<u>**Life stage comparisons in visual cognition abilities in *Gryllus pennsylvanicus***</u>

Name: Isaiah Morrow

Student Number: 300052581

Course: BIO4009

Supervisor: Dr. Gabriel Blouin-Demers

In Memory of Dr. Julie Morand-Ferron

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Department of Biology

Faculty of Sciences

University of Ottawa

Abstract

Spatial cognition is how individuals perceive and process their environment to identify, remember and navigate to biologically important stimuli. There is interspecific and intraspecific variation in spatial cognitive abilities. A reason for this intraspecific variation is the age of the individuals. Older individuals may develop better cognitive abilities as they gain life experience with age. Using pairs of same and different coloured stickers indicating which branch of a Y-maze contained a water reward, I studied if *Gryllus pennsylvanicus* was able to use colour cue association and differentiate between identical and different colours to locate the reward and, if they could do so, if there was a difference in accuracy (number of correct attempts) or speed (time to complete each trial) between adults and juveniles of the same species. Out of the 24 individuals who completed the test, only 10 successfully completed the maze. This suggests that *Gryllus pennsylvanicus* can’t learn the colour cue association task. There were no statistically significant differences in accuracy or speed to complete the test between the age groups. Further research is needed to further understand the intraspecific variation in spatial cognitive abilities.

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I would like to acknowledge the many people who have helped me throughout this past year that was not only tumultuous but presented its own set of unique challenges that I had to overcome. First and foremost, I would like to dedicate this honours thesis to Dr. Julie Morand-Ferron, who despite her health challenges, decided to become my thesis supervisor and guide me throughout the year. I will always be grateful for the opportunity she provided me. Her knowledge and the guidance she gave me throughout the short period we knew each other was immeasurable. She ensured that I felt on the same level as the rest of the laboratory group and gave credit where credit was due. With her passing, I hope that this thesis and the further work I'll be accomplishing in her laboratory may be used to honour her as a person and the scientific work she has brought to the realm of animal behaviour. I will always remember the meetings we had together even just a mere weeks before her unfortunate passing.

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Third, I would like to thank Dr. André Cyr who I worked with closely throughout the year to develop the experimental design and find ways to incorporate it into his overall project on cricket cognition and concept learning. Our frequent phone calls to brainstorm solutions to problems we encountered, discuss future steps, and discuss our results were very insightful. His eagerness and excitement for the project were contagious. Lastly, thank you to my friends and family that helped keep me motivated throughout this year despite the bumps in the road. I am infinitely

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Introduction

The study of cognition could be referred to as one of the founding pillars of animal behaviour. According to Shettleworth (2001), cognition is defined as “all ways in which animals take in information through the senses, process, retain and decide to act on it”. More specifically, cognition can be divided into subsets, including spatial cognition or spatial learning. Spatial learning is “the process through which animals encode information about their environment to facilitate navigation through space and recall the location of motivationally relevant stimuli” (Floresco, 2014). Organisms capable of processing their environment, recognizing spatial cues and understanding their relative position to other environmental stimuli have many fitness advantages such as being able to find and return to food/water sources more easily, to return to and from their home, avoid predators, locate mates, migrate to and from different habitats based on season and resource availability, etc. (Vorhees & William, 2014). Spatial learning and navigation are so important to the survival of living organisms that behaviours related to these spatial cognitive abilities can even be found in more ancestral phyla such as insects, notably ants and bees (see Wittlinger *et al*, 2006; Henry *et al*, 2012). Also in other invertebrates, where *Orconectes rusticus*, a species of crayfish, can remember their way through a maze, with a smaller number of errors (incorrect turns) after every attempt (Tierney & Andrews, 2012). Spatial cognitive abilities also vary in efficiency through life as animals adapt new life strategies and engage in more detailed exploration abilities (Shettleworth *et al*, 1988; Berger-Tal *et al*, 2014; Stephens & Dunlap, 2017). For example, in mountain chickadees, food-based information was not mentally updated as frequently in older individuals, as long as that information remained predictable (Benedict *et al*, 2021).

Outside of social insects (such as the Hymenopterans) and vertebrates, however, very little research has been done on spatial learning. I studied Orthopterans, more specifically *Gryllus pennsylvanicus*, the field fall cricket. One way of testing spatial learning in organisms is through the colour cue association test (see Brown & Gass, 1993). The colour cue association test involves coloured cues within a complex environment (in my study, a Y-shaped maze with colour cues in each branch) with a certain correct colour leading to a reward (motivationally relevant stimuli, water in my study). This test is thus designed to study how animals use cues in their environment to return to a certain reward. In prior studies using species of the *Gryllus* genus, Wessnitzer *et al* (2008) demonstrated that *G. bimaculatus* could remember where ideal temperatures were located in an open field test with a stronger accuracy after each attempt. Doria *et al* (2019) showed that *G. texensis* can return to the location where a food reward was located previously using relevant visual cues in a radial arm maze. In this study, I will be using four stickers, with two of the same colours in one branch indicating the reward branch and two of two different colours indicating the incorrect branch to test whether *G. pennsylvanicus* can distinguish between the colours in this increased complexity colour cue association test.

I expect that *Gryllus pennsylvanicus* will be able to successfully complete the colour cue association test. More specifically, I predict that the adult individuals will complete the maze test more precisely overall with fewer mistakes (number of trials where they entered the incorrect branch) than the juvenile individuals. I also predict that adult individuals will successfully complete the trials of the test more rapidly than their juvenile counterparts.

Materials and Methods

Species

Gryllus pennsylvanicus, commonly referred to as the fall field cricket, is a species of cricket widespread across southern Ontario (Capinera *et al*, 2004). It is mostly found in fields, forest edges, grassy disturbed areas and human habitats where they are capable of burrowing in the soil (Tennis, 1983). *G. pennsylvanicus* is a solitary opportunistic omnivore and feeds on vegetation as well as other invertebrates (Carmona *et al*, 1999; Burgess & Hinks, 1987).

For this study, adults and juveniles had to be distinguished for the sake of comparing their spatial association learning capabilities. Juveniles were individuals between 10 and 20 mm in length with no wing development (besides the presence of wing buds). These juveniles are typically 1-3 months of age in the lab. Adults are individuals over 20 mm in length with full development of wings and ovipositor (in the case of females). Adults are 3+ months old in the lab and live approximately 6 weeks in captivity once attaining maturity.

Pre-Trial Captivity

Before the Y-maze experiment could begin, *G. pennsylvanicus* were isolated for 48 hours without access to water. Since 5 μL of water is used as the reward, this isolation period ensured that the reward was enticing enough for the individuals to complete the seven trials. This time span was used as the standard isolation period to prevent complete desiccation and death, which occurred at a higher frequency (nearly 100% of the attempts) when the individuals were deprived for 72 hours. Each cricket was isolated individually in a circular transparent plastic container with a mesh lid. The container had a diameter of 11 cm, a circumference of 37 cm and a depth of 8 cm. Each container contained a 1.75 cm high dish of Harlan Teklad's Rodent Diet (8604 meal food) as well as a piece of egg carton for shelter. The isolation typically began at noon and ended at approximately the same time two days later. The containers were placed in the same area of the room where the trials were conducted. Only uninjured individuals were used in the study. The adults and juveniles that were used for the study came from larger colony bins. These colonies were in plastic bins (Rubbermaid totes, 45 cm x 55 cm x 40 cm, L x W x H) with multiple Harlan Teklad's Rodent Diet dishes, water vials, egg cartons and a sand dish for egg-laying. There were approximately 100 crickets per adult colonies and 200 crickets per juvenile colony.

<u>Experiment Setup</u>

To be able to test colour cue association learning, a Y-maze with removable stickers was used. The Y-maze was made of white plastic with a clear plastic cover to be able to see into the maze during the tests. Four mazes were used and alternated between each other between every single trial of the colour cue association test. The stickers were placed as seen in Figure 1. Avery Brand Colour Coding Labels were used for the test. Each sticker was 0.635 cm in diameter. Blue and green stickers were used for the trial as the yellow stickers were not visible in the dark lighting (blended into the white) and the red stickers weren't viable as insects generally do not see reds very well (Color Vision, n.d.). Two stickers were placed at the entrance of each branch, approximately 1 cm apart and 1 cm into the branch. Two stickers of the same colour (either two blue or two green stickers) in the same branch indicated the branch containing the water reward. Half of the individuals conducted the test with two blue stickers indicating the reward branch and the other half conducted the test with two green stickers indicating the reward branch. This was to account for possible one colour being more visible than the other to the crickets. 5 μL of water was placed approximately 3 cm from the end of the correct branch. The other branch was empty besides for the two stickers of two different colours (blue and green).

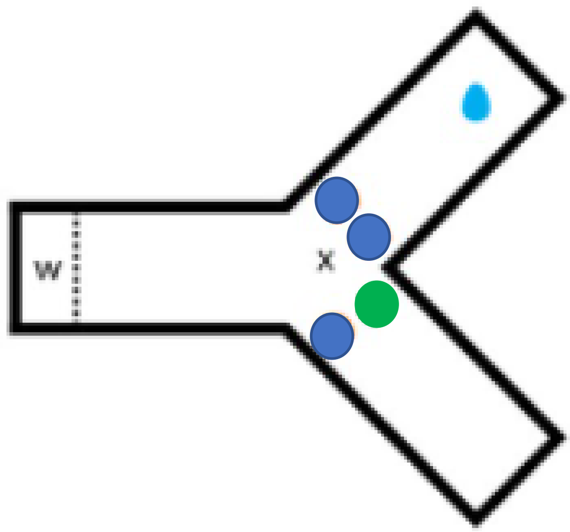

Figure 1. Schema of the Y-Maze and sticker placement for the colour cue association learning test for *G. pennsylvanicus*. The W represents the starting chamber, the blue dot is the water reward, and the 4 coloured dots are the stickers. The Y-maze had a length of 21 cm from starting point to the end of one of the branches with each branch having a length of 13 cm from the entry point to the end of the branch. The maze corridors have a width of 2.5 cm with the width of the end chambers being 5 cm. The maze had a depth of 10 cm.

Before the start of the trial, the cricket was carefully transferred to the starting chamber of the maze by using a plastic straw. The starting chamber was separated from the rest of the maze by a clear plastic divider. Once the cricket was transferred to the starting chamber, the maze was placed in the testing area where it could be recorded from the top down using a webcam and the free Open Broadcaster Software (OBS, https://obsproject.com/). Once the maze was placed correctly and no longer touched, the cricket went through a 3-minute habituation period to the new environment. Once this period was up, the colour cue association test could begin. The clear plastic divider was lifted and the timer (also being recorded by OBS) was started. Each individual had 15 minutes per trial to reach the water reward. The first branch that the cricket entered was noted (either left or right) as well as the time needed to reach the water reward. If the cricket entered the branch without the reward, the test continued until they found the water reward. Once the cricket started drinking the water, the trial was over, and the cricket was transferred to a new maze and the next trial began for a total of 7 trials. If the cricket did not leave the starting chamber after two trials, they were omitted from the results. Each maze was used once and afterwards the stickers were removed and the maze was sprayed with 70% ethanol to clean it. New stickers were added, and the maze was able to be reused again. The cleaning between trials was necessary to prevent possible scents or pheromones from causing a bias in the results. The branch containing the water reward and the two stickers of the same colour would alternate every trial to help dissuade any motor memory from occurring.

The room used for the isolation and the colour cue association test had an average temperature of 24.7 ± 0.88 C°, an average humidity level of 31.7 ± 4.47% and an average light intensity of 24.7 ± 13 lux. Measurements were taken at the beginning of each set of trials and can be found in Table S1. Each test was run midday between 10:00-14:00.

Analyses

Overall test success was defined as any individual who could enter the correct branch on the first try, three trials consecutively. This definition was required as from a biological perspective, it would be improbable for the cricket to pick the correct branch all 7 trials. They may no longer be thirsty or may want to explore the other branches of the maze for other possible rewards. Lower than 3 consecutive correct branch selections in a row was not possible to define success as it could be more likely due to chance that the cricket selected the correct branch twice. However, with 3 consecutive correct branch selections, it can be more ascertained that the cricket did in fact learn the colour cue association test.

To test for whether there was a difference between success rate and life stages (age), I will conduct a binomial regression generalized linear model. A binomial model was selected as there was only two outcomes to the dependent variable. Success will be the dependent variable with age group being the independent variable. Temperature, humidity and light intensity were covariates while sex and cue colour were fixed factors included in the model. To test the time differences between age groups, I will conduct a t-test between the successful and unsuccessful groups as well as a two-factor ANOVA with age and success as the two factors to see if there are any differences between the life stages of *G. pennsylvanicus*.

Results

Out of the 24 individuals who completed the test (omitting individuals who died during the isolation period or did not leave the starting chamber after 2 trials), 10 completed the Y-maze test successfully which gives a proportion of approximately 0.42. Dividing it among age groups, 5 adults and 5 juveniles out of 12 individuals in each age group completed the test successfully. Therefore, the proportion of adults, as well as juveniles, that completed the test does not differ from the overall proportion of successes at 0.42 for adults and juveniles respectfully.

After completing the GLM binomial model, age group was not significant, and neither were any of the other factors (Table 1). The generalized variance inflation factor was measured for every variable to account for multicollinearity to which no variables gave a GVIF value over 2. Backward stepwise regression was used to determine the most fitting model (Table 2). The final model of success ~ humidity was not significant. The success ~ sex + humidity model had the lowest AIC value of 30.712, albeit the simpler model had an AIC of 31.065, which means both models are of similar probability. In the model containing sex and humidity, the p-values of both variables were 0.1624 and 0.069 respectively while in the model containing only humidity, the p-value was 0.0939 (coefficient estimate = 0.5224).

Table 1. Summary of the full generalized linear model binomial regression. The full model was success ~ age + temperature + humidity + light intensity + sex + cue colour. This model was used to test if success rate in completing the Y-maze was a function of age (adult or juvenile) in *Gryllus pennsylvanicus*. The coefficient estimate, standard error, z value and p value are presented. This model serves as the starting point for the backward stepwise regression.

Variable	**Coefficient Estimate**	**Standard Error**	**Z Value**	**P-Value**
Age	0.99175	1.58381	0.626	0.5312
Temperature	0.33329	0.88688	0.376	0.7071
Humidity	0.77849	0.45567	1.708	0.0876
Light Intensity	-0.02605	0.05094	-0.511	0.6090
Sex	-2.29103	1.54380	-1.484	0.1378
Cue Colour	-0.41764	1.15269	-0.362	0.7171

Table 2. Stepwise regression of the GLM binomial model with success as the dependent variable. The backward stepwise regression began with the full model (success ~ age + temperature + humidity + light intensity + sex + cue colour) and the variable with the highest p-value was removed first. This method was used to determine whether age or another variable may become significant to explain success rate differences in completing the Y-maze in *Gryllus pennsylvanicus*. ANOVA (Chi-squared) test was used to compare the fits of the new model to the old model until a suitable model was found. The coefficient value, standard error, p-value, z-value of each variable being removed from the backward stepwise regression are presented. The deviance and p-value for the ANOVA comparing the old model and the new model without the variable indicated in the same row is also presented.

Backward Regression	**Coefficient Value**	**Standard Error**	**Z-Value**	**P-Value**	**ANOVA**	
					Deviance	**Pr(>Chi)**
Cue Colour	-0.41764	1.15269	-0.362	0.7171	0.13245	0.7159
Temperature	0.28569	0.88410	0.323	0.7466	0.10722	0.7466
Light Intensity	-0.01352	0.04285	-0.316	0.7523	0.1004	0.7513
Age	0.3787	1.0522	0.360	0.7189	0.13218	0.7162
Sex	-1.8859	1.3498	-1.397	0.1624	2.3526	0.1251

To determine if there's a relationship between the average time of trial completion and success rate as well as compare the speeds of the different age groups and between the successful and unsuccessful individuals, the average time to complete the 7 trials for each individual was taken and plotted based on age group and whether they successfully completed the test or not. In Figure 2, the time average for the 7 trials was plotted based on whether the individual succeeded in completing the test. The t-test between both groups indicated no statistically significant difference ($p = 0.2109$). In Figure 3, the average time to complete the Y-maze is plotted based on the age and whether they were successful or unsuccessful. Neither age ($p = 0.06499$), success (p= 0.22374) nor the interaction between both (p = 0.52335) were significantly different after completing the ANOVA.

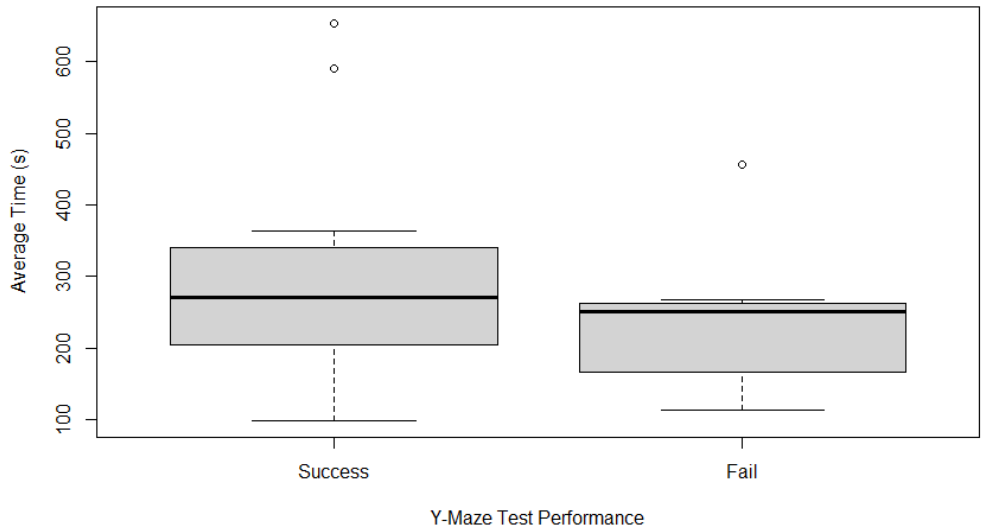

Figure 2. Average time (in seconds) to complete the 7 trials as a function of Y-maze test performance. Individuals that were deemed "successful" picked the correct first branch of the Y-maze containing the water reward 3 or more times consecutively. Individuals that failed the test were those who did not pick the correct first branch to enter to obtain the water rewards 3 or more times consecutively. The medians of the groups are represented by the black line with the 1st and 3rd quartiles indicated by the gray box. The whiskers indicate the maximum or minimum values that don't exceed 1.5 of the interquartile range. There is no statistical significance between both groups when it comes to average time differences.

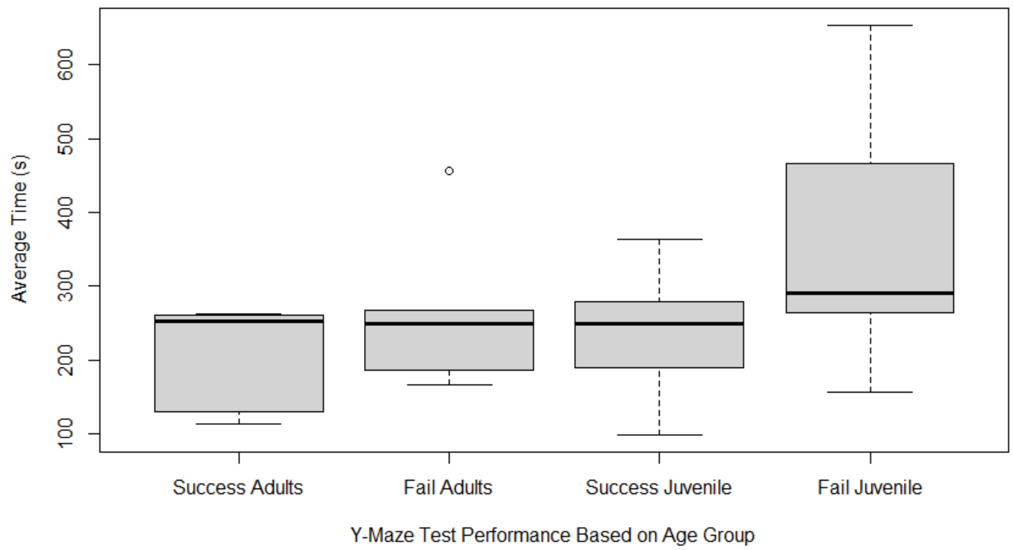

Figure 3. Average time (in seconds) to complete the 7 trials as a function of Y-maze test performance and age group. Individuals that were deemed "successful" picked the correct first branch of the Y-maze containing the water reward 3 or more times consecutively. Individuals that failed the test were those who did not pick the correct first branch to enter to obtain the water rewards 3 or more times consecutively. *Gryllus pennsylvanicus* individuals were categorized into their respective age groups based primarily on morphological traits (size, absence of wings, absence of ovipositor, etc.). The medians of the groups are represented by the black line with the 1st and 3rd quartiles indicated by the gray box. The whiskers indicate the maximum or minimum values that don't exceed 1.5 of the interquartile range. There is no statistical significance between both groups when it comes to average time differences.

Discussion

Based on the results summarized above, I can conclude that *Gryllus pennsylvanicus* were unable to complete the Y-maze colour cue association test nor was there any time differences between juveniles and adults. The final effect size is rather small for a typical behavioural study. I would require over 60% of the individuals to be successful to conclude that *Gryllus pennsylvanicus* could learn the simple visual pattern association task. This threshold is based on literature of similar spatial cognition/concept learning studies such as the honeybee Y-Maze concept learning study conducted by Avarguès-Weber *et al* (2012) presented in the introduction. It is also possible that the general linearized binomial model didn't have a strong enough statistical power with the current sample size to detect a meaningful effect size.

Since insects of the *Gryllus* genus are typically generalist hemimetabolous insects (Niwa *et al*, 1997), a possible explanation that could help to elucidate why there is no difference in spatial cognition capabilities between adults and juveniles is that even though they are at different life stages, their fundamental behaviours are still similar. Contrary to holometabolous insects that undergo a serious morphological (and often behavioural change) from juvenile to adult (Wiegmann *et al*, 2009), hemimetabolous insects typically have the same feeding and social behaviour as well as "personality traits" between juvenile and adults (Amat *et al*, 2018). This would thus be evidence that there's no need for spatial cognitive differences between adults and juveniles in the *Gryllus* genus as they need to process and understand their environment to the same extent to find the same biological stimuli (for example: food source, den, water source, etc.).

Albeit none of the results for this study were statistically significant, there may be some indicators of other mechanisms underlying the intraspecific variation in spatial cognition abilities. Outside of the 10 individuals who seemingly did use the colour cues to determine the correct branch of the maze containing the water reward, there may be evidence of *G. pennsylvanicus* individuals using other strategies such as spatial/motor memory to determine which branch of the maze contained the water reward using some form of "mental map". In Table S2, which summarizes the results of each test, some of the individuals that consecutively entered the incorrect branch first seemed to favour that kind of cognitive strategy to remember where the water reward was located. Since the trial didn't end until the individual found the water reward (regardless of what branch they picked), the crickets may have learned based on some form of spatial memorization of the maze, beginning from the starting point all the way to where the water reward was located. They thus headed back to that same branch based on this "mental map". However, since the water reward alternated between the left and right branch every trial, they entered the wrong first branch every trial as the branch they were choosing was where the water reward was located only in the previous trial. Another possible explanation is that they used items in the laboratory to orient themselves instead of the colour cues. However, the maze was deep enough that it would be highly unlikely for the crickets to see outside of the test chamber. Even though my study design and data collection method weren't designed to study this type of spatial cognition strategy, this may be indicative of a new research path to follow to elucidate the details surrounding how non-Hymenopterans perceive their environment and memorize key biological details within it. In other non-Hymenopteran species of insects, the same kind of visual memory strategy was seen to be used in hoverflies (Collett & Land, 1975), in *Parastrachia japonensis*, a species of shield bug (Hironaka *et al*, 2008), and in *Drosophila melanogaster* (Ofstad *et al*, 2011), among others, when it came to returning to or finding either specific areas of biological importance or a biological stimulus. Not to mention the *Gryllus bimaculatus* study that was used in the introduction of this thesis which indicated that the species could remember ideal temperature spots in an open field test which would be indicative of having some form of spatial memory abilities (Wessnitzer *et al*, 2008). Since this spatial cognition strategy is found in not only orthopterans but many other families, it may be possible that this strategy is more advantageous than simply relying on differentiating colour cues/patterns in their environment. However, more research is required to determine which strategy is favoured more in *Gryllus pennsylvanicus*.

A biological reason to explain the absence of overall success to complete the Y-maze test in this species could be that the colony bins are nowhere near as intricate as the natural conditions that *Gryllus pennsylvanicus* would typically be living in. They have a very simple, stable and predictable environment in the bins with easy access to food and water and thus it may be possible that they never developed the full extent of their spatial cognitive abilities as this kind of environment is not as cognitively demanding as a more complex wild environment. Complexity and quality of the environment has been linked to improved cognitive performance in many animals (Kotrschal & Taborksy, 2010; Pollen *et al*, 2007; Tebbich *et al*, 2010) and thus it may be interesting to compare wild-caught versus captive-raised *Gryllus pennsylvanicus* individuals as the environment they are raised in could be the mechanism that allows them to develop the visual cognition abilities required to complete the Y-maze colour cue association test.

In the general linearized binomial model, humidity seemed to be another mechanism that may have impacted the performance of the crickets during the tests. Even though humidity never became statistically significant throughout the backward stepwise regression, the p-value was always near or approaching significance throughout the model fitting. This means that there might be some possible correlation between an increasing success rate and an increase in humidity levels. As the crickets go through a 48-hour period without access to water, a possible explanation for this would be that the higher levels of humidity allow the cricket to withstand the effects of the lack of water more effectively during the tests and thus allows it to perform overall more precisely.

Conclusion

To summarize, I did not demonstrate that *Gryllus pennsylvanicus* can complete the colour cue association Y-Maze test. A small sample size may have restricted me from seeing the desired effect size. The results of this study are still useful as it helps eliminate a possible cause for intraspecific individual variation in *Gryllus pennsylvanicus*. Further research should focus on using new concepts and study designs to further understand the basis of cognition in this species as well as study the differences in spatial cognition between wild-caught versus captive-raised *Gryllus pennsylvanicus*

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Supplementary Material

Table S1. Temperature, humidity and light intensity of the test room at the start of each set of trials (test) conducted per life stage. The temperature (Celsius) and humidity (%) was recorded using a digital home use thermometer/hygrometer. The light intensity (lux) was measured using the Lux Light Meter app for Android devices. The light intensity was measured directly where the Y-Maze was placed to conduct the testing.

Life Stage	Test #	Temperature (C°)	Humidity (%)	Light Intensity (lux)
Juvenile	1	24.3	31	53
	2	25.5	30	47
	3	25.5	30	47
	4	24	30	36
	5	24.1	30	19
	6	25	30	19
	7	25	50	19
	8	25.2	29	10
	9	24.9	32	11
	10	23.8	31	13
	11	23.8	28	11
	12	25.2	29	11
Adult	1	25.5	30	19
	2	25.7	30	19
	3	26	28	10
	4	23.6	33	11
	5	25.2	32	11
	6	25.5	32	11
	7	25.5	32	11
	8	25.2	32	11
	9	25.3	33	18
	10	25.9	31	13
	11	22.3	25	11
	12	24.8	27	11

Table S2. Results of the colour cue association Y-Maze test separated by life stage, either adult or juvenile. The colour of the two stickers used to indicate the correct branch are indicated (B = blue and G = green) as well as the sex of the cricket (M= male and F = female). Note that a 1 means that the cricket first entered the correct branch containing the water reward and that a 0 means that they did not pick the correct branch on their first try.

Life Stage	Test #	Colour	Sex	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7
Juvenile	1	B	F	0	0	1	0	1	0	0
	2	B	F	1	0	1	1	1	0	1
	3	B	M	0	1	0	1	0	0	0
	4	B	F	0	0	1	0	0	0	0
	5	B	F	0	1	1	0	1	1	1
	6	G	F	1	0	1	1	1	1	1
	7	G	F	0	1	1	1	1	0	0
	8	G	M	0	1	0	1	0	1	1
	9	G	M	1	0	1	0	1	0	1
	10	G	F	1	1	1	0	0	0	0
	11	B	F	1	0	1	0	1	0	1
	12	G	F	1	0	1	0	0	0	1
Adult	1	G	F	1	1	0	1	1	0	0
	2	G	F	1	0	1	1	1	0	1
	3	G	F	0	0	1	0	1	0	1
	4	G	F	1	1	1	1	1	0	1
	5	G	F	0	1	0	0	0	1	0
	6	B	M	1	0	0	1	0	1	1
	7	B	F	0	0	1	0	1	1	0
	8	B	M	1	1	0	1	1	1	0
	9	B	F	0	1	1	1	0	0	0
	10	B	F	1	0	0	0	1	1	1
	11	G	F	1	0	0	1	1	0	0
	12	B	F	1	0	1	0	1	1	0