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A Time-Course Analysis of Behavioral Plasticity and Differential Gene Expression Patterns in Response to Density in Schistocerca americana (Orthoptera: Acrididae)

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A TIME-COURSE ANALYSIS OF BEHAVIORAL PLASTICITY AND DIFFERENTIAL GENE EXPRESSION PATTERNS IN RESPONSE TO DENSITY IN *SCHISTOCERCA AMERICANA* (ORTHOPTERA: ACRIDIDAE)

by

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ABSTRACT

Phenotypic plasticity is the ability of the genotype to express alternative phenotypes in response to different environmental conditions and this is considered to be an adaptation in which a species can survive and persist in a rapidly changing environment. Some grasshoppers and locusts are capable of expressing an extreme form of density-dependent phenotypic plasticity, known as locust phase polyphenism. At low population density, the individuals typically have a cryptic coloration as nymphs, are less active, and only seek out conspecifics for reproductive purposes. At high density, however, they develop a drastically different phenotype in which they have a conspicuous coloration, are much more active, and tend to stay together in large groups. The American Birdwing grasshopper, *Schistocerca americana,* is a non-swarming species related to the desert locust, *S. gregaria*, which shows density-dependent phenotypic plasticity in behavior, color, and morphology. In this thesis, I have identified the duration of crowding necessary for a 6th instar *S. americana* reared in the isolated condition to express the typical crowded behavior. The behavior changed after just one hour of crowding and the effect of crowding diminished after 48 hours to near-complete isolated behavior. In reverse, the crowded condition was isolated, but behavior did not significantly change over time. Gene expression of the following three genes suspected of having a role in behavior change were investigated based on studies of *S. gregaria*: protein kinase A (PKA), L-Tryptophan-5-monooxygenase (T-5), and Aromatic L-amino acid decarboxylase (Decarb). T-5 was up-regulated in the long-term isolated condition compared to the long-term crowded condition. T-5 and Decarb were up-regulated in

isolated individuals that were crowded for 10 hours compared to the long-term isolated condition. This study represents a novel contribution in the study of phenotypic plasticity as it establishes the time course of behavioral and molecular plasticity in a non-swarming grasshopper for the first time.

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CHAPTER I: GENERAL INTRODUCTION

It is important in the study of evolutionary biology to understand how species have evolved in a heterogeneous environment. Genes and phenotypes do not have just a one-to-one relationship, but in most cases there are several genes interacting with each other that can affect many aspects of a given phenotype (Zhong, 1999). Furthermore, the environment can have considerable influence on the expression of genes and the resulting phenotypes (Pigliucci, 2001; West-Eberhard, 2003). To study the ability of species that respond to different environmental conditions, the norms of reaction must be measured, in order to understand their adaptive phenotypic evolution. A reaction norm is a quantitative measure of a phenotypic trait that varies in expression across different environments (Weis and Gorman, 1990). This is typically measured experimentally by testing multiple individuals of essentially the same genotype in different environmental conditions such as temperature, acidity, light, predator presence, or population density and then quantifying how the phenotype may vary across the environmental gradients (Weis and Gorman, 1990). It is important to study reaction norms in order to investigate phenotypic plasticity, especially in species that have more visible plasticity and are well suited for testing.

There are multiple classic examples of studies of phenotypic plasticity for such species. For example, Pigliucci (1995) was able to find differences in the reaction norms of *Arabidopsis*, by testing the plant's responses to different environmental conditions. He found that nutrient levels in the soil resulted in the highest plastic response from the

plants measured by growth and leaf size. Phenotypic plasticity is also found in animals. A classic example is the water flea, *Daphnia pulex,* which has been studied in depth for its relatively small genome size (Colbourne, 2007), and largely because it exhibits predator-induced phenotypic plasticity where it grows teeth-like projections and enlarges itself to mitigate predation (Colbourne, 2011). Another example can be found in vetch aphids which develop a winged morph in high density or under colony predation in order to disperse to a better habitat. Currently, there are numerous undertakings to tease apart what genetic factors are at play in phenotypic plasticity. Only recently, has there been research investigating the processes that shape phenotypic plasticity at a molecular level, but there is still much left uncovered (Tollrian, 2010). Simon et al. (2011) argued that comparative studies between vetch aphids and water fleas can give more insight into the regulatory mechanisms of phenotypic plasticity that may be shared between these species. These vetch aphids and water fleas are excellent for studying phenotypic plasticity because they are easily reared in lab, have genomes partially sequenced and ongoing, and their plastic traits are inducible in a lab setting. It is suspected that entire gene networks and epigenetic variation allows for such plasticity (Bossdorf, 2008).

An even more striking display of density-dependent phenotypic plasticity (DDPP) is found in the swarming desert locust, *Schistocerca gregaria*. This species exhibits two very different phenotypes depending on population density, known as the solitarious and the gregarious phases, and this extreme form of DDPP is known as locust phase polyphenism (Pener, 1983; Uvarov, 1966). The solitarious phase occurs at low population densities and these individuals avoid each other except for reproductive purposes. They are typically cryptic, variably green in color, and have little black pattern as nymphs. The

gregarious phase occurs in high population densities where the individuals aggregate and march together. They are typically conspicuous, bright yellow in color, and have a large amount of black pattern as nymphs (Fig 1.) (Pener and Simpson, 2009; Uvarov, 1966).

Figure 1: Two Extreme Phases of the Desert Locust, *Schistocerca gregaria*
This figure depicts the 6th instar desert locust as the gregarious phase (left) that develops in high population density and the solitarious phase (right) that develops in a low population density. (Photo credit: Tom Fayle)

It was originally though that these were different species. Uvarov (1921) was among the first to realize that these were actually two different phases of the same species. He cautioned that plasticity such as this must be considered in future taxonomic work. His work led to numerous subsequent publications of others defining the differences between the phases, particularly for *S. gregaria* (Applebaum, 1997; Hassanali et al., 2005; Kang et al., 2004; Pener, 1991; Pener and Simpson, 2009; Roessingh and Simpson, 1994; Simpson et al., 1999; Simpson and Miller, 2007; Simpson et al., 2002; Sword, 2008; Tanaka, 2001, 2006; Verlinden et al., 2009). There are many changes involved in locust phase polyphenism such as behavior, biochemistry, morphometric ratios, pigmentation, sensory, and lipid content (Applebaum, 1997; Roessingh et al., 1993a). The list continues to grow as more density-dependent plastic reaction norms are discovered. The first change to occur between these phases, however, is behavior

(Simpson, 1999). When environmental conditions favor increase in local population densities, such as basking areas and clumps of vegetation, locusts will begin to aggregate and eventually march in groups (Buhl, 2006; Pener and Simpson, 2009; Steedman, 1990). These changes are stimulated by visual, olfactory, and tactile cues from interacting with conspecifics (Simpson, 1999). The stimulation of the mechanoreceptor hairs on the outer face of the hind femur has the strongest effect (Rogers, 2003). This sends a signal up the nerve of the femur to the brain (Rogers et al., 2004), which releases serotonin to the thoracic ganglia (Anstey et al., 2009). These stimulations cause solitarious locusts to become gregarious and a persistent high density causes color as well as many other underlying mechanisms to change (Tawfik et al., 1999). The initial changes cause a feedback loop where the nymphs will continue to aggregate and become more gregarious (Roessingh and Simpson, 1994). This polyphenism gave rise to the desert locust's ability to continue to swarm today in North Africa and devastate crops (Pener and Simpson, 2009). However, it is still under investigation though how this ability has evolved.

The genus *Schistocerca* has approximately 50 species and only four of these are known to be swarming locusts (*S. gregaria, S. piceifrons, S. cancellata,* and *S. interrita*) making the genus mostly made up of sedentary non-swarming species (Dirsh, 1974; Song, 2004). Recent molecular work has uncovered that the desert locust is basal to this genus and that this plasticity may have independently evolved multiple times (Lovejoy et al., 2006; Song, 2013; Song et al., 2013).

Study System: *Schistocerca americana*

Figure 2: The American Birdwing Grasshopper, *Schistocerca americana* (Adult) The adult with full wings develops this coloration whether it developed in the isolated or crowded condition. (Photo Credit: Tom Friedel)

The American Birdwing Grasshopper, *Schistocerca americana* (Fig. 2), is distributed across a wide range and is found throughout the eastern United States. They rarely form large outbreaks, but can have occasional devastating effects on crops, especially citrus groves (Kuitert and Connin, 1952). This species has not been known to be a swarming species, but it has been observed that they express DDPP in coloration and behavior (Gotham and Song, 2013; Sword, 2003). Recently, I explicitly quantified that *S. americana* expresses DDPP in behavior, morphology, and coloration, reminiscent of the desert locust (Gotham and Song, 2013). The fact that *S. americana* is phylogenetically related to the desert locust and expresses similar DDPP, but does not swarm, makes it an excellent system for studying the evolution of phenotypic plasticity in this genus. Furthermore, many of the proximate mechanisms that have been studied in the desert locust are completely unknown for *S. americana*, which would mean that there is a good

opportunity to find and characterize the differences in mechanisms between swarming and non-swarming species.

I have organized this thesis in the following way. In Chapter 2, I summarize what is known about behavioral plasticity and characterize the pattern of gregarization and solitarization in *S. americana* through a series of time-course behavior trials. In Chapter 3, I will provide a brief review on the studies regarding the molecular aspects of locust phase polyphenism, and reveal my differential gene expression results of *S. americana* between long-term isolated condition and long-term crowded condition, as well as the time-course of gregarization in the species. In Chapter 4, I will synthesize my findings and provide a general conclusion for this thesis.

CHAPTER II: A TIME-COURSE ANALYSIS OF DENSITY-DEPENDENT BEHAVIORAL PLASTICITY IN THE AMERICAN BIRDWING GRASSHOPPER

Introduction

Locust phase polyphenism consists of numerous density-dependent plastic reaction norms (Pener and Simpson, 2009), and the first reaction norms that respond to change in density is behavior (Ellis, 1963; Ellis and Pearce, 1962; Simpson, 1999). In the desert locust, *Schistocerca gregaria*, when individuals reared in isolation are exposed to high density, they quickly transform their behavior from being shy and avoiding each other to being highly active and gregarious (Uvarov, 1966). This plasticity in behavior can be considered an example of developmental plasticity or activational plasticity (Snell-Rood, 2013). Activational plasticity refers to differential activation of an underlying network in different environments such that an individual expresses various phenotypes throughout their lifetime, this would be the case with an individual that can rapidly change their phenotype given the environment. In this case, the ability to rapidly change behavior would be considered activational plasticity whereas, the other characteristics that ensue this changes would be considered developmental plasticity. This chapter investigates the initial activational plasticity and the long-term developmental plasticity of *S. americana* where developmental plasticity is defined as the capacity of a genotype to adopt different developmental trajectories in different environments (Snell-Rood, 2013).

For the desert locust, *S. gregaria*, there is robust literature on studies of the behavioral plastic reaction norms of its locust phase polyphenism (Pener and Simpson, 2009). The desert locust's behavior has been studied by direct observation and exposure to high-density stimulus of its conspecifics (Roessingh et al., 1993a). The behavioral phase state of *S. gregaria* dramatically changes after a solitarious individual is crowded significantly after 1 hour and for a maximal effect after 4 hours in that the solitarious individual will express typical gregarious behavior (Roessingh and Simpson, 1994). The reverse effect, where a gregarious individual expresses solitarious behavior, takes effect after 24 hours of being isolated (Roessingh and Simpson, 1994). The potential explanation given for these observed patterns is that locusts tend to aggregate at favored sites for basking and feeding (Ellis, 1959; Ellis and Pearce, 1962; Kennedy, 1939). This crowding imposed by the local environment causes behavioral gregarization, setting in motion a positive feedback loop, encouraging development of other phase characteristics as well as resistance to becoming isolated. If the locust loses contact with each other after short periods of crowding the acquired gregarious behavioral phase will be lost rapidly and the population once again shifts to the solitarious state (Roessingh and Simpson, 1994). The solitarious phase and gregarious phase are terms reserved for locusts only (Pener, 1983), so for the remainder of this work, I will refer to the solitarious phase of non-swarming grasshoppers as the isolated condition and the gregarious phase as the crowded condition.

The congeneric, *S. americana*, has also been observed to express a similar behavioral plasticity as *S. gregaria*, but to a lesser extent. Sword (2003) was the first to comparatively quantify the behaviors of *S. americana* and *S. gregaria* based on the assay

developed by Roessingh et al. (1993b). He did not find significant differences between density conditions of *S. americana* besides in time spent climbing the walls of the arena, at least not compared to the more extreme phase differences in *S. gregaria,* but he did find that there were behavioral differences in crowded condition of 1st instar *S. americana* in walk frequency, speed, and time between Texas and North Carolina populations of *S. americana*. He found that rearing density did not have a significant effect in the behaviors between the populations in the final instar though.

I revisited the study of behavioral plasticity in *S. americana* using a higher density for the stimulus chamber of the behavior arena for the 6th instar from a population collected in Pasco County, Florida. I found that there are significant density-dependent differences in color (Fig 3), behavior, and morphology (Gotham and Song, 2013).

Figure 3: Coloration differences in plasticity between treatments of *S. americana*
When reared in isolation, the nymphs develop and express cryptic coloration (left), but when reared in high density, they develop conspicuous coloration (right). (Photo: Steve Gotham)

My work was the first to take a more quantitative approach to validate the effect of rearing density in *S. americana,* which served as a foundation for understanding the effects of long-term isolation and crowding in this species. However, it is still unknown in *S. americana,* how rapidly behavior changes during the processes of gregarization and solitarization, such as when an isolated individual is crowded and when a crowded

individual is isolated. There may be species-level differences that can be correlated to the phylogeny that may give more insights into understanding the differences between swarming and non-swarming species within *Schistocerca*. In order to investigate the underlying physiological mechanisms involved, the behavioral time-course must first be determined (Roessingh and Simpson, 1994). This logical progression is essential to investigating the physiological basis of the change in density conditions. This may ultimately lead to a better understanding of locust population dynamics.

Understanding the time-course of behavioral gregarization and solitarization is imperative for understanding the proximate mechanisms of DDPP and the result of this work will be the basis for Chapter 3. In this Chapter, I specifically test the following hypotheses using a series of rearing experiments and behavioral assays:

H_0: There is no change in behavior of the 6th instar of *S. americana* with any duration of crowding or isolation.

When the isolated nymphs are crowded, we can expect the following two scenarios:

H_1: Crowding influences behavioral change in *S. americana.*

Prediction 1: The behavior of *S. americana* will change over time with crowding in a similar manner as *S. gregaria.*

Prediction 2: The behavior of *S. americana* will change over time with crowding in a different manner as *S. gregaria.*

When the crowded nymphs are isolated, we can expect the following two scenarios:

H_1: Isolation influences behavioral changes in *S. americana.*

Prediction 1: The behavior of *S. americana* will change over time with isolation in a similar manner as *S. gregaria.*

Prediction 2: The behavior of *S. americana* will change over time with isolation in a different manner as *S. gregaria.*

Materials and Methods

Study organism and rearing conditions

A population of *S. americana* was collected from Rock Springs Run State Reserve (Lake County, Florida) in January 2013 to establish a colony for this study. Wild caught grasshoppers were quarantined in environmental chambers and reared until they mated and oviposited. The substrate for oviposition was a mixture of 10 parts autoclaved play sand, 1 part peat moss, and 1 part water. The egg pods were harvested and the hatchlings were reared in dedicated quarantined environmental rooms specifically designed to study the effect of rearing density.

In order to quantify density-dependent behavioral plasticity, I established long-term colonies of isolated and crowded lines of *S. americana*. To rear grasshoppers in isolation, I placed individual hatchlings in separate opaque colored, inverted plastic cups (11.5 cm diameter bottom to 8.5 cm, height 8 cm) to keep them visually and physically isolated from each other. Each cup had constant positive flow of charcoal-filtered air to maintain olfactory isolation as well. This line was isolated for three generations to maximize the production of isolated traits. For the crowded treatment, I reared approximately 500-800 nymphs in large plastic bins (30 Gallon Storage Tote, 171,785 cm^3) in a separate room. All grasshoppers were reared under the regime of 16 h light at 30°C and 8 h darkness at 25°C and were fed Romaine lettuce and wheat bran daily.

Quantification of behavior

Roessingh et al. (1993a) were the first to develop a logistic regression model specifically for *S. gregaria* to predict whether an individual locust of unknown rearing history is displaying solitarious or gregarious behavior. They developed the model first by quantifying behavioral parameters of known solitarious and gregarious nymphs using an arena that had two stimulus chambers at each end of a rectangular arena. The model was based on the behavior observed for the long-term treatment of both phases. The model was based on solitarious nymphs that were never exposed to high-density crowding and gregarious nymphs that were never isolated. The arena constructed to observe behavior had one chamber that stimulated the high-density condition by containing 15 crowded 6^{th} instar locusts into a partition, and the other chamber stimulated the low-density condition by leaving the partition empty. Each individual locust was tested one at a time. The nymph as the test subject was introduced through a hole in the center of the arena and then observed for ten minutes. They quantified behaviors of the subject; such as how much they moved and how much time they spent in different sections of the arena. They used approximately 50 locusts from each phase, solitarious and gregarious, to develop the binary logistic regression model from a forward stepwise method to determine the best model from the given behavior variables. The model incorporated the data in a linear fashion as $\eta = \beta_0 + \beta_1\kappa_1 + \beta_2\kappa_2 + \ldots + \beta_\kappa\kappa_\kappa$ where η was density-dependent response variable between the extreme phase difference in behavior, β was the regression coefficient for each variable based on the model and κ was an individual test subjects' response to each variable, which gave the model the capability to

predict the phase of any nymph based on the response. The model predicts solitarious or gregarious behavior as the probability of being solitarious ($P(\text{solitarious}) = e^{\eta}/(1+e^{\eta})$). Once the model was established, they were able to predict the behavioral phase state of a subject of unknown rearing history. This was a powerful tool for measuring the behavior of locusts in an experimental setting, and a large series of studies were published using this method to tease apart the proximate mechanisms of behavioral gregarization.

To quantify the effect of rearing density in behavior of *S. americana*, I modified the behavioral assay developed by Roessingh et al. (1993a). I constructed the arena according to the exact specifications (57 x 31 x 11 cm) described in Roessingh et al. (1993a). Briefly, the arena had a stimulus chamber at each end, one simulating a low-density condition (no grasshoppers) and the other simulating a high-density condition (50 last nymphal instar reared in a crowded condition). A test subject (a grasshopper reared in either density condition) was introduced through a small hole in the center of the arena, and its behavior in response to the stimulus chambers was recorded using a video camera located directly above the arena for 10 min.

I used the software EthoVision (Noldus), which can track moving objects in live tracking or recorded videos by differencing images to locate the test subject, to video-track the behavior of the test subject. This enabled a more reliable means of quantification of movement and duration of time spent in different sections of the arena. The dimension of the arena was also programmed as a reference image in which overlays were built on top of the image (Figure 4).

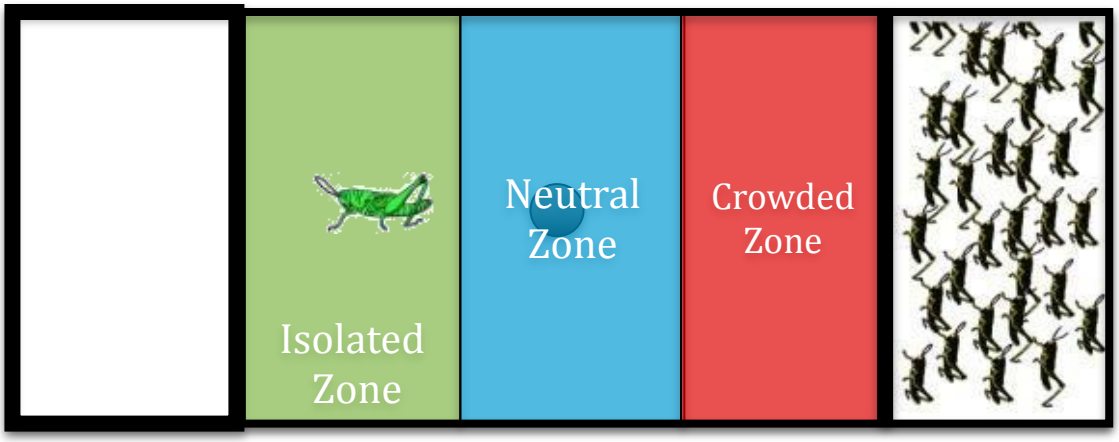

Figure 4: Arena Overlay for EthoVision Software
This is a graphical representation of the aerial view of the behavior arena. There are partitions sides in which one holds the crowded stimulus (50 conspecific crowded grasshoppers and the other is empty). The test area is split into three zones to track how much time is spent near the low and high density stimulus chambers partitioned to the side.

The test area within the stimulus partitions were segmented into different zones that took up a third of the test area each. The behavioral assay yielded many behavioral parameters for inclusion in analyses such as: (1) distance the subject moved (cm); (2) mean distance from a set area in the arena, in this case, the stimulus chamber with the 50 crowded grasshoppers (cm); (3) duration of the trial spent on the walls of the arena (sec); (4) Durations that the nymph was within zones of the arena (climb time, crowded zone, neutral zone, and isolated zone); (5) duration spent immobile, mobile, or highly mobile (the software defined mobility as the degree of movement of an animal's body independent of spatial displacement and did not incorporate the x,y coordinates of the animal's location throughout the video); (6) and duration of time spent moving or not moving. I used the default differencing settings for detection, where an image was grabbed before a grasshopper was introduced to the arena, and center-point tracking. The video files were saved after each trial and the tracks were later edited to ensure that the

software correctly tracked the subject's movement. For track smoothing, the "minimal distance used" setting at default was used, but not "smoothing lowess" because it ignored too much data and dramatically increased variance incorrectly for movement and mobility calculations. The following settings were used for the moving and mobility measurements: Moving and Not Moving of Center-point, averaged over 100 samples, with thresholds of 0.1 and 0.01cm/s and Immobile and Mobile, averaged over 150 samples, with thresholds of 80.00 and 0.00%. These settings reflected the movement of the test subjects more accurately and eradicated the majority of noise of the center-point moving to different segments of the grasshopper rapidly that may have caused the error originally in the software's calculations. Typically, isolated grasshoppers spent more time in the isolated zone and were repulsed by the crowded stimulus of 50 crowded conspecifics, and crowded grasshoppers moved much more and spent more time closer to the crowded stimulus (Gotham and Song, 2013). Because behavior was highly variable, there were occasionally individuals that behaved in the opposite manner than expected. To rectify this problem, a large sample size of behavior trials was required.

I built a logistic regression model specifically for this population of *S. americana* to predict the behavioral phase state of the test subject. I conducted 70 trials of each phase, long-term isolated and crowded, of *S. americana* to develop the model. I used a forward-stepwise procedure to identify which variables were appropriate for the model in RStudio ver. 0.97.449 (http://www.rstudio.com). The behavior variables recovered were the distance moved, mean distance from the stimulus chamber, duration of time spent in the crowded and isolated zone, the time spent climbing the walls of the arena, and

duration the grasshopper was mobile, immobile, moving, and not moving. The formula used for this model was $\eta = \beta_0 + \beta_1\kappa_1 + \beta_2\kappa_2 + \ldots + \beta_\kappa\kappa_\kappa$, with $p(\text{isolated}) = e^\eta / (1 + e^\eta)$.

Experimental set up for time-course analysis of behavioral plasticity

In order to study how long it took for isolated grasshoppers to change their behavior when crowded, and for crowded grasshoppers to lose their characteristic crowded behavior, I established the following experimental conditions. To characterize the time-course of gregarization, I crowded the sixth-instar isolated nymphs for 1, 2, 4, 6, 8, 10, 12, 24, and 48 hours. For crowding, I placed the isolated nymphs into a small cage (30 cm^3) with 50 crowded nymphs for the given time durations before assaying their behavior. To characterize the time-course of isolation, I isolated the sixth-instar crowded nymphs for the same periods of time as described above.

Marking was necessary in order to keep track of isolated individuals in the crowded treatment, especially for the ones that maintained some coloration and black pattern reminiscent of crowded nymphs although they had always been isolated. I tested whether marking the grasshopper' wing pads with an opaque water-based acrylic pigment marker altered an individual's behavior. For the isolated nymphs, I would remove one of the nymphs in the isolated treatment and then color its wings with the marker and then test it's behavior in EthoVision in the same method used to test the long-term isolated nymphs used in the behavior model. For the crowded nymphs, I would remove a crowded nymph from the crowded treatment stock and then color its wings with the marker and test it's behavior in EthoVision in the same method used to test the long-term crowded nymphs used in the behavior model. These individuals are not used again for any other

experiments. I compared the behaviors of 20 isolated and 20 crowded marked individuals with those of the unmarked long-term isolated and crowded individuals of the behavior model using pairwise T-Test in SPSS.

Statistical analyses

After all behavior tracks were first recorded for editing using a track editor implemented in EthoVision, I exported the resulting behavioral data into excel for further statistical analyses in IBM SPSS Statistics Ver. 20. For this study, the features used in the program were the chart builder for line and bar graphs, and "Univariate" which is a general linear model (GLM) Univariate Analysis. Univariant provides regression analysis and analysis of variance for one dependent variable by one or more factors and/or variables. In this case, the dependent variable used was the probability of isolation score from the model and the fixed factor was the Phase, crowded or isolated. The 69 isolated and 70 crowded *S. americana* used to construct the behavior model for this experiment are from the population collected at Rock Springs Run State Reserve (Seminole County, Florida) and are not the same individuals used to test behavior between the two treatments in the previous experiment (Gotham and Song, 2013). To ensure that marking the grasshoppers in order to keep track of them during crowding did not affect behavior, I ran two separate univariates with treatment as the factor and the probability of isolated behavior as the dependent variable between model isolated and crowded nymphs that were not marked against the isolated and crowded nymphs that were marked.

Results

Behavior Model

The behavior model consisted of 69 long-term isolated individuals and 70 long-term crowded individuals. One outlier was removed from the long-term isolated group using Grubbs outlier test. This sample size was sufficient for model accuracy and shows the significant difference in behavior between conditions (Figure 5). The best model incorporated 11 coefficients (Table 1) and was able to predict an individual's phase with 78% accuracy.

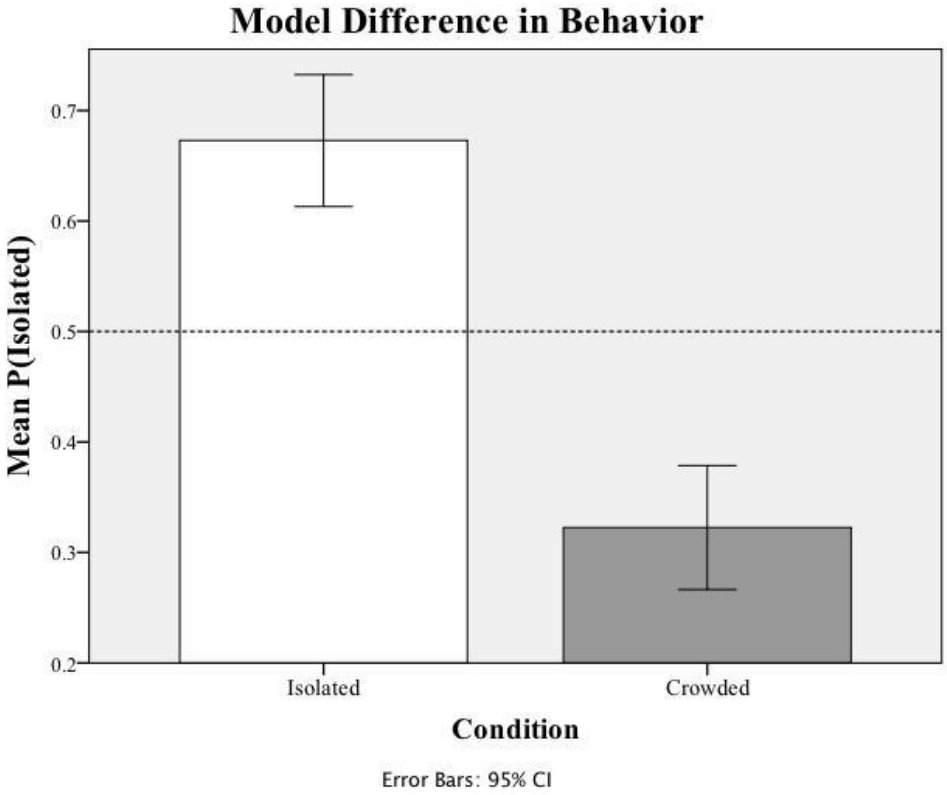

Figure 5: Probability of Isolated behavior in response to density treatment
This graph illustrates the effectiveness of predicting behavioral phase using logistic regression model. The model correctly predicts the behavior of isolated-reared nymphs as "isolated" and that of crowded-reared grasshoppers as "crowded." Above 0.5 (dash line) is the threshold to classify the behavior as "isolated". Isolated N=69, Crowded N=70, T-Test: t=8.531, P=0.000

Table 1: Coefficients for the behavioral parameters
Based on the most parsimonious logistic regression model (forward stepwise method). (9 of 19 possible parameters have been retained because these variables gave the model the most accurate predictions of the correct phase at 78%. Significance P-values upon removal of these variables are also shown. Model: $\eta = \beta_0 + \beta_1\kappa_1 z + \beta_2\kappa_2 + \ldots + \beta_\kappa\kappa_\kappa$, with $p(\text{isolated}) = e^\eta / (1 + e^\eta)$.)

Behavior Variables	Coefficient β	P-Value
Distance Moved (cm)	0.0002905	0.946499
Mean Distance from Stimulus (cm)	-0.2597289	0.022452
Time Spent Climbing (sec)	0.0027708	0.126932
Time in Crowded Zone (sec)	-0.0171384	0.000492
Time in Isolated Zone (sec)	-0.0025065	0.443687
Immobile (sec)	0.0020223	0.583245
Mobile (sec)	-0.0044600	0.143298
Moving (sec)	-0.0880540	0.146793
Not Moving (sec)	-0.0759328	0.185859
Constant	57.6041586	0.098813

Effect of marking

The isolated marked group (N=20) was significantly placed (t=-2.066, P=0.048) with a higher probability of being isolated than the isolated model group (N=49). This may be because there were more individuals within the model base whose behaviors seemed as crowded behavior by the model (Figure 6). Marking did not significantly affect the behavior of crowded individuals (t=0.831, P=0.413), and there was a similar trend between the marked individuals (N=20) and the model (N=50) since the model had more individuals (Figure 7).

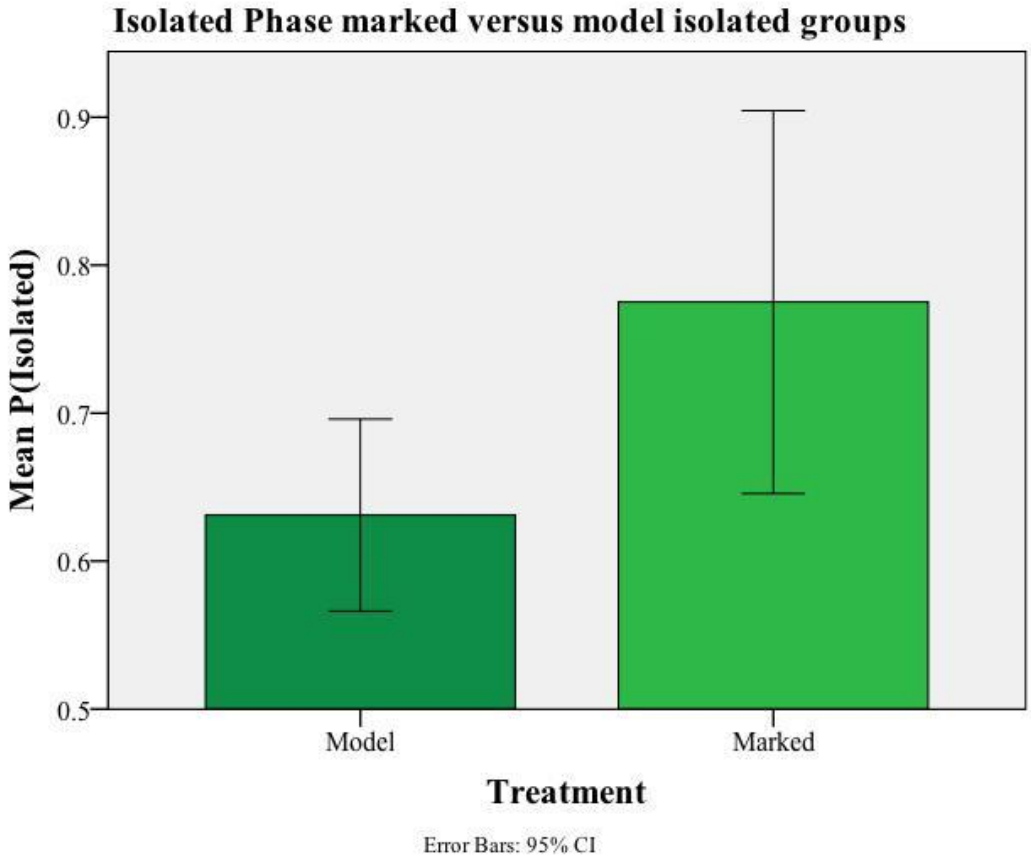

Figure 6: Isolated Condition Marked versus Unmarked
This graph illustrates that marking long-term isolated *S. americana* (N=20) does not significantly alter their behavior compared to long-term isolated *S. americana* (N=49) that were not marked. Pairwise T-test (t=-2.066, P=0.048)

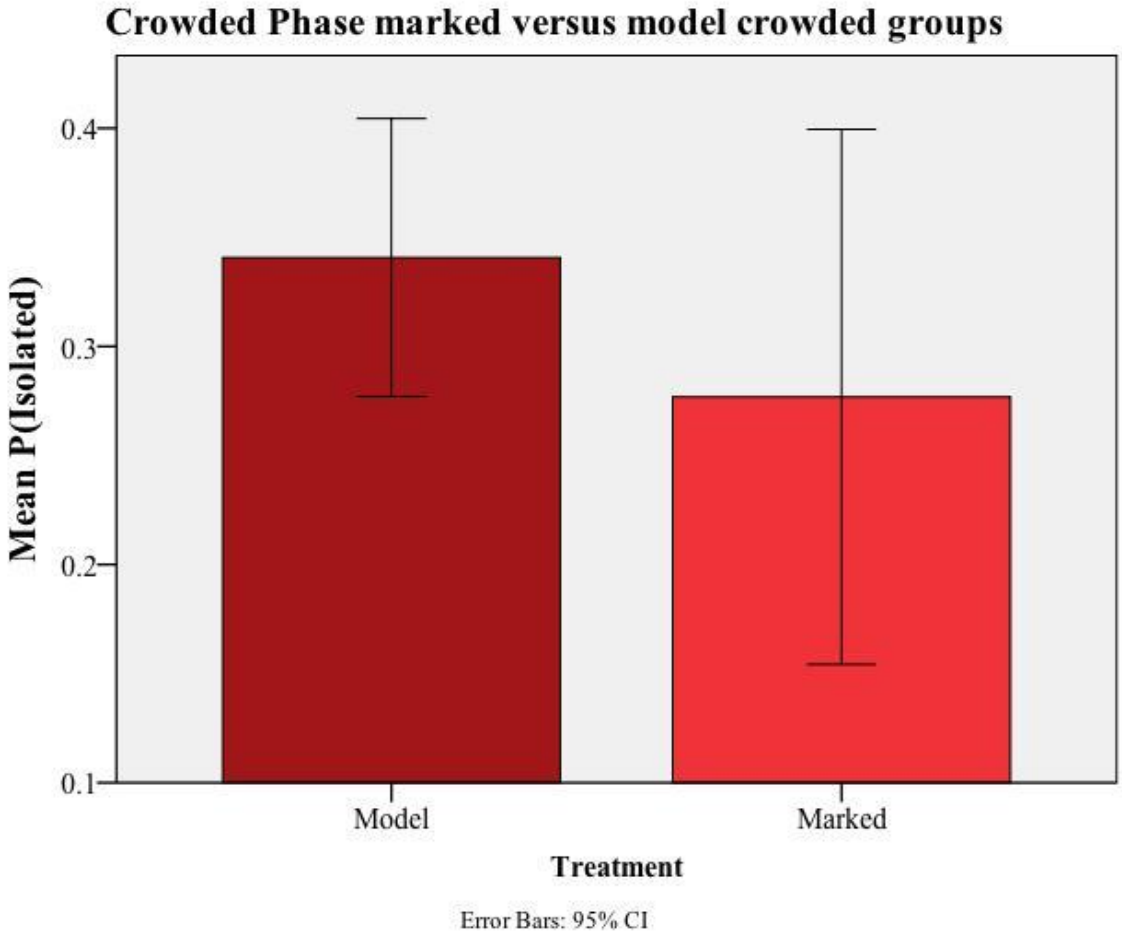

Figure 7: Crowded Marked versus Unmarked

This graph illustrates that marking of long-term crowded *S. americana* (N=20) also does not significantly alter their behavior in comparison to long-term crowded *S. americana* (N=50) that were not marked. Marking crowded individuals prior to isolation was not necessary; this was done to show that marking did not affect the behavior of either density treatment in *S. americana.* Pairwise t-test: (t=0.831, P=0.413)

Time course results

The time course results are split into two groups for clarity. The results for the change in behavior as the isolated condition experiences different durations of crowding is presented in (Table 2) and (Figure 8). Crowding of the isolated condition rapidly changed the behavior from typical isolated behavior to the typical crowding behavior after 1 hour. With longer durations of crowding, the crowded behavior remained and only marginally diminished after 48 hours of crowding. Dunnett test showed that each treatment group expressed behavior significantly different from the long-term isolated control group (0) (Table 2).

Table 2: Dunnett Test results between the isolated condition model and each treatment of isolated conditioned *S. americana* exposed to crowding for different durations.
Equal variances are not assumed since the model (0) has a larger sample size (N=70). This test accommodates the comparison of multiple treatments to one control and all treatments are found to be significantly different from the control except for after 48 hours of crowding**.**

Treatment: Hours Crowded	Mean Difference (P(Isolated) from control group 0 (N=70)	Std. Error	Sig.
1 (N=29)	0.4336	0.0675	0.000
2 (N=30)	0.5325	0.0416	0.000
4 (N=30)	0.4062	0.0667	0.000
6 (N=30)	0.3082	0.0675	0.002
8 (N=22)	0.3726	0.0824	0.005
10 (N=20)	0.4877	0.0586	0.000
12 (N=20)	0.3971	0.0781	0.001
24 (N=25)	0.4158	0.0628	0.000
48 (N=29)	0.1456	0.0743	0.870

There was an effect after 6 hours of crowding on the isolated condition that appeared to diminish the crowded effect, but more crowded behavior was expressed after 10 hours of crowding and did not seem to diminish again until after 48 hours of crowding.

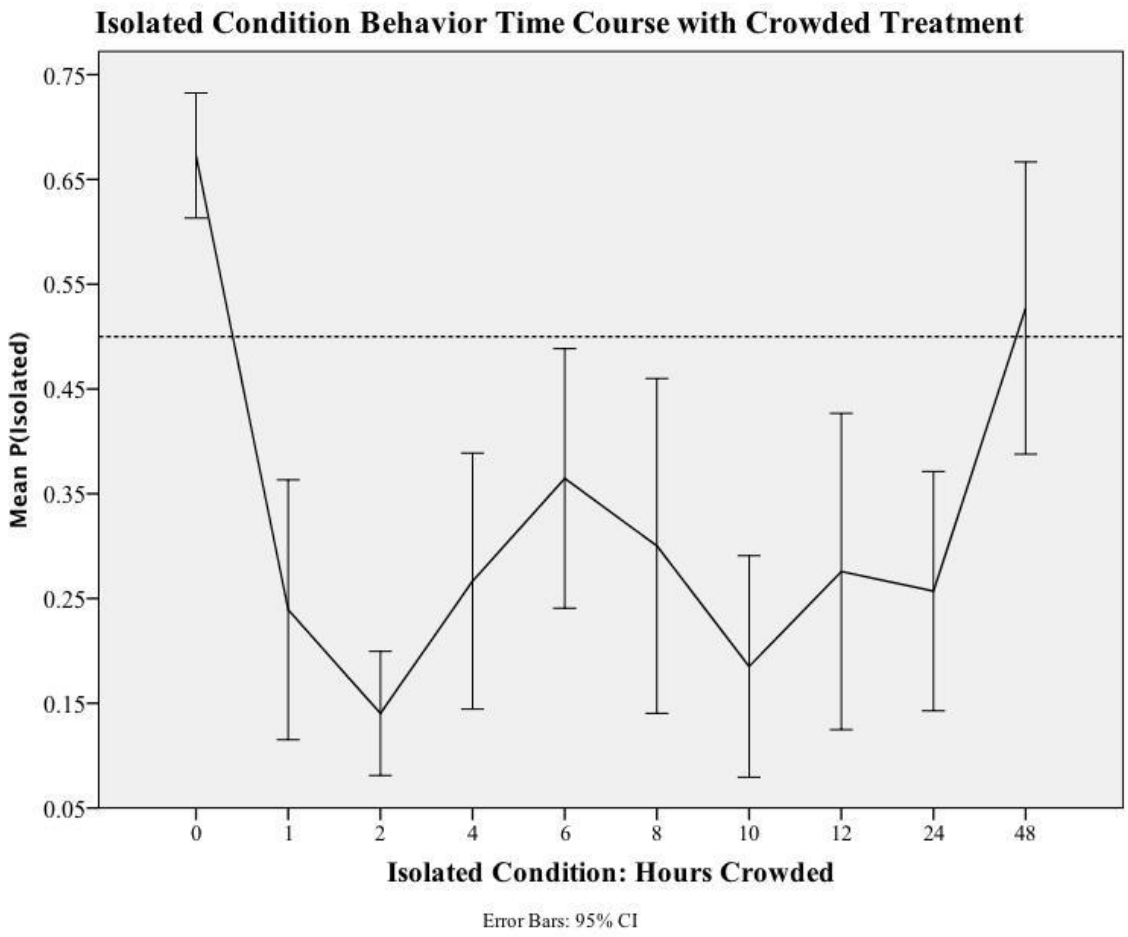

Figure 8: Isolated Condition Behavior Time Course
The y-axis represents the probability of isolated behavior with being over 0.5 (the dashed line) signifies expression of isolated behavior and below 0.5 signifies expression of crowded behavior based on the model shown as 0 on the x-axis. The hours on the x-axis represent the duration of crowded treatment to the isolated condition. This graph illustrates that behavior rapidly changed after the isolated condition was crowded.

The same pattern was not evident as the crowded condition was isolated for different durations (Table 3, Figure 9). None of the treatments of hours the crowded condition was isolated were significantly different from the long-term crowded condition that was never isolated. Although, the typical crowded behavior did seem to diminish after 48 hours of isolation.

Table 3: Dunnett test results between the crowded condition model and each treatment of crowded conditioned *S. americana* isolated for different durations
Equal variances are not assumed since the model (0) has a larger sample size (N=70)

Treatment: Hours Crowded	Mean Difference (P(Isolated) from control group 0 (N=70)	Std. Error	Sig.
1 (N=29)	0.3151	0.0886	0.065
2 (N=19)	0.1226	0.1279	1.000
4 (N=8)	0.1350	0.0641	0.746
6 (N=10)	0.1787	0.1076	0.942
8 (N=10)	0.2920	0.1346	0.709
10 (N=28)	0.1130	0.0729	0.989
12 (N=34	0.0070	0.0496	1.000
24 (N=28)	0.1051	0.0671	0.989
48 (N=23)	0.1903	0.0776	0.511

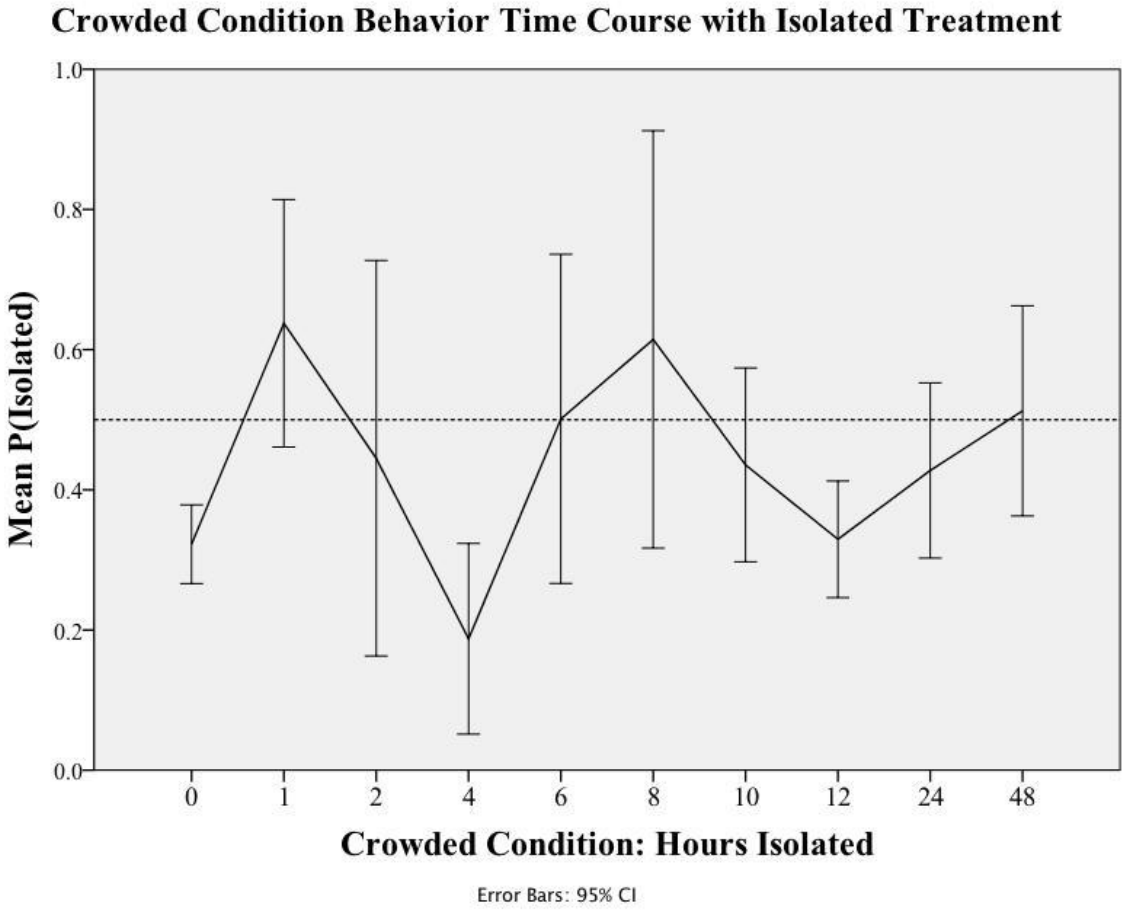

Figure 9: Crowded Condition Behavior Time Course
The y-axis represents the probability of isolated behavior with being over 0.5 (the dashed line) signifies expression of isolated behavior and below 0.5 signifies expression of crowded behavior based on the model shown as 0 on the x-axis. The hours on the x-axis represent the duration of isolation treatment to the crowded condition. This graph illustrates that behavior did not readily change as the crowded condition was isolated.

Discussion

The study of phenotypic plasticity is one of the final frontiers in evolutionary biology because it encompasses how genes can express different phenotypes in response to drastically different environmental conditions. Further testing of reaction norms at work and genetic differences can lead to more insights into the interplay between nature and nurture, and can eventually lead to modeling of how genes and thus, phenotypic traits, respond to the environment.

As discussed by Song (2005), the evolution of phenotypic plasticity in Acrididae (locust phase polyphenism in locusts and density-dependent phenotypic plasticity in non-swarming grasshoppers) needs to be studied from the perspective of reaction norms as well as the phylogenetic perspective. If the reaction norms are shared within a monophyletic group, it can be assumed that these traits are derived from a common ancestor. Furthermore, phenotypic plasticity is typically a complex syndrome that consists of numerous plastic reaction norms (Song and Wenzel, 2008), and it is often difficult to study it comparatively due to the difficulty in quantifying expression in plastic traits to obtain the needed data across many species (Pigliucci, 2001). Nevertheless, having a comparative perspective can lead to valuable insights.

In this study, *S. americana* is closely related to the swarming desert locust, *S. gregaria. S. americana* responds to change in density in a similar manner to *S. gregaria* in terms of behavior, color, and morphology (Gotham and Song, 2013). But, unlike *S. gregaria,* the behavioral time course has been unknown until this study. The time course must be known in order to be able to tie in any other physiology studies that occur with

the change in behavior. The understanding of the dynamics in behavioral phase change is a prerequisite for successful investigation of underlying physiological mechanisms (Roessingh et al., 1993a). In investigating these differences between swarming and non-swarming species in this manner, it may be possible to reveal how locust phase polyphenism has evolved.

In the time course of behavioral gregarization of *S. americana*, it is clear to see that the behavior rapidly changed to typical crowded behavior after one hour of crowding and longer durations of crowding maintained the crowded behavior (Table 2, Figure 8). There are interesting dips at 2 and 10 hours of crowded for the isolated condition, but this may be an artifact of low sample size. For this study, regarding when isolated nymphs are crowded, the evidence supports the rejection of the null hypothesis and agrees with the alternative hypothesis in which rearing density does influence crowding behavior (Figure 8). My second prediction is supported in that the behavior of *S. americana* changes over time after being crowded but in a different manner as seen in *S. gregaria.*

With respect to the behavioral solitarization, I cannot reject the null hypothesis since all durations of isolation do not have a significant difference in behavior from the model long-term crowded individuals that have not been isolated (Table 3). Nevertheless, it does appear that the crowded nymphs express less crowded behavior with longer periods of isolation. It is possible that it may require isolation earlier in development in order to achieve the typical isolated behavior. It is also interesting that after 1 hour of isolation the behavior shifted towards typical isolated behavior, but not significantly enough to be different from the long-term crowded behavior. I suspect this may be from an initial shock of going from being reared in a high-density environment, then being

suddenly isolated, and then being exposed to the behavior arena. It would be analogous to taking an urban person from a large city and then placing them in the middle of nowhere and observing their behavior response, which could be a very shocking experience for anyone. It is evident from this time course study that the transition in behavior from isolated to crowded is more rapid than from crowded to isolated behavior.

These trends in the behavior time course are somewhat similar to the findings of Roessingh and Simpson (1994) in their study of the behavior time course of *S. gregaria.* They also found in *S. gregaria* that the behavior of solitarious locusts significantly changed to the gregarious behavior after one hour of gregarization and there was a maximal effect after 4 hours of gregarization. In *S. americana,* I was unable to determine a maximal effect of crowding due to the higher variance in behavior of the species. It would require a much larger sample size for each time group in order to fully resolve when the maximal crowded effect takes place after the isolated condition of *S. americana* has been crowded. Also, for *S. americana,* the variance is much larger in behavior than it is with *S. gregaria.* Another difference between these studies is that with *S. americana,* the behavior peaks towards isolation at 6 hours of crowding than dips again and does not reach near isolation (P(Isolation)=0.5) until after 48 hours, whereas with *S. gregaria* the behavior reached near isolation after 12 hours. In this study, the crowded condition of *S. americana* did not reach near isolation until after 48 hours. For the behavioral time course of the gregarious phase of *S. gregaria,* the locusts only had near isolated behavior after 24 hours of isolation, but it did not reach 0.5 for the probability of being solitarious. There does not appear to be any significant difference between the behavior time course of the crowded condition being isolated between *S. americana* and *S. gregaria*. This may

be because it is equally difficult in both species to retain isolated behavior after long durations of crowding such as multiple developmental instars. I think that the ability to rapidly gregarize is fundamental and highly conserved in *S. americana* and possibly all species in Cyrtacanthacridinae that retain expression of density-dependent phenotypic plasticity in order to be able to aggregate and increase their survival if the environment changes enough to cause the isolated condition to have lower fitness.

These results are interesting because *S. americana* is not a swarming species and yet it appears to follow a similar trend of gregarization and solitarization as *S. gregaria*. Originally I suspected that it would take a longer duration of crowding for *S. americana* to express crowded behavior and I predicted that the crowded phase of *S. americana* would express isolated behavior with less hours of crowding. This is because as Sword (2003) has shown, the difference in behavior between treatment conditions of *S. americana* has less magnitude than the difference in behavior between phases of *S. gregaria*. The mechanisms responsible for rapid gregarization may be more conserved in *S. americana* than attributes of behavior itself in the species.

There are some improvements that could benefit this work. Primarily, since behavior between treatment conditions in *S. americana* is not as distinct as in *S. gregaria*, a much larger sample size is needed to accommodate the variance in behavior; this may also play a role in why *S. americana* does not swarm. Another aspect was the difficulty in the development of the behavior model for this species. Behavior models developed for the desert locust have extremely large sample sizes on the order of 100 gregarious and 120 solitarious. It may be possible that I would soon have reached the point of diminishing returns in my sample size in building the behavior model from our

population of *S. americana*, but it was beyond the scope of this work to build a stronger model which may have consisted of 200 or more of each condition. Also, this model is the same as was used for *S. gregaria*, therefore, there may be ways to mathematically improve the model to make it more suited for non-swarming species.

From observations alone, there are other *Schistocerca* species that may have behavioral density-dependent phenotypic plasticity, but the signal is too slight to be significant in this model. Behavior is more difficult to test and model because individual behavior is naturally highly variable. But, I was able to capture the expected trend and model it with 78% accuracy. Out of the 70 individuals from each phase, approximately 15 would express the opposite behavior as expected from their treatment merely by chance. Furthermore, any flaw in the model's accuracy further increases the variance in predictions for each group. Lastley, it was not tested in this work, but was assumed that further hours of crowding will only reinforce the crowded behavior. Also, it was not tested in this study what duration of isolation were required after individuals were crowded for different durations to once again express the isolated behavior as has been tested in the desert locust (Roessingh and Simpson, 1994). In conclusion, despite these possible pitfalls, this work does verify the nature of the behavioral time course for *S. americana* successfully.

CHAPTER III: DIFFERENTIAL GENE EXPRESSION PATTERNS RELATED TO DENSITY-DEPENDENT BEHAVIORAL PLASTICITY OF THE AMERICAN BIRDWING GRASSHOPPER

Introduction

Understanding the molecular basis of locust phase polyphenism is considered the final frontier in locust research (Pener and Simpson, 2009). Although many studies have been able to tease apart apparent external differences between phases (Applebaum, 1997; Hassanali et al., 2005; Kang et al., 2004; Pener, 1991; Pener and Simpson, 2009; Roessingh and Simpson, 1994; Simpson et al., 1999; Simpson and Miller, 2007; Simpson et al., 2002; Sword, 2008; Tanaka, 2001, 2006; Verlinden et al., 2009), very little is known about what changes are taking place between these phases at the molecular level. Considering the fact that density has a major effect on phenotypic expression in locusts, developmental plasticity (including behavioral plasticity of locust phase polyphenism) must have a molecular basis. So far, most of the published work on the molecular aspect of density-dependent phenotypic plasticity has largely focused on the desert locust (*S. gregaria*), the migratory locust (*Locusta migratoria*), and the Australian plague locust (*Chortoicetes terminifera*) to a lesser degree. Rather than exploring the functional genetics of locust phase polyphenism, most of these studies have focused on describing molecules that are differentially expressed with rearing density (Pener and Simpson, 2009). Below, I provide a brief review of what we know about the molecular aspects of locust phase polyphenism.

The earliest study was by Colgan (1987), who studied isozyme markers of phase in the haemolymph of *L. migratoria* nymphs and found differences between solitary and crowd reared nymphs in levels of two families of glycolytic enzymes. Wedekind-Hirschberger et al. (1999) also looked at haemolymph between phases using electrophorese to generate polypeptide maps and found 20 peptides to be displayed differentially. Clynen et al. (2002) used HPLC and MALDI-TOF mass spectrometry to compare peptide profiles between phases in the haemolymph and found a peak that was only in the solitarious phase as well as quantitative differences between phases in other peaks that had higher concentrations in the gregarious haemolymph. Rahman et al. (2002) continued this work and found different phase related patterns in the peptide profiles, but also found similar peaks, confirming the work of Clynen et al. (2002).

Differences have also been uncovered in a family of serine protease inhibitors, the pacifastins, found in the blood and central nervous system (CNS) of arthropods (Pener and Simpson, 2009). 8 pacifastin-like precursors encoding 22 different peptides have been identified in locusts (Hamdaoui et al., 1998; Simonet et al., 2005; Simonet et al., 2002a; Simonet et al., 2004; Simonet et al., 2002b; Simonet et al., 2002c; Vanden Broeck et al., 1998). Pacifastins inhibit the PO-activating system in *L. migratoria* (Boigegrain et al., 1992; Brehélin et al., 1991). Therefore, higher levels of pacifastins correspond with reduced immuno-competence in solitarious locusts (Pener and Simpson, 2009). Wilson et al. (2002) found higher survival in gregarious *S. gregaria* after topical application of entomopathogenic fungus *Metarhizium anisopliae* var. *acridum*, but did not find a difference in phenoloxidase activity even though gregarious locusts showed higher antibacterial activity. Both phases have similar behavioral fever responses. Breugelmans

et al. (2008) found higher levels of SGPP 2 and SGPP 4, pacifastin-like peptide precursors, transcripts in gregarious insects.

Another interesting study has focused on the expressions of heat shock proteins (Hsps), which help protect organisms from environmental stressors such as temperature, nutritional, and immune stress by being 'chaperones' for other proteins to help them maintain their folding state and function (Pener and Simpson, 2009). Higher copies of all Hsp gene families were found in the gregarious phase of *L. migratoria* (Kang et al., 2004). It is reasonable that Hsp genes have a higher expression in the gregarious phase due to increased competition and stress of a high-density environment, but Hsp, 90 in particular, may have properties that may be more relevant to the evolution of phase polyphenism (Queitsch et al., 2002; Rutherford and Lindquist, 1998; Williams et al., 2009). Hsp 90 are signal transducers involved in the regulation of cell-cycle and development, which may have the capacity to orchestrate biochemical elements involved in the induction and maintenance of phase polyphenism (Pener and Simpson, 2009). It is also thought that such rapid changes in the change from the solitarious phase to the gregarious phase are unlikely as a result from gene expression differences alone, but may be mediated by neuromodulation of existing neural circuitry (Rogers et al., 2004; Simpson et al., 1999).

In terms of neuro-chemicals, Rogers et al. (2004) compared various neurotransmitters and neuromodulators in the CNSs of solitarious and gregarious *S. gregaria* during behavioral phase transition. They used HPLC to quantify the levels of these molecules (aspartate, glutamine, glycine, GABA, arginine, taurine, acetylcholine, tyramine, citrulline, dopamine, serotonin, octopamine, and *N*-acetyldopamine) between

phases, and the most notable finding was that serotonin increased by nine fold during the 4-hour period as solitarious nymphs were crowded. Many of these studies have shown some insights on what changes are taking place as the phase state is changing, but there is still much to discover in order to start to put the pieces together. In order to gain a larger picture of locust phase polyphenism, there have been several efforts in sequencing a portion of the locust genome.

Sequencing a grasshopper genome is a daunting challenge compared to other model organisms. *S. gregaria* has a genome that has a mass of 9 picograms of haploid DNA content compared to the fruit fly, *D. melanogaster*, having a genome of 0.18pg, and the human genome is 3.5pg (Gregory, 2012). Sequencing the genome of a grasshopper thus, takes nearly three times the effort as the recent undertakings in sequencing the human genome. Biologists have found a way to still explore the genetic content of grasshoppers by sequencing transcriptomes. A transcriptome is a large sample of mRNA's that are being expressed by a specific organism's specific tissue at a specific developmental stage. For example, the transcriptome from antennae of an adult grasshopper would be different from the hind femur of a 4th instar nymph. Kang et al. (2004) were able to establish an expressed sequence tag (EST) library and database for *L. migratoria*. They used the whole body, samples of the head, hind legs, and the gut to derive the cDNA libraries for the database. They used this information to develop a public transcriptomic database (http://www.locustdb.genomics.cn.org) (Ma et al., 2006). Recently, the transcriptome for *S. gregaria* has been constructed (Badisco et al., 2011a). This study reported 12,709 unique transcripts and nearly 4,000 functionally annotated genes. Based on a microarray analysis, Badisco et al. (2011b) characterized 214

differentially expressed genes from the central nervous system between the two phases, and found that solitarious individuals up-regulated genes related to anti-oxidant systems, detoxification, and anabolic renewal, while gregarious individuals had more transcripts related to sensory processing and coping with stress and infection. With these transcriptomes, more genes can be revealed for further investigation.

More recent studies have used quantitative PCR and RNA interference to understand the functional genetics of a few candidate genes for locust phase polyphenism (Ott et al., 2011; Yang, 2014). Rahman et al. (2003) used qRT-PCR to identify genes that were differentially expressed between the solitarious and gregarious state of *S. gregaria*. Recently, in *S. gregaria*, protein kinase A (PKA) has been shown to be instrumental in phase transition. Ott et al. (2011) showed that PKA is controlled in a cyclic Adenine mono-phosphate (cAMP)-dependent equilibrium and when PKA is inhibited, the behavioral phase state of the solitarious locust does not change to gregarious after being crowded for an hour. It has also been shown in *S. gregaria* that when serotonin alone is topically applied to the thoracic ganglia, it can change the behavioral phase state of a solitarious grasshopper to the gregarious state (Anstey et al., 2009). Chapuis (2011) found heat shock proteins to be over-expressed with crowding in the Australian plague locust, *Chortoicetes terminifera*. Yang (2014) used microRNA to inhibit behavioral aggregation by lowering gene expression in two critical regulatory genes involved in dopamine synthesis in *Locusta migratoria*, the migratory locust.

My work is built upon these previous findings, but with an important distinction. *Schistocerca americana* is a non-swarming grasshopper, but is capable of expressing DDPP, reminiscent of the desert locust (Gotham and Song, 2013). I have also shown in

the Chapter 2 that the behavioral gregarization and solitarization in *S. americana* is somewhat similar to that of the desert locust. Does *S. americana* then have the same molecular basis for its DDPP despite the fact that it is non-swarming? I suspect that, like *S. gregaria*, *S. americana* will have similar molecular differences between phases because it shares a similar expression of DDPP as *S. gregaria*. But since the differences for *S. americana* are not as extreme as that of *S. gregaria*, there are most likely differences in how much the genes may be expressed.

In this Chapter, I use quantitative real-time PCR (qRT-PCR) to quantify the expression levels of three candidate genes that appear to be involved in DDPP in *S. americana*. The selection of the candidate genes was informed by previous work on behavioral gregarization in *S. gregaria*. Specifically, I searched for behavior related genes that may be differentially expressed between phases because the behavioral phase state is the first aspect to change from crowding or isolation. First, I chose to test the gene expression of protein kinase A (PKA), since PKA was found to be required for the behavioral phase state to change in the desert locust (Ott et al., 2011). Serotonin plays a significant role in the transition of the behavioral phase state (Anstey et al., 2009; Rogers et al., 2004). The synthesis of serotonin is a two-step process, in which the precursor L-Tryptophan is converted into 5-Hydroxy-L-Tryptophan by a rate-limiting enzyme L-Tryptophan-5-monooxygenase (T-5), which is then converted into serotonin by Aromatic L-amino acid decarboxylase (Decarb)(Figure 10).

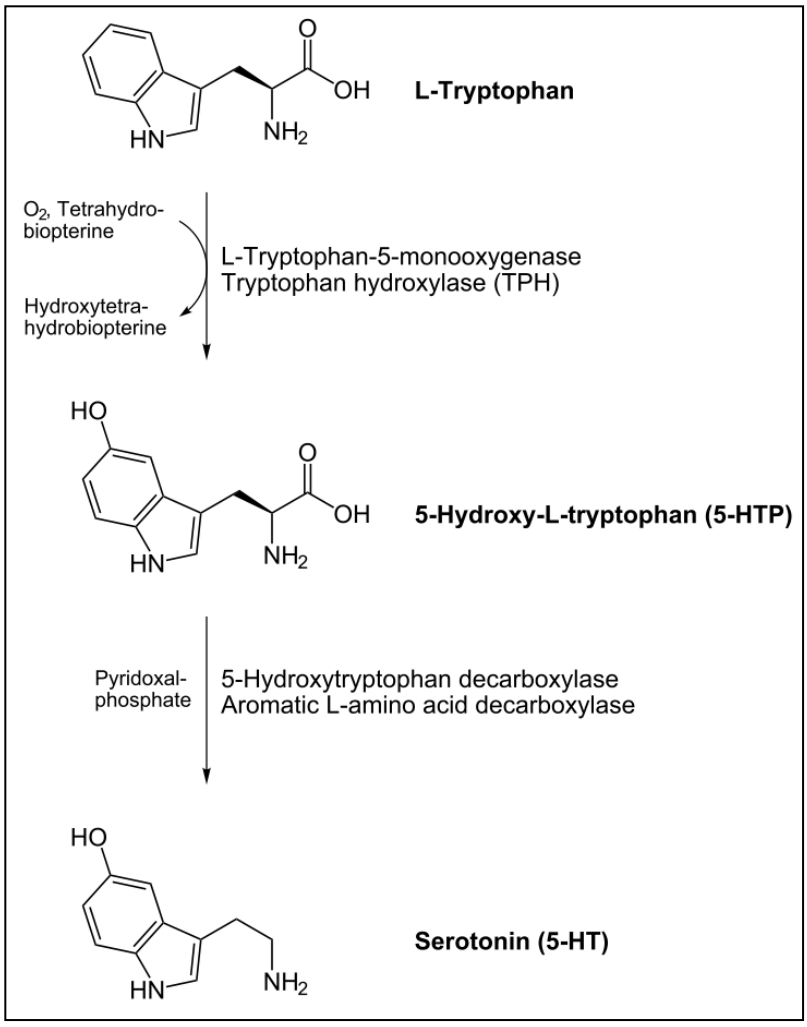

Figure 10: Serotonin Synthesis Pathway
This figure displays the initial components and enzymes that produce the final product, Serotonin. This study focuses on gene expression of the enzymes that catalyze the reaction. Previous work has only quantified the amount of the serotonin chemical in the solitarious and gregarious phase of *S. gregaria.* Figure Credit: Hojun Song

Thus, I also chose to explore the expression levels of these two enzymes involved in serotonin synthesis. Using qRT-PCR, I studied the differential gene expression patterns of these three genes in both long-term isolated and long-term crowded *S. americana*, as well as throughout the duration of behavioral gregarization at selected intervals.

Specifically, I test the following hypotheses in this Chapter:

H_0: Crowding does not influence the gene expression of PKA, T-5, and/or Decarb in *S. americana*.

H_1: Crowding influences the gene expression of PKA, T-5, and/or Decarb in *S. americana*.

Prediction 1: PKA is up-regulated, but T-5 and Decarb are down-regulated in the long-term crowded condition of *S. americana* compared to the long-term isolated condition.

Prediction 2: Expression of PKA, T-5, and Decarb increases as the duration of crowding increases.

Materials and Methods

RNA Extraction

For this study, I extracted RNA from the specimens used in the time-course study described in Chapter 2. To preserve RNA intact, the whole specimens of crowded and isolated 6th instar *S. americana* were stored in Ambion RNAlater® Solution and kept at 4°C overnight and the samples were then transferred to a freezer maintained at -20°C until dissection. The samples were dissected under a light microscope in a petri dish half filled

with RNAlater. To extract ganglia tissue as efficiently as possible, the thorax was opened from the ventral side where the thoracic ganglia rest against the thoracic plate. Then the head was opened to remove the optic lobes. Fat body tissues and gut tissues were avoided as much as possible. The extracted tissue sample was placed in 400µl of RNAlater solution and placed back into -20°C until RNA extraction became possible. In order to gain a high RNA concentration yield, I used the Qiagen RNeasy® Plus Mini Kit following the manufacture's protocol. I added β-mercaptoethanol to Buffer RLT as directed in the kit. For thorough homogenization of tissues, I used the Qiagen TissueRuptor®. After extraction, the RNA and DNA concentration of the sample was quantified using Qubit® 2.0 Fluorometer. At this step, RNA extractions were stored at -80°C until they were reverse transcribed. Subsequently, Ambion® TURBO DNA-free™ was used as directed to minimize DNA content in the RNA samples. RNA was reverse transcribed into cDNA using BIO-RAD iScript™ cDNA Synthesis Kit as directed as the resulting cDNA samples were stored at -20°C until they were ready for qRT-PCR.

Identification of candidate genes

A *de novo* assembled transcriptome from head tissues of *S. americana* was generated as a part of the ongoing project in Dr. Hojun Song's lab at the University of Central Florida. From a list of annotated transcripts from *S. americana* transcriptome, I obtained transcripts identified as protein kinase A (PKA), L-Tryptophan-5-monooxygenase (T-5), and Aromatic L-amino acid decarboxylase (Decarb). I searched for efficient primer sites from these transcripts and designed species-specific primers. As for a housekeeping gene, I used β-actin, based on reference housekeeping genes used for

S. gregaria, because it had the least variance between samples and phase difference (Van Hiel, 2009) (Table 4).

Table 4: Primer sequences
B-actin is used as the reference gene because it has equal expression in both the isolated and crowded condition. These are the optimal primer sequences for the target genes of this study.

Primer	Forward	Reverse
β-actin	AATTACCATTGGTAACGAGCGATT	TGCTTCCATACCCAGGAATGA
PKA	TGCTCCCCTTCCAACAACAA	TTCTTCCCCTGCCACTGTTC
T-5	GGTACAACTTGGTCAGCTC	GCCGATATTGCCTACAACTA
Decarb	AGGACAACCGTTTCGAGGTC	CCGAAGCATTTACAGCTGCC

qRT-PCR

The primers were optimized to be run on the same plate at 48° C. The efficiency I was able to obtain for the following primers were: (i) β-actin=0.996; (ii) PKA=0.964; (iii) T-5=0.984; (iv) Decarb=1.007. qRT-PCR was conducted using SsoAdvanced™ Universal SYBR® Green Supermix. All four genes were ran on each plate with the following PCR profile: 1 cycle of 95° C for 3 minutes followed by 40 cycles at 95° C for 15 seconds then 48°C for 30 seconds. qRT-PCR gives the Δct or number of cycles it takes for amplification to reach a readable threshold. I analyzed the resulting data using SPSS and REST 2009 (Pfaffl et al., 2002). REST is a program that analyzes gene expression data from qRT-PCR using a formula that runs iterations and implements efficiency of the primers into the calculation. The program calculated the expression ration as the summation of target gene to the power of the delta ct of the target gene times the control minus the treatment divided by the summation of the reference gene to the

power of the delta ct of the reference gene times the control minus the treatment. The equation was written out as $R = ((E_{target})^{\Delta CTtarget(control-treatment)})/((E_{ref})^{\Delta CTref(control-treatment)})$. In this study, the control was long-term isolated condition of *S. americana* and the treatments were the long-term crowded condition (LC), the isolated condition crowded for 2 hours (IC2), the isolated condition crowded for 10 hours (IC10), and the isolated condition crowded for 24 hours (IC24).

Results

Long-Term Phase Difference

Table 5 is the resulting output from REST 2009. P(H1) is the probability that the treatment group, crowded samples in this study, is significantly different from the control group, isolated samples. T-5 was down-regulated from the control group indicating that L-Tryptophan-5-monooxygenase is more expressed in the isolated phase than the crowded phase. PKA and Decarb were not found to be significantly different in gene expression between phases.

Table 5: qRT-PCR Results between Long-Term Crowded and Isolated
The only significant finding between the long-term conditions was that T-5 is down-regulated in the crowded condition compared to the isolated condition. This suggests higher gene expression of T-5, and thus serotonin levels, in the long-term isolated condition of *S. americana.* (Control: Long-term Isolated N=13, Treatment: Long-term Crowded N=13)

Gene	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
β-actin	0.996	1.000				
PKA	0.964	1.661	0.012-437.735	0.001-5,736.498	0.701	
T-5	1.0	0.295	0.048-1.554	0.011-19.469	0.043	DOWN
Decarb	1.0	0.659	0.174-2.667	0.069-9.462	0.355	

Gene Expression Changes Over Time-Course

After verifying the difference in gene expression between phases in the long term sample, I also quantified the difference in gene expression between phases in each of the time groups from the behavior time-course to observe gene expression change with behavior phase state change.

Table 6: qRT-PCR Results for Isolated Crowded for 2 Hours vs. Long-Term Isolated

There were no significant differences in gene expression between the long-term isolated condition and after the isolated condition has been crowded for 2 hours. (Control: Long-term Isolated N=13, Treatment: Isolated crowded for 2 hrs N=5)

Gene	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
β-actin	0.996	1.000				
PKA	0.964	1.724	0.111-5.759	0.055-8.195	0.601	
T-5	1.0	0.649	0.306-2.548	0.054-5.762	0.546	
Decarb	1.0	0.769	0.158-4.707	0.019-60.648	0.680	

Isolated conditioned grasshoppers that were crowded for only 2 hours did not have a change in gene expression compared to long-term isolated grasshoppers. There was an increase in gene expression of the three genes compared to the long-term crowded condition (Table 6).

Table 7: qRT-PCR Results for Isolated Crowded for 10 Hours vrs Long-Term Isolated

T-5 and Decarb were both up-regulated compared to the long-term isolated condition after the isolated condition had been crowded for 10 hours. This suggests that serotonin levels are increasing as the isolated are being crowded. (Control: Long-term Isolated N=13, Treatment: Isolated crowded for 10 hrs N=5)

Gene	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
β-actin	0.996	1.000				
PKA	0.964	0.864	0.026-51.433	0.055-8.195	0.601	
T-5	1.0	3.461	1.189-12.033	0.411-24.702	0.048	UP
Decarb	1.0	7.172	1.965-26.715	0.838-89.364	0.029	UP

After 10 hours of the isolation condition being crowded, T-5 and Decarb were up-regulated compared to the long-term isolated condition control group. This was a rapid difference in gene expression for the enzymes of the serotonin pathway compared to 2 hours being crowded. PKA dropped back down to equivalent gene expression with both long-term crowded and isolated conditions with a large variance (Table 7).

Table 8: qRT-PCR Results for Isolated Crowded for 24 Hours vs. Long-Term Isolated

After the isolated condition had been crowded for 24 hours it returned to gene expression levels that were near equivalent with the long-term isolated condition. (Control: Long-term Isolated N=13, Treatment: Isolated crowded for 2 hrs N=5,except for PKA, N=3)

Gene	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
β-actin	0.996	1.000				
PKA	0.964	0.207	0.004-6.339	0.003-425.606	0.704	
T-5	1.0	1.137	0.320-5.739	0.034-17.772	0.875	
Decarb	1.0	0.836	0.189-4.603	0.056-18.098	0.880	

After 24 hours of the isolated condition being crowded, PKA had a lower expression, but not significantly different from the long-term isolated condition control. T-5 and Decarb also decreased in gene expression and were no longer significantly different from the control. Decarb had a larger jump in expression after 10 hours of

crowding and a steeper depression of expression after 24 hours of crowding than seen in T-5 (Table 8, Figure 12)

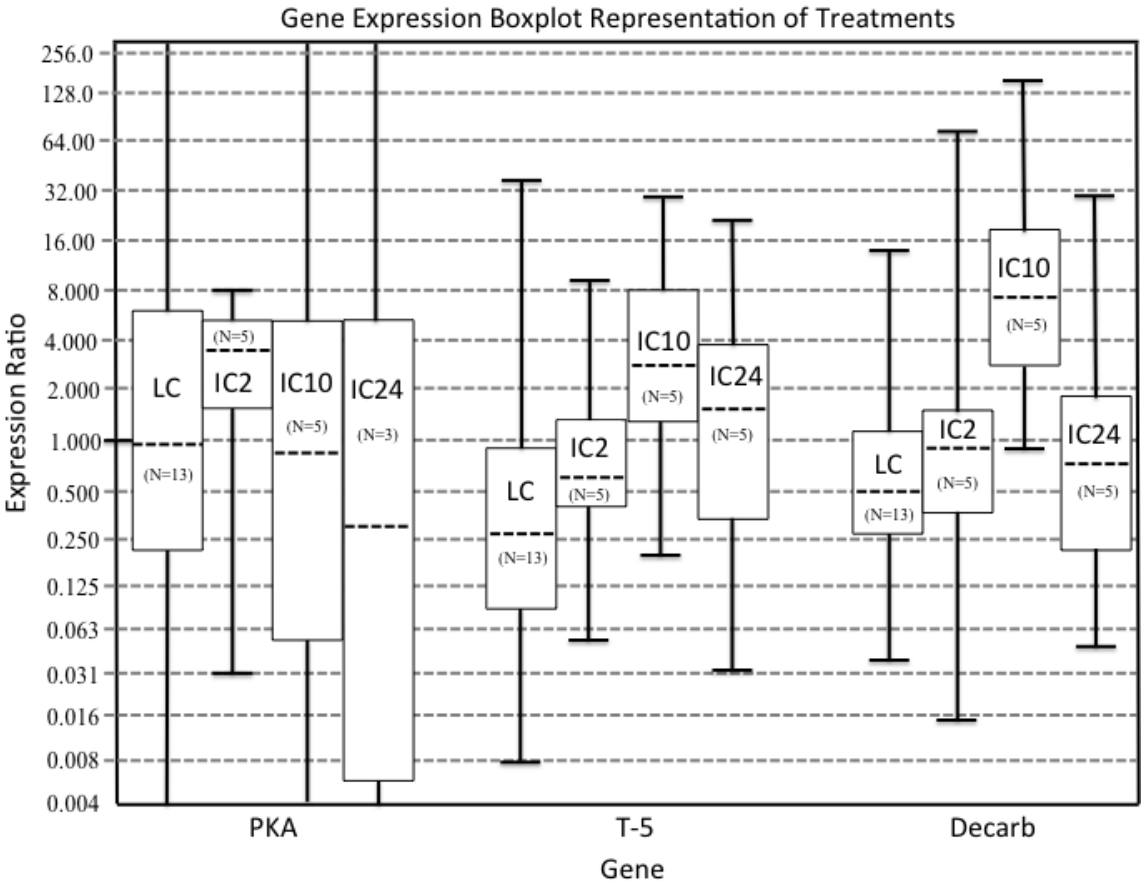

Figure 11: Gene Expression Boxplot of Treatments
Relative expression of genes with the treatment (LC-long-term crowded, IC2-Isolated condition crowded for 2 hours, IC10-Isolated condition crowded for 10 hours, and IC24-Isolated condition crowded for 24 hours) vs. the control long-term isolated condition. This boxplot illustrates the expression ratio for each treatment for each gene. The treatments are not comparable to each other because they are all being compared to the long-term isolated condition. Below and expression ratio of 1.000 signifies higher gene expression in the long-term isolated condition while above 1.000 signifies higher expression in the given treatment. The graph illustrates the high variance in PKA as well as the rise and fall in gene expression for T-5 and Decarb as the isolated condition is crowded over time.

The expression ratio for the genes of interest plotted with the treatments against the control, being long-term isolated condition grasshoppers are illustrated in Figure 12.

In this plot, below 1.000 on the y-axis represents a higher expression of the gene on the x-axis in the long-term isolated condition. Above 1.000 on the y-axis is a higher expression in a given treatment. The four treatments I conducted were long-term crowded individuals denoted as LC, isolated conditioned individuals that were crowded for 2 hours denoted as IC2, the same manner crowded for 10 hours as IC10, and crowded for 24 hours as IC24. Although, there was not a significant difference for the IC2 treatments against the control, it appeared that PKA gene expression had slightly increased after 2 hours of crowding. There was also more expression of T-5 in the IC2 treatment compared to the long-term crowded condition. A similar trend is apparent for Decarb for IC2. Gene Expression for PKA became insignificant after 10 and 24 hours. T5 and Decarb increased in gene expression after 10 hours and decreased after 24 hours.

Discussion

Significantly, T-5 had a higher expression in the long-term isolated condition in *S. americana* compared to the long-term crowded condition of *S. americana* (Table 5, Figure 11). This was reasonable given that T-5 is the first enzyme in the rate-limiting step of serotonin synthesis and Rogers et al. (2004) also found serotonin to have a more concentrated presence in the long-term solitarious phase of *S. gregaria* compared to the gregarious phase. After the isolated condition has been crowded for 2 hours, expression of all three genes also increased, but only PKA showed a higher expression in IC2 versus the long-term isolated condition. After 10 and 24 hours of crowding PKA dropped in gene expression and the variance dramatically increased. Anstey et al. (2009) effectively showed that inhibiting PKA prevented gregarization in the desert locust. But, in this

study, I have only quantified the amount of gene expression of PKA in *S. americana.* PKA may have such a large variation in expression because it serves multiple functions as it phosphorylates ion channels and synaptic proteins of existing neuronal machinery (Anstey et al., 2009). This signaling pathways affects neural plasticity in reflex sensitization, contextual fear conditioning, appetitive and aversive condition, and addiction (Abel and et, 1997; Bernier et al., 1982; Castellucci et al., 1982; Michel et al., 2008; Muller, 2000; Sanchez et al., 2010; Skoulakis et al., 1993). If PKA were to be inhibited in *S. americana* to the degree in which was done with *S. gregaria* (Ott et al., 2011), it is likely there would be a similar result in which an isolated *S. americana* would retain the typical isolated behavior after being crowded for an hour. It was notable that gene expression of T-5 and Decarb increased after 10 hours of crowding and then dropped after 24 hours. This suggested a spike in serotonin synthesis in the onset of crowding that diminished over time. Decarb had a sharper increase in gene expression after 10 hours and steeper decrease after 24 hours than T-5, which may be caused as a larger secondary effect from the increase and decrease of the enzymatic activity of T-5 since it is the first step. This finding in *S. americana* suggested that the expression of serotonin changed as the isolated condition was crowded which was also similar to what has been found with *S. gregaria.* Rogers et al. (2004) tested multiple neurochemicals, including serotonin, in *S. gregaria* as the gregarious phase was isolated and the as the solitarious phase was crowded. He found that serotonin production increased nine fold in the thoracic ganglia of solitarious *S. gregaria* after four hours of crowding, but serotonin decreased in the optic lobes at that time. After 24 hours of crowding, serotonin decreased back to 2 fold in the thoracic ganglia, but serotonin increased by four fold in the optic

lobes. When these locusts' hind femur mechanoreceptors were stimulated, a signal was sent to the thoracic ganglia that increased serotonin production in the thorax (Rogers et al., 2003). It is possible that there was a lag in serotonin production between the thoracic ganglia and the optic lobes. Rogers et al. (2004) concluded that large changes in serotonin are implicated in the early stages of gregarization in the thoracic ganglia, but in the brain during solitarization. This study did not account for the possibility of differences in serotonin production in separate ganglia of *S. americana* since the thoracic ganglia, brain, and optic lobes were dissected and extracted together per individual during the RNA extraction process. It would be interesting to tease this apart in *S. americana* to see if the same trend is seen. This study looked at serotonin indirectly by focusing on mRNA, but our results do suggest that *S. americana* may have a similar neurochemical pattern over the course of change in behavior. Gene expression of T-5 and Decarb may also have had a maximum peak of expression after the isolated condition has been crowded for four hours. *S. americana* would benefit from a similar HPLC study as has been done with *S. gregaria* to see if there is a key chemical difference between non-swarming and swarming species.

CHAPTER IV: GENERAL DISCUSSION AND CONCLUDING REMARKS

In this thesis, I have shown that the behavior of *S. americana* rapidly changes as the isolation condition was crowded, but not as much when the crowded condition was isolated. It may be that when the isolated condition of *S. americana* was exposed to crowding, it is not able to recover typical isolated behavior within the same stadia as noted by Roessingh and Simpson (1994) in the case of *S. gregaria.* I have also observed in *S. americana* that after 6 hours of crowding, the isolated condition had less crowded behavior, but not enough to be significant. This may be a half rate similar to the observation of *S. gregaria* showing less gregarious behavior after 24 hours of crowding, which was assumed because there was less interaction amongst them at scotophase, the dark period of an artificial light cycle, contributing to partial solitarization (Roessingh and Simpson, 1994). From my results, I would argue that there is a possibility of an underlying cyclic nature to the behavior time course of the isolation condition becoming crowded, rather than that loss of visual and physical stimulation during the scotophase is the cause for solitarization, despite being in a high density environment. The only treatment groups from my study that were crowded in the scotophase were the 24 and 48 hour treatment. This left the 6 hour treatment unexplained. It is also evident that the 6 hour treat group is not merely more solitarized by chance because the 4 hour treatment group started to solitarize in that direction and then sloped back down towards more crowded behavior with the 8 hour treatment group. If it were just a chance observation, it would appear more abruptly. I also increased the sample size for the 6 hour treatment

group to decrease the 95% confidence interval. Further study should look at more intervals with a larger sample size for each treatment group as well treatment groups falling between 12, 24, 48, and 60 hours to confirm this cyclic behavior.

I have shown that the nature of PKA during the behavior time course of *S. americana* cannot be determined from quantifying gene expression alone because of the numerous roles PKA takes part in of neural activities. I have also shown that both enzymes, T-5 and Decarb, both increased in expression after 10 hours of crowding and then decreased after 24 hours. This also corresponded with Rogers et al. (2004) findings of the rapid increase of serotonin in the thoracic ganglia and then the brain and optic lobes of the solitarious phase of *S. gregaria* after gregarization. Further HPLC work with *S. americana* may reveal more quantitative plastic traits that can be used to compare to other species of *Schistocerca* in order to gain a more phylogenetic perspective to this change in the balance of neurochemicals during the change in behavior.

Phenotypic plasticity is difficult to study in a phylogenetic framework because all species within the clade of interest need to be tested for the same adaptive plasticity (Song and Wenzel, 2008). The desert locust has undergone many studies concerning its reaction norms to density, but this is one of the few works that has begun to test these same reaction norms on another species within *Schistocerca, S. americana.* If we are to learn how this phenomenon has evolved, than all species within the clade of *Schistocerca* need to have their reaction norms quantified experimentally. Eventually, if more species serve as representatives within the family of Cyrtacanthacridinae, it will become clear how density-dependent phenotypic plasticity and locust phase polyphenism has evolved and been loss throughout the phylogeny (Doughty, 1995). A similar approach is often

taken in order to understand how ecological characters have evolved (Brooks and McLennan, 1991; Harvey and Pagel, 1991; Miller and Wenzel, 1995). The information that can be gleamed from this may inform us on how to better handle swarms, saving billions in crop loss, and to avoid the devastating secondary effects on using such large volumes of pesticide on the environment.

There is evidence that there are changes in gene expression as well as biochemistry with the change in behavior (Anstey et al., 2009; Ott et al., 2011). A curious result from this work is that *S. americana* maintains a similar pattern in how behavior changes as *S. gregaria* even though it is not a swarming species and the difference in behavior is not as extreme as it is in the desert locust. This may suggest that it is important in density-dependent phenotypic plasticity for these species to be able to readily adopt the crowded condition or gregarious phase sweep of characteristics for survival much more than it is necessary to maintain the cryptic coloration and behavior to avoid predation as typically seen with the solitary and isolated condition. Gene expression also seems to concur with this since it is evident that the gene expression does change even with a short amount of crowding. Fundamentally, there must be another reason why *S. americana* does not swarm as *S. gregaria* does. It is suggested that the prominent cause of swarming behavior in the desert locust is due to lack of vegetation in North Africa causing seasonal breeding in the populations there. But in the southeastern United States there is plenty of vegetation, so the incentive for swarming may not be present for *S. americana.* If this is the only reason for a lack of swarming behavior in *S. americana,* than we can be reassured that vegetation in the U.S is still plentiful enough to avoid the locust problems that plaque other countries.

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