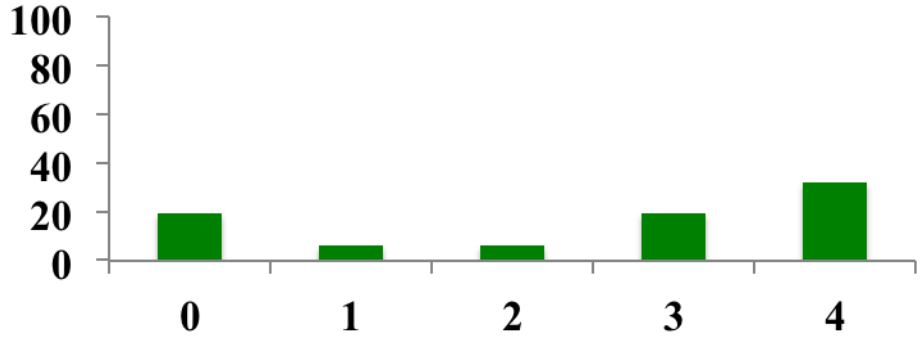
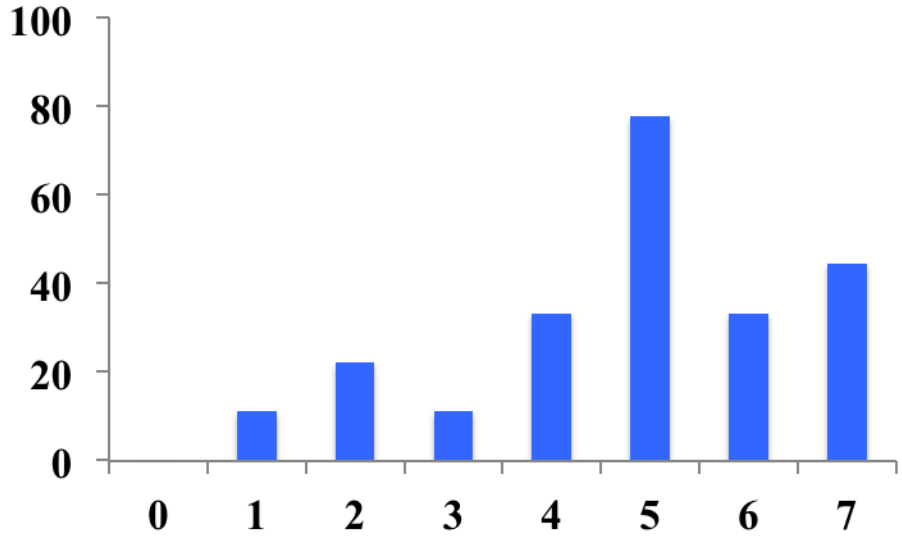


Figure 5: Percent of individual spiders exhibiting *bite* behavior at each shed within high threat. A) Females, sheds 0–4 ($N = 9$, $P = 0.017$). B) Females, sheds 0–7 ($N = 9$, $P = 0.007$). C) Pooled sexes, sheds 0–4 ($N = 31$, $P = 0.027$).

A



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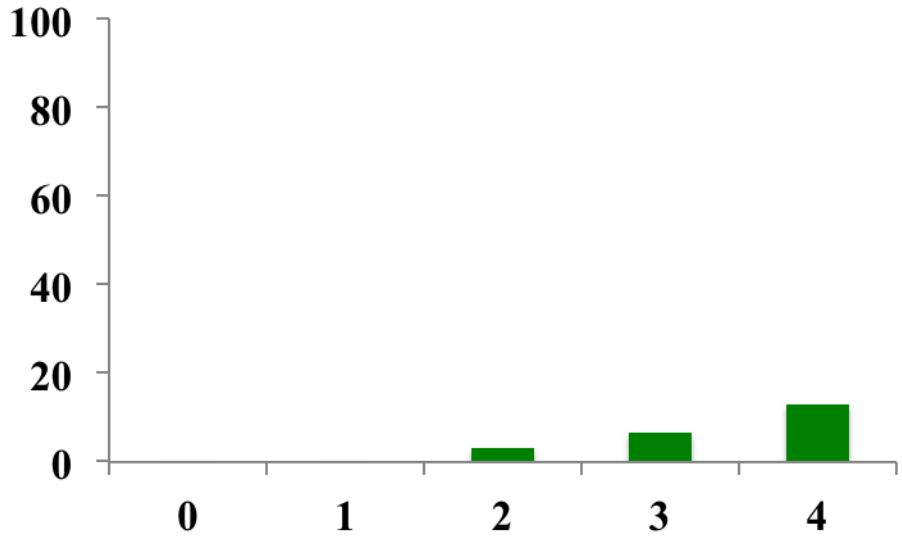

Figure 6: Percent of individuals exhibiting behavior at each shed within the high threat. A) Fang use among females, sheds 0–7 (. B) Silk flick for pooled sex shed 0–4.

Low Versus High Threat

Considering the negligible differences between sexes within low threat and within high threat, sexes were pooled for comparing defensive behaviors between low and high threat. Results are summarized in Table 11. Within the naïve group ($N = 14$), McNemar tests revealed that a higher proportion of individuals exhibited *bite* ($P = 0.008$) and *fang*

use ($P = 0.002$) during high threat. *Autotomize* ($P = 0.004$) appeared exclusively used in the high threat, with nine individuals (64%) losing a leg during the pinches.Within the experienced group ($N = 31$), *silk flick* was utilized most often in the low threat ($P = 0.002$), whereas *bite* ($P = 0.001$), and *fang use* ($P < 0.0001$) were more often employed in the high threat.

Naïve Versus Experienced

We compared experienced spiders repeatedly exposed to the experimental stimulus until attaining their final shed versus those that were naïve at the ultimate shed. Males and females were pooled for comparisons. Within the low threat treatment (Table 12), Mann–Whitney *U* tests indicated that naïve spiders exhibited *move* (frequency, $P = 0.001$), *bite* (time and frequency, both $P = 0.033$), and *retract* (frequency, $P = 0.002$) more often and/or for longer duration than experienced spiders. Within the high threat (Table 13), Chi-square tests showed that a significantly higher proportion of naïve spiders engaged in *bite* ($P = 0.042$) and *autotomize* ($P < 0.0001$) compared to experienced spiders.

Table 11: Comparisons of presence/absence of behaviors exhibited at low vs. high threat within experienced and naïve groups.

	Naïve Group (N = 14)					Experienced Group (N = 31)				
Behaviors	% low	% high	$X^2_{(df=1)}$	P-value	Φ	% low	% high	$X^2_{(df=1)}$	P-value	Φ
Play dead	57.1	21.4	2.29	0.125	0.27	51.6	54.8	0.00	1.000	0.00
Silk flick	14.3	7.1	0.00	1.000	0.00	54.8	16.1	8.64	0.002	0.53
Bite	14.3	71.4	6.13	0.008	0.45	12.9	54.8	9.60	0.001	0.56
Fang use	14.3	85.7	8.10	0.002	0.51	29.0	77.4	11.53	<0.0001	0.61
Autotomize	0	64.3	7.11	0.004	0.48	0	6.5	0.50	0.500	0.13
No reaction	21.4	21.4	0.00	1.000	0.00	64.5	80.6	1.23	0.267	0.20

McNemar's test and Phi (Φ) for effect size

Table 12: Comparison of naïve vs. experienced groups within low threat (N = 45, pooled sexes)

Behavior	Naïve (mean ± SE)	Experienced (mean ± SE)	Mann–Whitney U	P-value	r
Barrier (F)	0.6 ± 0.2	0.3 ± 0.1	279.5	0.053	0.29
Barrier (D)	2.6 ± 1.0	2.7 ± 1.4	275.5	0.073	0.27
Move (F)	7.5 ± 0.7	4.3 ± 0.5	350	0.001[a]	0.49
Move (D)	141.4 ± 7.7	117.5 ± 8.3	291	0.07	0.27
Play dead (F)	1.9 ± 0.6	1.5 ± 0.4	245.5	0.447	0.11
Play dead (D)	13.8 ± 5.7	14.9 ± 4.8	238	0.567	0.09
Slight move (F)	0.4 ± 0.2	0.7 ± 0.2	163	0.131	-0.23
Slight move (D)	6.4 ± 5.6	22.7 ± 7.6	151	0.068	-0.27
Retract (F)	3.9 ± 0.6	1.7 ± 0.3	340.5	0.002[a]	0.46
Retract (D)	10.7 ± 1.8	10.3 ± 2.1	258	0.313	0.15
On mouse (F)	0.9 ± 0.8	0.8 ± 0.3	233	0.664	0.06
On mouse (D)	3.5 ± 1.6	4.6 ± 1.3	215.5	0.968	-0.006
Silk flick (F)	0.1 ± 0.1	0.5 ± 0.2	201	0.556	-0.09
Silk flick (D)	0.9 ± 0.8	4.5 ± 2.2	203	0.607	-0.08
Bite (F)	0.1 ± 0.1	0	248	0.033[a]	0.32
Bite (D)	0.2 ± 0.1	0	248	0.033[a]	0.32
Bite attempt (F)	0	0	217	1	0
Bite attempt (D)	0	0	217	1	0
Fang flare (F)	0.1 ± 0.1	0	232.5	0.137	0.22
Fang flare (D)	0.2 ± 0.1	0	232.5	0.137	0.22
No reaction (F)	0.3 ± 0.2	0.3 ± 0.1	213.5	0.906	-0.02
No reaction (D)	0.5 ± 0.3	2.2 ± 1.0	206	0.711	-0.06

Mann–Whitney U tests and r for effect size ($z/\sqrt{N}$)
[a] Observed behavior greater in controls
F = frequency; D = duration

Table 13: Comparison of naïve vs. experienced groups within high threat (N = 45, pooled sexes).

Behaviors	% Naive	% Experienced	$X^2_{(df=1)}$	P-value[a]	Φ
Play Dead	21.4	25.8	0.10	1.000	0.05
Pull Free	100	100	—	—	—
Silk Flick	7.1	9.7	0.08	1.000	0.04
Bite	71.4	38.7	4.13	0.042[b]	0.30
Fang Use	85.7	61.3	2.68	0.165	0.24
Autotomize	64.3	6.5	17.47	<0.0001[b]	0.62
No Reaction	21.4	25.8	0.10	1.00	0.05

[a] = Fisher's Exact test and Phi (Φ) for effect size
[b] = observed behavior greater in naïve controls

Discussion

In this study, we experimentally tested three hypotheses regarding ontogenetic development of antipredator behavior in *L. hesperus*. First, we hypothesized that spiders would exhibit more non-combative behaviors when smaller during the early instars, and switch to more combative strategies as they aged and increased in body size. Second, we hypothesized that age would exert a stronger influence than sex for males and females within the range of equivalent size. Third, we hypothesized that spiders subjected to repeated testing would exhibit habituation upon reaching the ultimate (adult) shed, and therefore exhibit less responsiveness and combativeness than naïve spiders at the same age. We found experimental support for all three hypotheses, and will discuss each in turn.

Ontogenetic Shifts in Behavior

Our results showed clear shifts in behaviors used during the early stages of the

spider's life. Of the 14 stereotyped behaviors identified, nine were used differentially during different stages of the spider's life, or as a result of experience. In general terms, spiders relied more on non-combative behaviors when young, but switched to more combative behaviors as they grew in size.

Within the low threat, reliance on *barrier* declined with increasing age and size. *Barrier* is a form of hiding, as the spider moves in such a way as to place an object between itself and the stimulus. The barrier used during our observations was the single thin stick provided to aid in web construction. *Latrodectus hesperus* routinely constructs its webs within or around a retreat (Benjamin and Zschokke 2003; Blackledge and Zevenbergen 2007). Our spiders had limited space to construct their webs, being housed in a small round container, so no other retreats were available. During presentation of the threatening stimulus, spiders would often either move to areas of the web possessing more silk, or hide behind the stick, especially where the stick met the bottom or top of the container. This was observed at every age. However, young spiders would more often continue to hold on to the stick and reposition the stick between itself and the last direction of threat stimulation, whereas the older and larger spiders would drop from the web or stick, and engage in *play dead* (thanatosis). The size of the spider in relation to the stick may have influenced the spider's propensity to use *play dead* rather than *retract*. Future experiments should investigate variation in retreat and web architecture and the use of non-combative behaviors in response to threat.

Movement behaviors including *move, slight move*, and *retract* also changed during ontogeny, especially the frequency with which these behaviors were used by spiders in response to threat. *Slight move*, consisting of short-distance, whole-body

movements, occurred most often when young, whereas *move* and *retract* increased with increasing age and size. Movements of any kind have the potential to draw the attention of a predator; therefore, spiders during the more vulnerable early stages may rely more on hiding and crypsis until they attain a size where they can better defend themselves. In contrast to our findings, Uma and Weiss (2012) observed in *Parasteatoda tepidariorum* a decrease in movement (fleeing) behaviors with age and size. Smaller spiders were more likely to flee by moving to the periphery of their web or dropping from the web, whereas older spiders were more like to remain and fight by throwing silk onto the predator. Thus, there was an ontogenetic trade-off between *move* and *silk flick*.

As hypothesized, *L. hesperus* increasingly relied on more combative and energetically costly behaviors as they grew, particularly *silk flick* and *bite*. Spiders increasingly relied on *silk flick* as they aged, both in the low-threat and high-threat conditions. Vetter (1980) previously examined the ontogenetic development of *silk flick* or viscous web production, as he called it. Similar to our findings, he found that older and larger spiders used *flick silk* significantly more often than younger spiders (Vetter 1980). Vetter (1980) reported that no spider less than the 4th instar was observed to *flick silk*; however, we observed this behavior as young as the 3rd instar (one individual). Vetter (1980) also reported a significant difference between the sexes, with adult females relying more on *silk flick* than adult males. We did not directly compare adults because males attain maturity at an earlier age than females, and we limited comparisons of sex only among individuals of equivalent age. Spiders also relied increasingly on *bite* as they aged, but only in the high-threat condition. Similar to Nelsen et al. (Chapter 3), spiders very seldom bit when poked in the low-threat condition, but much more often bit when

pinched in the high-threat condition, which we interpret to be threat assessment. *Bite*, along with presumed venom use, appears to be a behavior relied on primarily as a last resort (Nelsen et al., Chapter 3). The ontogenetic increase we observed in combative behaviors matched the results we inferred from Troupe (2009), who reported an ontogenetic increase in aggressiveness (including *silk flick* and *bite* as high-ranking behaviors) in *L. mactans* responding to a pair of pokes. Adult females guarding eggs exhibited the highest levels of defensiveness.

Both silk and venom represent limited commodities (sensu Hayes 2008) that should be used judiciously. Assuming silk and venom expenditure correspond with number of flicks and bites, respectively, this study and Nelsen et al. (Chapter 3) suggest that *L. hesperus* possesses cognitive control of these defensive weapons, and delivers greater quantities when confronted with higher levels of threat. No study to date has investigated the metabolic cost of defensive silk use, but web construction from silk comes at a metabolic cost, and can influence decisions to relocate web sites (Craig et al. 1999; Prestwich 1977; Tanaka 1989). The metabolic cost of venom synthesis has been demonstrated in several species of snakes (McCue and Mason 2006; Morgensterna and King 2013; Pintor et al. 2010) and in the scorpion *Parabuthus transvaalicus* (Nisani et al. 2007, 2012). Future work should compare the metabolic cost of defensive silk and venom use.

Several constraints likely influenced the tendency of spiders to use silk or venom. Silk flicking can be initiated at a greater distance than biting, and involves contact via legs rather than the fangs and cephalothorax with its vulnerable organs. If a spider is grasped by the leg, it can autotomize the leg without substantially compromising its

fitness or survival (Brueseke et al 2001). Size and strength of jaws and fangs may also limit biting effectiveness (Vetter 1980), especially for younger spiders. Venom toxicity often changes with ontogeny in snakes (Mackessy 1988; Mackessy et al. 2006), and may do so in spiders as well (see Chapter 6) in ways that could influence envenomation effectiveness. Exoskeleton hardness may also have influenced the spider's willingness to engage in combative behaviors, especially biting. Vetter and Rust (2010) reported that the brown recluse spider (*Loxosceles reclusa*) returned to normal levels of hunting and feeding 48 hr after ecdysis. Our spiders had an additional 24-48 hrs following ecdysis to harden their exoskeleton before being subjected to the treatment; thus, we feel that exoskeleton hardness did not greatly impact the spider's behavioral repetoire.

Sex Differences

As hypothesized, ontogenetic changes were much more substantive than sex differences. We detected interactions between age and sex for several behaviors in the low-threat condition, suggesting an ontogenetic trajectory that may differ between sexes, but the number of significant pairwise comparisons between sexes at each age group (7% in low threat, 0% in high threat) was similar to that expected due to chance (5%, with alpha = 0.05). Clearly, sex differences were trivial compared to ontogenetic changes. We can think of no reasons why the two sexes should differ until they reach the ultimate shed, at which point females mature substantially larger than males. We did not compare the behavior of adults due to confounding age and size differences, but at this point male *L. hesperus* discontinues *silk flick* as a defensive measure when attacked by live rodents, whereas female spiders continue to rely on this behavior (Vetter, 1980). It would be

interesting to compare the defensive use of biting and venom between adult males and females.

Habituation

We compared naïve spiders and experienced spiders (repeatedly subjected to the threat conditions) after individuals in both groups reached their ultimate shed (adulthood). As hypothesized, naïve spiders relied significantly more on *bite* than experienced spiders, suggesting habituation of this particular behavior. Several other behaviors differed between the two groups, including *autotomize*, which occurred almost exclusively among the naïve spiders. Habituation to threatening stimuli has been reported previously in other spiders. Sitvarin and Rypstra (2012) found that males of *Pardosa milvina*, which habituated to the chemical cues of the larger wolf spider *Tigrosa helluo*, had increased survival in the presence of a live predator, whereas females showed no significant differences. Experience also improves hunting efficiency (Edwards and Jackson 1994), web relocation (Nakata and Ushimaru 1999), and web building and silk use (Venner et al. 2000). An unpublished study of thanatosis (play dead or death feigning) in *L. hesperus*, however, reported that experience had no measurable effect on spider willingness to use this behavior (Torres and Johnson 2008). The presence of habituation as a result of repeated exposure to a threat implies our spiders are capable of memory and learning. Future studies should seek to further investigate the role experience plays in shaping and modifying spider behavioral repertoires.

Statistical Inferences

Our analyses involved a high number of statistical tests, of which 5% (based on alpha of 0.05) would be expected to be significant by chance alone. Following Nakagawa (2004), we chose not to control for experimentwise error because doing so overemphasizes the importance of null hypothesis testing when effect size is more meaningful, and unacceptably increases the probability of making type II errors (i.e., the hyper-Red Queen phenomenon: the more research one does, the lower the probability that a significant result will be found; Moran 2003). In spite of the high experimentwise error, we feel that our conclusions are robust for each of the four major sets of analyses. For low threat, the proportion of significant main effects and interactions involving age (13 of 40 tests, 32.5%; Table 9) far exceeded the proportion expected by chance, and supports our conclusion that ontogenetic changes in antipredator behavior were substantial. The only sex differences apparent at low threat were detected from significant interactions, indicating that for a given behavior males and females differed among some but not all age groups. Only seven (7%) of the 100 non-parametric (Mann–Whitney *U*) tests comparing the sexes among the 20 dependent measures within the five age groups (results not supplied here) were significant, which was similar to that expected by chance and supports our conclusion that sex differences were comparatively negligible. At high threat, the proportion of significant main effects of age (five of 26 tests, 19.2%; Table 10) again far exceeded that expected by chance, whereas the proportion of significant main effects for sex (0 of 35 tests, 0%; results not supplied) was well below that expected by chance. For comparisons of low versus high threat (with sexes pooled), six of 12 (50%) tests were significant (Table 11), supporting our

conclusion that spiders exhibited different behaviors during the two levels of threat. Finally, for comparisons of naive and experienced spiders, six of 28 (21.4%) tests were significant, supporting our conclusion that behavioral habituation occurred during repeated testing.

Conclusions

Our findings suggest that *L. hesperus* exhibits ontogenetic changes in antipredator behavior that relate to constraints on the use of two important and energetically costly weapons: silk and venom. The spider relies more on non-combative behaviors during early instars, and switches to more combative behaviors, including silk flick and bite, as it increase in size. Age clearly exerts a greater influence on defensive behaviors than sex-related differences, at least for males and females of equivalent size. We further found that repeated exposure of the spiders to the threatening stimulus resulted in habituation, with experienced spiders behaving less combatively than naïve spiders at the same developmental stage. Collectively, these findings suggest that selection has favored size- or age-specific antipredator strategies that can be modified by experience.

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References

Baerg, W. J. (1923). The black widow: its life history and the effects of the poison. *Scientific Monthly*, *17*(35), 535–547.

Bednekoff, P. A. (2007). Foraging in the face of danger. In: *Foraging behavior and ecology. (eds. D.W. Stephens, J.S. Brown, & R.C. Ydenberg), University of Chicago Press, Chicago, Illinois, USA*, 305–329.

Benjamin, S. P., & Zschokke, S. (2003). Webs of theridiid spiders: construction, structure and evolution. *Biological Journal of the Linnean Society*, *78*(3), 293–305.

Bhatnagar, R. D. S., & Rempel, J. G. (1962). The structure, function, and postembryonic development of the male and female copulatory organs of the black widow spider *Latrodectus curacaviensis* (Müller). *Canadian Journal of Zoology*, *40*(3), 465–510.

Blackledge, T. A., & Wenzel, J. W. (2001). Silk mediated defense by an orb web spider against predatory mud-dauber wasps. *Behaviour*, *138*(2), 155–171.

Blackledge, T. A., & Zevenbergen, J. M. (2007). Condition-dependent spider web architecture in the western black widow, *Latrodectus hesperus*. *Animal Behaviour*, *73*(5), 855–864.

Brueseke, M. A., Rypstra, A. L., Walker, S. E., & Persons, M. H. (2001). Leg autotomy in the wolf spider *Pardosa milvina*: a common phenomenon with few apparent costs. *The American Midland Naturalist*, *146*(1), 153–160.

Caro, T. (2005). *Antipredator defenses in birds and mammals*. University of Chicago Press, Chiccago, Illinois, USA.

Craig, C. L., Hsu, M., Kaplan, D., & Pierce, N. E. (1999). A comparison of the composition of silk proteins produced by spiders and insects. *International Journal of Biological Macromolecules*, *24*(2), 109–118.

Cohen, J. (1988). *Statistical power analysis for the behavioral sciences*. 2nd ed. Lawrence Erlbaum Associates, Inc., Hillsdale, NJ, USA.

Deevey, G. B. (1949). The developmental history of *Latrodectus mactans* (Fabr.) at different rates of feeding. *American Midland Naturalist*, *42*(1), 189–219.

Ferrari, M. C., Sih, A., & Chivers, D. P. (2009). The paradox of risk allocation: a review and prospectus. *Animal Behaviour*, *78*(3), 579–585.

Glaudas, X., Winne, C. T., & Fedewa, L. A. (2006). Ontogeny of anti-predator behavioral habituation in cottonmouths (*Agkistrodon piscivorus*). *Ethology*, *112*(6), 608–615.

Green, S. B., and N. J. Salkind. (2005). *Using SPSS for windows and macintosh: analyzing and understanding data*. 4th ed. Pearson Prentice Hall, Upper Saddle River, New Jersey, USA.

Haight, K. L. (2008). Ontogeny of the defensive stinging behavior of the fire ant, Solenopsis invicta. *Journal of Insect Behavior*, *21*(3), 147–152.

Haight, K. L. (2010). Worker size and nest defense in *Solenopsis invicta* (Hymenoptera: Formicidae). *Annals of the Entomological Society of America*, *103*(4), 678–682.

Hayes, W. K. (1991). Ontogeny of striking, prey-handling and envenomation behavior of prairie rattlesnakes (Crotalus v. viridis). *Toxicon*, *29*(7), 867–875.

Hayes, W. K., Herbert, S. S., Rehling, G. C., & Gennaro, J. F. (2002). Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. *Biology of the vipers. Eagle Mountain Publishing, Eagle Mountain, UT*, 207–233.

Hayes, W. K., & Mackessy, S. P. (2010). Sensationalistic journalism and tales of snakebite: are rattlesnakes rapidly evolving more toxic venom? *Wilderness & Environmental Medicine*, *21*(1), 35–45.

Kardong, K. V., & Lavin-Murcio, P. A. (1993). Venom delivery of snakes as high-pressure and low-pessure systems. *Copeia*, *1993*(3), 644–650.

Lima, S. L., & Bednekoff, P. A. (1999). Temporal variation in danger drives antipredator behavior: the predation risk allocation hypothesis. *The American Naturalist*, *153*(6), 649–659.

Lima, S. L., & Steury, T. D. (2005). Perception of predation risk: the foundation of nonlethal predator–prey interactions. In: *Ecology of predator–prey interactions. Oxford University Press, Oxford*, 166–188.

Mackessy, S. P. (1988). Venom ontogeny in the pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. *Copeia*, *1988*(1), 92–101.

Mackessy, S. P., Sixberry, N. M., Heyborne, W. H., & Fritts, T. (2006). Venom of the brown treesnake, *Boiga irregularis*: ontogenetic shifts and taxa-specific toxicity. *Toxicon*, *47*(5), 537–548.

Mahmoudi, N., Modanu, M., Brandt, Y., & Andrade, M. C. (2008). Subtle pedipalp dimorphism: a reliable method for sexing juvenile spiders. *Journal of Arachnology*, *36*(3), 513–517.

McAlister, W. (1960). The spitting habit in the spider *Scytodes intricata* Banks (Family Scytodidae). *Texas Journal of Science*, *12*, 17–20.

McCue, M. D. (2006). Cost of producing venom in three North American pitviper species. *Copeia*, *2006*(4), 818–825.

Mooney, K. A., & Haloin, J. R. (2006). Spider size and guarding of offspring affect *Paraphidippus aurantius* (Araneae, Salticidae) response to predation threat. *Journal of Arachnology*, *34*(1), 98–103.

Moran, M. D. (2003). Arguments for rejecting the sequential Bonferroni in ecological studies. *Oikos*, *100*(2), 403–405.

Morgenstern, D., & King, G. F. (2012). The venom optimization hypothesis revisited. *Toxicon 63*, 120–128.

Nakagawa, S. (2004). A farewell to Bonferroni: the problems of low statistical power and publication bias. *Behavioral Ecology*, *15*(6), 1044–1045.

Nakata, K., & Ushimaru, A. (1999). Feeding experience affects web relocation and investment in web threads in an orb-web spider, *Cyclosa argenteoalba*. *Animal Behaviour*, *57*(6), 1251–1255.

Nisani, Z., Dunbar, S. G., & Hayes, W. K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *147*(2), 509–513.

Nisani, Z., Boskovic, D. S., Dunbar, S. G., Kelln, W., & Hayes, W. K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon*, *60*, 315–323.

Pintor, A. F., Krockenberger, A. K., & Seymour, J. E. (2010). Costs of venom production in the common death adder (*Acanthophis antarcticus*). *Toxicon*, *56*(6), 1035–1042.

Prestwich, K. N. (1977). The energetics of web-building in spiders. *Comparative Biochemistry and Physiology Part A: Physiology*, *57*(3), 321–326.

Riechert, S. E. (1979). Games spiders play: II. Resource assessment strategies. *Behavioral Ecology and Sociobiology*, 121–128.

Rowe, M. P., & Owings, D. H. (1990). Probing, assessment, and management during interactions between ground squirrels and rattlesnakes. *Ethology*, *86*(3), 237–249.

Rudolf, V. H., & Armstrong, J. (2008). Emergent impacts of cannibalism and size refuges in prey on intraguild predation systems. *Oecologia*, *157*(4), 675–686.

Sitvarin, M. I., & Rypstra, A. L. (2012). Sex‐Specific Response of *Pardosa milvina* (Araneae: Lycosidae) to Experience with a Chemotactile Predation Cue. *Ethology*, *118*(12), 1230–1239.

Suter, R. B., & Stratton, G. E. (2013). Predation by spitting spiders: elaborate venom gland, intricate delivery system. In *Spider ecophysiology* (ed. W. Nentwig). Springer Berlin Heidelberg, 241–251.

Tanaka, K. (1989). Energetic cost of web construction and its effect on web relocation in the web-building spider *Agelena limbata*. *Oecologia*, *81*(4), 459–464.

Torres, C. I., & Johnson, J. C. (2008). Death feigning in black widows: adaptive shyness or spider personality? Undergraduate Biology Enrichment Program, Undergraduate Research Poster Symposium. Poster #37. http://sols-fin.asu.edu/ubep/2008/37_torres.php

Troupe, J. E. (2009). Ontogenetic shift in agonistic behavior of the southern black widow spider, *Latrodectus mactans* (Araneae: Theridiidae). Thesis, University of Tennessee, Knoxville. (http://trace.tennessee.edu/utk_chanhonoproj/1330).

Uma, D. B., & Weiss, M. R. (2012). Flee or fight: ontogenetic changes in the behavior of cobweb spiders in encounters with spider-hunting wasps. *Environmental Entomology*, *41*(6), 1474–1480.

Venner, S., Pasquet, A., & Leborgne, R. (2000). Web-building behaviour in the orb-weaving spider *Zygiella x-notata*: influence of experience. *Animal Behaviour*, *59*(3), 603–611.

Vetter, R. S. (1980). Defensive behavior of the black widow spider *Latrodectus hesperus* (Araneae: Theridiidae). *Behavioral Ecology and Sociobiology*, *7*(3), 187–193.

Vetter, R. S., & Rust, M. K. (2010). Periodicity of Molting and Resumption of Post-Molt Feeding in the Brown Recluse Spider *Loxosceles reclusa* (Araneae: Sicariidae). *Journal of the Kansas Entomological Society*, *83*(4), 306-312.

Wisenden, B. D. (2000). Olfactory assessment of predation risk in the aquatic environment. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *355*(1401), 1205–1208.

Zar, J. H. (1996). Biostatistical analysis, 3rd Edition. Prentice Hall, Englewood Cliffs, New Jersey, USA.

Zeger, S. L., & Liang, K. Y. (1986). Longitudinal data analysis for discrete and continuous outcomes. *Biometrics*, *42*(1), 121.

CHAPTER FIVE

PREDATORY ETHOGRAM OF THE WESTERN WIDOW SPIDER *Latrodectus hesperus*: DETECTION, IMMOBILIZATION, AND PREY MANIPULATION

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Abstract

Predators often exhibit a complex suite of behaviors when subduing prey, especially when dealing with prey that are large or difficult to handle. Using silk and venom to rapidly immobilize their prey, spiders often secure food items that are much larger than otherwise possible. The purpose of this study was to develop an ethogram—an inventory of behaviors—of the adult female Western Widow (*Latrodectus hesperus*) capturing and subduing house cricket (*Acheta domestica*) prey averaging 1.5-fold larger in mass. The predatory sequence was divided into three phases: detection, immobilization, and prey manipulation. The detection phase (mean duration 387.6 sec) was characterized by initial detection of a potential prey item followed by behaviors that resulted in location of prey and subsequent approach of the spider. During the immobilization phase (mean duration 13.0 sec), spiders trapped and secured their prey largely by means of silk wrapping. During the subsequent prey manipulation phase (mean duration 1392.7 sec), spiders further secured, bit, prepared, and transported their prey off of the substrate. Spiders delivered an average of 15.2 (range 0–31) short bites, with initial bites primarily to a leg. In all, 21 behaviors were defined across phases. Three major behavioral loops involved a repeated set of behaviors, with a distinct loop exhibited in the detection phase and two loops in the prey manipulation phase. The behaviors and sequences observed were similar to those reported for other members of the Theridiidae family, with many behaviors also resembling those observed in other spider families. The findings provide a basis for designing future studies.

Introduction

Spiders have developed a multitude of different hunting methods, including trap doors, webs (cobweb, orb, sheet, funnel), net casting, lassoing, overpowering, mimesis, and more (Foelix 1996). These varied methods, often in conjunction with the use of special adaptations like silk and venom, allow spiders to prey upon animals that are larger than them. However, predation can be a costly undertaking in terms of time, energy expenditure, and risk of injury/death (Vollrath 1987). No matter the hunting method, predators must await, spot, identify, approach, immobilize, kill, transport, prepare, ingest, and digest prey (Robinson et al. 1969; Vollrath 1987). Thus, selection has likely acted on some or all of these stages of hunting to reduce cost and improve efficiency.

An ethogram is a catalogue of behaviors exhibited by an animal. The goal of an ethogram is to objectively stereotype the behavioral repertoire and sequence of behaviors performed by an organism as part of a specific life process (MacDonald et al. 2000; Peters et al. 2005). Often, an ethogram also contains quantitative data, including frequency of a behavior and probability of transitioning from one behavior to the next. Thus, an ethogram represents an ideal prerequisite for any study that seeks to investigate variation in behavioral patterns.

The western widow spider, *Latrodectus hesperus* (Chamberlin & Ivie, 1935), is a member of the family Theridiidae. These spiders are best known for their medically relevant venom, although fatal bites of humans are rare. They also construct seemingly chaotic three-dimensional webs. *Latrodectus hesperus* occurs throughout western North America, ranging from British Columbia into Mexico. Southern California has an abundant, synanthropic population, facilitating collection and field observations.

Latrodectus hesperus is a polyphagous predator (Salomon 2011), making it an excellent organism to study variation in prey capture behaviors and the role of experience in refining predatory events.

The purpose of this study was to describe the predatory sequence of *L. hesperus,* with emphasis on the use of silk and biting to subdue prey. As stated so well by Japyassu and Caires (2008), "We do not intend to describe the whole set of predatory responses, because we consider this an unending task: predatory behavior is dependent upon prey type and size (Robinson and Olazarri 1971; Coddington and Sobrevila 1987; Edwards and Jackson 1993), previous experience with prey (Jackson and Wilcox 1993; Jackson and Pollard 1996), development (Edwards and Jackson 1994), hungriness (Persons 2001), quality of the web (Rypstra 1982); it can be indirectly affected by the presence of predators (Persons et al. 2001) and, if we consider web building as part of a foraging bout, can be influenced by spider state (Witt and Baum 1960; Benforado and Kistler 1973; Vollrath 1987; Eberhard 1988; Higgins 1990; Japyassú and Ades 1998; Sherman 1994; Venner et al. 2000) and a multitude of environmental factors (see review in Thévenard et al. 2004)." A detailed description of the predatory sequence, even though not exhaustive, will allow us and others to test the aforementioned causes of variation, providing a basis for future comparisons.

Methods

Spider Husbandry

I collected spiders in the spring and summer months (generally May–September) from Redlands, Loma Linda, and Colton, California (San Bernardino County). Spiders

were individually housed in 540-mL plastic deli cups at 22° C on a 12-hr light-dark cycle. I provided spiders with a small stick to facilitate web construction, and offered house crickets (*Acheta domestica*) as food every 2 weeks. No water was provided, as it was deemed unnecessary. Collected spiders were sub-mature to mature females hatched that season, or older females that had over-wintered. All spiders used in this study were fully mature, in their ultimate instar.

Feeding Trials

I transferred each spider (mean mass 0.28 g; range 0.20–0.42 g) from its home container to a feeding arena 48 hr before observation. The feeding arena consisted of a transparent plastic Kritter Keeper box (36.8 x 22.3 x 24.4 cm L x W x H; Lee's Aquarium & Pet Products, San Marcos, CA, USA). A plastic mesh screen (1.5 mm mesh aperture, cut to 10.5 x 6 cm W x H) taped against the bottom half of one of the narrow sides of the cage allowed the spider to climb one of the walls and facilitated web construction. For the feeding trial, I transferred the cage from the housing room to the observation room, and gave the spider 30 min to acclimate. I conducted trials with 19 spiders over an 8-week period; one additional spider was excluded due to presence of an egg sac. All spiders were food-deprived for 2 weeks. The feeding session began when I dropped a cricket (*A. domestica*; mean ± 1 S.E. = 0.41 ± 0.02 g), averaging 1.5 times larger than the spider, directly into the web. I began recording behaviors when the spider performed the behavior *detect* (see Results for a list of behaviors and definitions). Feeding trials were conducted during the dark phase of the light cycle under minimal (red) light, and lasted up to 30 min. I videotaped each trial by positioning a camera (Leica Dicomar 3CCD,

Panasonic, Secaucus, NJ, USA) approximately 6 cm from the front of the cage. The camera was relocated as needed to keep the spider within the frame and positioned so that maximum behavioral detail was recorded.

Video Analyses

I subsequently reviewed the videos to quantify the presence, frequency, and duration of each behavioral act exhibited by the spider. Characterization of predatory behaviors was initially based on previous feeding observations, but was refined during slow motion playback of feeding sessions. The predatory sequence was divided into three phases, adapted from Japyassu and Caires (2008).

• *Detection phase* – This encompassed behaviors performed from the beginning of the sequence to when the spider began to immobilize prey via wrapping with visibly viscous silk.

• *Immobilization phase* – This began when the spider began to immobilize prey via wrapping with visibly viscous silk and ended when the spider first successfully affixed a strand of silk from the immobilized prey to the web or substrate.

• *Prey manipulation phase* – This phase began after the spider first successfully affixed a strand of silk from the immobilized prey to the web or substrate, and continued for the remainder of the ~30 minute observation period. Japyassu and Caires (2008) called this the "feeding phase," which began when the spider took the prey item to its retreat and continued arbitrarily for an additional 5 min. Spiders used in the present study did not reliably build retreats, and thus did not always relocate prey to a specific site. Further, the onset of feeding could also not be reliably ascertained, as the behavior

involving a long bite was sometimes indistinguishable from feeding. For these reasons, "prey manipulation" was chosen over "feeding" as it more appropriately described the behavior of *L. hesperus*.

Initially, I constructed separate tables to compile observations from each of the 19 spiders. Each table included the number of times a transition occurred from one behavior to the next, and the total times each behavior was performed. Data from individual tables were then compiled into a single frequency table, showing for all spiders the total number of times each behavior was performed and the total number of transitions. Based on this frequency table, I calculated a right stochastic matrix, also known as a Markov or first order transition matrix, using Excel version 14.3.1 (Microsoft, Redmond, WA, USA), in which conditional probabilities for each transition were calculated using the formula P(B|A) = P(A and B) / P(A) (Gilbert and Rayor 1985).

I converted the frequency and right stochastic matrixes into a flow diagram of predatory behaviors. In this graphic representation of behavioral transitions, relative size of text reflects the frequency of each behavior, and size of the transition arrows reflects the proportion of transitions. Behavior frequencies and transition proportions were categorized into five classes, with each class assigned a font or pixel size (Table 14).

Table 14. Size of text fonts and arrow pixel widths corresponding to Fig. 7.

Size of Text		Pixel Width of Arrows	
Frequency range	Font size	Probability of transition	Number of Pixels
0–60	14	0–20%	5
61–120	18	21–40%	10
121–180	24	41–60%	20
181–240	30	61–80%	30
240+	36	81–100%	40

Frequency range refers to total number of observations for each behavior

Descriptive statistics were calculated using SPSS (version 20, SPSS Inc, Chicago, IL, USA). Although each observation session lasted approximately 30 min (1800 sec), the sum of time spent in each behavior varied due to rounding error associated with multiple events. Thus, I proportionally corrected the sum of time in each behavior and phase relative to 1800 sec. All measures of central tendency are reported as mean ± one S.E.

Results

The entire predatory sequence encompassed three phases: detection, immobilization, and prey manipulation. Some behavioral acts were performed exclusively within a phase, while others were observed across two or more phases. Here, I begin by defining each behavior while placing it within one of the three phases or in a fourth, across-phase, category. Following Gilbert and Rayor (1985), each definition includes the structures used, actions, and context. I then describe each of the three phases in quantitative terms while providing data on frequencies of each behavior and transition.

Detection Phase

Detect

Structures used: Leg(s), with slight movements possible through whole body. *Actions:* Spider initially contracts one or more legs followed by relaxation/spreading of legs either to or beyond pre-detect positions. Contraction of leg(s) may occur while holding silk strand. *Context:* This behavior follows contact of prey with web strands and/or spider's body.

Search

Structures used: Whole body, especially legs I and pedipalps. *Actions:* Spider orients body with legs I toward area of last web disturbance or body contact. Spider moves toward last disturbance/prey contact in shorts bursts, but often pauses while legs I and/or pedipalps perform *probe* behavior (described below). *Context*: Following detect, spider employs a combination of behaviors that can collectively be called search, including *orient*, *probe*, *approach*, and *pause* (described below). Search continues until contact is made or *pause* lasts longer than 5 sec.

Initial contact

Structures used: Predominantly leg(s) I, but may also involve other legs or pedipalps. *Actions:* Spider typically extends leg(s) I with metatarsus and tarsus being flexed and re-extended, and makes contact with the prey. Spider may make a single contact with prey or multiple repeated contacts. *Context*: Following *search*, contact is made with leg I to un-captured prey, which I interpret as verifying position of prey item.

Immobilization Phase

Immobilization wrap (IW)

Structures used: Legs IV and spinnerets. *Actions*: Legs IV first move to the spinnerets where a strand of silk is grasped by the claw. Spider then extends legs IV toward the prey item; legs may be extended simultaneously or in alternating pattern. Upon contact with prey, silk is released from claws, and visibly viscous droplets of silk adhere to the prey item and other nearby structures. *Context*: After contact, and

orientation if needed, spider uses back legs to ensnare prey with visibly viscous silk. This behavior starts at an extremely rapid pace, then may slow to the post-immobilization wrapping pace before behavior is terminated by the onset of *fix*.

Fix

Structures used: Whole body, especially spinnerets. *Actions*: Spinnerets, or silk held by leg(s) IV, are pressed against the prey and silk is attached. With visible line of silk from prey to spinnerets, spider then attaches silk to the web or substrate by touching spinnerets against them. After silk has been attached to prey, the spider may move higher in its web before silk is finally attached to a web strand or to the substrate. The resulting silk strand is tightly stretched. *Context*: This behavior typically follows *IW* during the immobilization phase or *post-immobilization wrap* (described below) in the prey manipulation phase. Often combined with *hoist* (described next). Used during the immobilization stage to secure or move prey.

Hoist

Structures used: Whole body. *Actions*: After attaching silk strand to prey, using *fix*, the spider lifts prey higher into web. Lifting typically occurs as spider climbs the web, with silk strand stretched between prey and spinnerets. May also occur as spider pulls the prey higher, either directly pulling the prey with legs I or IV, or indirectly when spider pulls silk attached to the prey while the spider remains stationary. *Context*: This behavior most often occurs with *fix*, and is sometimes associated with *cut thread* (described below). This behavior repositions prey higher in web, often closer to the most heavily

fortified part, and moves the prey item away from the substrate or anything else that could be used by the prey as leverage for escape.

Prey Manipulation Phase

Post-immobilization wrap (PIW)

Structures used: Leg(s) IV and spinnerets. *Actions*: Similar to *IW*: legs IV, especially metatarsus and tarsus, first move to the spinnerets and then are extended towards the prey item. Legs IV are used in an alternating pattern, and when contact is made with the prey, silk is attached. *Context:* Similar to *IW* except alternating motion of legs is slower, and silk is not dotted with visibly viscous beads. Used after the prey has been initially immobilized. Can occur in the immobilization phase, but most often occurs in the prey manipulation phase. Associated with *fix* and combined as *fix/hoist*.

Manipulate

Structures used: Legs, particularly legs I, and pedipalps. *Actions*: Distal segments of leg or legs I, and/or pedipalps, are used to repeatedly contact the prey item. Spider may either actively move over captured prey or remain stationary while using leg(s) I and/or pedipalps to contact prey. *Context*: Typically follows *approach* or *orient* (described below), and precedes *bite* (described next) or *PIW*.

Bite

This behavior can be separated into two classes: short and long duration. Other behaviors may be performed while biting, particularly *probe*.

Short duration bite

Structures used: Chelicerae and fangs. *Actions*: Spider moves chelicerae close to prey, distal ends of chelicerae open laterally, and fangs are inserted into prey as chelicerae close medially. *Context*: Predominantly occurs after *manipulate*. A *short duration bite* lasts <6 sec and may be used singly or several in quick succession.

Long duration bite

Structures used: Chelicerae and fangs; may possibly include rostrum. *Actions*: Actions of spider are the same as *short duration bite*; however, spider appears to remain engaged in bite behavior for >6 sec. The rostrum appears to have a pair of spikes or stylets that have been observed in subsequent experiments to pierce prey in addition to, or in favor of, cheliceral fangs. Possibly opens a larger wound for the introduction of digestive enzymes during extra-oral digestion. *Context:* Typically appears later in the prey manipulation phase than *short duration bite(s)*, and most often follows *manipulation*. Although this behavior is easily distinguished from short duration bite(s), I could not reliably differentiate it from feeding.

Cut thread

Structures used: Chelicerae and leg(s) I. *Actions*: Silk thread is brought into the chelicerae by leg I, while anterior of prosoma is moved toward a specific thread. Chelicerae appeared to close on thread, resulting in cut. Fine details of this action could not be observed. *Context*: Prey item visibly changes position after this action. Used to cut any silk strand in the web, but usually one that is attached to the prey or attaches the prey

to the web. Behavior is associated with *manipulate* and precedes *fix/hoist*.

Web build

Structures used: Whole body. *Actions*: Spider moves around observation arena, periodically touching spinnerets against substrate or other silk strands. As spider continues to move, silk is visible dragged behind until dragged silk becomes affixed to either another substrate location or another silk strand. *Context:* This behavior is observed late in the prey manipulation phase.

Across-Phase Behaviors

Orient

This behavior occurs during all three phases. Within each phase the behavior is similar and can be divided into two classes: *forward orient* and *silk orient*.

Forward orient

Structures used: Whole body. *Actions*: Spider turns body so that legs I are facing direction of last web disturbance, incidental contact, or captured prey. *Context*: Observed during detection phase and prey manipulation phase; spider orients so that legs I are facing direction prey was last detected, or orients so that spider may *approach* captured prey.

Silk orient

Structures used: whole body. *Actions*: Entire body turns so that legs IV point

toward prey. *Context:* This often occurs very quickly, and often quite acrobatically, when performed as part of the transition from detection phase to immobilization phase. After contact, spider spins or flips into position so that legs IV are ready to begin *immobilization wrap*. A slower, more deliberate form of this behavior is also performed during the prey manipulation phase in connection with *PIW*.

Probe

Structures used: Legs, predominantly legs I and IV. *Actions*: Leg(s) raised from previous position and typically moved in a circular pattern while prosoma and opisthoma remain motionless. *Context*: This behavior appears during all phases, and can take place simultaneously with another behavior. Often observed as part of *search* and *bite*, but can also be performed singly. When performed in connection with *search*, the circular movement seems to increase chances of contact with prey.

Groom

Structures used: Legs, chelicerae, and other mouth structures. *Action:* Leg is brought into and passed through the chelicerae while the chelicerae are opened and closed in a chewing motion. Legs may also be used to pass over body (opisthoma, spinnerets, or other leg) in a combing motion, repeated several times, finally culminating in that particular leg being brought into and passed through the chelicerae as described previously. Pedipalps are moved over and across each other. *Context*: This behavior can be engaged in during any phase. If grooming appears during the immobilization phase, it is often associated with the removal of viscous silk from the distal segments of legs.

Pause

Structures used: Whole body. *Actions:* Spider stops all movement for a duration lasting 1 sec or longer. *Context:* Observed in all phases.

Approach

Structures used: Whole body. *Actions*: With prosoma oriented towards prey, spider moves whole body toward immobilized prey. Behavior ends when leg I makes contact with prey. *Context*: Most often follows *orient*, but may also follow *web build*.

Retract

Structure used: One or all legs. *Actions*: Spider moves contacted leg and/or other non-contacted legs from their starting position, medially, in toward the body. *Context*: Follows *contact* with prey and/or disturbance of web.

Nondescript behavior

Structures used: Whole body. *Actions*: Behaviors that do not meet any of the other behavioral definitions, or whole body movement that is not observed to be associated with web building or any other defined behavior. *Context*: Used as a catch-all category to categorize a behavior that, as of yet, has no discernible pattern or function.

Out of frame

Structures used: None. *Actions*: Spider has moved outside of camera frame. *Context*: Spider has moved to a position in which the camera cannot capture the behavior

being performed. Associated with *fix*, *fix/hoist*, and *web build*.

Predatory Sequence

The sequence by which spiders exhibited various predatory behaviors is summarized in Fig. 7, where frequencies of behaviors and behavioral transitions are indicated by relative size of text fonts and arrow pixel widths, respectively. Table 15 provides descriptive statistics on the frequency and duration of each behavior and phase.

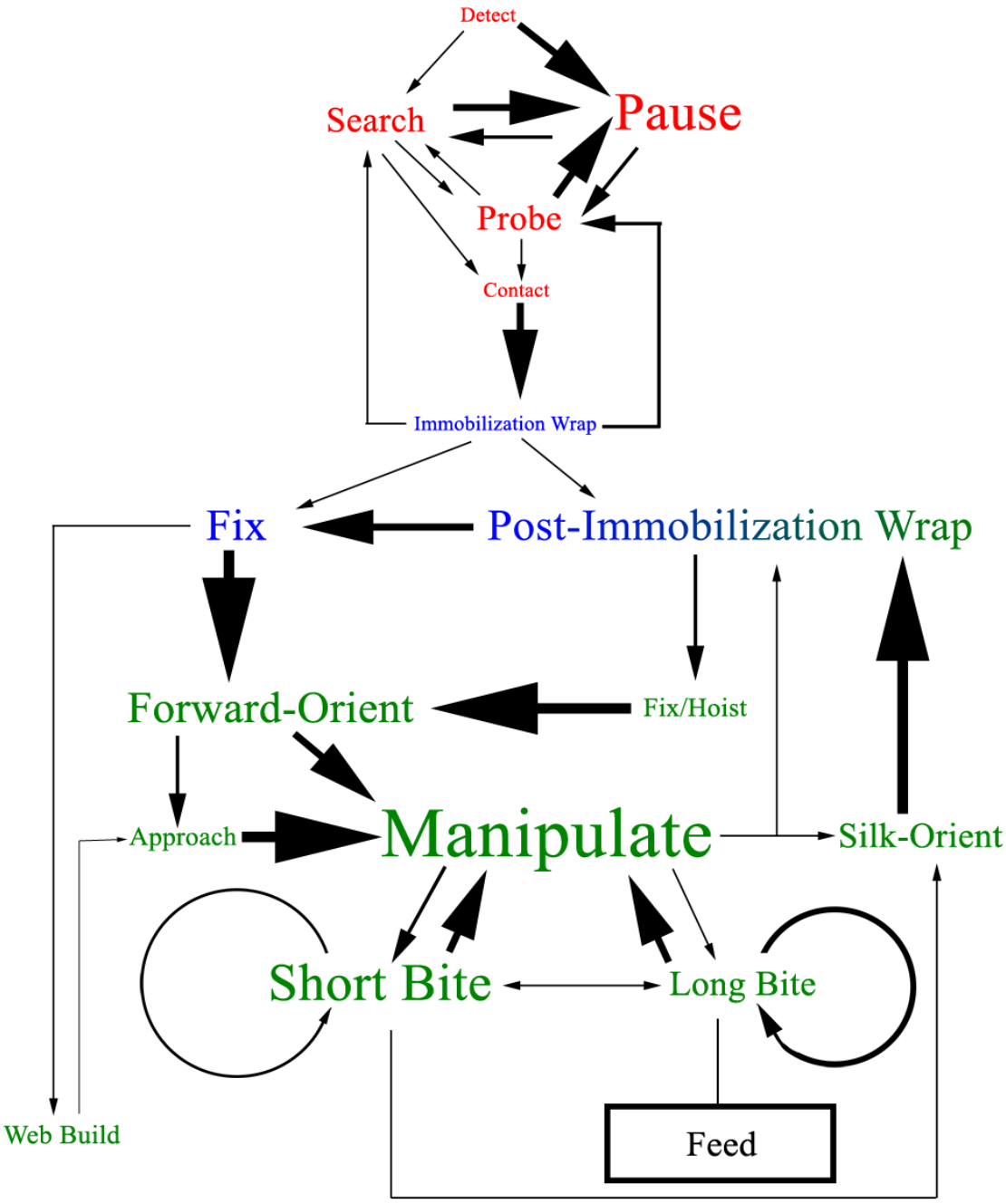

Fig. 7: Simplified diagram of the predatory sequence of the western widow spider *Latrodectus hesperus* (*N* = 19). Frequencies of behaviors and behavioral transitions are indicated by size of text fonts and arrow pixel widths, respectively (see Table 14). Color denotes phase of the predatory sequence: red = detection phase, blue = immobilization phase, and green = prey manipulation phase.

Table 15: Descriptive statistics of time spent in behaviors and phases for the predatory sequence of the western widow spider *Latrodectus hesperus* ($N = 19$)

Behaviors	Mean duration[a]	SD	SE	Min	Max	Mean duration[b]	# of spider performed	Mean time to appearance[b]
Detect	1.4	0.8	0.2	0.9	3.5	1.4	19.0	0.0
Search	62.7	91.1	20.9	0.0	305.2	70.1	17.0	112.0
Contact	2.8	3.7	0.8	0.0	15.0	3.2	17.0	201.9
IW	7.7	5.1	1.2	1.0	24.9	7.7	19.0	205.5
Fix	92.2	61.4	14.1	22.0	225.2	92.2	19.0	437.3
Hoist	1.5	5.2	1.2	0.0	21.9	14.7	2.0	157.0
Fix/hoist	52.4	40.3	9.2	0.0	118.7	62.3	16.0	572.8
PIW	179.0	88.8	20.4	41.9	401.8	179.0	19.0	410.1
Cut thread	16.7	31.3	7.2	0.0	122.6	24.3	13.0	1009.6
Web build	224.7	198.5	45.5	0.0	588.7	284.6	15.0	819.9
F-orient	22.8	16.6	3.8	5.2	78.2	22.8	19.0	300.9
Silk-orient	11.1	5.2	1.2	4.0	19.1	11.1	19.0	312.4
Probe	44.6	75.7	17.4	0.0	304.2	70.5	12.0	254.3
S-bite	31.8	18.4	4.2	0.0	74.9	33.5	18.0	444.2
L-bite	270.7	248.4	57.0	0.0	845.8	302.5	17.0	701.9
UD-Bite	14.3	31.0	7.1	0.0	120.6	33.9	8.0	751.0
Groom	84.4	94.1	21.6	0.0	357.3	94.3	17.0	378.6
Manipulate	228.0	90.0	20.7	50.9	386.5	228.0	19.0	430.8
NDB	18.2	32.3	7.4	0.0	138.2	31.4	11.0	710.5
OOF	42.0	44.4	10.2	0.0	143.7	49.9	16.0	477.4
Pause	373.9	322.8	74.1	0.0	1205.7	394.6	18.0	19.0
Approach	16.3	12.1	2.8	0.0	36.2	18.2	17.0	468.5
Retract	0.9	2.6	0.6	0.0	11.2	3.4	5.0	423.4
Phases								
Detection	387.6	390.1	89.5	2.1	1151.8	387.6	19.0	
Immobilize	13.0	8.9	2.0	3.0	41.4	13.0	19.0	
Manipulate	1392.7	386.3	88.6	626.3	1789.3	1392.7	19.0	
Total	1800.0	0.0	0.0	1800.0	1800.0	1800.0	19.0	

[a] indicates mean time calculated with all spiders included

[b] indicates mean time calculated with only spiders that performed behavior

Detection phase

This phase began when a spider first detected a prey item, and averaged 388 sec (range 2–1152 sec; Table 15). During this phase, spiders employed several behaviors to locate potential prey, mainly *pause*, *probe*, *search*, and *contact*. *Detect* was most often followed by *pause* (57.7%) and *search* (19.2%). Following the initial *detect*, spiders typically began in their first behavioral loop, referred to as the primary (1°) loop, performed an average of 8.8 ± 2.2 times. *Pause*, *search*, and *probe* could appear in any combination: *pause-search* (27.3%), *pause-probe* (21.1%), *search-pause* (50.8%), *search-probe* (13.7%), *probe-search* (16.1%), and/or *probe-pause* (51.6%). These three behaviors were repeated until *contact* was made with the prey. *Search* (16.1%) and *probe* (9.7%) most often preceded *contact*. *Contact* could also follow *detect* (7.7%), *immobilization wrap* (5.4%), and *pause* (5.4%); however, these connections were not included in the stereotyped diagram, as contact was incidental. During the *detect-contact* transition and the *immobilization wrap-contact* transition, contact was incidental with both occurring concurrently. No connection between *pause* and *contact* was depicted because *contact* was most often initiated by the cricket during a jump. *Contact* most often preceded the onset of *immobilization wrap* (41.2%) or *silk-orient* (23.5%); however, this did not always result in the transition from the detection to the immobilization phase. After *contact*, if the spider was not in position to begin *immobilization wrapping*, then *silk-orient* was first performed. Either during *silk-orient* or immediately following *contact* the crickets could jump away. The spiders would then perform *immobilization wrap* on the area where the cricket was previously. If *immobilization wrap* did not ensnare the cricket, then the 1° loop was again utilized to re-locate the prey:

immobilization wrap-search (19.6%) and *immobilization wrap-probe* (35.7%).

Immobilization phase

The immobilization phase began when *immobilization wrap* ensnared the cricket. This was the shortest of all phases, lasting an average of 13.0 ± 2.0 sec (range 3.0–41.4 sec; Table 15). During the immobilization phase, *fix* (16.1%) was the most common behavior to follow *immobilization wrap*. Occasionally, the frenetic pace of *immobilization wrap* noticeably slowed, transitioning into *post-immobilization wrap* (10.7%).

Manipulation phase

The performance of the first *fix* signaled the end of the immobilization phase and the onset of the prey manipulation phase. The prey manipulation phase averaged the longest at 1392.7 ± 88.6 sec (range 626.3–1789.3 sec; Table 15); however, much of the phase comprised the arbitrary continuation of observations beyond. At the beginning of this phase, the spiders engaged in a secondary (2º) behavioral loop consisting of *fix* or *fix/hoist*, *forward-orient*, *approach*, *manipulate*, *silk-orient*, and/or *PIW*. *Fix*, or the combination *fix/hoist*, were used following wrapping behaviors and occurred when the spiders attached a silk strand form the immobilized prey item to a strand of silk in the web. *Forward-orient* most frequently followed *fix* (59.6%). *Web build* could also follow *fix* (10.9%); however, *web build* usually occurred later in the prey manipulation phase (mean time to first appearance 819.9 sec) after the performance of several 2º loops. Following *web build*, *manipulate* (19.4%) and *approach* (14.5%) were the most common

behaviors that led back into the secondary and/or tertiary (3º) loops; the 3º loop consisted of *manipulate*, *short bite*, and *long bite*. *Forward orient* preceded either *manipulate* (59.4%) or *approach* (25.8%), which were followed by *manipulate* (25.8% and 70.2% respectively). *Manipulate* was a central behavior between the 2º and 3º loops. During the 2º loop, *manipulate* was either followed directly by *PIW* (15.2%), or *silk-orient* then *PIW* (10.0 % and 77.3% respectively). *PIW* was, in turn, followed by *fix* (49.4%) or *fix/hoist* (27.2%).

After the 2º loop had been performed a varying number of times (mean = 14.0 ± 1.2), the spiders then transitioned into an intermediate loop: the 2º/3º loop. During this portion of the manipulation phase, *manipulate* would be followed by *short bite* (37.8%), which was followed by *manipulate* (58.3%), and then reentry back into the 2º loop. *Short bite* could also be followed by *silk-orient* (10.1%) and then back into the 2º loop. This behavioral sequence was repeated numerous times until *manipulate* preceded *short bite* to initiate the 3º loop, performed an average of 18.4 ± 1.8 times. *Short bite* could then be repeated in succession several times (16.3%), or preceded *manipulate* (58.3%). *Short bite* would eventually lead into *long bite* (5.9%), followed by *manipulate* transitioning into *long bite* (17.8%), or a series of *long bites* (22.3%). *Long bite* could also be followed by *short bite* (7.8%), or *long bite-manipulate* (52.5%).

Several behaviors were not included in the ethogram: *cut thread, groom*, and *non-descript behavior*. Each of these behaviors was excluded to simplify the diagram. *Cut thread* was not a heavily utilized behavior, occurring only 28 times out of the 2954 behaviors observed. This behavior most often followed *fix* and *fix/hoist* (5.8%), and was most often followed by *fix* and *fix/hoist* (35.7%), *PIW* (17.9%), or *manipulate* (25.0%).

Groom was a commonly utilized behavior (121 occurrences), and was associated with 17 of the 23 behaviors, making its placement within the diagram problematic. *Non-descript behavior* was not included because its form and function defied stereotyping.

Another problem encountered during the course of these observations was that *long bites* could not reliably be differentiated form potential bouts of feeding. Although feeding was included in the ethogram diagram, no arrows were used to connect it to a specific behavior; instead, it was only associated with *long bite*.

Table 16 lists the frequencies of each behavior, with particular interest given to silk use and biting during the predatory sequence, which are critical in helping the spider secure large prey. The use of silk to immobilize prey, secure, and transport prey were performed throughout the predatory sequence. Spiders continued to wrap prey even after prey was immobilized and bitten. Silk use as *PIW* was performed an average of 12.6 ± 1.5 times. *Short bite* was also used numerous times (15.2 ± 1.9), and envenomation was assumed based on the cricket's eventual lack of struggle. Location of initial bite was recorded, with bites observed to the head, abdomen, leg, and ovipositor. Among the 19 spiders, a leg was bit first 11 times (57.9%). Other locations were bit less frequently: three to the abdomen (15.8%), two to the head (10.5%), and one to the ovipositor (5.3%). I was unable to determine the initial bite location for two of the spiders.

Table 16: Frequency of the predatory behaviors used by *L. hesperus* during the predatory sequence ($N = 19$)

Behaviors	Mean freq	SD	SE	Min	Max	Total freq
Detect	1.4	0.8	0.2	1.0	3.0	26
Search	6.5	8.8	1.9	0.0	34.0	124
Contact	2.7	3.3	0.8	0.0	13.0	51
IW	2.9	3.0	0.7	1.0	13.0	56
Fix	9.7	5.0	1.2	3.0	19.0	183
Hoist	0.3	0.8	0.2	0.0	3.0	5
Fix/hoist	5.4	3.5	0.8	0.0	10.0	102
PIW	12.6	6.7	1.5	4.0	34.0	239
Cut thread	1.5	1.8	0.4	0.0	7.0	28
Web build	3.6	2.8	0.6	0.0	9.0	62
F-orient	11.4	4.2	1.0	3.0	20.0	217
Silk-orient	6.7	2.6	0.6	4.0	13.0	128
Probe	6.5	10.6	2.4	0.0	43.0	124
S-bite	15.2	8.4	1.9	0.0	31.0	288
L-bite	9.6	7.9	1.8	0.0	31.0	179
UD-Bite	0.8	1.3	0.3	0.0	5.0	15
Groom	6.5	4.2	1.0	0.0	15.0	121
Manipulate	30.2	9.9	2.3	11.0	46.0	572
NDB	2.0	3.2	0.7	0.0	13.0	38
OOF	3.1	3.4	0.8	0.0	14.0	58
Pause	12.8	10.0	2.3	0.0	36.0	242
Approach	4.4	3.4	0.8	0.0	11.0	84
Retract	0.6	1.8	0.4	0.0	8.0	12

Discussion

Predators often exhibit a complex suite of behaviors when subduing prey, especially when dealing with prey that are large or difficult to handle. A thorough understanding of a particular process is required to understand and investigate patterns in ecology, phylogeny, biology, and plasticity. The predatory sequence of *L. hepserus* is undoubtedly similar to other spiders, particularly those within the family Theridiidae. The majority of behaviors observed during the prey capture sequence were synonymous, or very similar, to predatory behaviors previously described. *Detect* was previously

described by Ribeiro and Japyassu (2005); however, our definitions varied. Their definition seemed to allow for whole body movements toward the prey, whereas my observations showed a subtler tensing and relaxing of the legs without accompanying body movement. Prey also were initially trapped by gumfooted lines (if the translation from Portuguese is correct). Differences between our definitions may result from poor translation (from Portuguese), and/or the forceful nature with which the crickets in the present study initially made contact with the web or spider. *Detect* is also very similar to *retract*, a common defensive behavior (Nelsen et al. unpublished). It is possible that the forceful initial contact of the cricket with the web or spider, during my observations, was perceived as a possible threat, and influenced the characteristics of the behavior.

The *search* behavior described in this paper appears to be unique with respect to the literature reviewed. This, again, may be due to the way in which prey were initially trapped. Many prior studies reported that prey were initially trapped by adhesive silk strands that were part of the web (Garcia and Japyassu 2005; Japyassu and Caires 2008; Robinson and Mirick 1971; Robinson et al. 1969; Willey et al. 1992). However, my prey were rarely trapped by the web strands alone, and were often observed to move into and out of the web. Prey movement was not fully restricted until the spiders successfully performed *immobilization wrap*. Thus, *search* was often necessary to locate (sometimes repeatedly) the prey item.

Initial contact is similar to "tap" described by Gilbert and Rayor (1985), but different from the "tap" described by Li et al. (1999). I used this behavior to record the first times the spider made contract with the prey. Although there were instances when

spiders immediately went from *detect* to *immobilization wrap,* contact was usually a prerequisite to a successful *IW*.

Immobilization wrap was first described by Robinson and Olazarri (1971), who stated that the silk was pulled in sheets (see also Willey et al., 1992). Japyassu and Caires (2008) modified the definition to include a viscous silk texture and the spider holding the prey during wrapping with legs III, which they called "sswrap." My definition differed from the previous two in three ways: *L. hesperus* did not hold onto the prey during bouts of *immobilization wrap; IW* could occur within the web or while prey was on the ground; and *L. hesperus* used a highly viscous silk, distinct from the noticeably less viscous silk used during *post-immobilization wrap*. Hajer and Hruba (2006) also described a similar behavior in *Achaearanea tepidariorum* (Koch 1841) in their section called "wrap attack in capture site before catapulting of immobilized prey." Some authors differentiated between types of wrapping (Japyassu and Caires 2008; Robinson and Olazzari 1971; Willey et al. 1992), as I have done here with *IW* versus *PIW*. Other authors included all wrapping behaviors together into a single definition (Gilbert and Rayor 1985; Li et al. 1999). I observed that *L. hesperus* always performed both *IW* and *PIW*, and *IW* was essential early in the sequence for prey capture success.

Fix-like behaviors were described in several other studies: as "fix" (Japyassu and Caires 2008), "dragline attachment" (Gilbert and Rayor 1985), "attachment of prey to the hub" (Robisnon and Olazarri 1971), and possibly "attach threads" (Li et al. 1999). I interpreted this behavior as securing prey to the web to prevent prey escape or loss, and facilitating transport of the prey in conjunction with *hoist.*

Japyassu and Caries (2008) most recently described *hoist* as "carry on silk." Others have described similar behaviors: as "pull" (Li et al. 1999), and included with transportation of wrapped prey within the jaws (Robinson and Olazarri 1971; Willey et al 1992). I did not observe the transportation of prey within the jaws, and it has not been reported within the family Theridiidae by other studies.

Manipulate was first described by Robison and Olazarri (1971), and includes the use of short bites (see also Willey et al., 1992). Similar to Japyassu and Caires (2008), I separated short bites into its own discrete behavior. *Manipulate* was one of the most common behaviors performed, being central to both the 2^{o} and 3^{o} loops. It may be possible to divide *manipulate* in more discrete behaviors, but I found this unnecessary.

The majority of prior studies did not differentiate between short- and long-duration bites (Gilbert and Rayor 1985; Japyassu and Caires 2008, Robinson and Olazarri 1971; Willey et al. 1992). I chose to differentiate between them because I observed a difference between the initial use of very quick repeated bites and the subsequent appearance of more prolonged fang/prey interactions. Viera (1986) also distinguished between short (<20 sec) and long (>20 sec) bites. I chose to limit the duration of short bites to <6 seconds, as the early occurring short bites generally lasted 5 sec or less. I recognize, however, that the transition from short to long duration represents a continuum. Additionally, the distinction between bouts of long bites and feeding could not be reliably ascertained. Therefore, interpretations concerning long bites must be made cautiously.

Cut thread, also called "cut out" by others, has been observed in many spiders (Japyassu and Caires 2008; Li et al. 1999; Willey et al. 1992). My definition corresponds

well with the others; however, fine details concerning pedipalp and chelicerae movements could not be observed clearly, and further refinement may be necessary.

I observed spiders occasionally halt the predation sequence during the prey manipulation phase, and engage in web construction (*web build*). Individual bouts of *web build* were sometimes extensive. Japyassu and Caires (2008) described a similar behavior ("pay out line") occurring in connection with the retreat. However, *L. hesperus* spiders engaged in more extensive web building over the entire web, adding new strands from the top of the existing web to areas further away than any previous silk strand. The amount of time spent engaged in this behavior (224.7 ± 45.5 sec) suggests its importance to repair damage incurred to the web during prey capture, or to reinforce the web to provide greater protection to the spider during feeding. Future studies should investigate this behavior further.

Because of the posture taken during *IW* and *PIW* (spiders were positioned with spinnerets pointed towards prey and legs IV extended), and after the performance of *fix,* spiders had to *orient* themselves in relation to the prey in order to continue the predation sequence. Many previous studies seem to have taken this behavior for granted without mention of spider orientation. Japyassu and Caires (2008) mentioned a change in orientation as part of the behavior “fix and rotate.” However, I did not observe the dabbing of silk in an arc (i.e., "rotate") during the performance of *orient*. I believe my definition is more general, and undoubtedly distinct from “fix and rotate.”

I often observed *probe* as a part of the search routine. Leg(s), typically legs I, were moved in a circular pattern. This behavior further emphasizes the need of spiders to locate prey after initial detection. Gilbert and Rayor (1985) described a similar behavior

called “reach and roll.” Surprisingly, previous studies of members of Theridiidae made no mention of a behavior similar to this (Garcia and Japyassu 2005; Hajer and Hruba 2006; Japyassu and Caires 2008). As remarked earlier, *L. hesperus* had to perform *IW* in order to immobilize prey, as crickets were able to move into and out of the web. It is possible that spiders in the present study were not given sufficient time to construct complete webs, and therefore lacked certain structures like gumfooted lines. Alternatively, the crickets used in this study could have been too large to be severely restricted by the webs. However, in one instance a cricket was initially trapped by the web itself, being pulled off the substrate after making contact with a gumfooted line. Whatever the reason, I often observed *L. hesperus* trying to locate prey, sometimes with considerable search effort.

Groom (Garcia and Japyassu 2005; Gilbert and Rayor 1985; Japyassu and Caires 2008; Robinson and Olazarri 1971; Willey et al. 1992), *pause* (Garcia and Japyassu 2005; Japyassu and Caires 2008; Robinson and Mirick 1971; Robinson and Olazarri 1971; Willey et al. 1992), and *approach* (Garcia and Japyassu 2005; Japyassu and Caires 2008; Robinson and Olazarri 1971) were common behaviors reported in other predatory ethograms. All definitions are fairly synonymous with mine; however, *groom* has been referred to as “clean,” and *pause* as “rest.” Duration of cessation from movement necessary to be classified as *pause* has varied among studies, and *approach* was not always formally defined.

Retract was a common defensive behavior observed in *L. hesperus* (Nelsen et al. Chapter 3). Although Japyassu and Caires (2008) described a somewhat similar behavior called “withdraw,” characterized by the spider pulling all the legs inward towards the

body. *Retract* was not performed often, and when performed was often associated with the cricket making sudden contact with the spider. I suggest that this behavior was a defensive response to contact initiated by the cricket.

I described several miscellaneous behaviors, including *nondescript behavior* and *out of frame*. Because some actions performed by the spiders were not clearly visible or definable, *nondescript behavior* was used to designate movements that did not meet the criteria of the other definitions and defied attempts at stereotyping. As implied by the name *out of frame*, spiders sometimes wandered outside of the camera frame, and subsequent behaviors could not be classified. These behaviors seldom occurred (1.3% and 2.0% of all behaviors observed respectively), but needed to be accounted for in a quantitative description of the predatory sequence.

In conclusion, the predatory ethogram for *L. hesperus* provides a starting point and basis of comparison for future studies. One could, for example, more readily examine behavioral plasticity of *L. hesperus* when dealing with different types of prey, or when feeding under various constraints such as predation risk (Jackson and Pollard 1996; Robinson and Mirick 1971) or venom availability (Wullschleger and Nentwig 2002). One could also test the effects of experience on improving prey capture efficiency (Edwards and Jackson 1994).

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References

Benforado, J., & Kistler, K. H. (1973). Growth of the orb weaver, *Araneus diadematus*, and correlation with web measurements. *Psyche*, *80*(1–2), 90–100.

Coddington, J. A., & Sobrevila, C. (1987). Web manipulation and two stereotyped attack behaviors in the ogre-faced spider *Deinopis Spinosus* Marx (Araneae, Deinopidae). *Journal of Arachnology*, *15*(2), 213–225.

Eberhard, W. G. (1988). Behavioral flexibility in orb web construction: effects of supplies in different silk glands and spider size and weight. *Journal of Arachnology*, 16, 295–302.

Edwards, G. B., & Jackson, R. R. (1993). Use of prey–specific predatory behaviour by North American jumping spiders (Araneae, Salticidae) of the genus *Phidippus*. *Journal of Zoology*, 229(4), 709–716.

Edwards, G. B., & Jackson, R. R. (1994). The role of experience in the development of predatory behaviour in *Phidippus regius*, a jumping spider (Araneae, Salticidae) from Florida. *New Zealand Journal of Zoology*, *21*(3), 269–277.

Foelix, R. F. (1996). *Biology of spiders*. Oxford University Press, USA.

Garcia, C. R. M., & Japyassú, H. F. (2005). Estereotipia e plasticidade na seqüência predatória de *Theridion evexum* Keyserling 1884 (Araneae: Theridiidae). *Biota Neotropica*, *5*(1A), 27–43.

Gilbert, C., & Rayor, L. S. (1985). Predatory behavior of spitting spiders (Araneae: Scytodidae) and the evolution of prey wrapping. *Journal of Arachnology*, *13*, 231–241.

Higgins, L. E. (1990). Variation in foraging investment during the intermolt interval and before egg-laying in the spider *Nephila clavipes* (Araneae: Araneidae). *Journal of Insect Behavior*, *3*(6), 773–783.

Jackson, R. R., & Pollard, S. D. (1996). Predatory behavior of jumping spiders. *Annual Review of Entomology*, *41*(1), 287–308.

Jackson, R. R., & Wilcox, R. S. (1993). Spider flexibly chooses aggressive mimicry signals for different prey by trial and error. *Behaviour*, *127*, 1–2.

Japyassu, H. F., & Ades, C. (1998). From complete orb to semi-orb webs: developmental transitions in the web of *Nephilengys Cruentata* (Araneae: Tetragnathidae). *Behaviour*, *135*(7), 931–956.

Japyassú, H. F., & Caires, R. A. (2008). Hunting tactics in a cobweb spider (Araneae-Theridiidae) and the evolution of behavioral plasticity. *Journal of Insect Behavior*, *21*(4), 258–284.

Li, D., Jackson, R. R., & Barrion, A. T. (1999). Parental and predatory behaviour of *Scytodes* sp., an araneophagic spitting spider (Araneae: Scytodidae) from the Philippines. *Journal of Zoology*, *247*(3), 293–310.

Macdonald, D. W., Stewart, P. D., Stopka, P., & Yamaguchi, N. (2000). Measuring the dynamics of mammalian societies: an ecologist's guide to ethological methods. In (ed. M. C. Pearl) *Research techniques in animal ecology: controversies and consequences*, 332–388.

Sherman, P. M. (1994). The orb-web: an energetic and behavioural estimator of a spider's dynamic foraging and reproductive strategies. *Animal Behaviour*, *48*(1), 19–34.

Persons MH (2001) Hunger effects on foraging responses to perceptual cues in immature and adult wolf spiders. *Animal Behavior*, *57*(1), 81–88.

Persons, M. H., Walker, S. E., Rypstra, A. L., & Marshall, S. D. (2001). Wolf spider predator avoidance tactics and survival in the presence of diet-associated predator cues (Araneae: Lycosidae). *Animal Behaviour*, *61*(1), 43–51.

Peters, J. F., Henry, C., & Ramanna, S. (2005). Rough ethograms: Study of intelligent system behavior. In *Intelligent information processing and web mining* (pp. 117–126). Springer Berlin Heidelberg.

Robinson, M. H., Mirick, H., & Turner, O. (1969). The predatory behavior of some araneid spiders and the origin of immobilization wrapping. *Psyche*, *76*(4), 487–501.

Robinson, M. H., & Mirick, H. (1971). The predatory behavior of the golden-web spider Nephila clavipes (Araneae: Araneidae). *Psyche*, *78*(3), 123–139.

Robinson, M. H., & Olazarri, J. (1971). Units of behavior and complex sequences in the predatory behavior of *Argiope argentata* (Fabricius):(Araneae: Araneidae). *Smithsonian Contributions to Zoology, 65*, 1–36.

Rypstra, A. L. (1982). Building a better insect trap; an experimental investigation of prey capture in a variety of spider webs. *Oecologia*, *52*(1), 31–36.

Salomon, M. (2011). The natural diet of a polyphagous predator, *Latrodectus hesperus* (Araneae: Theridiidae), over one year. *Journal of Arachnology*, *39*(1), 154–160.

Thévenard, L., Leborgne, R., & Pasquet, A. (2004). Web-building management in an orb-weaving spider, *Zygiella x-notata*: influence of prey and conspecifics. *Comptes Rendus Biologies*, *327*(1), 84–92.

Venner, S., Pasquet, A., & Leborgne, R. (2000). Web-building behaviour in the orb-weaving spider *Zygiella x-notata*: influence of experience. *Animal behaviour*, *59*(3), 603–611.

Viera, C. (1986). Comportamiento de captura de *Metepeira* sp. A (Araneae, Araneidae) sobre *Acromyrmex* sp.(Hymenoptera, Formicidae) en condiciones experimentales. *Aracnologia, Montevideo*, *6*, 1–8.

Vollrath, F. (1987). Kleptobiosis in spiders. In *Ecophysiology of spiders* (ed wolfgang Nentwig), (pp. 274–286). Springer Berlin Heidelberg.

Vollrath, F. (1987). Altered geometry of webs in spiders with regenerated legs. *Nature*, *328*(6127), 247–248.

Willey, M. B., Johnson, M. A., & Adler, P. H. (1992). Predatory behavior of the basilica spider, *Mecynogea lemniscata* (Araneae, Araneidae). *Psyche*, *99*(2–3), 153–168.

Witt, P. N., & Baum, R. (1960). Changes in orb webs of spiders during growth (*Araneus diadematus* Clerck and *Neoscona vertebrata* McCook). *Behaviour*, *16*, 309–318.

Wullschleger, B., & Nentwig, W. (2002). Influence of venom availability on a spider's prey–choice behaviour. *Functional Ecology*, *16*(6), 802–807.

CHAPTER SIX

ONOTGENETIC AND SEXUAL VARIATION IN THE VENOM OF THE WESTERN WIDOW SPIDER (*Latrodectus hesperus)*

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Abstract

Venom comprises an essential tool for survival for the organisms that employ them, but also requires a metabolic and biological cost. Thus, selection often acts on animal venoms, resulting in substantial variation with regard to geographic distribution, diet, season, sex, and ontogeny. In this study, we investigated ontogenetic and sexual variation in the venom of the western widow spider *Latrodectus hesperus*, with emphasis on the vertebrate specific toxin α-latrotoxin. We analyzed venom changes during the ontogeny of males (3rd, 5th, and mature instars) and females (3rd, 5th, 6th, and mature instars), and compared venoms of the two sexes within groups. We sexed older spiders by the presence of an enlarged tarsus of the pedipalps in males. However, we sexed 3rd instar individuals by pedipalpal tibia width (males > females) using cutoffs that yielded a high positive predictive value (PPV) for males (100%) and a high negative predictive value (NPV) for females (92.7%). We assessed venom variation using reverse-phase fast protein liquid chromatography (RP-FPLC), liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI). Our initial results suggest the presence of both ontogenetic and sexual venom variation, with female venom becoming increasingly complex with age, and the hydrophobic components of male venom becoming less complex with maturity. However, because of limitations to the existing venom databases and techniques used to identify specific protein components, we expect to subject additional venom samples to LC-MALDI and may also run digested whole venom directly on LC-MS/MS (a shotgun approach) to confirm the ontogenetic and sexual differences that our limited analyses suggest.

Introduction

Venom comprises an essential tool of survival for the organisms that employ them. However, the synthesis, storage, and deployment of venom requires metabolic (McCue and Mason 2006; Nisani et al. 2007, 2012; Pintor 2010) and biological costs (Hayes et al. 2002). Therefore, venoms should be under high selective pressure to reduce their cost. Yet, venoms can be extremely complex, often containing hundreds to thousands of peptides, proteins, and other constituents (Escoubas et al. 2006; Nascimento et al. 2006), often with surprising redundancy (Morgenstern and King 2012).

Variation in venom composition has been documented in association with geographic distribution (Alape-Giron et al. 2008; Creer et al 2003; Daltry et al. 1996; Tsai et al. 2001), diet (Daltry et al. 1996; Mackessy 1988; Underwood and Seymour 2007), season (Atkinson and Walker 1985; Chippaux et al. 1991), sex (Atkinson and Walker 1985; Binford 2001; de Oliveira et al. 1999; Escoubas et al. 2002; Herzig and Hodgson 2009; Malli et al. 1993), and ontogeny (Alape-Giron et al. 2008; de Andrade et al. 1999; Escoubas et al. 2006; Herzig et al. 2004; Mackessy 1988; Mackessey et al. 2006; Malli et al. 1993; Underwood and Seymour 2007). Venom variation has often been linked to selection derived from dietary differences (e.g., McClounan and Seymour 2012; Richards et al. 2012), but other factors must also shape variation (Gibbs and Chiucchi 2011), especially since venom can serve multiple purposes in some species (Deslippe and Guo 2000) and in others is used exclusively for defense (e.g., caterpillars, fish).

Among spiders, the venoms of tarantulas and species that cause medically relevant envenomations have been the most commonly studied (for reviews of spider venoms see Escoubas et al. 2000; Kuhn-Nentwig et al. 2011; Nentwig and Kuhn-Nentwig

2013; Saez et al. 2010). Venoms of the spider genus *Latrodectus* have been studied extensively, most often focusing on mode of action (Bettini 1971; Frontali et al. 1976; Luch 2010; Rosenthal et al. 1990), clinical aspects (Andrews et al. 2011; Monte 2012; Thatcher and Janes 2012), and components (Grishin 1996; Rohou et al. 2007; Ushkaryov et al. 2008). Venoms of *Latrodectus* and other members of family Theridiidae uniquely include large molecular weight proteins in the 110–140 kDa range (Kuhn-Nentwig et al. 2011). *Latrodectus* venom consists of seven major taxon-specific proteins in three categories: (1) alpha-latrotoxin (α-LTX), which is vertebrate specific with a molecular mass of 130 kDa; (2) five latroinsectotoxins (α, β, γ, δ, ε-LIT), which are insect specific at 110–140 kDa; (3) alpha-latrocrustotoxin (α-LCT), which is crustacean specific at 120 kDa. The venom also includes numerous low molecular weight proteins (Gasparini et al. 1994; Volkova et al. 1995). Venoms from different *Latrodectus* species are structurally and functionally similar, making distinguishing toxins across species difficult (Rohou et al. 2007; Kuhn-Nentwig et al. 2011). However, differences are apparently sufficient that an anti-a-LTX monoclonal antibody made from *L. tridecimguttatus* fails to neutralize the neurotoxicity of *L. mactans, L. hesperus*, and *L. hasselti* venoms (Graudins et al. 2012).

Several studies have reported that spider venom varies with age (de Andrade et al. 1999; Escoubas et al. 2002; Herzig et al. 2004; Malli et al. 1993) and sex (Atkinson and Walker 1985; Binford 2001; de Oliveira et al. 1999; Escoubas et al. 2002; Herzig and Hodgson 2009; Malli et al. 1993). Female spiders are generally larger than males, and thus have larger venom glands and venom supply. Mature males and females also often differ in reproductive life histories, as males typically move more through their habitats

more in search of females. Thus, age and sex differences in venom may reflect diverging pressures related to body size and reproductive differences.

Among spiders, sexual differences in venom have involved the presence or absence of one or several toxins, with the majority of toxins common to both. Unfortunately, relative toxicity of male and female venoms depends on choice of animal used to test toxicity (Binford 2001; De Oliveira 1999; Herzig 2009; Malli 1993), which makes generalizations tenuous.

The purpose of this study was to investigate ontogenetic changes and sexual differences in the toxin components of *L. hesperus* venom. Because *Latrodectus* has taxon-specific toxins, including vertebrate and crustacean toxins, and because the earliest instars are incredibly small, we hypothesized that the vertebrate-specific toxin (α-LTX) will not be expressed in the youngest instars. We also hypothesized that mature males may stop producing certain toxins and put all their energy into mating activities, similar to their discontinuation of defensive silk use and possible degradation of silk glands as adults (c.f., Vetter 1980). To test these hypotheses, we sexed spiders beginning at the 3rd instar and collected venom through successive instars via dissection. We then subjected the venoms to proteomic analysis to compare their composition.

Methods

Spider Husbandry

We collected spiders in the spring and summer months (generally May–September) from Redlands, Loma Linda, and Colton, California (San Bernardino County). Spiders were housed in 540-mL plastic deli cups within a small room in the

laboratory at 22° C on a 12-hr light-dark cycle. We provided spiders with a small stick that facilitated web construction, and offered house crickets (*Acheta domestica*) biweekly. No water was provided, as it was deemed unnecessary.

Some wild caught females produced egg sacs. We removed the hatched spiderlings from their natal container and housed them individually in 350-mL plastic deli cups under conditions identical to the adult females. Instars 2–3 were fed fruit flies (*Drosophila melanogaster* or *Drosophila* sp.) twice a week, and instars 4–6 were fed once per week (same diet). Instars 7+ were treated the same as adult female spiders. Spiders of all ages were housed within the same room.

Aging and Sexing Spiders

Spiders are aged according to their sequence of ecdysis events (sheds). We called the first shed observed outside the egg sac shed 1; this corresponds to the spider becoming a 3rd instar, as the first molt occurs within the egg sac before emergence (Deevey 1949). Adult males can be recognized by the presence of an enlarged tarsus of the pedipalps; however, this feature cannot be readily observed in spiderlings. Sex identification of spiderlings followed a modified protocol first described by Mahmoudi et al. (2008). I photographed the pedipalps of 3rd instar spiders using a Cannon EOS 60D (Canon U.S.A Inc., Melville, NY, USA) attached to a Nikon SMZ-10A dissection scope (Nikon Instruments Inc., Melville, NY, USA). Spiders were not anesthetized, but were coaxed into a position within their web that allowed for a clear view of the dorsal surface of the pedipalps (Fig. 8). I adjusted brightness and contrast for each photo using Adobe Photoshop (CS3, Adobe Systems Inc., New York, NY, USA) so that the margins of the

pedipalps were clearly visible. I then measured pedipalpal tibia width to the nearest 0.001 mm (using the average of three measurements) via ImageJ software (http://rsbweb.nih.gov/ij/index.html). When the spiders reached their ultimate (adult) shed, I confirmed their sex by the aforementioned criterion. I tested reliability of sexing by computing the positive and negative predictive values (PPV and NPV; Altman and Bland 1994). The PPV is the proportion of positive test results that are truly positive: PPV = *N* of true positives / (*N* of true positives + *N* of false positives). Male was designated the positive result. The NPV is the inverse of PPV: NPV = *N* of true negatives / (*N* of true negatives + *N* of false negatives).

A

B

Figure 8: Comparison of pedipalpal tibia width of 3rd instar *L. hesperus*. A) Female B) Male

Dissection of Venom Glands

To procure venom glands, I anesthetized each spider with CO_2 for 10 min, then pinned it to a dissection plate, and immersed it in spider saline (160 mM NaCl, 7.5mM KCl, 1 mM $MgCl_2$, 4 mM $NaHCO_3$, 4 mM $CaCl_2*H_2O$, 20 mM $C_6H_{12}O_6$ at pH 7.4). I grasped the chelicerae with modified fine-point forceps (22-327-379, Fisher Scientific, Pittsburgh, PA, USA), and then used a dissection knife (Ref 961502, Surgistar, Vista,

CA, USA) to cut the soft connective tissue connecting the chelicerae to the carapace. I then gently pulled the chelicerae from the spider, which typically removed both venom glands along with the chelicerae. I then placed the tissues in a drop of spider saline in the cut-off top of a 1.5-mL snap cap tube. Once all spiders to be included in a single tube (typically $N = 6$) had been dissected, I separated the venom glands from the chelicerae, and removed the chelicerae from the saline drop. I then cut open the venom glands within the saline drop using a pair of 00 insect pins (Bioquip Products, Rancho Dominguez, CA, USA), gently agitated the glands, and then removed them. The venom-saline mixture was stored at -80°C until analysis.

Venom Fractionation

I fractionated the pooled venom samples using reverse phase-fast protein liquid chromatography (RP-FPLC). Separations were performed on an AKTA FPLC instrument (GE Healthcare Biosciences, Pittspurgh, PA, USA). Buffer A (0.065% trifluoroacetic acid (TFA), 2% acetonitrile (ACN) in water) was added to the venom sample to make a total volume of 200 mL and centrifuged at 10,000 x *g* for 10 min. Two Source 15 RPC ST 4.6/100 polystyrene/divinyl benzene reverse phase columns (GE Healthcare Biosciences, Pittspurgh, PA, USA), connected in series, were equilibrated in Buffer A, and 100 µL of the diluted sample was injected onto the column. Proteins were eluted at a flow rate of 0.5 mL/min, in a 40-column-volume linear gradient of 0-100% Buffer B (0.05% TFA, 80% ACN in water), and the elution monitored at 214 nm using Unicorn 5.0 software (GE Healthcare Biosciences, Pittspurgh, PA, USA). Fractions were manually collected during some of the FPLC runs for further analysis.

Chromatograms were compared for qualitative differences. We compared chromatograms of different ages within each sex, between sexes at each specific age, and all groups compared to the adult female standard.

Trypsin Digest

I adopted the trypsin digest protocol from Stone et al. (1989). Prior to digestion, samples were reduced and alkylated using 45 mM dithiothreitol and 100 mM iodoacetamide. The proteins were digested with 1 µg of trypsin overnight at 37°C (the amount of trypsin was empirically determined to be sufficient to adequately digest the samples over a 24-hr period). After digestion, the reaction was stopped by adding TFA sufficient to lower the pH of the sample to 2. Samples were then frozen at -80°C until desalting.

De-salting of Samples

The peptides were purified with Zip Tip_{C18} (Millipore Corp., Billerica, MA, USA) following manufacturer's guidelines, then evaporated to dryness and re-suspended in 20 µL of 2% ACN 0.1% formic acid (FA) in water, the same starting buffer used in LC-MS analysis.

LC-MS/MS

We subjected the tryptic peptides to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a ThermoFinnigan LCQ Deca XP spectrometer (Thermo, Waltham, MA, USA) equipped with a PicoView 500 nanospray apparatus with

Xcalibur software (ver. 1.3; Thermo, Waltham, MA, USA) for instrument control and data acquisition. Separation was performed on a 10 cm x 75 μM i.d. C_{18} Biobasic bead column (New Objective, Woburn, MA, USA), by injecting 20 μL samples. Mobile phase B consisted of 98% acetonitrile, 2% water, and 0.1% formic acid. The gradient program was: 0% B at 0.18 mL/min for 7.5 min, 0% B at 0.35 mL/min for 0.5 min, linear gradient to 20% B at 15 min at 0.35 mL/min, linear gradient to 75% B at 55 min at 0.3 mL/min (flow rate constant for remainder of program), linear gradient to 90% B at 60 min, hold at 90% B until 85 min, linear gradient to 0% B at 90 min, hold at 0% B until 120 min. Spectra were acquired in positive ion mode with a scan range of 300–1500 m/z. MS/MS data were converted into peaklist files using Extract_msn implemented in Bioworks (version 3.1; Thermo, Waltham, MA, USA) using the following parameters: peptide molecular weight range 300–3500, threshold 100000, precursor mass tolerance 1.4, minimum ion count 35. Following LC, some fractions were further separated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI), while other sample results were converted into output files and analyzed with a Mascot MS/MS database search.

LC-MALDI

We reduced and alkylated the venom in vapor using the method described in Hale et al. (2004). Lyophilized venom was alkylated in vapor using 20 mM iodoethanol and 10 mM triethylphosphine in 50% MeCN and 50 mM ammonium carbonate pH 10.5 for 2 hr at 37°C. We reconstituted the sample in 20 μl digestion solution (0.5 ug trypsin in 40 mM ammonium bicarbonate pH 8.3), and left it to digest for 30 min at room temperature

followed by 2 min of heating in a 100-W microwave. We then dried the sample and reconstituted it in 20 μl 5% MeCN 1% formic acid. We loaded 1 μg of sample (determined by NanoDrop™ 1000 Spectrophotometer, Thermo Scientific, Wilmington, DE, USA) for mass spectroscopy (MS) analysis using the following setup: samples were injected on a Vydac C18 238TP column (0.075 x 150 mm, 3 μm, 300A pores; Grace, Deerfield, IL, USA) and eluted using a 40-min gradient from 5–40% MeCN (0.1% FA) on to a 5600 Triple-TOF (AB SCIEX, Framingham, MA, USA) equipped with a nano source, scanning at 20 MS2/sec. We reconstituted the LC fractions in 5 μl of 30% MeCN/0.2% formic acid, and 0.5 μl were spotted at a 1:1 ratio with sinapinic acid (10 mg/mL in 50% MeCN) on a polished metal MALDI plate, and allowed to dry in open air. Data were collected on a 4700 MALDI-TOF/TOF system (AB SCIEX, Framingham, MA, USA) at 1 Kv positive linear mode, in a 10,000 Da bounds, between 80–150 kDa. If no data were acquired in the initial run, the spots were then re-spotted by adding to each of them 3 x 0.5 μl of sample. The samples were then treated the same way as before.

Database Search and Matching

We conducted LC-MS/MS database searches using Mascot search engine (licensed, version 2.2, Matrix Science, Boston, MA, USA) against the SwissProt (51.6) database in the taxa Metazoa with a parent tolerance of 1.20 Da, fragment tolerance of 0.60 Da, and two missed trypsin cleavages allowed. Carbamidomethylation of cysteine and oxidation of methionine were specified in MASCOT as fixed and variable modifications, respectively.

We analyzed LC-MALDI data using ProteinPilot 4 (AB SCIEX, Framingham, MA, USA) against a spider toxin database extracted from Arachnoserver (2.0) and Uniprot (release 2012_08).

Results

Sex Determination

Table 17 provides the results of the PPV and NPV analysis of sex identification. We photographed the pedipalps of 125 un-anesthetized 3rd instar spiders. Initially, pedipalpal tibia widths <0.102 mm were predicted to be female, and those >0.108 mm were assumed to be male (Mahmoudi et al. 2008). Given these criteria, we calculated PPV and NPV, and found that we were able to correctly predict the male sex 100% of the time and the female sex 82.5% of the time. Although 82.5% is a high NPV, we felt that it was not adequate, and narrowed the range for females to <0.097 mm. With the new criteria, we were able to predict the female sex 92.7% of the time. We subsequently used these predictions to sex 3rd instar spiders and to assign their venom samples to appropriate venom pools.

Table 17: Positive predictive value (PPV) of sex identification of the western widow spider (*Latrodecuts hesperus)*.

A. Criteria: pedipalpal tibia width male ≥ 0.108, females ≤ 0.102 mm; *N* = 125

		Actual		
		Male (+)	Female (-)	
Predicted	Male (+)	62	0	62
	Female (-)	11	52	63
		73	52	125
PPV (M)	0.100			
NPV (F)	0.825			

B. Criteria: pedipalpal tibia width male ≥ 0.108, females ≤ 0.096 mm; *N* = 103

		Actual		
		Male (+)	Female (-)	
Predicted	Male (+)	62	0	62
	Female (-)	3	38	41
		65	38	103
PPV (M)	0.100			
NPV (F)	0.927			

LC-MS/MS

We collected RP-FPLC fractions of adult female venom (pooled *N* =16; Table 18). Tryptic-digested and de-salted fractions were then subjected to LC-MS/MS separation and fragmentation. Of the 38 fractions analyzed, Mascot searches of the SwissProt database revealed that 16 fractions contained previously described toxins: α-LIT, δ-LIT, α-LCT, α-LTX, and alpha-latrotoxin low molecular weight accessory protein (α-LTX-LMWP). In addition to the described proteins, Mascot found that 22 of the fractions had no significant hits. Table 18 reports the toxin components of each fraction,

and Fig. 9 shows the position of each toxin within the original RP-FPLC chromatogram. The majority of described toxins were hydrophobic, all coming off the column in close succession. The notable exception was alpha-LTX-LMWP, which came off the column much earlier. All results reported in Table 18 are based on peptide sequence scores higher than the probability-based Mowse score (P <0.05). In general, individual ion scores had to be greater than ~38. This reduced the number of unique peptides that could be reported, thereby reducing the percent coverage for each protein. Our percent coverage ranged from as low as 0.6% (1 unique peptide) to as high as 26.4% (32 unique peptides). Possible reasons for the low percent coverage are discussed further.

Table 18: Results of LC/MS/MS database search using Mascot, adult female *L. hesperus*

Fraction	Accenssion #	Protein	# Unique	Score	% Cov	Ion-score
AM	LITA_LATMA	Alpha-LIT	9	476	8.8	>38
AL	LITA_LATMA	Alpha-LIT	27	1349	22.4	>38
	LATA_LATMA	Alpha-LTX	3	116	2.7	
AK	LITA_LATMA	Alpha-LIT	32	1570	26.4	>37
	LATA_LATMA	Alpha-LTX	12	601	10.8	
AJ	LCTA_LATMA	Alpha-LCT	18	694	19.1	>38
	LITD_LATMA	Delta-LIT	2	131	2.5	
AI	LITA_LATMA	Alpha-LIT	2	70	1.6	
	LCTA_LATMA	Alpha-LCT	25	1876	21.7	
Ah[ab]	LITA_LATMA	Alpha-LIT	1	52	0.9	
AG[a]	LITD_LATMA	Delta-LIT	2	129	3.3	
AF[a]	LITD_LATMA	Delta-LIT	3	253	1.6	
	LCTA_LATMA	Alpha-LCT	5	360	3.8	
AE	LITD_LATMA	Delta-LIT	1	41	1.1	
AD[a]	LITD_LATMA	Delta-LIT	9	474	6.6	
	LATA_LATMA	Alpha-LTX	8	345	6.2	
AC[a]	LATA_LATMA	Alpha-LTX	1	46	0.9	
AB[ac]	LCTA_LATMA	Alpha-LCT	5	221	5.4	
AA[a]	LCTA_LATMA	Alpha-LCT	2	103	1.6	>39
	LITA_LATMA	Alpha-LIT	10	344	9	>38
Z	LITA_LATMA	Alpha-LIT	6	233	6.2	
	LCTA_LATMA	Alpha-LCT	2	126	2.4	
Y	LCTA_LATMA	Alpha-LCT	1	50	0.9	
X[b]	unknown		1	63	0.6	>36
W	unknown					>38
V	unknown					
U	unknown					
T	unknown					
S	unknown					
R	unknown					
Q	unknown					
P	unknown					
O	unknown					
N	unknown					

[a] Contains hemocyanin

[b] Contains muscle protein

[c] *Crotalus ruber* contamination from previous column use

Table 18: Results of LC/MS/MS database search using Mascot, adult female *L. hesperus* Continued

Fraction	Accenssion #	Protein	# Unique	Score	% Cov	Ion-score
M	TXALA_LATMA	Alpha-LTX-LMWP	2	582	20.5	>39
L	unknown					>38
K	unknown					>38
I	unknown					>38
H	unknown					>38
G	unknown					>38
F	unknown					>38
E	unknown					>38
D	unknown					>38
C	unknown					>38
B	unknown					>38
A	unknown					>38

[a] Contains hemocyanin

[b] Contains muscle protein

[c] *Crotalus ruber* contamination from previous column use

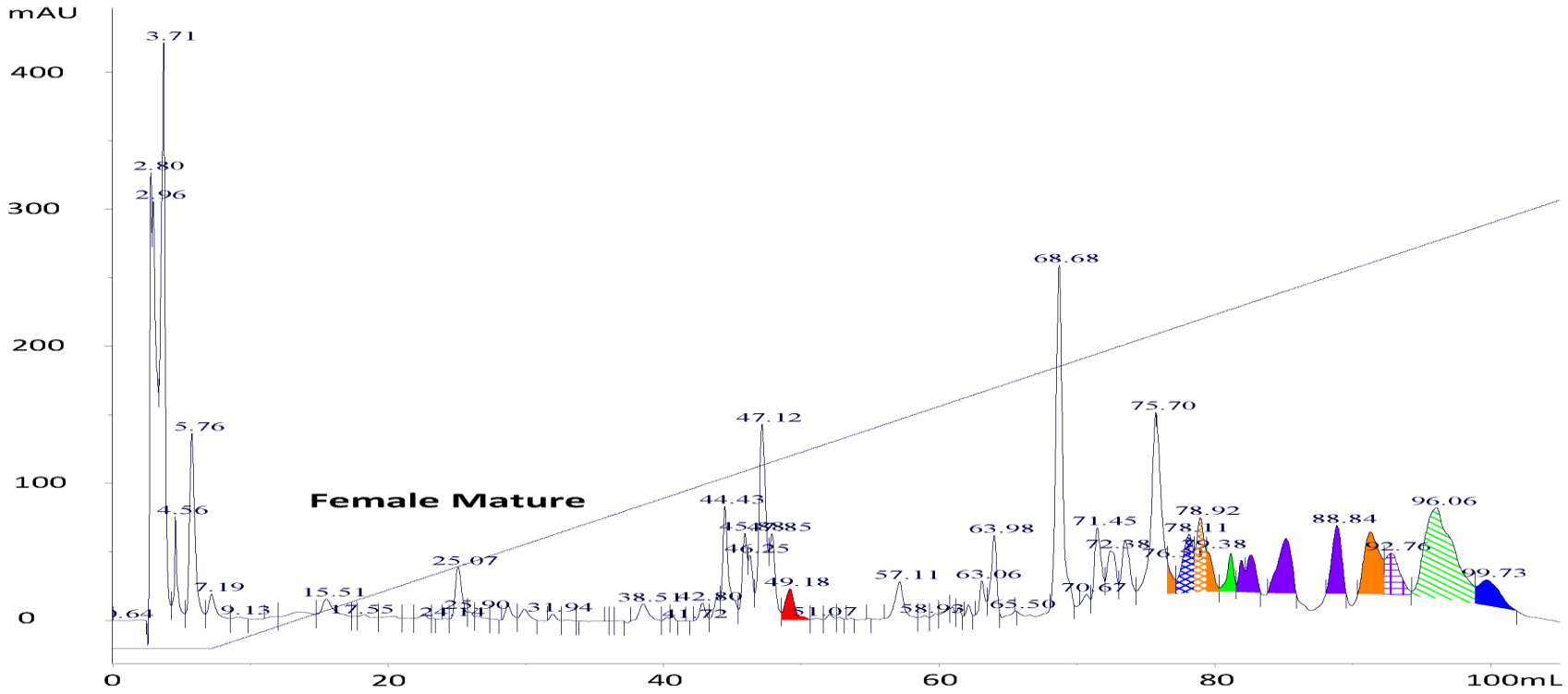

Figure 9: LC-MS/MS labeled RP-FPLC chromatogram of adult female venom, *L. hesperus* (pooled venom sample *N* = 16).
Red = α-LTX-LMWP
Orange = α-LCT
Green = α-LTX
Purple = δ-LIT
Blue = α-LIT
Checkered = α-LCT & δ-LIT (Color represents stronger signal of the two toxins)
Slash = α-LIT & α-LTX
Crisscrossed = α-LCT & α-LIT

FPLC

The RP-FPLC chromatograms were analyzed for qualitative differences between sexes within a particular age class, between sexes within each age class, and all groups compared to our standard (adult female). Concerning age classes, all differences are shown with respect to the older instar, i.e., differences are only highlighted when the older instar possesses a peak that is not evident in the younger instar. This is because older instars tend to have more venom, and thus peaks with higher mAU. Larger peaks tend to hide smaller peaks either within themselves or within the baseline. In some instances, younger instars appeared to possess peaks missing in the older instars, but the validity of this claim demands further testing, and will not be commented on here.

Comparisons of age classes within each sex were performed on 3rd, 5th, and mature instars for within males and females, and additionally for the 6th instar within females only. In females, we found four sets of potential differences between the 3rd and 5th instar (Fig 10C). One difference was observed in the hydrophobic region, around retention volume 93–94 mL. The 3rd versus 6th (N = 15 zip tipped) also showed similar age-related differences, with four sets highlighted in figure 10B. In both the 3rd vs. 5th and 3rd vs. 6th, the older instars possessed new or more pronounced peaks. The 3rd vs. adult also showed similar differences, with one additional change (5 sets; Fig. 10A). Around retention volume 49 mL the peak containing alpha-LTX-LMWP, seen in the mature females, was missing from the 3rd instar. These differences are, thus far, corroborated by LC-MALDI results (Table 19). Comparisons of the 5th vs. 6th instar females (Fig. 11A) revealed a reduction in the number of differences overall, compared to the 3rd instar. Based on superficial appearance alone, chromatograms of the 5th and 6th instars look

more similar to each other than either does to the 3rd instar. The first highlighted difference, around retention volume 49 mL, indicates the possible appearance of alpha-LTX-LMWP in the 6th instar. Another difference can be seen in the hydrophobic region around retention time 81–82 mL. Comparing both the 5th instar vs. mature female, and the 6th instar vs. mature female reveals a general reduction in the obvious differences, as compared to the 3rd instar vs. mature females (Fig 11B, 11C, and 10A respectively).

Males initially showed a similar trend to females with increasing number of distinct peaks within the more hydrophobic region of the chromatogram from the 3rd to 5th instar. We highlight three potential differences in Fig. 12B, with two sets appearing in the hydrophobic region around 72 mL and 91–95 mL retention volume. Unlike the female trend of increasing number of unique peaks across the entire chromatogram with maturation, there are noticeable decreases in the number of distinct peaks observed within the more hydrophobic portion from the 5th to the mature instar in males, observed around retention time 76–105 mL. The hydrophilic region, however, still shows a greater number of distinct peaks (Fig. 12C retention volumes 27–40 mL and 51–62 mL).

Comparisons of sexes within a particular age class were performed on 3rd, 5th, and mature instars. We found two potential differences between the 3rd instar males (N = 102, zip tipped) and females (N = 43, zip tipped; Fig. 13A): females possessed a series of small peaks at retension volume 61 mL, and small peak at ~73 mL. Two possible differences were also found between 5th immature instar males (N = 32, zip tipped) and females (N = 20, zip tipped; Fig. 13B), with female possessing an additional peak at ~64 mL, and males possessing as small peak within the known toxin range around retention volume 92 mL. Maturity of 5th instar males was based on pedipalp morphology. During

the course of the study, we observed that when male pedipalps first appear greatly enlarged, they retain their yellow color. The subsequent shed results in the pedipalps turning black in color, with the embolus coil clearly visible. Fifth instar males with yellow pedipalps were deemed immature or submature as the ultimate shed had not been reached. Mature males (N = 20, zip tipped) and females (N = 16, zip tipped) showed the greatest potential variation, with three sets of differences found (Fig. 13C). Major differences can be observed in the hydrophobic region of the chromatogram, from retention volume ~81–105 mL. No comparison was made between sexes at the 6th instar, as results of the male chromatogram were suspect.

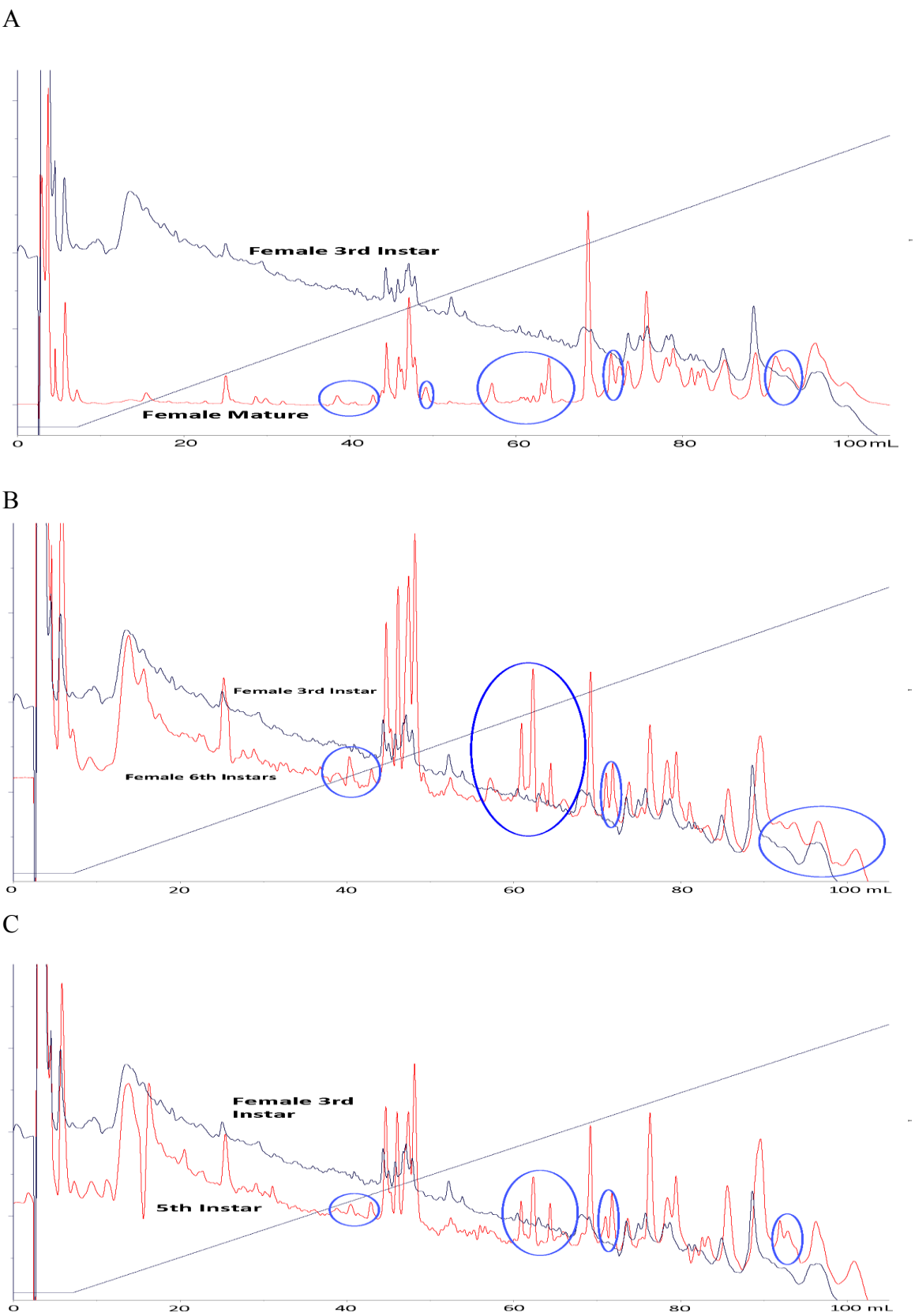

Figure 10: Within sex (female) comparison of RP-FPLC chromatograms, *L. hesperus*

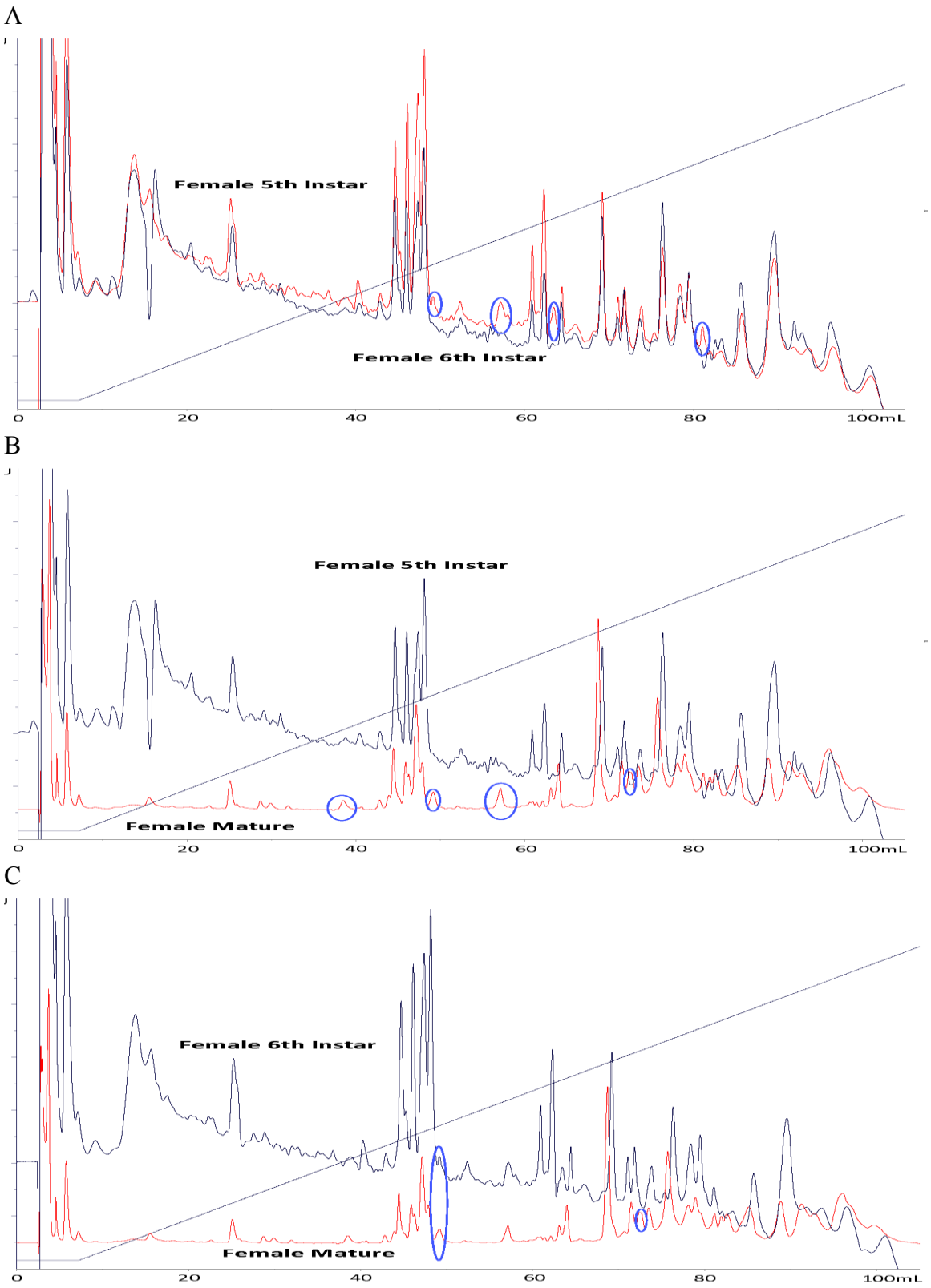

Figure 11: More within sex (female) comparison of RP-FPLC chromatograms, *L. hesperus*

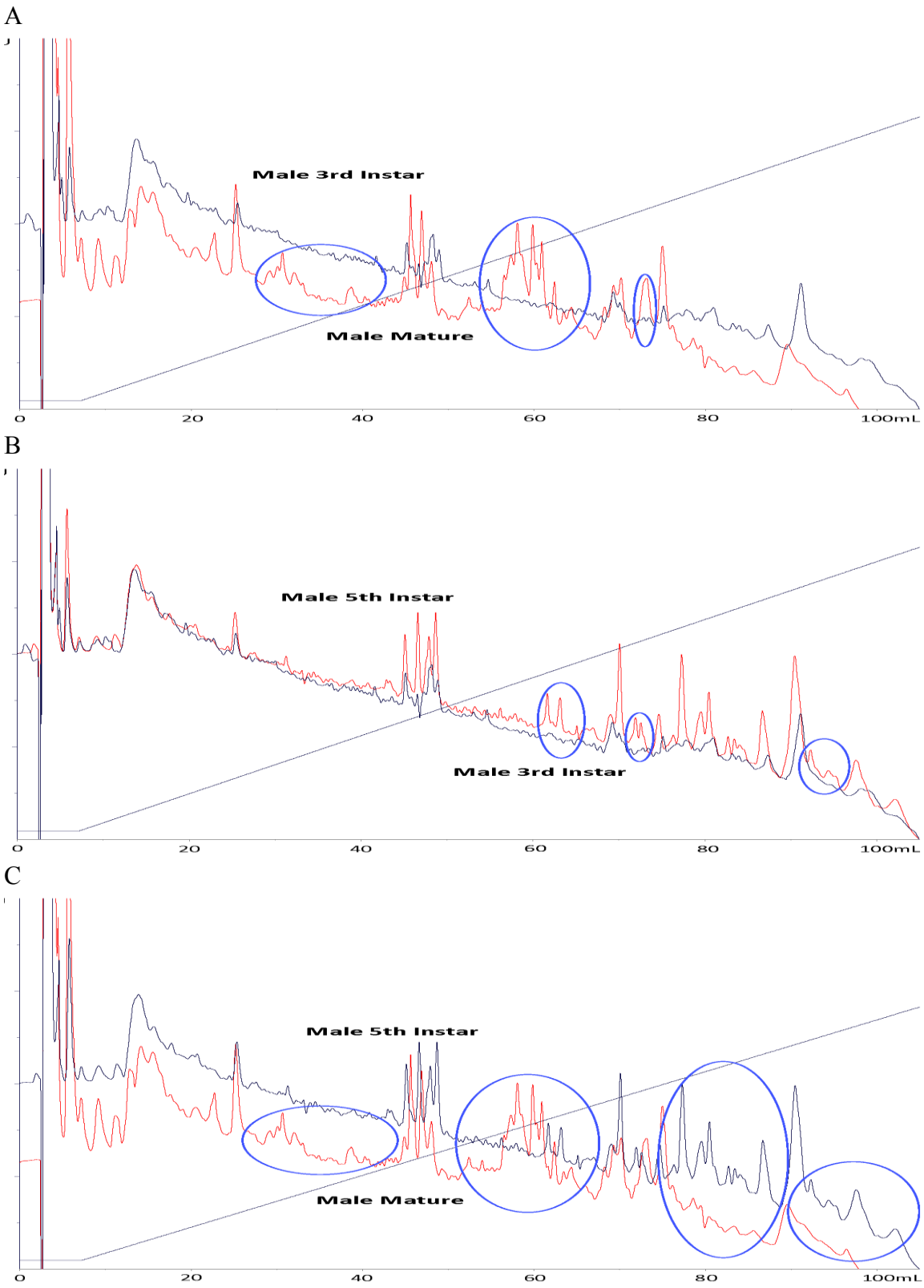

Figure 12: Within sex (male) comparison of RP-FPLC chromatograms, *L. hesperus*

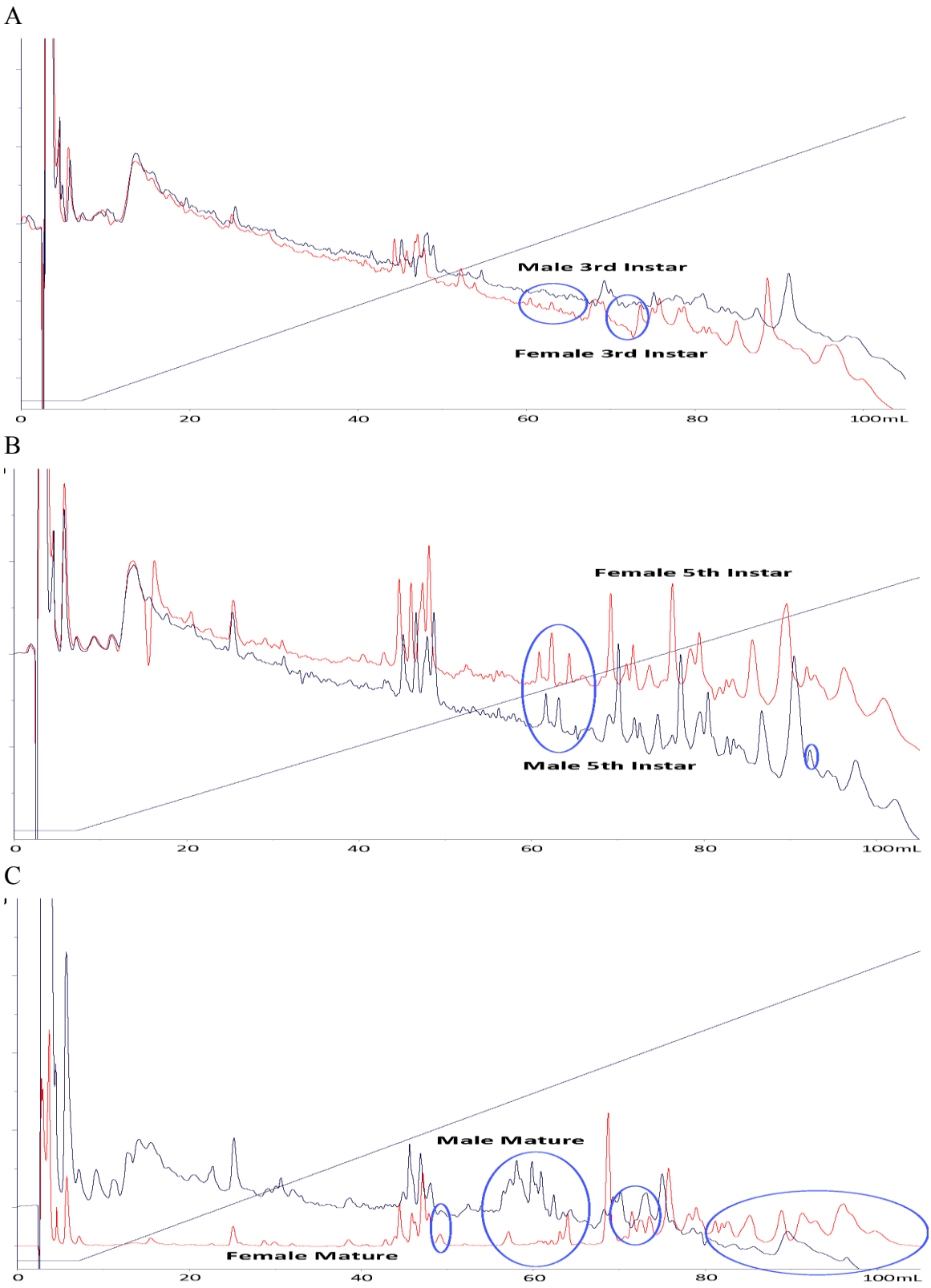

Figure 13: Between sex comparisons of spiders at the same developmental stage, comparing RP-FPLC chromatograms, *L. Hesperus*

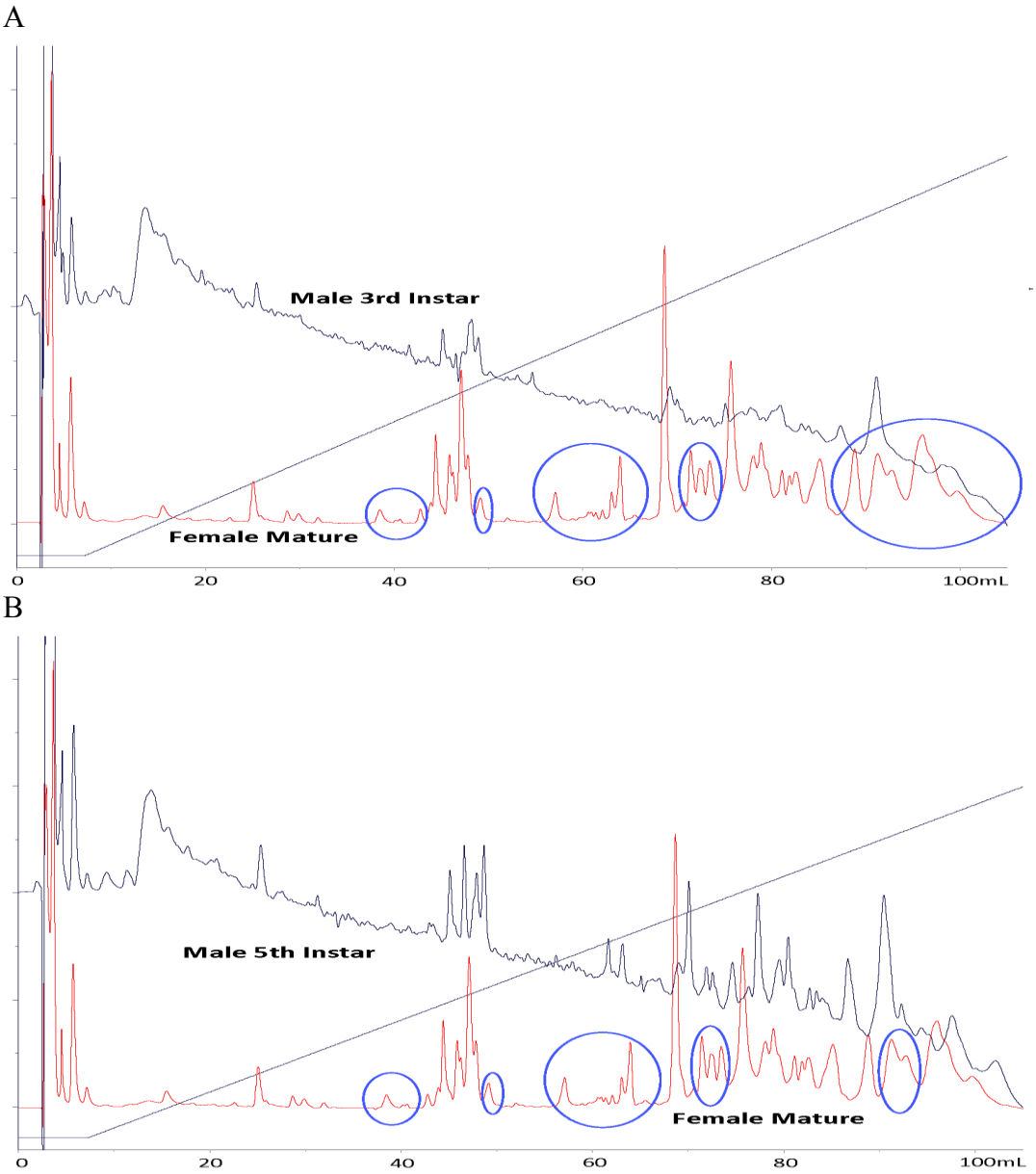

Figure 14: Comparison of the RP-FPLC venom profile of different age and sex classes to mature female, *L. Hesperus*

LC-MALDI

Unfortunately, we consider the current results (Table 19) to be inconclusive. We used Arachnoserver and UniProt databases to search against, as compared to SwissProt for the LC-MS/MS results. This should not have influenced the results as all databases

contain all formally described toxins for the genus *Latrodectus*. Database searches returned matches for *L. tredecimguttatus*, the Mediterranean black widow, instead of the appropriate species *L. hesperus*. Of the protein matches that were found, the percent coverage per protein hits were generally very small: ranging from 0.57% to 53.41% within the most liberal criteria for acceptable results. Protein hits for each age and sex were also sporadic, as evidenced by the lack of hits within the mature females. Adult females have the full range of toxins, but our search results only came back with a hit for alpha-latrotoxin-associated low molecular weight protein 2. We found hits for toxins never before reported within the genus *Latrodectus*. The protein U3-agatoxin-Ao1b, a toxin from the venom of *Agelena orientalis*, was found in several samples. In total, toxins from three different spider species were returned as possible matches for components within our venom samples: *Agelena orientalis, Kukulacania hibernalis,* and *Loxosceles intermedia*; with A. orientlais being an entelegyne and the latter two haplogyne spiders.

Table 19: Results from LC-MALDI database search, *L. hesperus*

Age/Sex	Protien	% Cov	% Cov (50)	% Cov (95)	Species
3rd/Female	Delta-Latroinsectotoxin	5.189	3.46	2.389	*Latrodectus Tredecimguttatus*
	U3-agatoxin-Ao1b	9.459	9.459	0	*Agelena orientalis*
3rd/Male	Delta-Latroinsectotoxin	0.7414	0.7414	0	*Latrodectus Tredecimguttatus*
	U3-agatoxin-Ao1b	9.459	9.459	0	*Agelena orientalis*
	Sphingomyelinase D	2.961	0	0	*Loxosceles intermedia*
4th/Female	Alpha-latrotoxon-associated LMWP2	53.41	38.64	28.41	*Latrodectus Tredecimguttatus*
	Delta-Latroinsectotoxin	5.848	2.059	2.059	*Latrodectus Tredecimguttatus*
	Alpha-latrotoxin	2.213	0	0	*Latrodectus Tredecimguttatus*
4th/Male	Astacin-like Metalloprotease toxin	5.682	0	0	*Loxosceles intermedia*
	Alpha-latrocrustotoxin	0.566	0	0	*Latrodectus Tredecimguttatus*
	Alpha-latroinsectotoxin	0.567	0	0	*Latrodectus Tredecimguttatus*
5th/Female	Delta-Latroinsectotoxin	15.9	8.32	2.718	*Latrodectus Tredecimguttatus*
	Alpha-latroinsectotoxin	7.016	1.843	1.843	*Latrodectus Tredecimguttatus*
	Alpha-latrotoxon-associated LMWP2	53.41	53.41	27.27	*Latrodectus Tredecimguttatus*
	U3-agatoxin-Ao1b	9.459	9.459	0	*Agelena orientalis*
5th/Male	Delta-Latroinsectotoxin	5.766	0.7414	0	*Latrodectus Tredecimguttatus*
	U1-filistatoxin-Kh1b	7.631	0	0	*Kukulacania hibernalis*
	Delta-Latroinsectotoxin	8.567	2.965	2.965	*Latrodectus Tredecimguttatus*
	Alpha-latrotoxon-associated LMWP2	37.5	22.73	12.5	*Latrodectus Tredecimguttatus*
6th/Female	Alpha-latrotoxon-associated LMWP2	53.41	53.41	53.41	*Latrodectus Tredecimguttatus*
	U3-agatoxin-Ao1b	9.459	9.459	0	*Agelena orientalis*
	Alpha-latrotoxin	4.568	0.857	0	*Latrodectus Tredecimguttatus*
6th/Male	Delta-Latroinsectotoxin	8.237	5.189	5.189	*Latrodectus Tredecimguttatus*
	Alpha-latrotoxin	1.713	0.857	0	*Latrodectus Tredecimguttatus*
	U3-agatoxin-Ao1b	9.459	0	0	*Agelena orientalis*
7th/Female	Alpha-latrotoxon-associated LMWP2	53.41	12.5	0	*Latrodectus Tredecimguttatus*
Adult/Female	Alpha-latrotoxon-associated LMWP2	53.41	53.41	53.41	*Latrodectus Tredecimguttatus*
Adult/Male	Alpha-latrotoxin	2.998	0	0	*Latrodectus Tredecimguttatus*

Discussion

Our results show that pedipalp width is a reliable means of sexing 3rd instar spiderlings, at least within the genus *Latrodectus*. Tentative results, particularly based on RP-FPLC, further suggest that age- and sex-related variation exists in the venoms of *L. hesperus*. However, more research is needed to confirm the exact proteins that differ among groups.

Validation of Sexing Techniques

Our results confirm the sexing method described by Mahmoudi et al. (2008). We were able to accurately predict the sex of unanesthetized 3rd instar spiders 92.7% of the time when using a restricted palp width for females (<0.096 mm). This method can be used in future research, provided that an adequate picture of the pedipalps is taken, and a sample is tested for PPV and NPV. Whether temperature or other factors associated with development might affect the choice of criteria remains unclear. Future studies should seek to expand this method to other species, as this could be a useful tool for any study of spider ontogeny.

RP-FPLC Peaks and Hydrophobicity—The Hide and Go Seek of Protein Identification

Concerning the RP-FPLC results, several caveats must discussed. First, the protein concentrations of some samples were reduced via zip tip$_{C4}$ prior to separation on the RP-FPLC. Although this reduced the mAU found during the separation, the resulting chromatograms lead us to feel that our preliminary results were not otherwise greatly affected. Second, because of scaling differences we only evaluated the chromatograms

for qualitative differences. Finally, all qualitative differences are speculative, pending further testing, as peaks thought to be missing may only be hidden. Better quantitative and qualitative results are currently being assessed using other methods. As mentioned previously, RP-FPLC alone cannot be used to determine if a peak is missing or is only hidden. With RP-FPLC separation based on hydrophobicity, proteins that have similar hydrophobic properties will be difficult to separate. If a peak is hidden within another, they cannot be differentiated, and subsequent qualitative analysis is prone to errors. Previous researchers, such as Graudins et al. (2012), first separated crude venom using size exclusion, and then followed with anion-exchange FPLC prior to separation using RP-chromatography. During each independent separation step, a portion of the sample was lost. Because of this, and the fact that the venom supply of the 3rd instar of *L. hesperus* is extremely small, we avoided the first two separation steps. However, this omission renders interpretation of our results tenuous. The reduction in signal strength, due to removal of protein by zip tip, does not seem to have altered the chromatograms greatly (Fig 13A); however, resulting changes cannot be ruled out.

LC-MALDI as a Partner in Crime to RP-FPLC

It was never our intent to rely on RP-FPLC solely. The LC-MALDI results, in conjunction with the RP-FPLC findings, should be able to determine the presence/absence of toxins and quantity of toxins present. However, as indicated by the results thus far, further analysis is needed. Nonetheless, there are some interesting lessons to be learned.

First, our results returned matches for *L. tredecimguttatus*, and not the correct species, *L. hesperus*. The versions of the databases used were dominated by *L. tredecimguttatus*, not surprisingly since this has been the major species studied for venom. Over time, expansion of the venom databases following studies of other species will further improve the quality of venom studies and reduce the level of misidentification.

Second, when a protein is digested, the resulting peptides ionize differently depending on the method used. For example, the peptides that ionize during LC-MS/MS may be different than the peptides that ionize during MALDI-TOF. It is possible that *Latrodectus* venom does not ionize well during MALDI and our general lack of hits (finding vey few of the toxins that should be there) may be the consequence. Interestingly, the only other study to attempt MALDI-TOF on *Latrodectus* venom (Duan et al 2006) reported that "no latrotoxin was identified in this analytical method." However, at the time of publication (2006), very few major toxins had been described—but the situation is rapily changing.

Third, if LC-MALDI proves to be an appropriate method to evaluate *Latrodectus* venom, then the general lack of protein coverage could be the result of sequence divergence. Since the matches returned were for *L. tredecimguttatus,* it is not surprising that the percent coverage was poor. A search of *L. hesperus* sequences specifically should improve our percent coverage. Currently, a colleague has done exhaustive sequencing of *L. hesperus* venom, using a population collected from southern California, and is in the process of submitting the sequences. Access to these sequences should immensely improve our coverage.

Fourth, our results found the presence of toxins from other spider families. This is almost certainly incorrect, but further analysis is necessary. If these toxins are confirmed to be present, this would represent a substantial discovery. Heretofore, the venom of Theridiidae has been thought to be unique among spiders, with all major toxins being high molecular weight proteins, 110–140 kDa (Kuhn-Nentwig et al. 2011).

Conclusions

We found evidence which suggests that age- and sex-related differences exist in the venom of *L.hesperus*. However, the techniques used to identify specific protein components and the existing venom databases leave room for doubt. We are presently collecting additional venom samples, and will begin analyzing them using LC-MALDI, including a quantitative analysis. The concentration of each sample has been increased. We may also run digested whole venom directly on LC-MS/MS (a shotgun approach). These two approaches (LC-MALDI and LC-MS/MS), in combination, should be able to confirm the ontogenetic and sexual differences that our limited analyses suggest.

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References

Abdel-Rahman, M. A., Omran, M. A. A., Abdel-Nabi, I. M., Ueda, H., & McVean, A. (2009). Intraspecific variation in the Egyptian scorpion *Scorpio maurus palmatus* venom collected from different biotopes. *Toxicon*, *53*(3), 349–359.

Alape-Girón, A., Sanz, L., Escolano, J., Flores-Díaz, M., Madrigal, M., Sasa, M., & Calvete, J. J. (2008). Snake venomics of the lancehead pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations. *Journal of Proteome Research*, *7*(8), 3556–3571.

Altman, D. G., & Bland, J. M. (1994). Statistics Notes: Diagnostic tests 2: predictive values. *British Medical Journal*, *309*(6947), 102.

Andrew, A. M., Bucher-Bartelson, B., & Heard, K. J. (2011). A US perspective of symptomatic *Latrodectus* spp. envenomation and treatment: a national poison data system review (December). *Annals of Pharmacotherapy*, *45*(12), 1491–8.

Atkinson, R. K., & Walker, P. (1985). The effects of season of collection, feeding, maturation and gender on the potency of funnel-web spider (*Atrax infensus*) venom. *Australian Journal of Experimental Biology and Medical Science*, *63*, 555–561.

Bettini, S., (1971). On the mode of action of *Latrodectus* spp. venom. *Annali dell'Istituto Superiore di Sanità*, *7*(1), 1–7.

Binford, G. J. (2001). An analysis of geographic and intersexual chemical variation in venoms of the spider *Tegenaria agrestis* (Agelenidae).*Toxicon*, *39*(7), 955–968.

Chippaux, J. P., Williams, V., & White, J. (1991). Snake venom variability: methods of study, results and interpretation. *Toxicon*, *29*(11), 1279–1303.

Creer, S., Malhotra, A., Thorpe, R. S., Stöcklin, R. S., Favreau, P. S., & Chou, W. S. H. (2003). Genetic and ecological correlates of intraspecific variation in pitviper venom composition detected using matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) and isoelectric focusing. *Journal of Molecular Evolution*, *56*(3), 317–329.

Daltry, J. C., Wuester, W., & Thorpe, R. S. (1996). Diet and snake venom evolution. *Nature*, *379*(6565), 537–540.

de Andrade, R. M. G., De Oliveira, K. C., Giusti, A. L., da Silva, W. D., & Tambourgi, D. V. (1999). Ontogenetic development of *Loxosceles intermedia* spider venom. *Toxicon*, *37*(4), 627–632.

de Oliveira, K. C., Gonçalves de Andrade, R. M., Giusti, A. L., da Silva, W. D., & Tambourgi, D. V. (1999). Sex-linked variation of *Loxosceles intermedia* spider venoms. *Toxicon*, *37*(1), 217–221.

Deslippe, R. J., & Guo, Y. J. (2000). Venom alkaloids of fire ants in relation to worker size and age. *Toxicon*, *38*(2), 223–232.

Duan, Z. G., Yan, X. J., He, X. Z., Zhou, H., Chen, P., Cao, R., Xiong, J.X., Hu, W.J., Wang, X.C., & Liang, S. P. (2006). Extraction and protein component analysis of venom from the dissected venom glands of *Latrodectus tredecimguttatus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *145*(3), 350–357.

Escoubas, P., Corzo, G., Whiteley, B. J., Célérier, M. L., & Nakajima, T. (2002). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and high-performance liquid chromatography study of quantitative and qualitative variation in tarantula spider venoms. *Rapid communications in mass spectrometry*, *16*(5), 403–413.

Escoubas, P., Diochot, S., & Corzo, G. (2000). Structure and pharmacology of spider venom neurotoxins. *Biochimie*, *82*(9–10), 893.

Escoubas, P., Sollod, B., & King, G. F. (2006). Venom landscapes: mining the complexity of spider venoms via a combined cDNA and mass spectrometric approach. *Toxicon*, *47*(6), 650–663.

Frontali, N., Ceccarelli, B., Gorio, A., Mauro, A., Siekevitz, P., Tzeng, M. C., & Hurlbut, W. P. (1976). Purification from black widow spider venom of a protein factor causing the depletion of synaptic vesicles at neuromuscular junctions. *The Journal of Cell Biology*, *68*(3), 462–479.

Furtado, M. F. D., Travaglia-Cardoso, S. R., & Rocha, M. M. T. (2006). Sexual dimorphism in venom of *Bothrops jararaca* (Serpentes: Viperidae). *Toxicon*, *48*(4), 401–410.

Gasparini, S., Kiyatkin, N., Drevet, P., Boulain, J. C., Tacnet, F., Ripoche, P., Forest, E., Grishin, E., & Menez, A. (1994). The low molecular weight protein which co-purifies with alpha-latrotoxin is structurally related to crustacean hyperglycemic hormones. *Journal of Biological Chemistry*, *269*(31), 19803–19809.

Graudins, A., Little, M. J., Pineda, S. S., Hains, P. G., King, G. F., Broady, K. W., & Nicholson, G. M. (2012). Cloning and activity of a novel α-latrotoxin from red-back spider venom. *Biochemical Pharmacology*, *83*(1), 170–183.

Grishin, E. V. (1996). Neurotoxin from Black Widow Spider Venom Structure and Function. In *Natural Toxins II* (eds. B.R. Singh and A.T. Tu) (pp. 231–236). Springer, New York, New York, US.

Hale, J. E., Butler, J. P., Gelfanova, V., You, J. S., & Knierman, M. D. (2004). A simplified procedure for the reduction and alkylation of cysteine residues in proteins prior to proteolytic digestion and mass spectral analysis. *Analytical Biochemistry*, *333*(1), 174–181.

Hayes, W. K., Herbert, S. S., Rehling, G. C., & Gennaro, J. F. (2002). Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. *Biology of the Vipers. Eagle Mountain Publishing, Eagle Mountain, UT*, 207–233.

Herzig, V., & Hodgson, W. C. (2009). Intersexual variations in the pharmacological properties of *Coremiocnemis tropix* (Araneae, Theraphosidae) spider venom. *Toxicon*, *53*(2), 196–205.

Herzig, V., Ward, R. J., & dos Santos, W. F. (2004). Ontogenetic changes in *Phoneutria nigriventer* (Araneae, Ctenidae) spider venom. *Toxicon*, *44*(6), 635–640.

Kuhn-Nentwig, L., Stöcklin, R., & Nentwig, W. (2011). Venom composition and strategies in spiders: is everything possible?. *Advances in Insect Physiology*, *40*, 1.

Luch, A. (2010). Mechanistic insights on spider neurotoxins. In *Molecular, Clinical and Environmental Toxicology* (ed. A. Luch) (pp. 293–315). Birkhäuser Basel.

Mackessy, S. P. (1988). Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. *Copeia*, *1988*(1), 92–101.

Mackessy, S. P., Sixberry, N. M., Heyborne, W. H., & Fritts, T. (2006). Venom of the brown treesnake, *Boiga irregularis*: ontogenetic shifts and taxa-specific toxicity. *Toxicon*, *47*(5), 537–548.

Mackessy, S. P., Williams, K., & Ashton, K. G. (2003). Ontogenetic variation in venom composition and diet of Crotalus oreganus concolor: a case of venom paedomorphosis? *Copeia*, *2003*(4), 769–782.

Mahmoudi, N., Modanu, M., Brandt, Y., & Andrade, M. C. (2008). Subtle pedipalp dimorphism: a reliable method for sexing juvenile spiders. *Journal of Arachnology*, *36*(3), 513–517.

Malli, H., Vapenik, Z., & Nentwig, W. (1993). Ontogenetic changes in the toxicity of the venom of the spider *Cupiennius salei* (Araneae, Ctenidae). *Zoologische*

Jahrbücher. Abteilung für allgemeine Zoologie und Physiologie der Tiere, *97*(2), 113–122.

McClounan, S., & Seymour, J. (2012). Venom and cnidome ontogeny of the cubomedusae *Chironex fleckeri*. *Toxicon*, *60*(8), 1335–1341.

McCue, M. D. (2006). Cost of producing venom in three North American pitviper species. *Copeia*, *2006*(4), 818–825.

Menezes, M. C., Furtado, M. F., Travaglia-Cardoso, S. R., Camargo, A., & Serrano, S. M. (2006). Sex-based individual variation of snake venom proteome among eighteen *Bothrops jararaca* siblings. *Toxicon*, *47*(3), 304–312.

Minton, S. A., & Weinstein, S. A. (1986). Geographic and ontogenic variation in venom of the western diamondback rattlesnake (*Crotalus atrox*).*Toxicon*, *24*(1), 71–80.

Monte, A.A., (2012). Black widow spider (*Latrodectus mactans*) antivenom in clinical practice. *Current Pharmaceutical Biotechnology*, *13*(10), 1935–1939.

Morgenstern, D., & King, G. F. (2012). The venom optimization hypothesis revisited. *Toxicon 63*, 120–128.

Nascimento, D. G., Rates, B., Santos, D. M., Verano-Braga, T., Barbosa-Silva, A., Dutra, A. A., Biondi, I., Martin-Eauclaire, M.F., De Lima, M.E., & Pimenta, A.M.C., (2006). Moving pieces in a taxonomic puzzle: venom 2D-LC/MS and data clustering analyses to infer phylogenetic relationships in some scorpions from the Buthidae family (Scorpiones). *Toxicon*, *47*(6), 628–639.

Nentwig, W., & Kuhn-Nentwig, L. (2013). Spider venoms potentially lethal to humans. In *Spider Ecophysiology* (pp. 253–264). Springer Berlin Heidelberg.

Nisani, Z., Boskovic, D. S., Dunbar, S. G., Kelln, W., & Hayes, W. K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon*, *60*, 315–323.

Nisani, Z., Dunbar, S. G., & Hayes, W. K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *147*(2), 509–513.

Richards, D. P., Barlow, A., & Wüster, W. (2012). Venom lethality and diet: Differential responses of natural prey and model organisms to the venom of the saw-scaled vipers (*Echis*). *Toxicon*, *59*(1), 110–116.

Rohou, A., Nield, J., & Ushkaryov, Y. A. (2007). Insecticidal toxins from black widow spider venom. *Toxicon*, *49*(4), 531–549.

Rosenthal, L., Zacchetti, D., Madeddu, L., & Meldolesi, J. (1990). Mode of action of alpha-latrotoxin: role of divalent cations in Ca^2 (+)-dependent and Ca^2 (+)-independent effects mediated by the toxin. *Molecular pharmacology*, *38*(6), 917.

Saez, N. J., Senff, S., Jensen, J. E., Er, S. Y., Herzig, V., Rash, L. D., & King, G. F. (2010). Spider-venom peptides as therapeutics. *Toxins*, *2*(12), 2851–2871.

Stone, L. S., LoPresti, M. B., Crawford, J. M., DeAngelis, R., & Williams, K. R. (1989). Enzymatic digestion of proteins and HPLC peptide isolation. In *A practical guide to protein and peptide purification for microsequencing* (ed. P.T. Matsudaira) pp. 33–47. Academic, San Diego. USA.

Thatcher, L., & Janes, R. (2012). Latrodectism: case report of a katipo spider (*Latrodectus katipo*) bite and review of the literature. *Journal of the New Zealand Medical Association*, *125*(1351).

Tsai, I. H., Chen, Y. H., Wang, Y. M., Liau, M. Y., & Lu, P. J. (2001). Differential expression and geographic variation of the venom phospholipases A_2 of *Calloselasma rhodostoma* and *Trimeresurus mucrosquamatus*. *Archives of Biochemistry and Biophysics*, *387*(2), 257–264.

Underwood, A. H., & Seymour, J. E. (2007). Venom ontogeny, diet and morphology in *Carukia barnesi*, a species of australian box jellyfish that causes Irukandji syndrome. *Toxicon*, *49*(8), 1073–1082.

Ushkaryov, Y. A., Rohou, A., & Sugita, S. (2008). α-Latrotoxin and its receptors. In *Pharmacology of Neurotransmitter Release* (eds. T.C. Sudhof and K. Starke) (pp. 171–206). Springer Berlin Heidelberg.

Volkova, T. M., Pluzhnikov, K. A., Woll, P. G., & Grishin, E. V. (1995). Low molecular weight components from black widow spider venom. *Toxicon*, *33*(4), 483–489.

Yamaji, N., Dai, L., Sugase, K., Andriantsiferana, M., Nakajima, T., & Iwashita, T. (2004). Solution structure of IsTX. *European Journal of Biochemistry*, *271*(19), 3855–3864.

Zelanis, A., Travaglia-Cardoso, S. R., & De Fátima Domingues Furtado, M. (2008). Ontogenetic changes in the venom of *Bothrops insularis* (Serpentes: Viperidae) and its biological implication. *South American Journal of Herpetology*, *3*(1), 43–50.

CHAPTER SEVEN

CONCLUSIONS

In this dissertation, I examined the behavioral ecology of venom use and venom variation in the western widow spider (*Latrodectus hesperus*), emphasizing ontogenetic and sexual differences. To date, my work represents the most comprehensive study of defensive venom use within spiders. In this chapter, I will revisit the most salient conclusions from each chapter.

In Chapter 2, I presented a revision to the definitions of venom, and in doing so also defined poisons and a new class of toxic biological secretions, toxungens. I defined each as:

● ***Poison*** – A toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that results in self-induced toxicity or is passively transferred without a delivery mechanism from one organism to the internal milieu of another organism without mechanical injury, usually through ingestion, inhalation, or absorption across the body surface.

● ***Toxungen*** – A toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that is actively transferred via a delivery mechanism from one organism to the external surface of another organism without mechanical injury

● ***Venom*** – A toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that is passively or actively transferred from one organism to the internal milieu of another organism via a delivery mechanism and mechanical injury.

I further proposed a system to classify toxic organisms with respect to delivery mechanism (absent versus present), source (autogenous versus heterogenous), and storage of toxins (aglandular versus glandular).

In Chapter 3, I investigated risk assessment and venom use in the context of threat. In order to test this, I performed two experiments. In experiment 1, spiders were subjected to three different threat levels. I found that poking at the lowest threat level elicited primarily avoidance responses ("move" and "retract"), but silk-flicking increased at moderate threat with repeated prodding. Pinching at the highest level of threat provoked significantly more biting. In experiment 2, spiders modulated venom expenditure by delivering 2.2-fold more venom per bite when pinched on the body compared to having a leg pinched. Spiders also expended 2.3-fold more venom when the target presentation was separated by a long interval (5 min) rather than a short interval (5 sec). Because silk and venom require a metabolic cost to replace, they can be viewed as limited commodities that should be used judiciously. Collectively, these findings support the growing body of literature showing that animals have the ability to cognitively meter their venom. This is also the first study to demonstrate that spiders actively control venom expulsion during defensive interactions.

In Chapter 4, I investigated ontogenetic and sexual variation in defensive behaviors, as well as possible habituation, testing three hypotheses. Consistent with the first hypothesis, spiders relied largely on non-combative behaviors early in life and switched to more combative behaviors, including silk flicking and biting, as they increased in size. As predicted by the second hypothesis, age exerted a much greater influence than sex differences for males and females within the range of equivalent body

sizes. Consistent with the third hypothesis, spiders habituated to the repeated testing by exhibiting fewer combative behaviors than naïve spiders upon reaching adult size. Collectively, these findings suggest that selection has favored age-specific antipredator strategies that can be modified by experience.

In Chapter 5, I developed an ethogram of *L. hesperus* prey capture of the house cricket *Acheta domestica*. The predatory sequence was divided into three phases: detection, immobilization, and prey manipulation. The detection phase (mean duration 387.6 sec) was characterized by initial detection of a potential prey item followed by behaviors that resulted in location of prey and subsequent approach of the spider. During the immobilization phase (mean duration 13.0 sec), spiders trapped and secured their prey largely by means of silk wrapping. During the subsequent prey manipulation phase (mean duration 1392.7 sec), spiders further secured, bit, prepared, and transported their prey off of the substrate. Spiders delivered an average of 15.2 (range 0–31) short bites, with initial bites primarily to a leg. In all, 21 behaviors were defined across phases. Three major behavioral loops involved repeated sets of behaviors, with a distinct loop exhibited in the detection phase and two loops in the prey manipulation phase. The behaviors and sequences observed were similar to those reported for other members of the Theridiidae family, with many behaviors also resembling those observed in other spider families. The findings provide a basis for designing future studies.

In Chapter 6, I investigated ontogenetic changes and sexual differences in the venom of *L. hesperus*. Initial results suggest that female venom becomes increasingly complex with age, while the hypdrophobic components of male venom become less complex with maturity. However, because of limitations to the existing venom databases

and techniques used to identify specific protein components, we expect to subject additional venom samples to LC-MALDI and may also run digested whole venom directly on LC-MS/MS (a shotgun approach) to confirm the ontogenetic and sexual differences that our limited analyses suggest.

Future Directions

The findings of this study have raised many interesting questions that merit further investigation. I am most interested in the following future projects. We have barely begun to understand the cognitive ability of animals, and silk and venom use offer an excellent opportunity to investigate how animals think, learn, and/or interact with their environment. Venom metering has been studied in numerous organisms; however, silk metering has not. Future studies should investigate silk metering, both in a defensive and predatory context. I would like to investigate learning, by testing how the capture of novel prey items changes with experience, again focusing on silk and venom use. Great variation in the amount of venom expended during a bite was observed in Chapter 3. Future research should investigate if the toxins contained in biologically relevant doses of venom changes as the spider depletes its venom gland. Venom heterogeneity is well documented for scorpions, with the first portion of venom that emerges being potassium rich to induce pain, and subsequent venom being protein rich and more toxic (Inceoglu et al. 2003; Nisani and Hayes 2011). Recent work has suggested that venom heterogeneity may also be present in spiders (Morgenstern et al. 2012). Many more studies may be inspired, at least in part, from this dissertation; this will be a task to keep me and perhaps others busy for years to come.

References

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., Pessah, I. N., & Hammock, B. D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proceedings of the National Academy of Sciences*, *100*(3), 922–927.

Morgenstern, D., Hamilton, B., Sher, D., Jones, A., Mattius, G., Zlotkin, E., Venter, D., & King, G. F. (2012). The bio-logic of venom complexity. In *Toxicon* (Vol. 60, No. 2, pp. 241–242). Pergamon, Philadelphia, Pennsylvania, USA.

Nisani, Z., & Hayes, W. K. (2011). Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering. *Animal Behaviour*, *81*(3), 627–633.