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Exploring the Relationship Between Behaviour and Neurochemistry in the Polyphenic Spider,  
*Anelosimus studiosus* (Araneae: Theridiidae)

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A dissertation

Presented to

the faculty of the Department of Biomedical Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

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by

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August 2016

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## ABSTRACT

Exploring the Relationship Between Behaviour and Neurochemistry in the Polyphenic Spider,  
*Anelosimus studiosus* (Araneae: Theridiidae)

by

Jennifer Bryson Price

The importance of social behaviour is evident in human society, but there are both costs *and* benefits associated with cooperation and sociality throughout the animal kingdom. At what point do the benefits outweigh the costs, and when do selective pressures favour sociality and colonization over solitude and independence? To investigate these questions, we have focused on an anomalous species of spider, *Anelosimus studiosus*, also known now as the northern social spider. Throughout its broad range, *A. studiosus* is solitary and aggressive, but recently, colonies of cooperative and social individuals have been observed at northern latitudes. This leads to two research questions: 1) what characteristics differentiate the two variants behaviourally, and, 2) how are they different physiologically? Colonies and individuals were collected from multiple populations throughout the Tennessee River watershed area and maintained in a laboratory environment for quantitative and qualitative assessment of behavioural traits as well as specific neurochemical analysis by high performance liquid chromatography with electrochemical detection. After classifying individuals as social or aggressive, I looked at the influence of factors such as age, reproductive state, nutritional state, and time of day on behaviour and neurophysiology. I found correlations between social behaviours and serotonin, aggressive behaviours and octopamine (invertebrate counterpart of norepinephrine), and several other compounds associated with an increase or decrease in aggression. These studies combine techniques from multiple disciplines to contribute to the greater understanding of the proximate

control of social and aggressive behaviours as well as factors influencing the evolution of sociality.

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## CHAPTER 1

### INTRODUCTION

“I have been increasingly impressed with the functional similarities between invertebrate and vertebrate societies and less so with the structural differences that seem, at first glance, to constitute such an immense gulf between them.” ~E.O. Wilson, Sociobiology, 1975

#### Preface

Animals, including humans, display an amazing range of behaviors. Why do some behave in an aggressive manner while other members of the same species cooperate? Behavior is influenced by many external and internal factors but is ultimately governed by complex neurological and physiological processes. Studying both significant and subtle behavioral differences among members of the same species, along with the corresponding underlying changes in neurochemical levels, can lend insight into the complex relationship between neurobiology and behavior. Our lab uses an arthropod model - spiders - to study ethology, behavioral ecology, circadian biology, behavioral neurobiology, and comparative physiology. Spiders have not extensively been used as model systems for behavioral neuroscience, but we have found them to be ideal models for several reasons mentioned in this chapter. We believe that our investigations of this model system will provide insight into the proximate mechanisms of social and aggressive behaviors in spiders and, ultimately, into the evolution of sociality.

#### Concepts from Sociobiology

The systematic investigation of the gradual development of social systems within species requires at least moderate knowledge and consideration of many concepts in Biology and social sciences. The study of biological factors influencing social interactions among organisms, known as sociobiology, combines information and methods from multiple fields such as integrative

neurophysiology, evolutionary biology, behavioral ecology, ethology, and sociology (Wilson 1975b), among others. Edward O. Wilson consolidated information, theory, and analytical tools from these fields to construct a more solid framework within which researchers could study social behavior and delineate mechanisms of social evolution. As Wilson did in his writings, I believe it is useful to clarify the social terminology used heretofore. Following is a table of terms and definitions (Table 1) adapted from Wilson’s book, Sociobiology (Wilson 1975b).

**Table 1.1: Terminology from Sociobiology**

Society	Group of individuals of the same species, organized in a cooperative manner
Aggregation	Group of individuals of the same species, gathered in the same place but not organized or engaged in cooperative behavior
Colony	A society of organisms which are highly integrated and fixed in one locality
Group	A set of organisms of the same species remaining together for any period of time while interacting with one another to a much greater degree than with other conspecifics
Deme	A local population in which breeding is completely random
Demographic Society	A society that is stable enough through time for the processes of death and birth to play a significant role in its composition.
Casual Society	A temporary group of individuals within a society; unstable and open to new members and losing old members at a high rate
Population	Group of organisms of the same species occupying a clearly delimited area at the same time
Communication	Action of one organism that alters the behavior of another organism in an adaptive fashion
Coordination	Interaction among units of a group, without leadership being assumed by any one of them, in such a way that overall effort is divided among units
Hierarchy	A system in which higher levels control, at least to some extent, the activities of the lower levels in order to integrate the group as a whole; sometimes involves dominance of one unit over another
Sociality	The combined properties and processes of social existence
Behavioral Scaling	Range of forms and intensities of a behavior expressed in an adaptive fashion by a society or individual organism

A trait, such as a particular behavior, may be considered adaptive or nonadaptive, depending on environmental circumstances. If the trait or behavior consistently increases fitness of the individuals possessing it under environmental conditions that are normal or typical for that species, it is considered adaptive. Conversely, if the trait or behavior decreases fitness it is considered nonadaptive or maladaptive (Wilson 1975b). But what happens when environmental factors change, or members of a species migrate out of their traditional range into geographical areas where different environmental conditions exist? The trait that was previously considered “abnormal” or maladaptive may then be beneficial or suddenly considered adaptive. In this case, flexibility in response, or phenotypic plasticity, can increase the survival, and therefore the fitness, of the individual or group. This concept is central to understanding the role of natural selection in the evolution of sociality.

### Primary Factors in Social Evolution

There are two forces which play major roles in the development of social behavior within a population: phylogenetic inertia and ecological pressure. In general, social evolution occurs when a population responds genetically to ecological pressures within the boundaries set forth by its phylogenetic inertia (Wilson 1975b).

*Phylogenetic inertia* is a combination of genetic factors and previous adaptations, or preadaptations, which make certain evolutionary changes more or less likely to occur. A preadaptation is the existence of a particular behavior which has been beneficial in some context in past and can lay the foundation for that behavior to be amplified or developed further to provide even more of a benefit for the individual or group exhibiting it. In order for a group or population to respond to selection, it must possess a degree of phenotypic variability which is heritable. The more heritable variation in a population, the more quickly it can respond to



selection and evolve. As is implied by the inclusion of the word *inertia*, high phylogenetic inertia is associated with greater resistance to evolutionary change, and low phylogenetic inertia is associated with lower resistance to evolutionary change. In reference to a particular trait, low phylogenetic inertia would be associated with a preadaptation.

*Ecological pressure* refers to all of the environmental factors which may act as selective forces upon a population. In the case of social evolution, certain benefits can be derived from group living and cooperation: defense against predators, competitive advantage over other species in resource acquisition, ability to obtain and feed upon more or larger prey, entrance into and ownership of novel habitat, increased reproduction and survival, better population stability, and alteration of physical environment to better serve individual and group needs (Wilson 1975b).

If, at some point, the ecological pressures that influenced selection of social behavior are removed or relaxed, or if the more antisocial behavior is favored by the environment, the effects of social evolution may be reversed, and the population or species may move back toward the less social or antisocial state. This has occurred in two species of bees, one in the genus *Exoneurella* (Michener 1964) and one in halictine sweat bees (Michener 1969), and in a species of weaverbirds (Crook 1964), among others.

The understanding of these three concepts (phylogenetic inertia, ecological pressures, and reversibility in social evolution) is crucial to appreciating the overarching questions and hypotheses of this dissertation.

## Concepts from Behavioral Neurobiology

Sometimes, an organism's response to an environmental stimulus is automatic and fixed, as in a reflex. However, organisms are capable of varied responses to the same environmental stimulus. Neural networks control an organism's responses to stimuli, and they do not always operate in a direct input→ output relationship. Control and mediation by internal factors can alter the output response. This influence is referred to as *motivation*. "Motivational changes in behavior are possible only if the neural network underlying this behavior exhibits the potential for neural plasticity. This is not the case in 'ideal reflex'." (Zupanc 2004) One of the major categories of neural mechanisms responsible for mediating motivational changes is termed *biochemical switching of neural networks*.

Studies of the American lobster (*Homarus americanus*) have focused on mechanisms of biochemical switching in a relatively simple nervous system. Release of different neurotransmitters and neuromodulators, including serotonin, from different presynaptic neurons serve as differential input signals to the same postsynaptic neuron, and therefore produce different firing patterns and different output responses. This shows that a single neural network is capable of producing variable responses under different circumstances.

Effects of neuromodulators themselves can be modulated by changes in the number of their specific receptors at different sites within the nervous system. This can be influenced by social experience and learning, as was found with a study in crayfish in which the modulatory effect of serotonin on the lateral giant interneuron (controlling escape) increased the tendency to escape in dominant crayfish, while it decreased that tendency in subordinate crayfish (Yeh et al. 1997).

## Behavior and Biogenic Amines in Arthropods

Neurotransmitters are chemicals within an organism which can promote or suppress signaling within the nervous system. Biogenic amines fall within this category and are biological compounds capable of inducing broad or specific effects. They can act as excitatory or inhibitory effectors upon the central and/or peripheral nervous system, causing an increase or decrease in particular physiological processes (Slaughter 2002). There are several biogenic amines associated with modulation of behavior: norepinephrine, epinephrine, dopamine, 5-hydroxytryptamine (5-HT or serotonin), and octopamine (Kakimoto and Armstrong 1962; Panksepp et al. 2003; Schulz et al. 2003; Waymire 2013).

Octopamine and serotonin are known to modulate aggression-related behaviors in invertebrates (Kravitz 1988). It has been suggested that octopamine modulates almost every physiological process in invertebrates, and it is considered homologous to the noradrenergic system in vertebrates (Roeder 1999). The effects of these neurochemicals can vary across taxa. For instance, octopamine increases aggression in *Drosophila* (Baier et al. 2002; Hoyer et al. 2008), crickets (Stevenson et al. 2005), the spider *Larinioides cornutus* (Jones et al. 2011), and some species of crayfish (Kravitz and Huber 2003b), but it decreases aggression in other crayfish species (Kravitz and Huber 2003b) and in squat lobsters (Antonsen and Paul 1997). Octopamine is released when energy-demanding behaviors such as sustained flight (in flying insects), fights, predatory attacks, or anti-predator escape maneuvers are needed (Roeder 1999). Octopamine has a broad range of effects in honeybees, including modulation of dance behavior (Barron et al. 2007), sucrose responsiveness (Scheiner et al. 2002), and age-related division of labor (Schulz and Robinson 2001). In some cases, serotonin has been shown to have an opposite modulatory effect. In crayfish, octopamine enhances an escape response, but serotonin suppresses the

response (Glanzman and Krasne 1983). Exogenous administration of serotonin into crayfish hemolymph increases their levels of aggression and lengthens the time of aggressive interactions (Huber et al. 1997; Huber and Delago 1998; Kravitz and Huber 2003b).

Both social and non-social invertebrates offer powerful model systems for studying the effects of biogenic amines on aggression (Kravitz and Huber 2003a). Octopamine null *Drosophila* mutants showed greatly reduced aggression (Baier et al. 2002), and in the same study, serotonin was found to have no effect on aggression. However, another study found that increased levels of serotonin in the *Drosophila* brain increased aggressive behavior (Dierick and Greenspan 2007). Serotonin is responsible for an extreme behavioral transformation in desert locusts, causing a switch from solitary to group behavior within a matter of hours, with the major behavioral change being loss of aversion to conspecifics (Anstey et al. 2009).

## Spider Behavior

### Sociality in Spiders

Spiders form a diverse group of organisms with an impressive array of behaviors. We are particularly interested in ultimate and proximate causes of social behavior. Of the currently known 45,756 species of spiders (Platnick 2015), the vast majority are solitary, aggressive, and even cannibalistic, but approximately 25 species across eight families have evolved to exhibit varying levels of social behavior (Agnarsson et al. 2006; Bilde and Lubin 2011). Most of these are quasisocial (Table 2), meaning that there is cooperation in brood care among adults of the species (Batra 1966; Wilson 1975a; Aviles 1997; Furey 1998). Nearly all of these ~25 social species are constrained geographically to tropical and subtropical regions, but in 1998, the first quasisocial species in a temperate region was described in East Tennessee (Furey 1998):

*Anelosimus studiosus*.

**Table 1.2: Social Terminology for *Anelosimus studiosus***

Quasisocial	Species exhibits cooperation among adults in brood care.
Docile	Used to describe behavioral type of an individual which is tolerant of conspecifics, shares web space, and cooperates in brood care and prey capture.
Aggressive	Used to describe behavior of individual spider which is territorial and aggressive toward conspecifics.
Social	Colony type consisting of multiple adult female <i>A. studiosus</i> and their adult and subadult offspring.
Subsocial/Solitary	Colony type consisting of a single adult <i>A. studiosus</i> and her subadult offspring.

*Anelosimus studiosus*

*Anelosimus studiosus* is a quasisocial species (*sensu* Wilson, 1971), and it is unique because it consists of both docile and aggressive individuals which form either social or subsocial (solitary) colonies. All adult females of the species will guard their egg sacs and care for their broods until near adulthood, at which time the offspring from subsocial colonies will disperse from the natal web, while offspring in social colonies will remain in close proximity and share web space with female relatives. The docile individuals within this species differ from the aggressive individuals only by the degree to which they tolerate conspecifics. More simply stated, the “aggressive” behavioral type in *A. studiosus* are more bold, assertive, and combative - sometimes fatally so - toward other adult females, her own adult (and sometimes sub-adult) offspring, as well as potential mates. The docile individuals display greater tolerance for one another, and therefore can form colonies consisting of anywhere from two to over 100 adult females and their offspring (Furey 1998; Jones and Riechert 2008). The females within these colonies cooperate in brood care, web maintenance, and prey capture. However, colonies are composed of individuals with varying degrees of tolerance and aggression. Some docile individuals are more aggressive than other docile individuals, and some aggressive individuals

are more passive or tolerant than other aggressive individuals. Social colonies can be composed of both docile and aggressive individuals (Pruitt and Riechert 2011), and docile individuals can be founders of subsocial colonies (Jones et al. 2010).

*Anelosimus studiosus* spans a large geographic area, from Argentina in South America to New England in North America, but, interestingly, social colonies are only found north of ~26° N Latitude. The proportion of social colonies increases with distance from the equator (Jones et al. 2007; Riechert and Jones 2008), a phenomenon known as behavioral cline. Anecdotal evidence suggests a similar occurrence in the southern hemisphere (Carmen Viera), but there are not yet empirical data to support this observation.

#### Animal Personality and Behavioral Syndromes

The study of individual variation in behavior among members of a population or a species has led to a recent increase in the number of publications focusing on ecological implications of animal personality and behavioral syndromes. Behavioral syndromes are arrays of behaviors that are correlated across situations. Evidence suggests that aggressiveness is associated with boldness; i.e., a spider which is aggressive toward a mate or a conspecific is more likely to attack an intruder of another species in its web and recover more quickly from a perceived attack (Sih et al. 2004; Sih et al. 2012; Johnson and Sih 2007; McElreath et al. 2007). Likewise, a spider that exhibits greater social tolerance is likely to be more fearful of intruders and remain in a huddled anti-predator posture following a perceived attack (Riechert and Hedrick 1993; Pruitt et al. 2012). This has been shown in *Anelosimus studiosus* (Perkins et al. 2007; Pruitt et al. 2008; Pruitt and Riechert 2009; Price et al. 2010; Pruitt and Riechert 2011; Pruitt et al. 2011; Pruitt et al. 2012; Watts et al. 2013; Keiser and Pruitt 2014), among other spider species, and is corroborated by findings of studies presented in this dissertation.

These dispositions (behavioral phenotypes) have been shown to be, at least in part, heritable (Duncan et al. 2010; Jones et al. 2010; Pruitt and Riechert 2011). The most recent heritability estimate is 0.32 (Pruitt et al. 2008; Pruitt and Riechert 2009).

### Spiders as Behavioral Model Systems

Animal models are not usually studied as a means of elucidating the mechanisms responsible for observed behaviors in that species alone; they are investigated to provide researchers with basic knowledge which may apply to many, or even all, animal systems (Zupanc 2004). Nature has a way, after all, of finding a limited number of solutions to any given problem. Animals with nervous systems ranging from the most simple to the most complex have given us a grand foundation of basic knowledge and inspired the formulation of multitudes of important observations and hypotheses. Let us not discount spiders on the account of a general disdain for their existence.

Due to the relatively small size of their brains and the belief that their behaviors are reflexive and innate, spiders have historically been underestimated and underappreciated as suitable models for behavioral studies. On the contrary, spiders, like other important animal models, exhibit behavioral plasticity and individual variability in situational contexts (Herberstein 2011). Moreover, there are several practical reasons that spiders serve as excellent model organisms: 1) Spiders are somewhat easy to collect and inexpensive to maintain in the laboratory, 2) research involving spiders does not require the involvement of the IRB or UCAC, and 3) spider behavior is easily quantifiable.

### Quantifying and Classifying Behaviour in Spiders

Latency to attack (LTA) is a measure of the amount of time in seconds (s) that passes from the moment a prey item contacts the spider's web until the moment the spider contacts the

prey item. Huddle duration (HD) is a measure of the amount of time in seconds (s) that a spider remains in thanatosis in response to a stimulus (a puff of air), also known as antipredator behavior. Social tendency (ST) is assessed by placing an individual of unknown disposition into a 16cm X 16 cm square container with a known docile/social individual for 24 hours. If the unknown spider positions itself in the same corner as the docile individual, it is likewise labeled as docile, but if it rests in an adjacent or opposite corner, it is labeled as aggressive/solitary. Due to these distinct behavioral differences, and for the sake of clarity, we will henceforth refer to the subsocial behavioral type as “aggressive” and the social behavioral type as “docile.”

### Behavioral Neurochemistry in Spiders

Not much research has been focused on neurochemistry of spiders, especially as it relates to behavior. Outside of studies conducted in our lab or in collaboration with our lab, only one study has focused on biogenic amines and behavior in spiders (Punzo and Punzo 2001). A few others have looked at specific neurotransmitters (Seyfarth et al. 1993), but not as they relate to behavior.

In 2010, we used high performance liquid chromatography with electrochemical detection (HPLC-ECD) to analyze and compare *A. studiosus* biogenic amine levels between behavioral types at the individual level, the colony level, and the population level. Significant differences in specific biogenic amines (octopamine and serotonin) were found between aggressive and docile individuals, between social and subsocial colonies, and among populations (Price et al. 2010). In general, serotonin was higher in social colonies and docile individuals, and octopamine was higher in subsocial colonies and solitary individuals. There were also significant differences in these biogenic amines among populations.



In other studies, alteration of biogenic amine (octopamine and serotonin) levels were found to induce significant changes in anti-predator behavior in *Larinioides cornutus* (Jones et al. 2011), and octopamine was also found to be correlated with more direct and aggressive male mating tactics in the spider *Rabidosa punctulata* (Hebets et al. 2015).

## Questions and Hypotheses

### Overarching Concept

Based on the above observations, we formulated an overarching question: Do variable internal and external factors influence neurochemical and behavioral changes in individual spiders, or do they possess genetically predetermined, static levels of aggression and biogenic amine production? *Our overarching hypothesis is that different conditions (i.e. time of day, nutritional state, reproductive state, age) elicit adjustments in behaviors, and these differences reflect underlying changes in neurochemistry.*

### Experimental Foci

In order to focus on separate factors, we asked several specific questions:

Q1: Does the relationship between an external factor such as prey density and an internal factor (nutritional state) influence social/aggressive behavior and biogenic amine levels?

Q2: Do natural oscillations in both behaviors and neurochemical levels occur throughout the day? Are there diel rhythms in neurochemistry?

Q3: Do behaviors and neurochemicals change with reproductive state?

Q4: Does behavior and neurochemistry change as a spider develops to determine its eventual personality and social characteristics?

Nutritional State (Q1). Studies have shown that when spiders are deprived of food, they perform riskier behaviors (Walker and Rypstra 2003), become more cannibalistic (Petersen et al. 2010), and are less likely to reproduce (Ebert 1998). Although there have been a few studies on the effects of hunger on behavior in spiders, there have been none investigating the associated changes in neurochemistry. We hypothesize that poor nutritional state will increase aggressiveness, while satiety will decrease aggressive behavior. These behavioral changes will be accompanied by changes in neurochemistry.

Diel Rhythms (Q2). A recent study by Watts *et al.* (2013) has shown that *A. studiosus* are more aggressive in the evening than in the morning. For our study, we hypothesized that aggression-related neurochemicals should be higher at the time of day when it most benefits the spider to be aggressive (which should coincide with the time of greatest prey density), and they should be lower at the time of day that it most benefits the spider to be fearful (coincides with the greatest predator density).

Reproductive State (Q3). The study by Watts *et al.* (2013) also found that brooding female *A. studiosus* are more aggressive than their nonbrooding counterparts. We hypothesize that aggression-related neurochemicals will be higher in brooding females (guarding egg case) than in non-brooding females, because the brooding females must be very vigilant and protective of their greatest investment.

Ontogeny (Q4). Female *A. studiosus* exhibit extended maternal care by maintaining their young in the natal web and regurgatively feeding them for the first few instars (molts). At that time, the young will either disperse as solitary, independent individuals, or they will remain nearby as part of the social colony, depending on their genetic makeup. Up until time of dispersal, the offspring must tolerate one another and be, to some degree, social. We hypothesize

that behavior and neurochemistry of both genotypically docile and genotypically aggressive variants will remain similar during the first 3 to 4 instars, at which time there will be a gradual divergence toward higher aggression levels and higher aggression-related neurochemicals in the individuals that are to become solitary/aggressive phenotypes, while there will be a less dramatic change in the social/docile individuals.

### General Research Methods

Colonies of *A. studiosus* were collected from multiple populations along waterways within the Tennessee River watershed area and maintained in our laboratory facility in 12:12 light: dark conditions at 20-22 degrees Celsius. Spiders were either separated into individual containers or kept together as colonies in large containers, depending upon the experiment in which they were included. All spiders were misted with water every 2-3 days and fed a diet of termites according to individual experimental protocol. Behavioral assays were conducted in the laboratory as described in each of the following chapters to classify and quantify behaviors of individual spiders. At the end of each behavioral study, spiders were weighed (body mass, g), frozen in liquid nitrogen, and prepared for HPLC analysis according to protocols described in each experimental methods section of this dissertation. Statistical analysis of behavioral and neurochemical data was performed using Excel (Microsoft Office 2013), Minitab 16, and SPSS 21 (IBM).

### Evolution of Sociality in Spiders: Motivation for Studying *Anelosimus studiosus*

“True understanding of present developments is possible only by knowing the past.”

~Gunther Zapanc

In the family Theridiidae, the family to which *Anelosimus studiosus* belongs, sociality has evolved independently eight to nine times. The trait of extended maternal care exhibited by this

clade is most likely the preadaptation which makes the transition to sociality more easily achieved. The social groups in this family tend to become highly inbred and have female-biased sex ratios. Short-term benefits of group living and sociality are eventually outweighed by the evolutionary costs of inbreeding, and the social colonies collapse and eventually die out (Agnarsson et al. 2006). The relatively recent appearance of social colonies of the species *Anelosimus studiosus*, coupled with observations stated in the following pages, lead us to believe that this species is currently evolving sociality in cooler climates. Evidence of past extinctions of social sister species may indicate that this phenomenon of emerging sociality will be short-lived in our study populations. We have a unique, and perhaps fleeting, opportunity to investigate internal and external forces which may drive the evolution, and eventual disappearance, of sociality in an ancestrally subsocial species.

## CHAPTER 2

# NUTRITIONAL STATE, REPRODUCTIVE STATE, AND TIME OF DAY ARE ASSOCIATED WITH CHANGES IN NEUROCHEMICAL LEVELS AND AGGRESSION IN BOTH DOCILE AND AGGRESSIVE VARIANTS OF THE SPIDER, *ANELOSIMUS STUDIOSUS* (ARANEAE:THERIDIIDAE)

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### Abstract

Spiders alter their behaviors in response to changing environmental factors and physiological states, making them excellent models for the study of behavioral plasticity. Several biogenic amines have been suggested to play a role in modulating behaviors in other arthropods. We hypothesized that spiders deprived of prey would be more aggressive than their well-fed counterparts, and behavioral differences associated with nutritional state, reproductive state, and time of day would be correlated with differences in endogenous levels of one or more biogenic amines. We tested these hypotheses using both docile and aggressive variants of the spider *Anelosimus studiosus* found in the southeastern United States. Adult females were collected from waterways along the Tennessee River watershed, conditioned in our laboratory, and behaviorally scored. Spiders were divided into “well-fed” and “starved” subsets and brooding and nonbrooding subsets. Whole-body extractions of biogenic amines were analyzed for neurochemical differences using high pressure liquid chromatography. Subjects of the reproductive state study were sacrificed at either dawn or dusk to assess neurochemical

differences attributable to time of day. Behavioral differences were found between well-fed and starved spiders and between brooders and nonbrooders. Biogenic amines were different between groups and from dawn to dusk. Octopamine was generally associated with increased aggression, while serotonin was correlated with decreased aggression and increased wariness. Studies of this nature can help to explore the physiological effects of environmental factors and gain insight into the proximate mechanisms of behavior.

## Introduction

### Spider Behavior

Spiders, like many animals, must balance the constant challenges of attaining prey and avoiding predation. They must have the ability to be both aggressive and wary. For instance, variation in prey density can influence spiders to become more or less reactive and/or aggressive in their pursuit and capture of individual prey items. Observations of the polyphenic spider, *Anelosimus studiosus*, have shown that they are more aggressive at certain times of the day, more defensive at other times of the day, and that brooding females are generally more aggressive than nonbrooding females. However, underlying physiological mechanisms responsible for this dynamic approach to survival and factors influencing these behavioral changes have not been well-studied. Reliable methods for evoking, observing, and quantifying both aggressive and defensive (protective) behaviors in spiders have been developed and used to study several spider species, including *Anelosimus studiosus*. Findings from those studies, along with results from several of our recent experiments involving biogenic amines in spiders, served as the basis for these investigations.

Latency to attack prey (LTA), time it takes for a spider to make physical contact with a prey item in its web, can be used as a continuous measure of a spider aggressiveness. When a

prey item is introduced into the web of a docile or more fearful spider, the spider will take longer to assess the situation and attack time will be delayed compared to that of a more aggressive spider. Another measure of aggressiveness and/or fearfulness is huddle duration (HD). When spiders perceive a threat or attack from a potential predator, they display thanatosis, or a huddle response, in which they draw in their legs and remain motionless for a period of time. Aggressive spiders typically display this posture for shorter durations than docile spiders, and in some cases may not huddle at all.

### Biogenic Amines and Aggression

Biogenic amines are physiological compounds that can have excitatory or inhibitory effects on the central or peripheral nervous systems of animals, causing an increase or decrease in particular physiological processes (Slaughter 2002). There are several biogenic amines known to affect behavior: norepinephrine, epinephrine, dopamine, 5-hydroxytryptamine (5-HT or serotonin), and octopamine (Kakimoto and Armstrong 1962; Panksepp et al. 2003; Schulz et al. 2003; Waymire 2013).

Octopamine and serotonin have been shown to modulate aggression-related behaviors in invertebrates (Kravitz 1988), but the effects of these neurochemicals can vary across taxa. For instance, octopamine increases aggression in *Drosophila* (Baier et al. 2002) (Hoyer et al. 2008), crickets, *Gryllus bimaculatus* (Stevenson et al. 2005), the spider *Larinioides cornutus* (Jones et al. 2011), and some species of crayfish (Kravitz and Huber 2003b), but it decreases aggression in other crayfish species (Kravitz and Huber 2003b) and in squat lobsters (Antonsen and Paul 1997). Octopamine is released when high-energy behaviors such as sustained flight (in flying insects), fights, predatory attacks, or evasion maneuvers are called for (Roeder 1999). Octopamine has several effects in honeybees (*Apis mellifera*), including modulation of dance

behavior (Barron et al. 2007), sucrose responsiveness (Scheiner et al. 2002), and age-related division of labor (Schulz and Robinson 2001). In some cases, serotonin has been shown to oppose the effects of octopamine. In crayfish, for instance, octopamine enhances an escape response, but serotonin suppresses the response (Glanzman and Krasne 1983). However, another study found that increased levels of serotonin in the *Drosophila* brain increased aggressive behavior (Dierick and Greenspan 2007). Serotonin (5-HT) has been shown to increase aggression in some invertebrates and decrease it in others. Serotonin increases aggression competitive interactions of stalk-eyed flies *Teleopsis dalmanni* (Bubak et al. 2013) and fruit flies *Drosophila melanogaster* (Alekseyenko et al. 2010), but it induces a switch from solitary to gregarious behavior in the desert locust *Schistocerca gregaria* (Anstey et al. 2009). Octopamine and serotonin have opposite effects on anti-predator behavior in the spiders *Larinioides cornutus* (Jones et al. 2011) and *Anelosimus studiosus* (Jones, unpublished data). In a previous study, we found that social variants of the spider *Anelosimus studiosus* had higher naturally-occurring levels of serotonin and lower levels of octopamine than solitary variants within the same local population (Price et al. 2010).

Invertebrates displaying social and/or aggressive behaviors can be strong model systems for studying the effects of biogenic amines on aggression (Kravitz and Huber 2003a) and perhaps offer insight into mechanisms underlying evolution of sociality.

### Nutritional State

Energy budgets demand that acquisition of food and conservation of energy be priorities for many animals. Web-building spiders typically sit and wait for prey to enter their web, while other spiders wander and hunt for prey. Spiders build their webs or hunt in areas where they are most likely to find suitable prey, but natural variation in factors such as prey abundance and



density of predators competing for the same prey can affect the availability of food sources and, therefore, the overall nutritional state of a spider. How does this variation affect the behavior of a spider? When prey is scarce, and nutritional state diminishes, is it advantageous for the spider to become more aggressive and expend energy that it may not be able to replenish? Or is it a better strategy to decrease energy expenditure by sitting and waiting for a closer encounter and higher probability of capture?

Studies have shown that when spiders are deprived of food, they perform riskier behaviors (Walker and Rypstra 2003), become more cannibalistic (Petersen et al. 2010), and are less likely to reproduce (Ebert 1998). Although there have been a few studies on the effects of hunger on behavior in spiders, there have been none investigating the associated changes in neurochemistry.

With these findings in mind, we ask, 1) how do changes in nutritional state influence a spider's responsiveness to prey, and 2) are there associated changes in aggression-related biogenic amine levels? We hypothesize that poor nutritional state will increase responsiveness to, and aggressiveness toward, prey, while satiety will decrease responsiveness, and these behavioral changes will be accompanied by changes in biogenic amine levels.

In this study, we look for differences in aggressive behavior and neurochemistry associated with differences in nutritional state between groups. We focus specifically on serotonin and octopamine because of their well-studied effects in other invertebrates and because our previous studies implicate them as possible modulators of social and aggressive behavior in this species, *Anelosimus studiosus*.

## Diel Rhythms and Reproductive State in Spiders

Ideally, spiders would always be aggressive toward prey and defensive when predators are present, but there exist overlaps in the presence of predators and prey. Since spiders rely heavily upon vibrations for the detection of both, the identity of the source is not always immediately clear. Moving to attack and secure prey can draw unwanted attention from predators, so spiders must have a strategy in order to minimize risk and maximize gain. The response to vibratory cues needs to be modulated. They must be alert and aggressive at times of the day when prey encounters are most likely, but wary and defensive when predators are abundant.

*Anelosimus studiosus* is a unique species that displays one of two basic behavioral types, aggressive/subsocial or docile/social. Aggressive/subsocial individuals are typically solitary but exhibit extended maternal care of offspring, while docile/social individuals continue to cooperate as adults in prey capture, web maintenance, and brood rearing. A recent study (Watts et al. 2013) showed that adult females of both behavioral types the species *Anelosimus studiosus* display a diel rhythm in aggressiveness, being more aggressive in the evening and less aggressive in the morning. These aggression scores were shown to correlate with prey densities. The same study (Watts et al. 2013) showed that brooding females (those with egg cases) are more aggressive than their nonbrooding counterparts.

With these findings in mind, we hypothesize that there are differences in biogenic amine levels between brooders and nonbrooders. More specifically, we predict that octopamine will correlate with aggressive behavior and that serotonin will correlate with docile behavior. We also hypothesize that the levels of octopamine and serotonin will change from dawn to dusk in both

brooding and nonbrooding females. We expect that there will be differences between spiders from social and subsocial colonies.

In this portion of the study, we quantified behavioral tendencies of 78 adult female *Anelosimus studiosus* and then used high performance liquid chromatography with electrochemical detection (HPLC-ECD) to analyze whole-spider extractions of biogenic amines. We compared behavior scores with amine levels of social and subsocial brooders and nonbrooders, at different times of day (dawn and dusk) in order to determine whether or not neurophysiological changes might accompany observed behavioral changes.

## Methods

### Nutritional State Study

#### Collection and Conditioning

Female *A. studiosus* were collected from both social and subsocial colonies of three geographically separate populations in May 2014 and were assigned individual identification numbers. In order to ensure that all spiders had reached maturity and would begin the trials in a similar healthy condition, they were each fed one termite worker twice per week for a minimum of three weeks prior to the start of the experiment, with two days between the last feeding and the start of trials. Spiders that produced eggs sacs or offspring, and spiders that died, were eliminated from the study.

Spiders were randomly assigned (using the list randomizer at Random.org) to either a “well-fed” (F) group (N=30) or a “starved” (S) group (N=30). All spiders were maintained at 21°C in lab in 12:12 L:D conditions (light from 7:30 AM to 7:30 PM, with 30 minutes of

ramping at “dawn” and “dusk”). Initial body mass (g) was recorded before the first day of the trials.

### *Behaviour Assessment*

Latency to attack prey (LTA) of 60 spiders was measured (in seconds) on day 1 of the experimental period. One live termite worker was placed on each web, approximately 2 cm from the spider, using Biogrip lightweight forceps. A stopwatch was used to measure time from the moment the termite contacted the web until the moment the spider contacted the prey, and this time was reported as the LTA score for each spider. Well-fed spiders were allowed to keep and consume the prey, but termites were immediately removed from starved spiders subsequent to attack. Well-fed spiders continued to be fed and watered every two days throughout the study. Starved spiders were allowed to consume only water on those days. Latency trials were performed between 12 PM and 2 PM each time to control for circadian behavioral variation. LTA was measured on the final day of the study, with prey offered and then removed from all spiders subsequent to attack in order to eliminate gut contents from neurochemical analysis. Spiders that did not attack within 5 minutes (300 seconds) were offered a second prey item between 30 minutes and one hour later.

Huddle durations (HDs) were measured, in seconds, on the day prior to the start of the study period (Huddle Pre) and again on the day prior to the last day of the study period (Huddle Post), so as not to be conducted on feeding (LTA) days. HDs were conducted by delivering a puff of air from a suction bulb aimed directly at the spider. A handheld stopwatch was used to measure the number of seconds each spider remained in the huddled posture before relaxing or repositioning the legs, and this was reported as the HD score for each individual.

### Sample Preparation

After the final HD and LTA measurements, ending body mass (g) was measured. Individual spiders were euthanized and frozen using liquid nitrogen before being homogenized in 700 ul of 0.2 M perchloric acid buffer containing 10 ug/ml DHBA as an internal standard for catecholamines and  $\alpha$ -methylserotonin as an internal standard for serotonin. Homogenization was performed in a 2 mL tube containing a ¼” ceramic bead using a FastPrep 24 Tissue Homogenizer set at 4.0 m/s for 2 x 20 seconds. Samples were centrifuged at 13K rpm for 10 minutes, 500 ul supernatant was transferred to 0.22 um Nylon filter tube (Costar Spin-X by Corning) and centrifuged again for 6 min at 13K rpm. 20 ul of each sample was diluted with 180 ul 0.2 M perchloric acid buffer to give 200 ul of 10% sample for HPLC.

### Analysis of Biogenic Amines

Neurochemical analyses were performed using HPLC-ECD (Antec, Leyden) with an ALF-115 C-18 column and a VT-03 electrochemical flow cell with an ISAAC electrode. Samples were kept at 4°C in vials in autosampler tray. Column and flow cell were housed at 35°C. Mobile phase was composed of 10% methanol with 50 mM phosphoric acid, 50 mM citric acid, 0.1 mM EDTA, 500 mg/L OSA, and 8 mM KCl, with a pH of 3.25. Samples were analyzed at 200 nA for 65 minutes each. Chromatogram peaks were identified by comparison with known standards, and peak heights were normalized by corresponding internal standards. Statistical analyses were performed using Microsoft Excel and Minitab 16.

### Reproductive State and Diel Rhythm Study

#### Collection and Rearing

Male and female *A. studiosus* from both social and subsocial colonies were collected from multiple locations in East Tennessee (I.C. King Park, Boone Lake, and Fort Patrick Henry

Lake) in early June 2013. Half of the females from each colony type were housed individually in 4.0 oz clear plastic Solo® deli containers, and the other half were paired with sexually mature males in the same type of container to encourage mating. All spiders were maintained in the laboratory at 22°C under controlled light/dark cycle (12:12 L:D) for a minimum of two weeks, with lights ramping up from 7:00 AM to full brightness at 8:00 AM and ramping down from 7:00 PM to total darkness at 8:00 PM. Times of 7:30 AM and 7:30 PM, for the purposes of this study, correspond to “dawn” and “dusk,” respectively. The spiders were each fed one termite twice per week and watered concurrently during the entrainment period.

### Grouping

After several weeks of 12:12 L:D entrainment, controlled temperature environment, and regular feeding to eliminate behavioral variation caused by natural habitat differences in the field, and to allow adequate time for mating and egg sac production, 80 individuals were divided into groups as follows (Table 1): 1) social or subsocial colony type, 2) reproductive state (brooding or nonbrooding), and 3) dawn (AM) or dusk (PM) biogenic amine levels (randomly assigned within the first two groups).

**Table 2.1: Experimental groups for reproductive state and diel rhythm study. Group classifications based upon reproductive stage, time of day of sacrifice, and behavioral phenotype of the colony of origin. Group sizes are indicated in parentheses.**

	<i>Brooders</i>	<i>Nonbrooders</i>
<i>AM</i>	Social (10)	Social (10)
	Subsocial (10)	Subsocial (10)
<i>PM</i>	Social (10)	Social (10)
	Subsocial (10)	Subsocial (10)

### Behavioral Assessment

We performed behavioral assays on brooding and nonbrooding adult female *Anelosimus studiosus* to quantify individual aggressive and defensive tendency. Spiders underwent behavioral trials to assess latency to attack prey (LTA) as a measure of aggressive behavior and huddle duration (HD) to assess defensiveness.

### Sample Preparation

Spiders designated as “AM” subjects were euthanized at 7:30 AM, and “PM” subjects were euthanized at 7:30 PM, which corresponded with the entrained dawn and dusk, respectively. Brooding females were deprived of their egg cases. Egg cases were weighed and frozen for a future study. Body masses of adult females were measured on an analytical balance, and individuals were flash frozen in liquid nitrogen and placed in 2.0 ml centrifuge tubes along with a ¼ inch ceramic bead and 700 µl chilled buffer (0.2M perchloric acid containing 10 µg/ml DHBA and 30 µg/ml α-methylserotonin as internal standards for catecholamines and serotonin, respectively). Spiders were homogenized using a Fast Prep tissue grinder operated at 4.0 m/s for 40 seconds. Homogenate was then centrifuged at 4°C for 10 minutes at 13,000 RPM. Approximately 500 µl of each supernatant was transferred to a Costar Spin-X filter tube (0.22 µm, cellulose acetate) and centrifuged for 6 minutes at 13,000 RPM at 4°C. Filtered samples were vortexed and kept on ice while each was diluted to 10% their original concentration using chilled 0.2M perchloric acid in 300 µl HPLC vials (20 µl of sample + 180 µl of 0.2 M perchloric acid). The vials were loaded into a refrigerated (4° C) autosampler tray.

### HPLC Analysis

HPLC-EC was performed using an Antec Alexys LC-EC analyzer with a Decade II electrochemical detector. The detector contained a VT-03 micro flow cell with an ISAAC

reference electrode and a C18 column, heated to 35° C. Mobile phase consisted of 50 mM phosphoric acid, 50 mM citric acid, 0.1 mM EDTA, 10 % methanol, 500 mg/l octane sulfonic acid (OSA), and 8 mM potassium chloride with a pH of 3.25. 5 µl of each sample was analyzed for 80 minutes at 100 nA. Any sample that produced an out-of-range peak was re-analyzed at 200 nA. Peaks were identified by comparison of retention times to known standards, which were analyzed immediately prior to the biological samples.

### Data and Statistical Analysis

Peak height (nA) of each biogenic amine was normalized by the peak height of its corresponding internal standard and was used as the measure of relative concentration of each biogenic amine contained within a sample. HPLC-ECD was used to detect biogenic amines in individual spiders. Peak height (in Na) was used to determine relative concentration of each biogenic amine. The peak height of each biogenic amine was normalized by its corresponding internal standard (DHBA for catecholamines, synephrine for octopamine, and  $\alpha$ -methylserotonin for serotonin).

A one-way ANOVA showed that there was no effect of body mass on results, and there were no significant differences in body mass between groups, so peak heights were not normalized by body mass. For regression analysis, octopamine and serotonin levels were normalized by performing a Johnson transformation. Statistics were performed using Microsoft Excel, Minitab, and SPSS.

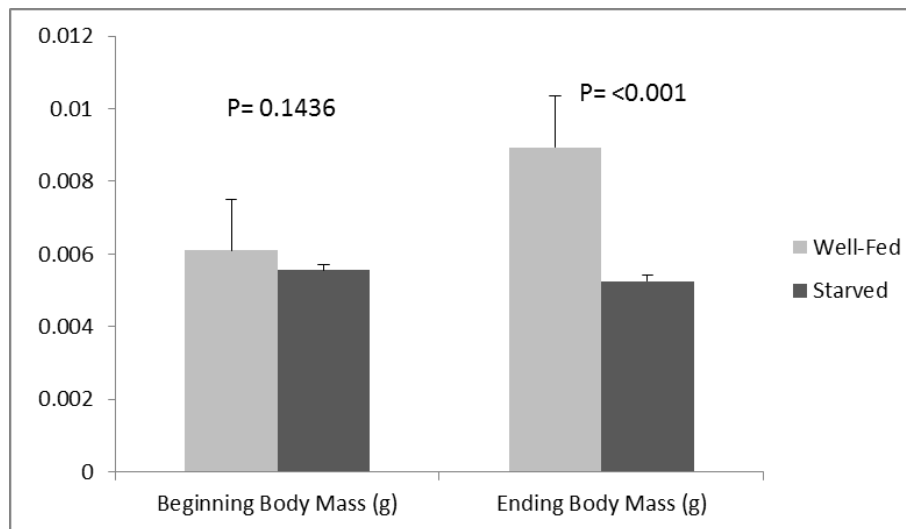


## Results

### Nutritional State Study

#### Body Mass

Body mass was measured at the beginning and at the end of the study. At the start of the trials, after receiving equal feedings for several weeks, there was no significant difference in mean body mass between groups (Figure 1 and Table 2; T-test,  $p = 0.14$ ), but as expected, there was a significant difference between the well-fed group and the starved group by the conclusion of the experimental period (T-test,  $p < 0.001$ ). Additionally, the well-fed spiders showed a significant gain in body mass during the experiment ( $p < 0.001$ ), with the mean mass shifting from 6 mg to nearly 9 mg. Meanwhile, the starved spiders showed a slight decrease in mean body mass, going from 5.6 mg to 5.3 mg (Table 2).



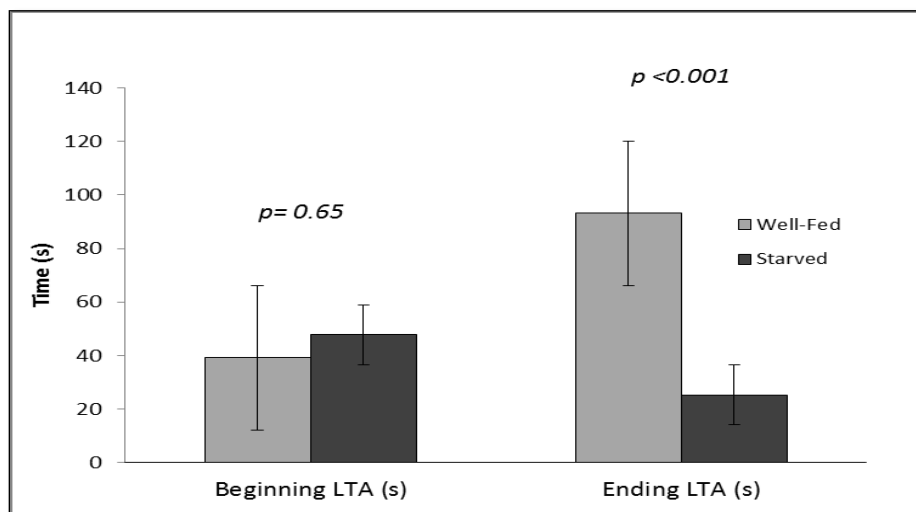
**Figure 2.1: Comparison of Beginning and Ending Mean Body Mass. There was no difference in mean body mass between well-fed and starved spider groups at the beginning of the study period ( $p=0.14$ ), but after 10 days, the difference in body mass was highly significant ( $p<0.001$ ).**

**Table 2.2: Mean Body Mass Comparison and T-test Results**

	Beginning Mean Body Mass (g)	Ending Mean Body Mass (g)	<i>p</i> value
Well-Fed	0.006096	0.008933	<b>&lt;0.001</b>
Starved	0.005552	0.005258	<b>0.374</b>
<i>p</i> value	<b>0.144</b>	<b>&lt;0.001</b>	

Behavior

Latency to attack (LTA) scores were measured at the beginning of the study and again at the end of the study (Fig. 2; Table 3). There was no difference between the fed group and the starved group at the beginning of the study (T-test;  $p = 0.65$ ), but there was a highly significant difference between them after 10 days (T-test and ANOVA;  $p < 0.001$ ). LTA scores for the well-fed group more than doubled (attack times were 2.38 times longer than initial attack times), while the score for the starved group decreased by almost half (ending attack times were 0.53 times as long as initial attack times).



**Figure 2.2: Bar graph of mean latency to attack (LTA) scores for well-fed versus starved *Anelosimus studiosus* at the beginning of the study period (Beginning LTA) and at the end of the**

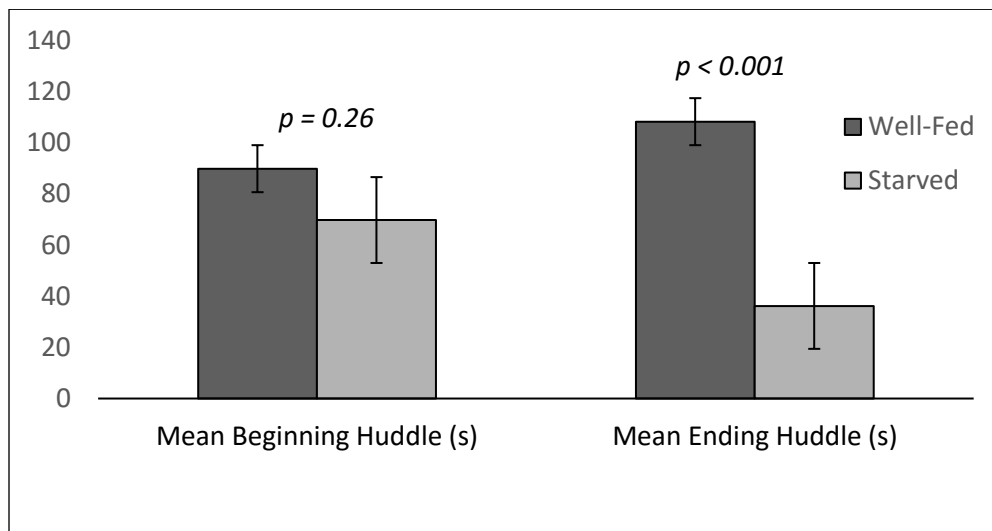
study period (Ending LTA), as measured in seconds. No difference existed between the groups initially ( $p=0.65$ ), but by the end of the study, latency times were significantly lower in the starved group than in the well-fed group ( $p<0.001$ ). Whiskers represent standard error.

**Table 2.3: Mean Latency to Attack (LTA) Scores and Differences**

	Mean Beginning LTA (s)	Mean Ending LTA (s)	<i>p value</i>	Start to Finish Difference (s)
Well-Fed	39.16	93.20	<b>&lt;0.01</b>	+54.04
Starved	47.82	25.38	<b>0.130</b>	-22.44
<i>p value</i>	<b>0.646</b>	<b>&lt;0.001</b>		

Huddle duration (HD) was also measured at the beginning of the study and again at the end.

Little difference was found between groups prior to treatment (ANOVA,  $p=0.26$ ), but the difference at the end of the study was highly significant ( $p<0.001$ ), with starved spiders showing a greatly reduced HD compared to well-fed spiders (Fig.3; Table 4).



**Figure 2.3: Bar graph of mean huddle duration (HD) scores for well-fed versus starved *Anelosimus studiosus* at the beginning of the study period (Mean Beginning Huddle) and at the end of the study period (Mean Ending Huddle), as measured in seconds. No difference existed between the groups**

initially ( $p=0.26$ ), but by the end of the study, HDs were significantly lower in the starved group than in the well-fed group ( $p<0.001$ ). Whiskers represent standard error.

There was an average increase of 18.38 seconds in HD within the well-fed group over the duration of the study, but it was not a significant change (T-test,  $p=0.34$ ). However, the HDs for the starved group decreased by nearly half, with the average dropping by 34 seconds, which was a significant change (Table 4; T-test,  $p=0.02$ ).

**Table 2.4: Mean Huddle Duration (HD) Scores and Differences**

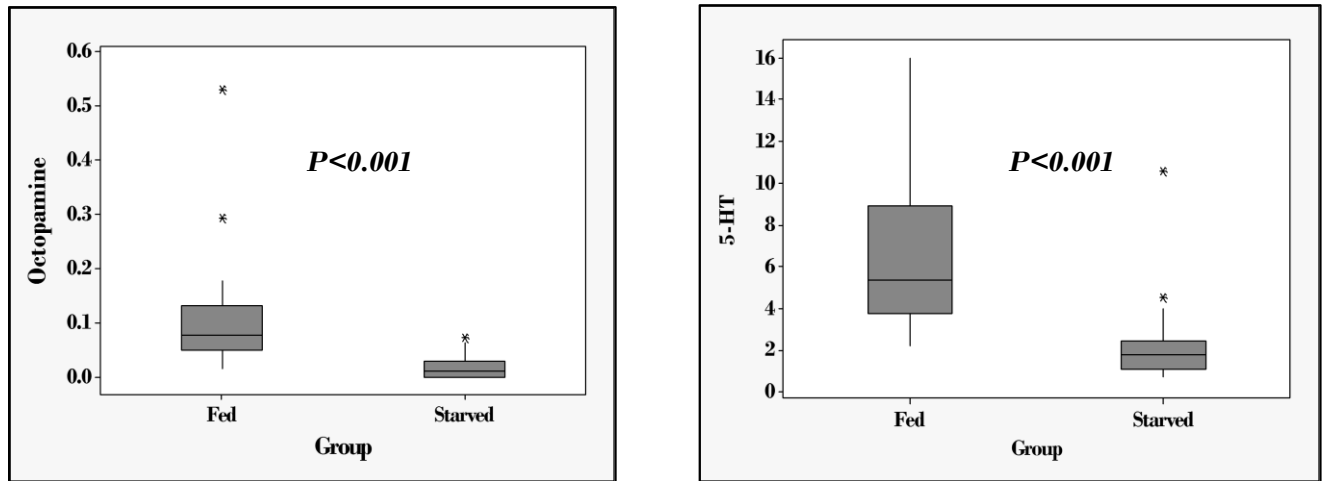
	Mean Beginning Huddle (s)	Mean Ending Huddle (s)	<i>p-value</i>	Start to Finish Difference (s)
Well-Fed	89.87	108.25	<b>0.335</b>	+18.38
Starved	69.78	36.21	<b>0.018</b>	-33.57
<i>p-value</i>	<b>0.26</b>	<b>&lt;0.001</b>		

#### HPLC Detection of Biogenic Amines

Highly significant differences in several of the biogenic amines were found between treatment groups (Table 5; Fig. 4). Well-fed *A. studiosus* showed significantly higher levels of serotonin (5-HT;  $p<0.001$ ), epinephrine ( $p<0.001$ ), and octopamine ( $p<0.001$ ). They also had higher levels of norepinephrine, but the difference was not significant ( $p=0.10$ ). Starved spiders had a higher mean level of dopamine, but not enough to be significant ( $p=0.41$ ). None of the HPLC samples from starved spiders (0 of 30) showed the presence of epinephrine, while over half of the samples from well-fed spiders did (16 of 30). Most of the starved spider samples (24 of 30) produced peaks for dopamine, and that amine was present in less than half of the well-fed spider samples (13 of 30).

**Table 2.5: Mean Biogenic Amine Levels in Well-Fed versus Starved *Anelosimus studiosus***

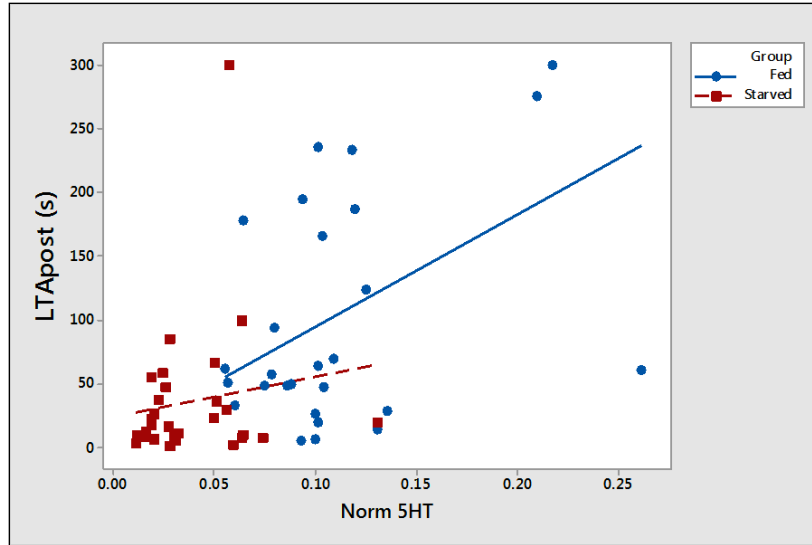
	Norm NE Height [nA]	Norm E Height [nA]	Norm OA Height [nA]	Norm DA Height [nA]	Norm 5-HT Height [nA]
Well-Fed Mean	3.745	0.005	0.009	0.003	0.113
Starved Mean	2.930	0	0.002	0.005	0.038
<i>p</i> value	<b>0.104</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.413</b>	<b>&lt;0.001</b>



**Figure 2.4: Boxplots of octopamine and serotonin (5-HT) in fed and starved groups. Octopamine (left) was significantly higher in fed spiders than in starved spiders (T-test;  $p < 0.001$ ), and serotonin (right) was also significantly higher in fed spiders than in starved spiders (T-test;  $p < 0.001$ ).**

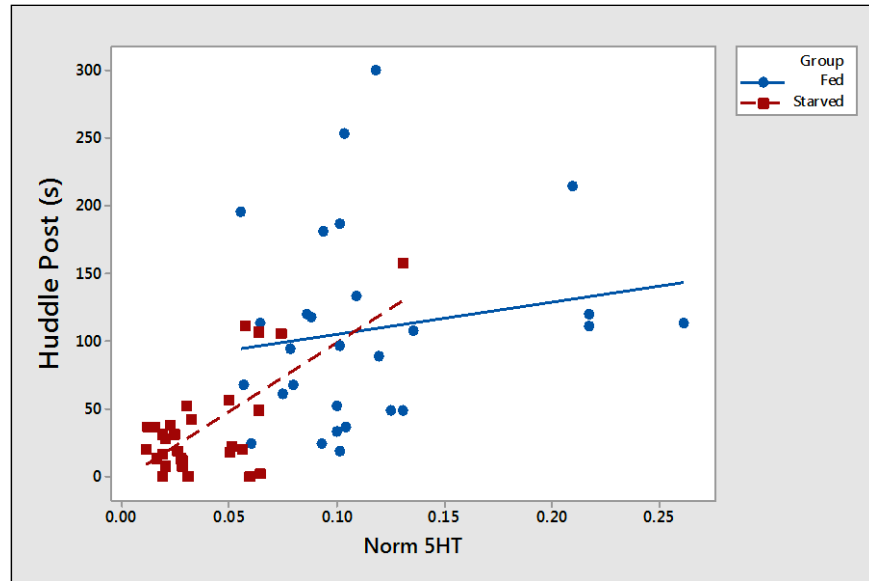
### Serotonin and Behavior

Latency (LTA) scores at the end of the treatment period were strongly correlated with serotonin (5-HT) levels in individual *A. studiosus* (Fig. 5; regression analysis,  $p < 0.001$ ,  $R^2 = 35.1\%$ ). Longer latency times were exhibited by spiders with higher levels of 5-HT in both groups. The trend was stronger in well-fed spiders than in starved spiders (Fig. 5;  $R^2 = 18.5\%$  and  $13.6\%$ , respectively).



**Figure 2.5: Scatterplot showing relationship between latency (LTA) times and normalized serotonin (5-HT) values in individual well-fed (blue dots) and starved (red squares) *Anelosimus studiosus*. Higher 5-HT is correlated with longer latency times (higher LTA). Starved spiders had shorter latency times and lower 5-HT levels than well-fed spiders. Regression analysis,  $p < 0.001$ , Well-fed R-Sq = 18.5% and starved R-Sq=13.6%.**

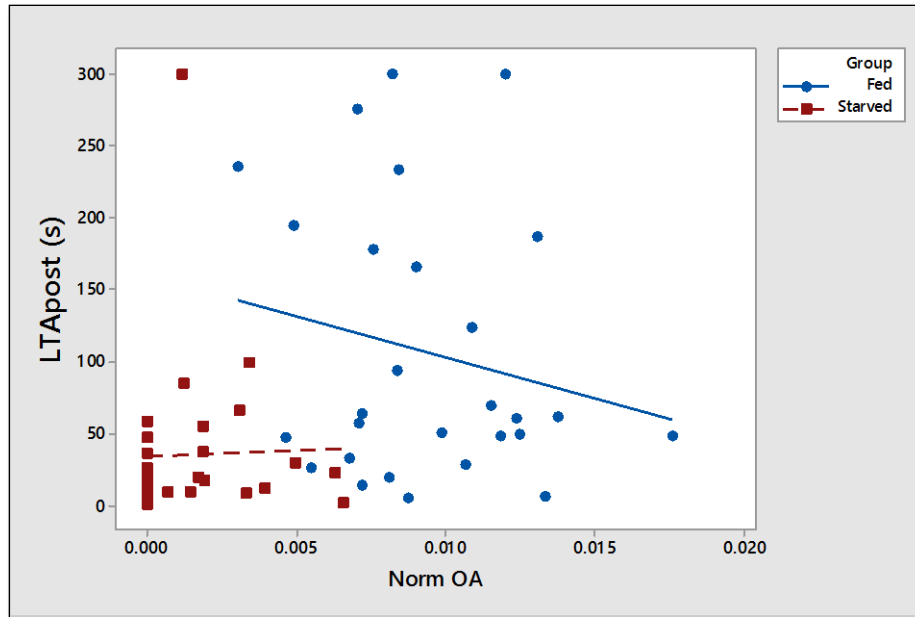
Huddle durations (HDs) were also positively correlated with serotonin (5-HT) levels (regression analysis;  $p < 0.001$ ) in both groups, but more strongly so in starved spiders (Fig.6; R-Sq well-fed =3.1% and R-Sq starved = 47.4%). Starved spiders (Fig. 6, red squares) had shorter huddle durations and lower levels of 5-HT than well-fed spiders, which had longer huddle durations and more 5-HT in general. Longer HDs are correlated with higher 5-HT in both groups.



**Figure 2.6:** Scatterplot showing relationship between ending huddle duration (Huddle Post) and normalized serotonin (Norm 5-HT) values in individual well-fed (blue dots) and starved (red squares) *Anelosimus studiosus*. Longer HDs are correlated with higher 5-HT in both group. Starved spiders had shorter HDs and lower 5-HT levels than well-fed spiders. R-Sq well-fed =3.1% and R-Sq starved = 47.4%.

### Octopamine and Behavior

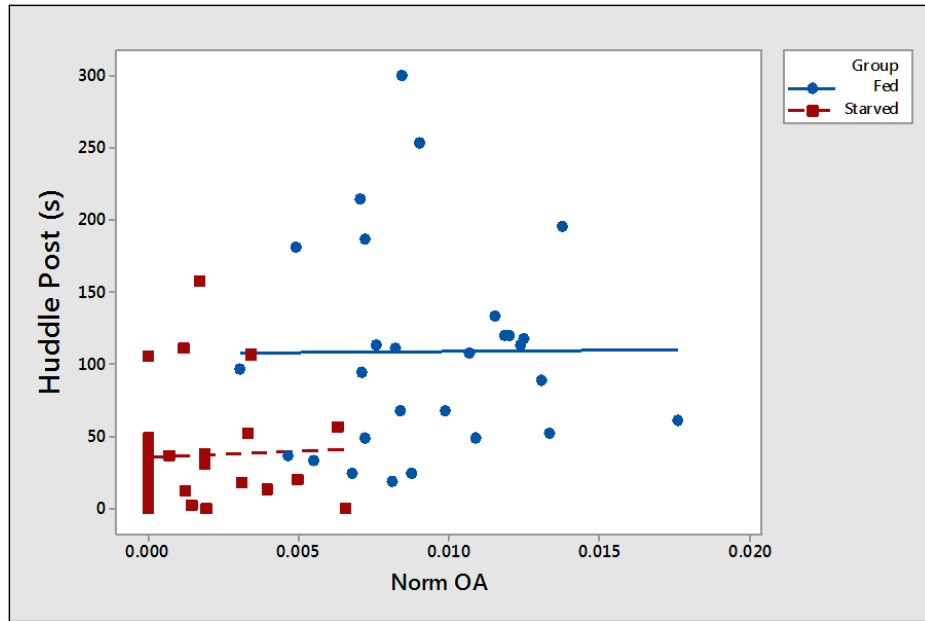
Lump analysis of all individuals indicated an overall significant relationship between octopamine (OA) levels and latency (LTA) scores (regression analysis,  $p=0.01$ ,  $R\text{-Sq}=11.4\%$ ). However, when separated by treatment group, the data reveal differing trends: In well-fed *A. studiosus*, higher levels of octopamine were correlated with shorter latency times, while there appeared to be no relationship between LTA and octopamine in the starved spiders (Fig.7).



**Figure 2.7:** Scatterplot showing relationship between latency (LTA) times and normalized octopamine (OA) values in individual well-fed (blue dots) and starved (red squares) *Anelosimus studiosus*. Higher OA is correlated with shorter latency times (lower LTA) in well-fed spiders. Starved spiders had overall shorter latency times and lower OA levels than well-fed spiders, but there was not a significant correlation between OA and LTA. Regression analysis, well-fed R-Sq = 1.9% and starved R-Sq=0.2%.

Starved spiders had shorter huddle durations and lower levels of octopamine than well-fed spiders, and well-fed spiders had longer latency times and higher octopamine levels than starved spiders, but no relationship was found between huddle durations and octopamine in either well-fed or starved spiders (Fig. 8).



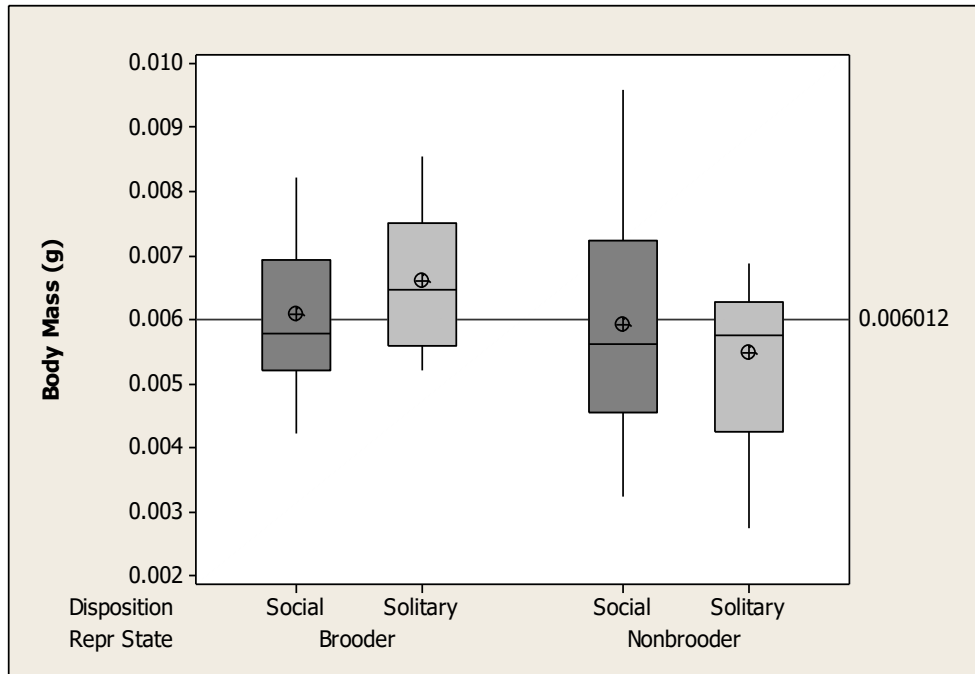


**Figure 2.8: Scatterplot showing relationship between huddle duration times at the end of the study period (Huddle Post) and normalized octopamine (Norm OA) values in individual well-fed (blue dots) and starved (red squares) *Anelosimus studiosus*. Well-fed spiders and starved spiders occupy different ranges on the plot and had significant differences in huddle times and octopamine levels, but there was not a strong correlation between HD and OA in either group.**

### Reproductive State and Diel Rhythm Study

#### Body Mass

Average body mass of all adult female *A. studiosus* included in the study (N=80) was 6 mg (0.0060 g). There was no overall difference in body mass between spiders from social colonies and those from subsocial colonies ( $p=0.97$ ), nor between social and subsocial spiders within brooding and nonbrooding groups (Fig. 9;  $p=0.44$  and  $p=0.17$ , respectively). However, overall body mass difference between brooders and nonbrooders was significant ( $p=0.05$ ), with brooders weighing 6.6 mg, on average, and nonbrooders weighing 5.5 mg.



**Figure 2.9: Boxplot of body masses among groups. No significant difference in body mass was found between spiders from social and solitary colonies, overall or within groups (overall social vs. solitary  $p=0.97$ , brooding social vs. solitary  $p=0.44$ , and nonbrooding social vs. solitary  $p=0.17$ ). The difference in body mass between brooders and nonbrooders was significant ( $p=0.05$ ).**

### Behavior

Behavior scores for latency to attack prey (LTA) and huddle duration (HD) were measured in seconds (s) for all spiders involved in the study and then compared. LTAs and HDs were measured 4 times per spider over the course of the study and averaged to give a final mean LTA and HD for each individual. LTA is an accepted measure of aggressiveness, and HD is a measure of defensiveness.

Adult female *Anelosimus studiosus* from social colonies (including both brooding and nonbrooding females) had shorter LTAs than those from solitary colonies by an average of 21.5 seconds (Table 6; T-test;  $p=0.14$ ) and longer HDs than those from solitary colonies by 18 seconds (Table 6;  $p=0.13$ ), but the overall differences were not significant.

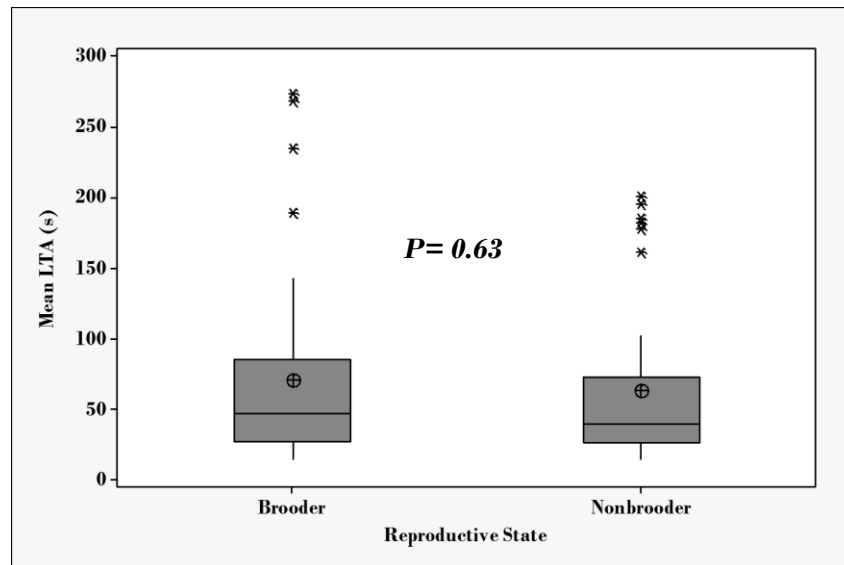
**Table 2.6: Behavior of *A. studiosus* from Social vs. Solitary Colonies**

<i>Assay</i>	<i>Social</i>	<i>Solitary</i>	<i>p-value</i>
Latency to Attack (s)	56.13	77.60	0.14
Huddle Duration (s)	50.46	32.08	0.13

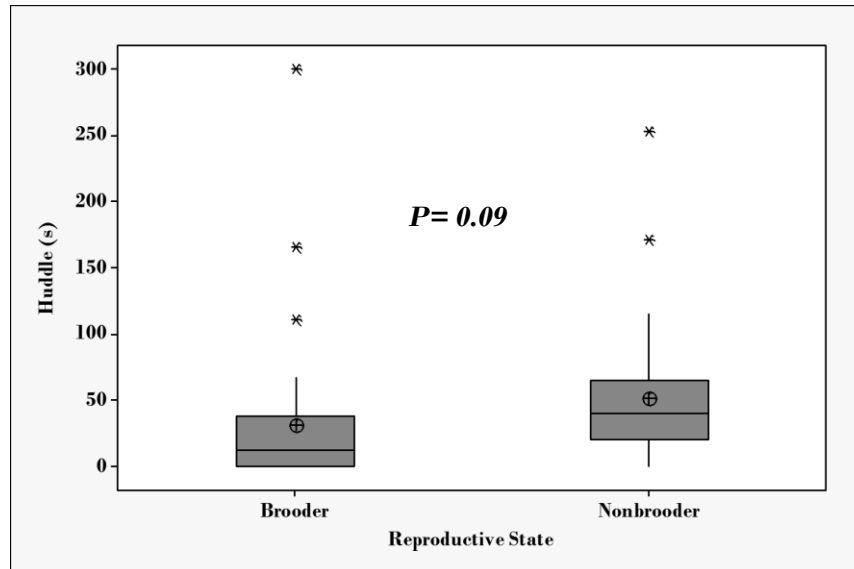
Mean LTAs for brooding females were ~70 seconds, slightly longer than those for nonbrooding females (Table 7 and Fig. 10;  $p=0.64$ ), but HDs for brooders were 20 seconds shorter than for nonbrooders (Table 7 and Fig. 11;  $p=0.09$ ).

**Table 2.7: Behavior and Reproductive State**

<i>Assay</i>	<i>Brooders</i>	<i>Nonbrooders</i>	<i>p-value</i>
Latency to Attack (s)	70.44	63.50	0.64
Huddle Duration (s)	31.06	51.43	0.09

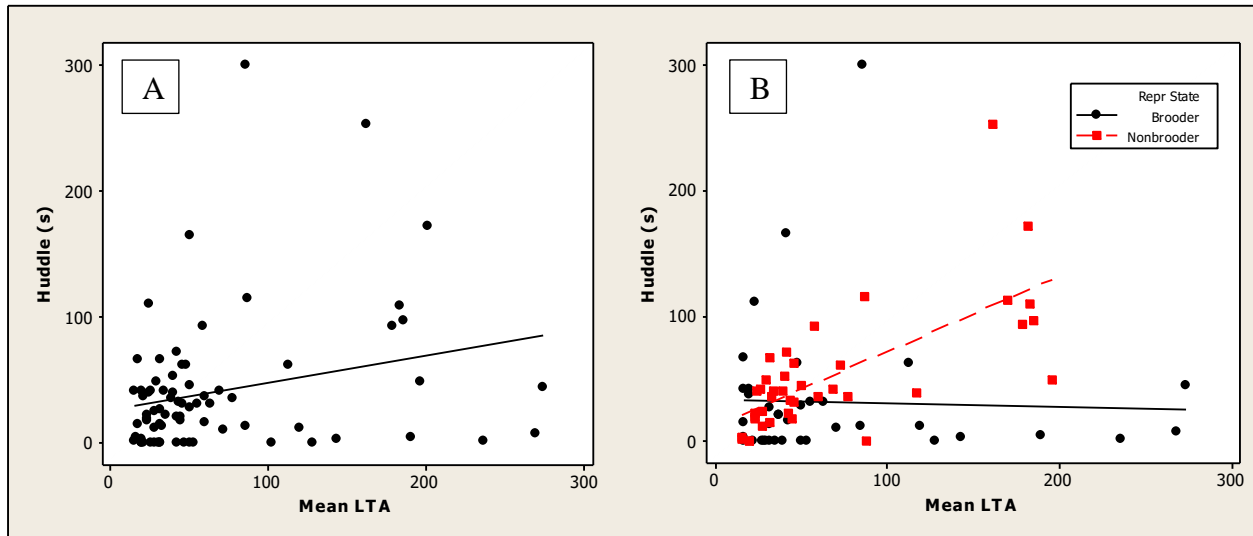


**Figure 2.10: Boxplot of mean latency to attack (LTA) times (in seconds) of brooders and nonbrooders. There was no significant difference between groups (T-test;  $p=0.63$ ).**



**Figure 2.11: Boxplot of mean huddle durations (in seconds) of brooders and nonbrooders. Huddle durations were longer in nonbrooders than in brooders, but the difference was not significant (T-test;  $p=0.09$ ).**

Longer latency times were associated with longer huddle durations across all groups (Fig. 12A and Table 8; regression analysis,  $p=0.027$ ,  $df=1$ ,  $F=5.07$ ,  $R-Sq=6.5\%$ ). When separated into groups by reproductive state, we found that nonbrooding spiders followed a more predictable trend ( $R-Sq=48.6\%$ ) than brooding spiders (Fig. 12B;  $R-Sq=0.1\%$ ). Multiple regression analysis (Table 9) showed significant correlation between HD and LTA ( $p=0.02$ ), with no significant difference between brooders and nonbrooders ( $p=0.09$ ).



**Figure 2.12:** Scatterplots of huddle duration versus mean latency to attack. Each dot represents the paired HD and LTA scores from an individual spider. *A*) Overall, HDs were positively and significantly correlated with LTAs ( $p=0.027$ ,  $R\text{-Sq}=6.5\%$ ). *B*) HD and LTA in nonbrooding spiders (red squares,  $R\text{-Sq}=48.6\%$ ) and brooding spiders (black diamonds,  $R\text{-Sq}=0.1\%$ ).

**Table 2.8: Results of general regression: HD vs. LTA**

Source	DF	F	p-value
Regression	1	5.07	0.03

**Table 2.9: Results of multiple regression: HD vs. LTA in brooders vs. nonbrooders**

Source	DF	F	p-value
Regression	2	4.05	0.02
Mean LTA	1	5.62	0.02
Reproductive State	1	2.9	0.09

### Biogenic Amines

Serotonin (5-HT) was not significantly different between brooders and nonbrooders (Fig. 13 and Table 10; GLM,  $p=0.88$ ), but there was a slight trend of higher 5-HT at dusk than at dawn, though not statistically significant ( $p=0.16$ ). We detected the highest 5-HT levels in

brooders at dusk and lowest levels in brooders at dawn. There was no significant interaction between reproductive state and time of day ( $p=0.32$ ).

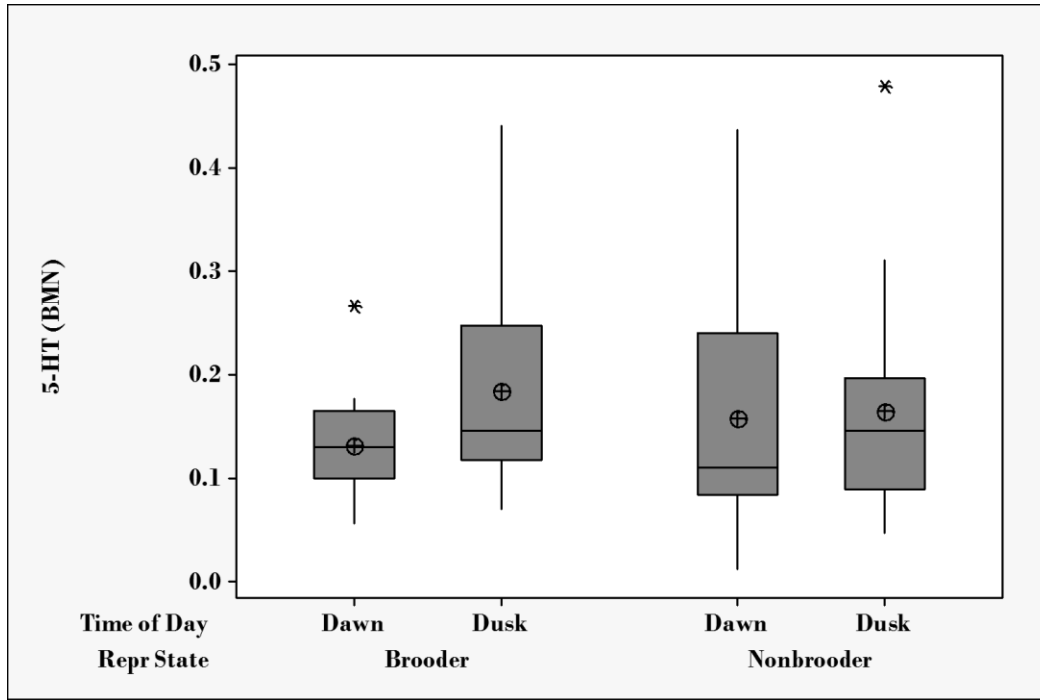


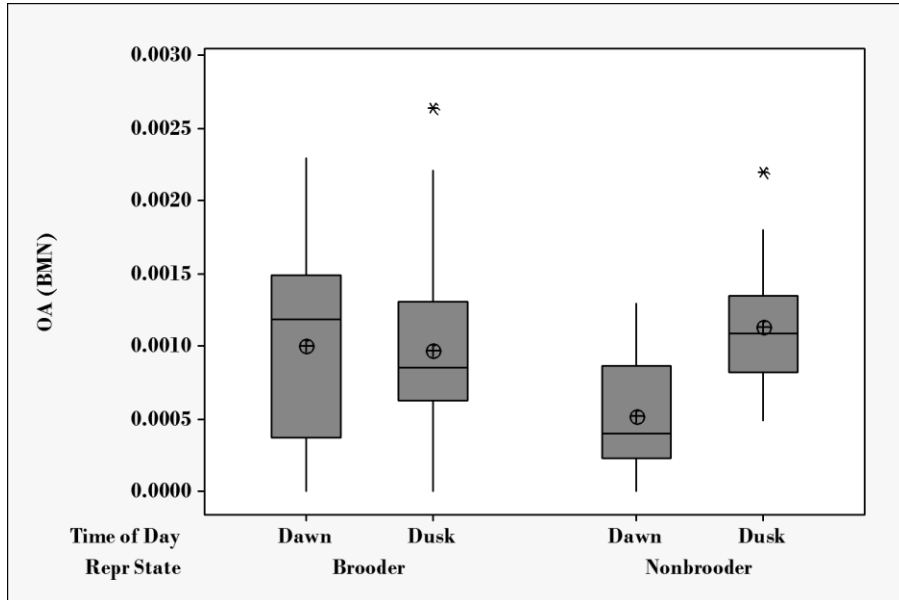
Figure 2.13: Boxplot of body-mass-normalized serotonin by reproductive state and time of day.

Table 2.10: Results of general linear model (GLM) comparing serotonin (5-HT) levels based on reproductive state and time of day.

Source	GLM		
	DF	F	p-value
Reproductive State	1	0.02	0.88
Time of Day	1	1.98	0.16
Reproductive State*Time	1	1.02	0.32

Octopamine was found to be significantly higher at dusk than at dawn in nonbrooders (Fig. 14 and Table 11; GLM,  $p=0.02$ ). OA did not change from dawn to dusk in brooders and was not significantly different overall between brooders and nonbrooders ( $p=0.21$ ). However,

there was a significant interaction between reproductive state and time of day influencing OA levels ( $p=0.01$ ).



**Figure 2.14: Boxplot of body-mass-normalized octopamine versus reproductive state and time of day.** There was no significant difference in OA based on reproductive state ( $p=0.21$ ), but OA was significantly different according to time of day ( $p=0.02$ ). There was also a significant interaction between reproductive state and time of day ( $p=0.01$ ). OA was higher at dusk than at dawn.

**Table 2.11: Results of general linear model (GLM) comparing octopamine levels by reproductive state and time of day.**

Source	GLM		
	DF	F	p-value
Reproductive State	1	1.61	0.21
Time of Day	1	5.38	0.02
Reproductive State*Time	1	6.51	0.01

## Discussion and Conclusions

### Nutritional State Study

#### *Body Mass*

This experiment was conducted at a time of year when females are undergoing a final molt to become sexually mature. After maturation, females will continue to increase in size and mass provided they have access to adequate nutrition. All spiders in this study were confirmed as sexually mature adult females, and, as expected, those in the well-fed group experienced a significant increase in body mass over a period of 10 days. The starved spiders were unable to increase their overall body mass, but they were nearly able to maintain it due to regular water intake.

#### *Behavior and Nutritional State*

After the three-week laboratory conditioning period consisting of regular feedings and programmed light cycles, and before the start of the experimental period, there were no significant differences in the behavioral scores between treatment groups (T-test, LTA  $p=0.65$ , HD  $p=0.26$ )

Over the 10-day treatment period, a highly significant divergence occurred between the well-fed group and the starved group in responsiveness to prey. Latency times for the well-fed group significantly increased 240% (by an average of 54 seconds), from 39 to 93 seconds (T-test,  $p<0.01$ ). A few of the well-fed spiders even ran away from the prey before coming back to subdue it, but then did not consume the prey. Mean latency times for the starved spiders were decreased (by approximately half) after the 10-day period of starvation, going from an average of 48 seconds to an average of 25 seconds (a decrease of 22 seconds). The final difference in LTAs between groups was ~70 seconds, a highly significant difference (T-test,  $p<0.001$ ). These results



suggest that nutrient deprivation can certainly increase responsiveness and aggression toward prey and that satiety can have the opposite effect.

Huddle durations increased slightly over the study period in the well-fed group by an average of 18 seconds (going from an average of 90 to 108 seconds) but as expected, the difference was not significant (T-test,  $p=0.34$ ). In the starved group, however, there was a marked decrease in HDs, going from a beginning average HD of 70 seconds to an ending average HD of 36 seconds (T-test.  $P=0.02$ ). By the end of the study, the difference in average HDs between groups was 72 seconds, with starved spiders emerging from huddled postures more than a full minute sooner than well-fed spiders, on average. This highly significant change in huddle duration (T-test,  $p<0.001$ ) indicates that starvation decreases defensive response, or wariness, in *Anelosimus studiosus*.

#### Biogenic Amines and Nutritional State

Due to the extremely small body size of individual *Anelosimus studiosus*, it was not possible to extract neurochemical samples for analysis at the beginning of the study for comparison, but HPLC analysis of biogenic amine levels at the conclusion of the study showed drastic between-group differences in whole-body levels of 3 of the 5 amines tested (serotonin, octopamine, and epinephrine).

Because serotonin has been associated with a decrease in aggression in *A. studiosus* (Price et al. 2010), and because it is synthesized from tryptophan, which is obtained from the consumption of protein, we expected that it would be higher in the well-fed spiders, and our data support this hypothesis. The complete absence of epinephrine from the starved spiders was surprising, as was the higher mean level of octopamine in the well-fed spiders. Since octopamine has shown correlation with higher aggression in this species (Price et al. 2010), we expected it to

be significantly higher in the starved spiders, but it was actually higher in the well-fed spiders. The starved spiders had a higher mean level of dopamine than well-fed spiders.

As predicted, behavioral responses to prey and predators can change bidirectionally according to nutritional state. These behavioral changes might be due, at least in part, to neurophysiological modulation of aggression. In this study, we have shown that, with all other factors being equal (age, sex, temperature, light cycle, time of day, and water intake), drastic behavioral differences can appear in as few as 10 days.

Serotonin and octopamine appear to be correlated with differences in aggression-related behaviors. Our data support our hypothesis that serotonin may be associated with a decrease in aggression and an increase in wariness, or anti-predator huddling response, while octopamine may be related to an observed increase in aggressiveness and a decrease in wariness.

Taken together, these results support our hypothesis that an environmental variable such as prey abundance may influence spider behavior by way of neurophysiological changes. These behavioral changes, if maintained over time in certain environments, could play a role in intraspecific interactions leading to formation of social groups or dispersal of aggressive individuals.

### Reproductive State and Time of Day Study

#### *Body Mass*

As expected, since all spiders in this study were maintained in identical laboratory conditions during their final molt and maturation, no overall difference in body mass was noted between spiders from social and solitary colonies, but there was a significant difference in body mass between brooding and nonbrooding females, with the brooding females weighing, on

average, a milligram more than nonbrooding females. Masses of the spiders were measured after egg cases had been produced and tended for a period of approximately 7 to 10 days. Because all spiders were fed and watered the same amount and on the same schedule, the difference in body mass associated with reproductive state could potentially be due to the fact that the brooding females (prior to brooding) were kept in containers with sexually mature males with which to mate, and some of the females killed (and in some cases, consumed) the inhabitant males after mating, providing an additional source of nutrient intake.

### Behavior

There is a significant positive relationship between latency time and huddle duration, overall. However, latency to attack prey is slightly longer in brooding female *A. studiosus* than in nonbrooding female *A. studiosus*, which may suggest that brooding females are more hesitant to leave their egg cases in order to attack prey until they have carefully interpreted vibratory cues induced by the prey item. Huddle duration is significantly shorter in brooders than in nonbrooders, indicating that brooding females respond to a perceived predatory threat by rapidly preparing to actively defend their egg cases and themselves from predation or parasitism. These results support our hypothesis that reproductive state (whether or not a female is actively defending an egg case) substantially influences a spider's behavior as it may relate to protecting her greatest investment.

### Biogenic Amines

Serotonin and octopamine are known to play roles in invertebrate aggression. In this study, we observed no significant change in serotonin associated with reproductive state or time of day. However, our results indicate that octopamine could be associated with the overall increased level of aggression we have observed in brooders compared to nonbrooders in a

previous study, as well as the spike in aggression observed in nonbrooders at dusk. This suggests that octopamine plays a role in behavioral plasticity associated with reproductive state and aggression-related diel rhythms.

The results presented here allude to a complex interaction among neurochemical compounds and their effects on the nervous systems, and behavior, of these spiders. Based on past findings in our lab, I suspect that we could break these groups down even further based on population of origin and further tease apart neurochemical differences. For now, these data suggest that there *are* quantifiable differences in neuroactive biogenic amines associated with behavioral changes. Predictable fluctuations in these effectors are strongly indicative of a diel rhythm in neurochemistry, which corresponds with prey and predator abundance, and aggressive behaviors associated with reproductive state.

#### Future Considerations

Environmental factors such as prey abundance and scarcity can be simulated in a laboratory environment while controlling for other variables in order to study behavioral and physiological effects of changes in nutritional state. In a preliminary version of this study (2013, unpublished data), we determined that starved *A. studiosus* began to undergo a marked attrition after approximately 10 days without food. A similar experiment with a hardier species might provide even greater insight into the long-term behavioral and neurochemical effects of food-deprivation or prey scarcity. Additionally, the relationship between nutrition and the synthesis pathways of biogenic amines in spiders should be considered in future investigations.

We plan to reanalyze serotonin levels in these samples using a different HPLC column which is more specific for serotonin detection. We are, as of now, unsure of the roles of several biogenic amines in aggression-related behavior in spiders, but a study along the lines of this one

with a larger sample size and identification of other variables would most likely prove fruitful. If I were to repeat this study, in addition to the considerations I just mentioned, I would conduct the behavioral assays at dawn and dusk, in addition to midday, for an additional level of comparison. I would also either collect all of the spiders from the same population or separate them into groups according to the population from which they were collected to control for genetic variation in behavior and neurophysiological characteristics. Evidence gained from these experiments will inspire further exploration of the relationship between behavior and neural controls.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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## CHAPTER 3

### BEHAVIOURAL AND NEUROCHEMICAL CHANGES ASSOCIATED WITH MATURATION IN THE NORTHERN SOCIAL SPIDER, *ANELOSIMUS STUDIOUSUS* (ARANEAE: THERIDIIDAE)

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#### Abstract

Nearly all spiders are solitary and aggressive, but a few species are unusual in their capacity to exhibit varying degrees of sociality. *Anelosimus studiosus* is a cobweb spider which consists of both social and solitary behavior types. All young spiders within the species begin life in the same way. As they grow, a behavioral divergence occurs. Spiderlings born to subsocial (solitary) mothers will begin to separate from siblings, demand more space, and show more aggression than spiderlings born to social mothers. Before the final molt, most of the solitary offspring have dispersed from the natal web to become solitary adults themselves. What physiological changes are occurring during this period of maturation, and are neurotransmitters and neurohormones affecting these observed behaviors? We used high performance liquid chromatography to analyze biogenic amine levels in developing social and subsocial spiderlings. We looked for relationships between biogenic amines (specifically, octopamine and serotonin) and quantified aggressive and defensive behaviors. As hypothesized, there was a significant relationship between octopamine and serotonin. Juveniles from social colonies tended to have more serotonin and less octopamine than juveniles from subsocial colonies. Serotonin and

octopamine levels were correlated with opposing behavioral trends. Higher serotonin was correlated with lower aggression and greater wariness, while higher octopamine was correlated with greater aggression and less wariness. Differences in octopamine levels also occur between social and solitary juveniles and change throughout development. We believe that these results can offer insight into the neurochemical changes that may accompany evolutionary shifts from solitary toward social behavior.

### Introduction

The comb-footed cobweb spider, *Anelosimus studiosus*, is behaviorally polymorphic; individuals can be classified as either docile or aggressive. Subsocial female individuals of this species are typically solitary but exhibit extended maternal care of offspring, while social individuals cooperatively capture prey, maintain conjoining web spaces, and raise their young in a communal setting. The two phenotypes are heritable (Pruitt and Riechert 2009), so offspring of solitary parents will be solitary, and offspring of social parents will be social. In the case where a social and a subsocial/solitary spider mate, their offspring will exhibit intermediate behaviors.

As the spiderlings of a solitary mother must remain in the natal web together and receive regurgitative feedings to survive to an age of independence (Viera et al. 2005), it is necessary for them to tolerate one another and exhibit a degree of sociality while sharing prey and close quarters. As they mature, they begin to wander farther from the mother and farther from each other within the web until they eventually disperse to mate or build their own webs. As adults, they will remain solitary with the exception of mating and rearing young. Offspring of social parents begin life in the same way as their subsocial counterparts, but as they mature, they remain tolerant of one another and maintain close proximity to conspecifics throughout their lives.

It is remarkable to find such stark differences within the same species, and this behavioral variation was only first observed in 1998 (Furey 1998) in Tennessee (a temperate climate). It is because of this unique occurrence that the species has recently received the nickname of the Northern Social Spider. Throughout the rest of the range, *Anelosimus studiosus* colonies are all subsocial/solitary. This phenomenon presents a unique and powerful opportunity to study the environmental and physiological factors influencing the evolution of sociality (Viera et al. 2007).

Behavioral assays used in this study (latency to attack prey and huddle duration) are reliable methods for assessing the aggressive or docile tendencies of individual spiders. For instance, a correlation has been shown between the amount of time it takes for a spider to attack a prey item and that spider's general temperament, including behavior toward conspecifics.

## Methods

### Collection and Rearing

Colonies of social and solitary *Anelosimus studiosus* were collected from three distinct populations in different geographic locations along the Tennessee River in June 2015: Fort Patrick Henry Lake in Kingsport, TN, Melton Hill Lake south of Knoxville, TN, and Lake Guntersville near Guntersville, AL. Six social colonies and six solitary colonies were maintained in the laboratory at 22°C under controlled light/dark cycle (12:12 L:D), with lights ramping up from 7:00 AM to full brightness at 8:00 AM and ramping down from 7:00 PM to total darkness at 8:00 PM. The spiders were fed worker termites twice per week and watered concurrently. Colonies were kept with their original foliage in large transparent plastic containers.

Brooding females were kept with their egg cases until spiderlings emerged, and they were allowed to rear them until the 4<sup>th</sup> instar, at which point the spiderlings are capable of capturing prey and feeding independently. The mothers were removed at this point, scored for huddle

duration and latency to attack prey, and then weighed and sacrificed by submersion in liquid nitrogen.

Beginning at the 3<sup>rd</sup> instar, and following each molt through the 6<sup>th</sup> instar, 3 spiderlings were removed from each colony, scored for huddle duration and latency to attack prey, and then weighed and sacrificed by submersion in liquid nitrogen. Due to their extremely small size, the spiderlings were pooled by colony and age for HPLC analysis of neurochemicals (3 spiderlings per sample). Adults were analyzed individually (1 per sample).

#### Preparation of samples for HPLC analysis

After expiration in liquid nitrogen, spiders were transferred to microcentrifuge tubes containing 700  $\mu$ l chilled 0.1M perchlorate with 1  $\mu$ g/ml synephrine as an internal standard for octopamine and 3  $\mu$ g/ml alpha-methylserotonin as an internal standard for serotonin. Each tube contained a 0.25” ceramic bead used to homogenize the spiders for whole-body extraction of neurochemicals. This was accomplished by agitation of the tubes for 2 x 15 seconds at a speed of 4 using a NextAdvance Air Cooling Bullet Blender Storm 24. The tubes were then chilled on ice for 5 minutes and centrifuged at 4°C for 10 minutes at a speed of 13K rpm. Supernatant was transferred to 0.22  $\mu$ m Nylon filter Costar Spin-X tubes (Corning) which were pre-washed with 700  $\mu$ l 0.2 M perchlorate to remove/minimize extractables. Supernatant was filtered by centrifugation in the Spin-X tubes for 6 minutes at 13K rpm at 4°C. Filter cartridges were removed, and filtrates were vortexed at high speed for 20 seconds. Aliquots (300  $\mu$ l) of each filtered sample were transferred to polypropylene HPLC autosampler vials (12x32 mm, MicroSolv). Vials were vortexed and tapped to remove air bubbles prior to loading in refrigerated (4°C) autosampler tray.

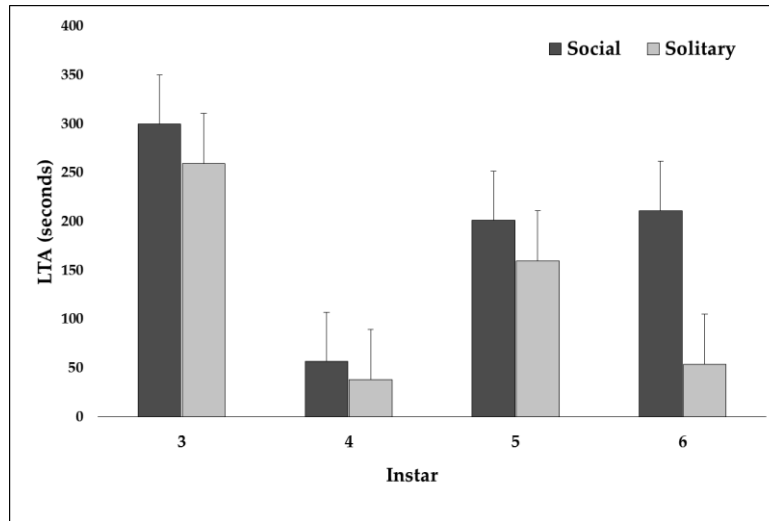
## HPLC analysis of neurochemicals

Samples were analyzed for neurochemical content by HPLC-ECD (high performance liquid chromatography with electrochemical detection; Antec, Leyden) with an ALF-115 C-18 column and a VT-03 electrochemical flow cell with an ISAAC electrode. Samples were kept at 4°C in vials in autosampler tray. Column and flow cell were housed at 35°C. Mobile phase was composed of 10% methanol with 50 mM phosphoric acid, 50 mM citric acid, 0.1 mM EDTA, 500 mg/L OSA, and 8 mM KCl, with a pH of 3.25. Five µl of each sample was injected and analyzed for 60 minutes at a range of 50 nA. Chromatograph peaks were identified by comparison with known standards, and peak heights were normalized by corresponding internal standards. Statistical analyses were performed using Microsoft Excel and Minitab 16.

## Results

### Behavior

Latency to attack prey (LTA, in seconds), as a measure of aggressiveness, was longer in spiderlings from social colonies at all instars than in spiderlings from solitary colonies (Fig. 1). There was a significant effect of colony type ( $p < 0.01$ ) and instar ( $p < 0.001$ ) on LTA, as well as a significant interaction between colony type and instar (Table 1; GLM;  $p = 0.04$ ).

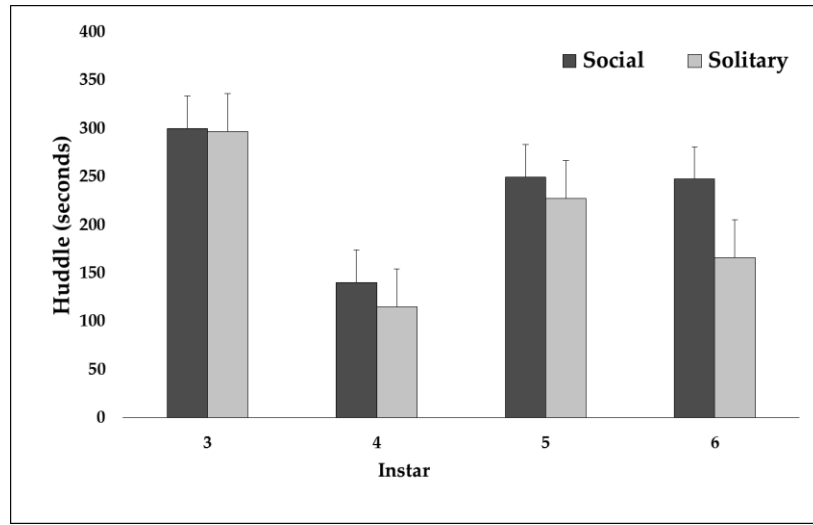


**Figure 3.1: Bar graph comparison of latency to attack prey (LTA) between phenotypes and among instars of developing *Anelosimus studiosus*. Error bars indicate standard error.**

**Table 3.1: Results of general linear model (GLM): LTA vs. colony type and instar.**

GLM			
Source	DF	F	p-value
Colony Type	1	8.38	<0.01
Instar	3	17.09	<0.001
Colony Type * Instar	3	2.95	0.04

Huddle duration (HD), as a measure of wariness or defensiveness, was longer in spiderlings from social colonies at all instars than it was in spiderlings from solitary colonies (Fig. 2). There was a significant effect of colony type ( $p < 0.01$ ) and instar ( $< 0.001$ ) on HD, but there was no significant interaction between colony type and instar (Table 2; GLM;  $p = 0.19$ ).

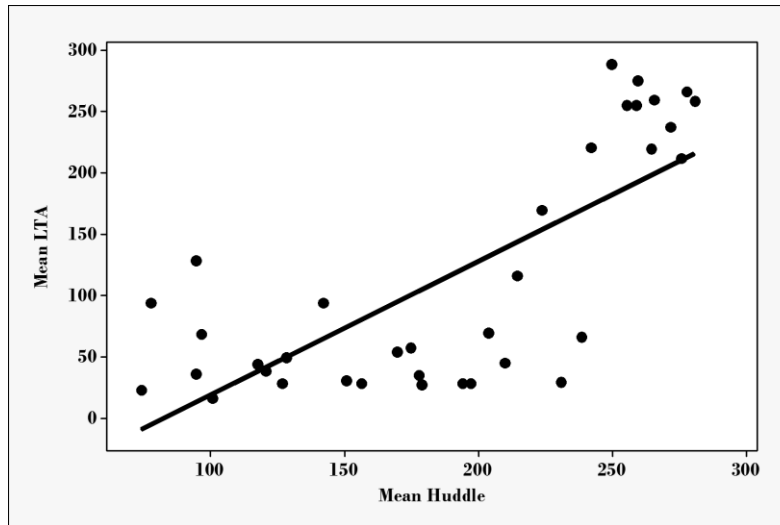


**Figure 3.2: Comparison of huddle durations between phenotypes and among instars in developing *Anelosimus studiosus*.**

**Table 3.2: Results of general linear model (GLM): HD vs. colony type and instar.**

GLM			
Source	DF	F	p-value
Colony Type	1	7.59	<0.01
Instar	3	26.97	<0.001
Colony Type * Instar	3	1.67	0.19

A strong and significant correlation was found between latency to attack (LTA) and huddle duration (HD) scores of developing spiderlings (Fig. 3; regression analysis; DF=1, F=128.51,  $p < 0.001$ ). Spiderlings with longer latency times tended to also have longer huddle durations.

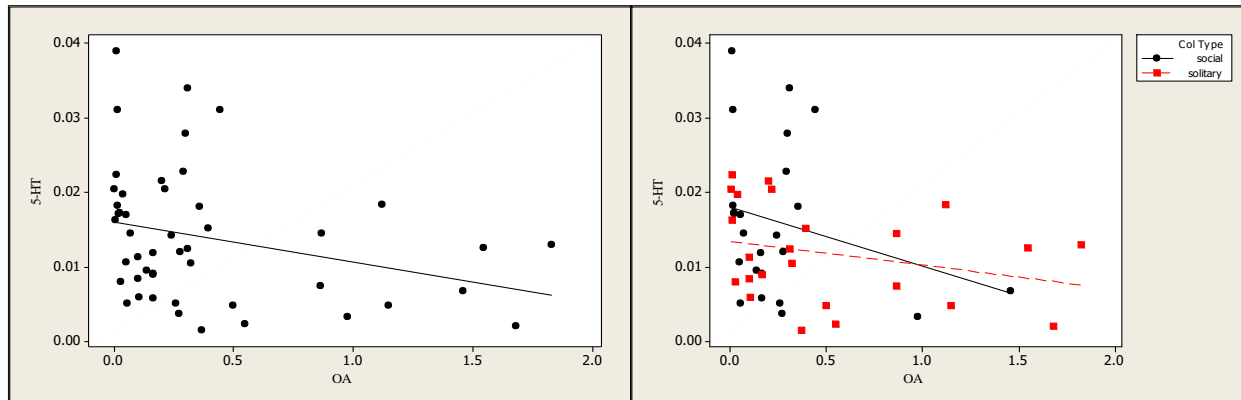


**Figure 3.3: Scatterplot of paired latency to attack (LTA) and huddle duration (HD) scores for developing *Anelosimus studiosus*.**

### Biogenic Amines

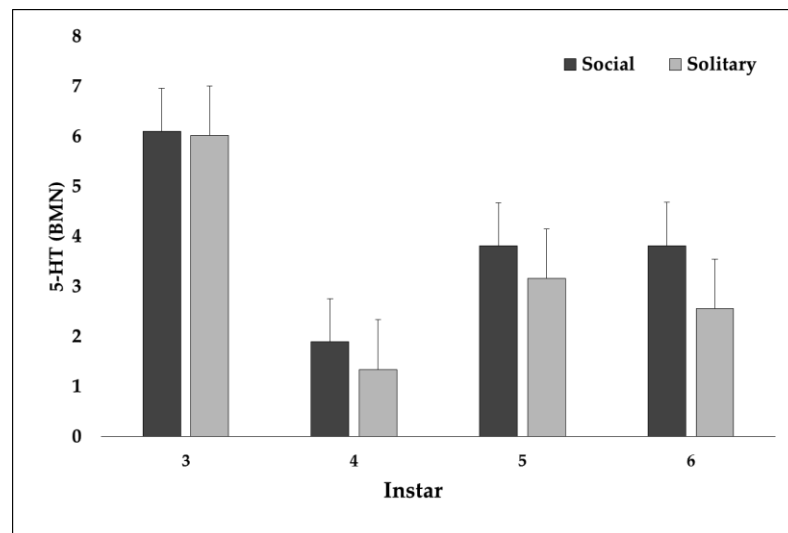
Regression analysis revealed a negative relationship between serotonin and octopamine (Fig. 4, left panel;  $p=0.045$ ) among juvenile *Anelosimus studiosus*. Generally higher serotonin (5-HT) levels were found to occur with lower octopamine (OA) levels. Looking at juveniles in the context of their maternal phenotype (colony type), we see that social and solitary juveniles occupy slightly different ranges on the scatterplot (Fig. 4, right panel), with higher 5-HT levels in spiderlings from social colonies and higher OA levels derived mostly from spiderlings from solitary colonies.





**Figure 3.4: Scatterplots of serotonin (5-HT) vs. octopamine (OA). (Left) Regression analysis indicates a significant negative relationship ( $p=0.045$ ). In general, higher serotonin levels are associated with lower octopamine levels in juvenile *A. studiosus*. (Right) The highest 5-HT measurements were generally measured from social juveniles (black circles), and the highest OA measurements were generally from solitary juveniles (red squares).**

Serotonin (5-HT) was higher in spiderlings from social colonies than those from solitary colonies throughout the observed developmental period (Fig. 5). There was a marked decrease in serotonin at the 4<sup>th</sup> instar and an increase at the 5<sup>th</sup> instar for both social phenotypes. There was a divergence at the 6<sup>th</sup> instar, with social juveniles experiencing an increase and solitary juveniles showing a decrease in 5-HT.



**Figure 3.5: Comparison of body-mass-normalized 5-HT levels between social and solitary phenotypes and among instars (ages) of immature spiderlings. Social phenotype and age are factors**

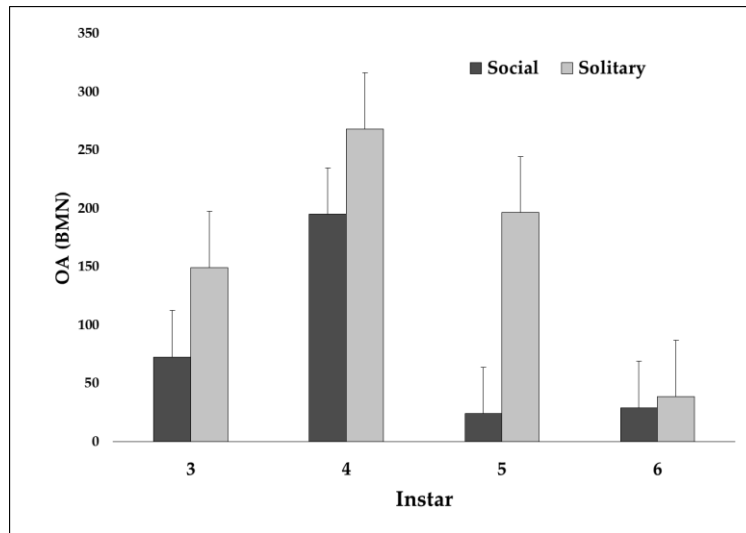
associated with significant differences in 5-HT levels, but there is not a significant interaction between these factors (p=0.49). Error bars indicate standard error.

Analysis by General Linear Model suggests that both colony type (social or solitary, p<0.001) and instar (p=<0.001) have significant effects on 5-HT levels in juvenile *A. studiosus* (Table 3). The interaction of colony type and instar had no significant effect on 5-HT (p=0.49).

**Table 3.3: Results from general linear model (GLM): 5-HT vs. colony type and instar.**

GLM			
Source	DF	F	p-value
Colony Type	1	14.63	<0.001
Instar	3	31.59	<0.001
Colony Type * Instar	3	0.81	0.49

Octopamine (OA) levels of social spiderlings were lower at all instars than those of solitary spiderlings (Fig.6). However, analysis by general linear model suggests that colony type does not have a significant effect on OA level (Table 4; GLM; p=0.41), but instar (age) was associated with highly significant differences in OA production per gram of body mass (Fig. 6; Table 4; p<0.01). There was no significant interaction of colony type and instar (p=0.24).



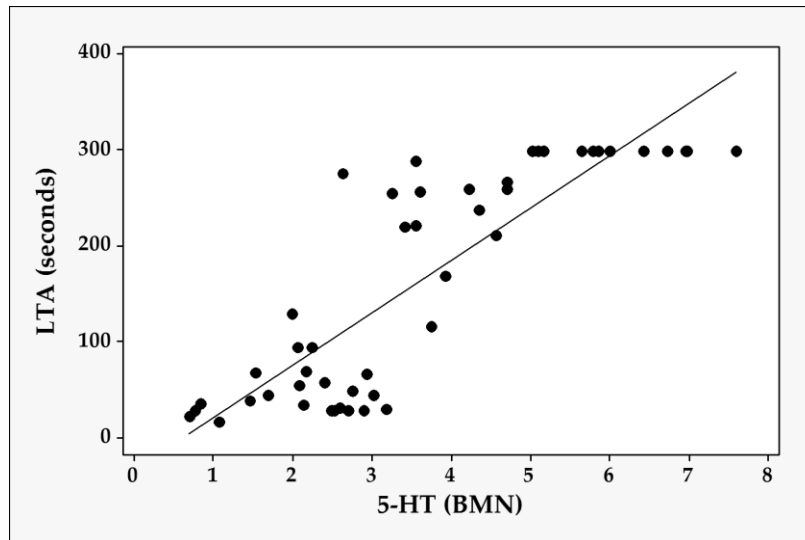
**Figure 3.6: Comparison of body-mass-normalized octopamine (OA) levels between social and solitary phenotypes and among instars (ages) of immature spiderlings. There is no significant difference between phenotypes ( $p=0.41$ ), but OA does differ significantly depending upon instar ( $p=0.001$ ). Interaction between phenotype and age was found to have no significant effect on OA levels ( $p=0.24$ ).**

**Table 3.4: Results of general linear model (GLM): OA vs. colony type and instar.**

GLM			
Source	DF	F	p-value
Colony Type	1	0.7	0.41
Instar	3	5.3	<0.01
Colony Type * Instar	3	1.47	0.24

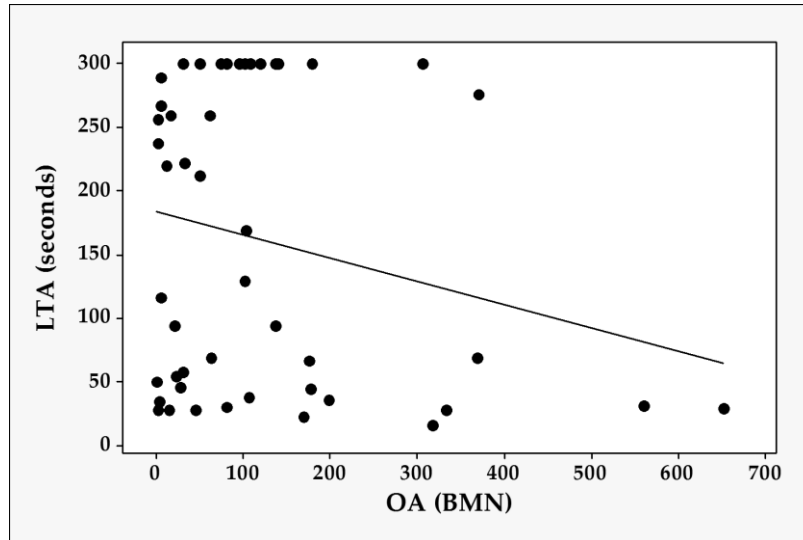
Latency to attack prey is a measure of time which can be used to assess aggressiveness in spiders. Serotonin (5-HT) is correlated with increased defensive behavior (wariness) and decreased aggressive behavior. Lower 5-HT levels are found in juveniles with shorter latency to attack (LTA) times (more aggressive), and higher 5-HT levels occur in juveniles with longer latency times (Fig. 7; Regression Analysis,  $p<0.001$ ). This trend is found in both social and

solitary juveniles. Most 3<sup>rd</sup> instar juveniles did not respond to the introduction of prey, which resulted in a latency score of 300 seconds (the maximum time allowed for attack of prey).



**Figure 3.7: Scatterplot of 5-HT levels vs. Latency to Attack (LTA) times (s). Higher 5-HT corresponds with longer latency times, and lower 5-HT is associated with shorter latency times. Regression Analysis,  $p < 0.001$ .**

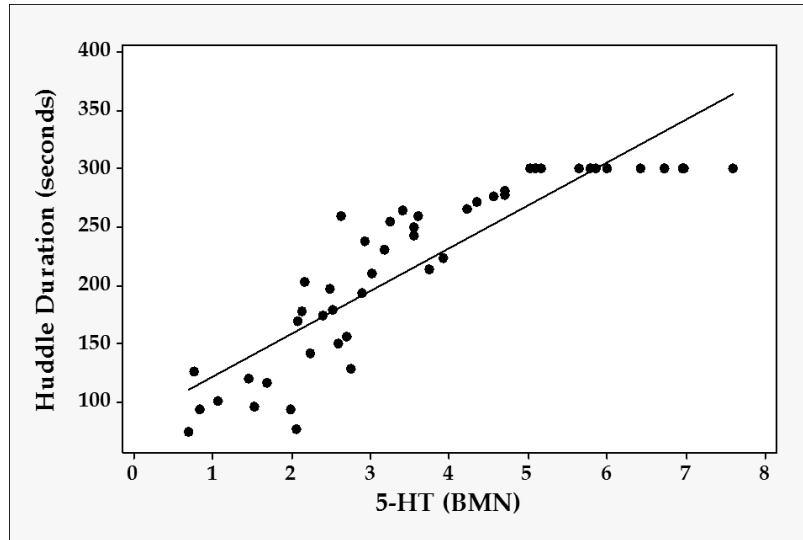
Regression analysis shows that octopamine (OA) has a weaker correlation with latency times than does 5-HT, at least in immature spiders (Fig. 8;  $p = 0.12$ ), but we still see that in general, longer latency times are associated with lower levels of OA, and the samples with the highest levels of OA came from spiders which attacked quickly.



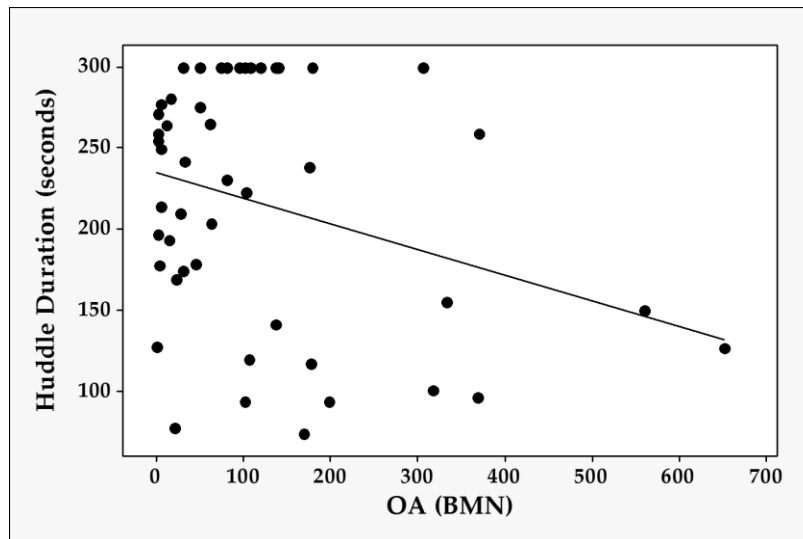
**Figure 3.8: Scatterplot of OA level vs. Latency to Attack Prey (s). OA levels are generally lower in spiders that take the longest amount of time to attack, and the highest OA levels are found in spiderlings that attack more quickly, but the correlation is not significant ( $p=0.12$ ).**

Mean huddle duration (a measure of defensive behavior) is shorter in spiders with lower 5-HT and longer in spiders with higher 5-HT. There is a strong positive correlation between 5-HT level and huddle duration (Fig. 9; Regression Analysis,  $DF=1$ ,  $F=167.12$ ,  $p<0.001$ ). This case is seen in both social and solitary juveniles. As with latency times, 3<sup>rd</sup> instar spiderlings displayed thanatosis (huddled) for the maximum duration (300 seconds).

Mean huddle duration is negatively and significantly correlated with octopamine (Fig. 10; regression analysis;  $DF=1$ ,  $F=4.68$ ,  $p=0.04$ ). Higher OA is associated with shorter amount of time spent in thanatosis (decreased wariness).



**Figure 3.9: Serotonin (5-HT) and mean huddle duration. Serotonin (5-HT) levels, normalized by body mass (BMN), are correlated with increased defensive behavior and decreased aggressive behavior. Mean huddle duration is shorter in spiders with lower 5-HT and longer in spiders with higher 5-HT, and the correlation is strong (regression analysis,  $p < 0.001$ ).**



**Figure 3.10: Octopamine (OA) levels and mean huddle duration. Higher octopamine levels (normalized by body mass) are significantly correlated with shorter mean huddle durations (regression analysis,  $p = 0.04$ ).**

## Discussion

Behavioral assays conducted on spiderlings of the 3<sup>rd</sup> instar illuminated little behavioral variation among individuals. At this age, spiderlings are not yet responsive to the introduction of prey items, and they respond to air puffs by remaining huddled for much longer than the cutoff measurement time of 5 minutes (300 seconds).

Serotonin and octopamine are believed to influence aggressive and defensive behaviors in invertebrates. Levels of these neurochemicals change throughout development of immature social and solitary spiderlings of the species *Anelosimus studiosus*. Serotonin is highest in the earliest developmental stages of both social and solitary spiderlings, when they are most dependent on their mothers and when it is most vital for them to coexist in the natal web. We see a major shift in serotonin and octopamine levels as the spiderlings transition to an age at which they begin to attack and consume prey, and these neurochemical differences are accompanied by significant behavioral changes (latency to attack prey and huddle durations). These shifts continue throughout development, and we see a significant difference in serotonin in social and solitary subadults (6<sup>th</sup> instar). Throughout development, serotonin is higher in social spiderlings than in solitary spiderlings, and octopamine is higher in solitary spiderlings than in socials.

As hypothesized, higher serotonin levels are associated with longer latency times and longer huddle durations, and lower serotonin levels occur with shorter latency times and shorter huddle durations, suggesting that serotonin may, in fact, influence a spider's tendency to be more docile or aggressive. Likewise, octopamine levels may also affect these tendencies. Shorter huddle times and shorter latency times are observed in spiders with greater octopamine levels, and longer times (more docile behavior) are associated with lower levels of octopamine.

Levels of 5-HT are more similar in social and solitary phenotypes during early stages of development, but we see a great divergence as the spiderlings mature. Conversely, our results show that octopamine levels are quite different between phenotypes earlier in development but become more equal before spiderlings reach maturity.

These data suggest that both serotonin and octopamine may be playing important roles in behavioral development of social and solitary *Anelosimus studiosus*, but it seems that serotonin may be a key player in determining the primary social tendency of an individual at the time of maturity and dispersal from the natal web.

We believe that these preliminary results, along with future studies, can offer great insight into the physiological differences and neurochemical controls that may accompany evolutionary shifts in sociality.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.



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## CHAPTER 4

### DISCUSSION

The evolution of social behavior is perhaps one of the most important events in the history of life on earth. Yet, many animal species remain solitary. Sociality is rare among spiders. Yet it has evolved independently across taxa multiple times. In the face of changing environmental factors, it is impossible not to wonder what neural mechanisms allow a species to transition from being solitary or aggressive to being cooperative and social. Little research has been done on behavioral neurochemistry of spiders, and spiders are an ideal model system for investigating questions like this.

Our model species, *Anelosimus studiosus*, offers a rare opportunity to study major *intraspecific* behavioral differences which are influenced by genotype and environmental conditions. Individuals can be classified as social or subsocial, and they form colonies which are either social (multiple adult females and their offspring) or subsocial (a solitary adult female and her subadult offspring). Juvenile offspring of both phenotypes are extremely tolerant of one another in early stages of development, but as they grow closer to sexual maturity, individuals of the solitary/aggressive behavioral type become less tolerant, while the docile/social individuals seem to maintain the tolerance to some degree.

Subsocial females are typically more aggressive toward conspecifics, while social females are more tolerant. Social females cooperate in web maintenance, prey capture, and brood rearing. It has been suggested that increased tolerance of conspecifics is one of the characteristics of a species evolving toward eusociality.

Aggressive behavior can change in response to hunger (Walker and Rypstra 2003), temperature (Pruitt et al. 2011), reproductive state, time of day (Watts et al. 2013), and age. In the research presented in this dissertation, we used high performance liquid chromatography with electrochemical detection (HPLC-ECD) to analyze biogenic amine levels of spiders experiencing different conditions, and we determined that specific neurochemical changes are coinciding with observed behavioral changes.

We found that nutritional state affects both behavior and neurochemistry. Starved spiders were significantly more aggressive in responding to prey than well-fed spiders. Well-fed spiders became significantly less aggressive, with latency times more than doubling, and starved spiders became more aggressive, with latency times decreasing by half. Levels of several biogenic amines (epinephrine, octopamine, and serotonin) were significantly higher in well-fed spiders than in starved spiders, but dopamine was higher in starved spiders. We expected higher levels of serotonin and epinephrine in well-fed spiders and higher dopamine levels in the starved spiders, but the octopamine trend was surprising.

Behavioral and neurochemical profiles change throughout the day and with reproductive state. Brooding adult females are significantly more aggressive than nonbrooding females. And all females are more aggressive in the evening than in the morning. An unidentified neurochemical compound appears to correlate with these changes in aggression, along with several known biogenic amines: serotonin, norepinephrine, and dopamine.

Finally, we conducted an ontogenetic study of *Anelosimus studiosus*, comparing behavior and neurochemistry of offspring from subsocial and social colonies at different stages of development. The aim of this investigation was to determine whether or not there is a gradual switch in behavior-related biogenic amines as spiderlings mature into adults. It was expected that

a neurochemical divergence between social and subsocial juveniles would occur, and our hypotheses were supported by our findings.

In the studies contained within this dissertation, we compared quantifiable behaviors with levels of neurochemicals known to affect aggression and social behavior. We looked at effects of development and age, nutritional state, reproductive state, and time of day. Studies of this nature can help to explore the physiological effects of environmental factors and gain insight into the proximate mechanisms of behavior that may lead to the evolution of sociality.

“How profoundly human it is; how deeply characteristic of our species, ethologically speaking – to wonder at, to study and investigate behavior of our own and other species – toward understanding, in the sense of accounting for, the actions, the appetites, the drives, alternative modes, and sensory guidance that we observe.”

~ Gunther Zupanc, Behavioral Neurobiology, 2004

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