

# **SEX DIFFERENCES IN CUE USE DURING PLACE LEARNING IN TÚNGARA FROGS**

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## **ABSTRACT**

Robert E. Ventura: Sex differences in cue use during place learning in túngara frogs  
(Under the direction of Sabrina S. Burmeister)

The adaptive specialization hypothesis posits that differences in cognitive demands between sexes can arise in response to differential requirements to solve ecological problems. In this thesis, the túngara frog is considered due to behavioral differences between males and females observed during mate choice, possibly leading to the development of advanced cognitive abilities in females to allow for them to accurately assess males. A previous study found support for this hypothesis, with females having learned to associate a red cue with the exit of a maze, while males relied on inconsistent left or right (egocentric) cues. These experiments retested this hypothesis, eliminating egocentric cues and solely providing red and yellow visual cues. Males appeared to learn at an equivalent rate as females, although a preference for red cues over yellow cues emerged, and differences in errors seemed to hint that males were not learning as effectively as females.

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## TABLE OF CONTENTS

<b>LIST OF FIGURES</b> .....	vii
<b>CHAPTER 1: INTRODUCTION</b> .....	1
<b>CHAPTER 2: MALE FROGS EXHIBIT LEARNING IN THE ABSENCE OF EGOCENTRIC CUES</b> .....	6
<b>Summary</b> .....	6
<b>Materials and methods</b> .....	6
(a) Animals.....	6
(b) Apparatus .....	7
(c) Procedure.....	8
(d) Statistical Analysis .....	9
<b>Results</b> .....	10
(a) Individual Success without Error .....	10
(b) Group Performance .....	11
(c) Differences in Performance by Cue .....	12
(d) Differences in Position Errors by Cue.....	12
(e) Evaluation of Potential Boldness Effects .....	13
<b>Discussion</b> .....	13
<b>CHAPTER 3: ELIMINATION OF CUE BIAS BY USING MONOCHROMATIC CUES</b> .....	25
<b>Summary</b> .....	25
<b>Materials and methods</b> .....	25

(a) Animals.....	25
(b) Apparatus .....	26
(c) Procedure.....	26
(d) Statistical Analysis.....	27
<b>Results.....</b>	<b>27</b>
(a) Individual Success without Error .....	27
(b) Group Performance and Sex Differences.....	28
(c) Probe Trials .....	28
(d) Sex Differences in Errors.....	29
(e) Evaluation of Potential Boldness Effects .....	29
<b>Discussion.....</b>	<b>29</b>
<b>CHAPTER 4: CONCLUDING REMARKS &amp; FUTURE DIRECTIONS .....</b>	<b>38</b>
<b>REFERENCES.....</b>	<b>41</b>

## LIST OF FIGURES

Figure 2.1. Photograph of two-arm maze apparatus .....	17
Figure 2.2. Success rates of males vs. females .....	18
Figure 2.3. Success rates of red cue vs. yellow cue trained frogs.....	19
Figure 2.4. Success rates of male frogs (red cue vs. yellow cue) .....	20
Figure 2.5. Success rates of female frogs (red cue vs. yellow cue) .....	21
Figure 2.6. Mean position errors of red cue vs. yellow cue trained frogs .....	22
Figure 2.7. Mean position errors of female frogs (red cue vs. yellow cue).....	23
Figure 2.8. Mean position errors of male frogs (red cue vs. yellow cue).....	24
Figure 3.1. Photograph of two-arm maze apparatus .....	32
Figure 3.2. Success rates of males vs. females .....	33
Figure 3.3. Proportion of time of frog location during probe trials .....	34
Figure 3.4. Mean position errors of males vs. females .....	35
Figure 3.5. Non-contingent error rates of males vs. females .....	36
Figure 3.6. Example monochromatic cues for potential future studies .....	37

## CHAPTER 1: INTRODUCTION

In the 19<sup>th</sup> century, Charles Darwin famously mused about the bright plumage of male peacocks and their ostentatious fan-like tails, wondering why females lacked any such coloration. In doing so, Darwin proposed the now well-known theory of sexual selection, suggesting that the progression of these sex differences occurred in response to evolutionary change. Put simply, the variation of physical characteristics between females and males arises in concert with the development of female preferences for particular male traits. The implications of this theory have had widespread implications on the field of evolution and has led to substantial research on the costs and benefits of sexual selection. In fact, the explosion of evolutionary research that has occurred during the 20<sup>th</sup> and 21<sup>st</sup> centuries has greatly improved our understanding of the factors that contribute to and result from sexual selection and sex differences. These factors include genetic correlations between certain traits and fitness (Hedrick & Temeles, 1989), modalities used by females to assess mate quality (Gibson & Langen, 1996), and differences in parental care roles between sexes (Fairbairn *et al.*, 2007). However with each new finding, there are often several more questions raised, along with the issue that sexual selection is an extremely complex process with several interacting variables to account for when studying it. This thesis focuses on cognitive differences between sexes that may arise due to sexual selection, and how these variations may lead to differences in learning and behavioral abilities within a species.

The adaptive specialization hypothesis is the accepted explanation for differences in cognitive ability that can develop between animals under differential environments and/or

demands for cognition (Gaulin & Fitzgerald, 1997; Pravosudov & Clayton, 2002; Dalla & Shors, 2009). A well-known example of this hypothesis is displayed in the black-capped chickadee (*Poecile atricapilla*), which inhabit the greater part of North America. Separate populations of these birds from Alaska and Colorado show significant differences in brain size and cognitive ability, with chickadees from Alaska having larger hippocampal volumes and displaying a more efficient and expansive memory than chickadees from Colorado. This is presumably a result of the climatic differences and an increased requirement for Alaskan chickadees for storing and remembering food caches to return to during lengthy winters (Pravosudov & Clayton, 2002, Croston *et al.* 2015). Additionally, in the polygamous meadow voles (*Microtus pennsylvanicus*), males exhibit an increase in spatial memory over females due to the natural behavior of expanding their home range during breeding season, balancing energetic risks with reproductive rewards (Gaulin & FitzGerald, 1989). However, female meadow voles and monogamous prairie voles (*M. ochrogasters*) do not show this behavior and perform more poorly on spatial memory tasks in comparison to male meadow voles (Gaulin & FitzGerald, 1989; Gaulin, FitzGerald & Wartell, 1990).

As displayed by meadow voles, it is possible for the adaptive specialization hypothesis to explain differences in cognitive ability between sexes due to differences in mating behavior and mate choice decisions (Gaulin & FitzGerald, 1989; Gaulin, FitzGerald & Wartell, 1990), with these differences leading to variable requirements for cognitive abilities between males and females. Given the vast implications of effective mate selection by females, which includes direct individual female survival and fecundity due to the particular male they select, as well as the indirect genetic consequences of choosing “good genes” to pass on to their offspring, it is understandable why cognitive abilities may develop in the face of these decisions. As such, the

need for individuals to assess mate quality or attract members of the opposite sex in some manner is pervasive in animals. Male bowerbirds (*Ptilonorhynchus violaceus*) that are capable of building more complex courtship displays have higher mating success, and in turn perform better on cognitive tasks than males who are less successful in mating and build less complex displays (Keagy, Savard & Borgia, 2009, 2011) and many males in anuran species display differential mating success due to qualities in male vocalizations (Arak, 1983).

Most recently, it has been proposed that the natural differences in male and female behaviors of the túngara frog (*Physalaemus (=Engystomops) pustulosus*) could be responsible for sex differences in place learning ability (Liu & Burmeister, 2017). Túngara frogs' mating behaviors rely on the female's pursuit, assessment, and eventual choice of males, with males remaining stationary in a breeding pond and broadcasting their individual breeding calls (Ryan, 1985). This behavior is expected to depend on the increased ability of females to remember the locations or patterns of calls of particular males to make mating decisions (Ryan, Akre, & Kirkpatrick, 2009). It is known that female túngara frogs use a variety of modalities in order to assess and locate mates (Wilczynski, Rand & Ryan, 1995; Cummings *et al.*, 2008; Taylor & Ryan, 2013) and are able to remember locations of specific males using these modalities (Akre & Ryan, 2010). Due to these noted behavioral differences between male and female túngara frogs, it is suggested that over time, female túngara frogs have adapted to have greater place memory and cognitive abilities than their male counterparts.

In order to investigate this possible occurrence of the adaptive specialization hypothesis, it is necessary to evaluate the cognitive skills of túngara frogs. Anurans are known to be able to use visual cues to navigate artificial environments (Daneri *et al.*, 2011, 2015), as well as using geometric differences (Sotelo *et al.*, 2015) and patterns (Liu *et al.*, 2016) in order to spatially

orient themselves to a goal. Most notably, female túngara frogs have been shown to use a cue-taxis strategy in order to associate a visual cue with a goal to escape a two-arm maze more effectively than male túngara frogs, who appear to rely on egocentric cues (or body-centered turning cues) (Liu & Burmeister, 2017). Given how place memory may be more useful to female túngara frogs over males during the mating process, this result makes sense in context. As previously mentioned, females assess several males before returning to a final mating choice, a task that likely requires memory and the awareness of a male's various cues in order to choose and navigate to a particular male (Ryan, 1985; Akre & Ryan, 2010). Males, on the other hand, do not rely on this ability to learn or remember another animal's cues at any point in their lives.

Research pertaining to cue use in amphibians is largely unexplored, but navigation using egocentric cues is generally thought to be the simplest learning strategy employed by animals and has been observed in most major vertebrate groups (Schmajuk *et al.*, 1980; Rodriguez *et al.*, 1994; Day, *et al.*, 2003). It has been suggested that there is a tendency within vertebrates to use egocentric cues rather than place memory of visual cues when the two cues are conflicting (Daneri *et al.*, 2011). Given that túngara frogs have no apparent demands related to spatial navigation, often only observed in animals exhibiting territoriality, food caching, or repeated return to immobile offspring during parental care (Brodbeck, 1994), it is expected that if a sex difference in túngara frogs were to be observed, it would be in either an egocentric or cue-taxis strategy.

The results of Liu & Burmeister (2017) are therefore consistent with the hypothesis that females have adapted to possess enhanced place memory abilities as compared to males. This explains why females displayed a superior ability in place learning by successfully learning to exit a two-arm maze in the presence of inconsistent egocentric cues. However, the preference

exhibited by male frogs to use egocentric cues over visual cues is puzzling and could be considered in two different ways. It is possible that male túngara frogs may rely on egocentric cues either due to an inability to use a cue-taxis strategy, or possibly due to an inability to eschew egocentric cues when they are presented in combination with visual cues.

The first experiment conducted in fulfillment of this thesis (Chapter 2) aimed to determine how male performance might be altered in the absence of inconsistent egocentric cues. It was hypothesized that females would be able to once again utilize a cue-taxis strategy as seen in Liu & Burmeister (2017), while males would either employ a similar strategy in the absence of egocentric cues and exhibit an ability for place learning, or fail to learn the task entirely, demonstrating a reliance on egocentric cues to display learning. In Chapter 3, unexpected inconsistencies in colored cue preference were addressed, with the goal of observing both males and females exhibiting a cue-taxis strategy without any bias displayed towards learning either of the visual cues.

## **CHAPTER 2: MALE FROGS EXHIBIT LEARNING IN THE ABSENCE OF EGOCENTRIC CUES**

### **Summary**

In a direct follow-up to Liu & Burmeister (2017), sex differences during place learning in the túngara frog were explored using a two-arm maze. In an attempt to retrospectively understand the previously observed interaction of egocentric and visual cues, this experiment utilized the exact visual cues used in the prior study while removing egocentric cues, releasing frogs in the maze randomly rather than facing left or right. Both sexes displayed evidence of having learned the task of approaching either the red or the yellow door. The group success rate surpassed 80% and a majority of individuals achieved success without error in at least six out of nine consecutive trials. No significant interaction was observed between sex and day on success rate in a repeated-measures ANOVA, indicating that there was no sex difference in place learning. This result also makes sense due to the lack of statistical differences in mean success rates observed between sexes. Frogs trained to exit via the red door performed significantly better than those trained to exit via yellow, and this difference was more obvious in males than females. This could suggest that in the absence of egocentric cues, frogs exhibit a previously unanticipated preference to the red door.

### **Materials and methods**

#### (a) Animals

For this experiment, 24 sexually mature túngara frogs (12 males, 12 females) naïve to any behavioral experiments were used. These animals were 2-6 generations removed from native

populations collected in Panama. Animals were maintained in conditions similar to their native tropical climate at 23-25 °C and 65-75% relative humidity (RH) at a 12:12 light:dark cycle (lights on at 0800 hours). They were separated into 12 same-sex terraria (22 × 14 × 13 cm) and we distinguished between two frogs in one terrarium by applying colored non-toxic nail polish to the frogs' dorsal sides. The frogs were fed fruit flies (*D. melanogaster*) dusted with calcium and vitamins three times per week. The University of North Carolina's Institution for Animal Use and Care Committee approved all procedures (protocol 14-026).

#### (b) Apparatus

Frogs were trained in a two-arm maze constructed from white fiberboard (Figure 2.1). Each arm was approximately 19 cm by 8 cm and attached to a central chamber (18 cm by 21 cm). The walls of the maze were 9 cm high, and at the end of each arm was a 9 cm by 9 cm door with either red or yellow poster board taped to it. Door colors were chosen based on preference test data and evidence of discrimination of red and yellow in túngara frogs from Liu & Burmeister (2017). The incorrect door in each trial was held immobile by a brick, and the correct door was able to be slowly slid out of the maze with a string to reveal three shelters that would be used to transport the frog back to its home terrarium once it had exited the maze. The maze was also covered completely with a pane of glass during trials to prevent frogs from escaping over the maze's walls and surrounded with a 1.4 m high white curtain to prevent any external cues from being used by the animals. Given that the frogs' preferred environments are shaded and moist, the maze was kept as a bright, hot and dry environment (approximately 35°C and 10-20% RH) in order to motivate the frogs to exit the maze. This was done by placing the maze on a light table to provide a bright, shadow-free environment and placing space heaters behind each door for the first seven days of trials, which were then moved to either side of the central

chamber for the remainder of the trials. A laboratory soaker paper was placed over the light table and replaced after each day of trials to ensure that olfactory cues could not be used.

### (c) Procedure

#### *Acclimation*

Frogs were acclimated to the environment of the maze before acquisition trials began with 2 trials per day over 2 consecutive days. During acclimation, both red and yellow doors to the maze were removed, leaving both arms open to a covered shelter. The frog was released in the middle of the starting chamber at a random orientation and given three minutes to exit the maze via either arm and enter a shelter, upon which the frog would be transported back to its home terrarium. If the frog had not exited the maze after three minutes, a small straw would be used to gently nudge the frogs from under the maze walls (in order to leave the glass on top of the maze) towards the nearest exit.

#### *Acquisition*

On the day immediately following the conclusion of acclimation, the frogs began training with three trials per day. Frogs were trained for 16 consecutive days and trained in the same order (subjects 1-24 consecutively) for all three trials, with an inter-trial interval of approximately 45 minutes. With both exits of the maze closed off, half of the frogs (6 males and 6 females) were trained to exit via the yellow door, with the other half being trained to exit via the red door. The maze itself remained in the same orientation on the light table for the duration of acquisition, but door location was alternated every trial in relation to the maze to ensure that frogs were responding to the color of the door rather than any other cues that may have been associated with the maze location. Frogs were gently corralled from their home environment into a small cup, which was held upside down with a small piece of posterboard on the bottom. The

entire cup was then placed in the center chamber and lifted up at the beginning of each trial to release the frog, and due to the unknown position of the frog at the time the cup was lifted, the frog always began the trial at a random orientation in relation to the maze exits at the beginning of each trial. Frogs were given three minutes to approach the exit of the maze, and a trial was recorded as successful if the frog approached the correct door to within approximately 2 cm within the allotted three minutes. If the frog failed to enter either of the arms within three minutes, the trial was recorded as a non-contingent error. A position error was recorded each time the frog approached the incorrect door to within approximately 2 cm during the trial. Success without error was also recorded and defined as the frog approaching the correct door within 3 minutes without committing a position error. At the conclusion of three minutes, the trial was recorded as unsuccessful, the correct door was opened and the frog was gently nudged in the correct direction if it had not moved towards the exit 30 seconds after the conclusion of the trial. In every trial, frogs were only transported from the maze back to their home cage after entering one of the shelters at the end of the correct arm of the maze.

#### (d) Statistical Analysis

In order to quantify learning for the purpose of ANOVA testing, the mean number of successful trials per day, or success rate, was calculated. Only the first 10 days of training were analyzed due to the evidenced decline in success in both sexes after day 10. Position errors and non-contingent errors were also analyzed for any insight into learning patterns. A repeated-measures ANOVA was used to analyze the effects of door color (“cue”) and sex on success and errors, in addition to analyzing the effect of day on success to determine whether success rates significantly improved across days. Due to the fact that success rates are proportions that are not normally distributed and therefore violate that assumption of ANOVA, an arcsine transformation

was used on the success rates, as well as the rates of non-contingent errors. In the case of position errors, which unlike non-contingent errors, could occur more than once during a trial, no data transformation was done since the data was determined to be normally distributed via a Q-Q plot. In order to assess whether success rate improved over time for each sex individually, linear models were used to observe within-subject effects.

All data was initially recorded in Microsoft Excel and then uploaded in R Studio (Version 1.1.442, R Foundation for Statistical Computing, Vienna, Austria) to conduct repeated-measures ANOVA and linear modeling.

## **Results**

### (a) Individual Success without Error

Over the 16 days of acquisition trials, 17 of 24 frogs (7 of 12 males and 10 of 12 females) were able to approach the correct door without committing a position error in at least 6 out of 9 consecutive trials. In other words, a majority of the frogs trained in this experiment displayed the ability to orient to their trained door color in at least 66.7% of trials over three consecutive days at some point over the 16 days of acquisition. However, this proportion of success without error only equates to a p-value of 0.17 in comparison to random chance (50%) for a two-choice task, at which level we cannot readily say that the behavior occurred due to learning rather than chance (binomial test:  $n=9$ ,  $x = 6$ ,  $p = 0.17$ ). Only 7 of 24 frogs (4 of 12 males and 3 of 12 females) were able to approach their correct door without error in at least 7 out of 9 consecutive trials, but at this level, we can more significantly say that these frogs indicated the ability to learn (binomial test:  $n = 9$ ,  $x = 7$ ,  $p = 0.07$ ). Only 3 of 24 frogs (1 of 12 males and 2 of 12 females) were able to approach their correct door without error in at least 8 out of 9 consecutive trials, and this behavior undoubtedly indicates the ability to learn over indicating random chance (binomial

test:  $n = 9$ ,  $x = 8$ ,  $p = 0.02$ ). All frogs that were able to achieve success without error at least 6 out of 9 consecutive trials did so between days 6 and 12 of acquisition.

### (b) Group Performance

As a whole, group performance appeared to improve following the moving of the heaters from behind each door to the sides of the central chamber on day 7. We cannot say that the frogs' behavior as a group in terms of success without error was significantly different from random chance, as the peak of 61% on day 9 did not reach the criterion of 70% required for a difference from 50% with  $p = 0.05$  and 24 subjects (one-proportion z-test,  $n = 24$ ). However, the effect of day on success rate was significant, suggesting that the behavior of the animals as a group was altered in response to experience (repeated-measures ANOVA, day:  $F_{9,207} = 2.230$ ,  $p = 0.022$ ; linear trend:  $F_{1,23} = 3.785$ ,  $p < 0.001$ ). In addition, males reached a peak group success rates of 0.89 on day 9 and females reached a peak group success rate of 0.83 on day 10 (Figure 2.2). Both of these peak success rates surpassed the threshold of 0.79 required for a significant difference when comparing to 0.5 (random chance) given a sample size of 12 in both males and females (one-proportion z-test,  $n = 12$ ,  $p = 0.045$ ). However, the data suggests that the effect of sex on this change in success rate by day is not significant (repeated-measures ANOVA, sex  $\times$  day:  $F_{9,207} = 1.193$ ,  $p = 0.301$ ). This result is also supported by analyzing the effect of day on success rate individually for each sex, with both males (repeated-measures ANOVA, day:  $F_{9,99} = 1.717$ ,  $p = 0.095$ ; linear trend:  $F_{1,9} = 5.159$ ,  $p < 0.0001$ ) and females (repeated-measures ANOVA, day:  $F_{9,99} = 1.706$ ,  $p = 0.097$ ; linear trend:  $F_{1,9} = 3.001$ ,  $p = 0.001$ ) displaying a fairly significant effect of day on success.

### (c) Differences in Performance by Cue

There was an unexpected effect of cue on success rate, suggesting that the success rates of frogs trained to the red door were significantly higher than for those trained to the yellow door (repeated-measures ANOVA, cue:  $F_{1,207} = 9.946$ ,  $p = 0.002$ ; linear trend:  $F_{1,9} = 38.72$ ,  $p < 0.001$ ) (Figure 2.3). The effect of the interaction of day and cue on success rate was not significant (repeated-measures ANOVA, day  $\times$  cue:  $F_{9,207} = 1.327$ ,  $p = 0.224$ ), which can be interpreted as animals trained to both cues exhibiting similar increases of success across day. In addition, the effect of day on success rate can be analyzed individually for each cue. Frogs trained to exit the maze via the red cue (repeated-measures ANOVA, day:  $F_{9,99} = 2.122$ ,  $p = 0.034$ ; linear trend:  $F_{1,9} = 2.007$ ,  $p = 0.035$ ) and frogs trained to exit via the yellow cue (repeated-measures ANOVA, day:  $F_{9,99} = 2.125$ ,  $p = 0.034$ ; linear trend:  $F_{1,9} = 2.132$ ,  $p = 0.025$ ) both displayed a significant effect of day on success.

A fairly significant effect on success rate was also seen due to the interaction between sex and cue (repeated-measures ANOVA, sex  $\times$  cue:  $F_{1,232} = 3.330$ ,  $p = 0.069$ ). This result can also be understood in the context of Figures 2.4 and 2.5, which illustrate that the success rates within males trained to respond to the red cue and males trained to respond to the yellow cue (Figure 2.4) seems to differ more in comparison to the success rates within females trained to the red cue versus the yellow cue (Figure 2.5).

### (d) Differences in Position Errors by Cue

The interaction of day and cue appeared to have a significant effect on the number of position errors committed (repeated-measures ANOVA, day  $\times$  cue:  $F_{9,207} = 2.474$ ,  $p = 0.011$ ), which can be interpreted in concert with the appearance that frogs trained to the yellow door committed more position errors than frogs trained to the red door (Figure 2.6). Analyzing the

effect of the interaction between day and cue on position errors committed in females reveals a fairly significant result (repeated-measures ANOVA, day  $\times$  cue:  $F_{9,80} = 1.892$ ,  $p = 0.065$ ), and it appears that females trained to the yellow door committed substantially more position errors than females trained to the red door (Figure 2.7). The effect of the day and cue interaction in males is decidedly not significant (repeated-measures ANOVA, day  $\times$  cue:  $F_{9,80} = 0.984$ ,  $p = 0.459$ ), and it seems that males committed similar numbers of position errors regardless of the door color they were trained to (Figure 2.8).

#### (e) Evaluation of Potential Boldness Effects

In order to account for animals who may have exhibited decreased boldness in exploring the maze, I removed animals that committed a non-contingent error on the first day of trials (6 females and 4 males), as well as animals that committed more than 10 non-contingent errors over the 10 days of trials analyzed (2 females and 4 males). After replicating the repeated-measures ANOVA for both conditions, an interaction between sex and day was still not observed when frogs that committed a non-contingent error on the first day were removed (repeated-measures ANOVA, sex  $\times$  day:  $F_{9,99} = 1.111$ ,  $p = 0.362$ ), as well as this interaction not being observed for frogs that committed an average of more than 1 non-contingent error per day (repeated-measures ANOVA, sex  $\times$  day:  $F_{9,140} = 1.109$ ,  $p = 0.3604$ ). Evaluating these modified datasets further did not reveal any significant trends, and based on these comparisons, it was determined that the complete dataset with all 24 subjects was the most accurate method of analysis.

## **Discussion**

This experiment showed that túngara frogs indeed learned the task of place learning in the two-arm maze, based on the 17 of 24 frogs that were able to approach the correct door without error in at least 6 out of 9 consecutive trials over the course of acquisition. Given our

care in only exposing the frogs to the red and yellow cues, preventing them from using anything else to orient to the exit of the maze, we can say that this behavior is likely evidence of the túngara frogs demonstrating an ability to place learn. Especially considering that 4 males and 3 females out of 24 frogs total were able to achieve 7 successes without errors in 9 consecutive trials, which is a criterion of  $p = 0.07$ , it seems that at least at the simple level of the frog as a species exhibiting the ability to learn, it is possible.

Based on these individual proportions of success without error and the similar group mean success rates for males and females, it is clear that there were no sex differences observed in place learning. This was also supported by the repeated-measures ANOVA, which revealed that the interaction between sex and day didn't have a significant effect on success. However, the interaction between sex and cue supported evidence for an effect on success rate, suggesting that males and females had differential success in learning red versus yellow cues.

Effects of the interaction of day and cue on the average number of position errors per day were also observed. The repeated-measures ANOVA suggests that females trained to the yellow door committed more position errors over the course of the experiment than females trained to the red door. This effect was not seen in males, with no evidence that the interaction of cue and day had an effect on the number of position errors committed.

In whole, given the effect of the interaction between sex and cue on success rate, males appeared to be more successful when trained to the red door, possibly hinting at a preference for red. The interaction between day and cue did not have an effect on position errors in males, while females trained to yellow appeared to make more position errors than females trained to red. Therefore, the preference for red was seen in both sexes, but males make position errors

regardless of cue, hinting at a strategy of simply approaching a door, either red or yellow, until being rewarded with exit.

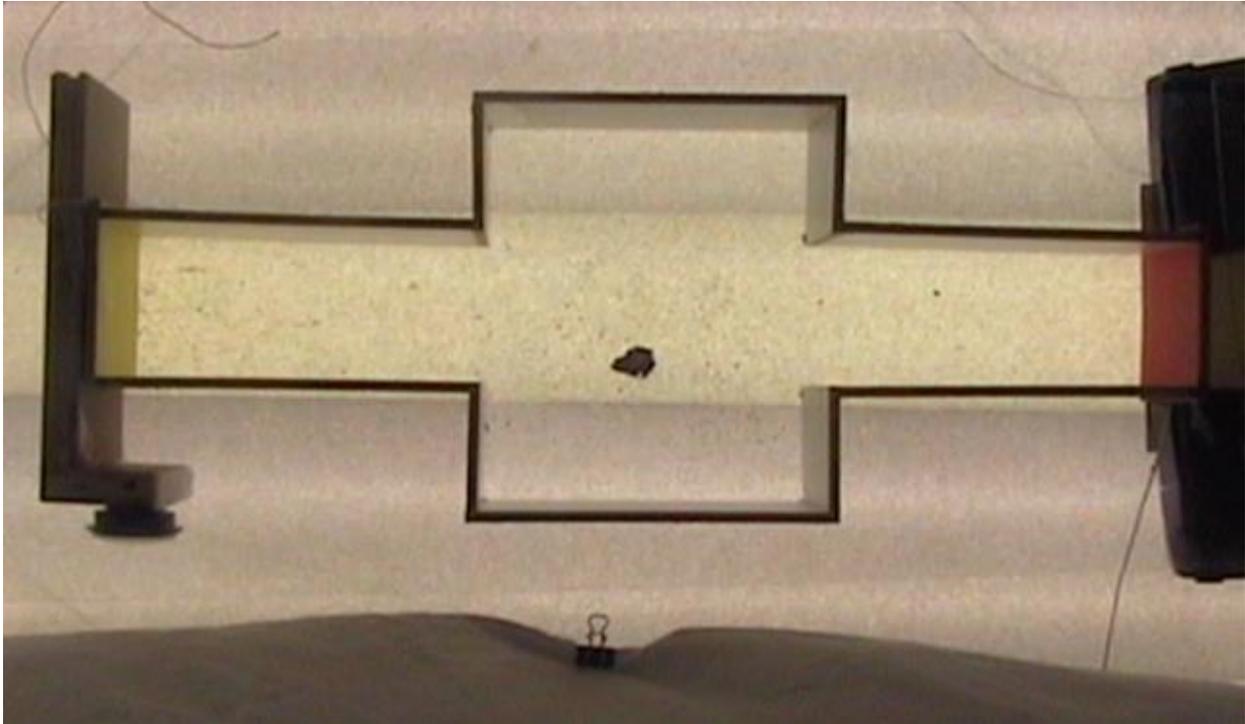
Previous evidence had suggested the presence of an adaptive difference in place learning between male and female túngara frogs (Liu & Burmeister, 2017). This particular study was complete in assessing initial differences in how the frogs behaved in the presence of both red and yellow doors by using a preference test, which statistically showed equivalent interactions with both colors by the frogs. However, during acquisition, frogs were only trained to the red door, and until the experiments described here were conducted, túngara frogs had only been proven to learn a task using visual cues by approaching a red door.

The results of this experiment exhibit no discernable difference between males and females in their ability to learn to escape the maze using solely visual cues, and túngara frogs displayed evidence of learning to approach both red and yellow cues. The differences in performance and position errors within male and female túngara frogs trained to each cue are understandably perplexing and seem to suggest that possible sex differences may have more to do with color preference and increased male impulsivity rather than a decreased ability to learn. Based on the evidence described here, it appears that male túngara frogs had relied on egocentric cues when presented due to an inability to ignore those egocentric cues, rather than due to an inability to utilize a cue-taxis strategy.

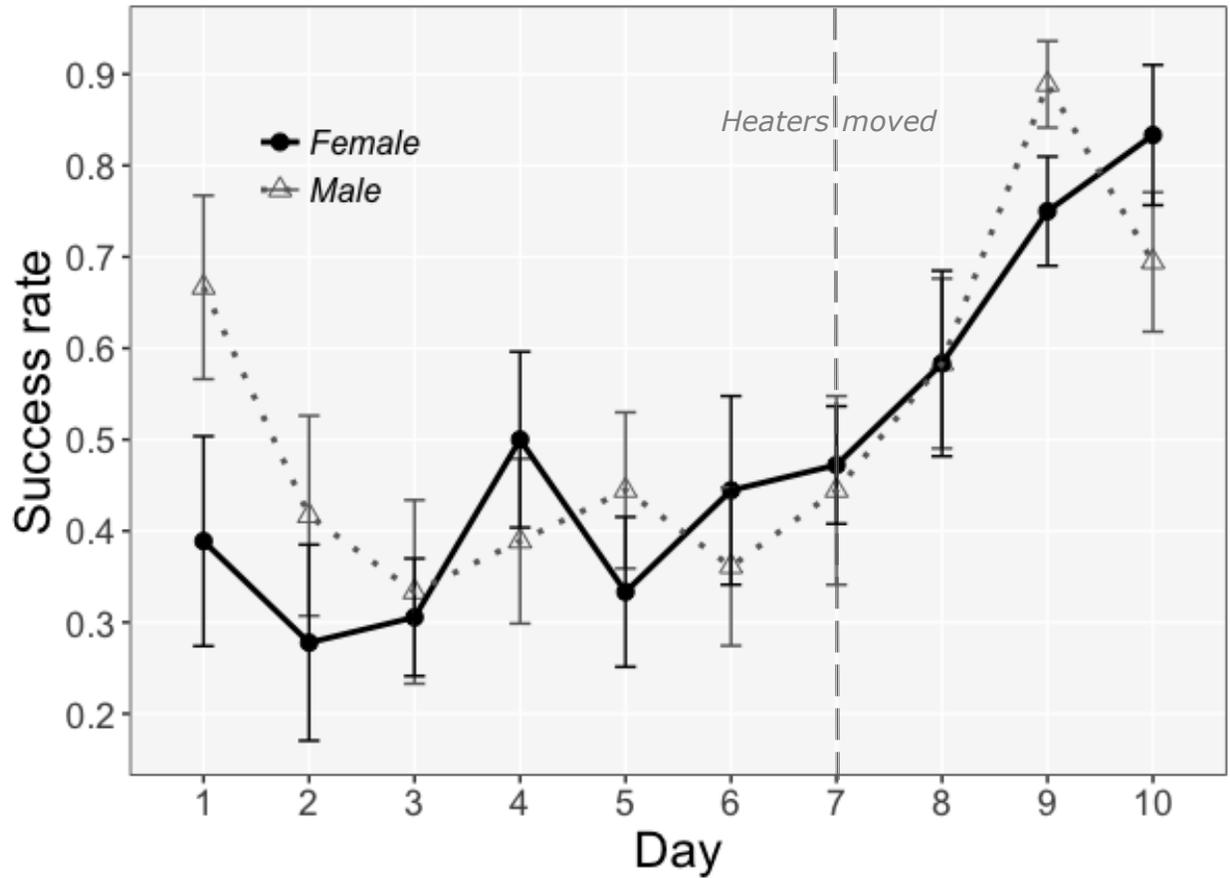
The observed differences in performance may have emerged due to several factors, but one factor that has appeared to differ between the abilities of males and females in both Liu & Burmeister (2017) as well as this study is the ability for female frogs to suppress a tendency to approach a random door within the maze soon after being released in the starting chamber in comparison to males. This ability of females may be a result of greater behavioral flexibility

(Ragozzino & Rozman, 2007) or lower levels of impulsivity, but the inhibition of these position errors exhibited by females might have led to the perceived sex differences in learning. Male amphibians have previously been seen to depend on egocentric cues in the Argentine toad (Daneri *et al.*, 2011). In addition, several studies in rats have suggested that egocentric cues may be a precursor to place learning abilities (Knierim *et al.*, 1998; Jeffery & O’Keefe, 1999). Overall, these results suggest that, compared to males, females have greater selectivity in a visually cued place-learning task and that these abilities may be due to a decreased impulsivity in females.

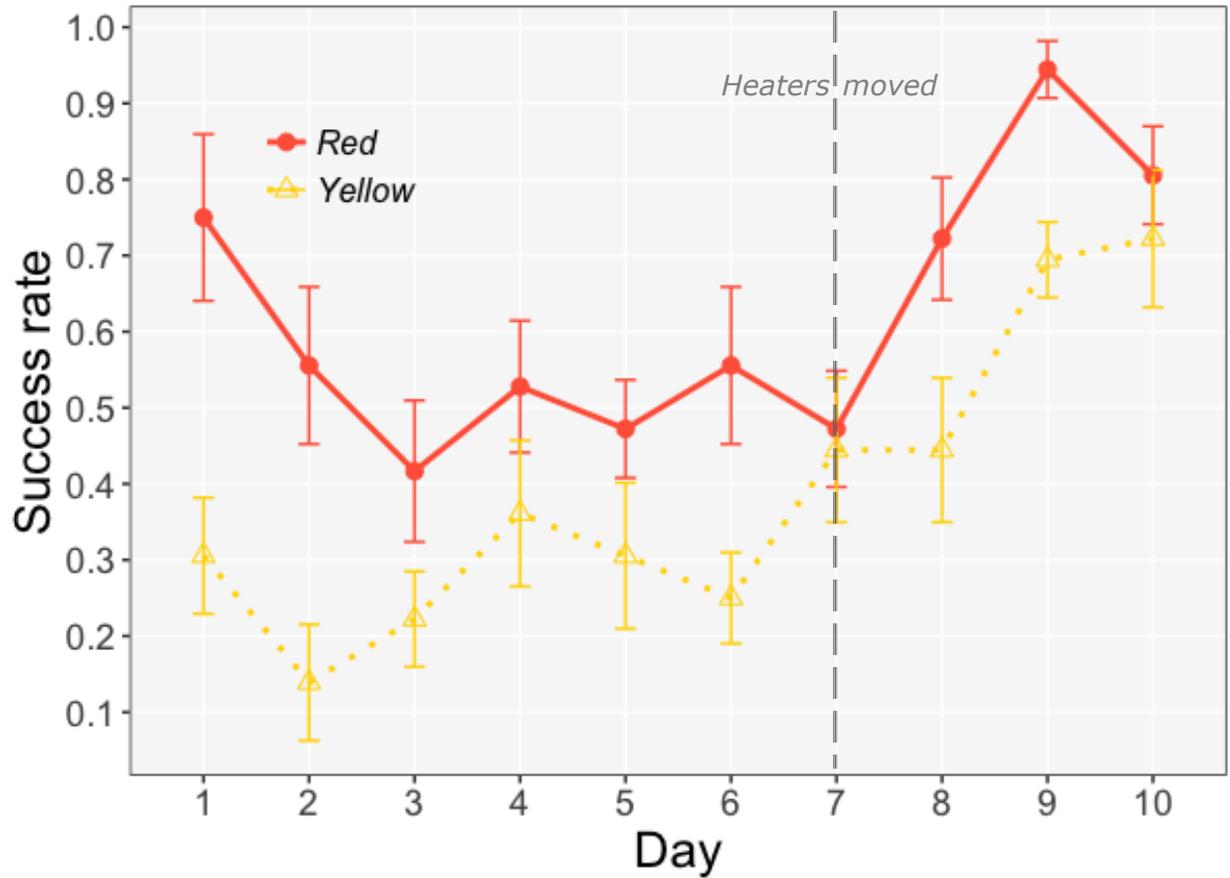
The few seemingly minor differences in experimental setup between this experiment and Liu & Burmeister (2017) may have also possibly contributed to some of the patterns observed in our data. Our experiment utilized a light table, and given the heat given off from the fluorescent lights that the frogs had to rest on while in the maze, as well as the proximity of bright light to the frogs may have contributed to issues with stress or visibility of cues. In addition, our experiment attempted to follow the guidelines for “hot and dry environment” used in the maze in Liu & Burmeister (2017) to the best of our abilities, but issues with excess heat persisted in our experiment, and a correlation with the moving of our space heaters from behind each door to facing both sides of the central chamber after day 6 of training seems to exist due to increased success rates occurring beginning on day 7.



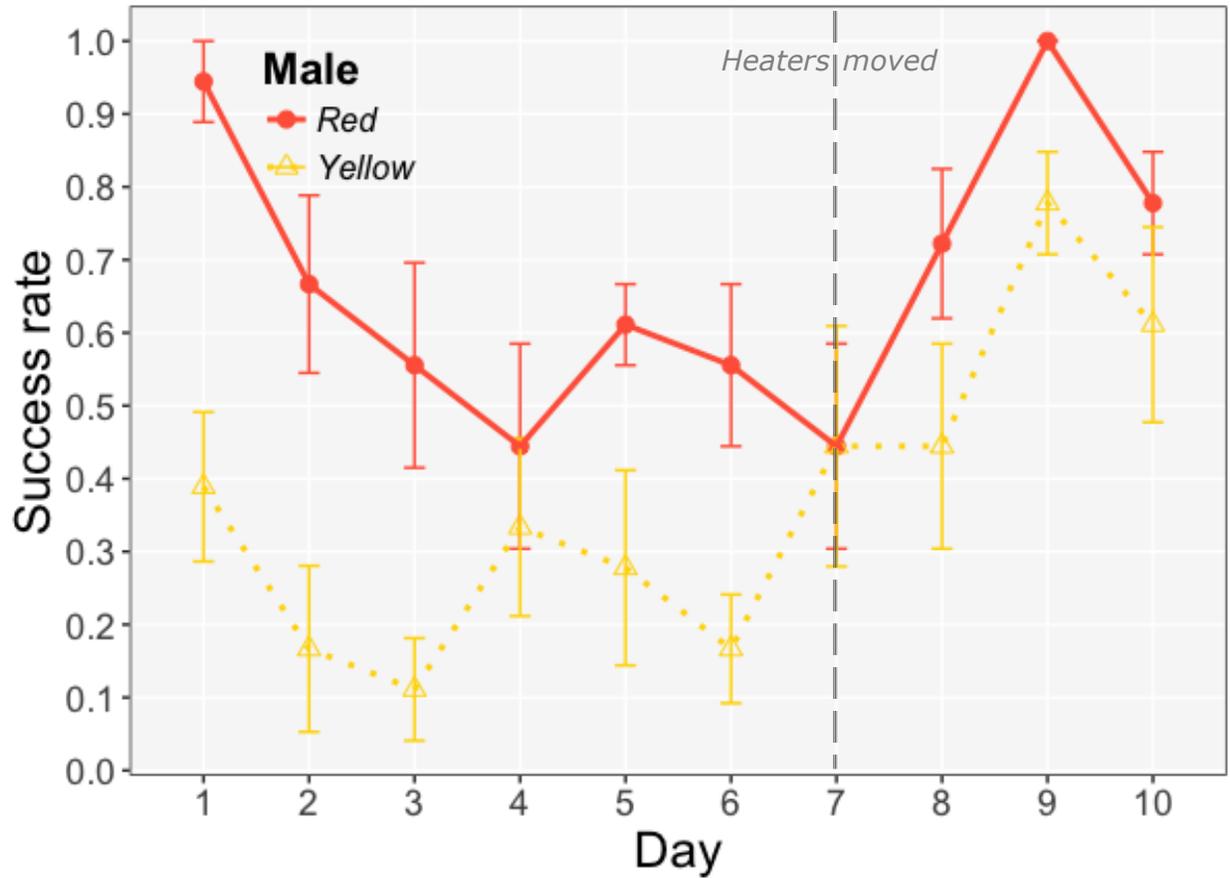
**Figure 2.1.** The two-arm maze used for the completion of these trials. The total length of the maze is about 60 cm. In this trial, the yellow door is blocked off and the red door can be slid open to reveal the dark shelters cut off from the image on the right.



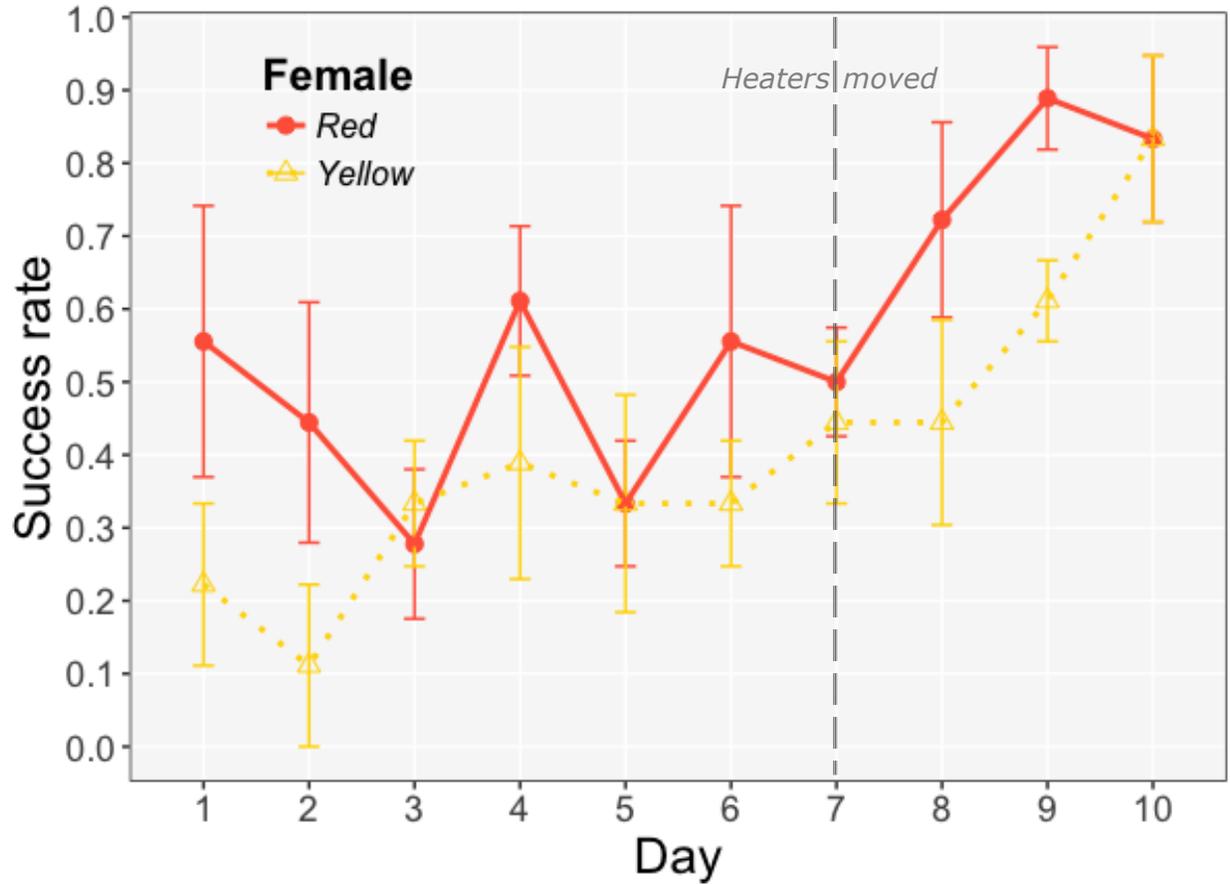
**Figure 2.2.** Variation in success rate (mean  $\pm$  SE) of male and female frogs during the first 10 days of acquisition. Success rate is calculated by day as the proportion of frogs that approached the correct door during their three 3-minute trials per day. The vertical dashed line at day 7 indicates the beginning of trials completed after moving the heaters from the ends of the maze behind the doors (days 1-6) to the sides of the central chamber (days 7-10).



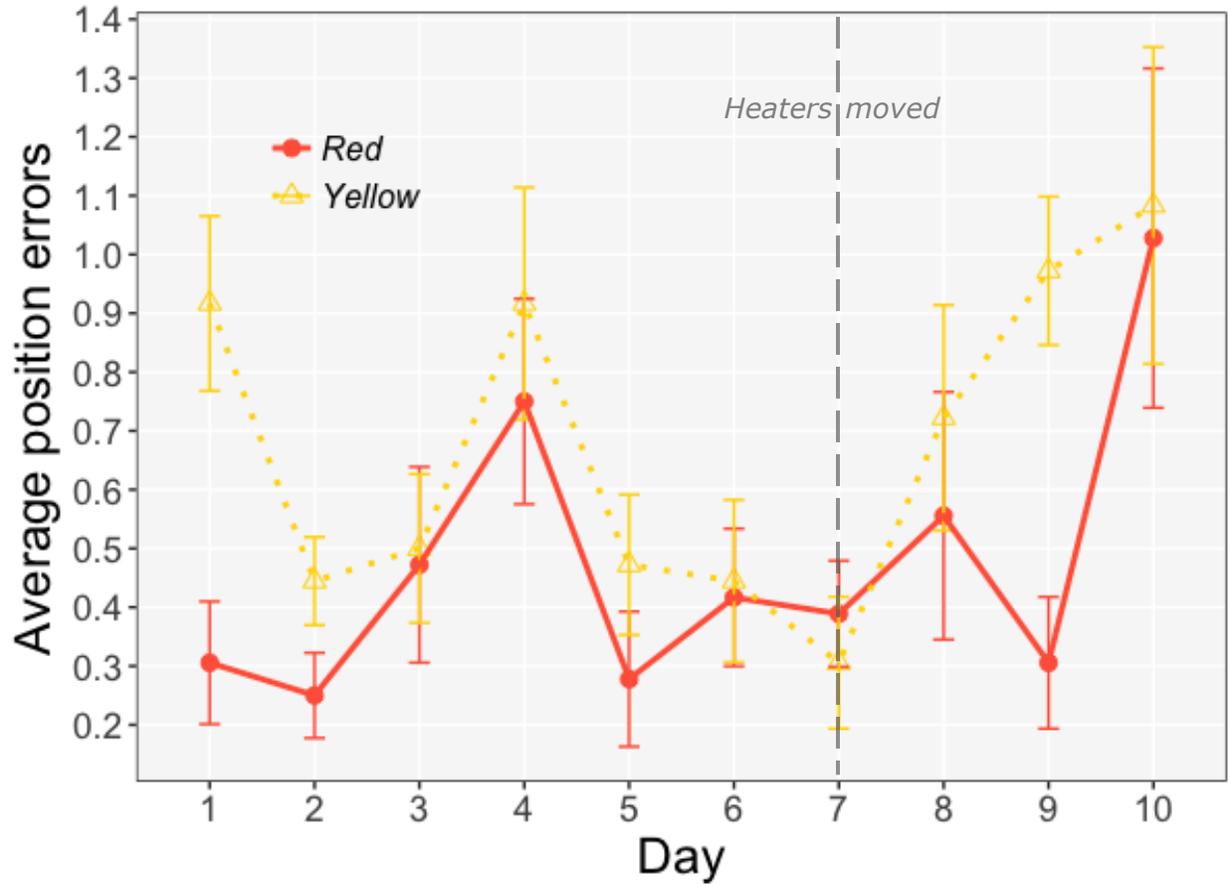
**Figure 2.3.** Variation in success rate (mean  $\pm$  SE) of both male and female túngara frogs as a group, separated by the door colors (cues) they were trained to associate with the exit of the maze during the first 10 days of acquisition. Success rate is calculated by day as the proportion of frogs that approached the correct door during their three 3-minute trials per day. The vertical dashed line at day 7 indicates the beginning of trials completed after moving the heaters from the ends of the maze behind the doors (days 1-6) to the sides of the central chamber (days 7-10).



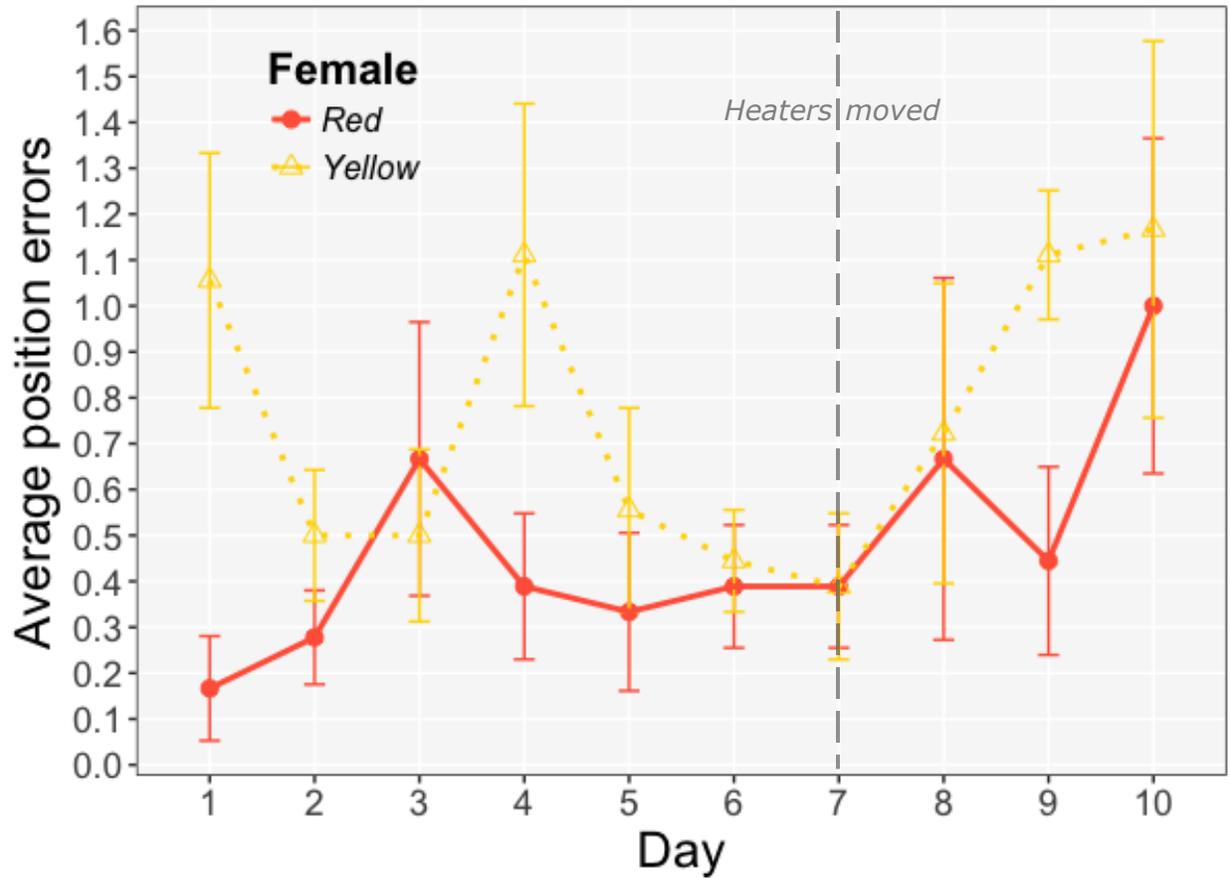
**Figure 2.4.** Variation in success rate (mean  $\pm$  SE) of male frogs, separated by the door colors (cues) they were trained to associate with the exit of the maze during the first 10 days of acquisition. Success rate is calculated by day as the proportion of frogs that approached the correct door during their three 3-minute trials per day. The vertical dashed line at day 7 indicates the beginning of trials completed after moving the heaters from the ends of the maze behind the doors (days 1-6) to the sides of the central chamber (days 7-10).



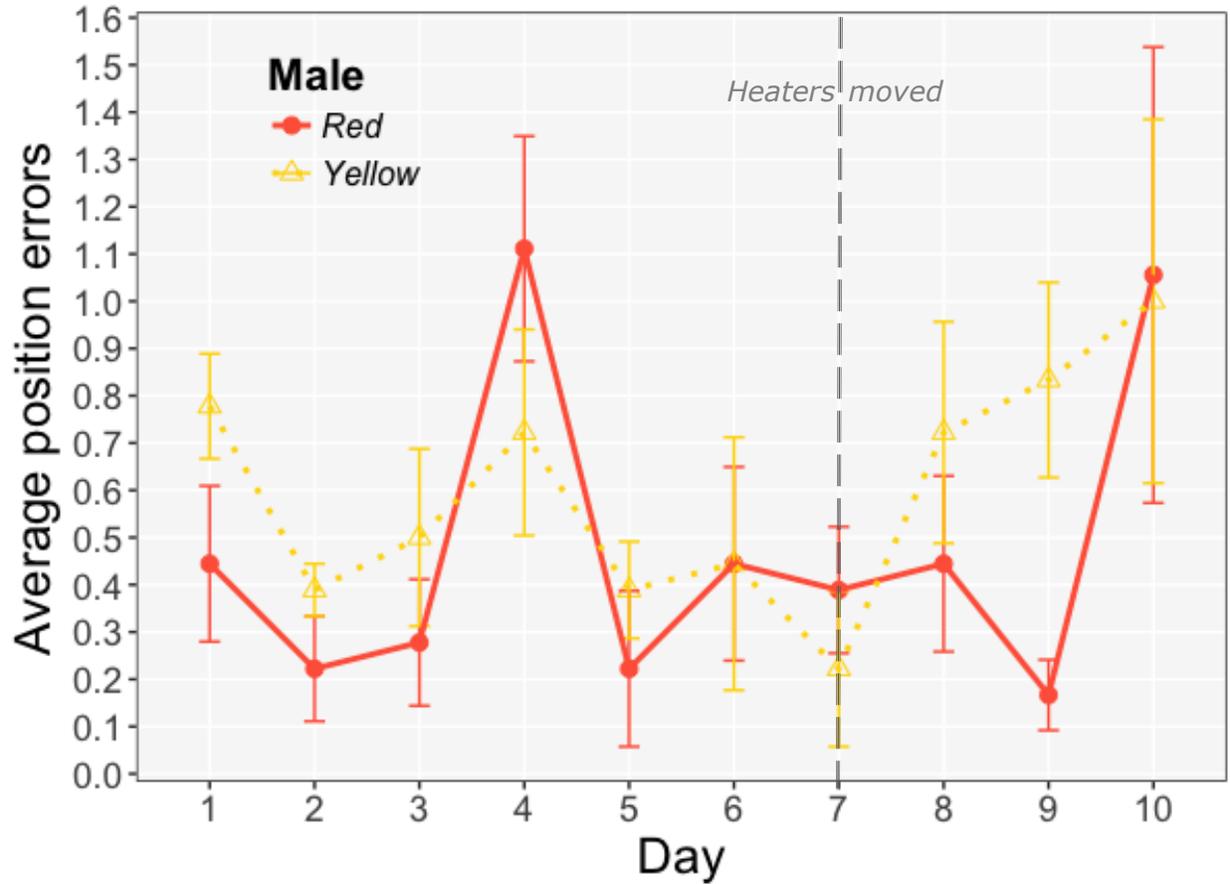
**Figure 2.5.** Variation in success rate (mean  $\pm$  SE) of female frogs, separated by the door colors (cues) they were trained to associate with the exit of the maze during the first 10 days of acquisition. Success rate is calculated by day as the proportion of frogs that approached the correct door during their three 3-minute trials per day. The vertical dashed line at day 7 indicates the beginning of trials completed after moving the heaters from the ends of the maze behind the doors (days 1-6) to the sides of the central chamber (days 7-10).



**Figure 2.6.** Variation in average position errors per day (mean  $\pm$  SE), separated by the door colors (cues) frogs were trained to associate with the exit of the maze during the first 10 days of acquisition. A position error was recorded each time that a frog approached the incorrect door during their three 3-minute trials per day. The vertical dashed line at day 7 indicates the beginning of trials completed after moving the heaters from the ends of the maze behind the doors (days 1-6) to the sides of the central chamber (days 7-10).



**Figure 2.7.** Variation in average position errors per day (mean  $\pm$  SE), separated by the door colors (cues) that female frogs were trained to associate with the exit of the maze during the first 10 days of acquisition. A position error was recorded each time that a frog approached the incorrect door during their three 3-minute trials per day. The vertical dashed line at day 7 indicates the beginning of trials completed after moving the heaters from the ends of the maze behind the doors (days 1-6) to the sides of the central chamber (days 7-10).



**Figure 2.8.** Variation in average position errors per day (mean  $\pm$  SE), separated by the door colors (cues) that male frogs were trained to associate with the exit of the maze during the first 10 days of acquisition. A position error was recorded each time that a frog approached the incorrect door during their three 3-minute trials per day. The vertical dashed line at day 7 indicates the beginning of trials completed after moving the heaters from the ends of the maze behind the doors (days 1-6) to the sides of the central chamber (days 7-10).

## **CHAPTER 3: ELIMINATION OF CUE BIAS BY USING MONOCHROMATIC CUES**

### **Summary**

In response to the differences in success rates and position errors that were observed between animals trained to exit the two-arm maze via the red door versus those that were trained to exit via the yellow door, this experiment aimed to eliminate cue bias to more accurately assess potential sex differences in place learning in túngara frogs. Besides for the removal of the light table from the maze apparatus and the changing of cues from red and yellow to two alternative grid-like monochromatic patterns, all other aspects of this experiment were held constant to the experiment described in Chapter 2. Using this maze iteration, there was a lack of evidence for either males or females having learned the task; however, trends in position errors seem to support the theories regarding male impulsiveness that were proposed at the conclusion of Chapter 2. Future studies should focus on choosing cues that rely on more obvious differences in visual acuity and contrast detection while using the action of opening the horizontally sliding door as a moving cue to its advantage. For instance, cues with alternative thick and thin monochromatic vertical bars might be more easily discriminated than the square and diamond grid cue that were used.

### **Materials and methods**

#### (a) Animals

For this experiment, 11 sexually mature túngara frogs (6 males, 5 females) naïve to any behavioral experiments were used. These animals were obtained from the same source as the animals used in Chapter 2 and housed and fed in the same manner mentioned on page 8. They

were housed in individual terraria (22 × 14 × 13 cm). The University of North Carolina's Institution for Animal Use and Care Committee approved all procedures (protocol 14-026).

#### (b) Apparatus

Frogs were trained in a the same two-arm maze used in Chapter 2. At the end of each arm was a 9 cm by 9 cm door with either a square grid pattern or a diamond “criss-cross” pattern taped to it (Figure 3.1). Door patterns were chosen based on inference of túngara frog visual acuity. The apparatus as mentioned on page 9 of Chapter 2 remained the same, and the same method of blocking one door while allowing the other to be slid away revealing the same shelters was used. The same space heaters were used to keep the maze as a bright, hot and dry environment, and they were placed on opposite sides of the central chamber as depicted in Figure 3.1. Instead of a light table, a simple plywood board was covered with laboratory soaker paper and replaced after each day of trials.

#### (c) Procedure

##### *Acclimation*

The methods for acclimation were the same as described in Chapter 2.

##### *Acquisition*

On the day immediately following the conclusion of acclimation, the frogs began training with three trials per day. Frogs were trained for 21 consecutive days and trained in the same order for all three trials, with inter-trial intervals of 100-120 minutes. With both exits of the maze closed off, half of the frogs (3 males & 3 females) were trained to exit via the door that had a monochromatic diamond “criss-cross” grid pattern cue on it, with the other 3 males and 2 females being trained to exit via the door with a square grid pattern (Figure 3.1). All other procedural details related to acquisition remained the same as described in Chapter 2.

### *Probe trials*

In order to determine whether a preference for the trained cue had emerged in our animals through training, probe trials were conducted for each animal in lieu of training on day 14, and again on day 22 at the conclusion of training. In these trials, both doors were blocked off in the maze, and the proportion of time that animals spent in each arm of the maze and in the central was recorded for a four-minute period. These proportions could then be analyzed and interpreted in regard to preference.

#### (d) Statistical Analysis

All methods used for statistical analysis were the same as described in Chapter 2. For the probe trials, a two-sample t-test was used in Microsoft Excel to evaluate which arm of the maze frogs spent more time in.

## **Results**

### (a) Individual Success without Error

Over the 20 total days of acquisition, with three trials per day, 7 of 11 frogs (3 of 6 males and 4 of 5 females) were able to approach the correct door without committing a position error in at least 6 out of 9 consecutive trials. Therefore, once again a majority of the frogs displayed the ability to orient to their trained door cue in at least 66.7% of trials over three consecutive days at some point over the 20 days of acquisition, presumably using the only provided visual cue. However, this proportion of success without error only equates to a p-value of 0.17 in comparison to random chance (50%) for a two-choice task, at which level we cannot readily say that the behavior occurred due to learning rather than chance (binomial test:  $n=9$ ,  $x = 6$ ,  $p = 0.17$ ). Only 3 of 11 frogs (1 male and 2 females) were able to approach their correct door without error in at least 7 out of 9 consecutive trials, and at this level, we can more significantly say that

these frogs indicated the ability to learn (binomial test:  $n = 9, x = 7, p = 0.07$ ). Lastly, only 1 of 11 frogs (a female) were able to approach their correct door without error in at least 8 out of 9 consecutive trials, at which level the ability to learn as compared to random chance is indubitable (binomial test:  $n = 9, x = 8, p = 0.02$ ). All frogs that were able to achieve success without error at least 6 out of 9 consecutive trials did so between days 4 and 12 of acquisition.

#### (b) Group Performance and Sex Differences

The effect of day on success rate was significant, suggesting that the behavior of the animals as a whole was altered in response to experience (repeated-measures ANOVA, day:  $F_{9,80} = 3.021, p = 0.004$ ; linear trend:  $F_{1,10} = 4.159, p < 0.001$ ). We cannot say that the frogs' behavior as a group in terms of success without error was significantly different from random chance, as the peak mean group rate of 61% on day 10 did not reach the criterion of 80% required for a difference from 50% with  $p = 0.05$  and 11 subjects (one-proportion z-test,  $n = 11$ ). Males only reached a peak mean group success rates of 0.56 on day 10 and females only reached a peak mean group success rate of 0.67 also on day 10 (Figure 3.2). Due to these success rates, we cannot be sure whether the frogs chose the correct door significantly more than random chance would cause. Despite this, the data still suggests that the effect of sex on this change of success rate by day is not significant (repeated-measures ANOVA, sex  $\times$  day:  $F_{9,80} = 0.431, p = 0.915$ ).

#### (c) Probe Trials

A probe trial was done after 13 days of training, and again after 21 days of training. In both instances, animals spent slightly more time in the arm containing the cue that they had been trained to, however these differences were not statistically significant (two-sample t-test:  $p = 0.29, p = 0.51$ ) (Figure 3.3).

#### (d) Sex Differences in Errors

Analysis of position errors revealed no results of note. There was a slight effect present of sex on mean position errors (repeated-measures ANOVA, sex:  $F_{1,70} = 7.075$ ,  $p = 0.01$ ), which can most likely be explained by the slightly increased amount of position errors committed by males as opposed to females (Figure 3.4). However, the interaction of sex and day had no effect on position errors (repeated-measures ANOVA, sex  $\times$  day:  $F_{9,80} = 0.371$ ,  $p = 0.945$ ). The interaction of sex and day also had no effects on non-contingent errors (repeated-measures ANOVA, sex  $\times$  day:  $F_{9,80} = 0.634$ ,  $p = 0.765$ ) (Figure 3.5).

#### (e) Evaluation of Potential Boldness Effects

In order to account for animals who may have exhibited decreased boldness in exploring the maze, I attempted to remove animals that committed a non-contingent error on the first day of trials, however since 8 of 11 subjects fit this criterion, I did not investigate this any further. In addition, I removed animals that committed 10 or more non-contingent errors over the 10 days of trials analyzed (2 males and 1 female). After replicating the repeated-measures ANOVA, this modified dataset didn't seem to reveal any unseen significant trends, and based on this comparison, it was determined that the complete dataset with all 11 subjects was the most accurate method of analysis.

### **Discussion**

Three túngara frogs did display evidence of place learning in this two-arm maze, one male and two females that was able to approach the correct door without error in 7 out of 9 consecutive trials over the course of acquisition for a p-value of 0.07, which demonstrates statistical significance in comparison to chance in a two-choice maze. Although 7 of 11 frogs were able to achieve success without error in at least 6 out of 9 consecutive trials, this is more

likely to be attributed to chance. These proportions are quite similar to those seen in Chapter 2, but in the light of mean success rates for both sexes being markedly lower, as well as the much smaller sample size, it is not clear that the túngara frogs were able to learn this task as a group.

This experiment also revealed that the group success rates of both male and female túngara frogs within a two-arm maze varied across ten days of training. Again, the absence of a sex difference was shown by the lack of an interaction observed between sex and day on success rate, and this also seems logical based on the visual interpretation of Figure 3.2.

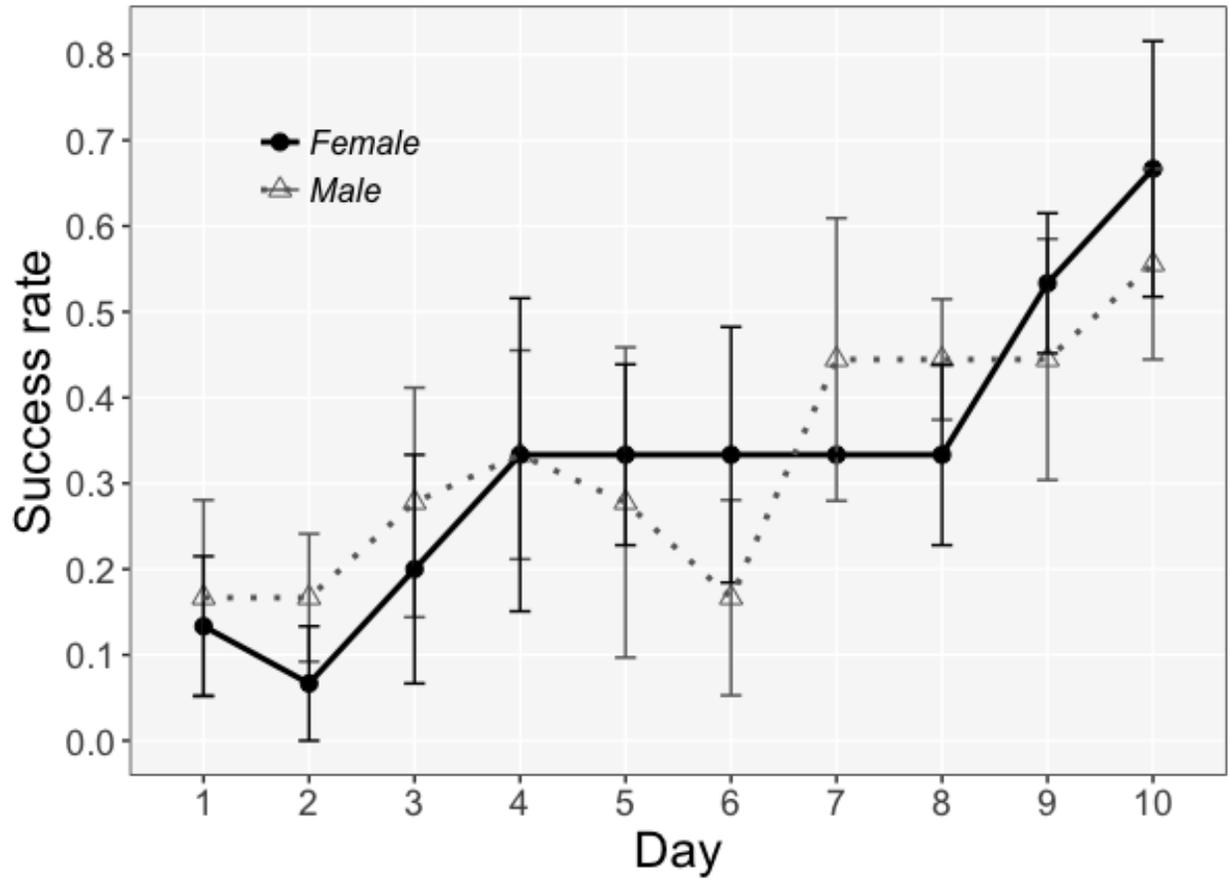
Probe trials demonstrated that the frogs had failed to associate their trained cue with the reward of exiting the maze, and although animals spent a larger proportion of time in the arm containing the cue they had been trained to than the arm containing the incorrect cue, the difference between these proportions was not significant, and in fact animals spent the most time in the central chamber, avoiding both arms, possibly suggesting that the frogs had failed to learn the task of the maze (to approach a door and exit) completely.

This particular experiment sought to address the apparent preference in color observed from the results of Chapter 2. Literature pertaining to color preferences in anurans is very limited, however it has been seen that anurans prefer blue in lower light settings, and in settings with increased light, tend to exhibit a preference for longer wavelengths, such as red or orange (Jaeger & Hailman, 1976). Based on our prior results and the limited knowledge from this experiment, it appears that future examinations of learning in túngara frogs should incorporate monochromatic cues in order to account for a possible color preference. Although the monochromatic cues used in this experiment were ineffective in retrospect due to the small patterning and potential requirement for advanced visual acuity, this should not prevent future studies from utilizing monochromatic cues for such tasks.

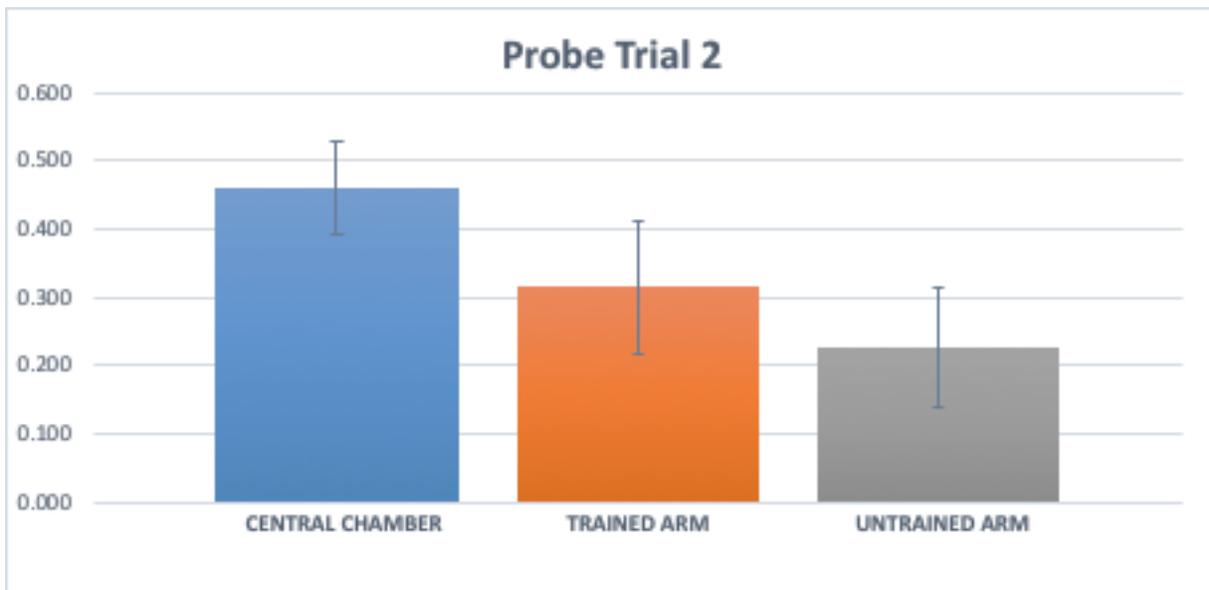
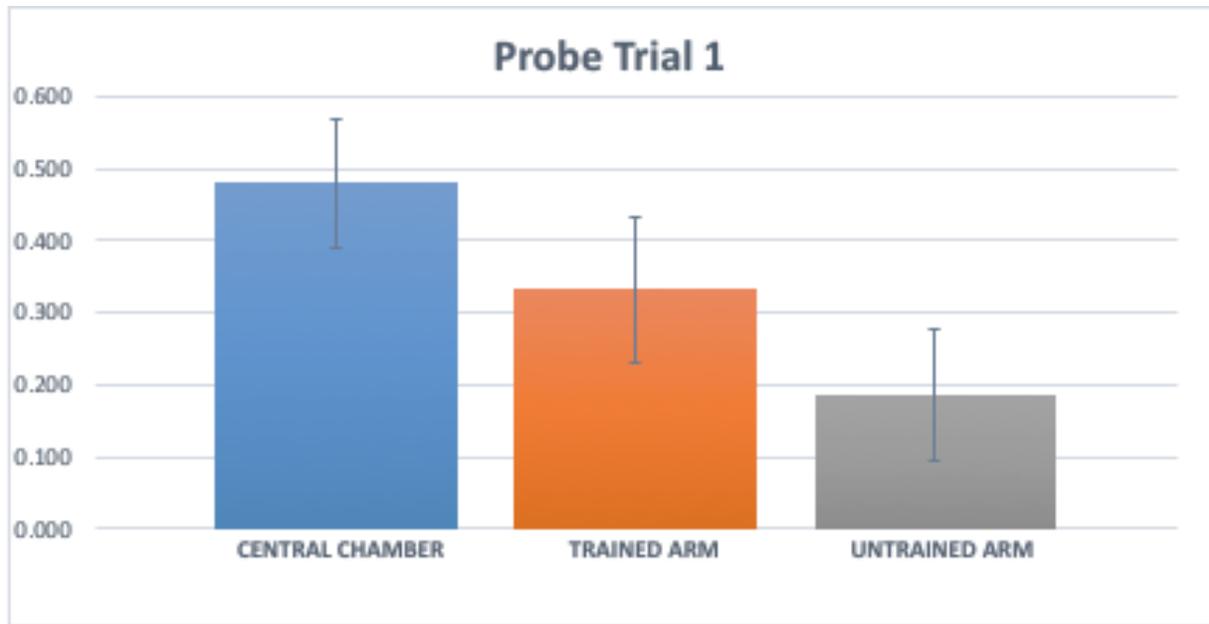
The most suitable monochromatic cues would appear to be ones that rely on basic visual acuity through contrast detection for differentiation. Although it is thought that frogs would prefer darker environments, and potentially prefer a monochromatic cue that contained more black than white, this can be accounted for by ensuring that each cue has equal areas of black and white. This could be done by using alternating black and white vertical bars. One cue could have wider bars and include approximately 5 vertical bars on the door itself, while the other cue could have much thinner vertical bars, including closer to 15 or 20 bars on the cue. Potential examples of these cues can be seen in Figure 3.6. These differences in bar thickness would be ideal as they would not depend on the ability of the frogs to perceive color, and with equivalent amounts of white and black, should also account for any preference that might exist for black or darkness. In addition, when the door was slid horizontally, it would provide for a perceived movement to the frogs' vision, which also may assist in its ability to recognize and differentiate the cues.



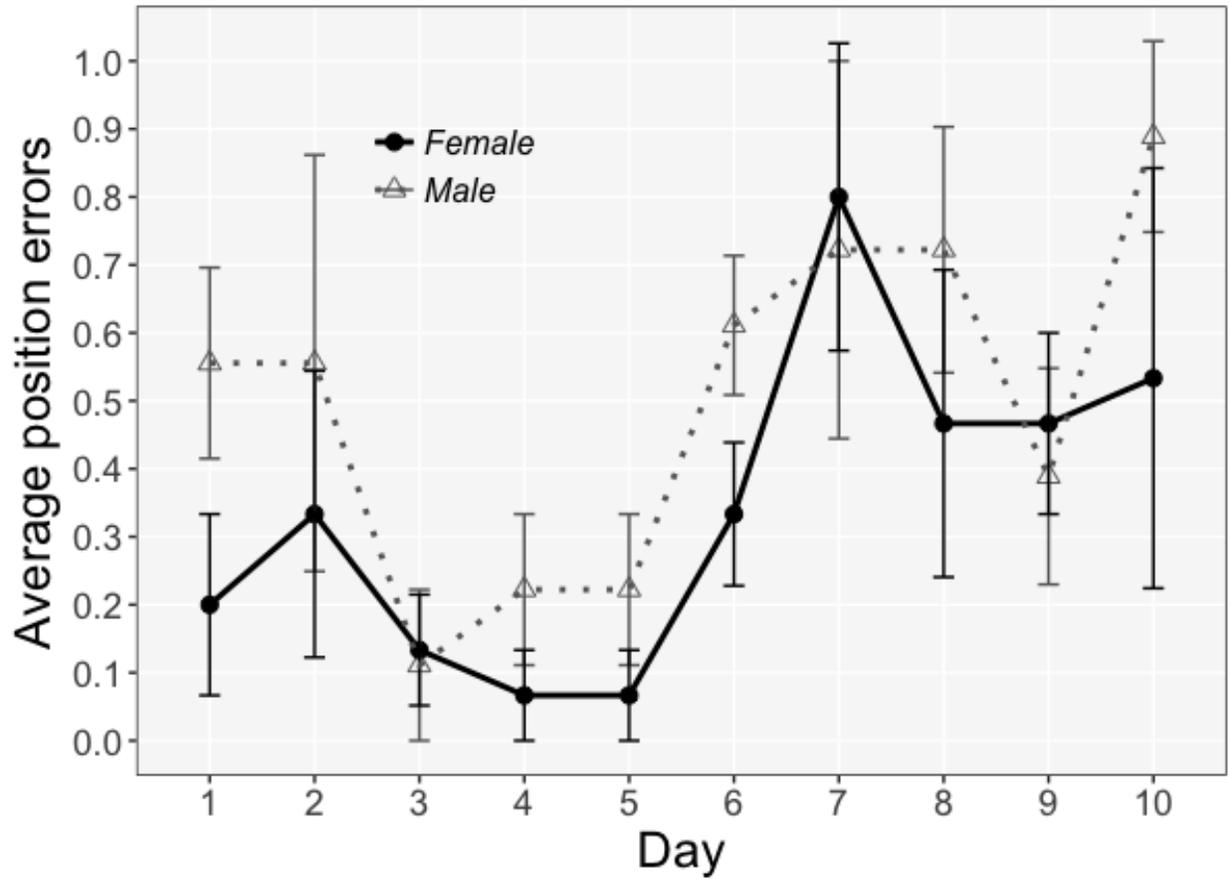
**Figure 3.1.** The two-arm maze used for the completion of these trials. The total length of the maze is about 60 cm. In this trial, the square gridded door is blocked off and the diamond gridded door can be slid open to reveal the dark shelters cut off from the image on the right.



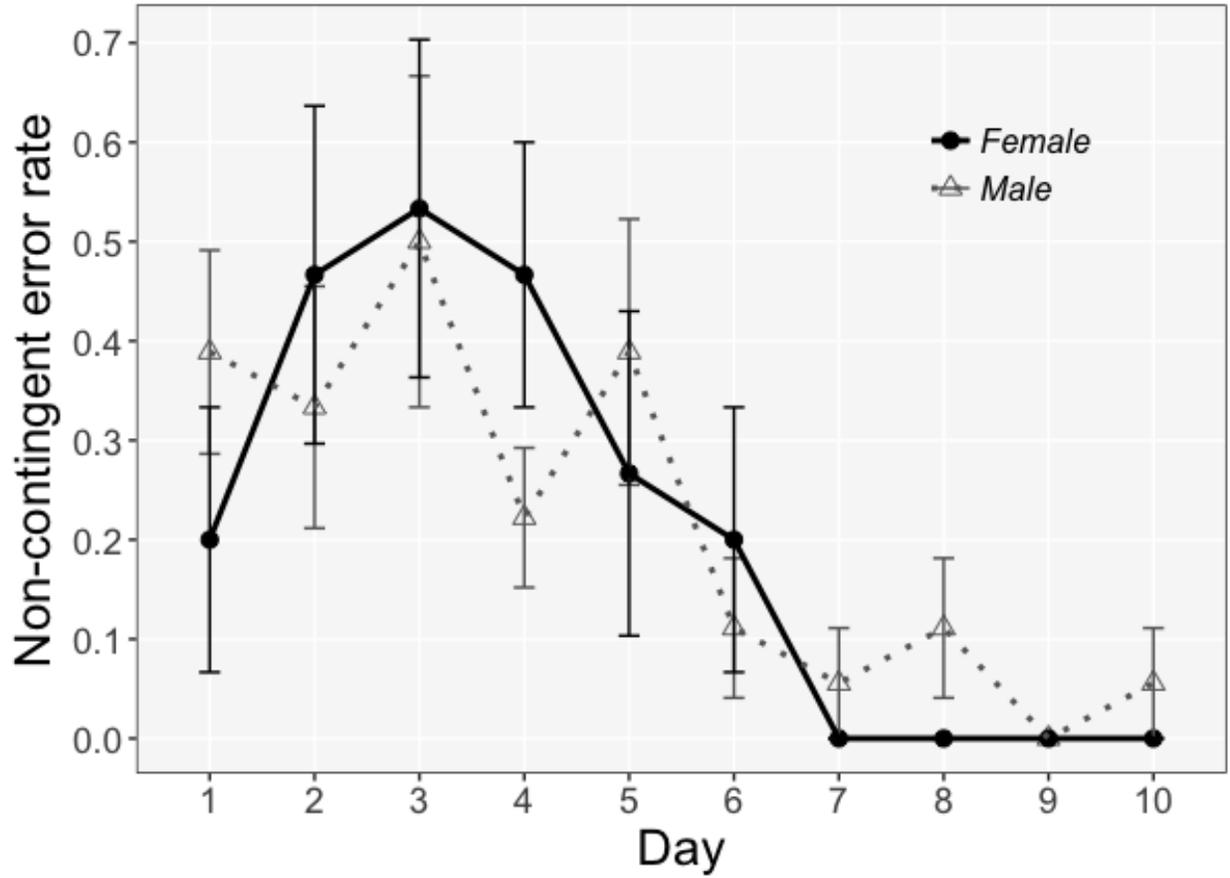
**Figure 3.2.** Variation in success rate (mean  $\pm$  SE) of male and female frogs during the first 10 days of acquisition. Success rate is calculated by day as the proportion of frogs that approached the correct door during their three 3-minute trials per day.



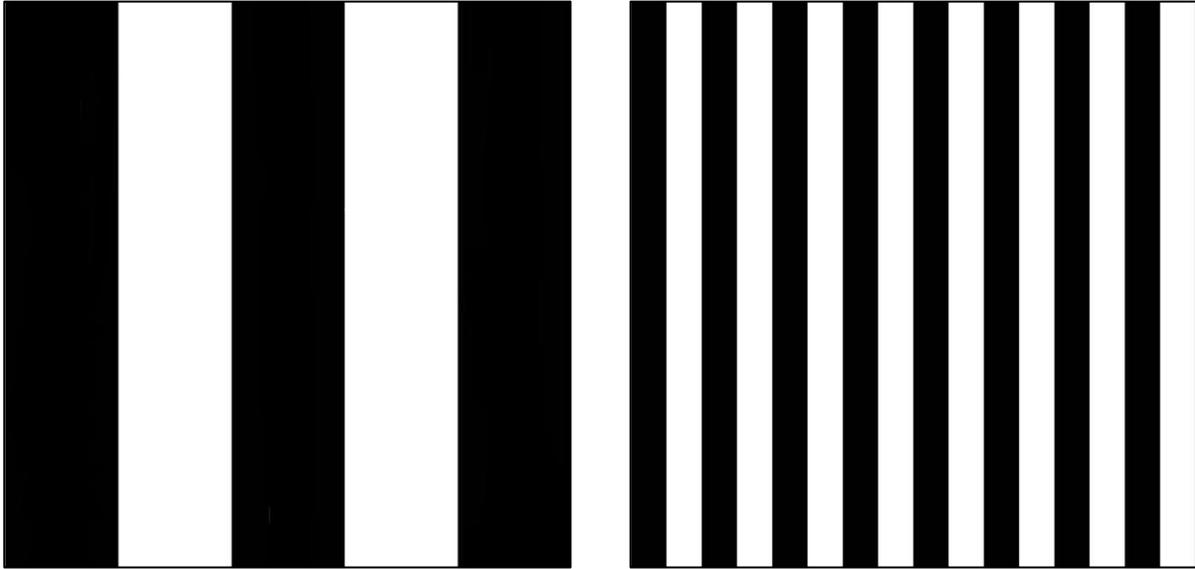
**Figure 3.3.** Histograms indicating the mean  $\pm$  SE proportions of a four-minute trial that frogs spent in either the central chamber, the arm containing the cue that they had been trained to, and the untrained arm. Probe trial 1 occurred on day 14 of acquisition trials, while probe trial 2 occurred on day 22.



**Figure 3.4.** Variation in average position errors per day (mean  $\pm$  SE), committed by each sex during the first 10 days of acquisition. A position error was recorded each time that a frog approached the incorrect door during their three 3-minute trials per day.



**Figure 3.5.** Variation in rate of non-contingent errors per day (mean  $\pm$  SE), committed by each sex during the first 10 days of acquisition. A non-contingent error was recorded each time that a frog failed to enter either arm of the two-arm maze for the entirety of their three 3-minute trial.



**Figure 3.6.** Example monochromatic cues for potential use during future studies. By relying on basic contrast detection as opposed to observing differing orientations in grid patterns, these cues would potentially allow for more successful differentiation by túngara frogs.

## CHAPTER 4: CONCLUDING REMARKS & FUTURE DIRECTIONS

The stated goal of this thesis was to resolve the observed differences in place learning ability between male and female túngara frogs. It was hypothesized that the source of learning disparity was either due to an inability of males to associate a visual cue with a rewarded goal (a cue-taxis strategy), or due to an inability of males to eschew egocentric cues when presented in combination with visual cues. Both experiments described in this manuscript failed to reveal a sex difference in success rates of exiting a two-arm maze. However, the interaction between sex and cue mentioned in Chapter 2 supported evidence for an effect on success rate, suggesting that males and females did not have equivalent success in place learning if they were trained to red cues as opposed to yellow cues.

In addition, the interaction between day and cue did not have an effect on position errors in males in Chapter 2, revealing that although males may have exhibited an ability to approach a door in order to exit the maze based on their success rates, they might have not learned to discriminate between red and yellow in order to choose a specific door, and seemed to commit an similar numbers of position errors regardless of whether they were trained to the red cue or the yellow cue, even though the males frogs trained to the red cue had higher success rates.

The unexplained preference in cue was troubling in that ideally, we would expect animals to perform equally no matter what cue they were trained to. Therefore, in Chapter 3, we aimed to resolve the differences that arose between frogs trained to different cues by repeating the experiment using monochromatic cues. Unfortunately, although the effect of the interaction of sex and day on success rate suggested that the behavior of the frogs did change significantly over

the course of the experimental trials, there was not enough evidence to say that the success that the frogs did exhibit was not due to chance. No sex differences were observed in success rate, average position errors, or non-contingent error rates, and this experiment would greatly benefit from being revisited with improved monochromatic cues to further assess the differences in learning between male and female túngara frogs.

In conclusion, this thesis attempts to understand sex differences in place learning in the context of sexual selection, and as mentioned previously, the ability for female túngara frogs to remember the location and/or calls of quality males is expected to directly affect their reproductive success (Ryan, 1980). On the other hand, no evidence currently exists suggesting that male túngara frogs may experience selection for place memory, even in the consideration of behaviors such as pond selection and migration or competition with other males. The strength of the interpretations made throughout the course of this thesis are somewhat limited by the lack of knowledge regarding the navigational ecology of túngara frogs, however this thesis does serve to add to the current knowledge about the types of cues and strategies that might be used by túngara frogs in place learning.

The major questions that remain in regards to this research pertain to the strategies of learning that are able to be used by male versus female túngara frogs. It appears that the source of sex differences in place learning is likely due to increased male impulsiveness; however due to it cannot be known for sure until more significant data correlating increasing success rates with decreasing error rates is obtained. It would be useful to conduct a two-arm maze solely utilizing egocentric cues, and in the case of frogs, who are known to exhibit personality variation in boldness and exploratory tendencies in their behavior (Brodin *et al.*, 2013; Raynaud &

Schradin, 2014), the more subjects that can be trained, the greater the opportunity to observe a trend among subjects.

Understanding the sex differences between túngara frogs in their learning strategies is relevant to the fields of evolution and comparative biology due to the implications for the evolution of learning as a behavior. At this juncture, there is still much more to be understood about this topic, and in using the túngara frog as a model species to grasp the effects of selection on cognitive ability, it is possible to better understand the proliferation of particular learning strategies and cue utilization in lower order vertebrates.

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