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Alexander E. DeMarco
East Tennessee State University

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Diel Rhythmicity Found in Behavior but Not Biogenic Amine Levels in the Funnel-Web Spider *Agelenopsis pennsylvanica* (Araneae, Agelenidae)

A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

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Master of Science in Biology

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by

Alexander Edward DeMarco

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Dr. Thomas Jones, PhD, Chair

Dr. Darrell Moore, PhD

Dr. Lev Yampolsky, PhD

Keywords: *Agelenopsis pennsylvanica*, animal personality, biogenic amines, diel rhythms, neurochemistry and behavior, spider behavior

ABSTRACT

Diel Rhythmicity Found in Behavior but Not Biogenic Amine Levels in the Funnel-Web Spider, *Agelenopsis pennsylvanica* (Araneae, Agelenidae)

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Quantifying individual differences in behavior and the extent that behavior is influenced by circadian control is of paramount importance in behavioral ecology. In addition, the proximate mechanisms underlying behavior are also critical in order to obtain a more complete picture of how behavior evolves. Biogenic amines (BAs) are simple nitrogenous compounds derived from amino acids and have been consistently and extensively linked to behavior. For this study, we analyzed temporal patterns of BAs in relation to the antipredator (boldness) and aggressive behavior in female *Agelenopsis pennsylvanica*, a funnel-web spider. Using HPLC-ED, we compared behavioral responses to temporal patterns of octopamine and serotonin, two BAs known to influence behavior in invertebrates. Our results suggest that, while there was a clear diel cycling pattern of both aggression and boldness, BAs do not follow this same pattern, suggesting that oscillations in absolute levels of BAs are not the underpinnings of behavioral oscillations.

DEDICATION

For my grandmother, Emma Napoli, and all the love and unwavering support she has given me throughout my entire life. Even though she can’t be here to see me complete this degree, I know she’ll always be proud of me. I will always love you. Rest in peace, Grandma.

For my parents, who have supported me throughout everything and have helped shape me into the person I am today. I love you both, and words cannot begin to express the depth and breadth of the gratitude and appreciation I have for the both of you.

For my friends, who have always stood by me and listened to me talk about my research even if they didn’t fully understand why I do what I do. You have all the thanks and appreciation I am able to muster.

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Lastly, I would like to thank Rebecca Wilson, my lab mate, roommate, and overall confidant. Without your help, I probably would not be as sane as I am at this point. Chronobiology does wonders for the psyche, doesn’t it?

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CHAPTER 1

INTRODUCTION

Animal Personality and Behavioral Syndromes

Since the inception of the fields of animal behavior (ethology) and behavioral ecology, many studies have opted to focus their attention on the effect of a singular behavior or behavioral pattern on an individual's overall fitness. While this is assuredly important, Dobzhansky (1956) noted that 'a trait has no adaptive significance in isolation from the whole pattern that the organism exhibits'. Phenomena such as pleiotropy and the genetic linkage of traits have led ethologists and behavioral ecologists to look at behavioral patterns and their relationships to other phenotypic traits, e.g. Riechert and Maynard Smith (1989) and Riechert and Hedrick (1993). To that end, there has been increasing focus in recent years on the subject of behavioral syndromes or "animal personality". This is the idea that, within a given population of organisms, there are inter-individual differences in behavioral tendencies that are consistent across behavioral contexts and time (Sih et al. 2004; Stamps and Groothuis 2010). For example, in a particular population some individuals may respond to potential predators with high levels of aggressive behavior. If a syndrome is present along this behavioral axis, those individuals will also respond with high levels of aggression towards potential prey and/or mates. These individuals have a more aggressive behavioral type (BT). Conversely, if individuals in the population respond with low levels of aggressive behavior towards predators, they will also respond to prey and/or mates with low levels of aggression and have a less aggressive BT. These behavioral tendencies are heritable (Riechert and Jones 2008; Pruitt and Riechert 2009) and do not change over the course of an individual’s life (Sih et al. 2004).

Personality is a concept that has been investigated in human psychology since at least the 1930s (Allport 1937). Psychologists have studied the developmental, social, emotional and environmental factors that influence the development of personalities in humans (McAdams and Olson 2010), but there is still much debate among psychologists as to whether personality is even a valid concept. It is worth noting that the study of human personality can be called into question, the reason for which being that there can always be some degree of uncertainty as to the truthfulness and sincerity of an individual’s responses. While nearly all organisms possess the capability to and/or actively engage in deceptive behavior, humans will sometimes use deceptive behavior with no tangible benefit to their own fitness. This could prevent researchers from obtaining responses that are as honest and objective as possible. The issue appears to be mitigated when studying personality using animal models. This fact alone makes personality an intriguing and worth investigating. Animal models allow us to obtain unbiased insights into the fitness consequences of personality and apply those principles to humans, underscoring its importance as a field of study.

Taking that into account, personality appears to be ubiquitous across many different taxa, encompassing both vertebrates and invertebrates. In vertebrates, personality has been studied in lizards (A. Carter et al. 2012), bighorn ewes (Réale et al. 2000), fish (Coleman and Sloan Wilson 1998; Brown et al. 2005), and humans (Sloan Wilson et al. 1994). In invertebrates, personality is widespread, from social insects (Jandt et al. 2014; Monceau et al. 2015) to crustaceans (Gherardi et al. 2012) and may other arthropod groups. One group where personality appears to be extremely important from an ecological perspective is spiders. Spiders have been the focus of much of the recent work focusing on animal personality because they are ecologically dynamic organisms, acting as both predator and prey in a given environment. This suggests that an individual's behavior most assuredly plays a large role in determining its ability to avoid predation, successfully forage for food, court and obtain mates, as well as participate in social interactions with conspecifics. This speaks to the immense ecological importance and implications on individual fitness of personality not only in spiders but many other taxa (Sih et al. 2012).

Behavioral syndromes have fitness implications not only at the individual level, but also at the population and species levels. Across a species' distribution, there are often populations that are locally adapted to survive in a particular ecological niche. The collective BT of a certain population may also be locally adapted to survive in a given environment. A prominent example of this is the desert spider, *Agelenopsis aperta* (Araneidae, Agelenidae). Susan Riechert has used this spider as the subject of extensive research into the fitness consequences of behavioral syndromes. *A. aperta* occurs primarily in the southwestern United States, occupying a range of different habitats, with two of the main habitat types being arid desert and riparian. Individuals in the desert habitat are prey-limited and have a more aggressive BT, while individuals that occupy the riparian habitat have abundant prey, higher levels of bird predation, and have a less aggressive BT. Individuals within each of these populations differ in their own levels of aggressiveness, and the difference in collective BT between these two populations has been found to have a genetic basis (Maynard Smith and Riechert 1984; Hedrick and Riechert 1989; Riechert and Hedrick 1990) and is likely the result of selection acting on the genes responsible for foraging ability. This claim is reinforced by the experimental manipulation of gene flow and predation performed in Riechert, 1993. Similar patterns of behavioral syndromes have been detected in many other spider families, including comb-footed spiders (Theridiidae) (Riechert and Jones 2008; Pruitt et al. 2010), nursery-web spiders (Pisauridae) (Johnson and Sih 2007), and orb weaving spiders (Nephilidae) (Kralj-Fišer et al. 2012). For a more complete list, see Pruitt and Riechert, 2012.

Based on the available research, it is clear that there are major consequences of personality on fitness in multiple levels of biological organization. It is important to continue this research track in order to derive further insights into (1) the effects behavioral phenotypes have on fitness at different levels of organization (i.e. community and others), (2) the different selection pressures that lead to the evolution of behavioral syndromes, and (3) the underlying proximate mechanisms that mediate changes in behavior and how those mechanisms are related to behavioral syndromes.

Biogenic Amines and Behavior

One of the underlying goals of ethology is to study the proximate mechanisms of behavior. One such proximate mechanism organisms employ to modulate behavior is the use of biogenic amines. Biogenic amines are a group of neurotransmitters that have been shown to have at least some influence on behavior in both invertebrates and vertebrates. In invertebrates, the biogenic amines octopamine (OA), serotonin (5HT) and dopamine (DA) are suspected to mediate behaviors in a variety of contexts including aggression/wariness (Adamo et al. 1995; Stevenson et al. 2005; Jones, Akoury, Hauser, Neblett, et al. 2011), courtship and mating (Hebets et al. 2015), social behavior (Barron et al. 2007; Cunningham et al. 2014), locomotion (Yellman et al. 1997; Cooper and Neckameyer 1999; Dacks et al. 2003; Fussnecker et al. 2006), and foraging (Erber et al. 1993; Scheiner et al. 2006). Despite the myriad of studies showing that biogenic amines influence behavior, there is still much to be understood about the specific mechanisms through which these compounds actually modulate these behaviors. Existing studies have shown that the manner in which biogenic amines are employed across different taxa is not uniform. Our knowledge base for this subject could be a result of the different techniques and study systems employed to measure and examine the influence of biogenic amines on behavior. These techniques include pharmacological manipulations of biogenic amines via topical, oral or injection pathways, as well as some genetic manipulation. These methods are not always reliable due to their use of manufactured analogues of the actual compounds. These analogues may interact differently within an organism's system, thus providing results that may be inaccurate or incomplete. This is something to consider when using manufactured chemicals to study the effects of biogenic amines on behavior.

Of the biogenic amines mentioned above, OA has been widely studied over the past several decades. This compound is an analog of norepinephrine found primarily in invertebrates and modulates a variety of physiological processes behaviors (Widmer et al. 2005). OA has been characterized as the "fight or flight" hormone due to its association with a myriad of aggression-related behaviors in arthropods (Orchard 1982). OA appears to have opposite effects between different arthropod taxa. At high levels, OA appears to decrease aggression in crustaceans. When OA is injected into freely moving lobsters, individuals assume a posture indicative of subordinate lobsters (Kravitz 1988). This posture is characterized by a raising of the legs and tail off the substrate, with the legs and tail extended toward the anterior end and the large claws are extended towards the posterior (Livingstone et al. 1980). OA appears to have the opposite effect in other arthropod taxa. In the field cricket *Gryllus bimaculatus*, it was found that circulating levels of OA in hemolymph increased significantly following agonistic interactions between male crickets, regardless of which individual won the contest (Adamo et al. 1995). In the orb-weaving spider *Larinioides cornutus*, the duration of the "huddle response", a common metric of "boldness" or wariness in spiders, significantly decreased following topical treatment with OA (Jones, Akoury, Hauser, Neblett, et al. 2011). In the wolf spider *Rabidosa punctulata*, males will utilize one of two different mating tactics: (1) a traditional courtship display employing the use of both visual and acoustic signals or (2) a direct mounting of the female, the more aggressive tactic of the two. Following mating trials, it was found that males that employed the direct mount approach had higher levels of circulating OA (Hebets et al. 2015). Lastly, in the Western Black Widow spider, *Latrodectus hesperus*, both the huddle response mentioned above and latency to attack prey, a commonly used metric of aggression in spiders (Hedrick and Riechert 1989; Pruitt et al. 2008; Kralj-Fišer et al. 2012), significantly decreased following topical treatment with OA (DiRienzo et al. 2015). Shorter duration of huddle responses and latency to attack prey are indicative of individuals that are bolder and more aggressive, supporting the claim that increased levels of OA result in higher levels of aggression.

Another biogenic amine, serotonin (5-HT), potentially has an opposite effect on behavior in arthropods. In the orb-weaving spider *L. cornutus*, exogenous dosing with serotonin resulted in a significant increase in the duration of the huddle response, indicating less bold individuals following treatment. This effect lasted for at least twenty-four hours, if not longer (Jones, Akoury, Hauser, Neblett, et al. 2011). Additionally, 5-HT was found to significantly increase the latency to attack prey in both *L. hesperus* and the funnel-web spider *Agelenopsis pennsylvanica*. In *L. hesperus*, 5-HT increased the duration of the huddle response, indicating a decrease in boldness for the treated individuals (DiRienzo et al. 2015). In *Drosophila melanogaster*, two behaviorally different fly lines (one line being more aggressive than the other) treated with precursor 5-HT showed higher frequencies of aggressive interactions than lines not treated with

5-HT (Dierick and Greenspan 2007). In *Gryllus bimaculatus*, chemical depletion of serotonin with the synthesis inhibitor α-methyltryptophan (AMTP) resulted in individuals developing an apparent hypersensitivity to aversive stimuli, thus lowering the threshold needed to elicit an antipredator response (Stevenson et al. 2000). In the case of locomotor activity, chemical depletion of 5-HT via the use of a 5-HT_2 receptor agonist caused locomotor activity in *Drosophila* to increase (Johnson et al. 2009). However, in the flesh fly *Neobellieria bullata*, injection with 5-HT saw not only a decrease in locomotor behavior, but also a decrease of all active behaviors across the board (Dacks et al. 2003). These examples suggest that the role of 5-HT in modulating behavior is still unclear, potentially modulating multiple behaviors at one time or working in concert with other neurochemicals to achieve the same effect. Clearly, the influence of biogenic amines on behavior is something that requires further study.

Diel Changes in Behavior

In addition to better understanding the proximate mechanisms of behavior, another goal of animal behaviorists is to better understand how abiotic factors influence changes in behavior. Many behavioral and physiological processes are controlled endogenously and operate over a period of approximately 24 hours. These processes are considered to be “circadian” and oscillate independently of external cues. They are, however, kept in sync with the 24 hour daily cycle (entrained) by external cues e.g. the rising or setting of the sun (Suter and Rawson 1968). Such rhythms are nearly ubiquitous among animals and are found in both vertebrates (Suter and Rawson 1968; Pita et al. 2011) and invertebrates (Refinetti 2000; Moore et al. 2016). It is widely believed that these diel rhythms are adaptive, with entrainment to cyclical environmental conditions serving to optimize behavioral and physiological responses for periods where those conditions are more favorable (Watts et al. 2014). This optimization enables an organism to anticipate multiple changes in biotic and abiotic factors rather than simply react to these changes and risk injury or death. However, the adaptive value of circadian rhythms is limited to environments where environmental conditions regularly cycle (Woelfle et al. 2004). In environments with either extremely low or high variation in abiotic conditions, the absence of cycling conditions would not select for individuals whose behavior changes regularly, instead selecting for individuals whose behavior is more constant over the course of the day.

Experimental Aims and Hypotheses

While most studies focusing on circadian rhythms tend to look at singular behaviors, e.g. boldness (Jones, Akoury, Hauser, and Moore 2011), locomotor behavior (Smith and Larimer 1979; Suter 1993), there are few studies that have looked at the influence of circadian rhythms on behavioral syndromes (Watts et al. 2014; Watts et al. 2015). If behavioral syndromes are indeed adaptive as the wealth of the research suggests, it could be argued that diel cycling of abiotic conditions is at least one of the primary factors driving selection on many behavioral traits within a population, rather than just the abiotic factors themselves. Many studies on personality do not take diel cycling into account, which leaves this area ripe for novel studies that examine the relationship between behavioral syndromes and diel rhythms, using proximate mechanisms such as the characterization of biogenic amines in model invertebrate systems.

The aims of this project are twofold. First, this project aims to examine how changes in light cycling affect different behavioral axes, specifically boldness and aggression, in the funnel web spider *Agelenopsis pennsylvanica*. Second, this project aims to examine if there is any correlation between biogenic amine levels, behavior, and diel cycling. We hypothesize that boldness, aggression and biogenic amine levels will cycle with time of day, underscoring the influence biogenic amines have on behavior and providing a window into the proximate underpinnings of behavior.

CHAPTER 2

MATERIALS AND METHODS

Organism Collection and Lab Maintenance

Agelenopsis pennsylvanica (Araneae: Agelenidae) is a large (body length: males 9–12 mm; females 10–17 mm), brown funnel-web spider that can be found in numerous habitats across the Northern United States and is the *Agelenopsis* species most common in the Northeast (Bradley 2013). Its web is commonly built near the ground in open grassy habitats and is composed of a sheet of non-sticky capture silk, an attached funnel that leads to a retreat contained in some feature of the habitat, and a vertical scaffold that assists in knocking down prey onto the sheet portion of the web. Like other funnel weavers, foraging behavior in this species consists of individuals waiting at the mouth of the funnel and running out to capture prey that fall down onto it. Adult female *A. pennsylvanica* were caught in Northeast Tennessee during the summer of 2016 and spring of 2017. These individuals were fed a diet of live crickets and misted with water 1-2 times a week. The spiders were entrained to and housed in a 12:12 h light-dark cycle with one-hour ramping transitions. The transition from dark to light began at 07:00 h and finished at 08:00; the transition from light to dark began at 19:00 h and finished at 20:00 h. This ramping cycle was used in order to better simulate natural light conditions. All assays were performed using the same group of spiders, in the order in which they are described and recorded with surveillance cameras equipped with infrared night vision capabilities (Q-SEE QT9316) in order to simultaneously collect data on multiple individuals.

Boldness Assay – Antipredator Behavior

"Boldness" is defined as the predilection of an individual to engage in risk-prone behavior (Coleman and Sloan Wilson 1998; Pruitt et al. 2016), especially in novel situations. Although the methods for measuring boldness can differ, it is used as a behavioral metric in many taxa, including spiders (Riechert and Hedrick 1990; Riechert and Hedrick 1993) and other arthropods (Wilson et al. 2010; Niemelä et al. 2012), as well as many vertebrates such as reptiles (A.J. Carter et al. 2012) and fish (Brown and Braithwaite 2004). For this experiment, boldness was characterized by measuring the amount of time an individual takes to resume normal behavior following the administration of an aversive stimulus (simulating the approach of a predator).

Assay and Data Collection.

Boldness trials were performed every three-hours over a 24-hour period, starting at 01:00 h. Subsequent time points tested were 04:00, 07:00, 10:00, 13:00, 16:00, 19:00, and 22:00 h. Individuals were removed from their home container, placed in an open-top, six-quart plastic box (Sterilite) and allowed to acclimate to their new environment for a period of 60 s. After acclimation, two puffs of air were applied to the anterior prosoma of the spider using an infant nose-cleaning bulb. This resulted in the spider drawing its legs towards its body in a "death-feign" or "huddle" posture. Video cameras were used to record the spiders for the duration of the trial. Trials were determined to be complete when the spider successfully moved one body length from its original position or after 600 s, whichever occurred first.

Videos were reviewed following recording using an Apple MacBook Pro laptop computer and VLC media playing software. Recording for each trial began immediately prior to acclimation, and videos were not scored until after evidence of the aversive stimulus being administered. For each spider, we recorded (1) whether the spider fled from the stimulus after its administration, (2) the latency to emerge from the huddle, and (3) the latency move one body length from its original position, a procedure modified from previous experiments (Pruitt et al. 2008; Jones, Akoury, Hauser, Neblett, et al. 2011).

Latency to Attack Assay – Aggression

Like boldness, the methods for measuring aggressive behavior can differ across taxa. However, for the purposes of this study, aggression was measured using latency to attack a prey item. This particular method has been used as a metric of aggressive behavior in spiders (Pruitt et al. 2008; Pruitt et al. 2010; Keiser and Pruitt 2014) and other taxa. Aggressive tendencies were estimated by measuring the amount of time required for an individual spider, following the introduction of a prey item into its web, to emerge from its retreat and make contact with the prey item.

Assay and Data Collection.

Individuals were randomly selected into two groups and tested at two different time points within a 24-hour period: 01:00 h (mid-scotophase) and 13:00 h (mid-photophase). These time points in particular were chosen because we observed in other laboratory experiments that the largest difference in behavioral response occurred between these two time points. Individuals were provided a cricket as a meal approximately 48 hours prior to testing in order to control for hunger state. We then removed individuals from their original containers, placed them in plastic vials and allowed them to construct a retreat in the vial for a period of 12 hours. Vials were fastened into the side of a six-quart plastic box (Sterilite), and after the initial 12-hour period the lid of the vial was removed, allowing access to the entirety of the larger container and controlling for the location of the spider's retreat. The spider was then given another 24 hours to construct the sheet portion of its web. At the start of a trial, the large container lid was removed, the spider was given 60 s of acclimation time and a cricket was introduced to the web at a point approximately 25-30 cm from the spider's location in the container. Trials were deemed to be completed when the spider made first contact with the cricket or after 600 s, whichever occurred first. Twenty-four hours after their respective trial, individuals were sacrificed for biogenic amine collection.

Videos were reviewed using an Apple MacBook Pro laptop computer and VLC media playing software. Recording began prior to acclimation, and videos were not scored until after the cricket made initial contact with the web. For each spider, we recorded the length of time between the cricket's initial contact with the web and the spider's initial contact with the cricket.

Biogenic Amine Quantification

Biogenic Amine Extraction.

Modified from Hebets et al. 2015, hemolymph was collected at 01:00 and 13:00. Each spider was anesthetized in a clean plastic chamber using CO_2. The right second walking leg of each individual was cut approximately in the middle of the tibia with dissecting scissors. We collected 5uL of hemolymph using a micropipette from the cut leg and immediately transferred the hemolymph to an Eppendorf tube (pre-labeled with each spider's unique identification number) with buffer solution (0.2 M perchloric acid with 1 μg/ ml synephrine as an internal standard for OA and 2ug/ml alpha methyl serotonin as an internal standard for 5-HT). Eppendorf tubes were immediately vortexed for 5 seconds and then filtered using a Costar Spin-X Centrifuge tube with a 0.22um cellulose acetate filter (centrifuged for 6 minutes at 13,000RPM). Filtered hemolymph samples were then stored in a -20 °C freezer until analysis.

Immediately after hemolymph collection, the cephalothorax was separated from the abdomen using dissecting scissors. All walking legs and pedipalps were removed. The separated cephalothorax was flash frozen using liquid nitrogen, massed, and then immediately placed in an Eppendorf tube (pre-labeled with each spider's unique identification number) with buffer solution (0.2 M perchloric acid with 10 μg/ ml synephrine as an internal standard for OA and 20ug/ml alpha methyl serotonin as an internal standard for 5-HT). Cephalothorax samples were homogenized with the buffer solution using ceramic beads and a bead grinder system for 2 minutes and then placed over ice. Cephalothorax samples were centrifuged for 10 minutes at 13,000 RPM to pellet all physical debris. The supernatant was then filtered using a Costar Spin-X Centrifuge tube with a 0.22um cellulose acetate filter (centrifuged for 6 minutes at 13,000 RPM). This final filtered cephalothorax sample was then stored at -20 °C until analysis was performed.

HPLC-ED Analysis.

Cephalothorax and hemolymph samples were analyzed using high-performance liquid chromatography with electrochemical detection (HPLC-EC; Alexys Monoamines Analyzer). The mobile phase consisted of 10% MeOH with 50 mM phosphorc acid, 50 mM citric acid, 500 mg/ml 1-octane sulphonic acid sodium salt, at a pH of 3.25 (Antec, Boston, MA, USA 180.7050A rev 02). Samples were injected into the instrument using an AS110 autosampler at a rate of 50ul/min at 191.7kg/cm through an ALF-115 microbore column (150 x 1mm) with porous silica c(18) 3 um particle size at 4 C. Biogenic amine detection in hemolymph samples was acquired at a 5 nA range for 90 min with a VT-03 cell set at 850 mV. Peaks were confirmed against known standards and by spiking samples with an analyte. Chromatogram analysis was performed with Clarity software (Solihull, U.K.). Chromatogram peaks of OA and 5-HT in lymph were identified by comparison with known standards, and peak heights were normalized by the corresponding internal standard synephrine. We then took into account the fact that the hemolymph was diluted to 5% in each sample to determine actual amounts of OA per sample.

CHAPTER 3

RESULTS

Antipredator Behavior

There was a significant effect of the time of day on both the latency to break from the huddle response (Kruskal-Wallis test, $P<0.0001$) and the latency to move one body length from the original position (Kruskal-Wallis test, $P<0.001$). In both cases, the pattern of behavioral response showed a clear oscillation, with individuals being least bold (shyest) at 16:00 h and boldest at 07:00 h (**Figures 1a & 1b**).

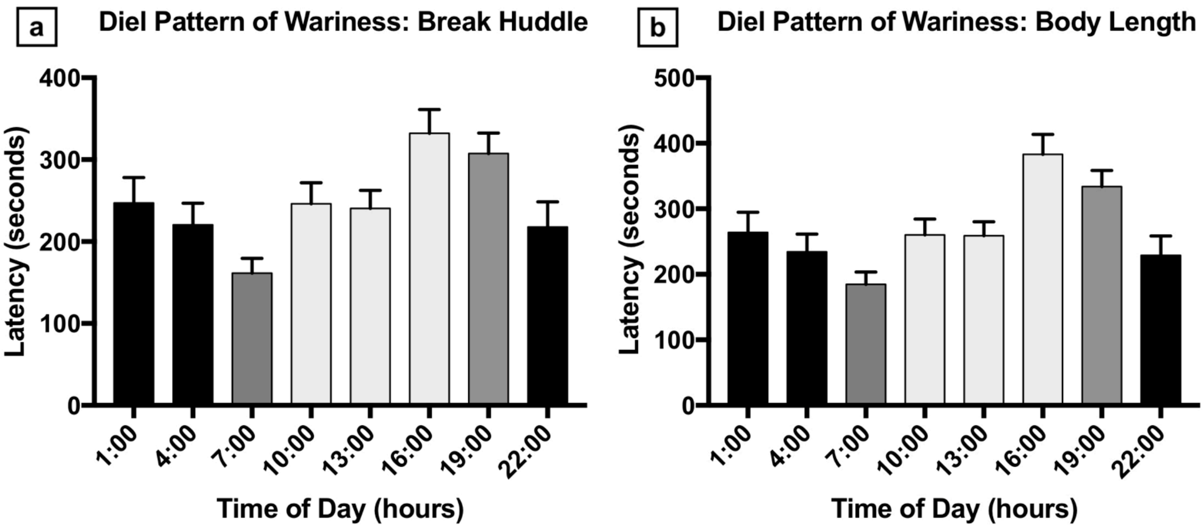

Figure 1. Diel Pattern of Antipredator Behavior. 1a. Mean latency to break out of huddle response in seconds following an aversive stimulus. 1b. Mean latency to resume normal movement in seconds following an aversive stimulus.

Aggressive Behavior

There was a slightly significant effect of time of day on an individual's latency to attack a prey item (Kruskal-Wallis test, $P=0.0452$). The latency to attack a cricket was significantly shorter at 01:00 h than at 13:00 (**Figure 2**).

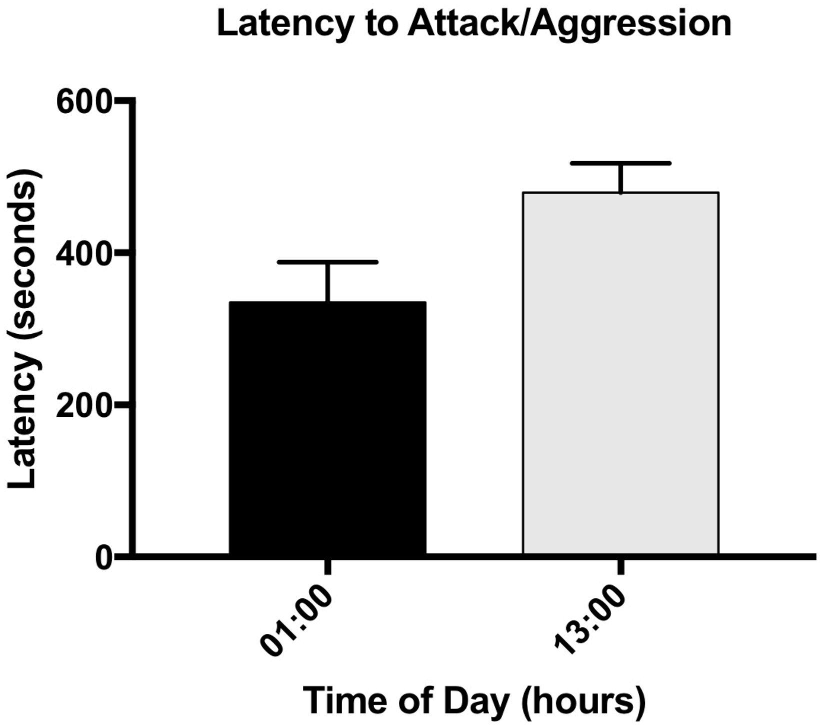

Figure 2. Diel Pattern of Aggressive Behavior. Mean time between introduction of a cricket into an individual's web and the moment of first contact by the spider.

Amine Quantification

There was no significant difference in levels of octopamine (OA), serotonin (5-HT) or the ratio of OA/5-HT at either time point tested (Figure 3). The lack of significance between the time points suggests that amines do not cycle in the same way that behavioral traits do. It is worth noting that the ratio of OA/5-HT increases in the cephalothorax and decreases in the hemolymph between 01:00 h and 13:00 h. This change is not significant but can still provide insight into the role the amines play in other behavioral contexts, i.e. locomotor activity.

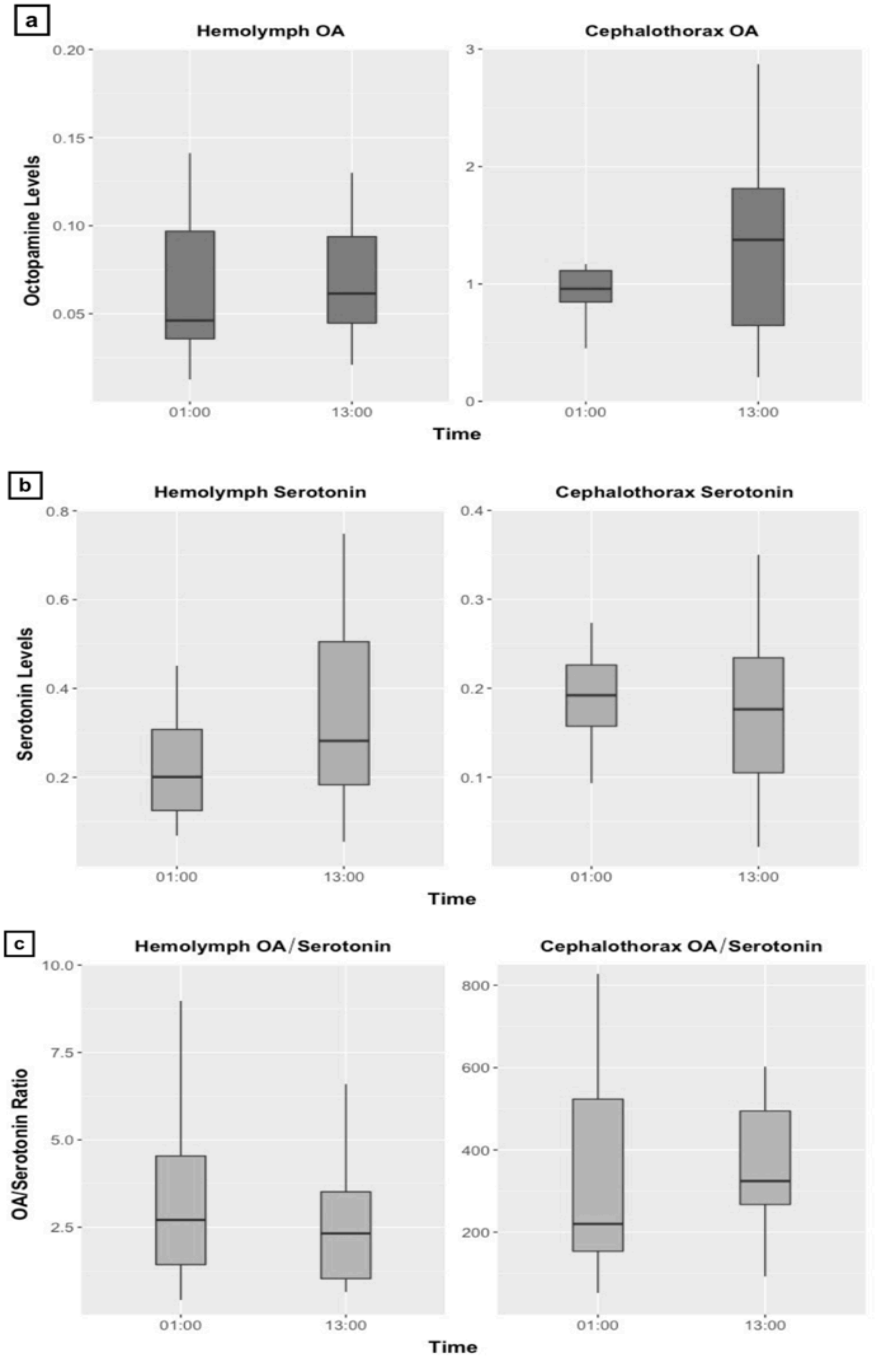

Figure 3: Biogenic Amine Levels at Different Times of Day. Bars represent the mean levels of each octopamine (Figure 3a), serotonin (Figure 3b), and the ratio between the two compounds at the time of measurement (Figure 3c). There were no significant differences in levels of octopamine (One-way ANOVA, H: $p = 0.7932$; C: $p = 0.7171$), serotonin (One-way ANOVA, H: $p = 0.2077$; C: $p = 0.167$), or the ratio between the two (One-way ANOVA, H: $p = 0.2609$; C: $p = 0.6464$) at either time point measured.

Individual Variation in Biogenic Amines

Table 1 shows p-values of the correlations between individual behavioral categories and different biogenic amine levels. There were no significant correlations between any behavioral category and biogenic amine level. Neither amine sampling location showed a significant correlation with antipredator or aggressive behavior.

Table 1. Correlations between behavioral categories and amine levels

Behavioral Category	Heme OA	Heme 5-HT	Heme OA/5-HT	Ceph OA	Ceph 5-HT	Ceph OA/5-HT
Break Huddle	0.8097	0.3203	0.9694	0.4399	0.3766	0.3621
Body Length	0.5809	0.5373	0.7369	0.4339	0.3952	0.3042
Latency to Attack	0.8399	0.5210	0.4569	0.8019	0.7247	0.5649

CHAPTER 4

DISCUSSION

Diel Changes in Behavior

In line with what was expected based on the results from other published studies (Watts 2014), antipredator behavior in A. pennsylvanica appears to be under significant influence of diel rhythmicity. Both measurements of antipredator behavior show a clear and significant oscillation across the eight different time points sampled. For the purposes of these results, an individual who is considered "bold" has lower latencies to exit the huddle and resume normal movement, and an individual who is considered "shy" has higher latencies. A. pennsylvanica individuals were boldest at 07:00 h, with boldness decreasing (increasing latencies) at subsequent time points, reaching a peak at 16:00 h. Following this time point, boldness increases until 07:00 h. This suggests that, as is the case with most diel rhythmicity in behavior, selections pressures such as predation and resource abundance have necessitated individuals of this species to modify their behavior in order to increase their chances of survival (Watts et al. 2014). It is worth noting that the magnitude of the difference in boldness for A. pennsylvanica individuals between 01:00 h and 13:00 h is smaller than the magnitude of the aggressive response between those same time points, which could be explained by examining prey abundance and predator presence throughout the day.

Like antipredator behavior, aggressive behavior also appears to be under the influence of diel rhythmicity. Again, the time points tested were chosen because it was between these two that the largest difference in behavioral response was seen during lab experiments with other species. Despite this, it may have been more prudent to test aggressive behavior and the subsequent amine levels based on the results of the antipredator behavior assays. This is because the largest difference between behavioral responses in this particular group of spiders was seen between two different time points. Ideally, it would have been more prudent to perform these trials at all eight time points, however there are two significant problems with this approach. The first issue is the use of a live prey item. While this method is useful for determining a baseline level of aggressive behavior for an individual, issues arise when performing the same assay across multiple time points during the day, such that if individuals are allowed to fully consume the prey item (usually a cricket), it can irreparably alter the results of subsequent time points. This same issue appears if one was to perform this assay with a live prey item on consecutive days instead of at multiple time points throughout the course of one day. One method to mitigate this would be to use a controlled stimulus to mimic the presence of a prey item in the spider's web (Pruitt et al. 2016). However, this runs the risk of individuals becoming habituated to the stimulus (as observed in Watts et al…), provided that the vibrational pattern is not randomized. These issues can all be mitigated with careful planning and experimental design.

The results discussed above all support the hypothesis that both boldness and aggression both operate under diel, and possibly circadian control. Further research is needed to examine the possibility that the patterns in these behavioral types are under circadian control and not just functioning under the influence of light cues.

Diel Changes in Biogenic Amine Levels

Contrary to what was hypothesized, amine levels in *A. pennsylvanica* do not appear to follow any kind of diel pattern. The differences between amine levels at both time points was not significant, and while this was not expected, it does fall in line with other observations found in unpublished studies. One observation of note is that the ratio of OA:5-HT in the hemolymph increases from 01:00 h to 13:00 h. This could suggest that larger quantities of OA and 5-HT act as hormones, entering and circulating through the body via the hemolymph (Adamo et al. 1995; Hebets et al. 2015) and could be linked to daily locomotor activity behavior.

A drawback of the format of this project was that only two different time points were sampled and that amine levels were measured in the cephalothorax as a whole. Only two time points were chosen for amine collection for several reasons. The first coincides with the reasoning behind measuring aggressive behavior at two time points. The second is that, due to the relatively small sample size of this study and the large amount of individual variation in amine levels, it was necessary to pre-select time points in order to account for this variation, maximizing the number of samples tested at each time point. Examining more time points would assuredly provide a clearer picture of the cycling pattern of these amines, if one is indeed present. Such a pattern has been shown in other studies (Levenson et al. 1999; Schulz and Robinson 1999; Carrington et al. 2007a), with these studies taking advantage of a larger range of time points than what was utilized in this study.

Another potential drawback of this study was the use of whole cephalothorax samples for biogenic amine sampling. By using whole cephalothorax samples, it is entirely plausible that there were certain changes in individual regions of the brain that were missed due to this particular sampling method. Alternatively, there could be areas within the brain where biogenic amines could be sequestered. During the sample preparation process, those pockets could have been ruptured, providing an inaccurate reading of amine levels in that particular region of the individual's body. Other studies have performed micro-dissections, looking at amine levels in different regions of the brain, seeing differences between individual regions (Levenson et al. 1999; Carrington et al. 2007b) but no difference when using entire cephalothorax samples (Schulz and Robinson 1999). If there is no diel cycling of amines, it is reasonable to infer that the variation seen with these samples is a result of individual variation and is not under circadian control.

Individual Variation in Biogenic Amine Levels

One of central tenets in the field of behavioral ecology is that there is variation in not only behavior (Sih et al. 2004; Stamps and Groothuis 2010), but other phenotypic traits, among individuals within a population. In addition to examining the effects of time of day on behavior and biogenic amine production, it was prudent during the analysis to assess the extent to which there were differences in biogenic amine production between individuals in the sample. To achieve this, we averaged the boldness scores from each of the eight time points for each individual, as well as the latency to attack measurements, and compared them to the levels of OA, 5-HT, and the ratio between the two amines. The results of this can be found in Table 1. As the table shows, we found that there was no significant relationship between overall biogenic amine levels and individual personality metrics. Furthermore, there was also no significant relationship between biogenic amine levels and personality when taking into account the area of the spider's body where the sample was being taken. Not shown in Table 1 is the correlations between biogenic amine levels and personality measurements taken by time point or treatment group. Similarly, there was no significant relationship when separating the individuals according to time of day.

Conclusion

This study presents us with somewhat mixed results. On one hand, the clear oscillation pattern exhibited by antipredator and aggressive behavior demonstrated the adaptive benefit of diel and circadian rhythms and the need for an organism to modify its behavior over its evolutionary history in order to increase its chances of survival and reproduction. We expected to observe differences in amines at different times of day, as the literature previously suggested. However, this was not the case, as amine levels did not significantly differ between the measured time points. Amine levels also did not significantly differ on an individual basis, suggesting that there could be some other factor in play with regards to differences in amine levels. This lack of a relationship between diel rhythmicity and biogenic amine is somewhat puzzling and requires further investigation, perhaps by sampling amines at a wider array of time points or sampling individual regions of the brain. The changes in OA and 5-HT levels in the different regions of the spider's body may be indicative of a link between daily activity patterns rather than personality, so investigating those patterns in this species would be prudent.

Future Directions

There are multiple avenues that can be pursued as future directions from this project. The first, and most logical direction would be to follow a similar procedure to determine if these behavioral axes are under circadian control in addition to the very obvious diel influence. To accomplish this, we would perform the same experiments over a 10-14-day period, entraining the spiders to the normal light cycle and performing assays every three hours, transitioning the light cycle to constant darkness after a period of 5-7 days. As mentioned above, the latency to attack a prey item procedure would need to be altered in order to account for habituation to the stimulus.

Likewise, samples would need to be collected every three hours as well, providing a clearer picture of any pattern of cycling that may exist in these neurotransmitters.

Another potential avenue for future direction would be to include a third behavioral axis, locomotor behavior. During the night, these spiders are generally found outside of their retreats, repairing their web and defending it from intruders. As stated above, it stands to reason that there may be a link between an individual's boldness and their activity levels during different parts of the daily light cycle. If a link between activity, boldness, and biogenic amine cycling was able to be established, it would go a long way towards shedding more light on the relationships between selection pressures (i.e. predation and prey availability), behavior, and the neurophysiology behind behavior.

Alternatively, manipulation studies are another potential avenue for examining the relationship between biogenic amines, behavior, and diel rhythmicity. By exogenously dosing individuals with biogenic amines and subsequently observing and measuring their behavioral responses, it may open avenues of research not previously considered and could also act in a confirmatory function for the results of this study and future studies of a similar nature.

Lastly, studying receptor expression instead of the levels of individual amines could help clear up what can be conservatively called a blurry picture. Using certain immunohistochemical techniques could highlight certain areas of the brain where these receptors are being up- or down-regulated, and this regulation pattern could potentially be under diel or circadian control. Likewise, western blots could be used to determine overall level of receptors in a sample, potentially indicating individual variation in receptor development, which would explain the inherently large amount of variation in biogenic amine levels.

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VITA

ALEXANDER E. DEMARCO

Personal Data: Date of Birth: 20 September, 1989
Place of Birth: Pittsburgh, Pennsylvania
Marital Status: Single

Education: Schenley High School, June 2007, Pittsburgh Public Schools
B.S. Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 2015
M.S. Biology, Biology Concentration, East Tennessee State University, Johnson City, Tennessee 2018

Professional Experience: Graduate Assistant, East Tennessee State University, College of Arts and Sciences, 2015-2017