THE CONSERVATION OF VARIATION IN *GRYLLODES SIGILLATUS* AND CLOSELY

RELATED CRICKET SPECIES

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Jeremy David Dalos

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota

State University's regulations and meets the accepted standards for the degree of

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SUPERVISORY COMMITTEE:

Ned Dochtermann

Chair

Erin Gillam

Jason Harmon

Approved:

4/9/2020	Kendra Greenlee
Date	Department Chair

ABSTRACT

The ability to adjust behaviors to a particular environment has been well documented across taxa. Our understanding of behavioral plasticity is largely based on experiments in which individuals have a single exposure to an environment. Observed behavioral changes are then traditionally measured in small windows of responsiveness in a single population or species. In this project I investigated the effects of prolonged exposure to predator cues in *Gryllodes sigillatus* and also tested for the presence of trans-generational effects of this prolonged exposure. I found there were no differences in anti-predator behaviors when measured in subsequent assays compared to control individuals. These results were paired with a comparison of differences in average expressions of behaviors, differences in variances, and behavioral correlations of five closely related cricket species, including *G. sigillatus*. Our results showed that species differ in average behaviors and plasticity but did not significantly differ in behavioral correlations.

ACKNOWLEDGEMENTS

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INTRODUCTION

The ability to adjust a behavior to an environment, i.e. behavioral plasticity (Relyea 2001), can have ecological and evolutionary benefits such as increased survival and increased reproductive fitness. These changes are caused by experiencing a certain environment, then altering aspects of behavior to better suit the same or a similar environment (Pallier et al. 1997). The changes involved in behavioral plasticity can be permanent or temporary (Tabashnik et al. 1981, Lynch and Walsh 1998, Piersma and Van Gils 2011). Theory suggests that the better a behavior is suited to an environment, the higher resulting fitness of those individuals displaying that behavior. The genetic components of traits for individuals with high reproductive fitness, e.g. plasticity of a certain trait, will result in higher observed frequencies in subsequent generations. Plastically customizing behaviors to an environment can potentially increase survival and fitness but only if the current environment matches the environment for which the behavior was modified. If an individual plastically changes its behavior to suit one environment, then lives in another or behaviors are genetically conserved to match a previously experienced ancestral environment, this can have negative consequences on survival and fitness (Emlen et al. 1984, Snell-Rood 2012).

Behavioral plasticity can be expressed within a single generation or over multiple generations (Fjeld et al. 1998, Storm and Lima 2010). When a behavior is permanently changed in response to cues of an environment, this is known as developmental or irreversible plasticity. These changes are consistent throughout an individual's entire lifetime or at least the entire time period of measurement (West-Eberhard 2003). For example, Tessier and Leibold (1997) showed that environmental factors affecting morphological size differences in *Daphnia pulicaria* clones are also connected to differences in habitat depth preferences. Daphnia that were smaller at

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maturity utilized shallower depths, which led to lower fecundity when compared to larger clones who preferred larger depths. In comparison to natural populations, daphnia preferring shallower depths were more prone to predation. Larger daphnia are benefited by these developmental effects by being less prone to predation, but only if the predation pressures of that environment stays consistent. Additionally, the daphnia were only able to induce these permanent behavioral changes due to their genetic components interacting with environmental cues.

Behaviors can remain constant for an individual's entire lifespan through irreversible plasticity or genetic components. Based on similar genetic components, populations that are closely related have a shared phylogenetic history and previous adaptations to past environments can cause constraints in the expression of behaviors in the current environment (McKitrick 1993). This contradicts traditional views that behaviors are highly plastic in their response to an environment. This conservation of behavior can be beneficial if the environment remains constant as individuals will not have to experience an environment to be suited to it, potentially avoiding consequences of experiencing the environment, e.g. predation (Albecker and Vance-Chalcraft 2015). Alternatively, these restrictions can limit populations from properly adapting to an environment if that environment differs from the one genetically adapted to. Evidence of this is shown in Royauté et al. (2019b), where different populations of field crickets (*Gryllus integer*) displayed genetically conserved behavioral correlations despite being captured in differing environments. The conservation of behaviors that are not well suited for an environment can limit the effectiveness of natural selection.

Natural selection will favor individuals that have traits best suited for an environment, whether these traits are underpin by genetic components or an interaction with an environment (Hajduk et al. 2020). When traits such as behaviors are matched with an environment, e.g.

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through direct experience or passed on from previous generations, this can be adaptive in nature (Holland 1992). By not having to pay potential costs of learning in an environment, this can increase survival in a stable environment. For example, in Storm and Lima (2010) the authors exposed gravid *Gryllus pennsylvanicus* (fall field crickets) to *Hogna carolinesis* (wolf spiders) with waxed chelicerae to simulate a direct encounter with a predator. The resulting encounter led to offspring of these exposed individuals to display freezing behavior 27% more often when compared to unexposed individuals in subsequent behavioral trials. Furthermore, these exposed crickets exhibited significantly greater longevity and survival probability when exposed to *Hogna* spiders in survival assays. These results matched wild populations that were under natural predation from wolf spiders, when compared to populations without the presence of these predators. This study provided strong evidence that multiple generations can be influenced by environmental conditions, resulting in offspring exhibiting behavioral adaptations without having to directly experience that environment.

In this study I investigated the effects of prolonged exposure to cues of predator presence and potential trans-generational effects on behaviors related to predator avoidance. *Gryllodes sigillatus* (tropical house cricket) were reared in the presence of *Eublepharis macularius* (leopard geckos) until maturity. Once mature, individual's latency to emerge from shelter followed by response to cues of predator presence were measured, followed by pre-determined matings to asses any potential trans-generational effects of this chronic exposure to predator presence. Latency to emerge a shelter is a repeatable measure of boldness (Beckmann and Biro 2013) and may be affected by lengthened predator exposure. Activity in the presence of cues of predator presence was measured by exposing *G. sigillatus* to cues created from leopard gecko excreta and has been shown to increase the activity of crickets during exposure (Royauté and Dochtermann

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2017). This activity can indicate anti-predator responses and may also be affected by chronic exposure during development. The behavioral differences were estimated relative to control individual's reared under similar conditions, except for the presence of the leopard gecko, for both generations. The results of this study can help shed light on the lengths of plastic behavioral responses caused by chronic exposure to predator stimulus and the effects on offspring behavior.

Paired with the effects of chronic exposure to predator stimulus on behavioral expressions I evaluated the differences in behavioral aspects of five closely related cricket species, including *G. sigillatus*. The average behaviors, behavioral variances, and correlations of behavior of exploratory behavior and activity in the presence of predator cues was measured for all five species. The potential differences in average expression of behavior can be an indicator of different optimal behaviors present in each environment. Variances of behaviors can show the effects of genetic and environmental contributions to behaviors. Finally, behavioral correlations can show how behaviors are expressed in relation to one another and if these behaviors are being constrained genetically. Overall, the results of this study can shed light on similar, or different, evolutionary pathways of closely related species present in different environments.

Matching a behavior to an environment can increase an individual's survival and have positive effects on fitness (Snell-Rood 2013, Krause et al. 2017). The expression of behaviors can be affected by genetic and environmental contributions captured in measurements of variances (Dochtermann et al. 2015, Westneat et al. 2015). The effects of environmental influences can have short or long-term effects on the expressions of behaviors via reversible and irreversible plasticity (Tabashnik et al. 1981, Lynch and Walsh 1998, Piersma and Van Gils 2011). The consequences of these changes on survival and fitness are dependent on how frequently the environment changes. A relatively stable environment would benefit individuals

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with irreversible plasticity as the continued changes in behavior would match the environment. For a constantly changing environment, individuals that can quickly change any behavioral alterations would be better suited. Behaviors can be passed on to offspring either through genetic components or trans-generational effects (Fisher 1930, Storm and Lima 2010). Shared genetic components can prevent individuals from freely adapting behaviors to best suit an environment and reduce the effectiveness of natural selection (Sih et al. 2004, Royauté et al. 2019b). Overall, this study sheds light on the effects of genetic and environmental contributions to behaviors and to what extent these behaviors can be inherited by future offspring. These comparisons are performed not only intraspecifically but also interspecifically as well. By comparing differences in behaviors both among- and within-species this study also reveals ecological and evolutionary pathways and how freely species can adapt to a certain environment.

CHAPTER 1: DIRECT AND TRANS-GENERATIONAL EFFECTS OF CHRONIC EXPOSURE TO CUES OF PREDATOR PRESENCE IN *GRYLLODES SIGILLATUS*

Introduction

The ability to adjust to a changing environment can increase reproductive fitness in an individual or population. Not only can adjustments in morphological traits be beneficial, but changes in behavioral expressions can also increase fitness, i.e. behavioral plasticity. Behavioral plasticity is when an individual changes its behavior to better suit its environment (Relyea 2001). These changes are caused by experiencing a certain environment, then altering aspects of behavior to better suit that same or similar environment (Pallier et al. 1997). Environmentally customized behavioral changes can potentially increase survival but only if the current environment matches the environment for which the behavior was modified. If an individual adapts to one environment, then lives in another or the environment changes too quickly, plasticity can have negative consequences on survival and fitness (Snell-Rood 2012).

Plasticity can be expressed in one of two ways, reversible and irreversible plasticity (Gabriel 2006, Snell-Rood 2012). Reversible or passive plasticity, is the amount of temporary change in a particular trait within a certain individual (Westneat et al. 2015). This type of variation is observed in labile phenotypes and is typically well suited for a frequently changing environment, e.g. varying levels of predation pressures (Relyea 2001), and can also fall under the label "phenotypic flexibility" (Piersma and Van Gils 2011). For example, Binz et al. (2014) showed that the same species of wood cricket (*Nemobius sylvertis*) expressed different levels of activity based on the hunting strategies of the predator cues they were exposed to. Cues of diurnal predators resulted in increased activity levels and nocturnal predator cues elicited a reduction in activity. These contrasting anti-predator responses were presumably driven by the

hunting strategies of predators and gives evidence of how behaviors can quickly reverse plastic responses to an environment or situation.

The length of exposure to a stimulus can affect how an individual responds to that stimulus. A single exposure to cues of predator presence has been shown to cause individuals to subsequently behave more cautiously in arthropods (Storm and Lima 2008), fish (Holmes and McCormick 2010), mammals (Orrock et al. 2004), and birds (Griffin 2004). However, there is room for further investigation in the literature for the prolonged effects of predator cue exposure and its effects on predator responsiveness. Chronic exposure to a stressor can result in altered forms of behavioral expressions (Weiss et al. 1975). When individuals acclimate to a stimulus one of two behavioral changes can occur: habituation or sensitization (Peeke 1969, DiFranza and Wellman 2007, Blumstein 2016). Habituation is a reduced response to a stimulus from the expected response levels, while sensitization is an increase in responsiveness when compared to the norm. For example, Owen et al. (1991) has shown that after an acclimation period to a cold environment, the heat-seeking behavior of *Rattus norvegicus domestica* (laboratory rats) were significantly lower than before the acclimation period. This indicates a lessened response to a colder environment, i.e. habituation. Conversely, in a review, DiFranza and Wellman (2007) documented replicable sensitization processes in response to nicotine (stimulus). Repeated exposure to the same dose of a stimulus, in this case nicotine, caused an increase of locomotor activity. However, the sensitization response is not consistent across stimulants, which may suggest that this exaggerated response may be adaptive in nature (DiFranza and Wellman 2007). Biotic stimuli, including those related to predator presence have been shown to have similar effects on behavioral responses (Peeke 1969). While changes in behavioral responses have been

observed in response to predator stimulus, whether these changes in behavior are temporary (i.e. reversible) or permanent (i.e. irreversible) is less well understood.

The benefits of temporary versus permanent changes in a phenotype are dependent on how frequently the environment experienced changes. Irreversible or developmental plasticity is when the phenotype of an organism is, effectively, permanently changed in response to environmental cues. This change persists throughout an individual's entire lifetime or at least the entire time period of measurement (West-Eberhard 2003). Irreversible plasticity and permanent environmental effects are well described for morphological traits. For example, Tollrian (1995) has shown that exposure to predator cues induced permanent morphological changes in *Daphnia pulex* by developing small spines in the neck region as a form of predatory defense. The presence of this morphological feature has been shown to reduce mortality in the presence of invertebrate predators (Krueger and Dodson 1981, Dodson and Havel 1988). Permanent environmental effects similarly affect behaviors: Tessier and Leibold (1997) has shown that the environmental factors leading to morphological size differences in *Daphnia pulicaria* clones leads to differences in habitat depth preferences in multiple populations. Individuals that were smaller at maturity displayed tendencies to utilize shallower depths and led to lower fecundity when compared to larger clones. In comparison to a natural population, daphnia that were consistently found in shallower depths were more prone to predation than those at higher depths. Irreversible plasticity, e.g. permanent environmental effects, can be beneficial, in this case to larger daphnia clones at larger depths, but only in a stable environment (Tollrian 1995). If these permanent phenotypic responses are made to suit one environment and the environment changes, this can have negative impacts on fitness, also known as the environmental matching hypothesis (Krause et al. 2017).

The presence of predators often changes the behavior of individuals (Relyea 2001) and customizing responses to predators is advantageous to survival (Laurila 2000). However, these behavioral changes may be specific to environments and predator densities. For example, Relyea (2001) showed that when exposed to predators during development numerous species of Anurans displayed plasticity in either their behavioral responses to predators or exhibited morphological changes. More specifically, these plastic responses were predator-specific, demonstrating that plasticity can match morphological and behavioral responses to the appropriate predator in different environments.

Plasticity can also indirectly affect offspring that have not directly experienced a particular environment via trans-generational inheritance. Environmental conditions that parents are exposed to can have a strong impact on the behavior and phenotype of their offspring, which can directly affect their fitness. While both maternal and paternal effects can match the phenotype of their offspring to the current environment (Marshall and Tobias, 2007), maternal effects in particular have been shown to have a greater effect on offspring. This has been observed in lizards (Uller and Olsson 2006) and birds (Groothuis and von Engelhardt 2005). This is due to the increased influences during the embryonic deposition period that the mother is in contact with the eggs. Different types of stimuli and stressors in an environment can be catalysts for changes in offspring traits via epigenetic responses, i.e. alterations of gene expression not involving changing underlying DNA sequences (Owen et al. 2018). Encounters with predators can influence the morphological traits of offspring: Body size and limb length can be influenced by predation stress, which can result in offspring being able to run faster and escape more frequently than offspring that do not have these special traits suited for a specific environment

(Owen et al. 2018). These changes in phenotype can lead to greater survival rates and can lead to increased fitness.

Plasticity and trans-generational effects on anti-predator behaviors in response to perceived threat have been studied in multiple taxa (Relyea 2001, Storm and Lima 2010, McGhee et al. 2012). Perhaps the most comprehensive examination was by Storm and Lima (2010) who exposed gravid *Gryllus pennsylvanicus* (fall field crickets) to *Hogna carolinesis* (wolf spiders) with waxed chelicerae to create extreme non-lethal encounters with predators. The encounter resulted in offspring of these exposed crickets displaying freezing behavior 27% more than unexposed crickets in subsequent behavior trials. Additional testing also showed that offspring of exposed crickets had significantly greater longevity and survival probability when exposed to Hogna spiders in survival assays. While only speculative, the results of this study suggest that maternal investment in the eggs altered predator avoidance since deposited eggs exposed to predators did not show altered forms of anti-predator responses. Furthermore, these patterns of freezing behavior matched wild populations that are under predation threats of these wolf spiders, when compared to populations without Hogna spiders (Storm and Lima 2010).This study not only lends support that the displayed behaviors are consistent with wild ecosystems containing these cricket-spider interactions, but that the resulting increase in survival suggests that these trans-generational effects are adaptations that can increase survival and intern, reproductive fitness. Despite this evidence of the effects of acute exposure to cues of predator presence or predators themselves, the effects of habituation or sensitization on anti-predator responses remain unclear.

Here I reared groups of *Gryllodes sigillatus* (tropical house cricket) in the presence of *Eublepharis macularius* (leopard geckos) until maturity. Once mature, individuals had their

latency to emerge from shelter and response to cues of predator presence measured, followed by selective matings. In so doing, I addressed the following questions:

1. Will rearing *G. sigillatus* with constant exposure to predator stimulus influence latency and anti-predatory behaviors?

2. Will chronic exposure to predator stimulus result in trans-generational effects of behaviors related to predator avoidance?

Materials and Methods

F_0 Rearing Conditions

G. sigillatus used in this study were from an outbred line established from individuals initially caught in California in 2017 and currently being maintained in Fargo, ND. Hatchlings were initially reared in a single group housing container until individuals reached 1 cm in size. After reaching the targeted size, *G. sigillatus* were separated into three groups in relation to relative size (small, medium, large) and distributing 5 individuals per group into 10, 37.9-liter terraria, with 5 terraria acting as the control and 5 terraria acting as the treatment terraria, totaling 150 individuals at the start of the exposure treatment. There was, however, an initial die off of individuals and fewer crickets survived to maturity (Table 1). Sex of individuals was unknown before maturity and sex and mass were determined at the time of testing. All terraria were divided into two sections, a cricket and gecko area. The dimensions of the gecko section were 38 cm x 25 cm and the dimensions of the cricket area were 12.5 cm x 25 cm (Figure 1). The control terraria were similarly divided but without the gecko present. During rearing, the gecko and cricket areas were switched weekly to increase exposure to cues of predator presence. All terraria were kept under a 12:12 light: dark photoperiod with an average temperature of 29°C during the course of exposure. Each cricket portion of the terraria included a food source, shelter, and glass

vials with cotton balls as the water source. All individuals were exposed to a diet of ad libitum food (commercially purchased chicken feed) (Royauté et al. 2015).

All Leopard geckos used in this study were housed according to standards of the Institutional Animal Care and Use Committee of North Dakota State University (Protocol A14006, A17015, and A19067). Once mature, individual crickets were isolated in individual housing for 24 hours. Individual housing consisted of a 0.71-liter container with transparent cover that included the food source, shelter, and glass vials similar to the F_1 generation. After the isolation period, individuals were then run through behavioral assays.

F_1 Mating and Rearing Conditions

After behavioral assays (see details below), matings were performed with pairings established within treatment groups to explore potential trans-generational effects of prolonged predator exposure. Random mating pairs were generated and paired male and female crickets were housed in 5.7-liter containers with water, food source, and shelter for 24 hours and allowed to mate. After the mating period, both individuals were removed and placed back into their respective individual housing. Despite maturity checks every 48 hours, the use of unmated females and known sire could not be guaranteed, only treatment exposure. Therefore, only intra-treatment matings were performed to control for treatment effects. F_1 hatchlings were reared in the same 0.71-liter container with shelter, food, and water as their mother until reaching 1 cm in size. Once the targeted size was reached, individuals were isolated into individual housing until maturity was reached. Each individual housing container was also 0.71-liters with transparent covers that included the food source, shelter, and glass vials similar to the F_0 generation and the hatchling rearing period. The F_1's were also reared under a 12:12 light: dark photoperiod at a temperature of 25-28˚C. Once mature all individuals were also run through identical behavioral

assays as the F_0 generation. 150 individuals were isolated into individual housing after reaching the targeted size of 1 cm and very little die off was observed before maturity and testing (Table 1).

Behavior Trials

To measure latency to emerge from a shelter and anti-predatory responses I recorded individuals' time to emerge from a shelter followed by their responses to cues of predator presence created from diluted *E. macularius* excreta (see details below). The F_0 generation were measured between May 2019 and July 2019 and the F_1 generation were measured between August 2019 and October 2019. I used a plastic arena (60 cm x 60 cm and 15 cm high) with a Plexiglas lid for both behavioral trials. The testing apparatus was split into four 30 cm x 30 cm arenas separated by a Plexiglas divider, allowing for up to four crickets to be tested at one time. For latency trials, a smaller container with an artificial burrow was placed inside each of the four arenas. All individuals were run through latency assays then through the anti-predator assays with a minimum 1-minute acclimation period between the trials. After the completion of the anti-predator assay, each arena was thoroughly cleaned with 70% ethanol wipes to avoid accumulation of any chemical traces of conspecifics and mass was recorded to the nearest 1 mg.

Latency Trials

Cricket species such as *G. sigillatus* use small burrows or crevices for refuge from predators and to which they retreat when under threat of predation. As a result, latency to emerge from a shelter after a disturbance can be considered a proxy for risk-taking behavior or "boldness" (Kortet et al. 2007). In this study, I conducted latency tests where individuals were transferred from their home containers to small artificial burrows (40 cm³) placed within a 34.6 cm× 21 cm arena. These artificial burrows were capped so that individuals could not

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immediately emerge. Crickets were forced to remain in the artificial burrow for two minutes after which a cap was removed from the burrow. Crickets were then allowed six minutes and thirty seconds to emerge from the artificial burrow. During this test I recorded how long it took for an individual to emerge (in seconds). Individuals that did not emerge were given a maximum latency of 390 seconds (Royauté et al. 2019c).

Antipredator Response

To measure the responses to cues of potential predator presence, I collected excreta from three adult *E. maculariu*s, that were fed a diet of *G. sigillatus.* The excreta that was collected was frozen and then finely ground and diluted with deionized water (1 ml H_2O: 5 mg of excreta). This solution was then applied to 15 cm diameter filter paper disks with a 5 cm diameter central cutout that allows crickets to be left to rest unexposed to the predator cues (Royauté and Dochtermann 2017, Royauté et al. 2019a). Each predator cue was left to dry for a minimum of 2 hours then stored at 4˚C between trials but was allowed to warm to room temperature before use in antipredator trials and discarded after a single use. I placed the predator cue disk at the bottom of a 15 cm diameter arena and left the cricket to rest for a minimum of 60 seconds under a 5 cm diameter cup in the nontreated central cutout. I then allowed the cricket to move freely for 220 seconds and estimated the distance travelled in cm (AP distance) using the Ethovision XT software package. Previous studies with this protocol have shown that crickets had heightened activity levels in the presence of this diluted gecko excreta compared to water controls (Royauté and Dochtermann 2017). Greater activity during the antipredator response assays was therefore interpreted as a greater responsiveness to predator cues.

Data Analysis

To assess treatment effects of prolonged exposure to predator stimulus I used mixed-effects models (Dochtermann and Dingemanse 2013) with generation, sex, temperature, mass, number of days between maturity and testing date, and arena as fixed effects. For random effects, container ID was used for the F_0 generation to account for the 10 terraria in which subjects were reared and pair ID used for the F_1 generation. For the F_1 generation, individuals were individually reared but all F_1 individuals were given the same dummy container ID. Using unique container IDs for F_1 individuals resulted in the within-container variation being improperly estimated. Assigning individual container IDs to individuals of the F_1 generation also decreased the fit of our models due to an inability to properly estimate within-factor variances. F_0 individuals were all assigned the same dummy pair ID due to their unknown pedigree. While assigning a single value for pair ID for all individuals in the F_0 generation did not significantly reduce model fit, using unique pair IDs for all F_0 individuals again misestimated the within-pair variation estimation. During post-hoc analysis, differences in mass were estimated using mixed-effects models (Dochtermann and Dingemanse 2013) with generation, sex, temperature, number of days between maturity and testing date, and arena as fixed effects. Similar to mixed-effects models assessing behavioral differences, pair IDs and container IDs were also used for both generations in estimating differences in mass for treatment effects.

Results

There were no effects of treatment or generation for anti-predator and latency to emerge behavioral trials (Figure 2 and 3, Table 2 and 3). Male *G. sigillatus* males did move 86 cm less than females (Table 2) during anti-predator trials. Due to natural deaths and unable to determine sex when assigning exposure groups resulted in unequal sex rations of treatment groups.

Additionally, male crickets overall are known to be less active during predator cue exposure (Royauté and Dochtermann 2017). Differences in mass were seen for treatment, among generations, sex, and between generations for the treatment group (Table 4). Individuals in the treatment group weighed 38 mg less than the control individuals (Figure 4). The F_1 generation overall weighed 42.7 mg more than the F_0 generation, regardless of assigned treatments (Figure 4). Male *G. sigillatus* weighed 81.4 mg less than females (Table 4), but this is consistent with sexual size dimorphism observed across *Insecta* (Teder and Tammaru 2005, Fairbairn et al. 2007). Finally, F_1 treatment group individuals weighed 55.5 mg more than the F_0 treatment individuals (Table 4).

Table 1. Number of individuals by treatment and sex ratio for both F_0 and F_1 generations. F_0 unequal sex ratio was caused by unknown sex at treatment assignment. Unequal F_1 control individuals is due to lack of F_0 control females used in mating trials.

F_0 Individuals	Female	Male	Total	F_1 Individuals	Female	Male	Total
Control	7	11	18	Control	23	24	**47**
Treatment	12	16	28	Treatment	50	51	**101**
Total	**19**	**27**	**46**	**Total**	**73**	**75**	**148**
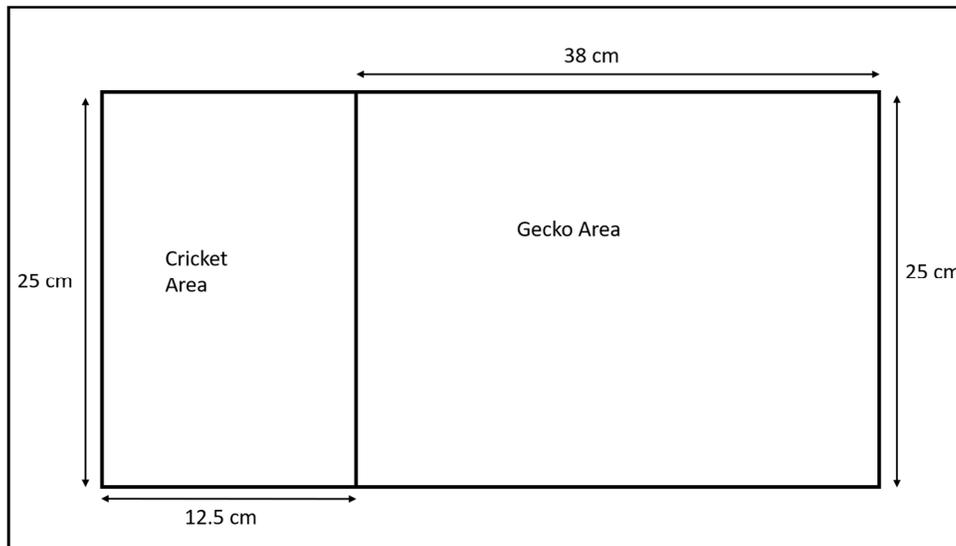
Figure 1. Dimensions of terraria used in rearing F_0 generation.
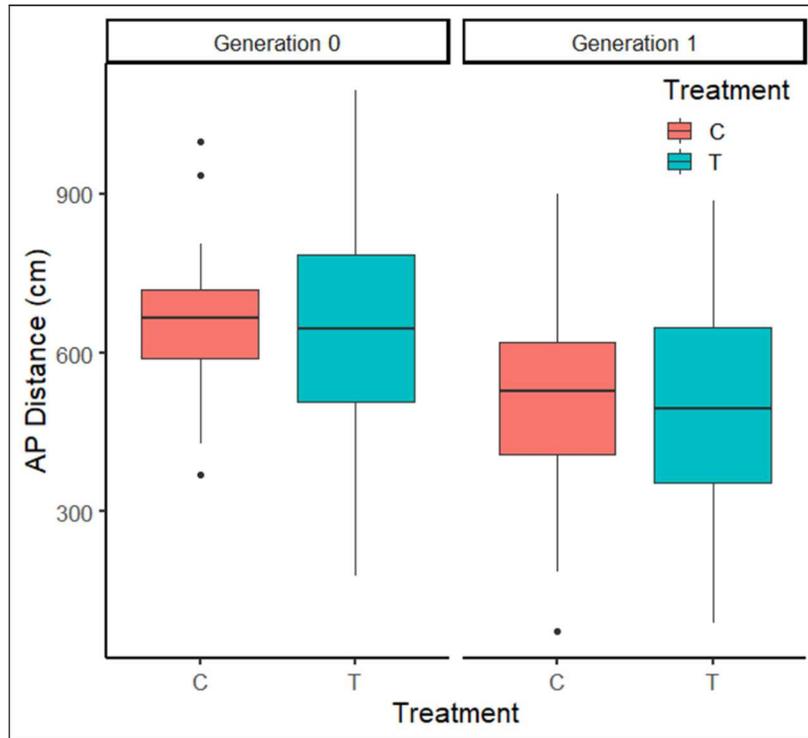
Figure 2. Differences in average distance traveled when exposed to cues of predator presence shown for control and treatment individuals. Boxes indicate the lower and upper quartiles; horizontal lines within the boxes indicate the median, whiskers extend to 1.5 interquartile range of the box, and points indicate outliers ($F_{1,\ 157.08}$=0.03, P=0.87).
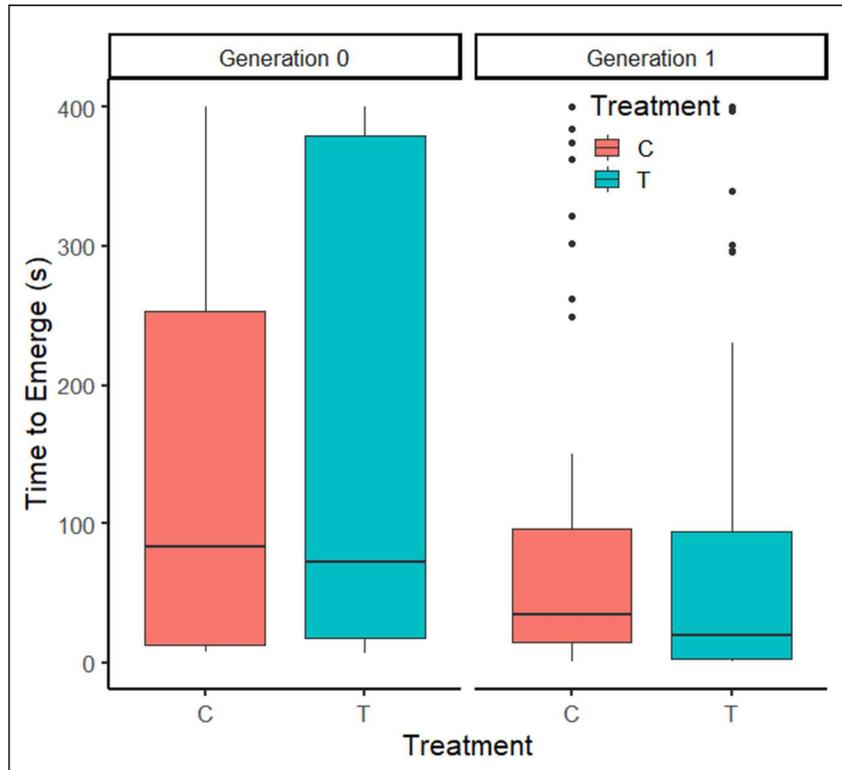
Figure 3. Differences in average latency to emerge from a shelter shown for control and treatment individuals. Boxes indicate the lower and upper quartiles; horizontal lines within the boxes indicate the median, whiskers extend to 1.5 interquartile range of the box, and points indicate outliers ($F_{1,8.95}=0.12$, $P= 0.74$).

Table 2. ANOVA table for the effects of treatment, generation, sex, temperature, mass, days since maturity, arena, and the interaction between treatment and generation on antipredator distance. Significant effects were found for sex in males, who on average moving 86 cm less than females.

Fixed Effect	DF	Den DF	F Value	Prob (>F)
Treatment	1	157.08	0.025	0.87
Generation	1	7.41	5.17	0.06
Sex	1	168.8	5.38	**0.02**
Temperature	1	171.02	1.22	0.27
Mass	1	171.65	0.54	0.47
Days Since Maturity	1	146.77	0.03	0.87
Arena	1	168.8	0.68	0.56
Treatment* Generation	1	53.98	0.01	0.91

Table 3. ANOVA table for the effects of treatment, generation, sex, temperature, mass, days since maturity, arena, and the interaction between treatment and generation on latency to emerge a shelter. No significant results of any fixed effects were found.

Fixed Effect	DF	Den DF	F Value	Prob (>F)
Treatment	1	8.95	0.12	0.74
Generation	1	6.73	0.73	0.42
Sex	1	163.5	0.03	0.86
Temperature	1	168.51	0.01	0.94
Mass	1	171.17	0.84	0.36
Days Since Maturity	1	131.98	0.001	0.97
Arena	1	170.13	0.70	0.55
Treatment* Generation	1	15.28	0.31	0.59

Table 4. ANOVA table for the effects of treatment, generation, sex, temperature, days since maturity, arena, and the interaction between treatment and generation on mass (mg) at maturity. Significant results for treatment, generation, sex, and the interact between treatment and generation on mass (mg) were found. Treatment individuals of both generations on average weighed 38 mg less than control individuals. F_1 individuals weighed on average 42 mg more than F_0 individuals. F_1 treatment individuals weighed on average 56 mg more than F_0 treatment individuals.

Fixed Effect	DF	Den DF	F Value	Prob (>F)
Treatment	1	169.03	10.59	**0.001**
Generation	1	3.93	31.19	**0.01**
Sex	1	187.88	185.46	**<0.001**
Temperature	1	163.49	1.15	0.29
Days Since Maturity	1	92.25	6.80	**0.01**
Arena	1	189.03	0.88	0.45
Treatment* Generation	1	62.36	13.53	**0.001**
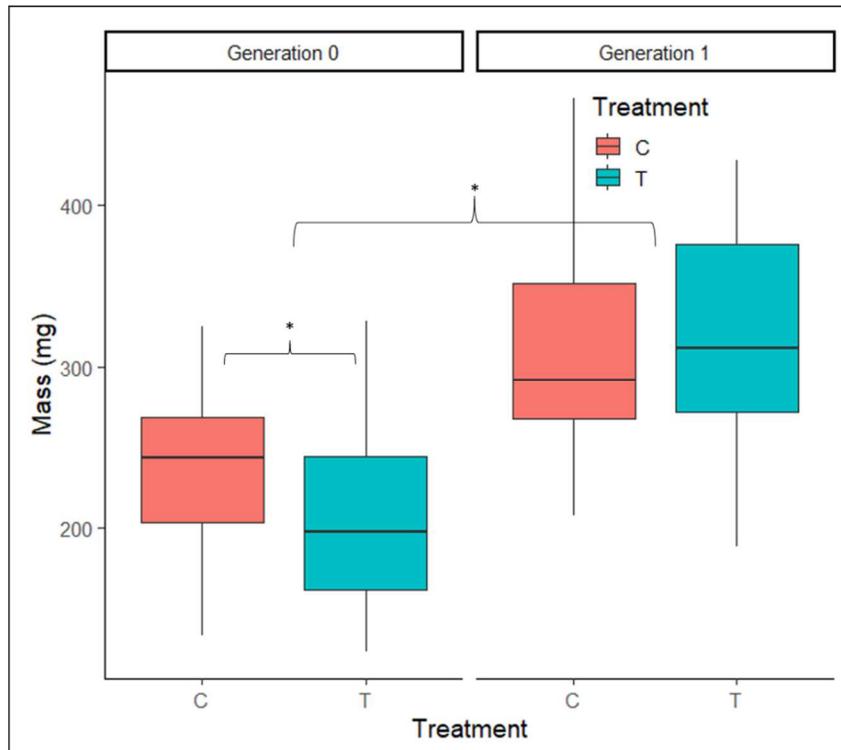
Figure 4. Differences in average mass shown for control and treatment individuals. Boxes indicate the lower and upper quartiles; horizontal lines within the boxes indicate the median, whiskers extend to 1.5 interquartile range of the box, and points indicate outliers (Treatment effects: $F_{1,169.03}$=10.59, P=0.001;interaction between Treatment and Generation: $F_{1,62.36}$=13.53, P=0.001).

Discussion

To investigate the direct and trans-generational effects of prolonged exposure to cues of predator presence on behaviors related to predator avoidance I exposed the parental generation to cues of predator presence throughout development. The offspring were not exposed to predator cues and were measured at maturity. Overall, there were no significant differences between treatments in behavioral responses for latency to emerge a shelter or distance traveled in the presence of predator cues for either generation (Figures 2 and 3). There were however multiple effects found for differences in mass among treatment groups and generations (Figure 4). This may suggest that there was not an effect of prolonged exposure to predator presence on anti-predatory behaviors nor trans-generational effects of the subsequent generation. Alternatively, since F_0 individuals were only tested once at maturation and not tested throughout development,

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to prevent repeated exposure to the cue for the control groups, subjects may have had an initial response to the presence of the leopard geckos and, as exposure continued, behavioral responses habituated back to normal response levels compared to a control group. If accurate, this is an example of reversible plasticity in anti-predator behavioral responses. Such a reversal of behavior could be due to a lack of direct encounters with a predator during prolonged exposure causing these behavioral responses to habituate back to expected levels (Peeke 2012). Consistent with this explanation, Bucklaew and Dochtermann (In Review), tested *G. sigillatus* in latency trials and activity trials before and after being exposed to live leopard geckos. In their study *G. sigillatus* were released into an arena with a leopard gecko and removed once attacking behavior by the gecko was observed. This exposure to a live predator caused significant increases in activity levels and reduction in the proportion of individuals that emerged in latency trials measured in the post exposure testing. Although this is a more extreme and acute exposure than performed in this study, it provides evidence that *G. sigillatus* are at least initially responsive to the presence of live leopard geckos.

Differences in mass at maturity can indicate the effects of stress caused by the direct exposure to cues of predator presence during development. The physiological effects of stress on development are well document (DeVries et al. 1997, Mishra et al. 2011, Kriengwatana et al. 2013, Royauté et al. 2019a) and significantly lower body masses could be an indicator of a stressful developmental environment caused by the presence of live leopard geckos (Kriengwatana et al. 2013). Evidence of F_0 treatment individuals initially responding to the presence of the leopard geckos is seen in this experimental group having significantly lower masses at maturation compared to the F_0 control individuals (Figure 4). These potential permanent environmental effects on mass during development did not result in any trans-

generational effects on offspring mass as F_1 treatment individuals weighed significantly more than the previous generation (Figure 4) and were not exposed to any cues of predator presence until behavioral assays at maturation. Significantly greater masses overall of the F_1 generation compared to the F_0 generation is most likely due to the increased quality of rearing conditions as the F_1 generation was reared in individual containers and without the presence of a live predator (Monaghan 2008). However, the effects of prolonged exposure to predator cues during development needs to be further investigated as the finding of differences in mass were not part of our *a priori* questions and were instead discovered during post-hoc data exploration.

Our results combined with recent findings suggest that after initial responses to predator presence prey will revert altered behaviors back to baseline levels of responsiveness if not reinforced by direct consequences of predator interaction. Further evidence of this is observed in Pilakouta and Alonzo (2014), where the authors observed that female *Xiphophorus helleri* (green swordtail fish) changed their mate preference from males with longer swords to males with shorter swords when in the presence of predators as compared to a control group. This altered mate choice only lasted for 24 hours after predator exposure and female preference shifted towards males with longer swords, which was consistent with control individual preference. These results lend further support that behavioral responses can be influenced by predator exposure and without continued exposure these temporary behavioral changes can revert back to normal levels before predator exposure.

The lack of apparent behavioral response to chronic exposure of predator cues raises several questions. First, if, as our results and the behavior of the species in other contexts suggests, habituation resets behaviors back to naïve levels, at what point in exposure duration does the habituation occur? Second, since a lack of differences in behavioral responses of

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treatment groups contradicts responses to extreme, acute exposure examples found in the literature (Storm and Lima 2010, Bucklaew and Dochtermann In Review) the effects of prolonged exposure to predation stimulus remains unclear.

CHAPTER 2: A COMPARITIVE APPROACH TO BEHAVIORAL DIFFERENCES IN CLOSELY RELATED CRICKET SPECIES

Introduction

Behavioral responses affect fitness via both survival and reproductive success. For example, in Great tits (*Parus major*), Dingemanse et al. (2004) found that both survival and number of offspring were affected by exploratory behavior, e.g. number of flights and hops, in a novel environment. As the environmental pressures changed from year to year, so too did the relative frequencies of these behaviors in the studied population. Individuals with the highest reproductive fitness were those performing behaviors that best suited the environment they were experiencing. Similar effects have been observed across taxa, with variation in behavior explaining roughly 6% of observed variation in survival (Moiron et al. 2020). Meta-analysis of standardized selection coefficients has likewise found that behavior is under stronger directional selection than are other classes of traits (Kingsolver and Diamond 2011), although there were considerably fewer estimates for selection on behavior than for other traits.

When behavior is under selection, theory suggests that behaviors leading to higher fitness will be expressed more frequently. Inferring similarities of selective pressures across species can therefore be done by comparing the average expression of certain behaviors along with variation of those behaviors (Price and Schluter 1991). Selection can shape the distribution of phenotypes, in this case behavior, in many ways. Primarily it is predicted that selection should drive a species' mean phenotype towards a fitness optimum (Robertson 1966, Price 1970, Hajduk et al. 2020). This optimum is created from a number of different trade-offs with the goal to maximize the net rate of return of resources (Emlen et al. 1984). This optimum is positively selected for and should lead to higher fitness for a species (Emlen et al. 1984). Alternatively, there are three

main reasons why a measured average behavior would not be a true optimized behavior for the current environment. 1) behaviors are genetically conserved behaviors, this is known as the phylogenetic constraint's hypothesis (McKitrick 1993). This hypothesis is characterized as any result or component of the phylogenetic history of a lineage that prevents an anticipated course of evolution in that lineage (McKitrick 1993). Simply put, phylogenetic constraints are a result of closely related species or populations that have shared phylogenetic history and previous adaptions have caused constraints in expressing behaviors in their current environment. These constraints will limit these species abilities to properly adapt and find a true fitness optimum to the current environment (Emlen et al. 1984). 2) frequency-dependent selection is acting on a species (Emlen et al. 1984). This is when the most commonly expressed phenotype is negatively selected for (Emlen et al. 1984). 3) the conflict between a local and global optimum behavior (Emlen et al. 1984); which is when a certain behavior would be optimal for a population or portion of a species while another behavior might be optimal for the species as a whole.

How a behavior is expressed is dependent on two things: the genome of an individual and the interaction of that genome with the environment. If a group of individuals are experiencing the same environment then they may behave in a similar manner, even if they have very different genomes. Alternatively, individuals experiencing similar environments may behave differently due to the restrictions in behavioral expressions caused by their genomes. Regardless of the cause, the behaviors conferring highest fitness in an environment will be selected for and as generational turnover continues the frequencies of those behaviors will increase (Fisher 1930). Any variation of behavior measured in an individual is known as behavioral plasticity (Relyea 2001, Relyea 2003).

Regardless of the cause, if a fitness optima is reached, variation can be reduced around the average expression of behavior and narrow the distribution of this trait as sequential generations are exposed to similar selective pressures, i.e. stabilizing selection (Robertson 1956). This reduction in variation occurs at the among-individual variation level as selection acts on the genetic differences between individuals, which is one contributor to differences observed between individuals (Dochtermann et al. 2015). Assessing genetic contributions to behavior can be difficult due to the expression of behavior being caused by the interaction of genes with the currently experienced environment. Also captured in the between individual differences lies any permanent environmental effects experienced by an individual before measurement. Permanent environmental effects are when the phenotype of an organism is permanently changed in response to environmental cues from selective pressures. This change is throughout an individual's entire lifetime or at least the entire time period of measurement, also referred to as active irreversible plasticity (West-Eberhard 2003). However, phenotypic measures may be used as indicators of genetic components, an approach known as the phenotypic gambit (Grafen 1984, Hadfield et al. 2007, Dochtermann 2011, Dochtermann et al. 2015). As selective pressures continue to act on the distributions of behaviors in a species; additive genetic variation is reduced, which leads to lower heritability of that particular trait and, in turn, slows the effectiveness of stabilizing selection (Roff 1993). Although not as responsive to selection as morphological traits due to lower hertitabilities of traits, behavioral traits are still influenced and shaped by selective pressures such as stabilizing selection (Kingsolver et al. 2001). If a species shows relatively low levels of among-individual variation for a behavior when compared to other species, one potential cause of this would be that the behavior has reached a fitness optima and stabilizing selection has been reducing variation around the average for a relatively longer period

of time (Roff 1993). Differences in these variances can therefore give us an idea of how behavior has been shaped by selection.

While variation observed among-individuals is caused by genetic variation and permanent environmental effects, the variation observed within-individuals is attributed to temporary environmental variation (Dingemanse and Dochtermann 2014, Westneat et al. 2015) and these components contribute to the total phenotypic variation observed in individuals and species. Within-individual variation occurs when a labile phenotype, e.g. behavior, physiology, and certain morphological traits, deviate from a norm-of-reaction within the same individual (Westneat et al. 2015). This interaction is where phenotypic plasticity occurs and can be responsive to selection (Berdal and Dochtermann 2019). Phenotypes are created by genes being expressed in a certain environment. It is this interaction with the environment that creates plastic trait expression (Westneat et al. 2015). While variation among individuals is necessary for selection to occur, within-population or species patterns affects units of measurements when quantifying selection at higher levels (Westneat et al. 2015). Environmental influences can contribute to the expression of a phenotype in the following ways: *Active reversible plasticity*, is when a reversible change in an individual's phenotype is expressed in response to environmental cues from selective pressures. These changes vary within individuals and are also referred to as phenotypic flexibility (Piersma and Drent 2003, Piersma and Van Gils 2011). *Passive plasticity*, which instead of responding to selective pressures, phenotypes are being altered by environmental conditions (Whitman and Agrawal 2009). Finally, *Organismal error*, this occurs when changes in an individual's phenotype are created through failures to process an environmental cue correctly. This can be either reversible or irreversible organismal error (Berdal and Dochtermann 2019). Since this within individual variation is necessary for the

expression of a phenotype it can reveal biological processes linking phenotype and environmental interactions. Within-individual variation of different species can also indicate how adaptive certain species are to a changing environment. Species showing higher levels of behavioral plasticity could potentially be under selective pressures from a frequently changing environment and selection would favor behavioral traits that show more flexibility when being expressed in an environment.

How individuals differ from themselves over time and from others in one particular trait can indicate many things, but how two or more traits covary in a population or species can also reveal valuable information, e.g. the presence of phylogenetic constraints. Of increasing interest in the scientific community are the ecological and evolutionary impacts of behavioral syndromes. Behavioral syndromes are suites of correlated behaviors across situations (Sih et al. 2004). Potential causes of these correlated behaviors are genetic pleiotropy or linkage disequilibrium in gene expression (Sih et al. 2004). Pleiotropy is the expression of one gene affecting multiple traits, while linkage disequilibrium is when two loci that affect the expression of phenotypes are closely located on an allele and are less likely to be separated during recombination (Sih et al. 2004). A common example of behavioral syndromes is aggression carrying over across different situations: Some individuals may be more aggressive in certain situations like male-male competition or territory defense and these same individuals may also show high aggression in situations where aggression has negative impacts on fitness like mating behaviors (Sih et al. 2004). The genetic contributions to these correlations of behaviors in behavioral syndromes implies limited plasticity in behavior and can potentially conserve less than optimal behaviors. This contradicts the traditional concept that behaviors are infinitely plastic to an environment. The conflict between the optimization of one behavior in an

environment and the genetic linkages to other behaviors can create trade-offs that can affect an individual's fitness. For example, these trade-offs can limit dispersal of species if the affected behaviors influence the adaption to a new environment, i.e. behaviors related to resource allocation, while negatively affecting predator avoidance (Emlen et al. 1984). By looking at how behavioral correlations differ between closely related species we can investigate how behavioral syndromes are being conserved or reduced as phylogenic trees expand.

In this study we analyzed data collected from five closely related cricket species (*Gryllus integer, Gryllus assimilis, Gryllus lineaticeps, Gryllodes sigillatus,* and *Acheta domesticus*) measuring exploratory behavior via unique zones traveled and activity in the presence of a predator cue (Dingemanse et al. 2002, Dingemanse et al. 2007, Royauté et al. 2015, Royauté and Dochtermann 2017). Despite the fact that both of these behavioral assays are reliant on general activity of our crickets, independently they can convey ecological information relative to selective pressures being imposed on the different cricket species (Dingemanse et al. 2002, Dingemanse et al. 2007, Royauté et al. 2015).

With the data collected during these behavioral assays we asked the following questions:

1. Does the average expression of behavior differ among species? This can indicate selective pressures driving behavioral frequencies to potential fitness optimums.

2. Do the among-individual variances differ among species? This can indicate the rate of stabilizing selection imposed of the different species and potential genetic differences in behavioral expression.

3. Do the within-individual variations differ among species? These variances indicate temporary environmental effects on behavior and the magnitude of phenotypic plasticity in behaviors.

4.   Do behavioral covariances differ among species? This indicates if the strengths of

behavioral syndromes differ among closely related species.

Materials and Methods

Cricket Acquisition, Housing, and Rearing Conditions

Data used in this study was originally collected for various studies investigating the

presence of behavioral constraints and behavioral syndromes (Royauté and Dochtermann 2017,

Royauté et al. 2019a, Royauté et al. 2019b). *A. domesticus* males and females were obtained as

nymphs (~ 1 mm in size) from a commercial supplier (Fluker's Cricket Farm, Port Allen, LA,

U.S.A.) in 2015 and were measured once mature. *G. integer* females were captured in Aguila,

AZ, *G. lineaticeps* males and females were caught in Dunnigan, CA, and the *G. assimilis* males

and females were caught in Maricopa County, AZ. These species were all captured during 2017.

G. sigillatus individuals were taken from an outbred population established by S. Sakaluk and

currently maintained in Fargo, ND. For *G. lineaticeps* and *G. assimilis*, the same individuals that

were caught in the field were measured, while lab reared offspring of *G. integer* were measured.

All species were reared under a 12:12 light: dark photoperiod at a temperature of 25-28˚C. All

individuals were housed in 0.71-liter containers with transparent covers that included food,

shelter, and water filled glass vials plugged with cotton balls. *A. domesticus* were exposed to a

mixture of high and low quality diets used in Royauté et al. (2019a), while all other species

included in this study were fed ad libitum food (commercially purchased chicken feed).

Behavior Trials

To measure exploratory behavior and anti-predator responses we repeatedly recorded

individuals' activity levels in an open field arena, followed by their responses to cues of predator

presence created from diluted *Eublepharis macularius* excreta (see details below). *A. domesticus*

were measured between March 2015 and October 2016, *G. lineaticeps* were measured from August 2017 to September 2017, *G. assimilis* were measured between September 2017 and October 2017, *G. integer* were measured between May 2018 and June 2018, and *G. sigillatus* were measured in May 2019. We used a plastic testing apparatus (60 cm x 60 cm and 15 cm high) with a Plexiglas lid for both behavioral trials. The apparatus was split into four 30 cm x 30 cm arenas separated by a Plexiglas divider, allowing for up to four crickets to be tested at one time. Open field trials were always conducted first followed by antipredator response trials either immediately after or on another day to minimized potential carryover effects from exposure to cues of predator presence. After each behavioral assay arenas were thoroughly cleaned with 70% ethanol wipes to avoid accumulation of any chemical traces of conspecifics and mass was recorded to the nearest 1 mg. All individuals were measured in each assay for a maximum of three repetitions (Table 5).

Open Field Behavior

Individuals were left to rest for 30 seconds in a 5 cm diameter cup introduced into the lower left section of the arena (Figure 5). We then allowed the cricket to move freely through the arena for 220 seconds. We measured each individual's exploratory propensity by calculating the number of *unique zones* visited by the cricket with Ethovision X (Noldus Information Technology, Wageningen, The Netherlands). This behavioral protocol has previously been used with *A. domesticus* and *G. integer* to evaluate individual differences in activity and exploratory behaviors (Royauté et al. 2015, Royauté and Dochtermann 2017, Royauté et al. 2019a).

Antipredator Response

To measure responses to cues of potential predator presence, we collected excreta from three adult leopard geckos, *Eublepharis macularius*, that were fed a mixed diet of *A. domesticus,*

G. sigillatus, G. lineaticeps, *G. integer*, and *G. assimilus* were used in this study. Leopard geckos were housed according to standards of the Institutional Animal Care and Use Committee of North Dakota State University (Protocol A14006, A17015, and A19067). The excreta that was collected was frozen and then finely ground and diluted with deionized water (1 ml H_2O: 5 mg of excreta). This solution was then applied to 15 cm diameter filter paper disks with a 5 cm diameter central cutout that allows crickets to be left to rest unexposed to the predator cues (Royauté and Dochtermann 2017, Royauté et al. 2019a). Each predator cue was left to dry for a minimum of 2 hours then stored at -23°C until needed for trials. Predator cues were allowed to warm to room temperature before use in antipredator trials and discarded after a single use. Between each trial, sets of cues were stored at 4°C where they were kept for a maximum of 14 days. We then placed the predator cue disk at the bottom of a 15 cm diameter arena and left the cricket to rest for a minimum of 30 seconds under a 5 cm diameter cup in the nontreated central cutout. We then allowed the cricket to move feely for 220 seconds and estimated the distance travelled in cm (AP distance) through Ethovision (Figure 5). Previous studies with this protocol show that crickets had heightened activity levels in the presence of this diluted gecko excreta compared to water controls (Royauté and Dochtermann 2017). This greater activity during the antipredator response assays was interpreted as a greater responsiveness to predator cues.

Data Analysis

All analyses were conducted in R 3.4.4 (Team 2018))

Univariate Models

To assess differences in behavioral responses between species for means and variances we analyzed behavioral data using univariate mixed-effects models for unique zones traveled and AP distance (distance traveled was square root transformed) using the MCMCglmm library

(Hadfield 2010). We included species, temperature (Celsius, mean centered), mass (using between- and within-individual centering (Van de Pol and Wright 2009)), and sex as fixed effects. Individual ID was included as a random effect.

To determine whether species differed in their average behavior we compared the fit of two models for each of the behaviors. The first model allowed average behavior to differ by species while the second did not. Both models allowed variances to differ by species (see below). These models were specified using the MCMCglmm package for Bayesian mixed models (Hadfield 2010) and fit using Markov-chain Monte Carlo (MCMC) chains with 1.3 million iterations, 300,000 iteration burn-in, a thinning interval of 1000, and an inverse-Wishart prior. AP distance and unique zone models were fit with Gaussian and Poisson error distributions, respectively. We then compared the deviance information criterion (DIC) between models. The model with the lowest DIC value was considered the best model and a Δ DIC>5 was considered a substantively poorer fit. Models with ΔDIC<5 were considered as having equivalent support compared to the best model (Barnett et al. 2010). These ΔDIC values were then used to determine whether there was support for the inference that average behavior differed among species.

To determine whether species differed in either their among-individual or within-individual variances, we compared the fit of four univariate mixed models structured as follows:

1) Model 1: $V_i =$ & $V_w =$ A null model where the among- (V_i) and within-individual (V_w) variances were kept constant between species.

2) Model 2: $V_i \neq$ & $V_w =$ A model where the among-individual variance differed between species, but the within-individual variance was kept constant.

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3)   Model 3: V_i = & V_w ≠ The within-individual variance differs between species, but the among-individual variance was kept constant.

4)   Model 4: V_i ≠ & V_w ≠ Both the among and within-individual variances were allowed to vary between species.

All models were specified with the same fixed effect structure as specified above to prevent biased estimates of variance components and repeatability (Spiegelhalter et al. 2003, Nakagawa and Schielzeth 2010, Westneat et al. 2011). We also fit these models using the same prior and chain specifications as used for the evaluation of species average behaviors. Support for the presence or absence of species differences in among- or within-individual variances was determined based on ΔDIC values according to the criteria described above.

Bivariate Models

Behavioral syndromes were assessed using bivariate mixed-effects models with unique zones traveled and AP distance as response variables, also using the MCMCglmm library (Hadfield 2010), and analyzed separately for each individual species. We fit models using temperature (Celsius, mean centered), mass (using between and within-individual centering on subjects (Van de Pol and Wright 2009)), and sex as fixed effects and individual was fit as a random effect. These models were fit with 2.6 million iterations, a 600,000 burn-in period, a thinning interval of 2000, and a prior that was flat for correlations. Among-individual correlations were estimated for all species, while within-individual correlations were only assessed with species data where individuals were measured for unique zones traveled and antipredator activity during the same testing period. Consequently, we were unable to assess within-individual covariation of *G. lineaticeps* and *G. assimilis* due to the fact that these species

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were not measured in succession. Differences in behavioral correlations among species were assessed based on whether HPD intervals overlapped.

Results

Differences in Average Behavior Among Species

Species differed in average behaviors, this is observed by the inclusion of species as a fixed effect improving model fit for both behaviors (Table 6). For AP distance the DIC when including species as a fixed effect was 7763.82 compared to 7780.94 without species (ΔDIC: 17.12) (Table 6). A mixed-effects model of unique zones including species as a fixed effect of species had a DIC of 8338.21, while a model without had a DIC of 8344.88 (ΔDIC: 6.67) (Table 6). Differences in average behavior showed no apparent phylogenetic pattern (Table 7, Figure 6). *G. sigillatus* was most active when exposed to cues of predator presence (Table 7, Figure 6) and *G. assimilis* was shown to be the least active (Table 7, Figure 6). Similarly, *G. assimilis* visited the least number of unique zones during open field assays and *G. sigillatus* explored the most unique zones (Table 7, Figure 6).

Differences in Variances Among Species

The best fit model for unique zones was Model 4. Model 4 allowed both among and within-individual variances to vary between species. All other models were poorly supported (ΔDIC>8; Table 8). This indicates that both among- and within-individual variances differed among species in open field trials. For AP distance Models 3 and 4 fit comparably well (Table 8). Both of these models support differences among species in within-individual variances for AP distance. The difference between the models suggests mixed support for species differences in among-individual variances.

Neither the among- nor within-individual variances showed apparent patterns of phylogenetic relationships (Table 7 and 9, Figure 6). *G. assimilis, G. integer,* and *G. lineaticeps* exhibited the lowest among-individual variation for AP distance (Table 9, Figure 6), while *A. domesticus* and *G. sigillatus* showed the lowest for unique zones (Table 9, Figure 6). While *G. sigillatus* exhibited the highest among-individual variation in AP distance and *G. integer* showed the highest for unique zones (Table 9, Figure 6). *A. domesticus* and *G. sigillatus* had the lowest within-individual variation for AP distance and unique zones traveled, respectively (Table 10, Figure 6). *G. integer* also had the highest within-individual variation for AP distance and *G. assimilis* had the highest within-individual variation for unique zones (Table 10, Figure 6).

Differences in Behavioral Correlations Among Species

Among-individual behavioral correlations were of similar magnitude for *A. domesticus, G. assimilis, G. lineaticeps*, and *G. sigillatus* (Table 11, Figure 7) while the correlation for *G. integer* was estimated much higher (Table 11, Figure 7). Importantly, the lower bounds of the HPD intervals for *G. assimilis, G. integer, G. lineaticeps*, and *G. sigillatus* also overlapped with 0 (Table 11, Figure 7). Behavioral correlations at the within-individual level were similar for *A. domesticus* and *G. integer*, with G. integer having the lower bound of HPD intervals overlapping with 0 (Table 11, Figure 7). The overlapping of 0 can indicate that there is no behavioral syndrome connecting these behaviors in this species. Behavioral correlations at either level did not show any pattern of phylogenetic relatedness and were not significantly differ across species (Table 11, Figure 7). This suggests that behavioral correlations are genetically constrained at the species levels.

Table 5. Count of individuals by species and sex included in this study.

Species	Rep 1	Rep 2	Rep 3	Total
Acheta domesticus	281	263	225	769
Gryllus assimilis	16	16	16	48
Gryllus integer	91	91	74	256
Gryllus lineaticeps	23	16	13	52
Gryllodes sigillatus	50	50	49	149
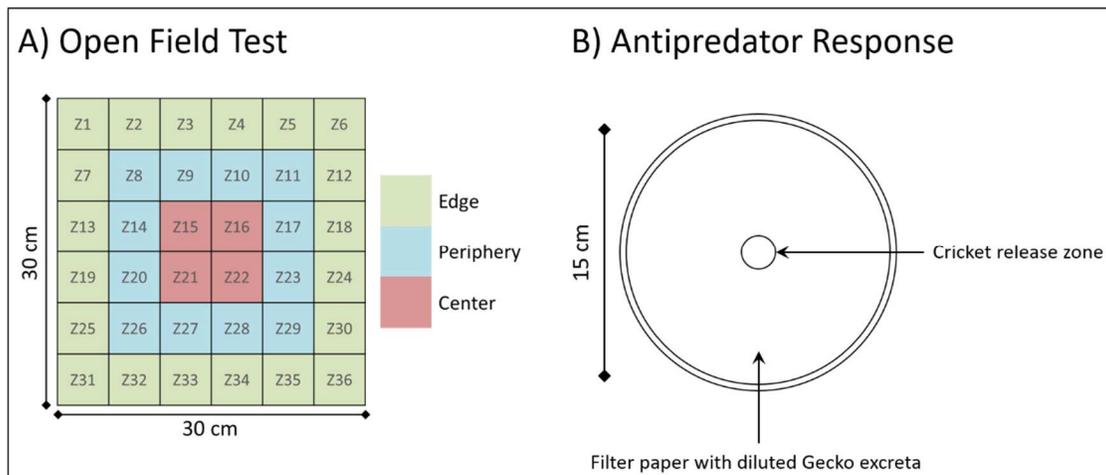
Figure 5. Arena designs for open field activity and antipredator trials. (A) Open field arena. Individuals were introduced into the bottom-left quadrant (Z31) and allowed to 220 seconds to explore the arena. Number of unique zones explored was measured during behavioral analysis. (B) Antipredator arena. Individuals were introduced into the center circle, which did not contain cues of predator presence, and then allowed to move freely through the arena for 220 seconds.

Table 6. DIC and ΔDIC values for models with and without species as a fixed effect.

Behavior	DIC with Species	DIC without Species	ΔDIC
AP Distance	7763.82	7780.94	17.12
UZ	8338.21	8344.88	6.67

Table 7. Average unique zones visited and antipredator distances by species along with upper and lower HPD bounds.

Gryllodes sigillatus
Gryllus lineaticeps
Gryllus integer
Gryllus assimilis
Acheta domesticus
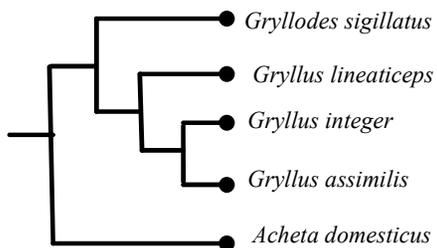
Species	HPD lower	Unique zones visited Posterior-modal values	HPD upper	HPD lower	AP distance Posterior-modal values	HPD upper
GS	22.01	**24.7**	28.28	515.15	**596.21**	677.4
GL	6.79	**10.15**	14.73	166.73	**275.48**	392.61
GI	7.59	**9.2**	10.87	368.4	**425.22**	485.26
GA	3.18	**5.21**	9.58	151.2	**240.81**	315.75
AD	17.62	**18.87**	19.76	264.22	**284.57**	312.02
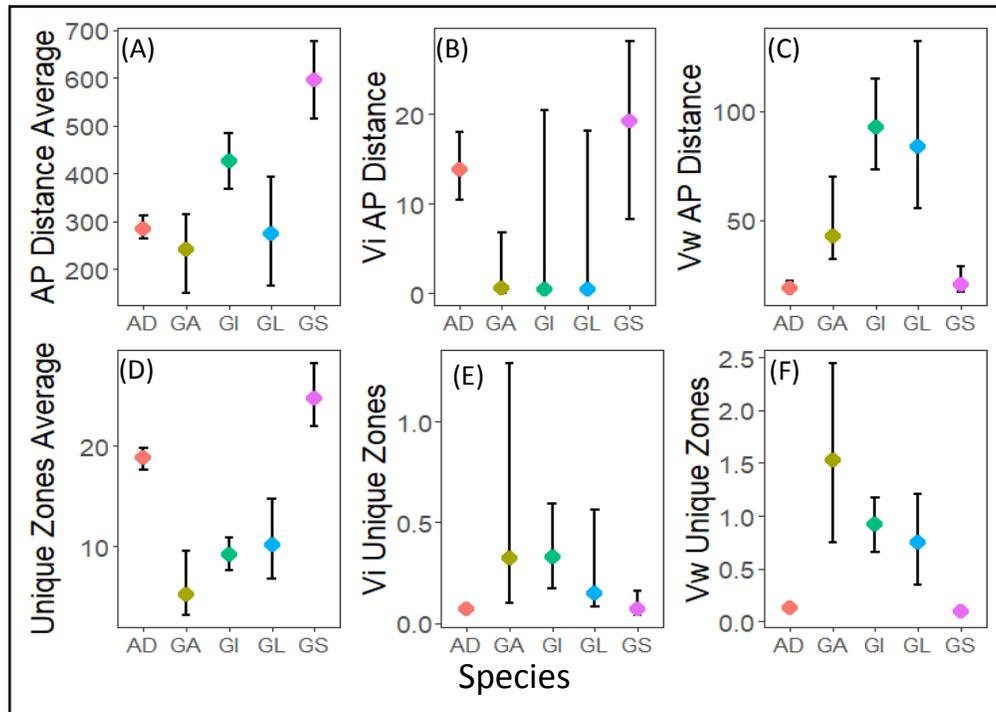
Figure 6. Species posterior-modal values with HPD credibility intervals. (A) Average antipredator behavior. (B) Among-individual variances in antipredator activity. (C) Within-individual variances in antipredator activity. (D) Average unique zones visited. (E) Among-individual variances in unique zones traveled. (F) Within-individual variances in unique zones traveled.

Table 8. DIC and ΔDIC values of model fit for AP distance and unique zones traveled.

	Behavior	DIC	ΔDIC
Model 1	AP Distance	8025.51	263.31
	UZ	8982.97	644.76
Model 2	AP Distance	8010.04	247.84
	UZ	8420.69	82.48
Model 3	AP Distance	7762.20	0
	UZ	8346.44	8.23
Model 4	AP Distance	7763.82	1.62
	UZ	8338.21	0

Table 9. Among-individual variances for unique zones visited and antipredator distances by species along with upper and lower HPD bounds.

Gryllodes sigillatus
Gryllus lineaticeps
Gryllus integer
Gryllus assimilis
Acheta domesticus
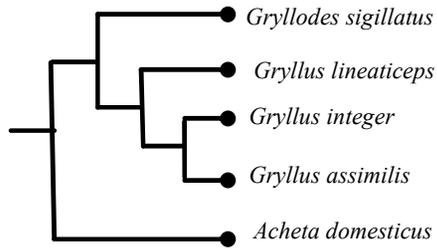
Species	HPD lower	Unique zones visited Posterior-modal values	HPD upper	HPD lower	AP distance Posterior-modal values	HPD upper
GS	0.04	**0.07**	0.16	8.30	**19.18**	28.17
GL	0.08	**0.15**	0.56	0.12	**0.47**	18.09
GI	0.17	**0.33**	0.59	0.10	**0.43**	20.43
GA	0.10	**0.32**	1.29	0.09	**0.54**	6.73
AD	0.05	**0.07**	0.09	10.38	**13.86**	18.04

Table 10. Within-individual variances for unique zones visited and antipredator distances by species along with upper and lower HPD bounds.
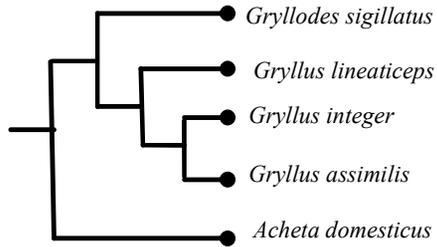
Species	HPD lower	Unique zones visited Posterior-modal values	HPD upper	HPD lower	AP distance Posterior-modal values	HPD upper
GS	0.06	**0.09**	0.13	16.78	**20.51**	28.75
GL	0.35	**0.75**	1.21	55.14	**83.57**	132.18
GI	0.66	**0.92**	1.17	73.33	**92.45**	114.54
GA	0.75	**1.53**	2.45	31.98	**42.72**	70.03
AD	0.11	**0.13**	0.16	16.66	**18.52**	21.79

Gryllodes sigillatus
Gryllus lineaticeps
Gryllus integer
Gryllus assimilis
Acheta domesticus

Table 11. Behavioral correlations of among-individual differences for all five species for unique zones visited and antipredator distances by species along with upper and lower HPD bounds. Behavioral correlations of within-individual differences for *G. sigillatus*, *G. integer*, and *A. domesticus* for unique zones visited and antipredator distances by species along with upper and lower HPD bounds. *G. lineaticeps* and *G. assimilis* correlations were not calculated due to behavioral trials not being performed in succession.
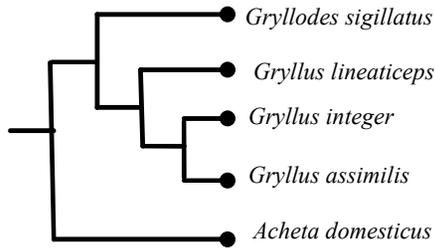
Species	HPD lower	Among-individual correlation Posterior-modal values	HPD upper	HPD lower	Within-Individual correlation Posterior-modal values	HPD upper
GS	-0.05	**0.30**	0.82	0.12	**0.35**	0.53
GL	-0.42	**0.49**	0.78	N/A	**N/A**	N/A
GI	-0.01	**0.66**	0.82	0.10	**0.10**	0.25
GA	-0.63	**0.29**	0.75	N/A	**N/A**	N/A
AD	0.21	**0.37**	0.54	0.08	**0.18**	0.28

Gryllodes sigillatus

Gryllus lineaticeps

Gryllus integer

Gryllus assimilis

Acheta domesticus
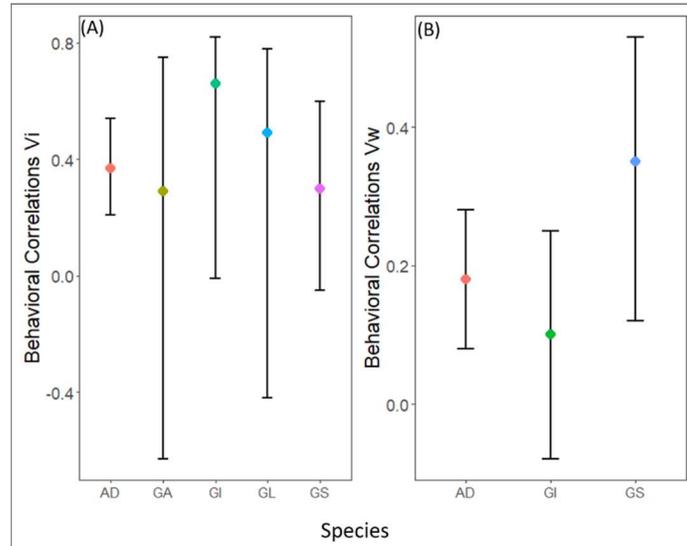
Figure 7. Species posterior-modal values with HPD credibility intervals. (A) Among-individual behavioral correlations of unique zones traveled and antipredator activity. (B) Within-individual differences of behavioral correlations of unique zones traveled and antipredator activity. Within-individual correlations for *G. assimilis* and *G. lineaticeps* were not calculated due to behavior trials not being performed in succession.

Discussion

Differences in Average Behaviors

Species differed from one another in average behaviors and variances and these differences were not consistent with patters of phylogenetic relationships. For example, the most distantly related species, *G. assimilis* and *A domesticus*, had the most similar values in AP distance (Table 7, Figure 6). *G. sigillatus* and *A domesticus* also showed the most similar variances at both levels for unique zones and *G. integer* and *G. lineaticeps* showed similar average within-individual variances for AP distance (Table 7, 9, and 10, Figure 6). Overall, there was no clear pattern relating the average measurements to phylogenetic relationships. This suggests that when expressing these behaviors individually, these species are not phylogenetically constrained. However, when looking at how these behaviors are being expressed relative to each other, behavioral correlations did not significantly differ among

species. This indicates that the joint expression of these behaviors is linked at the genetic level and in this manner are phylogenetically constrained.

Since the average expression of these behaviors are not clearly phylogenetically constrained among species, this suggest that these species are not genetically restricted in expressing different levels of behavior. The unrestricted expression of these behaviors have led to different average levels of expression for open field and anti-predatory behaviors of these species. When phenotypes are not being constrained, selection can act more freely upon the variation available and optimize behaviors for the current environment. With optimized behaviors being expressed in higher frequencies, this can lead to species having higher average fitness. Alternatively, the differences observed among species could be attributed to stochastic processes such as genetic drift. Drift would reduce the genetic variation of a trait and selection would act on whatever variation is still present, favoring the best suited behavior to an environment. The behavioral averages measured in this study could be the best available behavior in each environment and could be misinterpreted as indicative of different fitness optima. Regardless if stochastic processes are affecting behavioral averages, these behaviors are still not phylogenetically constrained, as a loss in genetic variation would still show conserved genetic contributions to a phenotype that would lead to this constraint on behaviors.

The ecological relevance of exploratory behavior has been well documented (Wilson et al. 1993, Dingemanse et al. 2002). Exploratory behavior is used when individuals are acclimating to a novel environment and attaining new information relevant to resource allocation, predation threats, and potential mating opportunities (Wilson et al. 2010). Optimized exploratory behaviors can allow species to more efficiently assess new environments and gain valuable resources related to reproductive fitness. Whether an environment is novel or not,

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assessing and responding to threats of predation is necessary for survival. The optimization of anti-predatory responses can increase surviving these threats, which can lead to more mating opportunities and positively affect fitness. Selection can act on specific anti-predator behaviors, e.g. amount of activity, and reduce variation around the level of expression that is most suited for a particular environment. For example, Binz et al. (2014) showed that wood crickets (*Nemobius sylvetris*) expressed different levels of activity when exposed to chemical cues of the presence of different predators. Crickets either increased or decreased activity levels based on the type of chemical predator cue they were exposed to. Exposure to chemical cues of native nocturnal predators resulted in a reduction of activity when compared to baseline activity levels. While being exposed to diurnal predator cues caused the wood crickets to increase activity levels. These contrasting anti-predator strategies were presumably driven by the hunting strategies of the specific predators. Quantitative genetic theory suggests stabilizing selection will continue to reduce the variation surrounding these alternative behavioral strategies, higher frequencies of optimized behaviors will be present in this species resulting in higher survival rates and leading to increased fitness opportunities.

Differences in Among-Individual Variation

The species we examined here also differed in among-individual variation in exploratory (unique zones) and anti-predator (AP distance) behaviors, but with no patterns of phylogenetic relationship (Table 9, Figure 6). Quantitatively, among-individual variation represents the combination of genetic and permanent environmental influences to a phenotype, e.g. behavior (Dochtermann et al. 2015). Differences observed in among-individual variation could be attributed to selection, or potentially genetic drift, acting upon these species by reducing the amount of variation among individuals in successive generations. This is caused by the reduction

of additive genetic variance that contributes to the phenotypic variation observed in these behaviors (Falconer 1960, Mousseau and Roff 1987). As mentioned above, the longer stabilizing selection acts upon a trait, additive genetic variation is reduced and intern slowing the strength of selection on that particular trait (Roff 1993). The amount of among-individual variation observed can indicate the length to which stabilizing selection has been acting upon a particular trait. Individuals of a species with low amounts of variation among individuals potentially have reached an optimized behavior for an extended number of generations and stabilizing selection has been lowering this type of variation for these traits. This may be the case particularly for *A. domesticus* and *G. sigillatus* for unique zones measured and for *G. assimilis, G. integer* and *G. lineaticeps* for AP distance. These species showed the lowest amounts of among-individual variation relative to the other species in this study (Table 9). Genetic drift may also have been a contributing factor for the low levels of among-individual variation observed in unique zones trials for *A. domesticus* as individuals in this study were measured from a captive population where successive inbreeding potentially could have taken place over multiple generations (Table 9). These species are also susceptible to founder effects (Ladizinsky 1985), resulting in a reduction in additive genetic variation. Furthermore, *G. sigillatus* showed the highest amount of among-individual variation for unique zones and *G. integer* for unique zones trials(Table 9, Figure 6). This can be potentially explained in the following ways; 1) stabilizing selection is not acting upon these traits for as many generations relative to the other species; or 2) Portions of our sample populations could have experienced permanent environmental effects, affecting these behaviors before being measured. This is most likely not the case due to the fact that individuals of these species were reared in the lab and experienced limited environmental variation among individuals.

Alternatively, environmental variability that contributed to any permanent environmental effects can increase amounts of among-individual variation along with the genetic variation captured in this level of variation. This would be predicted to particularly be the case for measurements of *G. assimilis* and *G. lineaticeps*, as they were measured directly from the field and any permanent environmental effects experienced would influence the among-individual variance measured. As mentioned previously, this is not the case according to our results, as they showed lower among-individual variation relative to the other species (Table 9, Figure 6). This indicates that permanent environmental effects were not a major contributor to increased variation among individuals.

Differences in Within-Individual Variances

Selection acts on the among-individual variation of species, while within-individual variation shows how individuals differ from themselves. Behavioral plasticity can be measured at the within-individual variation level. By repeatedly measuring individuals, any variation captured within the same individual is caused by a labile phenotype's interaction with an environment. This variation allows individuals to respond flexibly to an environment (Westneat et al. 2015). Individuals showing high levels of plasticity have the ability to be more adaptive to a changing environment by being able to alter aspects of their behavior to the current situation (Relyea 2001). This is primarily beneficial to individuals experiencing a frequently changing environment. For individuals in a stable environment, paying the energetic cost of showing variation in traits can have negative consequences on fitness (Snell-Rood 2012). Our results show no phylogenetic relationship relative to levels of within-individual variation measured (Table 10). While the range of plasticity expressed can be acted upon by selection (Scheiner and Callahan 1999) as mentioned above the cost of plasticity can also be selected for and individuals

who pay lower costs to be plastic can more easily vary in the expression of a trait (Snell-Rood 2013).

If behaviors are being constrained due to shared genetic components then closely related individuals should behave in a similar way. Although *G. assimilis, G. integer* and *G. lineaticeps* are three of the more closely related species in our phylogeny and show the highest levels of plasticity (Table 10, Figure 6), this relationship is not consistent throughout our results. This may indicate that these species are not being constrained by genetic contributions and are able to more freely expressed behaviors that are suited to their specific environments. By having high levels of variation at the within-individual level species are not as limited in expressing these behaviors to match an environment relative to the other species (Scheiner and Callahan 1999). Furthermore, the potential cause for showing higher levels of behavioral plasticity could be attributable to exposure to a frequently changing environment and selection favoring individuals able to show higher levels of within-individual variation (Relyea 2001).

Differences in Behavioral Correlations

Populations and species can exhibit suites of correlated behaviors across different situations, i.e. behavioral syndromes (Sih et al. 2004). Behavioral syndromes can have major evolutionary consequences by conserving less than optimal behaviors that are correlated with other aspects of behaviors that do increase reproductive fitness. For example, exploratory behaviors individually can be optimized to increase survival and indirectly increase fitness, but behavioral syndromes can affect other behaviors not initially measured with one another that negatively affect fitness, like aggression in mating behaviors (Sih et al. 2004). Traditionally these behaviors have been measured and assessed independently, but with the increased appreciation of behavioral syndromes, multiple aspects of behavior are being assessed in relation to one

another to identify any potential correlations of behavior that can limit the effectiveness of selection. The leading contributor to causing these suites of correlated behaviors is thought to be at the genetic level, more specifically genetic pleiotropy (Sih et al. 2004). As a single gene is expressed to affect one aspect of behavior, other behaviors are affected by the same gene expression.

Due to among-individual correlations not differing among species, our results support the constraints hypothesis (Dochtermann and Dingemanse 2013), i.e. the correlations of two behaviors acting as a constraint from either behavior reaching optimization (Schluter 1996), despite differing in variances and average expressions of behaviors (Figure 6). This indicates that for the expression of exploratory and anti-predatory behaviors, the range at which these behaviors can be expressed is potentially being constrained on the genetic level instead of responding to local selective pressures (Dochtermann and Dingemanse 2013). Even though individual behaviors are not apparently being constrained when comparing them to other closely related cricket species, behavioral syndromes can be present at a higher phylogenetic level and may not be optimized to maximize fitness (Sih et al. 2004). The genetic contributions to behavioral syndromes can limit the ability of species to diverge from one another in response to selective pressures (Dochtermann and Dingemanse 2013). Based on quantitative genetic theory, if these behavioral syndromes are stemming from pleiotropic effects, populations will be constrained to diverge along shared evolutionary pathways. The direction of this divergence will follow the covariation of traits that contain the most genetic variation, rather than solely fit to an environment (Schluter 1996). This is due to the fact that when a population is under the influence of a single fitness optimum, selection is not evolving in the direction of greatest fitness increase, but initially aligns in the direction with the greatest amount of genetic variation (Schluter 1996).

This is due to the fact that as adaptive radiation occurs selection favors the "path of least resistance" and larger amounts of genetic variation allows selection to more easily act upon that variation (Schuett et al. 2011). As selection reduces genetic variation in the expression of these correlative behaviors, the alignment should move in the direction of the fitness optimum, creating a curved path of adaptation (Figure 1 in (Schluter 1996)). Moreover, this correction in adaptive alignment caused by selection can be limited by the genetic mechanisms underpinning the correlations of these behaviors, i.e. pleiotropy (Royauté et al. 2019b). As a result, this evolutionary constraint will conserve these correlations among species containing less than optimal traits (Schluter 1996). Alternatively, if these genetic correlations were caused by previously favored or random trait combinations, e.g. linkage disequilibrium, the divergence of populations would be relatively unconstrained as the genetic correlations would quickly breakdown as selective pressures change, favoring genetic modifiers that alter or limit the joint expression of a gene and decouple this correlation (Roff 1997, Conner 2002, Sih et al. 2004, Saltz et al. 2017). Since the trait correlations in our study were consistent across species, this supports the inference that genetic pleiotropy is underpinning these correlations.

Conclusion

Differences in average expressions of behaviors and variances of these behaviors tell us how species differ genetically and environmentally from each other and from themselves. Different species might have optimized expression of behaviors to better maximize reproductive fitness in specific environments. Here, average behavioral response was not constrained suggesting that these species can adapt to their specific environment and optimize fitness when expressing these behaviors independently. Our species also showed differing levels of among-individual variation, possibly indicating exposure to differing levels of stabilizing selection

around a fitness optima. Species showing lower levels of among-individual variation can be an indicator that an optimal behavior has been reached and that stabilizing selection is acting upon this trait by removing genetic variation around the average expression of that trait. For species showing higher levels of plasticity (within-individual variation), individuals who show a greater variance in the expression of a behavior have the potential to be more flexible in the current environment. Finally, when looking at how behaviors are being expressed in relation to one another, correlations of behaviors do not significantly differ among species. This supports that these behaviors *are* being genetically constrained relative to one another across these different species. The correlations of behaviors can potentially impact other aspects of behaviors that can affect fitness.

Further, if entire species are phylogenetically constrained then behaviors may be relatively optimized to have the highest fitness possible given these constraints, but fitness may not be truly maximized in an environment. Overall, the species measured in this study significantly differed in average expression of behaviors in open field behavior and activity in the presence of a predator cue (Figure 6). They also differed in the within- and among-individual variances in these behaviors (Figure 6). They did not, however, significantly differ in among-individual (co)variances for all species and within-individual (co)variances for *A. domesticus*, *G. integer*, and *G. sigillatus*(Figure 7). Similar results were found in Royauté et al. (2019b), where populations of *G. integer* also differed in average behaviors and additive genetic variation of open field and anti-predator behaviors. The Royauté et al. (2019b) analysis did differ as their analysis was a (co)variance matrix comparison of the additive genetic variance attributing to these behaviors. Other studies comparing the behavioral correlations of multiple cricket species have also previously been performed. Blankers et al. (2017) compared the phenotypic variances

and (co)variances of 7 calling traits of multiple cricket species (including *G. lineaticeps*, which was included in our study). Their results showed that the phenotypic (co)variances differed among cricket species. This is contrary to the results of this study as here behavioral (co)variances did not significantly differ among cricket species. A potential explanation for these contradicting results could be because they measured the phenotypic expressions of these calling traits, which conflates multiple sources of variance (Royauté et al. 2015). In contrast, our study captured the within-individual variances that can help tease apart some of the conflated traits captured in phenotypic expressions. This is a novelty that has not previously been examined in species or population comparisons. For example, while behavioral (co)variances have been shown to be constrained (White et al. 2019), only the among-individual (co)variance matrices were compared among seven freshwater fish species and within-individual (co)variances were not estimated. In conclusion, our results suggest that even when average behaviors differ among species and are not apparently phylogenetically constrained, syndrome structure may still be preserved across species boundaries.

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