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# Biogenic Amine Levels Correlate with Time of Day, Age, Light Cycle, and Aggressive State in the Flesh Fly, Sarcophaga crassipalpis

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# Biogenic Amine Levels Correlate with Time of Day, Age, Light Cycle, and Aggressive State in the Flesh Fly, Sarcophaga crassipalpis

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ABSTRACT

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The biogenic amines serotonin (5HT), dopamine (DA), and octopamine (OA) have been indicated in the regulation of behaviors, including aggression. The flesh fly, Sarcophaga crassipalpis, was used to investigate ontogenetic and circadian changes in amines and aggression. Heads of male flies were analyzed for amine content using high performance liquid chromatography with electrochemical detection (HPLC-ECD) at 3 time points on each of 4 consecutive days in 2 light cycles, 12:12 LD and 15:9 LD. Both DA and OA levels decreased with age. Light-cycle dependent differences were observed for all amines in overall levels and patterns of change throughout the day. A behavioral assay quantified interactive and aggressive behaviors at three time points in the light period for 2 age groups. The daily changes in behavioral profiles differed dependent on age. Correlations from these data can be made between changes in amine levels and time of day, photoperiod, age, and aggressive state.

# DEDICATION

For my husband because he has carried me emotionally and supported me intellectually through this process. He has been there as an editor, encourager, and an extra brain.

For my parents, brother, and sisters for supporting me and shaping me into who I am today.

For my son who has made me stronger than I ever thought possible.

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# CHAPTER 1

# INTRODUCTION

## Biogenic Amines

The biogenic amines serotonin (5HT), dopamine (DA), and octopamine (OA) are potential mediators of a wide range of behaviors and physiological systems in invertebrates. Roles for these amines have been identified in locomotion (Yellman et al. 1997; Dacks et al. 2003; Neckameyer and Weinstein 2005; Fussnecker et al. 2006), feeding (Erber et al. 1993; Scheiner et al. 2002), aggression (Hoyer et al 2008; Johnson et al. 2008; Zhou et al. 2008; Alekseyenko et al. 2010), social status (Bloch et al. 2000), and reproduction (Bloch et al. 2000; Certel et al. 2010). The amines have also been found to fluctuate during and mediate changes in circadian rhythms (Cymborowski 2003; Wildt et al. 2004). Despite the large numbers of studies on the roles of these amines, there remains much confusion as to the role of biogenic amines in the control of behavior and physiological processes. Even within studied and proposed interactions, there remains conflict between studies. This is likely due to differing techniques and organisms of interest. Aminergic manipulation, such as injection, oral and topical administration, and genetic alterations have all been used to study the actions of biogenic amines. Often the same amine will act differently dependent upon the manipulation method (i.e. Scheiner et al. 2002; Barron et al. 2007b). Zera criticized using manipulation methods of hormones as the sole investigation of hormonal roles. Changes in hormones, or amines, with time of day or age could be problematic in manipulation methods that do not control strictly for such factors.

Manufactured chemicals have differences from naturally occurring neurotransmitters or hormones and may also breakdown during storage. Use of analogues should be interpreted with caution as analogues may act differentially. There should also be considerations for manipulating substances outside of physiologically relevant levels. Following administration, levels should be confirmed not only to confirm that dosages are relevant but also to ensure that the dosage method is effective (Zera 2007). A major gap in our understanding is the natural circadian rhythmicity of the amines and how those fluctuations may be affecting the results of behavioral and manipulation assays.

Biogenic Amines in Behavior

Locomotor behavior has been well studied because of the ease of quantification and the ability to gather large amounts of data in short periods of time. Biogenic amines have been linked to the circadian rhythmicity of locomotor activity and the total amount of time spent in locomotor bouts. For example, in the blow fly a reduction of 5HT using 5,7-DHT created an overall reduction in locomotor activity. This reduction also shifted the phase of free-running flies in a manner that was dependent upon time of injection. If the injection was performed early in the respective day the phase was delayed, whereas an injection late in the respective day advanced the phase. Regardless of injection time, the period was lengthened from approximately 21 hours in control flies to 24 hours in injected flies. (Cymborowski 2003). The administration of a $5HT_2$ receptor agonist, (R)-1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI), in Drosophila yielded an increase in locomotor activity (Johnson et al. 2009) providing support for the positive relationship between 5HT and locomotor activity. Conversely, a study using 5HT

injection in the flesh fly, Neobelliara bullata, found 5HT to decrease walking activity and to, in fact, cause an overall depression of all active behaviors (Dacks et al. 2003). A loss-of-function mutation of the D1 dopamine receptor in Drosophila has been indicated to decrease arousal, increasing sleeping, and therefore decreasing locomotor activity (Lebetsky et al. 2009). Yet another study in Drosophila found that application of 5HT, DA, and OA to the nerve cord of decapitated flies all increased locomotor activity relative to controls (Yellman et al. 1997). Increased levels of OA and tyramine in the honey bee have been linked to a decrease in walking, but the same study found that an antagonist to these amines created the same effect. The patterns of the decrease, however, were different in both the amine and antagonist groups (Fussnecker et al. 2006) indicating differential roles for different amines in the control of a single behavior.

Aggression is widespread throughout the animal kingdom. It is a survival tool important for territoriality, mate competition and acquisition, predator defense, and prey acquisition. Biogenic amines have been identified as modulators of aggressive behavior. Again, the results have been somewhat contradictory dependent upon the method of manipulation and the organism of interest. Genetic depletion of OA has been found to decrease high-intensity aggressive behavior (i.e. lunging) in Drosophila melanogaster. The flies are still capable of reaching high-intensity aggressive acts, but the likelihood of occurrence is greatly decreased (Hoyer et al. 2008). Further supporting the positive correlation between OA and aggression, a study in D. melanogaster found that genetically reducing OA decreases aggressive behaviors, while enhancing OA increases aggressive behavior in socially reared flies (Baier et al. 2002; Zhou et al. 2008). Measuring levels of OA in the hemolymph of crickets showed that OA increased after aggressive encounters (Adamo et al. 1995). Depleting OA and DA from crickets created a depressed system, decreasing willingness to escape or participate in aggressive interactions

(Stevenson et al. 2000). Balance of amines is likely an important part of the regulation of behaviors. While most invertebrates tend to show increased aggression with the addition of OA, some assays using crustaceans may indicate the opposite. Injection of OA into the hemolymph created a subordinate posture and an individual that is less willing to enter aggressive encounters (review, Huber et al. 1997; Tricarico and Gherardi 2007). Barring the examples in crustaceans, OA seems to increase aggressive behavior in most invertebrates. A separate study in Drosophila, however, found that enhancing OA, again through a genetic mutant, could actually create males that exhibit courtship behavior towards other males rather than aggression, even in situations where competition would support aggressive responses (Certel et al. 2010). The same research group performed a similar experiment, however, where OA levels were abolished, and the males exhibited similar courtship behavior towards other males (Certel et al. 2007). This is further indication that balance of amines within physiological systems is important and that manipulating levels beyond naturally occurring ranges creates behaviors outside of the norm (Certel et al. 2010).

5HT has controversial results in the literature pertaining to its role in the control of aggression. Earlier research pertaining to 5HT in Drosophila reported that pharmacologically altering levels via feeding had no effect on aggression, and therefore a role for 5HT in aggression was dismissed (Baier et al. 2000). A study of crickets released the same year also supported this finding, reporting that depleting 5HT had no effect on aggressive state (Stevenson et al. 2000). Further research into this subject illuminated a possible role for 5HT in aggressive behavior. In 2007, a study found that while 5HT may not be necessary for aggressive encounters to occur, pharmacologically or genetically increasing levels of 5HT created higher levels of aggression in Drosophila (Dierick and Greenspan, 2007). This established 5HT as at least a modulator of

antagonistic behaviors. The role of 5HT has become more complicated as different receptor subtypes have been investigated. Use of a 5HT2 receptor agonist, (R)-1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI), caused a decrease in lunging, a high-intensity aggressive behavior, and boxing and created an overall decrease in aggressive interactions. Conversely, a 5HT1A/7 receptor agonist, 8-hydroxy-2-dipropylaminotetralin hydrobromide (8- OH-DPAT), caused an increase in wing threats and fencing and created an overall increase in aggressive interactions. These results support 5HT involvement in the control of aggression in Drosophila, but the mechanism still remains somewhat unclear; likely there are interactions between different receptors, and the researchers proposed a sort of "homeostasis" of receptor up-regulation (Johnson et al. 2009). This supports the idea that amines, and their receptors, need to be at certain levels to maintain normal behaviors. Disruption of 5HT production through enzyme manipulation via transgenic lines of Drosophila created male flies that did not escalate aggressive encounters above low-intensity behaviors that lasted for brief intervals and did not result in dominance relationships. An increase in 5HT resulted in pairings of flies that had higher intensity interactions that escalated quickly and almost always resulted in dominance relationships (Alekseyenko et al. 2010). In several studies of crustaceans, 5HT consistently induced dominant postures, increased willingness to fight, and decreased willingness to retreat (Huber et al. 1997a; Huber et al. 1997b; Tricarico and Gherardi 2007). Escape behavior in the cricket is enhanced by 5HT depletion (Stevenson et al. 2007), so while aggression may not be directly affected by 5HT in crickets, perhaps 5HT helps to create crickets that are less likely to retreat from an aggressive or predatory encounter. A separate study supported this hypothesis by finding that after an aggressive dominance relationship had been formed; subordinate male crickets had lower levels of brain 5HT (Murakami and Itoh 2001). In a looser correlation, 5HT

levels were found to increase with age in minor workers of the ant Pheidole dentate. Minor workers of this species move from brood care to foraging as they age. Given that foraging takes higher levels of agonistic behavior to be able to obtain prey and defend food sources, a correlation between aggression and higher 5HT levels can be made (Seid and Traniello, 2005).

Because 5HT and DA share a common catalytic enzyme, these amines could be manipulated simultaneously via that enzyme. When they were both targeted in this manner in Drosophila, amine levels were decreased, and mid- and high-level aggression was completely abolished in pairings. However, when only DA was targeted, no aggressive interactions occurred, likely due to the resulting hyperactive flies. These hyperactive flies had an increase in speed and quantity of locomotor activity. These flies also became uninterested in courtship behavior (Alekseyenko et al. 2010). These results indicate that 5HT, more than DA, seems to be a dominant factor in the control of aggression, although, when dopamine is disrupted, its effects on behavior also appear to disrupt aggression. This illustrates the delicate natural balance of amines and how disruption of one amine can affect the control another exerts. A separate study found that when levels of DA were elevated to high levels, aggressive encounters were decreased (Baier et al. 2002). These opposing effects between studies could be due to DA being manipulated too far outside of biologically relevant levels and emphasizes the importance of being familiar with endogenous levels (Zera, 2007).

While flies, especially Drosophila, have been commonly used as a model system for aggression, there are fewer studies on bee aggression. It follows that even fewer studies have looked at how biogenic amines are involved in the control of aggressive behaviors in bees. A study of reproduction and social behavior in the bumble bee found that dominant, egg-laying workers tended to have higher OA levels than their nest mates. Levels of 5HT and DA,

however, were not significantly different between bees of high and low social status. Higher OA levels can be loosely correlated to an observed higher level of aggression in reproductively dominant bees (Bloch et al. 2000). Aggression, however, was not specifically assayed in this experiment, and so it cannot be directly linked to higher levels of OA.

Dance behaviors have been well studied in bees. OA appears to have a role in the modulation of honey bee waggle dance behavior by enhancing the representation of a floral reward. Oral and topical treatments of OA differentially affect waggle dancing. Oral treatments resulted in a higher likelihood of bees to perform a waggle dance in response to reward (sucrose, pollen, or a combination of both). Topical treatments affected behavior in a dose dependent manner. An intermediate dose of OA created the highest likelihood of a dance. A low dose increased the dance vigor. A high dose of OA increased the duration of the dance. These results were consistent whether a sucrose or pollen reward was presented to the bee. The use of an antagonist to OA blocked the behavioral effect of exogenously administered OA but did not create any significant effects in behaviors of control bees. These results indicate OA as a modulator of reward perception and, therefore, point to an indirect role for OA in the control of dance behavior (Barron et al. 2007a). As with most behaviors examined, there appear to be many factors that are involved in the control of dance behavior in the honey bee. This example also shows how different methods of manipulation can lead to varying results.

Feeding behavior has been used as a model behavior for many studies. It is, of course, well known for its role in classical conditioning. Proboscis extension response, or PER, is commonly used in insects as a way to quantify feeding behavior and to examine responses of individuals to food and odor stimuli in a dose dependent manor. In the honey bee, OA and DA, and their precursor tyramine, have been indicated in the control of PER. Sucrose solutions of

increasing concentrations were touched to the antennae of bees to determine if they would extend their proboscis. The same bees were then dosed with the respective chemical and then retested with the sucrose solutions. Injection or feeding of OA and tyramine resulted in an increased responsiveness to lower levels of sucrose. Injection of DA had an opposing effect by decreasing the responsiveness of bees to sucrose. Feeding of dopamine showed no significant change in sucrose responsiveness between bees before or after feeding. The DA receptor agonist, 6,7-ADTN, also decreased sucrose responsiveness, and the results were consistent whether injection or feeding was used (Scheiner et al. 2002). A review of the roles of OA and 5HT in the honey bee reported OA to consistently increase proboscis extension in response to water and odor but found that 5HT reduced proboscis extension in response to the same stimuli (Erber et al. 1993). A study in the flesh fly, Neobellieria bullata, found 5HT to be a system depressant, which included a decrease in feeding and associated feeding behaviors. In addition, an immunocytochemical analysis indicated that 5HT is released in response to feeding (Dacks et al. 2003). Perhaps these results are indicative of 5HT being involved in a satiation-like response.

Like all the behaviors above, grooming also appears to be under the control of these 3 amines. It might be expected for amines that positively affect locomotion (i.e. as the amines build up in the neural system, locomotor activity increases) to have a negative effect on a more stationary behavior such as grooming. The opposite, however, seems to be occurring in flies. Increases in DA, OA, and 5HT are all correlated with an increase in grooming in Drosophila (Yellman et al. 1997). While increased OA has also been identified with increased grooming in the honey bee, it also decreased walking, more consistent with what would be expected (Fussnecker et al. 2006).

Changes in Levels of Biogenic Amines

Biogenic amines have been found to change with age. Harris and Woodring used HPLC to detect all 3 amines in brains of honey bees of different ages. Their findings indicated that amine levels are lower in younger bees than randomly aged worker bees (Harris and Woodring 1992). Similar results were found in a separate study of honey bees. DA, 5HT, and OA were all found to increase with age within the mushroom bodies of older bees (Schulz and Robinson 1999). A study measuring differences in DA between 5-day and 11-day old nonforaging honey bees found DA to decrease with age (Carrington et al. 2007). This was also the case in Drosophila. After an initial trough (1 day after emergence) and peak (3 to 7 days after emergence) DA levels decrease with age. DA levels were also generally higher in male Drosophila than in females (Neckameyer et al. 2000). Similar assays in the bumble bee showed that by day 3 worker bees had a significant increase in OA (Bloch et al. 2000). The ant, Pheidole dentate, exhibited higher levels of 5HT and DA in older ants of the minor worker caste (Seid and Traniello, 2005). Differential responses in the levels of a precursor to DA, tyrosine hydroxylase, to stress were observed dependent upon age and sex of Drosophila (Neckameyer and Weinstein 2005), suggesting differences in endogenous levels and possibly receptor regulation. Amine levels also have been found to vary depending upon season and colony in the honey bee (Harris and Woodring 1992). All of these findings should be taken into account when conducting behavioral studies, as natural fluctuations dependent upon age, season, sex, and genetics may affect any exogenous manipulations or behavioral assays.

Not only do these amines change dependent upon factors such as age, their endogenous levels also appear to be on a circadian cycle. A locomotor study, previously mentioned, not only

saw that a reduction of 5HT could alter the free-running phase of the blow fly but also noted that optic lobe interneurons have a circadian rhythm of changes in neuronal size that can be induced by the addition of exogenous 5HT (Cymborowski 2003), suggesting that 5HT may also fluctuate in a circadian rhythm within optic lobes to regulate this process. DA levels in the optic lobes of the honey bee have a circadian cycling that can be altered with changes in the light-dark cycle. A study using forager bees from hives maintained outside under an approximate 14:10 LD cycle showed optic lobe levels of DA to peak just prior to the middle of the light cycle. When the light cycle was shifted by 8 hours, the DA peak shifted by 4 hours, resulting in a peak DA level in the dark period just before lights on (Carrington et al. 2007). This study supported that DA cycles in a circadian fashion and that it is dependent on the light cycle. Further analysis of forager bee optic lobes created a more interesting story. A more controlled study using forager bees that had been kept under a LD 12:12 cycle in an indoor colony found DA to be lower just prior to lights off, increase significantly after lights off, maintain this level until at least midway through the dark cycle, and then plummet to a trough just prior to lights on. Maintaining bees in a reversed light dark cycle resulted in no cyclic pattern of DA; no time point was significantly different from any other. Bees kept in DD did show cycling of DA. While levels were relatively low in relation to the other light cycles, a similar pattern to the original 12:12 LD cycle was observed (Carrington et al. 2007). Aplysia californica, a sea slug, exhibited a diurnal rhythm of 5HT within the hemolymph. A study assaying 7 time points throughout the 12:12 LD period found that 5HT is higher during the photophase and lower during the schotophase (Levenson et al. 1999). Unfortunately, in comparison to manipulation studies, little work has been done to look at the circadian rhythmicity of endogenous biogenic amine levels. This is an important control that should be considered before beginning any type of manipulations of biogenic amines.

The roles of biogenic amines within animals are vast. While there is significant evidence that OA, 5HT, and DA are all involved in behavioral control, there are still many questions to be answered as to what their exact roles are in that control. Many studies have been conducted to investigate the interactions between amines and behavior, but the current literature is contradictory, even when comparing studies of the same species. Increases in DA and 5HT are generally associated with increased locomotion in the fly (Yellman et al. 1997; Cymborowski 2003; Lebestky et al. 2009; Johnson et al. 2009). There are a few studies, however, showing the opposite (Dacks et al. 2003; Alekseyenko et al. 2010). OA has also been shown to increase locomotion in the fly (Yellman et al. 1997), but in the bee it can decrease locomotion (Fussnecker et al. 2006). The role of DA in aggression is debatable, while studies indicate both increased and decreased levels of DA to be associated with decreased levels of aggression; the answer to why this occurs is unclear (Baier et al. 2002; Alekseyenko et al. 2010). Whether DA is actually a regulator of aggression, or whether the changes we see in aggression are side effects of changes in other behaviors, such as increases in locomotion, is still to be determined. OA and 5HT have overwhelming evidence to suggest their positive correlation with aggression in Drosophila, crickets, and ants (Adamo et al. 1995; Bloch et al. 2000; Stevenson et al. 2000; Murakami and Hoh 2001; Baier et al. 2002; Seid and Traniello 2005; Dierick and Greenspan 2007; Stevenson et al. 2007; Hoyer et al. 2008; Zhou et al. 2008; Johnson et al. 2009; Alekseyenko et al. 2010). Even with so many studies suggesting this relationship, there are still several that contradict it and show a negative correlation (Johnson et al. 2009; Certel et al. 2010). The role of amines in other behaviors such as grooming, the honey bee waggle dance, and feeding, need to be researched more as there are few studies focusing on each amine. Differential manipulation methods, experimental biases, measurement differences, time of day,

age and season of the experimental animals, and individual interpretation of data have all led to a very convoluted view of the role of these amines. Other factors worth considering, such as the ratio of different amines to one another, regulation of receptors, and differential rates of breakdown have not been examined closely.

The majority of the studies above have examined behaviors through various aminergic manipulation methods. An important first step that is often ignored is to examine natural levels of the biogenic amines within the organism of interest and then ensure that manipulations are not pushing levels too far out of the physiological range (Zera, 2007). Studies that have taken the time to examine this often have not paid attention to patterns of aminergic change associated with age, time of day, or changes in other physiological conditions. In this study, the diel aggressive behaviors and circadian amine levels were examined in the flesh fly, Sarcophaga crassipalpis. Based on other studies, it was expected that 5HT, OA, and DA would exhibit diel patterns of change. Two light cycles were used, 12:12 LD and 15:9 LD. It stands to reason that a shift in the LD cycle would also cause a shift in the diel cycling of amines, similar to what has been identified in more dramatic shifts, complete LD reversals, in bees (Carrington et al. 2007). If amines that are so involved in the control of behaviors cycle in a circadian fashion, then the behaviors that are associated with the levels of these amines may also change throughout the day in a similar fashion. If this hypothesis is correct, then it follows that the aggressive behaviors of S. crassipalpis should exhibit a cyclical change in levels throughout the day in correlation with changing amine levels. Correlating endogenous levels of amines in the brain with changes in behavior is only a first step in the understanding of how behaviors are controlled. There are likely complicated mechanisms involved with in the physiology of organisms that all are required to maintain the intricate control behaviors. It is probable that receptor regulation is

involved in this process, and it is important to remember such mechanisms when interpreting experimental outcomes.

This study not only looked at the circadian cycling of the amines, but it is also investigated the ontogeny of changes that could also occur. Males, aged 1 to 4 days following eclosion, were used in the study to determine if there were differences in the levels of amines dependent on the age of the flies. Studies in bees have seen changes in amines based on age (Harris and Woodring 1992; Bloch et al. 2000). Between days 3 and 4 days posteclosion, S. crassipalpis males become territorial, sexually active, and increasingly aggressive toward other males (Paquette et al. 2008; Moore et al. in prep). This increase in aggression further supports the idea that fluctuations in amine levels would also occur with aging in S. crassipalpis, as these amines have been indicated in the control of aggression in other species of flies (see Table 1). As all 3 amines have been more often associated with an increase of aggressive behavior, it was expected that the endogenous levels would increase with age of S. crassipalpis. As mentioned previously, there may also be differences in receptor regulation with age that could alternatively be affecting the behaviors observed. This study is a significant first step in the study of behavior and aminergic control in the flesh fly, S. crassipalpis, and will set the stage for further use of the flesh fly as a model system for the study of aggression and biogenic amines.

**Table 1.** Summary of Correlations Between Amines and Aggressive Behaviors

| OA | | 5HT | | DA | |
|---|---|---|---|---|---|
| correlation | organism | correlation | organism | correlation | organism |
| + | Fruit Fly[13] | O | Fruit Fly[4] | + | Cricket[6] |
| + | Fruit Fly[14] | O | Cricket[6] | + | Fruit Fly[16] |
| + | Fruit Fly[8] | + | Fruit Fly[10] | - | Fruit Fly[8] |
| + | Cricket[1] | - | Fruit Fly[15] | | |
| + | Cricket[6] | + | Fruit Fly[15] | | |
| - | Crustaceans[2,12] | + | Fruit Fly[16] | | |
| - | Fruit Fly[17] | + | Fruit Fly[16] | | |
| + | Fruit Fly[17] | + | Crustaceans[2,3,12] | | |
| + | Bumble Bee[5] | + | Cricket[11] | | |
| | | + | Cricket[7] | | |
| | | + | Ant[9] | | |

'+' indicates a positive correlation. '-' indicates a negative correlation. 'O' indicates that no correlation was found. [1]Adamo et al 1995, [2]Huber et al 1997a, [3]Huber et al 1997b, [4]Baier et al 2000, [5]Bloch et al 2000, [6]Stevenson et al 2000, [7]Murakami and Hoh, 2001, [8]Baier et al 2002, [9]Seid and Traniello 2005, [10]Dierick and Greenspan 2007, [11]Stevenson et al 2007, [12]Tricarico and Gherardi 2007, [13]Hoyer et al 2008, [14]Zhou et al 2008, [15]Johnson et al 2009, [16]Alekseyenko et al 2010, [17]Certel et al 2010.

# CHAPTER 2

# MATERIALS AND METHODS

## Diel Changes in Interactive Behavior

### Flies

Flesh flies, Sarcophaga crassipalpis, were obtained from a lab-reared colony at East Tennessee State University maintained in an incubator under a 15:9 LD cycle and at a temperature of 25 °C. Male flies were collected on the day of emergence (Day 0) and placed into individual Petri dishes (9 cm x1.5 cm). The flies were cooled at -4 °C for 5 minutes and then marked with a dot of enamel paint on the dorsal thorax for ease of identification during video review. Petri dishes were supplied with a 1 ml microcentrifuge tube that contained water with a cotton ball tip and a 1 ml microcentrifuge-tube cap that contained granulated sugar. Opaque dividers were placed between the Petri dishes to visually isolate the flies. The flies were housed in an aluminum observation shed constructed within the laboratory. The shed's light cycle was the same as the rearing incubator (15:9) and the temperature (25 °C) was maintained by a thermostat-regulated space heater.

Behavioral Assay

Assays were completed on Day 1 and Day 4 following emergence. Three time points were selected during the photophase for observation (Hour 1, Hour 8, and Hour 14) representing early, mid, and late times in the photophase. Based on the previous finding that territorial behavior is not exhibited during the scotophase, this study only used photophase time points to assess interactive and aggressive behavior (Moore et al. in prep). At each time point, 2 flies were cooled at -4°C for 5 minutes and then placed into opposite ends of an experimental arena. The arena consisted of a Petri dish (9 cm x 1.5 cm) with an opaque divider inserted across the center. The arena was then placed into the observation shed on a table with legs stabilized in buckets of sand to reduce vibrations. Video cameras were used to record the flies for a total of 40 minutes. After the first 5 minutes of acclimation, the opaque divider was removed from the arena to allow the flies to interact. After 40 minutes, flies were removed from the arena and returned to the source colony. Each fly was only used once. The arena was cleaned between experiments with ethanol.

Data Collection

Videos were reviewed following filming using a Macintosh HD laptop computer and VLC media player software. The first 10 minutes of each video was not scored; the divider remained in the Petri dish for the first 5 minutes and the second 5 minutes were allowed for acclimation after the disruption of removing the divider. The remaining 30 minutes of video were analyzed for behavior based on an established ethogram (Moore et al. in prep). Behaviors

were divided into 3 categories: interactive (nonaggressive), low intensity aggression, and high intensity aggression. Interactive behaviors required no contact between individuals but were performed within 2 body lengths of an opponent. Low intensity aggression (low aggression) included behaviors that required minimal physical contact between opponents. High intensity aggression (high aggression) behaviors generally involved full-bodied behaviors that had a high probability of eliciting a response from the opponent (Moore et al. in prep). Behaviors were recorded by hand with a video-time stamp and then transferred into a Microsoft Excel file for analysis. Total amount of time spent in each behavioral category was used for analysis.

Statistical Analysis

Minitab-16 was used to perform all statistical analyses. Kruskal-Wallis (KW) was used to determine differences in time spent in each behavioral categoy by Hour within a Day and a Tukey-Type test for non-parametric data was used to compare differences between groups. General Linear Model (GLM) was used to assess interactions between Day and Hour and to determine differences between Days for each behavioral category. Significance was determined based on $\alpha=0.05$.

## Amine Quantification

### Flies

As in the behavioral assay, flies were obtained from a lab-reared colony at East Tennessee State University kept at 15:9 LD and 25 °C. For the experiments using a 12:12 LD cycle, larvae were removed from the 15:9 LD rearing incubator 3 days postlarviposition, to prevent them from entering diapause, and placed into an incubator in 12:12 LD and 25 °C for the remainder of the experimental period.

Male flies were collected on Day 0 within 4 hours of emergence and placed in individual Petri dishes (9 cm x 1.5 cm). Petri dishes were supplied with a 1 ml microcentrifuge tube that contained water with a cotton ball tip and a 1 ml microcentrifuge-tube cap that contained granulated sugar. Placing opaque dividers between and around Petri dishes visually isolated flies. Flies were kept in the rearing incubators (12:12 LD or 15:9 LD) until collection for amine extraction.

### Amine Extraction

Isolated flies were removed from the incubator at each respective time point. Collection of flies for decapitation began 1 hour after "lights on" ("lights on"=Hour 0) on Day 1. Replicate one collections took place on Days 1 and 4 only at Hours 1, 4, 8, 11, 13, 16, 20, and 23 for 12:12 LD and Hours 1, 4, 8, 11, 14, 16, 20, and 23 for 15:9 LD (Figure 1a). Ten flies were collected at each time point. Replicate 2 collections took place on Days 1, 2, 3, and 4 at Hours 1, 12, and 23

for 12:12 LD and Hours 1, 14, and 23 for 15:9 LD (Figure 1b). Five flies were collected at each time point. Flies were cooled at -4°C for 10 minutes and then decapitated. Heads were immediately frozen in liquid nitrogen and kept on ice for the remainder of the extraction. Heads were placed in 1.5 ml microcentrifuge tubes with a 1 cm ceramic bead and 400 µl of 0.2M perchloric acid buffer containing 10 ug/ml synephrine and 30 ug/ml alpha-methyl serotonin as internal standards. Heads were homogenized using a Fast-Prep24® tissue grinder (MP Biomedicals) at 4 m/s for two 5-second pulses. Homogenized samples were centrifuged for 10 minutes at 13000 rpm at 4°C. The resulting supernatant was siphoned into a Spin-X tube containing a 0.22 µm filter and centrifuged for 6 minutes at 13000 rpm at 4°C. Samples were stored at -4°C temporarily (<4 weeks) and at -80°C for longer term.

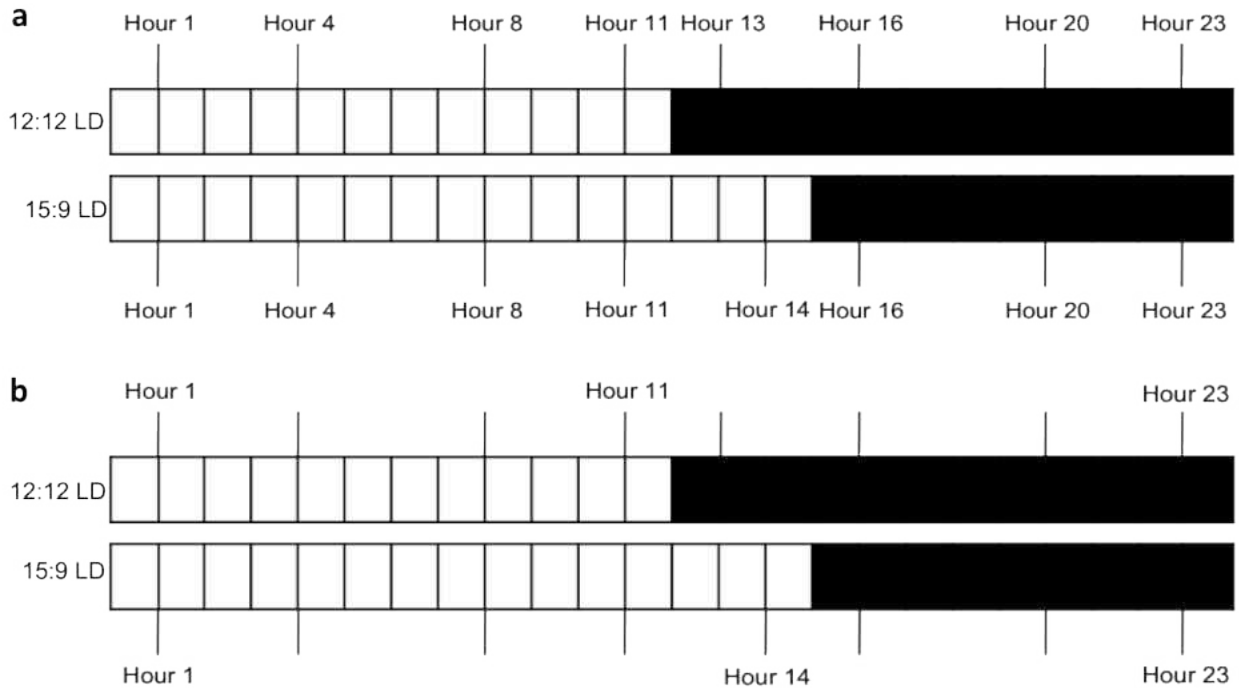


**Figure 1. Fly Collection Times for HPLC Analysis**. Hour 1 begins 1 hour after lights on. White sections on bar represent hours when lights were on; dark sections represent hours when lights were off. a. Replicate 1 collection times. b. Replicate 2 collection times.

HPLC-ECD Analysis

For HPLC-ECD analysis, 5µl of supernatant was mixed with 45 µl of 0.2M perchloric acid in a 100 µl HPLC tube. Amines were detected using a C-18 ALF-115 column (150 mm length, 1.0 mm diameter, 3µm particle size). The mobile phase consisted of 50mM phosphoric acid, 50mM citric acid, 0.1mM EDTA, 10% methanol, and 500 mg/L OSA. The pH was adjusted to 3.25 using sodium hydroxide. The mobile phase was degassed before use. The HPLC-EC was an Alexys Monoamines Analyzer (Antec) consisting of an Alexys LC 110 pump with a flow rate of 50 µL/min and pressure set to 180 bar, an AS 110 autosampler set to maintain samples at 4°C, a Decade II detector set to 20nA, and a VT-03 electrochemical flow cell set to a potential of 850mV. Chromatograms were produced and analyzed using Clarity™ software (DataApex). Amines were identified by external standards run before and after each set of samples. Internal standards (synephrine and α-methylserotonin) were used to verify retention times within runs. Standards, synephrine, serotonin hydrochloride, octopamine hydrochloride, and α-methylserotonin maleate salt were obtained from Sigma-Aldrich Co. Dopamine hydrochloride standard was obtained from Alfa Aesar.

Protein Quantification

A MicroBCA protein assay kit (ThermoScientific) was used to quantify protein amounts in samples from Replicate 2 (Appendix). Samples were read at 562nm using a Synergy HT microplate reader (BioTek) and Gen 5 software (BioTek). The mean of 3 replicates for each sample was used to create a ratio of amine to protein.

Statistical Analysis

All statistics were performed in MiniTab-16. A regression analysis was used to determine differences in amine levels at time points over the 4-day sampling period. GLM was used to compare differences between Hours and Days within each Light Cycle. Tukey's pairwise comparison was used to determine differences between groups. Analysis of Variance (ANOVA) was used to compare differences in amine levels between light cycles. Significance was determined based on $\alpha=0.05$.

# CHAPTER 3

# RESULTS

## Behavioral Assay

Behaviors of flies were analyzed using an ethogram of behaviors (Table 2). Behaviors were categorized into 4 types: 'Noninteractive', 'Interactive' (Nonaggressive), 'Low Aggression', and 'High Aggression'. A "radius of interaction" was determined to be approximately 2 fly-body lengths. Any of the behaviors scored as 'Interactive' occurred within this radius; any of the behaviors listed under 'Interactive' in Table 1 that occurred outside of this radius were scored as 'Noninteractive.' Aggressive behaviors also occurred within this radius and generally involved contact between the flies. High-intensity aggression required the entire body of the fly to be involved in the aggressive encounter whereas low-intensity aggression required only minimal contact between opponents (Moore et al. in prep). High intensity aggression, (high aggression) was described thusly because they generally elicited a response from the opponent fly, whereas low intensity aggression (low aggression) did not always elicit a response. Behaviors were scored individually and then grouped into respective categories for analysis.

**Table 2.** Ethogram of Fly Behaviors

| Behavioral Category | Description |
|---|---|
| **Interactive (Nonaggressive)** | |
| Purposeful Approach* | Fly advances toward opponent |
| Bob* | Fly quickly raises and lowers body multiple times |
| Crouch Down | Fly bends legs and crouches low |
| Stomp | Fly stomps forelegs while standing |
| High step | Fly walks toward opponent taking high steps with forelegs |
| Pop Up* | Fly rises from and returns to standing position |
| Lean Toward | Fly leans toward opponent |
| Retreat* | Fly quickly moves away from opponent |
| Stilt* | Fly rises from standing position and holds body up |
| Turn Toward* | Fly turns to face opponent |
| Avoidance* | Fly slowly moves away from opponent |
| Lean away | Fly leans away from opponent |
| Wing Flutter | Fly bats wings repeatedly |
| **Low Aggression** | |
| Chop* | Downward strike with foreleg |
| Fence* | Repeatedly strikes opponent with forelegs |
| Head Butt* | Pushing opponent with head |
| Back Kick* | Strikes opponent with back leg |
| Uppercut* | Upward strike with foreleg |
| Box* | Fly rears up on bag legs and repeatedly strikes opponent |
| **High Aggression** | |
| Immobilized* | Fly is held by opponent |
| Lunge* | Fly rears up and jumps toward opponent |
| Holding* | Fly grasps opponent with forelegs and attempts to immobilize |
| Wrestle* | Both flies grasp each other with forelegs and roll |
| **Noninteractive** | |
| Incidental approach | Fly advances toward opponent indirectly or as a result of exploring arena |
| Non-interactive | Fly is outside radius of interaction and/or is performing non-interactive behaviors such as grooming, walking, standing |

* Indicates a behavior previously described by Moore et al. in prep

Interactive Behavior

There was no significant interaction between day and hour for interactive behavior (GLM, P=0.372). There was a tendency for flies to spend more time in interactive behavior

later in the photophase (Figure 2a). On Day 1, interactive behavior was lowest in the Hour 1 time point and increased by the Hour 14 time point (KW, p=0.018). On Day 4, interactive behavior was again lowest at the Hour 1 time point, had increased by Hour 8, and by Hour 14 it had begun to decrease again, though not significantly (KW, p=0.005). Overall averages for each day showed that flies spent less time in interactive behavior during Day 4 than during Day 1 (Figure 2d, GLM, p=0.024).

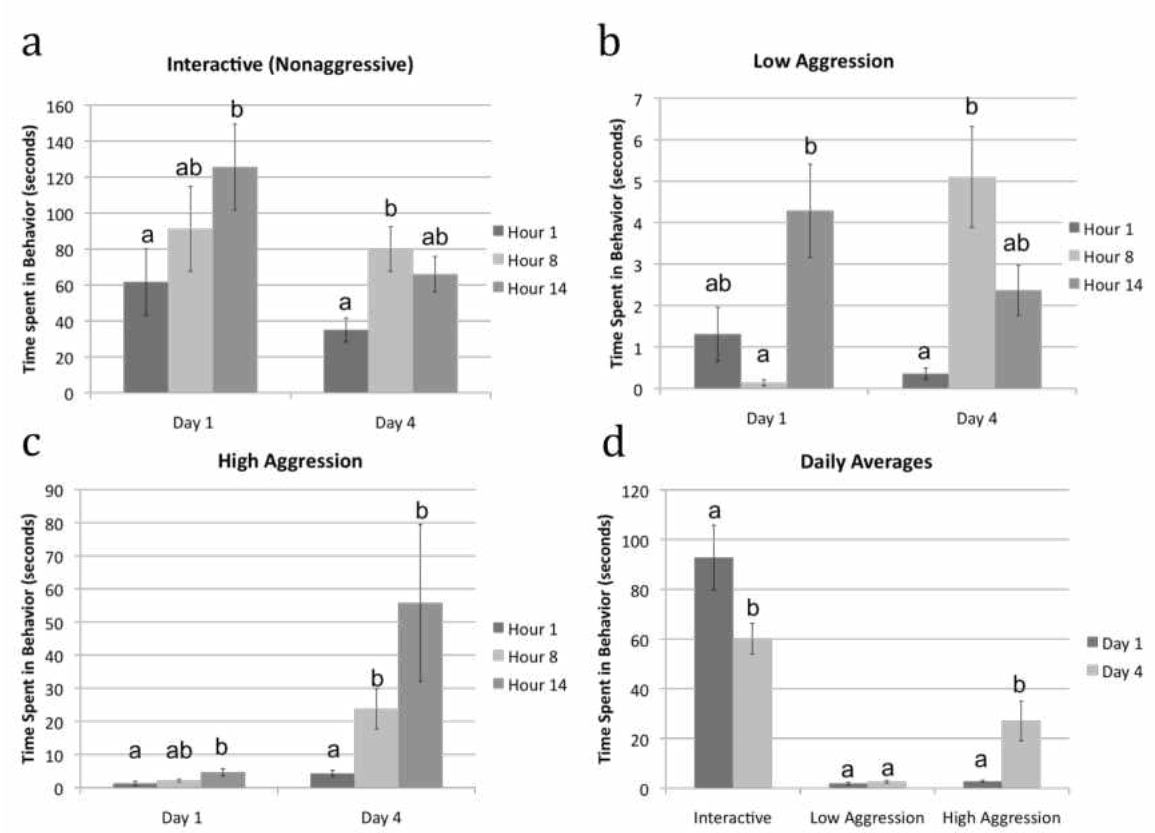


**Figure 2.** Time Spent in Behavioral Categories. a-c. Mean time flies spent in behavior by seconds for each behavioral category. Means are by hour for days of age 1 and 4 (days after emergence). Bars within a day that do no share a letter are significantly different; differences were determined by Kruskal Wallis followed by a Tukey type test for nonparametric data. d. Mean of time flies spent in behavioral categories by day. Bars within each behavioral category that do not share a letter are significantly different. Differences were determined by GLM.

Low Intensity Aggression

The time that flies spent in low aggression was dependent upon both day and hour (GLM, p=0.000). The pattern of time spent in low aggression behavior differed greatly from Day 1 to Day 4 (Figure 2b). On Day 1, Hour 8 was the lowest amount of time flies spent in low aggression and Hour 14 was the highest (KW, p=0.004). On Day 4, however, Hour 1 was the lowest and Hour 8 was the highest (KW, p=0.000). There was no significant difference between the overall levels on Day 1 versus Day 4 (Figure 2d, GLM, p=0.273).

High Intensity Aggression

As with low aggression, there was also a significant interaction between day and hour for high aggression (GLM, p=0.036). While both days show an increase in high aggression from Hour 1 to Hour 14 (Figure 2c), the increases from hour to hour were much more pronounced on Day 4 (KW, p=0.000) than Day 1 (KW, p=0.007). Overall, flies spent more time in high aggression on Day 4 than on Day 1 (Figure 2d, GLM, p=0.001).

## Amine Quantification

### Replicate 1

OA levels in either light cycle did not have an obvious diel rhythm (Figure 3a-b). Although there were differences when analyzing both days by hour (12:12 LD, GLM, f=5.41, p=0.000; 15:9LD, GLM, f=6.98, p=0.000), the patterns were not obvious, and were influenced mostly by changes seen in Day 1. In the 12:12 LD cycle, the most obvious difference was a peak in OA levels at Hour 11, which was 1 hour before lights off (Figure 3a). Day 4 did not show any obvious pattern, but OA levels were overall higher than Day 1 (Figure 3c, GLM, f=6.05, p=0.015). In the 15:9 LD cycle, Day 1 shows a steady increase of OA levels throughout the day that begins to show significance at Hour 16 and peaks at Hour 23 (Figure 3b; RA F=48.99, p=0.000). Day 4 levels are lower than Day 1 (Figure 3d, GLM, f=105.57, p=0.000), but no time points throughout Day 4 are significantly different from any others (Figure 3b). Levels of OA in the 12:12 LD cycle were significantly lower than those in the 15:9 LD cycle (note scale in graphs for each light cycle in Figure 3; ANOVA, f=335.70, p=0.000). Light cycle also had significant effects on levels of OA by day and hour (ANOVA, f=5.91, p=0.000).

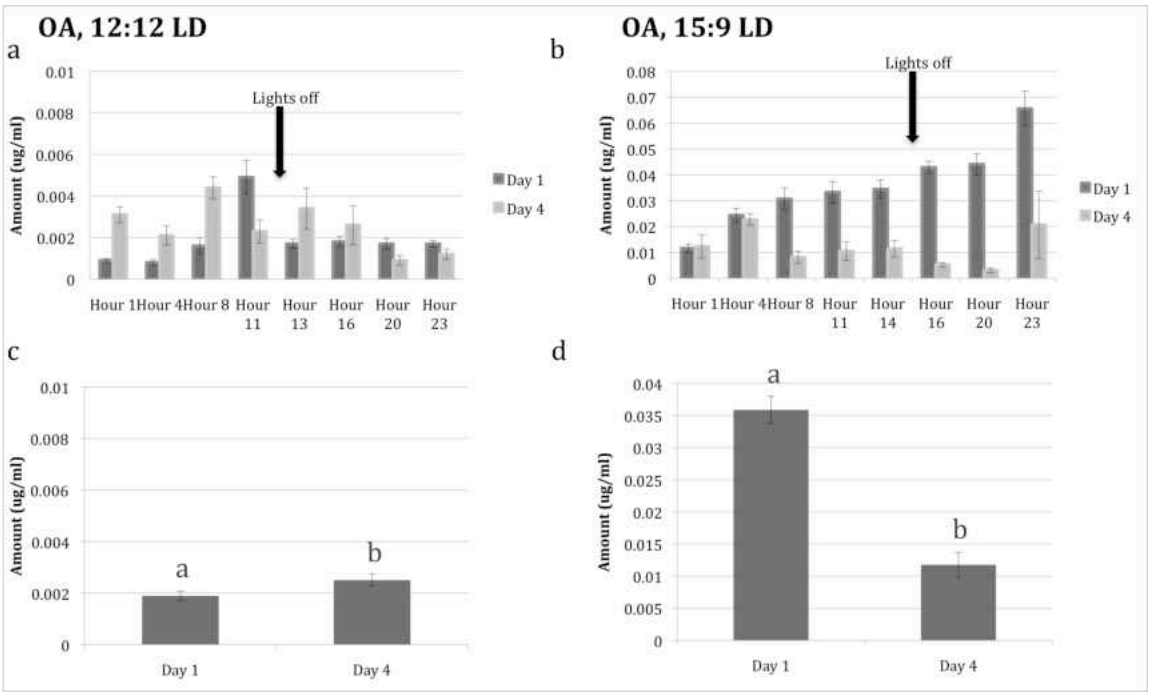


**Figure 3.** Replicate 1 Levels of OA. Bars represent mean OA present in samples. Error bars represent SEM. a. Mean OA by hour for Day 1 and Day 4 in 12:12 LD. n=10 for each time point. RA, f=3.04, p=0.083. b. Mean OA by hour for Day 1 and Day 4 in 15:9 LD. n=10 for each time point. RA, f=48.99, p=0.000. c. Mean OA for each day in the 12:12 LD cycle. n=60 for each day. Columns that do not share a letter are significantly different. GLM, f=105.57, p=0.000. d. Mean OA for each day in the 15:9 LD cycle. n=60 for each day. Columns that do not share a letter are significantly different. GLM, f=105.57, p=0.000.

Levels of 5HT in the 12:12 LD cycle showed a peak at Hour 11 in Day 1, similar to what was seen with OA (Figure 4a, RA, f=31.40, p=0.000). All other time points in the 12:12 LD cycle for both days were similar and did not have an obvious pattern. Levels of 5HT in 12:12 LD for Day 4 were lower than Day 1 (Figure 4c, GLM, f=54.18, p=0.000). In the 15:9 LD cycle, there were no significant differences between any hour or day (Figure 4b,d). Levels of 5HT in the 15:9 LD cycle were significantly higher than in the 12:12 LD cycle (ANOVA, f=6.13, p=0.014). Light cycle did not have a significant effect on day or hour.

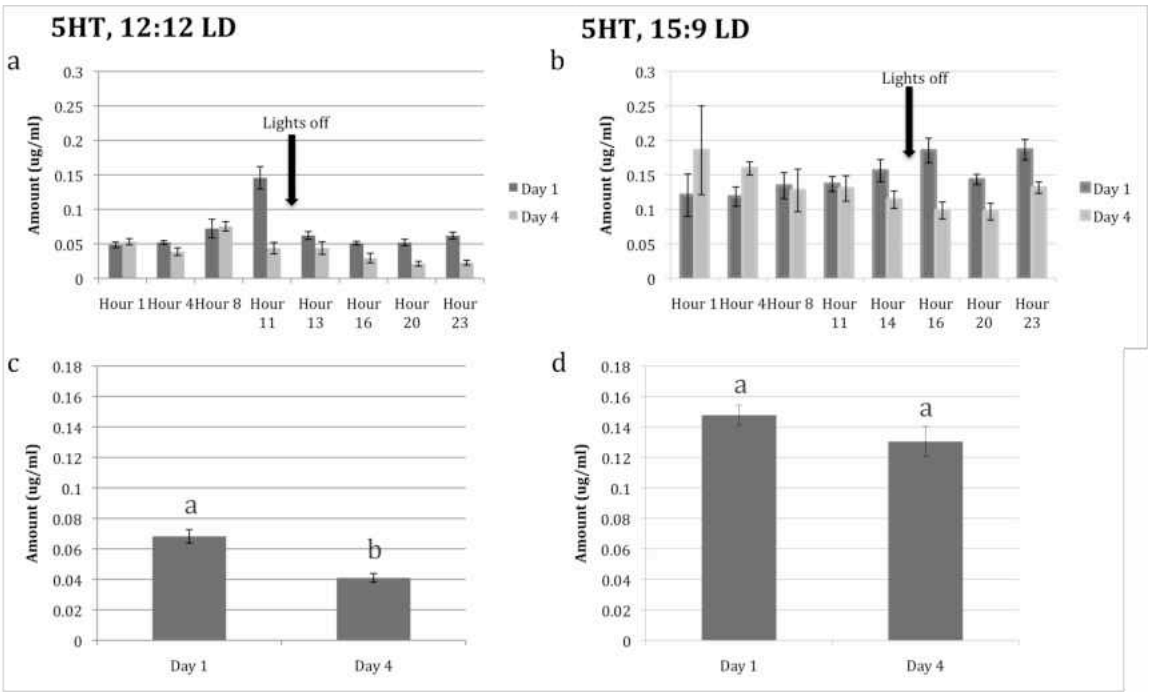


**Figure 4.** Replicate 1 Levels of 5HT. Bars represent mean 5HT present in samples. Error bars represent SEM. a. Mean 5HT by hour for Day 1 and Day 4 in 12:12 LD. n=10 for each time point. RA, f=31.40, p=0.000. b. Mean 5HT by hour for Day 1 and Day 4 in 15:9 LD. n=10 for each time point. RA, f=1.26, p=0.264. c. Mean 5HT for each day in the 12:12 LD cycle. n=60 for each day. Columns that do not share a letter are significantly different. GLM, f=54.18, p=0.000. d. Mean 5HT for each day in the 15:9 LD cycle. n=60 for each day. Columns that do not share a letter are significantly different. GLM, f=0.71, p=0.401.

Levels of DA in the 12:12 LD cycle changed very little within Day 1 and there was no difference between any hour in Day 4 (Figure 5a). The only hour that exhibited any significant difference on Day 1 is Hour 11. This is interesting, as OA and 5HT were also highest at this time point (Figure 3a and Figure 4a). In the 15:9 LD cycle, Day 1 levels were lowest at Hour 1 and highest at Hour 23, although Hour 23 was only significantly different from Hour 1 and Hour 4 (Tukey's multiple comparison, Figure 5b). Day 1 was significantly higher than Day 4 for both light cycles (12:12 LD, GLM, Figure 5c-d). There was no significant difference in the overall level of amines between light cycles, but light cycle did affect the amount of DA present within hours and days (ANOVA, f=11.84, p=0.000).

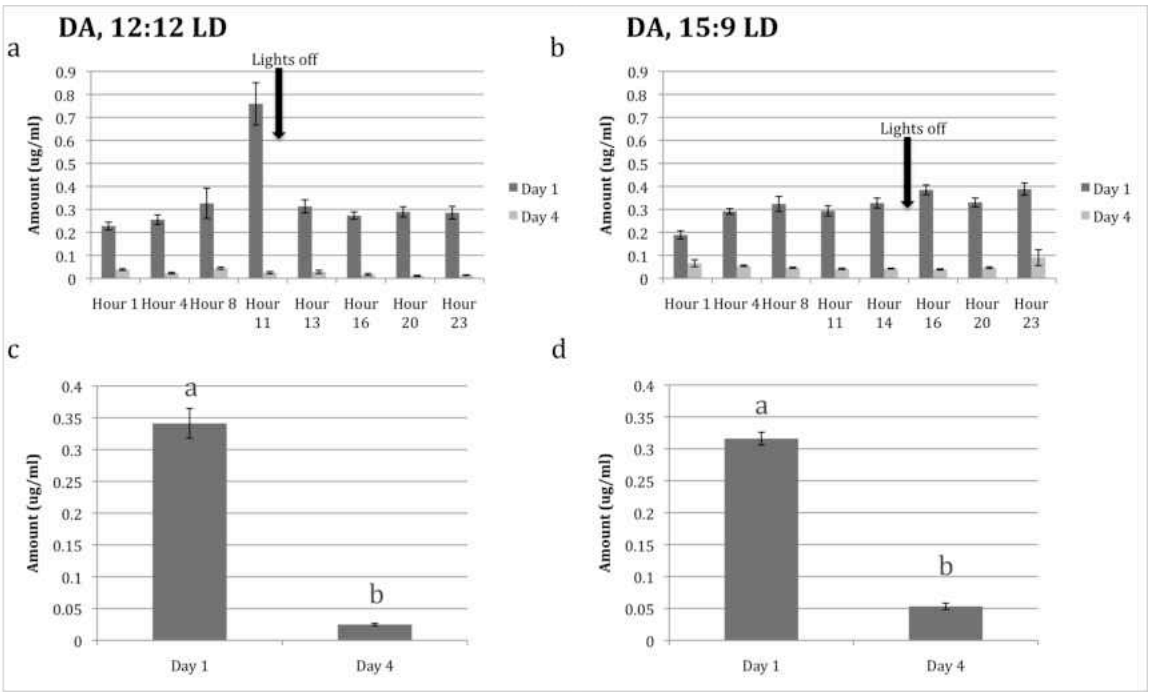


**Figure 5.** Replicate 1 Levels of DA. Bars represent mean DA present in samples. Error bars represent SEM. a. Mean DA by hour for Day 1 and Day 4 in 12:12 LD. n=10 for each time point. RA, f=167.77, p=0.000. b. Mean DA by hour for Day 1 and Day 4 in 15:9 LD. n=10 for each time point. RA, f=352.50, p=0.000. c. Mean DA for each day in the 12:12 LD cycle. n=60 for each day. Columns that do not share a letter are significantly different. GLM, f=400.25, p=0.000. d. Mean DA for each day in the 15:9 LD cycle. n=60 for each day. Columns that do not share a letter are significantly different. GLM, f=804.73, p=0.000.

Replicate 2

OA levels did not exhibit a diel pattern in the 15:9 LD cycle (GLM, f=1.48, p=0.206, Figure 6a), but in the 12:12 LD cycle a diel pattern was possibly emerging (GLM, f=5.00, p=0.011). In the 12:12 LD cycle, OA was generally higher at Hour 1 and lowest at Hour 23 (Figure 6b), but the pair-wise differences within each day were not significant (Tukey's pair-wise comparison, α=0.05). There were changes between hours over sampling days for each light cycle (15:9 LD, RA, f=34.03, p=0.000; 12:12 LD, RA, f=170.14, p=0.000). The mean level of OA for the 12:12 LD cycle was higher than in 15:9 LD (ANOVA, f=18.92, p=0.000, Figure 7), and there was a significant effect of light cycle on the daily level of OA (ANOVA, f=4.82,

p=0.004). Regardless of light cycle, mean OA levels for each day decreased from Day 1 to Day 4 (15:9 LD, GLM, f=12.19, p=0.000; 12:12 LD, GLM, f=53.34, p=0.000; Figure 6c-d).

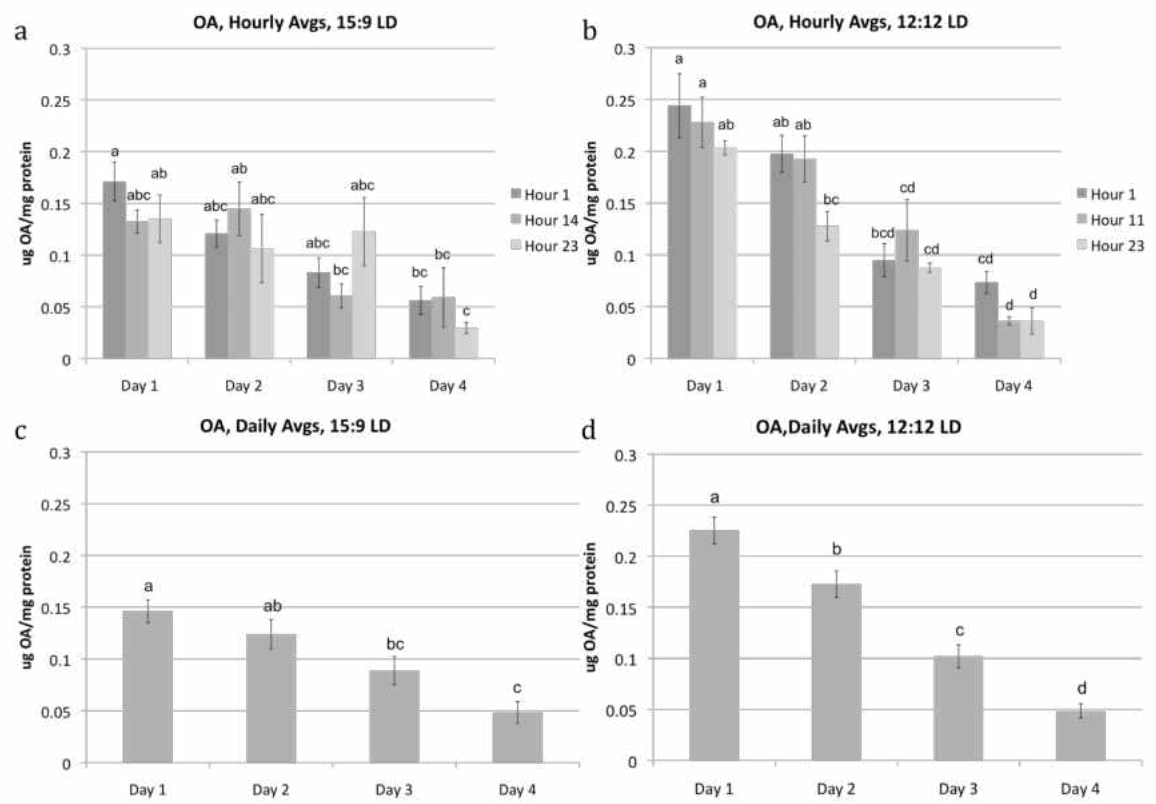


**Figure 6.** Replicate 2 Levels of OA. Bars represent mean OA in µg divided by mg of protein present in each sample at each time point or day. Error bars represent SEM. Bars that do not share a letter are significantly different. a. Means for each sample time in the 15:9 LD cycle, n=5. (RA, f=34.03, p=0.000). b. Means for each sample time in the 12:12 LD cycle, n=5. (RA, f=170.14, p=0.000). c. Means by day for the 15:9 LD cycle, n=15 (GLM, f=12.19, p=0.000). d. Means by day for the 12:12 LD cycle, n=15 (GLM, f=53.34, p=0.000).

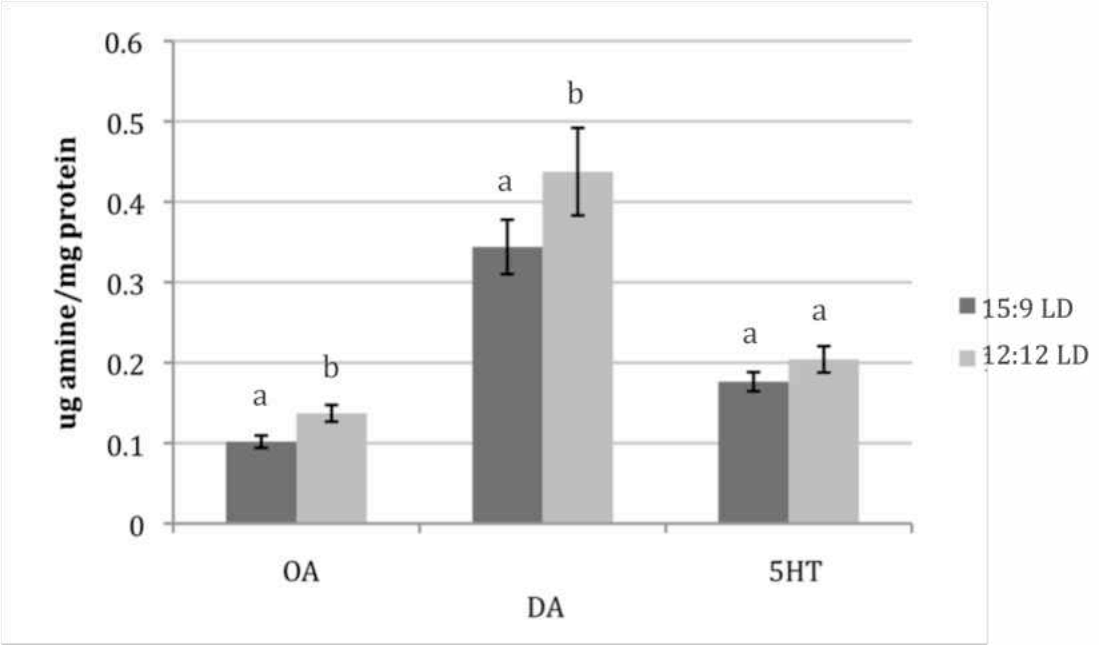


**Figure 7.** Light Cycle Affects Amine Levels. Bars are means for each amine in the 12:12 LD and 15:9 LD cycles. Error bars represent SEM. n=60 for each group. Bars that do not share a letter for each amine are significantly different (Tukey's pair-wise comparison, α=0.05). OA levels differ among light cycles (ANOVA, f=18.92, p=0.000). DA levels differ among light cycles (ANOVA, f=11.68, p=0.001). 5HT levels do not differ among light cycles (ANOVA, f=3.27, p=0.074).

5HT levels did not exhibit a diel pattern in the 15:9 LD cycle (GLM, f=1.31, p=0.278, Figure 8a), but in the 12:12 LD cycle, as with OA, a diel pattern was possibly emerging (GLM, f=5.62, p=0.006). In the 12:12 LD cycle, 5HT was generally higher at Hour 1 and had lowered by Hour 23 (Figure 8b), but the pair-wise differences within each day were not significant (Tukey's pair-wise comparison, α=0.05). There were changes between hours over sampling days for the 12:12 LD cycle (RA, f=57.62 p=0.000). The 15:9 LD cycle, alternately, did not show a significant regression (RA, f=0.63, p=0.429), but an ANOVA comparing the hours over all the days was significant (ANOVA, f=3.05, p=0.004), indicating there were some differences between the sample time points over the four day sampling period. The 15:9 LD cycle also had a significant interaction between day and hour for 5HT (GLM, f=4.43, p=0.001), indicating that the age of the organism affected the hourly level of 5HT. Daily averages of 5HT levels decrease from Day 1 to Day 4 in the 12:12 LD cycle (GLM, f=16.98 p=0.000; Figure 8d),

but in the 15:9 LD cycle no differences between days were observed (GLM, f=1.44, p=0.242, Figure 8c). There was a significant effect of light cycle and day on the hourly averages (ANOVA, f=3.59, p=0.003). Although these differences in pattern were observed, overall levels of 5HT between the two light cycles were not significant (ANOVA, f=3.27, p=0.074; Figure 7).

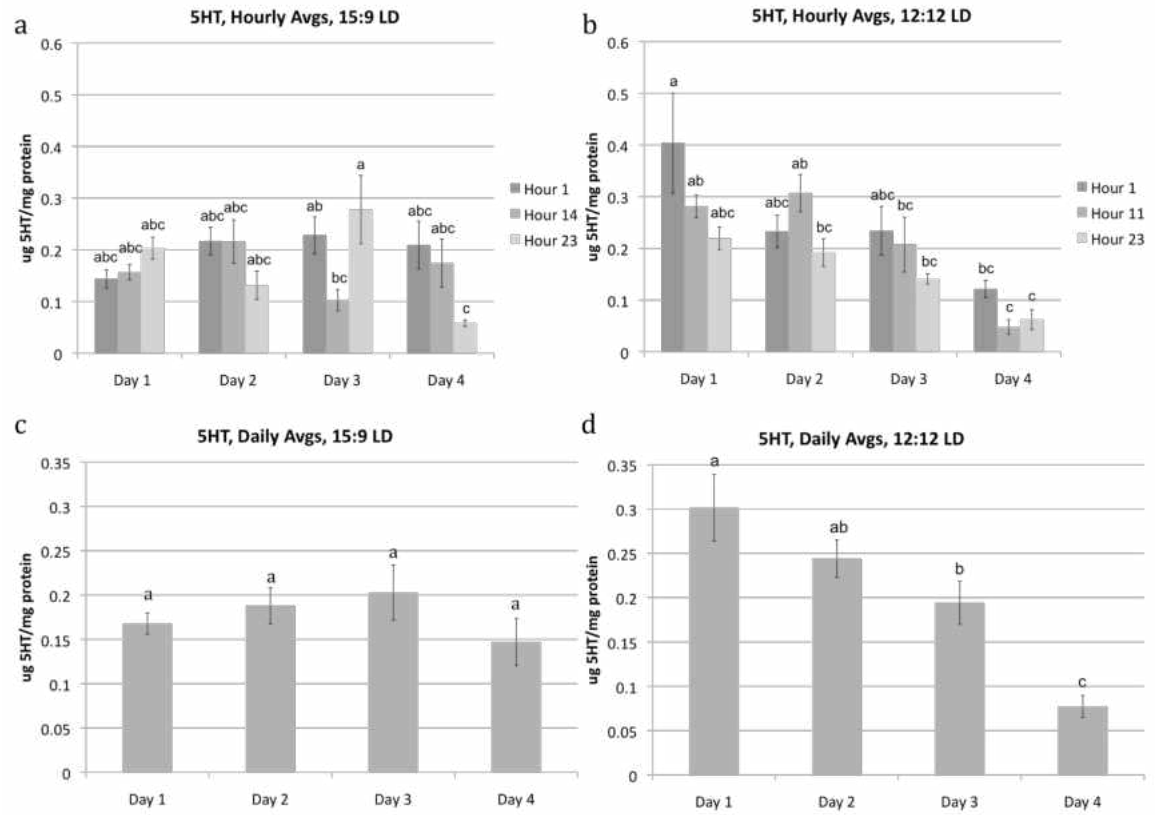


**Figure 8.** Replicate 2 Levels of 5HT. Bars represent mean 5HT in µg divided by mg of protein present in each sample at each time point or day. Error bars represent SEM. Bars that do not share a letter are significantly different. a. Means for each sample time in the 15:9 LD cycle, n=5. (ANOVA, f=3.05 p=0.004). b. Means for each sample time in the 12:12 LD cycle, n=5. (RA, f=57.26, p=0.000). c. Means by day for the 15:9 LD cycle, n=15 (GLM, f=1.44, p=0.242). d. Means by day for the 12:12 LD cycle, n=15 (GLM, f=16.98, p=0.000).

DA levels did not exhibit a diel pattern in the 15:9 LD cycle (GLM, f=1.43, p=0.249, Figure 9a), but as with the other amines in the 12:12 LD cycle, a diel pattern was emerging (GLM, f=15.38, p=0.000). In the 12:12 LD cycle, DA was generally higher at Hour 1 and had lowered by Hour 23 (Figure 9b), but the pair-wise differences within each day were not

significant (Tukey's pair-wise comparison, α=0.05). There were differences between hours over sampling days for each light cycle (15:9 LD, RA, f=57.96, p=0.000; 12:12 LD, RA, f=147.96, p=0.000), and day had a significant interaction on hourly means of DA, (15:9 LD, GLM, f=2.89, p=0.017; 12:12 LD, GLM, f=7.13, p=0.000). Regardless of light cycle, daily means of DA levels decreased from Day 1 to Day 4 (15:9 LD, GLM, f=29.62, p=0.000; 12:12 LD, GLM, f=141.14, p=0.000; Figure 9c-d). While the pattern of decrease from Day 1 to Day 4 was similar for both light cycles, DA levels overall were higher in 12:12 LD than in 15:9 LD (ANOVA, f=11.68, p=0.001; Figure 7). There was also a significant effect of light cycle on day and hour (ANOVA, f=2.91, p=0.012).

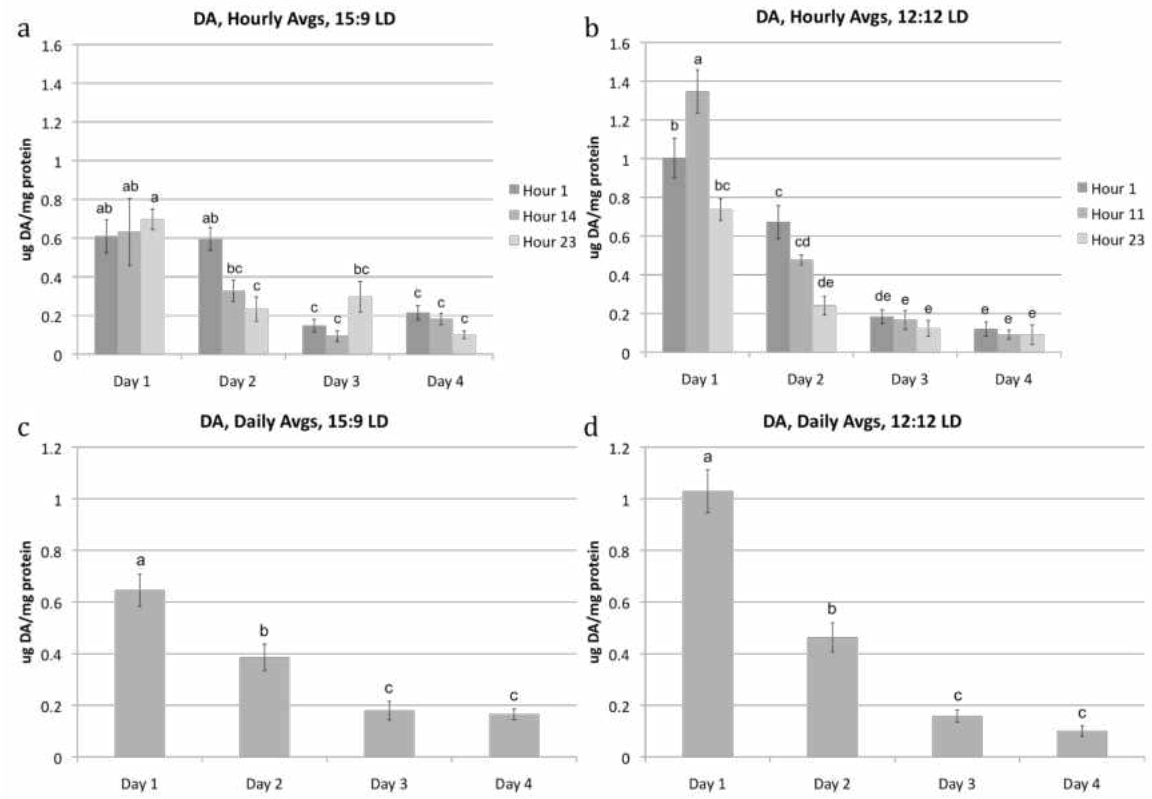


**Figure 9.** Replicate 2 Levels of DA. Bars are mean DA in µg divided by mg of protein present in each sample at each time point or day. Error bars represent SEM. Bars that do not share a letter are significantly different. a. Means for each sample time in the 15:9 LD cycle, n=5. (RA, f=57.96, p=0.000). b. Means for each sample time in the 12:12 LD cycle, n=5. (RA, f=147.96, p=0.000). c. Means by day for the 15:9 LD cycle, n=15 (GLM, f=29.62, p=0.000). d. Means by day for the 12:12 LD cycle, n=15 (GLM, f=141.14, p=0.000).

# CHAPTER 4

# DISCUSSION

## Diel Behavioral Changes

All of the behavioral categories investigated showed S.crassipalpis had diel changes in behavior and that the pattern of diel change differed dependent upon age. It had previously been found that aggressive behavior increased from Day 1 to Day 4 (Moore et al. in prep), but the present study looked more closely at how changes in behavior occurred throughout each day. There were slight differences in the daily rhythm of change of interactive behaviors between Day 1 and Day 4, but in general, interactive behavior increased from Hour 1 to the later time points. High aggression, like interactive behavior, showed an increase from Hour 1 to Hour 14. Focusing on the hourly changes, the correlation between an increase in interactive behavior and an increase in high aggression could be made. The daily averages, however, would indicate the opposite relationship between interactive and high aggression. Low aggression did not change in total levels from Day 1 to Day 4 but had the most profound difference in daily patterns of change.

Many studies to date have not strictly controlled for age of an organism or for the time of day that behavioral assays take place. It is important to understand how an organism's behaviors change with age and time before beginning behavioral studies. If there are diel or ontogenetic changes that occur, differences seen between individuals or between replicates could be due to these factors. For example, it has been noted that the cricket, Gryllus bimaculatus, does not

exhibit aggressive behavior during specific times of day, high humidity conditions, or specific seasons (Stevenson et al. 2000). Drosophila males will not enter consistently into aggressive encounters until 3 days after emergence (Chen et al. 2002). While these behavioral changes have been noted within the laboratory setting, no studies have been performed to further investigate them in detail. Changes with age are even less controlled for within studies. Social insects that change caste with age have been strongly studied for ontogenetic changes in behavior, and amines, because the behavioral changes are so pronounced (Bloch et al. 2000; Sied and Traniello, 2005). Unfortunately, this has been less studied in other invertebrates, aside from changes in locomotor behavior.

## Amine Quantification: Replicate 1 versus Replicate 2

The 2 replicates of the amine quantification differed greatly in the amount of amine present per hour, the pattern of change from Day 1 to Day 4, and from 12:12 LD to 15:9 LD. The first replicate had several points of uncertainty associated with the HPLC-ECD system. The first replicate's samples were run over a time span of 8 months. While consistency can be obtained with HPLC, the system used in this study was new and knowledge of its components was below par. Chemical components of the system, such as the mobile phase, were not maintained appropriately to keep the system at peak performance. Additionally, the 8-month time span could have led to breakdown in some of the samples, even though they were stored at an appropriate temperature (-80°C). The second replicate was run over a 2-month span under more controlled chemical conditions, and the results appear more consistent. For these reasons, Replicate 2's results were used for the interpretation of the data and for all further discussion.

## Changes in Amine Levels

### Diel Rhythmicity

Contrary to what was expected, amine levels in S.crassipalpis do not appear to have a daily rhythm. The 12:12 LD possibly had some diel rhythmicity emerging for each of the amines, but the patterns were not robust. Some of the studies that have seen diel or circadian rhythmicity in amines examined more time points within the light cycle and have looked at specific areas of the brain (Carrington et al. 2007, Levenson et al. 1999). The current study only examined 3 time points and analyzed the entire head capsule. It is possible that peaks or troughs in amine levels could occur between those 3 sampling times within each day. Studies that have taken advantage of a broader range of time points have shown diurnal rhythmicity to exist for biogenic amines (Carrington et al. 2007; Levenson et al. 1999; Schulz and Robinson, 1999). Another possibility is that by sampling the entire head capsule changes that occur within various regions within the brain were masked. Such was the case in a study of honey bees, where differences were seen between age and behavioral state in the antennal lobe and mushroom bodies, but no differences were seen when analyzing the brain as a whole (Schulz and Robinson, 1999). Biogenic amines also can act as hormones, entering the hemolymph to circulate throughout the body (Adamo et al. 1995; Levenson et al. 1999). In future studies of S. crassipalpis, brain dissection, hemolymph collection, and more extensive sampling could all lend further insight into the complicated changes that may occur throughout the day.

Changes in Relation to Age

All amines in both light cycles decreased with age of the flies except for 5HT in the 15:9 LD cycle. Both bees and ants show correlations with amine levels and age (Seid and Traniello, 2005; Schulz and Robinson, 1999; Harris and Woodring, 1992). Because both of these organisms are social insects, they have strong behavioral roles that change with age in support of colony life. It is not as simple of an aging process as may occur in many solitary organisms, but in the case of S. crassipalpis, there may be a similar mechanism occurring. S. crassipalpis males show a strong behavioral change with age, becoming aggressive, territorial, and sexually active 3 to 4 days after emergence (Paquette et al. 2008; Moore, personal observation; Moore, in prep). These behaviors, however, have only been studied within the 15:9 LD cycle, so it is not necessarily valid to assume that the changes seen in amine levels in the 12:12 LD cycle are also associated with the same behavioral ontogeny. One way to test the hypothesis that the aminergic changes are associated with the behavioral ontogeny of S. crassipalpis would be to perform a similar amine quantification experiment using females. Females of this species do not have the same ontogeny of behavior that is exhibited in males. If the same age-related aminergic changes are exhibited in females then it would indicate that these changes in amine levels are not associated with the behavioral changes seen in males.

Another hypothesis that has stemmed from these results is that there may be an association of higher levels of these amines with either development or emergence. Again, similar amine quantification experiments in females could support this hypothesis if females show a similar pattern of change with age. Dopamine is involved in sclerotization, and thusly, large quantities of dopamine are generally found immediately following eclosion to aid in the

hardening of the exoskeleton (Neckameyer et al. 2000), lending a strong developmental role associated with eclosion to DA. This phenomenon would not explain the high levels seen of the other amines, and other studies have shown that the high levels of DA immediately after eclosion are sequestered and altered for use in the exoskeleton structure by Day 1 (Neckameyer et al. 2000). Additionally, further supplementing the current study by looking at pre-eclosion levels of amines could explain the high levels seen on Day 1. If the high levels of amines on Day 1 are associated with eclosion, then it may indicate that there is a "ramp-up" of amine levels leading up to eclosion (Day 0). It would also be of interest to sample beyond our current end point of Day 4 to investigate whether the amines continue to decline or level off after Day 4.

Endogenous amine levels may exert control over behaviors, but receptor regulation is also an important component. Amines may change their pathway of action based on receptor regulation. There are several different types of receptors for each amine and the receptors may be up- and down-regulated over time. 5HT receptors in the crayfish brain show circadian rhythmicity (Calderon-Rosete et al. 2006). Similar changes may also be occurring with age, leading to different pathways of action for amines. There was no change from Day 1 through Day 4 for 5HT in the 15:9 LD cycle in this study, but that does not necessarily suggest that its role in aggression is null. It is possible that although the overall levels of 5HT remain constant, the receptor densities and types are being modified to accommodate physiological needs.

Light Cycle Affects Aminergic Changes

Light cycle had a significant effect on all investigated amines. OA had a significant interaction between light cycle and day. DA and 5HT both had a significant interaction of light cycle, day, and hour. Amines were all trending toward higher levels in the 12:12 LD cycle than in the 15:9 LD cycle. While the higher levels in 12:12 LD for 5HT were not significant, 5HT showed another obvious difference between the 2 light cycles. The daily averages in 15:9 LD for 5HT did not differ between any of the 4 days, but in the 12:12 LD cycle, there was a steady decrease from Day 1 to Day 4. These results did not fit with the expectations prior to beginning the study. The hypothesis was that there would be phase-shifts in aminergic changes rather than the pronounced differences that were seen. A possible explanation for the differences between light cycles is seasonal variation.

In a field study, bees showed seasonal fluctuations in amine levels; higher levels of OA, 5HT, and DA were detected during summer as compared to spring and fall (Harris and Woodring, 1992). The current study indicated that the opposite is true of S. crassipalpis; amine levels are higher in 12:12 LD than in 15:9 LD. The 15:9 LD cycle represents the longer photophase associated with summer, while the 12:12 LD cycle has a shorter light period representative of fall and winter. S.crassipalpis is most active during the summer while a shift in temperature and light cycle with the onset of fall induces diapause. For the purposes of this study, larvae were kept in 15:9 LD conditions for 3 days before placing them in 12:12 LD to prevent diapause. The increase in amine level from 15:9 LD to 12:12 LD may be representative of natural fluctuations onset by seasonal change. This would be difficult to verify outside of a

laboratory setting because S. crassipalpis do not enter the adult part of the life cycle under short photophase conditions.

## Conclusion

This study demonstrated that time of day, light cycle, age, and aggressive state can be correlated with aminergic changes. Time of day did affect aminergic levels, but diel rhythmicity of amines was not displayed as expected from previous literature. Light cycle had significant effects on the daily levels of amines, possibly indicating the environmental effect of seasonal variation on physiological plasticity. Differences in amine levels with age was observed in all groups except 5HT in 15:9 LD, demonstrating the importance of controlling for age of organisms in future studies. Behavioral profiles changed with time of day and age, further emphasizing the need for control of organism age. No clear hourly correlations emerged from this study, but overall daily averages suggest that decreased levels of OA and DA are associated with decreased interactive behavior and increased high aggression.

## Future Directions

Future studies of aminergic changes in S.crassipalpis should take a more detailed approach. HPLC-ECD is a useful tool for examining endogenous levels of amines. The system used in this study is specifically tailored for measuring monoamines in minute quantities; sections of single brains can be analyzed without the necessity to pool samples. To further investigate ontogenetic, diel, or circadian changes in amine, brain dissection techniques and more comprehensive sampling times should lead to a clearer picture of any patterns or

fluctuations that are occurring. Hemolymph samples could indicate if circulating levels of amines are fluctuating.

The current study found that in most cases amine levels were high on Day 1 and lowered by Day 4. Two possible hypotheses have emerged from this finding: 1. Decreases in amine levels are correlated with the increase of aggression, territoriality, and sexual behavior 2. The high levels of OA, 5HT, and DA on Day 1 are associated with eclosion. To begin testing these hypotheses, an HPLC analysis of female S. crassipalpis over the same 4-day period should be done. If females do not also exhibit the high levels of amines on Day 1 that is seen in males, then the hypothesis that higher levels of amines are associated with eclosion could be rejected. This result would also support that the changes in amines in males may be involved in male ontogenetic behavioral changes. A different approach would be to sample males for HPLC-ECD prior to eclosion up to Day 0 and at time points after Day 4, looking for changes leading up to eclosion and determining if amine levels continue to decline after Day 4.

The findings from this study have also prepared S. crassipalpis to be used as a model system for the study of aminergic control of aggression. The correlations between behavior and the investigation of the endogenous amine levels would be complemented nicely by an amine manipulation study. Exogenously applying OA, DA, and 5HT and examining effects on behavior could support correlations from this study.

In addition to manipulation methods, receptor studies could help to complete the story. Immunohistochemical techniques could illuminate in what areas of the brain receptors are being up- or down-regulated, or a western blot could be used to determine overall presence of receptors in a sample. Changes in receptor quantities or sub-types could also explain the strong behavioral changes that S. crassipalpis males exhibit.

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# APPENDIX

## Protein Assay

Sample Preparation

80 ul HPLC grade water + 10 ul 0.2M NaOH + 10 ul supernatant

Diluted Albumin (BSA) Standards: Thermo Scientific MicroBCA™Protein Assay Kit

| Vial | Volume of Diluent | Volume & Source of BSA | Final BSA Concentration |
|---|---|---|---|
| A | 0 | 300 µl of stock | 2000 µg/ml |
| B | 325 µl | 325 µl of stock | 1000 µg/ml |
| C | 325 µl | 325 µl of vial B dilution | 500 µg/ml |
| D | 325 µl | 325 µl of vial C dilution | 250 µg/ml |
| E | 325 µl | 325 µl of vial D dilution | 125 µg/ml |
| F | 900 µl | 100 µl of vial B dilution | 100 µg/ml |
| G | 400 µl | 100 µl of vial E dilution | 25 µg/ml |
| H | 900 µl | 100 µl of vial F dilution | 10 µg/ml |
| I | 900 µl | 100 µl of vial H dilution | 1 µg/ml |
| J | 400 µl | 0 | 0 µg/ml=Blank |

BCA Working Reagent:

BCA Reagent A (50 parts) + Reagent B (48 parts) + Reagent C (1 part)

# VITA

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