COMPARATIVE ANALYSES OF SPATIAL COGNITION IN FROGS

Yuxiang Liu

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Approved by:

Sabrina S. Burmeister

Keith W. Sockman

Kenneth J. Lohmann

Karen S. Pfennig

Kyle Summers

ABSTRACT

Yuxiang Liu: COMPARATIVE ANALYSES OF SPATIAL COGNITION IN FROGS
(Under the direction of Sabrina S. Burmeister)

Efficient navigation through space is important for animal survival and reproduction. Adaptive hypothesis argues that sex and species with higher levels of cognitive challenge imposed by the environment should outperform others. To date, major efforts to understand animal spatial cognition have focused on mammals and birds. As the branch with the most primitive traits of all tetrapods, the amphibian lineage provides valuable opportunities to understand the evolution of spatial cognition in vertebrates. However, we still know relatively little about spatial cognition in amphibians. Therefore, I studied spatial cognition in this group by asking the following questions: What cognitive strategies are used in place learning? What neurogenomic mechanisms of spatial cognition exist in amphibians? Do amphibians have cognitive abilities that are comparable to mammals and birds? I studied these questions by comparing sexes and species whose natural histories differ in their spatial demands. In túngara frogs, males call from a fixed position in breeding ponds while females visit multiple males before returning to the preferred mate. Thus, females are expected to process more complicated cognitive information than males. For species comparison, Poison frogs defend territories and carry out complex parental care that relies on complex interactions with the environment, while túngara frogs do not defend territories and have no long-term parental care. Based on adaptive hypothesis, female túngara frogs and poison frogs are expected to show better performance in cognitive tasks than males and túngara frogs, respectively. I found sex differences in the use of visual cues to do place learning in túngara frogs. Females were able to use visual cues to solve the two-arm maze task while males were not (Chapter 2). On the other hand, I found túngara frogs used a cue-taxis strategy, while poison frogs used a landmark strategy to learn the same two-arm maze (Chapter 2, 3, 5). Poison frogs outperformed túngara frogs in learning acquisition

and reversal training (Chapter 5). Both of sexes and species comparisons are consistent with adaptive hypotheses of spatial cognition. To understand the neurogenomic mechanisms behind the cognitive differences, I compared hippocampal transcriptomes between the two species. I found that genes related to learning and memory, neurogenesis, and synaptic plasticity were upregulated in poison frogs, while genes related to apoptosis and negative regulation of biosynthesis and metabolism were upregulated in túngara frogs. Therefore, species differences in place learning of frogs may, in part, result from differential expression of those genes in hippocampus. To determine if these species have advanced level of cognitive ability which is comparable to mammals in place learning, I trained poison frogs in a serial reversal task and a modified version of the Morris water maze. The results showed that poison frogs could use a rule-based strategy and cognitive map to learn the serial reversal task and Morris water maze respectively (Chapter 3 and 4). This is the first demonstration of a rule-based strategy and cognitive map in a non-mammalian or avian vertebrate. Given the advanced performance of poison frogs in both tasks, it is likely that poison frogs (and possibly other amphibians), have the neural architecture to generate advanced levels of spatial cognition. Future research may reveal how the complex behavior patterns encoded in mammalian and avian brains can be encoded in the neuroanatomically simpler amphibian brain.

To my families, who always support and understand me.

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CHAPTER 1: INTRODUCTION

Navigating through space in search of the resources necessary to survive and reproduce is an essential function of animal behavior. It is important for homing, migration, locating resources (e.g. food), defending territories, and reproduction. The study of spatial cognition has made significant contributions to a wide range of biological disciplines including behavior (Morris, 1984), ecology (Fagan et al., 2013), evolution (Gaulin, 1992; Rodrıguez et al., 2002), neuroanatomy (Krebs, Sherry, Healy, Perry, & Vaccarino, 1989), neurophysics (Fyhn, Molden, Witter, Moser, & Moser, 2004; O'Keefe & Nadel, 1978), pharmacology (Olton, 1987) and neurogenetics (Geary, 1995). However, most of these studies focused on mammals and birds, and my knowledge of other animal groups is quite limited. This is especially true of amphibians.

Amphibians were once thought to lack the ability to modify their behavior in a flexible manner (Hodos & Campbell, 1969; Thorpe, 1956). This claim has been countered by studies in which amphibians have demonstrated learning abilities in the context of various artificial tasks (Ellins, Cramer, & Martin, 1982; Schmajuk, Segura, & Reboreda, 1980). However, a consensus has remained that amphibians are hard to train due to their sedentary nature and to the difficulty of finding appropriate stimuli to motivate learning (Schmajuk et al., 1980; Sinsch, 2014). Recent progress on training amphibians in various tasks has caused people to question the consensus on amphibian place learning and spatial cognition (Lüddecke, 2003; Pašukonis et al., 2013; Stynoski, 2009), but the field remains under-investigated (Broglio et al., 2015). The lack of research on spatial cognition in amphibians represents a crucial gap in my knowledge of vertebrate cognition. Amphibians diverged from other vertebrate clades about 400 MYA, and so the evolution of spatial cognition in amphibians is independent of other vertebrate clades. Comparing spatial cognition in amphibians with that of other vertebrate clades will provide insights into

the neuroanatomical and neurophysiological mechanisms underpinning the evolution of spatial cognition. Amphibians have a relatively small and simple forebrain, prompting the question of how it can code for mammal-like spatial abilities.

A key starting point for the study of spatial cognition is to understand how animals use different cues to code for locations. Cues can be classified into different categories. In psychology, cues are mainly distinguished as egocentric and allocentric according to the source of cues (Shettleworth, 2009). Egocentric cues are generated by internal organs (e.g. proprioceptive and vestibular systems) of the animals themselves (Maaswinkel & Whishaw, 1999; McNaughton et al., 1996). This type of cue contains vector information (direction and/or distance) between the animal and the goal (Burgess, 2006; Müller & Wehner, 1988). Allocentric cues include any cues that are generated by the external world, and can be further classified into different sensory modalities (Burgess, 2006).

The types of cues determine the types of learning strategies an animal can use. Regarding egocentric cues, the body turn strategy and path integration (also known as dead reckoning) has been widely demonstrated in animals. The body turn strategy requires animals to remember the relative direction between themselves and the goal (Shettleworth, 2009). It is the simplest form of learning strategy, and it has been identified in almost all major vertebrate groups (Blodgett, McCutchan, & Mathews, 1949; Day, Ismail, & Wilczynski, 2003; Rodriguez, Duran, Vargas, Torres, & Salas, 1994; Schmajuk et al., 1980). Path integration is a strategy in which animals record the vector (including both direction and distance) of every movement and then calculate their position relative to previous positions in real time (Müller & Wehner, 1988). The most famous example of path integration occurs in desert ants, which can take a winding outbound pathway to locate food and then take an almost straight pathway to their starting points, without any input from external cues (Müller & Wehner, 1988). So far, path integration has been reported in some insects, mammals and birds (Etienne & Jeffery, 2004; Mittelstaedt & Mittelstaedt, 1982; Müller & Wehner, 1988).

Although allocentric cues may include any sensory modality, visual cues are the most commonly studied type of allocentric cues. Herein, I will mainly focus on visual cues to introduce strategies which employ allocentric cues. A cue-taxis strategy allows animals to use features of the goal itself to approach the goal (Day et al., 2003; Shettleworth, 2009), for example animals learn to use color of doors to exit a T maze. In a landmark strategy, animals learn a vector between a single cue and the goal (Day et al., 2003; Shettleworth, 2009), for example rats always use an object which is closest to goal for locating. Both cue-taxis and landmark strategies exist broadly in vertebrates (Cheng & Spetch, 1998; Daneri, Casanave, & Muzio, 2011; Lopez et al., 2000; Sovrano, Bisazza, & Vallortigara, 2007). A geometric strategy enables animals to associate shapes that are generated by a particular space with locations in that space (Cheng, 1986; Kelly, Spetch, & Heth, 1998). For example in a rectangular arena without any cue, if reward is associated with one corner, animals will learn to visit the reward corner and its diagonal corner in equal and higher frequency since the two corners are geometrically equal in the rectangular arena. While a geometric strategy has been well documented in mammals and birds, it has been largely ignored in other taxa. Recently, it was demonstrated that the Argentine toad can use the geometric shape of a rectangular space to locate a water resource (Sotelo, Bingman, & Muzio, 2015). A cognitive map strategy enables animals to learn the spatial relationships among multiple cues and then configure the shortest pathway from a random position to a destination (O'Keefe & Nadel, 1978; Shettleworth, 2009; Tolman, 1948). So far, convincing evidence for a cognitive map has only been found in mammals and birds (Jacobs, 2003; Shettleworth, 2009).

Although different types of cues and strategies have been distinguished, they also work together, or compete with one another, during place learning. For example, route learning requires animals to associate a series of landmarks (allocentric cues) with a corresponding set of correct directions (egocentric cues). Using this strategy, animals take fixed routes to locate goals (Shettleworth, 2009). This kind of compound strategy has never been reported in amphibians. In the real world, animals always have more than one type of cue or learning strategy to use for place learning (Maaswinkel & Whishaw, 1999).

Cue conflict experiments are designed to determine whether animals always select one cue or strategy over another. A conservative tendency across vertebrates is to use body turn strategy rather than cue-taxis strategy when the two strategies point to different locations (Daneri et al., 2011; Maaswinkel & Whishaw, 1999). Animals that have more navigational demands (e.g., territoriality, food caching) are more likely to use spatial relationships between cues and goals rather than a cue-taxis strategy for place learning (Brodbeck, 1994). This phenomenon has been found in both mammals and birds, but it is not clear if amphibians also share similar tendencies.

The cognitive map is an intriguing yet controversial concept in the field of animal cognition. Since it was proposed by Tolman (1948), the concept of cognitive map continues to stimulate empirical research in a broad range of disciplines, such as biology (O'Keefe & Nadel, 1978), psychology (Jacobs & Schenk, 2003), computer science (Stach, Kurgan, Pedrycz, & Reformat, 2005), education (Kevany et al., 2007), and philosophy (Mingers, 2003). However, the question of whether animals actually have a cognitive map has been disputed for about half century (Shettleworth, 2009). Rodents' performance in the Morris water maze and the discovery of hippocampal place cells and grid cells, support the existence of a cognitive map in animals (Brandeis, Brandys, & Yehuda, 1989). However, some people doubt that the cognitive map exists in the real world, since animals always have alternative ways to accomplish place learning in nature (Bennett, 1996; Brown, 1992). Some researchers have remained neutral, advocating that we should avoid the concept of cognitive map in animals, but instead pay more attention to the cues that animals actually use in place learning (Mackintosh, 2002). Although most cognitive scientists accept the existence of a cognitive map in mammals and birds (Jacobs, 2003), it is still not clear how broadly it exists in animals. In amphibians, there has been only one attempt to test for the presence of a cognitive map in a Morris water maze (Bilbo, Day, & Wilczynski, 2000). However, the results showed that the leopard frog, when placed in the maze, showed high levels of thigmotaxis to the maze wall and did not show appropriate response to the test. Thigmotaxis, which is defined as the orientation to touch stimuli, is broadly exist when animals are tested in artificial mazes. Hence it is unclear if leopard frogs in this

experiment did not learn the Morris water maze task due to the lack of a cognitive map or to a mismatch between the presentation of the maze task and the perceptual expectations of this species. My pilot work on training poison frogs in the classic Morris water maze found that they also showed strong thigmotaxis to the maze wall and neglected the visual cues when placed in the maze, swimming with their heads underwater (Liu, Day, Summers, & Burmeister, 2012). Thus, valid tests for the presence of a cognitive map requires an appropriate maze design is congruent with the perceptual requirements of amphibians.

The study of spatial cognition is focused on understanding how animals navigate through the environment and the mechanisms underlying those abilities. However, environments in the natural world are not constant. Behavioral flexibility is the ability of animals to change what they learned based on changes in the environment (Coppens, de Boer, & Koolhaas, 2010). It has been demonstrated in a number of species that animals facing more complex physical and/or social environments show higher behavioral flexibility, compared to species that do not face similar levels of environmental variation or complexity (Bond, Kamil, & Balda, 2007). Behavioral flexibility, which is generally measured by reversal learning (reward contingency reversal) in a discrimination task, has been demonstrated in some amphibians (Daneri et al., 2011; Ellins et al., 1982). A more broadly accepted measurement of behavioral flexibility is serial reversal learning, in which reward contingencies are reversed sequentially, each time an animal demonstrates learning. The hallmark that provides clear evidence for serial reversal learning is progressive improvement of performance across reversal sessions (Mackintosh, McGonigle, & Holgate, 1968). However, there are a couple of ways to achieve progressive improvement, including strengthening of learning the reward-stimuli contingency and increasing proactive interference (Mackintosh et al., 1968; Parker et al., 2012; Strang & Sherry, 2014). In contrast to those strategies, demonstration of a rule-based strategy requires animals to not only show progressive improvement but also to learn to cope with the rule (also known as learning to learn; (Shettleworth, 2009)). The type of rule-based strategy has only been demonstrated in mammals and birds (Mackintosh et al., 1968; Randall & Zentall, 1997; Rayburn-Reeves, Stagner, Kirk, & Zentall, 2013). So far, the only two demonstrations of serial reversal learning in

amphibians showed that they learned the task by increasing proactive interference, which is defined as the process of forgetting previously-learned associations in order to facilitate future learning (Elepfandt, 1985; Ellins et al., 1982). It is still not clear whether amphibians are able to accomplish rule-based learning.

Compared to the few studies on behavior of spatial cognition, there is a complete lack of studies on the neural mechanism of spatial cognition in amphibians. A fundamental key to understand the neural mechanism is the study of gene expression in the corresponding brain regions responsible for spatial navigation abilities. The hippocampus has been demonstrated to play an essential role in the spatial memory and behavioral flexibility of mammals and birds (Morris, Garrud, Rawlins, & O'Keefe, 1982; Sherry & Vaccarino, 1989). Comparative anatomy and embryonic development suggest that the neuroanatomy of this brain region is highly conserved across vertebrates (Butler & Hodos, 2005; Rodrıguez et al., 2002; Striedter, 2015). In amphibians, the medial pallium is considered to be the homologous brain region of the mammalian hippocampus (Butler & Hodos, 2005; Roth, Laberge, Mühlenbrock-Lenter, & Grunwald, 2007). Studying the hippocampal transcriptome provides a window into functional categories and co-expression networks of genes that are responsible for spatial cognition. So far, hippocampal gene expression profiles have only been studied in mammals and birds (Colangelo et al., 2002; Pravosudov et al., 2013), so we know nothing about the neurogenomics of spatial cognition in amphibians.

In my dissertation I studied the spatial cognition of amphibians by asking the following questions: what kind of cues and learning strategies do amphibians use during place learning? How flexible is their place learning? What are the neurogenomic mechanisms of spatial cognition in amphibians? Do they possess advanced levels of cognitive abilities (i.e. a rule-based learning strategy and cognitive map) that are comparable to those in mammals and birds? In order to address these questions, I studied two frog species that are sympatrically distributed in tropical rain forests of Central America. The green and black poison frog (*Dendrobates auratus*) defends territories and engages in complex patterns of

parental care, including egg attendance and tadpole transportation (Summers, 1989, 1990). Field studies show that poison frogs spend considerable time traveling to find pools with standing water for tadpole deposition (Summers, 1989). Poison frogs have to travel among egg sites, tadpole deposition pools, and resource patches (e.g. food and shelter), and thus have substantial and complex navigational demands on their cognition. In contrast, the túngara frog (*Physalaemus pustulosus*) is a lek breeder (Ryan, 1985) that shared a common ancestor with poison frogs about 110 MYA (Ruvinsky & Maxson, 1996). Males defend calling sites, but do not possess territories. Male and female túngara frogs aggregate in a pond for mating and then leave a foam nest without any on-going parental care (Ryan, 1985).The species differences in their natural history suggest that they might differ in spatial cognition. Therefore, I first compared species differences in learning strategy and behavioral flexibility to learn a two-arm maze task (Chapter 2, 3, and 5). I then tested whether poison frogs have rule-based learning (Chapter 3) in a serial reversal task and a cognitive map (Chapter 4) in a modified version of the Morris water maze. At last, I compared patterns of gene expression in their hippocampal transcriptomes using differential expression analyses of RNA-Seq transcriptome sequence data (Chapter 5).

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CHAPTER 2: SEX DIFFERENCES DURING PLACE LEARNING IN THE TÚNGARA FROG[1]

Summary

The adaptive specialization hypothesis states that sex differences in cognition are shaped by differences in cognitive demands to solve ecological problems. While it is widely accepted that female mate choice can lead to the evolution of exaggerated male traits, mate choice might also select for different cognitive abilities in males and females. In the túngara frog, males call from a fixed position in breeding ponds while females visit multiple males before returning to the preferred mate. Thus, I predicted that females have better place memory than males. I tested this prediction in a place-learning task in which the rewarded arm of a maze was associated with a visual cue. I found that females were able to use the visual cue to solve the task while males were not, even though both males and females could discriminate the cues in an optomotor test. In contrast, males attempted to solve the task using egocentric cues (remember body-turn direction) in spite of the fact that my training procedure interrupted their use of such cues. Finally, I found that males and females had similar motivation to solve the task but females showed a greater ability to inhibit incorrect responses, leading to improved learning. My finding that females could use a visual cue to remember locations in space is consistent with the idea that place memory could improve sequential mate assessment in túngara frogs.

Introduction

The adaptive specialization hypothesis suggests that sex differences in cognition are shaped by differences in cognitive demands to solve ecological problems (Dalla & Shors, 2009; Geary, 1995; Jonasson, 2005; Jozet-Alves, Modéran, & Dickel, 2008). For example, in meadow voles, males show an

[1] This chapter has been submitted to Animal Behaviour for review.

advantage in spatial memory likely because of selection for males who could hold bigger home-ranges (Gaulin & Fitzgerald, 1989), while in cowbirds, females show an advantage likely because the breeding success of females relies on their ability to relocate hosts' nests (Guigueno, Snow, MacDougall-Shackleton, & Sherry, 2014; Sherry, Forbes, Khurgel, & Ivy, 1993). In addition, mice show task-specific sex advantages due to differentially perceived information between males and females in appetitive and avoidance tasks (Dalla & Shors, 2009; Mishima, Higashitani, Teraoka, & Yoshioka, 1986).

One of the most important evolutionary processes for producing sex differences is mate choice (Andersson, 1994; Andersson & Simmons, 2006). In order to process, compare, and remember the information contained in the complex signals of males, it is expected that females should evolve correspondingly robust cognitive abilities (Ryan, Akre, & Kirkpatrick, 2009). Although this hypothesis has not been tested extensively, it has been suggested by a number of studies (Keagy, Savard, & Borgia, 2009, 2011; Minter, 2015) and cognitive ability has been confirmed as a trait under selection by mate choice (Boogert, Fawcett, & Lefebvre, 2011). Túngara frogs (*Physalaemus = Engystomops pustulosus*) mate in a lek-like breeding system in which males call from a fixed position within a breeding pond and females visit multiple males before choosing a mate (Ryan, 1985), a behaviour that likely depends on memory for the location, and/or displays, of particular males. Females use both acoustic and visual cues when evaluating (Lea & Ryan, 2015; Taylor, Klein, Stein, & Ryan, 2011; Taylor & Ryan, 2013) and locating males (Cummings, Bernal, Reynaga, Rand, & Ryan, 2008; Farris, Rand, & Ryan, 2002) and have been shown to remember the location of calls (Akre & Ryan, 2010). Thus, according to the adaptive specialization hypothesis, I hypothesized that female mate choice in túngara frogs has selected for place memory in females.

Animals can use a variety of cues to remember locations in space. Anurans are adept at using egocentric cues (e.g., turn left or right) (Brattstrom, 1990; Daneri, Casanave, & Muzio, 2011; Schmajuk, Segura, & Reboreda, 1980) and visual cues (Daneri et al., 2011; Jenkin & Laberge, 2010) to navigate to the rewarded arm of a Y- or T-maze. Interestingly, the Argentine toad prefers to use egocentric cues

rather than a visual cue when the two are in conflict (Daneri et al., 2011). Some anurans can use more complex visual cues (e.g., geometric cues (Sotelo, Bingman, & Muzio, 2015) and spatial cues (Liu, Day, Summers, & Burmeister, 2016). In túngara frogs, the ability of females to return to a preferred mate when sequentially assessing males (Ryan, 1985) is predicted to be improved by learning a simple association between a cue (e.g., the male's call or other cue in the environment) and the male's location (Akre & Ryan, 2010; Ryan et al., 2009). This type of strategy is considered a cue-taxis strategy for place learning (Day, 2003; O'Keefe & Nadel, 1978). Thus, I chose to test my hypothesis in a simple place-learning task in which the rewarded arm of a maze was associated with a visual cue. If female mate choice has selected for better place memory in túngara frogs, I predict that females would outperform males in this task. In contrast, if males have been selected to have better place memory (e.g., through stronger selection for remembering pond location) then I would predict that males would outperform females.

Materials and methods

(a) Animals

Túngara frogs are distributed in lowland neotropical forests of Central and South America (Ryan, 1985). Because they are not commercially produced, the availability of túngara frogs for laboratory study is limited. For my two-arm maze and colour preference tests, I used seven females and six males. For my optomotor test (described below), I gained access to an additional 10 females and 10 males. All my animals were sexually mature and were one or two generations derived from populations in Gamboa Panama. In addition, the frogs were naïve for any experiment.

I maintained the animals under conditions that approximated their natural habitat: 25° C, 80% relative humidity (RH), 12:12 light:dark cycle (lights on at 07:00 h). I housed the frogs in two same-sex terraria (50 × 25 × 27 cm) and, because I did not provide the animals with standing water, they remained non-reproductive throughout the duration of the study. I fed them fruit flies that were 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

I used a two-arm maze composed of six bricks that were painted white (Figure 2.1). The maze was 9.5 cm high and consisted of a central starting chamber (18 × 18 cm) and two channels (A and B, each 18 cm × 6.5 cm). I blocked the exits at the end of the channels with a red or yellow poster board to serve as doors, only one of which could be opened. I blocked the incorrect door from behind with a brick, which was not visible to the frog in the maze. The correct door could be opened with a rope attached to its reverse side. To prevent the frogs from escaping, I covered the maze with glass. I covered the floor of the maze with absorbent paper that I replaced every other day. I surrounded the maze with a 1.4 m-high white curtain in order to isolate other visual cues in the room. To motivate the frogs to locate the maze exit in order to be returned to their home cage, I created a bright, hot (37° C), and dry (10% RH) environment inside the maze. To maintain the maze temperature, I placed a heater along one longer side of the arena.

(c) Colour preference and test of discriminability

I selected the door colours based on the results of a preference test. I tested two pairs of colour: yellow vs. dark blue and yellow vs. red. In the preference test, I allowed each frog to approach one of the colours that were placed at the end of the two channels (Figure 2.1) after being released in the starting chamber with orientation perpendicular to the two arms. I did two trials to test each colour pair for each individual, alternating the location of the colours. The first colour that a frog touched was counted as the preferred colour for that trial. I assigned each frog a score (0, 1, or 2) based on the number of times the frog chose yellow in the two trials, and I used a one sample t-test assuming the frogs would select yellow once (half the trials) if there were no preference. When compared to yellow, the frogs preferred the dark blue door (yellow:blue = 3:10, blue:yellow = 11:2; $t_{12} = 2.9$, $P = 0.014$), while there was no preference between yellow and red (yellow:red = 7:8, red:yellow = 6:5; $t_{12} = 0.43$, $P = 0.673$). Thus, I chose the yellow and red as the door colours for my place-learning task.

While the frogs did not express a preference between the red and yellow doors, I could not determine from the preference test whether the two colours could be discriminated. To address this, I

capitalized on the optokinetic response of vertebrates, including túngara frogs (Cummings et al., 2008), in which an animal will move its head and body position to adjust its eye orientation coincident with moving visual stimuli (i.e., the animal moves in the direction of the moving stimuli). The optokinetic response is elicited in an optomotor test in which a spinning drum is lined with alternating panels. If the frogs are capable of discriminating the red and yellow poster board, I reasoned that an optomotor test with alternating red and yellow panels should elicit an optokinetic response. If the colours cannot be discriminated, then it would not be possible for them to elicit an optokinetic response.

The túngara frogs in the optomotor test were different from individuals in the two-arm maze and colour preference test. My optomotor device was based on one previously used to test túngara frogs (Cummings et al., 2008). I lined the spinning drum with 1.5-cm wide strips of the yellow and red poster boards that were the same as those I used for the maze doors. After allowing the frogs (n = 10 males and 10 females) to dark-adapt for 60 min, I adapted them in the optomotor device (stationary) for 5 min in the lighting conditions used in the place-learning task. Next, I tested each individual in two successive trials (2 min each) in which I assigned the drum spinning direction of one trial as clockwise and the other as counter clockwise in a pseudorandom manner. I quantified the angle moved in either direction (drum spinning direction or counter drum spinning direction) in each trial and averaged the moving angles of the two trials for each frog.

Túngara frogs demonstrated an optokinetic response to the yellow-red stripes by moving more in the drum spinning direction than the counter drum spinning direction (direction: $F_{1,36} = 11.4$, $P = 0.002$; Table 2.1). There was no evidence for a sex difference (sex: $F_{1,36} = 0.03$, $P = 0.87$; direction x sex: $F_{1,36} = 0.11$, $P = 0.74$; Table 2.1). Thus, males and females appear to have similar abilities to discriminate the red and yellow doors under the conditions tested in my task.

(c) Procedure

Acclimation

Before training began, I acclimated the frogs to the arena in two trials over two days. During acclimation, I removed the coloured doors, leaving both channels open. I released the frog in the middle

of the starting chamber, with the frog oriented 90° to the channels leading out of the maze. I alternated initial orientations in the two trials. All frogs exited the maze in a short time (mean ± SD: 137 ± 29). Once each frog exited the maze, I returned it to its home cage.

Acquisition

I closed the exits of the maze by placing the yellow and red doors at the end of channel A and B, respectively (Figure 2.1). During acquisition, the red door (correct door) could be opened while the yellow one was blocked. I trained the frogs in two trials per day for nine successive days with an inter-trial interval greater than 1 h. In the first trial of the day, I placed the frog in the starting chamber oriented perpendicularly to the two arms, with the direction determined pseudorandomly, and then alternated their orientation 180° for the second trial of that day, in order to prevent them from solving the task by remembering turning direction (i.e., egocentric cues). I defined the trial as successful if the frog knocked down the correct door directly, touched the correct door, or sat very close (less than 0.5 cm) to the correct door within three minutes. In the latter case, I pulled the rope to open the door. If the frogs failed to complete the task after three minutes, I defined it as a non-successful trial. Then I opened the door and allowed them to exit. In all cases, I returned the frogs to their home cage upon exiting the maze.

Reversal learning

During reversal learning, I used the same maze and procedure as acquisition, except that the red door was blocked while the yellow door could be opened. Hence, it required the frogs to reverse the associations they had learned during acquisition.

Running speed

I estimated velocity of movement speed by dividing the sum of visits to each area (channel A, B, starting chamber) with latency. Before learning has occurred, movement speed reflects baseline motivation to exit the maze. During periods of learning, movement speed likely reflects increased familiarity with the goal of the task (that is, to find an exit in order to be returned to the home cage) or development of a stronger association with the cue (door colour) and the reward (return to home cage).

Probe trials

I conducted two identical probe trials immediately after acquisition and reversal. Each probe trial consisted of one 3-min trial in which the doors were switched location and neither could be opened. I began the probe trials with initial random orientation perpendicular to the channels. Because door colour was confounded with place during training, the probe trials enabled me to determine which cues were used to navigate to the correct exit (e.g., door colour or some other unintended cue).

(d) Analysis and Statistics

I quantified behaviour from video recordings. I used success rate (mean number of successful trials per day) as the primary measure of learning across days. Because success rate is a proportion, I used an arcsine transformation on the data before analysis. I also recorded latency to exit the maze, but I do not report those data because, in all cases, they replicate success rate. To determine whether there was a sex difference in learning, I used repeated measures ANOVAs to examine the interaction between day and sex on success for all training days. I used within-subjects contrast (linear trend) for the effect of day on success for each sex separately to ask whether success rate improved. I used ANOVA to test for a sex difference in movement speed.

For probe trials, I quantified the duration frogs spent in each channel as a measure of channel preference. I used Wilcoxon signed-ranks test to determine if the frogs were using colour of the door to learn the task. A paired t-test produced identical conclusions.

I observed that the frogs had a tendency to turn left after release in the starting chamber, which may be related to right-handedness in amphibians (Bisazza, Cantalupo, Robins, Rogers, & Vallortigara, 1996). First, I used Fisher exact probability test to determine if male and female differ in the left-turn tendency in their first training trial. In addition, I examined the relationship between the left-turn tendency and performance in the maze throughout training. I used ANOVA to determine whether success rate differed depending on initial orientation (i.e., whether frogs were more likely to be successful if the channel to their left was correct) and whether this differed between the sexes (i.e., interaction between effects of sex and initial orientation on success rate). I used repeated measures ANOVAs to ask whether

the tendency to turn left or the probability of first visiting the correct channel changed over training or differed between the sexes (interaction between sex and day and effects of day within each sex). Because left turn rate and rate of initially correct channel are proportions, I used an arcsine transformation on the data before analysis.

Finally, I examined whether the frogs tried to use egocentric cues (remember last turn direction) to solve the maze by capitalizing on an unintended feature of my training protocol. I intended to prevent the use of egocentric cues by randomly determining initial orientation of release for the first trial of a day and then switching the orientation for the second trial that day. However, because the orientation of the first trial of a day was randomly determined, across days (trial 2 of previous day and trial 1 of current day), there were some pairs of trials in which release orientations were the same (and the frogs could potentially remember their previous turn direction) and some trials in which release orientations were different. If frogs were using egocentric cues, they should perform better in trials in which release orientation was previously the same compared to trials in which release orientation was different. [Note, this is independent of whether the correct channel was on the left.] I used repeated measures ANOVA to determine the effect of relative position of initial orientation (same or different than previous trial) on success rate and to determine if there was a sex difference in this effect (i.e. interaction between sex and effect of relative position on success rate).

All of the statistics were run in SPSS (v. 20, IBM, Armonk, NY).

Results

(a) General Performance

During acquisition, females performed better than males (sex × day: $F_{8,88} = 2.3$, $P = 0.03$; Figure 2.2). Specifically, females got increasingly better at solving the maze (day: $F_{8,48} = 3.1$, $P = 0.007$; linear trend: $F_{1,6} = 15.1$, $P = 0.008$) while males did not (day: $F_{8,40} = 0.6$, $P = 0.77$; linear trend: $F_{1,5} = 0.89$, $P = 0.39$). This was true even if one only considers the last five days of training (males: linear trend: $F_{1,5} = 1.07$, $P = 0.35$). However, in the reversal learning, females failed to improve their performance (day: $F_{8,48}$

= 0.6, P = 0.78). In fact, even after nine days of reversal, females continued to visit the previously correct channel (red channel) at a similar rate as during acquisition (see result of probe trial).

During acclimation, latency to exit the maze was similar for males and females (sex: $F_{1,11} = 0.19$, P = 0.68), indicating similar levels of motivation in the task. In addition, on the first day of acquisition, males and females had similar movement speeds (sex: $F_{1,11} = 0.056$, P = 0.82). However, females increased their movement speeds across acquisition (linear trend: $F_{1,6} = 6.5$, P = 0.04), while males did not (linear trend: $F_{1,5} = 0.27$, P = 0.63). This increased movement speeds during learning suggests that females were developing a greater familiarity with the task - that is, an understanding that the task is to approach the door in order to exit. However, during reversal learning, movement speeds remained steady for both males and females (male: linear trend: $F_{1,5} = 1.60$, P = 0.26; female: linear trend: $F_{1,6} = 0.04$, P = 0.85) and overall velocity was similar between males and females (sex: $F_{8,4} = 0.84$, P = 0.61). This supports the interpretation that motivation was similar between the sexes and that the increased movement speed of females during acquisition reflected learning.

(b) Probe Trials

During the first probe trial I switched the red (previously correct) and yellow doors so that they were now associated with different channels of the maze. During the probe, females spent significantly more time in the channel with the red door (T = 1, N = 7, P < 0.05; Figure 2.3a), indicating that they had learned to associate the red door with the exit, and not other place cues. In contrast, males did not show a preference for either channel (T = 8, N = 6, P > 0.05; Figure 2.3b), which is further indication that they failed to associate the red door with the exit.

The second probe trial followed reversal, during which the yellow door (channel B) was correct. In spite of nine days of reversal training, during the probe trial, females still preferred the channel associated with the red door (T = 2, N = 7, P = 0.05; Figure 2.3a), while male did not have a preference (T = 10, N = 6, P > 0.05; Figure 2.3b). This finding suggests that one reason for the failure of females to reverse their associations was an inability to extinguish what they had previously learned.

(c) Left-turn Bias

Túngara frogs showed a strong tendency to turn to their left side. Eleven of 13 of them made a left turn as their first action in the first trial of the experiment. There was no evidence of a sex difference in the left-turn bias (Fisher exact probability test: P = 0.731). Generally, success rate was significantly higher when the correct door was on the frog's left side compared to the right (side: $F_{1,11} = 17.2$, P = 0.002), indicating that they tended to enter the first channel they saw. However, this left-turn bias influenced the success of males more than females (sex x side: $F_{1,11} = 6.8$, P = 0.025; Figure 2.4*a*).

Given the strong effect of the turning bias on success, I asked whether females learned to solve the maze by modifying their first turn direction. In fact, females continued to turn left as their initial action across acquisition (day: $F_{8,48} = 0.41$, P = 0.55; linear trend: $F_{1,6} = 0.03$, P = 0.88; Figure 2.4*b*). In addition, there was no sex difference in the left turn tendency across training (sex x day: $F_{8,88} = 0.53$, P = 0.83; Figure 2.4*b*) or within males across days (day: $F_{8,40} = 0.14$, P = 0.99; linear trend: $F_{1,5} = 0.09$, P = 0.77; Figure 2.4*b*), indicating that differences in learning were not due to differences in the tendency to turn left. However, although they showed no plasticity in their left-turn rate, females appeared to be able to suppress the tendency to first visit the left channel (day: linear trend: $F_{1,6} = 6.0$, P = 0.05; Figure 2.4*c*). In other words, females increased the likelihood of first visiting the correct channel, even when it was on their right, in spite of turning left initially. In contrast, males showed no evidence of such plasticity (day: linear trend: $F_{1,5} = 0.32$, P = 0.59; Figure 2.4*c*).

(d) Effect of Egocentric Cues

Finally, although I interrupted the frogs' ability to use egocentric cues (remember last turn direction) to solve the maze, I found evidence that males tried to use such cues. In trials when the orientation of release was the same as the previous trial, males performed better than when the relative orientations differed, while female performed equally well in both situations (sex x relative orientation: $F_{1,11} = 8.1$, P = 0.02; Figure 2.5). In fact, when males were able to use egocentric cues, they performed as well as females (Figure 2.5).

Discussion

I found that female túngara frogs were able to use a visual cue to remember the location of the maze exit while males were not. However, females were unable to reverse these learned associations because they could not extinguish what they had learned during acquisition. Both males and females showed a tendency to initially explore their left side in the maze and while there was no initial sex difference on this tendency, this left-turn bias influenced the performance of males more strongly than females. Finally, although I prevented frogs from using egocentric cues to solve this maze, I found males still tried to use these cues.

The sex difference in performance in my two-arm maze could have emerged from sex differences in sensory abilities. I tested for the ability to discriminate the two doors in an optomotor test and found no evidence for a sex difference in discriminability in the lighting conditions used during training. Furthermore, there is no evidence to date for sex differences in visual sensitivity (Cummings et al., 2008), or any evidence from their natural history (Ryan, 1985) to suggest a sex difference in vision. Yet, I cannot strictly rule out the possibility that the doors, within the context of the maze, had differential discriminability by males and females. Nonetheless, give current evidence, this seems an unlikely explanation for the sex differences I observed.

Differences in performance could also be caused by differences in motivation to solve the task (Wise, 2004; Wulf & Lewthwaite, 2016). I used latency during acclimation and initial movement speed during acclimation as an estimate of the frogs' general motivation (Olarte-Sánchez, Valencia-Torres, Cassaday, Bradshaw, & Szabadi, 2015; Vorhees & Williams, 2006), and found no evidence for a sex difference. A sex difference in movement speed only emerged when females were learning the task, which likely reflects increased familiarity with the goal of the task.

Finally, the differences in performance I observed could have emerged from differences in cognitive abilities, such as learning ability, differential cue use, attention, and/or behavioural flexibility. One factor that appeared to be important in the ability of females to learn to associate the visual cue with the exit was their ability to suppress the tendency to visit the first channel they saw (usually the left

channel) after being released in the starting chamber. Males, in contrast, were unable to do so. The ability of females to inhibit incorrect responses may reflect greater behavioural flexibility (Floresco, Zhang, & Enomoto, 2009; Ragozzino, 2007) or lower levels of impulsivity (Anderson & Platten, 2011; Reynolds, Ortengren, Richards, & de Wit, 2006). Regardless, the ability to inhibit this tendency may have enabled females to associate the visual cue with the correct channel of the maze. In contrast, males relied more on egocentric cues (remember last turn direction) and they failed to associate the visual cue with the correct channel. This dependence on egocentric cues is reminiscent of findings from Argentine toads (Daneri et al., 2011), and suggest a significant difference in cue use in male and female túngara frogs. Overall, the evidence suggests that, compared to males, females have greater learning abilities in a visually-cued place learning task and that these abilities were related, at least in part, to less impulsivity or greater behavioural flexibility. Whether the sex differences in learning I observed can be generalized to other cognitive tasks or to place learning tasks that involve other cues (e.g., egocentric cues) will require further study, particularly given the relatively small sample sizes in my study. Given the apparent preference of males to use egocentric cues, it will be of particular interest to know whether the sex differences in learning persist when egocentric cues are associated with the maze exit.

Mate choice decisions can have a direct effect on reproductive success. In frogs, females generally prefer male calls with lower fundamental frequencies (Ryan, 1980) which, in turn are associated with larger male body size and, consequently, higher fertilization rates (Gibbons & McCarthy, 1986; Kruse, 1981). Thus, a female's ability to remember the location and/or calls of a preferred male could directly affect her reproductive success. In contrast, there is no evidence to date that male túngara frogs experience selection for place memory, such as during pond selection, migration, or competitive interactions with other males. However, it is important to acknowledge that little is currently known about the spatial ecology of túngara frogs and the strength of my interpretation is limited by that lack of knowledge.

Sex differences in cognition have been widely reported, particularly for spatial cognition (Geary, 1995; Halpern, 2013; Jones, Braithwaite, & Healy, 2003). In most cases, it is the males that experience

greater demands for spatial cognition and outperform females in laboratory tasks (Gaulin, 1992; Gaulin & FitzGerald, 1986; Jones et al., 2003). But in the female cowbird (Guigueno et al., 2014; Sherry et al., 1993) and the túngara frog (present study), females outperform males. These cases provide strong support for the adaptive specialization hypothesis, as they de-couple sex from the link between natural history and cognition. My study broadens the support for the adaptive specialization hypothesis somewhat further by putting sex differences in cognition into the context of sexual selection.

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Table 2.1. Optokinetic response to a drum with yellow-red stripes as measured by mean (± SE) degree of movement. Match refers to movements that were in the same direction as the spinning drum. Mismatch refers to movements that were in the opposite direction as the spinning drum.

	Match (degree)	Mismatch (degree)
Male	75 ± 25.25426	7 ± 2.134375
Female	79.5 ± 33.47843	5.5 ± 2.291288

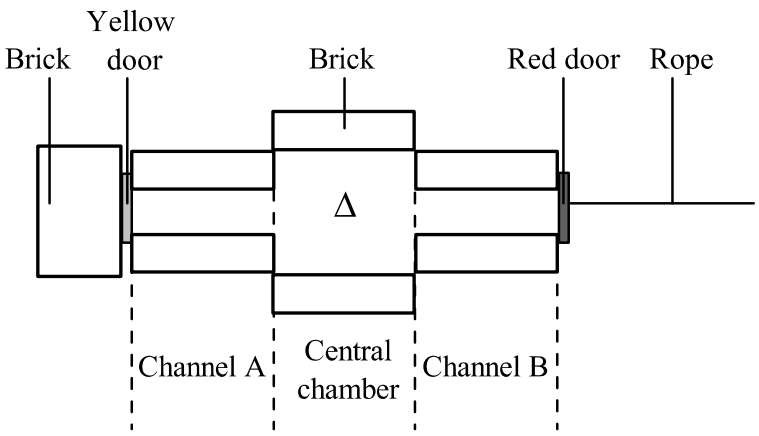

Figure 2.1. Schematic drawing of the two-arm maze (not to scale). The triangle indicates the release point of frogs; initial orientations were opposite between two trials in the same day.

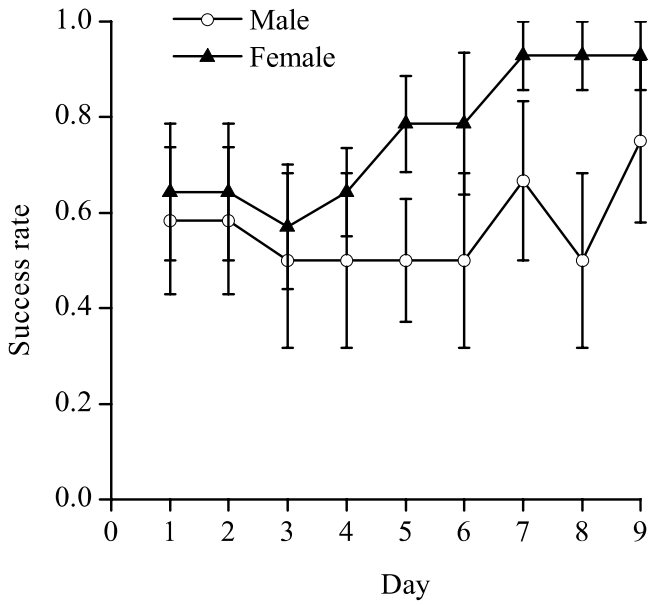

Figure 2.2. Variation in success rate (mean ± SE) of male and female túngara frogs during acquisition. Success rate was defined as the proportion choosing the correct (i.e., rewarded) door.

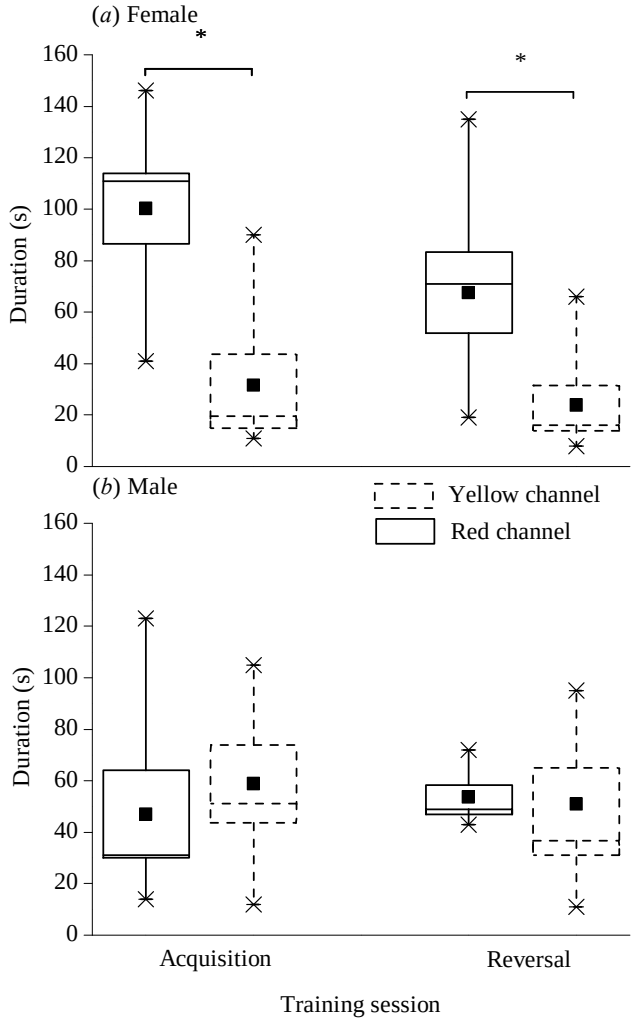

Figure 2.3. Channel preferences (duration) during the probe trials in (*a*) females and (*b*) males. Stars represent maximum and minimum values, the upper and lower border of the rectangles represent the standard error, solid squares represent the mean, and horizontal lines represent the median. The asterisk indicates significant differences of Wilcoxon test ($P < 0.05$) in time spent in the two channels.

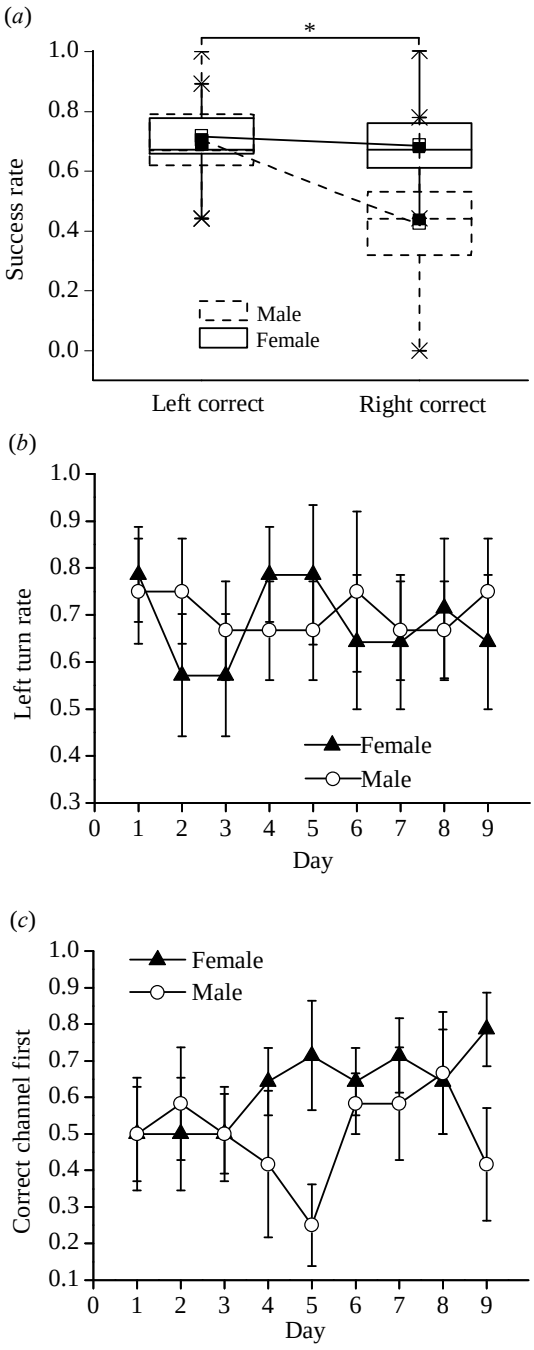

Figure 2.4. The effect of body turn tendency on performance. (*a*) The effect of initial orientation on success rate in males and females; left correct refers to trials in which the correct channel was on the animals' left side. Stars represent maximum and minimum values, the upper and lower border of the rectangles represent the standard error, solid squares represent the mean, and horizontal lines represent the median. The asterisk indicates a significant interaction between sex and initial orientation ($P < 0.05$). (*b*) Variation in left turn rate (mean ± SE) during the acquisition. (*c*) Variation in the rate of visiting the correct channel first (mean ± SE) during the acquisition.

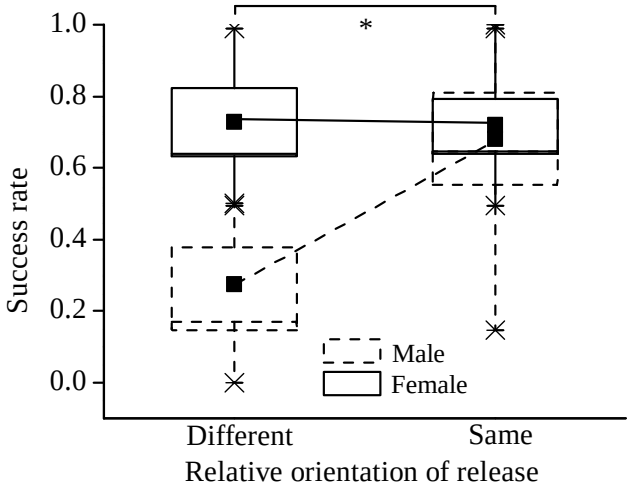

Figure 2.5. The effect of egocentric cues on success rate in male and female túngara frogs. The role of egocentric cues was assessed by comparing trials when a release orientation was different or the same compared to the preceding trial. When release orientation was the same as the preceding trial, an animal could potentially remember the previous turn direction (i.e., use egocentric cues). Stars represent maximum and minimum values, the upper and lower border of the rectangles represent the standard error, solid squares represent the mean, and horizontal lines represent the median. The asterisk indicates a significant interaction between sex and relative orientation ($P < 0.05$).

CHAPTER 3: LEARNING TO LEARN: ADVANCED BEHAVIOURAL FLEXIBILITY IN A POISON FROG[2]

Summary

Behavioural flexibility is essential for survival in a world with changing contingencies and the evolution of behavioural flexibility is linked with complex physical and social environments. Serial reversal learning, in which reward contingencies change frequently, is a key indicator of behavioural flexibility. While many vertebrates are capable of serial reversal learning, only birds and mammals have previously been shown to use rule-based decision strategies (e.g., win-stay/lose-shift) to become better at learning changes in reward contingencies across reversals. While the lifestyles of many amphibians have a degree of complexity, the evidence to date suggests limited levels of behavioural flexibility. Here, I show that the poison frog *Dendrobates auratus*, which has evolved complex parental behaviours that likely depend on remembering locations in a flexible manner, can use a win-stay/lose-shift strategy to increase their behavioural flexibility across sequential changes in the reward contingencies in a visual discrimination task. Furthermore, probe trials demonstrate that the frogs used the provided visual cues to spatially orient in the maze in a manner reminiscent of complex spatial cognition. My study provides the first evidence of serial reversal learning in frogs and is the first to demonstrate the use of a rule-based learning strategy in a non-avian, non-mammalian species.

Introduction

Behavioural flexibility is the ability to change one's behaviour according to variation in the environment, and it can enable animals to increase survivorship and reproductive success (Fagen, 1982;

[2] This chapter has been published on Animal Behaviour.

Snell-Rood, 2013). For example, species with greater levels of behavioural flexibility are more like to successfully invade a new environment (Tebbich, Sterelny, & Teschke, 2010; Wright, Eberhard, Hobson, Avery, & Russello, 2010). Serial reversal learning, in which animals progressively improve their performance in a task with frequently changing reward contingencies, is a standard laboratory method for measuring behavioural flexibility (Bitterman, 1965; Roth & Dicke, 2005), and the ability to perform serial reversals is more often found in animals that live in complex physical and social environments (Bond, Kamil, & Balda, 2007; de Waal & Tyack, 2003; Godfrey-Smith, 2002).

There are several mechanisms that allow animals to learn serial reversal tasks (Gonzalez, Behrend, & Bitterman, 1967; Mackintosh, 1974; Parker et al., 2012; Shettleworth, 2009; Strang & Sherry, 2014). Among them, lower-order processes, such as proactive interference (Bitterman, 1965; Mackintosh, 1974), involve involuntary learning and hence are thought to represent a lower level of behavioural flexibility (Parker et al., 2012; Shettleworth, 2009). These mechanisms have been discovered across a broad range of vertebrate taxa (Gaalema, 2011; Gonzalez et al, 1967; Mackintosh, McGonigle, & Holgate, 1968). In contrast, rule-based strategies, which indicate the ability of an animal to learn and use the underlying rule of the reversal task, represent a greater degree of behavioural flexibility (Parker et al., 2012; Shettleworth, 2009). For example, using a win-stay/lose-shift rule requires animals to make their current choice based on the reward from their previous choice (Mackintosh et al., 1968; Shettleworth, 2009). The optimal outcome of this strategy is the one-trial reversal in which animals make an error on the first trial of a reversal followed by all correct choices on subsequent trials of that reversal (Mackintosh et al., 1968). This type of rule-based strategy has only previously been found in mammals and birds (Mackintosh et al., 1968; Rayburn–Reeves, Stagner, Kirk, & Zentall, 2013; Rumbaugh, Savage-Rumbaugh, & Washburn, 1996; Shettleworth, 2009).

Amphibians, which include both aquatic and terrestrial lifestyles in their lifecycle, have to handle environments with a high degree of complexity. Yet, they were once thought to lack behavioural flexibility (Bitterman, 1965, 1975), and have been considered inflexible in learning tasks in artificial laboratory environments (Maier & Schneirla, 1935). More recent studies, however, show that amphibians

can solve mazes using local visual cues (i.e., visual cues that are directly associated with goal or are part of the goal), and body-centred motor strategies (remembering a place by learning to turn left or right), and geometric cues (i.e., the shape of the space) (Daneri, Casanave, & Muzio, 2011; Ellins, Cramer, & Martin, 1982; Sotelo, Bingman, & Muzio, 2015; Crane & Mathis, 2011; Heuring & Mathis, 2014). Furthermore, in simple discriminations, amphibians are capable of single reversals (Daneri et al., 2011; Ellins et al., 1982; Schmajuk, Segura, & Reboreda, 1980). Nonetheless, I still know relatively little about the cognitive strategies used by amphibians in reversal tasks and whether they are capable of the types of behavioural flexibility observed in mammals and birds.

While many frogs have relatively simple social behaviours, the poison frogs (Dendrobatidae) have evolved complex social and spatial behaviours reminiscent of many mammals and birds (Summers, 1989; Summers & Tumulty, 2013): they are territorial, show mate guarding and pair bonding (some are even monogamous; Brown, Morales, & Summers, 2010), and the parents of some species transport tadpoles to deposition sites (small pockets of water) in the forest canopy after hatching. Some species show homing abilities in the field that suggest advanced spatial cognition (Pasukonis, Warrington, Ringler, & Hödl, 2014). However, whether poison frogs can use spatial cues in a flexible manner and whether they use cognitive strategies similar to birds and mammals is unknown

I trained the poison frog *Dendrobates auratus* in a two-arm maze in which the position of the correct arm was associated with visual cues in the starting chamber. The visual cues could be reliably associated with the goal based on spatial relationships, but could not be used for direct guidance (e.g., an animal could not simply approach the visual cues to locate the goal). My study was designed to: 1) determine whether poison frogs could use visual cues to learn a complex spatial discrimination task; 2) investigate whether poison frogs are capable of serial reversal learning; and 3) identify the behavioural mechanisms underlying improvement during serial reversal.

Materials and methods

(a) Animals

I used ten (four male, six female) sexually mature *D. auratus* that were bred in captivity and were likely several generations removed from the wild (Indoor Ecosystems, LLC). In this species, males maintain territories and provide parental care (egg attendance, tadpole transport); females maintain territories and perform mate guarding, but do not provide parental care (Summers, 1989). I maintained the animals under conditions that approximated their natural habitat: 25° C, 80% relative humidity (RH), 12:12 light:dark cycle (lights on at 07:00 h). I housed the frogs individually in terraria and fed them fortified fruit flies 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

The maze consisted of a central starting chamber and two arms (Figure 3.1). The maze arms were uniformly white but the starting chamber had visual cues on each side (Figure 3.1). The frogs were required to use the visual cues in the starting chamber to spatially orient to the goal. I blocked the exits at the end of the arms with identical white doors, only one of which could be opened during a given trial. I attached a rope to the reverse side of the correct door and I blocked the other door from behind with a brick that was not visible to the frog in the maze. I used white absorbent paper, which was replaced every day, as the floor of the maze. Thus, any potential olfactory cues on the floor would be disrupted each day and would not be reliably associated with the goal. I covered the maze with Plexiglas and surrounded the maze with a 1.4 m-high white curtain in order to isolate extraneous visual cues in the room. I recorded trials using a camera above the arena (1.5 m-high). Experimenters, who were blind to the progress of each individual, sat outside the white curtain to record each training trial and open the door on the correct side. I provided five shelters outside the maze in which the frogs could find refuge after exiting the maze (Figure 3.1). To motivate the frogs to locate the exit in order to find shelter, I created a bright, hot (37° C), and dry (10% RH) environment inside the maze. The frogs are accustomed to a moist environment with ample shelter, similar to the forest floor, and, as such, they find the bright, open environment of the maze to be aversive. Therefore, the reward for finding the correct door was to gain access to a shelter and then the home cage.

(c) Procedure

Acclimation

Before training, I acclimated the frogs to the maze in two trials approximately 24 hours apart. During acclimation, both doors were open and no shelters were provided. I used a small, overturned pot with a cardboard floor to transfer and release the frogs in the middle of the starting chamber, resulting in an unpredictable orientation of the frog at the start of each trial. All frogs appeared highly motivated to leave the maze and successfully exited within 2 minutes.

Acquisition

For the initial learning trials (acquisition), I arbitrarily determined which door was correct. I trained the frogs with three trials per day with an inter-trial interval greater than 1 hour (from 60 min to 80 min). I wiped the apparatus with alcohol after all individuals finished one trial. As frogs could be in any position within the release chamber when trials began, the orientation of the frog at the start of each trial was unpredictable.

I defined three possible behavioural outcomes for each trial: Successful trials without error were those in which the frog approached within 0.5 cm of the correct door within two minutes of release without first moving halfway down the incorrect arm (i.e., committing a position error). Successful trials with error were those in which the frog first advanced at least half way toward the incorrect door (a position error) before approaching within 0.5 cm of the correct door within two minutes of release. Unsuccessful trials were those in which the frogs failed to complete the task after two minutes. In unsuccessful trials, I opened the correct door and allowed the frogs one additional minute to exit, after which I guided them to the exit by orienting them to face the exit and touching them to make an initial hop in the correct direction. After exiting, the frogs entered one of five small shelters that I used to return them to their home cage.

I operationally defined a learning criterion in order to determine when an individual frog's performance demonstrated sufficient evidence of learning. Because the threshold for success (0.5 cm of

correct door) and the threshold for a position error (halfway toward incorrect door) were not equidistant, their probabilities were not equally likely. Therefore, as is standard in similar studies (Le Bourg & Buecher, 2002; Landau & Spelke, 1988), I used the outcomes on the first day of training (i.e., in naïve animals) to estimate the random probability of success. This approach provides a more accurate measure of learning than using chance probability of turn choice or an arbitrary benchmark. In my case, I required that the animals perform a successful trial without error, and, in naïve animals, the probability of such an outcome was 17%. I then defined my learning criterion as seven successful trials without error in nine sequential trials (7/9 = 77.8%). Based on a binomial test, this performance criterion differs significantly from that of naïve frogs ($p = 1.1 \times 10^{-4}$). Thus, I could be confident that animals that reached the criterion had learned the task.

Reversal

Each time a frog reached criterion, I reversed the reward contingencies by switching the location of the correct door for five sequential reversals. I recorded the number of trials required for each frog to reach the criterion and used repeated-measures ANOVA to determine whether the number of trials to criterion decreased across reversals. The experimenters performing the trials were blind to the identity of the animal and the experimental expectation. Furthermore, they did not know which stage of the experiment each individual was in (acquisition, reversal 1, etc.) for a particular trial. After the trials were run, I collected additional data from the videos. During data collection, I was blind to the identity of the frog, the training session (acquisition, reversal 1, etc.) and which side was correct (that is, until the end of the trial when the door opened).

Probe trial

Although I only intended to provide the visual cues in the starting chamber, frogs could potentially use any available cue, including visual cues invisible to humans, olfactory cues and so on, which are unpredictable and hard to control. Therefore, to determine whether the frogs used the visual cues in the starting chamber when solving the maze, I ran two probe trials for each individual once that

frog achieved the learning criterion in acquisition and on the 5th reversal. In the probe trials, I blocked both doors and switched the two walls of the starting chamber to opposite sides. Thus, the contingency between the visual cues and the correct turning response was reversed from that during training. Because I left all other potential cues intact, the probe trials determine whether the frogs' behaviour was guided by the provided cues, and not other, uncontrolled cues. During the 3-min probe, I quantified time spent in each arm. I refer to the arm as spatial-correct if it was the correct side indicated by the visual cues, and as original-correct if it was the correct arm during acquisition. I used paired samples *t*-test to compare the duration in each arm in the probe trials. After the first probe trial, I retrained individuals to criterion before proceeding with the first reversal.

(d) Error Analysis

To examine the behavioural mechanisms underlying the improvement in reversal learning, I analysed the types of errors committed during each reversal. I defined position errors as cases in which a frog advanced half the length of the incorrect arm. I defined non-contingent errors as cases in which the frogs failed to approach either door. This error may reflect familiarity with the task (that is, an understanding that the task is to approach a door in order to exit) or a lack of motivation to complete the task. I defined perseverative errors as the number of position errors before the first success after a particular reversal. Perseverative errors reflect poor extinction (i.e., the inhibition of previously learned responses; Mackintosh et al., 1968; Strang & Sherry, 2014). Extinction is a critical step in learning a reversal task because an animal must inhibit previously learned responses in order to learn new associations and rapid extinction suggests the animal has learned the overall rule of the task -- that serial reversals are taking place.

In order to test if the frogs used a rule-based strategy (i.e. win-stay/lose-shift) to solve the serial reversal task, I created a choice matrix to categorize the choice pair of every two successive trials within individuals. For each trial, there were four types of choices: position error, non-contingent error, success (no error), and a position error in a successful trial (position error + success). I labelled each cell in the matrix with win-stay, lose-shift, win-shift, lose-stay or excluded (Table 3.1).

I recorded the frequency of each category and calculated the win-stay rate and lose-shift rate by taking their proportions out of the trials with win and lose as the previous choices respectively. Because the win-stay and lose-shift data are proportions that cannot be normally distributed, I used an arcsine transformation before statistical analysis. I used repeated measures ANOVA to determine whether errors (non-contingent, perseverative) or decision strategies (win-stay rate and lose-shift rate) changed across reversals. All of the statistics were run in SPSS (v. 20, IBM, Armonk, NY).

Results

All frogs learned to find the correct door, reaching the criterion for learning in seven to 22 days (Figure 3.2a). Furthermore, the first probe trial demonstrated that they used the visual cues in the starting chamber when remembering the location of the correct door (Figure 3.2b; $t_9 = 2.30$, $p = 0.047$). The second probe trial showed that they continued to rely on the provided visual cues to solve the maze after five reversals ($t_9 = 3.20$, $p = 0.011$; data not shown). During five sequential reversals, the poison frogs reached the learning criterion more quickly each time they reversed, indicating a grasp of the experimenter-imposed rule of the task – that serial reversals are taking place (Figure 3.2c; $F_{4,36} = 4.14$, $p = 0.007$).

A combination of mechanisms contributed to the increased flexibility across reversals. First, I found a decrease in the perseverative errors (Figure 3.3a; $F_{4,36} = 3.31$, $p = 0.021$), reflecting the frogs' ability to inhibit responses to the previously correct door. Second, non-contingent errors declined (Figure 3.3b; $F_{4,36} = 2.19$, $p = 0.090$; linear contrast: $F_{1,9} = 6.62$, $p = 0.030$), suggesting that increased motivation or familiarity with the task contributed to the improved performance. Third, I found that the frogs increased the rate of lose-shift trials across reversals ($F_{4,36} = 3.12$, $p = 0.026$) while the rate of win-stay trails remained stable (Figure 3.3c; $F_{4,36} = 1.62$, $p = 0.190$), indicating that the frogs used a rule-based decision strategy similar to that of birds and mammals.

Discussion

I found that the poison frog *D. auratus* learned to find the maze exit by associating the correct orientation with the visual cues in the starting chamber, demonstrating they are capable of complex

spatial learning. Furthermore, I found that they were able to reverse their associations in a highly flexible manner, improving their performance across five reversals. To my knowledge, this is the first evidence of serial reversal learning in frogs. Importantly, I also found that they increased the lose-shift rate across reversals, which indicates they could use a rule-based decision strategy to flexibly respond to changing reward contingencies. While previous studies indicate that amphibians (Ellins et al., 1982), fish (Woodward, Schoel, & Bitterman, 1971), and reptiles (Gaalema, 2011; Kirkish, Fobes, & Richardson, 1979) are capable of serial reversal, none have demonstrated the use of a rule-based strategy as shown here in *D. auratus*. Thus, my study is the first to demonstrate this greater level of behavioural flexibility outside birds and mammals.

In addition to using a rule-based strategy (i.e. win-stay/lose-shift), I found that non-contingent errors declined across reversals, indicating that increased motivation and/or familiarity with the task contributed to the improved performance. This change in motivation and/or familiarity is consistent with attentional processes previously described in rodents (Mackintosh, 1974; Mackintosh et al., 1968), indicating that the frogs also used lower-order processes to increase flexibility during the serial reversal task. Therefore, I conclude that *D. auratus* is able to use both attentional processes and a rule-based strategy to flexibly adapt to an unpredictable world.

One of the hallmark behaviours in dendrobatid frogs is tadpole transportation, in which a parent transports recently hatched tadpoles from the clutch site on the forest floor to small pockets of temporary standing water in the forest canopy (Summers, 1989). Dendrobatids tend to deposit only one tadpole in one water pocket in order to increase survivorship (Summers, 1990). Since the pockets of water are a highly unpredictable resource, frogs spend considerable time locating them (Summers, 1989, 1990). However, pools can dry out or become unsuitable for other reasons, creating a highly dynamic landscape. Hence, an essential element of reproductive success is the ability of an individual to update its memory of the available deposition sites in real time. The most efficient way to maintain an accurate mental map of useful deposition sites would be to use spatial memory in a flexible manner, not unlike that demonstrated by my serial reversal task. Thus, the high level of behavioural flexibility demonstrated by *D. auratus* in

this serial reversal task is likely to have adaptive significance in nature and suggests that greater behavioural flexibility might have evolved in dendrobatids as they adapted to a terrestrial lifestyle.

The ability of dendrobatids to revisit tadpole deposition sites based on location (Stynoski, 2009) and to return to home territories after displacement (Pasukonis et al., 2014) have led to the speculation that poison frogs have spatial memory – that is, memory for locations based on the spatial relationships among distal visual cues. However, up until now, whether poison frogs could use visual cues in such a complex manner was unknown. Previous work has shown that anurans are able to use local visual cues, but, like most other vertebrates (Bitterman, 1965; Day, Ismail, & Wilczynski, 2003; Morris & Hagan, 1986; Murray & Ridley, 1999), they prefer to use a body-centred motor strategy (e.g., turn left or right) when visual cues and turn cues are in conflict (Daneri et al., 2011). Leopard frogs in a water maze appear to be incapable of using, or prefer not to use, distal spatial cues for orientation (Bilbo, Day, & Wilczynski, 2000). While my task is not as spatially complex as those using multiple distal cues in a configuration to cue the goal, such as the Morris water maze, my study is among the first, to my knowledge, to show that an amphibian can use non-local visual cues in such a complex manner when orienting in space.

In summary, I found that *D. auratus* could use visual cues in a complex spatial discrimination and they were able to update their visual associations in five sequential reversals using a rule-based decision strategy (win-stay/lose-shift). Their ability to learn the underlying rule of the serial reversal task demonstrates an advanced cognitive ability (Brown & Bowman, 2002) and indicates a degree of behavioural flexibility that until now was exclusively associated with birds and mammals.

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Table 3.1. Choice matrix to categorize the choice pair of every two successive trials within individuals

		Previous choice			
		Position error	Non- contingent error	Success	Position error + Success
Current choice	Position error	Lose-stay	Excluded*	Win-shift	Win-shift
	Non- contingent error	Excluded*	Lose-stay	Win-shift	Win-shift
	Success	Lose-shift	Lose-shift	Win-stay	Win-stay
	Position error + Success	Lose-stay	Excluded*	Win-shift	Win-shift

* These choice pairs were excluded from the error analysis because both the previous choice and current choice involved an error.

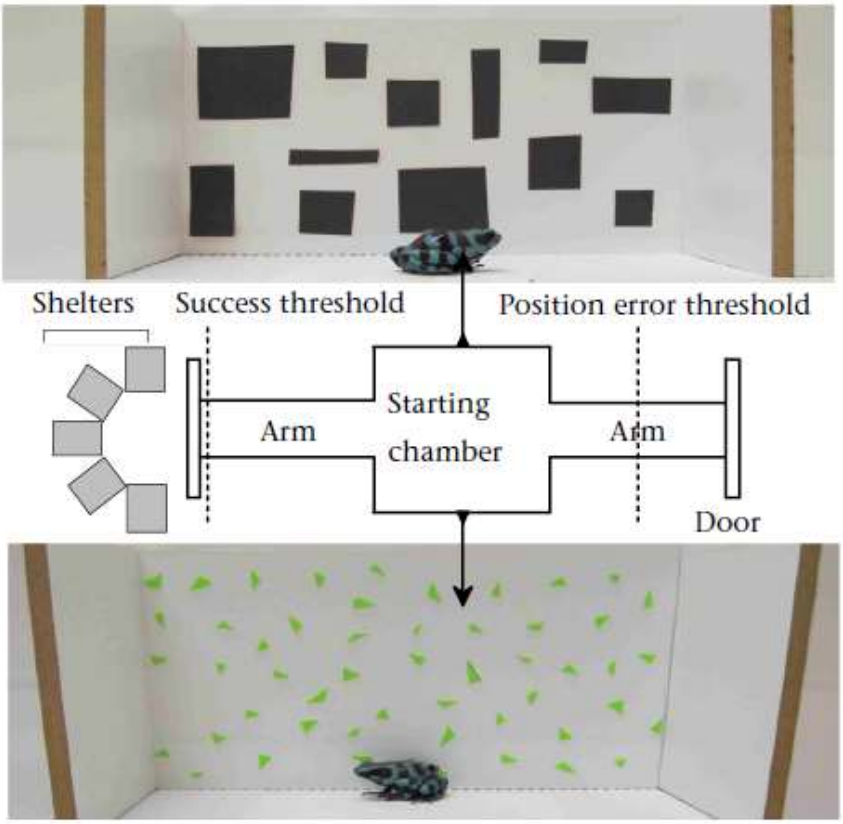

Figure 3.1. Schematic drawing of the two-arm maze [54 cm (L) x 18 cm (W) x 9.5 cm (H)] and photos of the visual cues on the interior walls of the starting chamber.

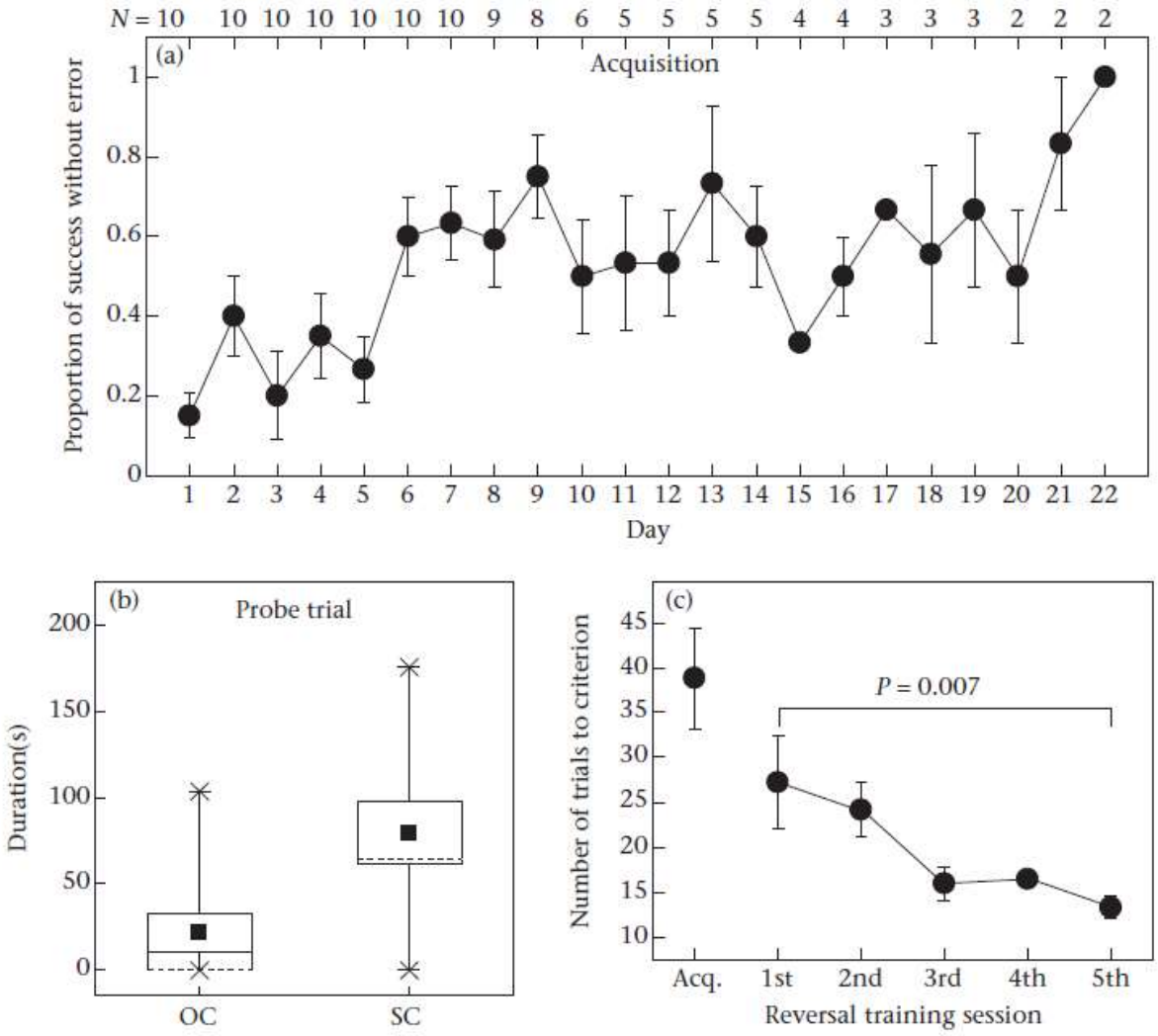

Figure 3.2. (a) Variation in the mean (± SE) proportion of successful trials without error over successive days for individual frogs during the acquisition training period. (b) Duration that frogs spent in the spatial-correct arm (SC) and original-correct arm (OC) during the first probe trial. Stars represent max and min values, solid squares represent the mean, dashed lines represent the median, upper and lower border of open rectangle represent S.E. (c) Number of trials (mean ± SE) to criterion during acquisition (Acq.) and across five reversals.

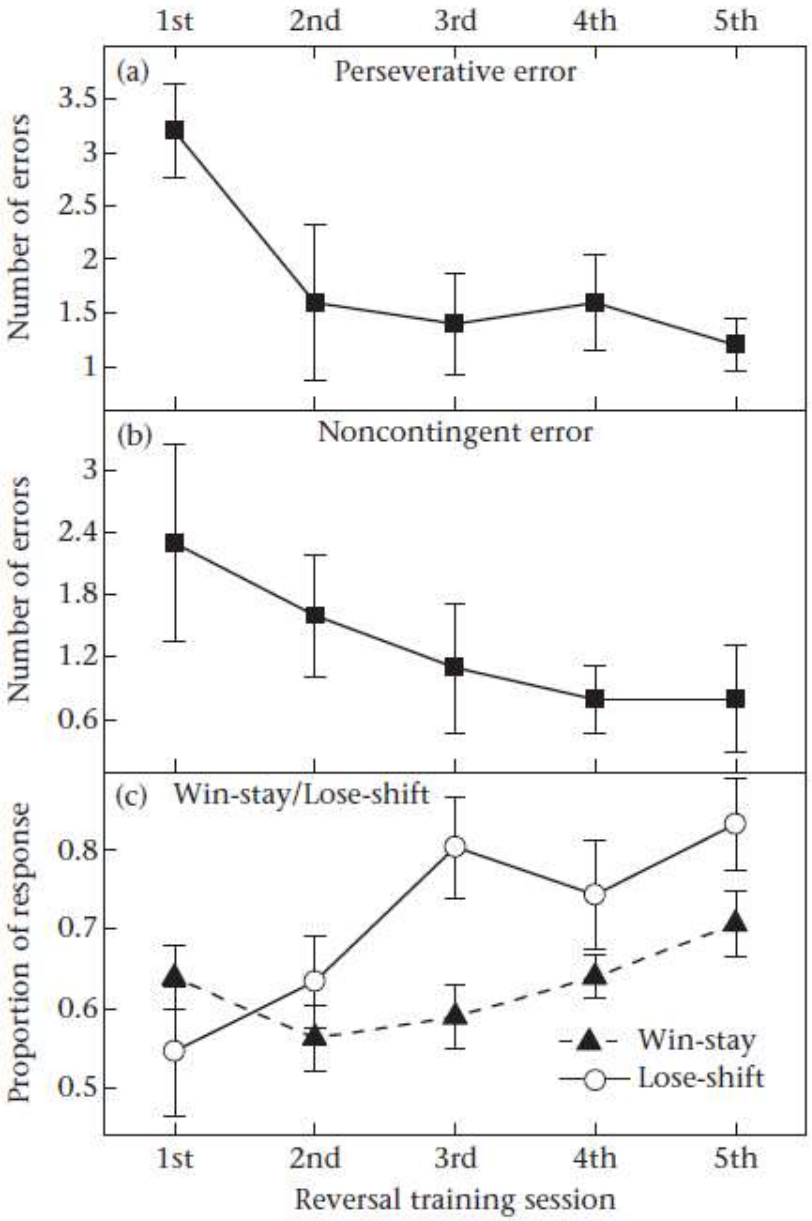

Figure 3.3. (a) Perseverative errors (mean ± SE) across five reversals; (b) Non-contingent errors (mean ± SE) across five reversals; (c) Mean (± SE) proportion of win-stay and lose-shift responses across five reversals.

CHAPTER 4: A MODIFIED MORRIS WATER MAZE PROVIDES EVIDENCE FOR COGNITIVE MAP IN POISON FROGS[3]

Summary

A fundamental question in spatial navigation is whether an animal can use a cognitive map, which is characterized by the ability to use a mental representation of the external world, and knowledge of one's place in this world, to determine efficient routes to any destination. It is well established that both birds and mammals possess a cognitive map, but whether the cognitive map is broadly represented among other vertebrates is less clear. Amphibians are capable of using beacons, gradients, and landmarks when navigating and many are proficient at homing, but whether they possess a cognitive map has received scant attention. In fact, only one prior study has directly tested for a cognitive map in amphibians and found the species lacking. Whether amphibians are capable of using a cognitive map has important implications for my understanding of the evolution of spatial cognition in vertebrates. Here I used a Morris water maze to show that the green poison frog was able to use a configuration of visual cues to choose the shortest path to a goal, fulfilling the definition requirement for a cognitive map in amphibians for the first time. The behavior of the frogs in the maze was qualitatively similar to that of mammals and homologies between the mammalian hippocampus and the anuran medial pallium suggest that the two mapping systems may share neural circuits. Poison frogs are unique among amphibians, having unusually complex social and spatial behaviors that enabled the evolution of a terrestrial lifestyle. Thus, a cognitive map likely has adaptive significance for this group.

[3] This chapter is under final revision of colleagues and almost ready to submit to PNAS.

Introduction

In 1948, Tolman proposed the concept of a cognitive map, which he defined as a mental representation of the external world (Tolman, 1948). Since then, the concept of the cognitive map has inspired research in biology and psychology (Burgess, 2006; O'Keefe & Nadel, 1978; Shettleworth, 2009), computer science and mechanical engineering (Georgopoulos, Malandraki, & Stylios, 2003; Kosko, 1986), education (Kevany et al., 2007), and management (Langfield-Smith, 1992). However, the controversy about whether it truly exists in animals continued for about half century (Bennett, 1996; Brown, 1992; Mackintosh, 2002; Shettleworth, 2009; Wehner & Menzel, 1990). Although evidences of using cognitive map have been provided in some mammals (Boesch & Boesch, 1984; Foo, Warren, Duchon, & Tarr, 2005; Singer, Abroms, & Zentall, 2006; Wills, Cacucci, Burgess, & O'keefe, 2010) and birds (Bingman, Ioale, Casini, & Bagnoli, 1990; Kamil & Jones, 1997), whether it is a broadly shared cognitive ability among vertebrates remains unclear. Amphibians retain more primitive characters of common ancestor of all tetrapods and comparisons of both extant and fossil species of tetrapods and lobe-finned fishes suggest that their emergence from an aquatic to a terrestrial environment was associated with the evolution of a more complex forebrain (Butler & Hodos, 2005; Northcutt, 1995, 2002). Yet, the question of whether amphibians possess a cognitive map has been almost ignored, with one exception (Bilbo, Day, & Wilczynski, 2000).

It is widely accepted that the hippocampal formation is the seat of the cognitive map (Jeffery, 2015; Morris, Garrud, Rawlins, & O'Keefe, 1982; O'Keefe & Nadel, 1978). According to the parallel map theory, the integrated map of the hippocampal formation consists of two mapping systems (Jacobs, 2003; Jacobs & Schenk, 2003). The bearing map encodes cues that provide directional information such as environmental gradients and compass marks; evidences of bearing maps have been broadly found in amphibians, including the use of magnetic fields (Diego-Rasilla, Luengo, & Phillips, 2015; Shakhparonov & Ogurtsov, 2016) and sensory beacons (Sinsch, 1990, 2014). The sketch map, in contrast, stores topographical information by recording geometric relationships of position cues and corresponds to the classic definition of the cognitive map; as such, I use sketch map and cognitive map interchangeably here.

A hallmark of sketch maps is that they enable animals to use spatial relationships among allocentric cues to configure the shortest pathway from any novel location to a goal (Bennett, 1996; Gallistel, 1990; Jacobs & Menzel, 2014; O'Keefe & Nadel, 1978; Shettleworth, 2009). The sketch map has never before been demonstrated in amphibians. Only one study, of which I are aware, has directly tested for a sketch map in amphibians, and this study found the leopard frog did not show spatial learning (Bilbo et al., 2000), but whether the results were due to maze design, choice of species within the amphibians, or overall lack of a sketch map in amphibians was is unclear.

The poison frogs (Dendrobatidae family) are an unusual group of anurans that has evolved sophisticated parental care that requires complex use of space. Mothers deposit eggs on leaf of forest floor and parents periodically return to hydrate the clutches. Once eggs hatch, parents transport tadpoles, one or two at a time, to pools of water that form in tree holes and in epiphytes in the forest canopy (Roithmair, 1992; Summers, 1989; Wells, 1978, 2010; Weygoldt, 1980). Since the pools are ephemeral and unpredictable, frogs spend considerable time locating them (Summers, 1989, 1990; Summers, Weigt, Boag, & Bermingham, 1999; Weygoldt, 1987). In order to survive and successfully reproduce, a major daily task is to travel among sites in the environment -- shelters, egg clutches, water pools, and feeding locations (Ringler, Pašukonis, Hödl, & Ringler, 2013; Summers, 1989; Ursprung, Ringler, Jehle, & Hoedl, 2011). The most efficient way to travel among these locations would be a sketch map.

There is a growing understanding of the abilities of poison frogs to navigate and orient in the natural environment. *Oophaga pumilio* can use fine-scale place discrimination to locate tadpoles (Stynoski, 2009) and can accurately orient to their territories after displacement (Nowakowski, Otero Jiménez, Allen, Diaz-Escobar, & Donnelly, 2013). Yet, the cues they use to do so are unknown. *Allobates femoralis* has an accurate homing ability that does not depend on path integration (Pašukonis et al., 2013), but does appear to require familiarity with the environment (Pašukonis, Loretto, Landler, Ringler, & Hödl, 2014; Pašukonis, Warrington, Ringler, & Hödl, 2014), suggesting a role for learning. Yet, once again, the cues the frogs use to orient during homing are unknown. When navigating to familiar tadpole deposition pools, *A. femoralis* can use olfactory cues from tadpoles and directional cues (of unknown

source) (Pašukonis et al., 2016). The tadpole deposition pools in this manipulation were arranged linearly and had been established for many years (Ringler, Mangione, & Ringler, 2015). The frogs adopted direct trajectories among the pool positions when searching (Pašukonis et al., 2016), a behavior that is consistent with route learning. In sum, poison frogs have accurate place memory and can travel among locations along a direct path. These considerations suggest that poison frogs might use a sketch map to navigate in nature. However, evidence of sketch map cannot stand until alternative cognitive mechanisms, such as route learning and the use of beacons and vectors, are excluded (Bennett, 1996; Shettleworth, 2009). Given the fact that it is impossible to control all the necessary cues and the subjects' prior experiences in a natural environment, a laboratory experiment is necessary to establish the existence of a sketch map in poison frogs (Jacobs & Menzel, 2014).

A major challenge to test cognition in laboratory experiments is maze design, which must take into account the natural tendencies of the animal to be tested. While the Morris water maze has proven the most successful maze for testing the cognitive map in rodents (D'Hooge & De Deyn, 2001; Jacobs, 2003; Morris, 1984; Vorhees & Williams, 2006), it has been less successful in other vertebrates, including anurans (Bilbo et al., 2000). Both the leopard frog (Bilbo et al., 2000) and *Dendrobates auratus* (see Results) show a strong tendency to touch the walls of the maze (i.e., thigmotaxis), a common response of many vertebrates. As a consequence, the frogs spend little time in the center of the arena and they apparently fail to attend to distal visual cues (Bilbo et al., 2000), making it impossible to use the classic Morris water maze to test spatial memory in anurans. Therefore, I modified the Morris water maze by creating a shallow area in the center and a deep area on the edge to reduce thigmotaxis to the wall, allowing the frogs to explore the arena and attend to cues in the environment. Using my modified Morris water maze, I were able to ask whether *D. auratus*, which expresses a pattern of parental care typical of many poison frog species (Summers, 1989) and possesses remarkable flexibility in place learning (Liu, Day, Summers, & Burmeister, 2016), is likely to use a sketch map to locate a hidden platform.

Materials and Methods

(a) Animals

I trained five sexually mature *D. auratus* (3 male and 2 female) that were bred and raised in captivity (Indoor Ecosystems, LLC). They were likely 2-3 generations removed from the wild, although these frogs remain attentive parents even in captivity. I maintained the animals under conditions that approximated their natural habitat: 25°C, 80% relative humidity (RH), 12:12 light:dark cycle (lights on at 07:00 hours). I housed the frogs individually in terraria and fed them fortified fruit flies three times per week; all of them were in non-breeding state. The University of North Carolina's Institution for Animal Use and Care Committee approved all procedures (protocol 14-026).

(b) Apparatus

I used a white polyethylene cylindrical tank (diameter = 84 cm, height = 72 cm) as the arena. A white round table (diameter = 62 cm) divided the maze into two areas: a shallow area created by the table with 2 cm-depth of water and a moat, which was the annular area between the table and the wall, with 8 cm-depth of water (Figure 4.1). Because the frogs prefer the shallow area, thigmotaxis to the wall was reduced with pretraining. In addition, because the frogs could explore the shallow area by walking or hopping (instead of swimming), it allowed them to raise their heads and attend to the visual cues (Day & Schallert, 1996).

I divided the shallow area into four quadrants (NE, SE, SW, NW), and I provided four visual cues 5 cm above water level: red flashing light, yellow artificial flower, blue spinning fan, and green artificial leaves on the east, south, west, and north walls of the tank, respectively (Figure 4.1). I provided a white platform (diameter = 5 cm, height = 1.2 cm), which was submerged in opaque water, in the center of the SE quadrant. I increased the water temperature to 35°C to motivate the frogs to use the visual cues to find the platform in order to escape the water. I used a white curtain surrounding the maze to exclude cues outside of arena. I recorded the behavior of the frogs from a camera above the arena.

(c) Procedure

Pretraining

Before training frogs in the spatial task, I pretrained them in three trials per day for 10 days. During pretraining, the water was 1 cm above the table and there were no visual cues or platform. For

each trial, I released the frog in the shallow area and allowed three minutes for exploration. Gradually, the frogs learned to swim back to the shallow area after falling into the moat. By the end of pretraining, frogs spent most of the time in the shallow area.

Acquisition

I trained the frogs in five trials per day. I divided the area without the platform (i.e., NE, NW, SW quadrants combined) into five equal sections. For each trial in a day, I released the frogs in a different section and the order of sections was changed each day. I transported the frogs to the maze in a transparent cup that I rotated during transport to ensure that orientation at release varied unpredictably. I then released the frog into one of the above mentioned section on the table. As a result, release points and head direction were unpredictable and evenly distributed in the maze.

After the frogs' first movement, I allowed three minutes to find the platform. If a frog climbed onto the platform and stayed on it for 20 sec, the trial was counted as a successful trial. Latency in successful trials was the duration between the first movement and climbing onto the platform. When frogs did not find the platform within three minutes, I covered them with the transparent cup, moved the cup slowly to the platform, and kept the frog on the platform for 20 sec. Latency for these unsuccessful trials was recorded as 180 sec. After 20 seconds on the platform, I transferred frogs to their home cage. I stirred the water after every trial to prevent the frogs from using olfactory cues to learn this task. Inter-trial intervals were around 40 min.

I tracked the success rate of individuals to determine when each frog learned the task. I defined my criterion for learning as four successful trials within one day (80%). After 10 days' training, four of the five frogs had reached the criterion at least once. The last frog reached the criterion on the 13th day. I monitored group performance by determining when success rate and latency reached asymptotic performance across three successive days (days 12-14). I stopped training on day 14. I used repeated-measures ANOVA to tests for changes in latency and success rate (after arcsine transformation) across days.

Probe trial

I conducted the probe trial on day 15 by removing the platform and moving the visual cues 180 degrees from their original position, leaving the rest of the maze unchanged. I released each frog in the SW or NE quadrant and tracked its movement for three minutes. I recorded the proportion of total time spent in each of the four quadrants and used repeated-measures ANOVA to test whether frogs were biased to particular quadrants. If the frogs used the spatial configuration of visual cues, they should prefer the NW quadrant, which is the quadrant indicated by the rotated visual cues.

(d) Pathway analysis

I determined the pathway of each frog in each trial using the MultiTracker plugin (Kuhn, 2001) in Image J (Abràmoff, Magalhães, & Ram, 2004) to extract coordinate data of the frogs' locations that, in turn, I used to generate vectors of each pathway. I then used circular statistics to examine the frogs' orientation toward the platform following a strategy used by Domenici et al. (Domenici, Booth, Blagburn, & Bacon, 2008), as follows. The frogs' pathways consisted of discrete movements (i.e., hops). I assessed orientation of a pathway by analyzing the angle between the vector of actual hops and the vector of perfect direction toward the centre of the platform (Figure 4.2). For pathways of successful trials, I averaged the angles from every hop in that pathway to determine whether the frogs as a group showed significant orientation using Hoetelling's one sample second order test (Batschelet, 1981; Zar, 1999). Hoetelling's test reflects whether frogs are significantly oriented ((i.e. non-random directions)), but does not directly test the hypothesis that they are oriented toward the platform itself. Therefore, I also calculated a Straightness Index (Mahan, 1991) that reflects whether or not the frogs were moving directly toward the platform. Straightness index (SI) could be represented by circular standard deviation (s) (Batschelet, 1981; Mahan, 1991; McCarthy, Heppell, Royer, Freitas, & Dellinger, 2010):

$$s = \sqrt{2(1 - r)} \qquad \text{(Eq.1)}$$

r is the length of mean vectors (Batschelet, 1981). It is a measure of concentration of vectors. Deviation (*s*) will decrease as *r* increasing, so a more straight pathway. However, this equation only tests if vectors concentrated to any direction but without a predicted direction which is the direction to platform in this study. So I calculated *R* to justify *r* in Eq.1 (Batschelet, 1972):

$$R = \mathrm{r}\cos(\theta) \tag{Eq.2}$$

is the deviation of each hop to most efficient direction. Then Eq.1 could be converted into:

$$s = \sqrt{2(1-R)} \tag{Eq.3}$$

Since Eq.2 adjusted *r* based on deviation of each hop, *R* will decrease as the deviation increase. Therefore Eq.3 considers both concentration and deviation of vectors, and SI will increase as increasing of concentration and decreasing of deviation (Batschelet, 1972). Finally, I did a V-test plus 95% confidence interval (CI) (Batschelet, 1981; Fisher, 1995; Mardia & Jupp, 2009) to determine if each pathway of every frog was significantly orientated toward the platform during the last three days of training. In the V-test (unlike Hoetelling's test), the angle of each hop was the statistical unit. For Hoetelling's test and the V-test, I used Oriana 4 (Kovach Computing Services). For the straightness index, I used repeated measures ANOVA in SPSS 20 after feature scaling and arcsine transformation.

Results

My modified Morris water maze significantly reduced thigmotaxis during pretraining, enabling all the frogs to learn to locate the hidden platform during acquisition of the spatial task. After 10 days of training, four of the five frogs reached 80% success rate at least once. The last frog reached the criterion on the 13th day. As a group, learning was demonstrated by increasing success rate ($F_{13,52} = 8.8$, $p < 0.0001$; Figure 4.3a) and decreasing latency to find the platform ($F_{13,52} = 5.7$, $p < 0.0001$; Figure 4.3b).

A sketch map requires the animal to learn the location of a goal based on a configuration of cues in the environment. Therefore, I used a probe trial on the 15th day to directly test whether the frogs used

the provided visual cues by rotating the cues 180 degrees from their original position. The frogs spent significantly more time searching in the quadrant indicated by the rotated cues ($F_{3,12} = 18.5$, $P < 0.0001$; Figure 4.4).

A sketch map is characterized by the ability to take direct routes to a goal regardless of starting position, a prediction I tested by quantifying the pathways of the frogs during training. The frogs found the platform with orientations non-different from random at the beginning (Hotelling's test: $F = 2.1$, $p = 0.26$, $n = 5$; Figure 4.5a,b; Table 4.1), showed increasingly-more direct paths across training (ANOVA: $F_{13,52} = 4.2$, $p < 0.0002$; Figure 4.5c), and, by the end of training, they took significantly direct paths to the platform (Hotelling's test: $F = 24.4$, $p = 0.014$, $n = 5$; Figure 4.5d,e; Table 4.2). However, going straight from release point to the platform could be attributed to route learning that reflects learning a series of stimulus-response associations on particular tracks (O'Keefe & Nadel, 1978; Shettleworth, 2009). In contrast, a sketch map enables animals to take a straight pathway from any release point to the platform. To distinguish these two possibilities, I confirmed that the release points of frogs were distributed throughout the maze in the last three days, when learning had reached an asymptote (Figure 4.6a). Frogs took significantly direct pathways to the platform in 86.4% of these trials (V test: $p < 0.05$ and mean vector ϵ 95% CI; Figure 4.6a; Table 4.3) and, as a group, showed significant orientation to the platform (Hotelling's test: $F = 594.6$, $p = 0.0001$, $n = 5$; Figure 4.6b).

Discussion

My modified Morris water maze successfully eliminated thigmotaxis to the maze wall and the frogs were able to learn to find the hidden platform. The probe trial, in which the platform was removed, confirmed that frogs did not use a beacon associated with platform, or the area near the platform, to learn the task. Furthermore, the configuration of visual cues, which were distal to the platform, ensures that the frogs would not have been able to use a single cue as a beacon to accurately navigate to the platform, ruling out the use of vectors to navigate in the maze. Finally, I demonstrated that the frogs were able to take a direct pathway from multiple unpredictable locations. The performance of poison frogs is qualitatively similar to that of rodents in the classic Morris water maze (e.g., Morris, 1984). Together,

these findings represent the first demonstration of sketch map in an amphibian. Combined with the results of field experiments in *O. pumilio* and *A. femoralis* (Nowakowski et al., 2013; Pašukonis, Loretto, et al., 2014; Pašukonis et al., 2016; Pašukonis, Warrington, et al., 2014; Stynoski, 2009), I can conclude that poison frogs are likely to have an integrated cognitive map that includes both bearing and sketch mapping systems.

Several aspects of the natural history of poison frogs likely select for complex spatial cognition – including territoriality and mate guarding (Roithmair, 1992; Summers, 1989) – but it is their parental care that would appear to depend most heavily on a cognitive map. The male maintains the clutches while they develop and, during this time, must locate suitable tadpole deposition sites (typically tree holes) in the forest canopy tens of meters or more away from their territories (Summers, 1989, 1990; Ursprung et al., 2011). Environmental events (e.g., rainstorms) can dramatically change the landscape, causing rearrangements of leaf litter, branches, etc., which could affect normal routes and/or beacon to known sites. In addition, because tadpole deposition sites can dry out or become unsuitable for other reasons, they are highly unpredictable, requiring that frogs spend considerable time locating them (Summers, 1989, 1990; Summers & Tumulty, 2013; Wells, 2010; Weygoldt, 1987). T. As competent parents, poison frogs are required to either relocate tadpole deposition sites or return to territories from novel sites. An integrated cognitive map is likely to be the most efficient way to solve this task, suggesting that the sketch map demonstrated here by *D. auratus* had adaptive value as poison frogs evolved a terrestrial lifestyle.

One contribution of parallel map theory to the study of cognitive maps is to associate the bearing and sketch mapping systems to subdivisions of the hippocampal formation (Jacobs, 2003; Jacobs & Schenk, 2003). In mammals, sensory information travels from the septum to the dentate gyrus and CA3 of the hippocampus (septo-hippocampal pathway) to form the bearing map which encodes directional cues (Amaral & Witter, 1995; Brandner & Schenk, 1998; Jacobs & Schenk, 2003; Mizumori, McNaughton, Barnes, & Fox, 1989), while the sketch map relies on CA1 to code for position cues (Gilbert, Kesner, & Lee, 2001; Jacobs & Schenk, 2003; Morris, 1990). Finally, information from the two mapping systems are integrated in the subiculum (Morris, Schenk, Tweedie, & Jarrard, 1990) before being sent to the

entorhinal cortex (hippocampo-cortical pathway) to generate the integrated map (Jacobs & Schenk, 2003; Schenk & Morris, 1985). In amphibians, the medial pallium is the homologue of the mammalian hippocampal formation (Butler & Hodos, 2005). Generally, the medial pallium of frogs is divided into three subdivision: dorsal, intermediate and ventral portions (Neary, 1990; Roth, Laberge, Mühlenbrock-Lenter, & Grunwald, 2007; Westhoff & Roth, 2002). The dorsal and intermediate portions are the proposed homologues of the dentate gyrus and CA3 based on hodology and neurochemistry (Roth et al., 2007; Westhoff & Roth, 2002). The ventral portion of the medial pallium is the proposed homologue of the subiculum, as it connects with the lateral pallium whose caudle part is the homologue of entorhinal cortex (Roth et al., 2007). Thus, both the septo-hippocampal and hippocampo-cortical pathways are conserved in mammals and amphibians, which indicates that the cognitive map of poison frogs might share the same neural substrate as mammals. Nonetheless, while many features of the hippocampus are conserved among vertebrates, notable divergences are also evident (Striedter, 2015).

Although the primary neural circuits of the hippocampus are conserved between amphibians and mammals, it is not necessary to conclude that the sketch map is also conserved among amphibians. In fact, evidence suggests that an elaboration of the hippocampus in response to specific selective pressures is necessary to evolve a sketch map (Healy, 2006; Jones, Braithwaite, & Healy, 2003; Sherry, Jacobs, & Gaulin, 1992). Work from corvids, parids, and lineages of rock doves demonstrate that species, populations, or sexes that experience particularly strong demands on their ability to remember locations (e.g., caching food for later retrieval in order to survive the winter) will evolve neural and cognitive systems that enable a sketch map, which is typically associated with a larger relative hippocampal volume (Bond, Kamil, & Balda, 2007; Ebinger & Löhmer, 1984; Healy & Krebs, 1992; Rehkämper, Frahm, & Cnotka, 2007). Among amphibians, the only other species tested for a sketch map is the Northern leopard frog. While the Northern leopard frog has the ability to home toward natal ponds (Dole, 1968; Mazerolle & Desrochers, 2005), which likely utilizes a bearing map, they failed to use allocentric cues to locate a platform in a Morris water maze (Bilbo et al., 2000), perhaps because they do not possess a sketch map.

Why this might be so requires further study. A detailed comparison of cognition and neuroanatomy of a broad range of species is needed to truly understand the evolution of the cognitive map (Jacobs, 2003).

An important breakthrough in the present study was maze design. Although Morris water maze is the most powerful task to test cognitive map of rodents, it does not work well with frogs because of strong thigmotaxis (Bilbo et al., 2000). My modified Morris water maze largely reduced thigmotaxis. Thus, for the first time, a systematic study of spatial cognition in frogs is now possible. Most notably, thigmotaxis is a response that impairs performance of many animals in the water maze (Bilbo et al., 2000; Day & Schallert, 1996; McMahon, Patullo, & Macmillan, 2005; Vorhees & Williams, 2006). Rats show dysfunction of learning of water maze with non-stopping thigmotaxis after lesion or drug interruption (Devan, McDonald, & White, 1999; Hostetter & Thomas, 1967). These results suggest that a required step in learning of Morris water maze is to switch strategy from thigmotaxis to spatial navigation. Therefore, one possible reason for the success of my maze might be my modification helped frogs to release from thigmotaxis and reveal learning before overtraining effects (e.g. loss of motivation, exhausted)(Dickinson, 1998; Hosono, Matsumoto, & Mizunami, 2016).

While the natural history of poison frogs suggests that they may excel at spatial memory (Pašukonis et al., 2013; Pašukonis, Warrington, et al., 2014; Summers, 1989), cognitive map has never been explicitly tested. In this work, I reviewed previous studies of spatial learning of poison frogs, and then put them into the framework of two mapping systems of cognitive map (Jacobs, 2003; Jacobs & Schenk, 2003). In summary, previous studies demonstrated bearing map (directional information) (Shakhparonov & Ogurtsov, 2016; Sinsch, 1990, 2014), herein my modified Morris water maze provided evidences of sketch map (topographic information). This is the first evidence of cognitive map in amphibian. This result expands the existing of cognitive map to amphibian, an extant clade which is closest to the stem of all tetrapods. In addition, the two mapping systems are conservative in neuroanatomy between mammals and amphibian (Butler & Hodos, 2005; Neary, 1990; Roth et al., 2007; Westhoff & Roth, 2002), which suggests cognitive map might be conserved in evolution of tetrapods.

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Table 4.1. Orientation analysis of first successful trial of each frog

ID	N	Mean Vector (μ)	Length of μ (r)	S.D.	95% CI for μ		V Test	
					-	+	u value	P value
52	28	67.184	0.689	49.454	48.826	85.542	1.999	0.023
51	51	96.44	0.335	84.713	64.236	128.644	-0.38	0.647
27	11	326.486	0.767	41.762	298.2	354.773	2.998	8.83E-04
53	29	310.08	0.199	102.912	236.814	23.346	0.977	0.165
48	105	26.127	0.067	133.385	269.784	142.469	0.866	0.194

Table 4.2. Orientation analysis of last successful trial of each frog

ID	N	Mean Vector (μ)	Length of μ (r)	S.D.	95% CI for μ		V Test	
					-	+	u value	P value
52	15	351.155	0.794	38.92	329.499	12.811	4.297	1.03E-06
51	13	349.297	0.994	6.038	345.584	353.009	4.983	1.74E-07
27	18	3.381	0.527	64.839	330.564	36.199	3.157	5.85E-04
53	10	343.246	0.536	63.995	297.281	29.211	2.295	0.01
48	10	356.703	0.852	32.479	333.094	20.312	3.802	6.02E-06

Table 4.3. Orientation analysis of all successful trials of each frog in asymptote

ID	trial	N	Mean vector (μ)	Length of μ (r)	S.D.	95% CI for μ		V Test	
						-	+	u value	P value
	1	14	332.225	0.962	15.938	322.872	341.579	4.504	2.18E-07
	2	15	333.601	0.725	45.953	307.907	359.294	3.557	9.07E-05
	3	24	10.724	0.717	46.763	352.098	29.35	4.879	1.06E-07
	4	13	56.793	0.887	28.119	39.541	74.045	2.476	0.006
	5	12	329.734	0.721	46.353	299.905	359.563	3.05	7.49E-04
	6	22	28.171	0.602	57.712	3.12	53.222	3.521	1.41E-04
52	7	16	340.304	0.716	46.863	317.439	3.17	3.812	2.62E-05
	8	22	327.73	0.453	72.06	292.449	3.01	2.543	0.005
	9	18	0.361	0.865	30.811	346.169	14.553	5.192	4.48E-08
	10	14	340.284	0.826	35.377	319.686	0.882	4.117	2.82E-06
	11	24	10.754	0.713	47.142	351.963	29.546	4.852	1.23E-07
	12	15	345.808	0.931	21.585	333.674	357.942	4.946	1.16E-07
	13	27	347.088	0.373	80.496	307.583	26.594	2.67	0.003
	1	14	359.602	0.82	36.149	338.574	20.631	4.336	5.49E-07
	2	12	57.581	0.146	112.486	*****	*****	0.382	0.353
	3	12	8.718	0.697	48.661	340.587	36.848	3.376	1.74E-04
51	4	6	338.171	0.834	34.556	301.575	14.767	2.681	0.002
	5	15	5.54	0.664	51.842	338.476	32.603	3.62	6.60E-05
	6	19	35.187	0.926	22.436	25.105	45.268	4.666	2.27E-07
	7	37	51.297	0.507	66.787	27.34	75.253	2.727	0.003

	8	28	341.349	0.648	53.367	321.258	1.439	4.595	7.04E-07
	9	13	353.417	0.954	17.574	342.615	4.219	4.833	1.99E-07
	10	13	349.297	0.994	6.038	345.584	353.009	4.983	1.74E-07
	1	6	8.002	0.973	13.515	353.589	22.416	3.336	3.29E-06
	2	29	25.243	0.385	79.16	348.441	62.046	2.652	0.004
	3	13	7.76	0.945	19.306	355.895	19.625	4.773	2.01E-07
	4	14	24.321	0.652	52.969	355.481	53.16	3.145	5.51E-04
	5	13	25.491	0.61	57.006	352.349	58.633	2.806	0.002
	6	21	3.637	0.637	54.425	339.867	27.407	4.119	7.09E-06
27	7	21	299.231	0.523	65.256	268.552	329.91	1.654	0.049
	8	14	338.625	0.61	56.949	306.812	10.439	3.007	9.57E-04
	9	5	332.785	0.935	21.005	306.558	359.012	2.629	0.002
	10	5	303.707	0.873	29.873	266.484	340.93	1.532	0.065
	11	28	33.821	0.569	60.88	9.921	57.721	3.535	1.47E-04
	12	27	10.989	0.526	64.958	344.119	37.858	3.794	4.61E-05
	13	18	3.381	0.527	64.839	330.564	36.199	3.157	5.85E-04
	1	5	358.204	0.994	6.165	350.501	5.907	3.142	2.62E-05
	2	8	357.477	0.335	84.745	243.002	111.953	1.338	0.093
	3	8	319.482	0.528	64.735	265.801	13.162	1.606	0.055
53	4	7	308.619	0.773	41.11	270.274	346.965	1.805	0.035
	5	13	266.958	0.718	46.663	238.405	295.511	-0.194	0.576
	6	24	71.775	0.458	71.578	38.396	105.155	0.993	0.162

	7	6	0.408	0.982	11.008	348.667	12.148	3.401	-1.81E-05
	8	10	343.246	0.536	63.995	297.281	29.211	2.295	0.01
	9	15	14.104	0.104	121.931	*****	*****	0.552	0.293
	10	6	28.587	0.841	33.674	352.877	64.297	2.559	0.004
	11	5	341.551	0.698	48.58	296.662	26.44	2.094	0.016
	1	9	1.65	0.641	54.075	324.137	39.162	2.717	0.002
	2	21	41.85	0.376	80.161	357.449	86.251	1.814	0.035
	3	9	351.461	0.951	18.221	337.172	5.751	3.989	-2.68E-06
	4	10	18.936	0.487	68.758	326.186	71.685	2.059	0.019
	5	12	354.876	0.973	13.389	346.213	3.538	4.748	2.62E-07
	6	19	23.491	0.599	57.977	356.369	50.612	3.388	2.32E-04
48	7	36	326.218	0.362	81.642	290.946	1.49	2.555	0.005
	8	11	29.854	0.612	56.788	353.799	65.909	2.489	0.006
	9	10	5.162	0.619	56.153	327.804	42.519	2.755	0.002
	10	22	289.114	0.259	94.225	224.774	353.453	0.562	0.289
	11	9	4.94	0.951	18.104	350.742	19.138	4.021	-2.60E-06
	12	10	356.703	0.852	32.479	333.094	20.312	3.802	6.02E-06

***** indicates that a result could not be calculated because of low concentration.

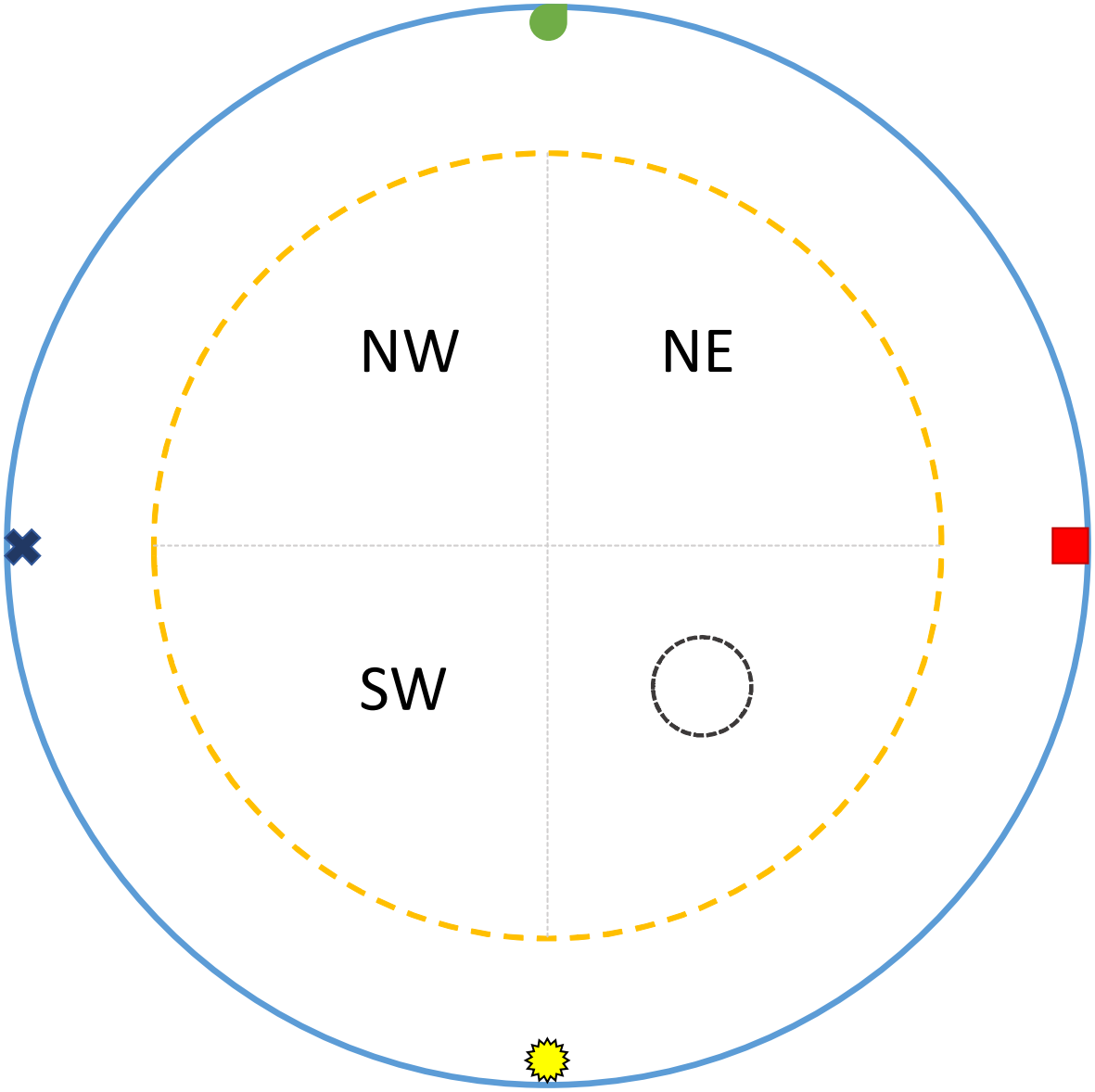

Figure 4.1. Diagram of the maze during training. I provided visual cues on the east (red flashing light), south (yellow artificial flower), west (blue spinning fan), and north (green artificial leaves) walls of the maze. I included kinetic cues (red flashing light and blue spinning fan) because frogs may attend better to moving visual stimuli than static stimuli. The blue spinning fan was potentially multi-modal, possibly generating auditory and somatosensory (air flow) cues in addition to the visual cue. The effect of using kinetic and/or multimodal cues on the ability of the frogs to learn the maze was outside the scope of the present manuscript.

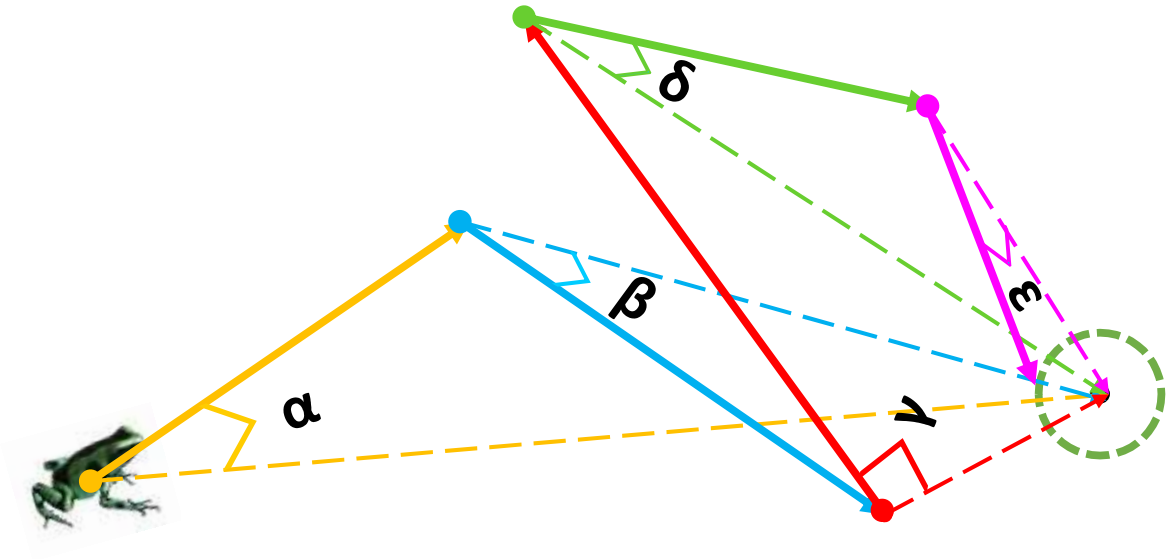

Figure 4.2. Quantification of orientation within single trial/pathway. The movement of frogs from release point to the platform or final position is composed of discrete hops. For each hop, there is an actual direction (concrete arrow) and a perfect direction toward the centre of the platform (dash arrow). I used the deviations between the two directions (i.e. α, β, γ, δ, and ε) in a V-test to see if the frogs were consistently oriented to the platform within one trial.

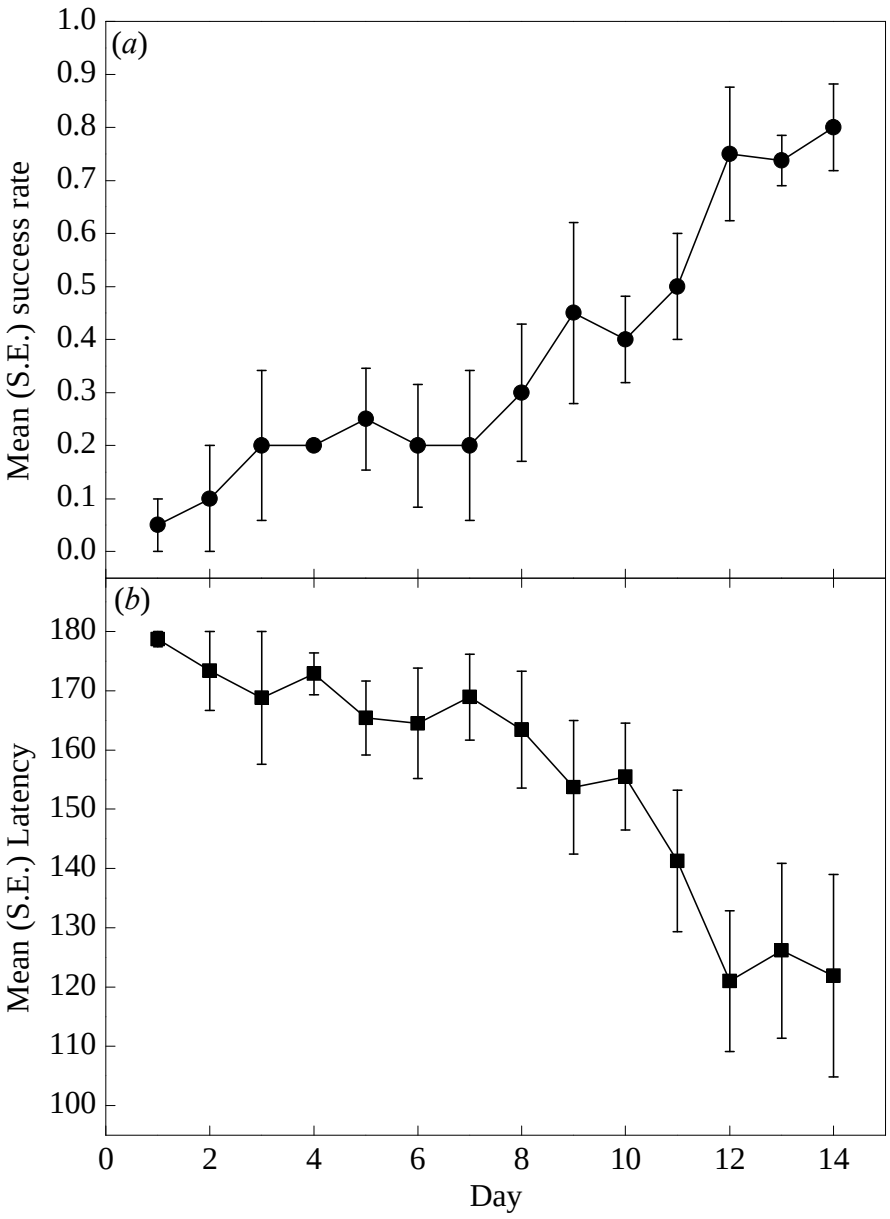

Figure 4.3. Frogs had increasingly greater success finding the platform (a) and found the platform more quickly (b) across 14 days of training.

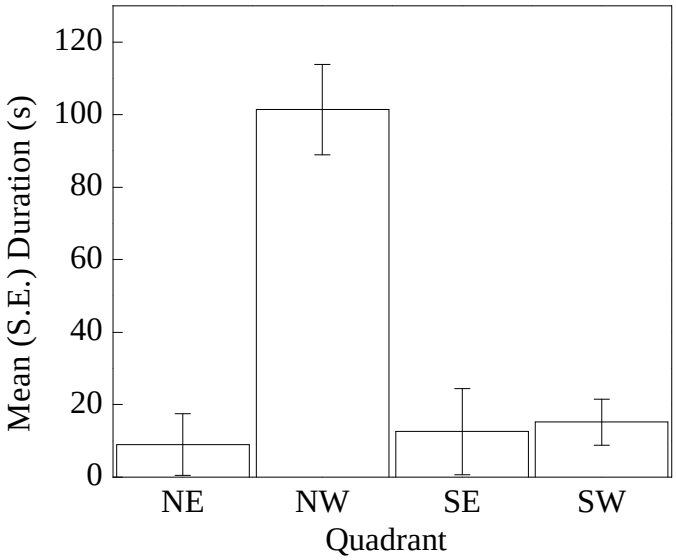

Figure 4.4. During the probe trial, frogs spent significantly more time in the NW quadrant, demonstrating that they used the spatial configuration of visual cues to find the platform.

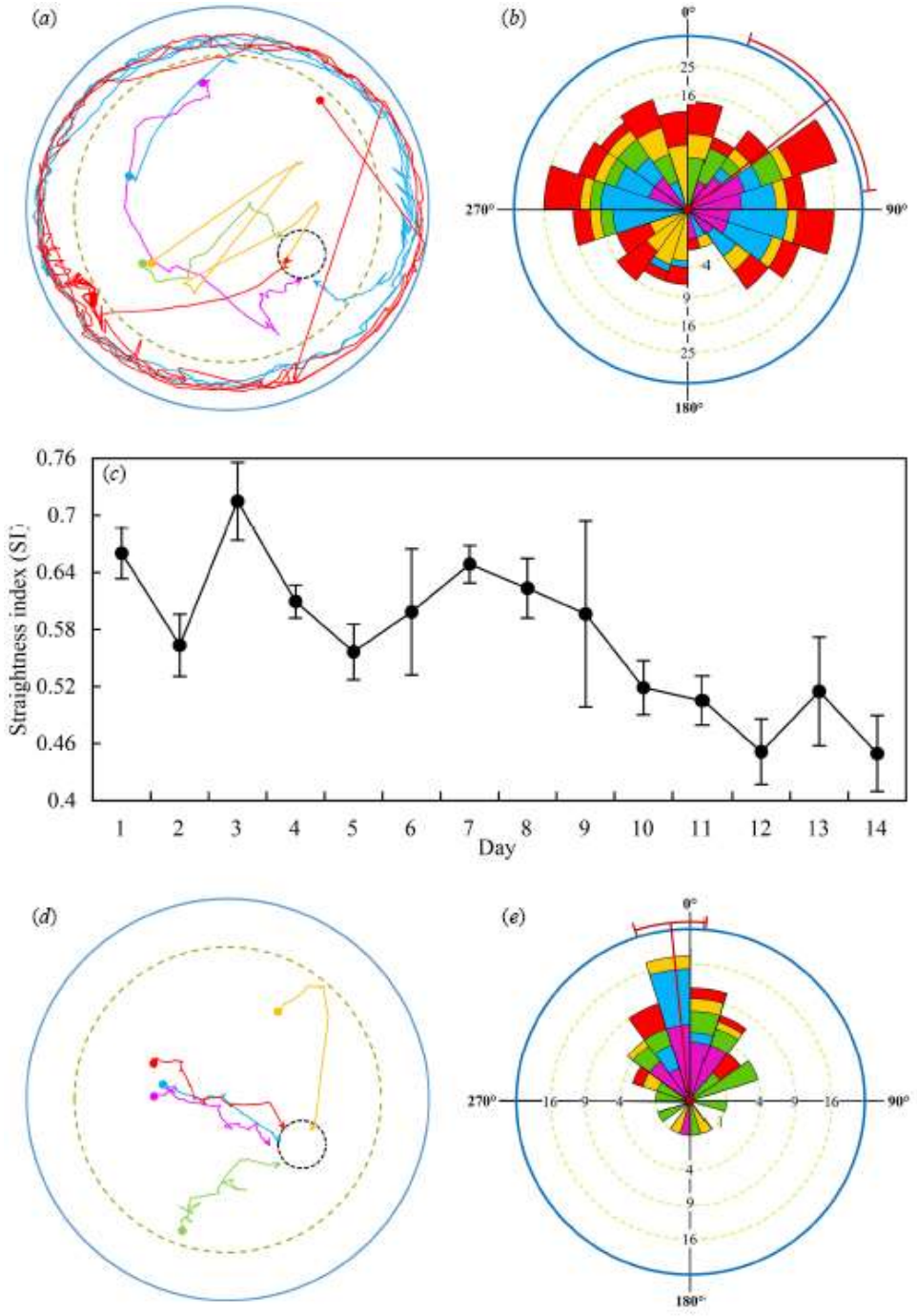

Figure 4.5. Pathway analysis shows that the frogs gradually established a mental representation of visual cues to solve the task. At the beginning, the frogs took indirect paths to the platform (a, b). Across training, pathways became more direct (c). At the end of training, the frogs chose direct pathways to the platform (d, e). Each color represents a different frog; dots indicate release points.

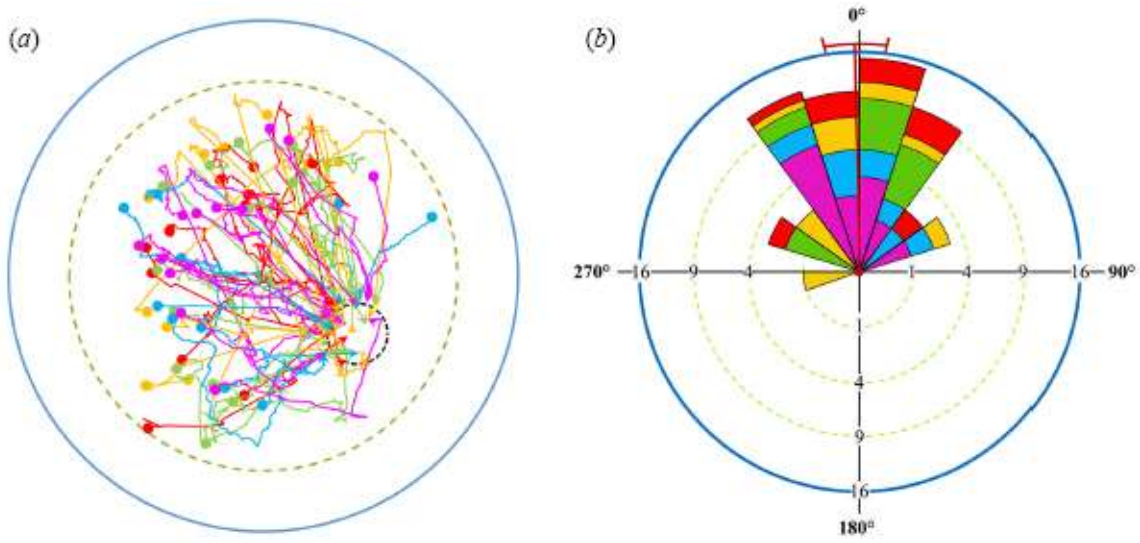

Figure 4.6. Pathway analysis demonstrates that the frogs took direct paths to the platform regardless of release point (a) and that they were significantly oriented to the platform (b) after reaching asymptotic performance (trial 12-14). Each color represents a different frog; dots indicate release points.

CHAPTER 5: HIPPOCAMPAL TRANSCRIPTOMES ARE ASSOCIATED WITH PLACE LEARNING ABILITY IN FROGS[4]

Summary

The complexity of an animal's interaction with its physical and/or social environment is associated with behavioral flexibility and cognitive sophistication. While there have been numerous studies on cognitive ability in an ecological context in birds and mammals, we still know little about these abilities in amphibians. Here, I compare spatial cognition and patterns of gene expression the hippocampus in two species of frog that have evolved in different ecological contexts. Poison frogs defend territories and show sophisticated parental care behaviors that involve complex spatial and social interactions, while the sympatrically-distributed túngara frog is a lek breeder that provides foam nests for offspring, without further parental care. In a first experiment, I found that poison frogs tended to use spatial cues (a landmark strategy) while túngara frogs tended to use local cues (a cue-taxis strategy) to solve the same two-arm maze. This result is consistent with the hypothesis that species experiencing environments that impose more complex demands on navigational skills should be more likely to rely on spatial cues for place learning. In a second experiment, I found that poison frogs could successfully learn a reversal task, whereas túngara frogs could not, demonstrating that the poison frogs have higher behavioral flexibility. One reason for the failure of reversal learning in túngara frogs is their higher rate of perseverative errors compared to poison frogs. An ability to use spatial cues, greater levels of behavioral flexibility, and lower levels of perseverance are all associated with hippocampual function. Thus, I compared hippocampal transcriptomes of poison frogs to túngara frogs using RNA-Seq. I found that genes related to learning and memory, neurogenesis, and synaptic plasticity were upregulated in poison

[4] This chapter is waiting for data from qPCR for data validation. I plan to submit to a journal which focuses on neurogenomics (e.g. Behavior Genetics or Neurogenetics)

frogs, while genes related to apoptosis and negative regulation of biosynthesis and metabolism were upregulated in túngara frogs. The species differences in cognition in these place learning tasks might stem, at least in part, from differential expression of those genes in hippocampus.

Introduction

Variation in animal cognition is generally associated with the complexity of the physical and/or social environments that the animals have to cope with (de Waal & Tyack, 2003; Godfrey-Smith, 2002). Some of the most prominent examples of this come from the study of spatial cognition. Substantial numbers of studies of mammals and birds have demonstrated that spatial learning ability and navigational strategy are correlated with environmentally imposed navigational challenges that are required for survival and reproduction (Brodbeck, 1994; Clayton & Krebs, 1994; Lavenex, Shiflett, Lee, & Jacobs, 1998; Macdonald, 1997; Shettleworth, 2009). Spatial adaptation theory attributes this correlation to selection for cognitive abilities required to solve specific spatial tasks (Gaulin, 1992; Sherry, Jacobs, & Gaulin, 1992). So far, studies in this field have mainly focused on mammals and birds, the groups that show the highest levels of complexity in terms of life history and forebrain neuroanatomy. It is not clear whether species of more primitive clades (e.g. amphibians) also show differences in their abilities and spatial strategies in the context of task learning, and, if so, what neurogenetic mechanisms generate these species differences.

Amphibians, particularly anurans, show a diversity of behaviors designed to cope with environmental change (Jacobs & Schenk, 2003). One of the most specialized and complex set of behaviors in this regard is the sophisticated parental care in Dendrobatid frogs (Kevany et al., 2007; Summers, Weigt, Boag, & Bermingham, 1999). These frogs lay eggs on leaves just under the leaf litter on the forest floor. In order to prevent the eggs from drying out and to find a suitable location to deposit the tadpoles, parents need to go back and forth among multiple locations (e.g. egg sites, deposition pools, and shelters). These tasks have been suggested to heavily rely on place memory (Pašukonis et al., 2013; Summers, 1989). In comparison, a closely-related species to the dendrobatid family (Ruvinsky & Maxson, 1996), the túngara frog, leaves a foam nest on the water, but does not provide any further

parental care (Ryan, 1985). Considering the difference between these species in their natural history and reproductive behaviors, a key question is: what are the differences in spatial strategy and learning ability during place learning, and what are the neurogenetic mechanisms behind the differences in spatial cognition?

Complex environments always provide more than one type of cue for place learning. Different types of cues correspond to different strategies to locate places (Day, Ismail, & Wilczynski, 2003; O'Keefe & Nadel, 1978; Shettleworth, 2009). A spatial strategy requires the animal to use spatial cues, which are not the features of the goal but have a fixed spatial relationship with the goal. In contrast, a cue-taxis strategy is linked to the use of local cues, which are features of and thus part of the goal (O'Keefe & Nadel, 1978; Shettleworth, 2009). Studies of mammals and birds show that species that regularly perform tasks that rely heavily on spatial memory prefer to use a spatial strategy rather than a cue-taxis strategy, while related species that lack those navigational demands showed no preference between a spatial strategy and a cue-taxis strategy (Brodbeck, 1994; Clayton & Krebs, 1994; Lavenex et al., 1998). My previous work has shown that túngara frogs used a cue-taxis strategy (Chapter 2) while poison frogs used a spatial strategy (Chapter 3) in different versions of a two-arm maze. Whether they will differ in strategies when trained in the same maze remains to be tested.

Behavioral flexibility is defined in terms of how efficiently animals can change their behaviors in response to a change in the environment. It is a useful indicator of learning ability in the context of place learning. A common view from previous work is that species that have the cognitive abilities to deal with complex social and physical environments show higher levels of behavioral flexibility (Jones, 2006; Robinson, 1990). Pools with standing water for tadpole deposition are a temporary and unpredictable resource (Summers, 1989, 1990). This requires poison frogs to update the information stored in their memories of available pools for navigation in real time, while túngara frogs seem to lack comparable challenges in their life history. The results from my work on túngara frogs and poison frogs are consistent with this prediction. Poison frogs were able to learn a serial reversal task (Chapter 3), while túngara frogs did not learn the reversal task in a nine days training (Chapter 2). However, since I only gave túngara

frogs nine days of reversal training, it is possible that túngara frogs could also learn the reversal task if I trained them for a longer period of time.

A fundamental way to understand mechanisms of behavior is to study the gene expression profile in the corresponding brain region (Valor & Barco, 2012). A well-known brain region for place learning and behavioral flexibility is the hippocampus in mammals and birds (Krebs, Sherry, Healy, Perry, & Vaccarino, 1989; O'Keefe & Nadel, 1978). The homologue of the mammalian hippocampus in amphibians is the medial pallium, which I will refer to as the hippocampus in this paper (Brodbeck, 1994; Gaalema, 2011). Experimental manipulations of candidate gene expression in the hippocampus have dramatically enhanced my understanding of gene function in the context of mammalian spatial cognition (e.g. Abel et al., 1997; Falkenberg et al., 1992; Silva, Paylor, Wehner, & Tonegawa, 1992). As a first step toward this goal, I used comparisons of hippocampal transcriptomes (using RNA-Seq) between the two frog species to reveal patterns of differential gene expression. Such data are designed to generate hypotheses for candidate gene manipulation in the future.

In short, I tested túngara frogs and poison frogs to compare their strategies and behavioral flexibility to learn the two-arm maze tasks in two behavior experiments. Then, I compared their hippocampal transcriptomes using RNA-Seq.

Animals

I used sexually mature and experimentally naïve green poison frogs (*Dendrobates auratus*) and túngara frogs (*Physalaemus pustulosus*).Poison frogs were bred in captivity and were likely several generations removed from the wild (Indoor Ecosystems, LLC). Túngara frogs were one or two generations derived from populations in Gamboa Panama. I maintained the animals under conditions that approximated their natural habitat: 25° C, 80% relative humidity (RH), 12:12 light:dark cycle (lights on at 07:00 h). I housed poison frogs and túngara frogs individually in terraria or two same-sex terraria, respectively. The reason to house them in different ways is because poison frogs defend territory whereas túngara frogs do not (). I fed both species fortified fruit flies three times per week. The University of

North Carolina's Institution for Animal Use and Care Committee approved all procedures (protocol 14-026).

Experiment I: Local cues provided

(a) Materials and methods

Behavior test

I trained eleven poison frogs (5 male and 6 female) and thirteen túngara frogs (6 male and 7 female) in the two-arm maze that was composed by six white-painted bricks and with red and yellow doors at the exits of two arms (see Chapter 2 for detail). I trained them with acclimation with both door open, acquisition in which red door was correct, and reversal sessions in which yellow door was correct (exactly the same as Chapter 2). Right after reversal training, I tested both in the first probe trial in which the two doors were switched positions and blocked with bricks (Chapter 2). After that, I trained poison frogs in an inter-probe training session which was the same as reversal training for three days. I then did the second probe trial on poison frogs by turning the whole maze for 180° except for the two color doors. The second probe trial was designed to test if poison frog tried to use cues on the wall of maze (bricks) to learn the task.

Data analysis and statistics

I quantified behaviors from video recordings. I used success rate (mean number of successful trials per day) as the primary measure of learning across days. I then used an arcsine transformation on the data before analysis. To determine whether poison frog learned this task, I used repeated measures ANOVAs to examine success rate across all training days. I also used repeated measures ANOVAs to examine the interaction between species and day on success rate across all training days (data of túngara frog from Chapter 2). For probe trials, I quantified the duration frogs spent in each channel as a measure of channel preference. I used a paired t test to determine if the frogs prefer to stay in channel which could be associated with particular cues. I then used repeated measures ANOVAs to examine the interaction between species and channel on time spending in probe trial (data of túngara frog from Chapter 2).

(b) Results

Poison frogs learned this two-arm maze task by showing an increasing success rate across training days ($F_{8,80} = 4.39$, $p < 0.001$; Figure 5.1). Compared with the learning curve of female túngara frog in the same maze (Chapter 2), the two species did not show difference in learning of acquisition (species × day: $F_{8,128} = 1.21$, $p < 0.301$). In the first probe trial, in which the two color doors have been switched, poison frogs still spent more time in the arm with yellow door which is the non-rewarded color door, although the statistical result is only moderately significant ($t_{10} = 2.01$, $p = 0.07$; Figure 5.2a). Moreover, there is significant species by color interaction ($F_{1,15} = 15.74$, $p = 0.001$). In the second probe trial, in which the whole maze, except for the two color doors, was turn 180°, poison frogs spent more time in the arm with yellow door which is spatially associated with rewarded exit in training ($t_{10} = 3.27$, $p = 0.008$; Figure 5.2b). These results indicate that poison frogs tend to use spatially-related cues for location rather than approaching or avoiding objects based on their features (e.g. colors).

Experiment II: Landmark cues provided

(a) Materials and methods

Behavior test

In this experiment, I constructed a two-arm maze by using white boards with uniform surface. Both doors were white, and visual cues (light green triangles and dark purple rectangles) were provided on the two walls of starting chamber (Chapter 3). I trained eight túngara frogs (3 male and 5 female) and ten poison frogs (4 male and 6 female) in the same maze at the same time. I trained them with acclimation with both doors open, acquisition in which arm on the left hand when frogs face wall with triangles is correct, and 1st reversal sessions in which the right hand when frogs triangles is the correct direction (Chapter 3). In order to make sure that túngara frogs could do reversal learning in this two-arm maze task, I trained them for twice the number of days for which each individual was trained to criterion in acquisition. After the 1st reversal session, I trained all túngara frogs to relearn acquisition task. I then did the first probe trial in which the two walls of the starting chamber were switched (Chapter 3). After that, I

trained them in an inter-probe training session which was the same as the acquisition for three days. I then did the second probe trial by turning the whole maze for 180°.

Data analysis and statistics

I set up the criterion the same as Chapter 3 to determine learning of particular session for each individual. I defined position errors, non-contingent errors, and perseverative errors the same as Chapter 3. Position errors and non-contingent errors were quantified in each individual of both training session as sum of session error divided by number of session trials. Perseverative errors were only recorded in the first reversal. I then used independent t test to compare position errors, non-contingent errors, and perseverative errors between túngara frogs and poison frogs (data are from experiment in Chapter 3). For probe trials, I quantified the duration frogs spent in each channel as a measure of channel preference. I used a paired t test to determine if the frogs prefer to stay in channel which could be associated with particular cues.

(b) Results

All túngara frogs reached the criterion in acquisition, but none of them reach the criterion in the reversal (Figure 5.3a). While poison frogs learned both acquisition and reversal tasks (Figure 5.3b). Compared with poison frogs, túngara frogs had similar numbers of non-contingency errors ($t_{16} = 1.36$, $p = 0.193$; Figure 5.4a), while they committed more position errors ($t_{16} = 3.28$, $p = 0.005$; Figure 5.4b) in learning of acquisition. Túngara frogs showed significantly higher preservative error than poison frogs in reversal learning ($t_{16} = 2.89$, $p = 0.010$; Figure 5.4c). Probe trials showed that they used neither the provided spatial cues which were on the wall of starting chamber ($t_7 = 1.23$, $p = 0.258$) nor any cue that was associated with other parts of maze to learn this task ($t_7 = 0.87$, $p = 0.413$).

Experiment III: Hippocampal transcriptome comparison

(a) Materials and methods

Sample preparation and RNA-Seq

Eight experimentally naïve poison frogs (4 male and 4 female) and túngara frogs (4 male and 4 female) were housed in my lab for at least one month after they were transported from commercial supply or collaborator's lab. Each individual was kept in its home cage for 1 hour without any interruption. I decapitated all frogs without anesthesia. I removed lower jaw, skull, and accessory organs (e.g. eyes and nose) from head, and immersed the skull in Tissue-Tek O.C.T compound (Sakura Finetek USA, Inc.) in NALGENE Cryogenic vial 1.8 ml (Naige Nunc Int. Corp.). The vial with the skull was frozen in liquid nitrogen immediately. I made 200 µm cross sections from rostral to caudal side of the brain, and then selected sections that contained the forebrain. I punched out these selected sections with hippocampus (Figure 5.5) and preserved in TRIzol Reagent (Invitrogen^{TM}) for RNA extraction. In order to have enough RNA to perform RNA-Seq, I pooled all four individuals of the same sex to one sample. Therefore, I only have one sample for each sex of each species. RNA was extracted from the hippocampus by using Invitrogen^{TM} RNA extraction protocol. The RNA concentration ranged from 21 - 31 µg/ml and the RNA integrity number (RIN) were higher than 8. I reverse transcribed each RNA library into cDNA with the Invitrogen^{TM} SuperScript II Reverse Transcriptase kit. I then sent these cDNA samples to a high-throughput sequencing facility at UNC-Chapel Hill. All samples were sequenced on the Illumina HiSeq 2000 platform with 50 bp paired end reads.

Transcriptome assembly and reciprocal blast

Figure 5.6 shows the bioinformatics pipeline. I filtered sequences with quality control criterion (quality cut-off = 20; minimal percentage = 90%) through Galaxy version 15.03 (Goecks, Nekrutenko, & Taylor, 2010). I then used these quality-controlled sequences to carry out *de novo* assembly of the reference transcriptomes. Samples from the same species were put together for *de novo* assembly with Trinity (Haas et al., 2013), yielding one assembled transcriptome for each species (the green and black poison frog and the túngara frog). In order to match the contigs from the reference transcriptomes of the two species, I ran a reciprocal blast search using the two assembled transcriptomes with an e-value

threshold of 1^{-10}. A match was only recognized when two contigs from different assembled transcriptomes always listed each other as the best hit.

Annotation, contigs assembly, and gene expression

I blasted the sequences of these commonly expressed contigs of the two species against *Xenopus tropicalis* protein sequences as the reference genome with an e-value threshold of 1^{-10}. Contigs that matched the same protein sequence were treated as exons of the same gene. These contigs were then assembled as one gene according to their corresponding positions on the reference genome. The secondarily assembled transcriptomes of the two species were compared again by using the results of the blast search against the *Xenopus* reference genome. For each gene, I trimmed out the parts of sequence that share the same fragments with *Xenopus* reference genome, these parts of each gene were secondarily assembled in both species for downstream analysis. I then used the trimmed and secondarily-assembled transcriptomes as references to call the expression levels of each gene, using the Burrows-Wheeler Alignment (BWA) tool (Li & Durbin, 2009). I then transformed the rough expression values to reads per kilobase of transcript per million mapped reads (RPKM) to normalize expression level based on contig/gene length and the amount of RNA in the samples (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008).

Differential expression analysis

I used the nbinom Test in the R Bioconductor package, DESeq2, to compare the expression levels of each commonly expressed gene between the poison frog and the túngara frog (Love, Huber, & Anders, 2014). I then used Benjamini–Hochberg procedure to adjust the p values for multiple comparisons (Benjamini & Hochberg, 1995). Given the small sample size (two for each species), I set the threshold for significant evidence for differential expression as an adjusted p value of less than 0.05 and a 5-fold (5x) change.

I matched the differentially expressed genes with their human homologues with DAVID (Huang, Sherman, & Lempicki, 2009) and bioDBnet (Mudunuri, Che, Yi, & Stephens, 2009). I then imported the

higher expressed genes of each species to DAVID for a gene ontology (GO) enrichment analysis with a threshold of p=0.05 for inclusion. Genes that belong to learning-associated GO terms (i.e. learn and memory, neurogenesis, synaptic plasticity, and apoptosis) were compared in terms of gene expression pattern between species.

(b) Results

Transcriptome assembly, annotation, and differential expression

De novo assembly of transcriptome returned 76,742 and 102,174 transcripts (contigs) in the túngara frog and the poison frog, respectively. The túngara frog and the poison frog had 55,265 contigs that matched with each other. In these matched contigs, 18,976 of the túngara frog and 28,939 of the poison frog contigs matched with a specific *Xenopus* protein in the blast search. The secondarily-assembled transcriptomes had 11,156 and 12,386 contigs (genes) in the túngara frog and the poison frog, respectively. Finally, I found that 9,566 genes were commonly expressed in both species. In these commonly expressed genes, 87 were upregulated in the túngara frog, while 143 were upregulated in the poison frog. However, 964 túngara frog contigs and 1,987 poison frog contigs did not match with any contig of the other species.

GO analysis

DAVID matched 64 and 121 human homologues for these upregulated genes in the túngara frog and the poison frog, respectively. The results of the enrichment analysis are shown in Table 5.1 and Table 5.2 for the two species. Upregulated genes were mainly enriched for the category of metal binding and transcription in the túngara frog, while they were enriched for axon extension in the poison frog. When I used learning-associated GO terms to categorize these differentially expressed genes, I found that all of the genes associated with learning and memory were upregulated in the poison frog: 20 out of 23 of the genes related to neurogenesis were upregulated in the poison frog, and all of the 18 genes related to synaptic plasticity were upregulated in the poison frog. In contrast, 20 out of 26 of apoptosis genes were downregulated in the poison frog, and all of the 14 genes that negatively regulate biochemical synthesis

and metabolism (e.g. cholesterol, fatty acid, and steroids), were also downregulated in the poison frog (Figure 5.7). Half of these differentially expressed genes are unknown function or hard to categorized (Figure 5.8).

Discussion

(a) Learning strategy

In experiment I, poison frogs used cues on the maze wall rather than features of the goal (i.e. door colors) to learn the task, while túngara frogs learned the same maze by using cues associated with the goal (i.e., door colors; Chapter 2). In experiment II, túngara frogs did not use the provided spatial cues or other cues in the maze. Poison frogs learned to navigate the same maze using spatial cues (Chapter 3). These results suggest that túngara frogs use a cue-taxis strategy while poison frogs use a spatial strategy during place learning.

My results are consistent with the prediction that species in which the environment imposes more challenging navigational demands should be more likely to learn a task by using spatial cues. This is also consistent with the results of previous work in other taxa (Brodbeck, 1994; Clayton & Krebs, 1994; Lavenex et al., 1998). A spatial strategy is adaptive for species that rely heavily on spatial memory because the changing environment could result in the loss of cues for a cue-taxis strategy, while the spatial relationship could still be configured by using the remaining cues (Brodbeck, 1994). For example, in the tropical forest of Central America, a heavy storm could fundamentally change the microhabitat on the ground. However, the basic landscape and big trees should still be there. Therefore, the evolutionary advantage of a spatial strategy in poison frogs is clear because it is more reliable for spatial navigation than a cue-taxis strategy. Compared with the poison frog, the túngara frog is an opportunistic breeder. They do not defend territories and, to date, there is no evidence of pond fidelity. It has even been reported that túngara frogs will make a foam nest in a paper cup (Ryan, 1985) or the impression of a boot in the mud, indicating that a cue-taxis strategy is a sufficient, and possibly more efficient, strategy to find the available place for breeding compared to a spatial strategy.

(b) Behavioral flexibility

In experiment II, I trained each túngara frog in reversal learning for twice the number of trials required by each frog for acquisition. However, none of them reached the learning criterion. In contrast, poison frogs learned the reversal task and showed progressive improvement in learning during serial reversal (Chapter 3). These results indicate that poison frogs have higher behavioral flexibility than túngara frogs. Further examination of the nature of the errors shows that túngara frogs committed more position errors than poison frogs, but their non-contingency error rates were similar during acquisition. These results suggest that the poison frogs outperformed the túngara frogs in learning the two-arm maze due to faster correction of position errors rather than to higher familiarity with the maze or higher levels of motivation, which are linked to non-contingency errors (Chapter 3). One reason for the failure of túngara frogs in reversal learning was their higher preservative error rates compared to poison frogs. Since preservative error is an indicator of extinction (Mackintosh, McGonigle, & Holgate, 1968; Strang & Sherry, 2014), this result is consistent with my previous results in which túngara frogs fixated on the reward color of acquisition after they were trained in a reversal session (Chapter 2).

(c) Hippocampal transcriptome comparison

The results from the behavioral comparisons show that the túngara frog and the poison frog differed in hippocampus-dependent learning abilities. Comparison between food caching and non food-caching birds show that food caching birds, which outperform non food-caching birds in spatial learning, have larger hippocampal sizes (Krebs et al., 1989). The higher volume of the hippocampus in food-caching birds has been partly attributed to a higher rate of neurogenesis in adults (Pravosudov & Smulders, 2010; Sherry & Hoshooley, 2010). Learning, especially in long-term memory formation, relies on dendrite growth, which is a type of neurogenesis (Aimone, Wiles, & Gage, 2006). Hence, a higher neurogenesis rate in the hippocampus could be associated with better spatial learning ability (Deng, Aimone, & Gage, 2010). Consistent with these findings, the results of the hippocampal transcriptome comparison showed that most of differentially expressed genes associated with neurogenesis are upregulated in the poison frog.

Apoptosis is defined as programmed cell death. Neuronal apoptosis has been associated with some mental illnesses characterized by degraded cognitive function (e.g. Alzheimer's disease) (Smale, Nichols, Brady, Finch, & Horton, 1995). Modifications to genes associated with neural apoptosis have effectively relieved cognitive degradation in adult mice (Choi-Lundberg et al., 1997; Nicholson, 2000; Thompson, 1995). Therefore, it is reasonable to speculate that neural apoptosis could be negatively correlated with cognitive ability among adult individuals of different species. Recent comparisons of gene expression in the hippocampus of chickadee populations under different ecological conditions supports this hypothesis by demonstrating the downregulation of apoptosis genes in the population with better spatial ability (Pravosudov et al., 2013). These results in chickadees parallel the results in poison frogs presented here.

Synaptic plasticity is defined as the ability to increase or decrease synaptic strength due to increases or decreases in synaptic activity. In the hippocampus, it is typically measured by long-term potentiation (LTP) (Bliss, 1979). It has been well established that LTP, which is associated with NMDA-receptor related cascade for protein synthesis and dendrite growth (Engert & Bonhoeffer, 1999), is required for long-term memory formation (Tsien, Huerta, & Tonegawa, 1996). I found that all differentially expressed genes of synaptic plasticity were upregulated in poison frogs. Similar results were found in chickadees, in that the population that showed better spatial memory upregulated most of synaptic process genes (Pravosudov et al., 2013).

Negative regulation of activity (a functional category: Fig 5.6), includes protein synthesis, steroid synthesis, cholesterol and fatty acid metabolism process. Protein synthesis in the hippocampus is critical in long-term memory formation (Davis & Squire, 1984). As the elements of bilayer lipid membranes, cholesterol and fatty acid are important material in neurogenesis (Das, 2003; Koudinov & Koudinova, 2001). Steroids are important regulatory chemicals that trigger biological processes (Rose, 1995). I found that these negative regulation genes were downregulated in the poison frog. This result indicates that better learning ability in the poison frog may also be associated with a higher level of biosynthesis and metabolism.

One shortcoming of my transcriptome data is the small sample size (1 male and 1 female per species) and lack of biological replicates. Either false positive or false negative problems could potentially bias the results of the transcriptome comparison. One way to address this problem is by data validation using qPCR (Fang & Cui, 2011). Another potential problem is in the GO enrichment analysis, in which I used human homologous genes instead of frog genes for analysis. The reason I used this approach is that there are too few genes that have well-known functions in amphibians; using human homologues provides substantially more information about potential functions. However, it will be important to test the functions of these genes in amphibians using gene manipulation methods (e.g. RNAi and CRISPR/Cas9) in the future.

In summary, my work on two frog species in learning to navigate the two-arm mazes showed that poison frogs used a spatial strategy and learned the serial reversal task, while túngara frogs used a cue-taxis strategy and did not learn the reversal task. These results are consistent with the navigational demands imposed by their respective natural histories, and this is consistent with the idea that spatial adaptation theory (Gaulin, 1992; Sherry et al., 1992) applies to amphibians. The hippocampal transcriptome analysis of differential gene expression suggests that better spatial learning ability in the poison frog might be associated with gene expression differences related to long-term memory formation (i.e. neurogenesis, synaptic plasticity, neural apoptosis, and biosynthesis activities), although further work is necessary to validate these preliminary data.

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Table 5.1. GO enrichment analysis of upregulated genes in túngara frog

GO term	Count	List Total	Pop Hits	Pop Total	Fold Enrichment	P Value
Mitochondrion	11	64	1116	20568	3.1676747	0.001968
Acetylation	19	64	3432	20568	1.779174	0.01269
region of interest: Beta-galactoside binding	2	64	7	20063	89.566964	0.021778
Transferase	11	64	1666	20568	2.1219238	0.029244
GO:0044822~poly(A) RNA binding	9	57	1129	16313	2.2814321	0.03785
hsa01100:Metabolic pathways	10	28	1228	6910	2.0096557	0.038083
hsa00920:Sulfur metabolism	2	28	10	6910	49.357143	0.038418
SM00276:GLECT	2	23	19	10071	46.091533	0.040735
SM00908:SM00908	2	23	19	10071	46.091533	0.040735
Lysosome	4	64	254	20568	5.0610236	0.042994
Lipid metabolism	5	64	432	20568	3.7196181	0.043355
GO:0055114~oxidation-reduction process	6	57	590	16787	2.9950045	0.046367
GO:0071257~cellular response to electrical stimulus	2	57	15	16787	39.267836	0.048907

Table 5.2. GO enrichment analysis of upregulated genes in poison frog

GO term	Count	List Total	Pop Hits	Pop Total	Fold Enrichment	PValue
Alternative splicing	91	121	10594	20568	1.460119	1.04E-07
splice variant	72	121	7760	20063	1.538443	4.20E-06
Disease mutation	33	121	2539	20568	2.209316	1.58E-05
Acetylation	38	121	3432	20568	1.882101	9.05E-05
GO:0030424~axon	7	113	235	18202	4.798117	0.003333
Nucleotide-binding	21	121	1787	20568	1.997567	0.003446
Metal-binding	34	121	3637	20568	1.589067	0.004961
Phosphoprotein	63	121	8250	20568	1.298056	0.006668
Mental retardation	7	121	295	20568	4.033506	0.007736
metal ion-binding site: Zinc 1	4	121	75	20063	8.843196	0.010307
metal ion-binding site: Zinc 2	4	121	76	20063	8.726838	0.010685
Cytoskeleton	14	121	1126	20568	2.113471	0.014268
GO:0031965~nuclear membrane	6	113	234	18202	4.130247	0.014863
GO:0006611~protein export from nucleus	3	107	30	16787	15.68879	0.015313
RNA-binding	10	121	666	20568	2.552304	0.016101
mutagenesis site	22	121	2191	20063	1.66491	0.019664

GO:0005829~cytosol	31	113	3397	18202	1.469964	0.021591
Neurodegeneration	6	121	286	20568	3.566087	0.026265
GO:0007190~activation of adenylate cyclase activity	3	107	40	16787	11.76659	0.026361
Coiled coil	27	121	3044	20568	1.507738	0.027249
GO:0030819~positive regulation of cAMP biosynthetic process	3	107	43	16787	10.94566	0.030149
GO:0031175~neuron projection development	4	107	107	16787	5.864966	0.030269
Zinc	22	121	2351	20568	1.590658	0.030751
Sodium transport	4	121	117	20568	5.811401	0.031166
ATP-binding	15	121	1391	20568	1.833035	0.032023
Sodium	4	121	124	20568	5.483338	0.036094
Epilepsy	4	121	126	20568	5.396301	0.037571
GO:0009267~cellular response to starvation	3	107	49	16787	9.605379	0.03832
compositionally biased region: Poly-Pro	7	121	421	20063	2.756934	0.040958
GO:0030659~cytoplasmic vesicle membrane	4	113	126	18202	5.11364	0.042825
GO:0005938~cell cortex	4	113	127	18202	5.073375	0.043671
compositionally biased region: Lys-rich	4	121	131	20063	5.062898	0.044013
GO:0042802~identical protein binding	9	107	615	16313	2.231092	0.046938
GO:0005634~nucleus	43	113	5430	18202	1.275585	0.049016

Nucleus	40	121	5234	20568	1.299071	0.049715
GO:0005049~nuclear export signal receptor activity	2	107	8	16313	38.11449	0.050827
Cell projection	9	121	701	20568	2.182384	0.05294
GO:0030529~intracellular ribonucleoprotein complex	4	113	139	18202	4.635385	0.054465
Protein biosynthesis	4	121	152	20568	4.473249	0.059504

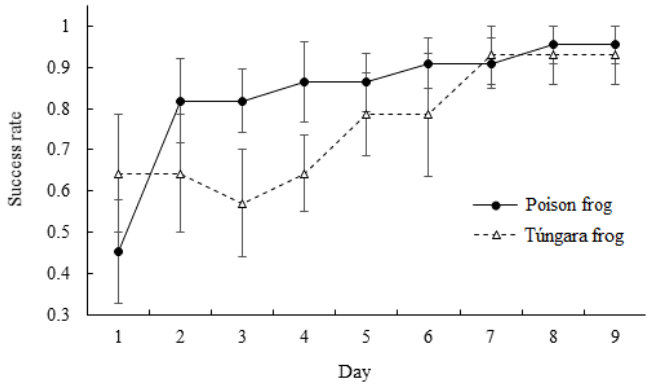

Figure 5.1. The success rate of poison frog (solid circles with solid line) and túngara frog (opened triangles with dash line) during acquisition of experiment I.

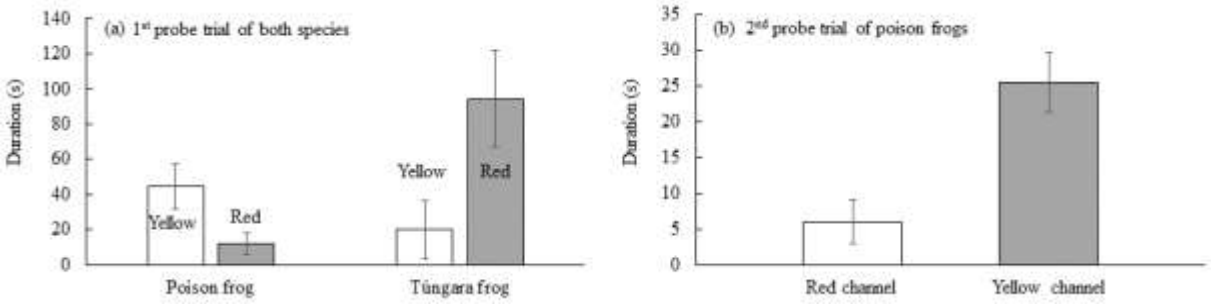

Figure 5.2. Duration that frogs spent in the arm with yellow door (yellow) and the arm with red door (red) during experiment I. (a) the first probe trial includes both poison frog and túngara frog; (b) the second probe trial on poison frogs.

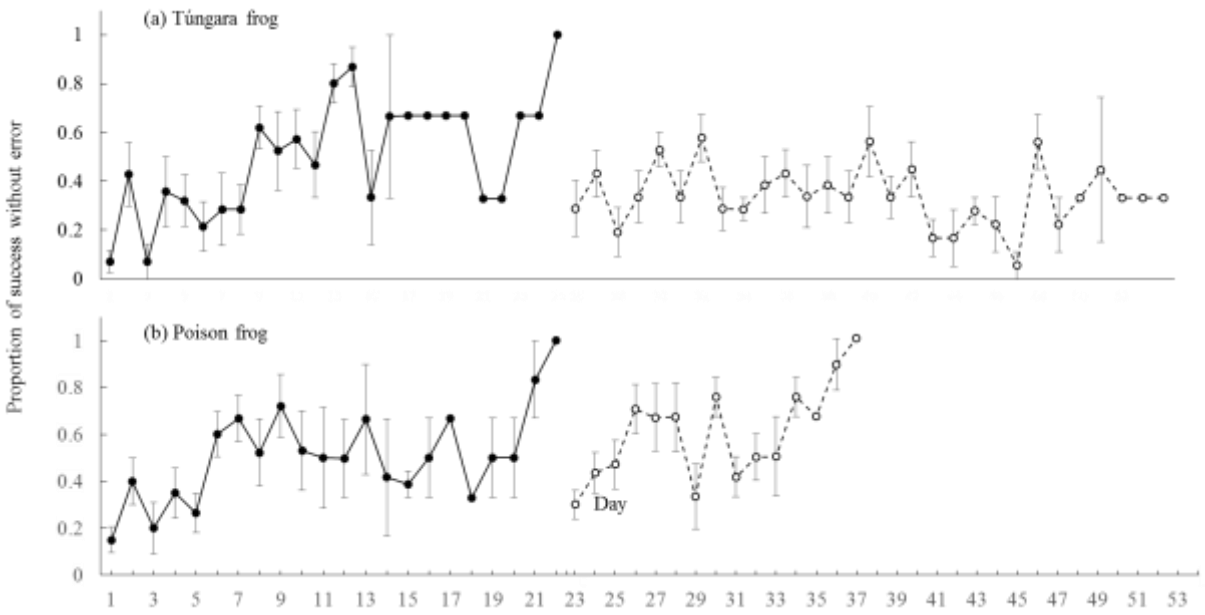

Figure 5.3. Variation in the mean ± SE proportion of successful trials without error over successive days for frogs during acquisition (solid line with solid circles) and reversal (dash line with opened circles) in Experiment II. (a) túngara frog; (b) poison frog.

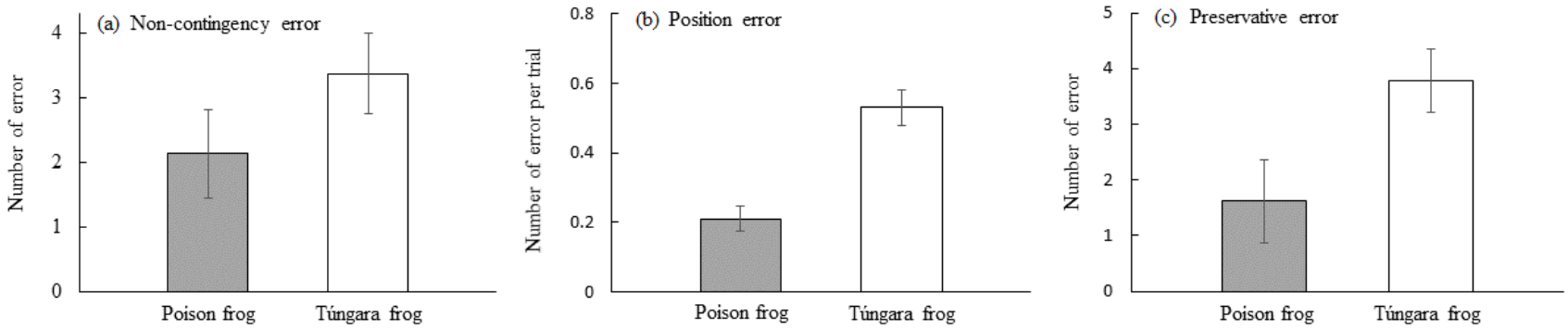

Figure 5.4. Comparison of errors between túngara frog (white box) and poison frog (grey box) in experiment II. (a) non-contingency error of acquisition; (b) position error of acquisition; (c) preservative error of reversal learning.

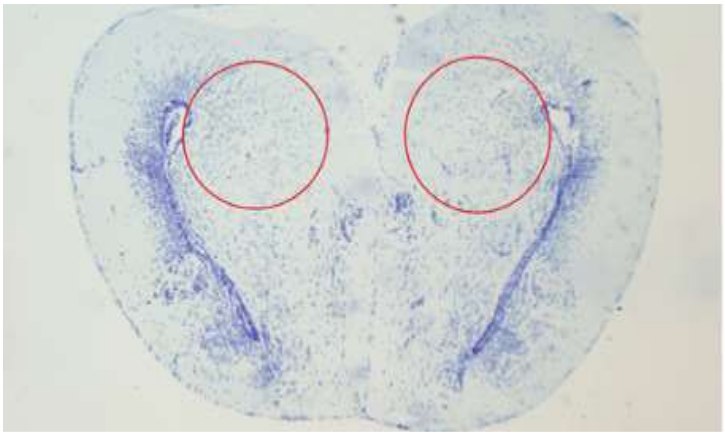

Figure 5.5. Slide of cross section of túngara frog forebrain. The two red circles are the medial pallium where I took samples from.

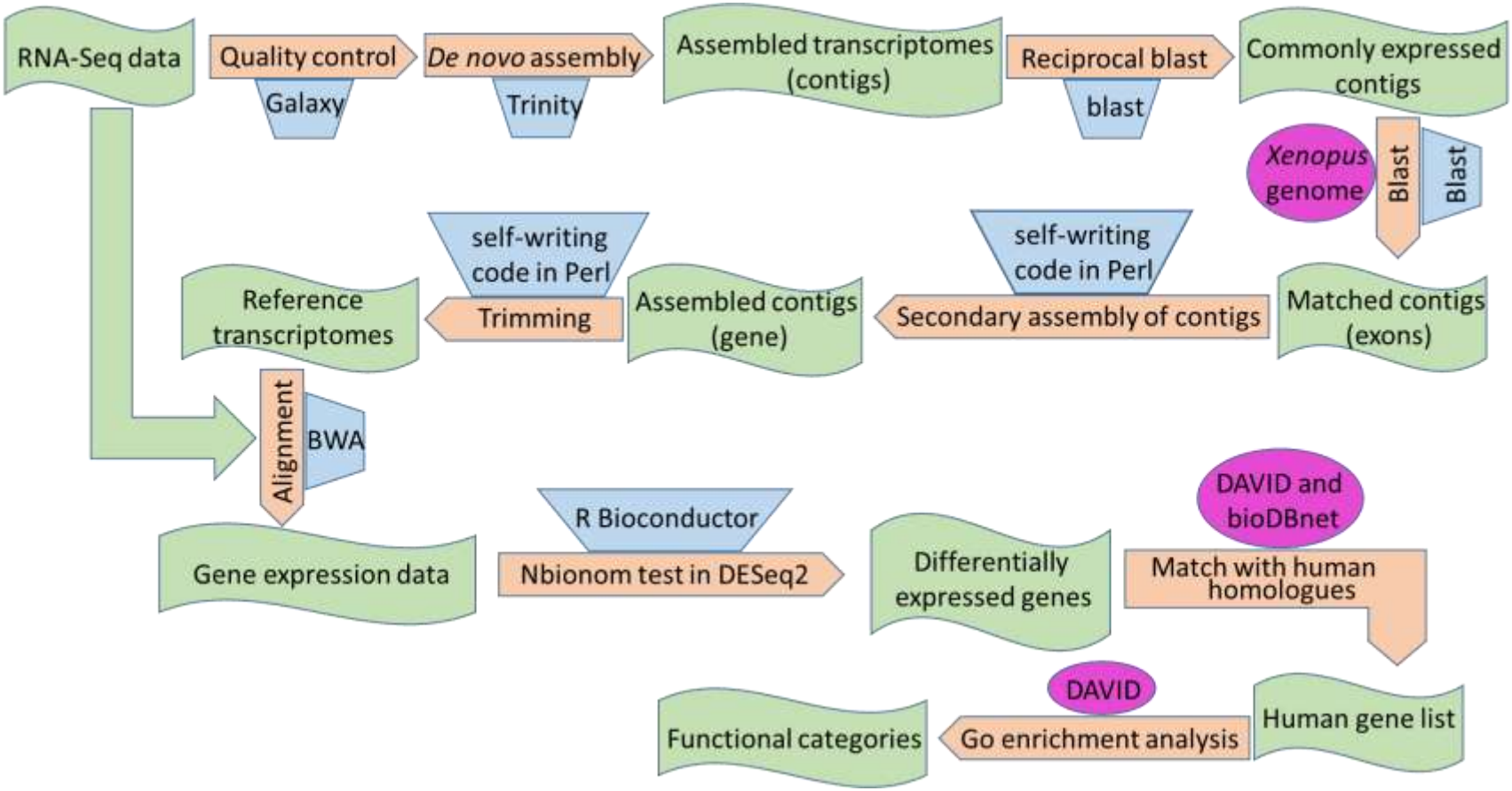

Figure 5.6. Flow diagram of bioinformatics pipeline. Green wave tape represents input data or output result of each process. Orange pentagon represents each analysis process. Blue trapezoid represents the software in each process. Purple oval represents external database.

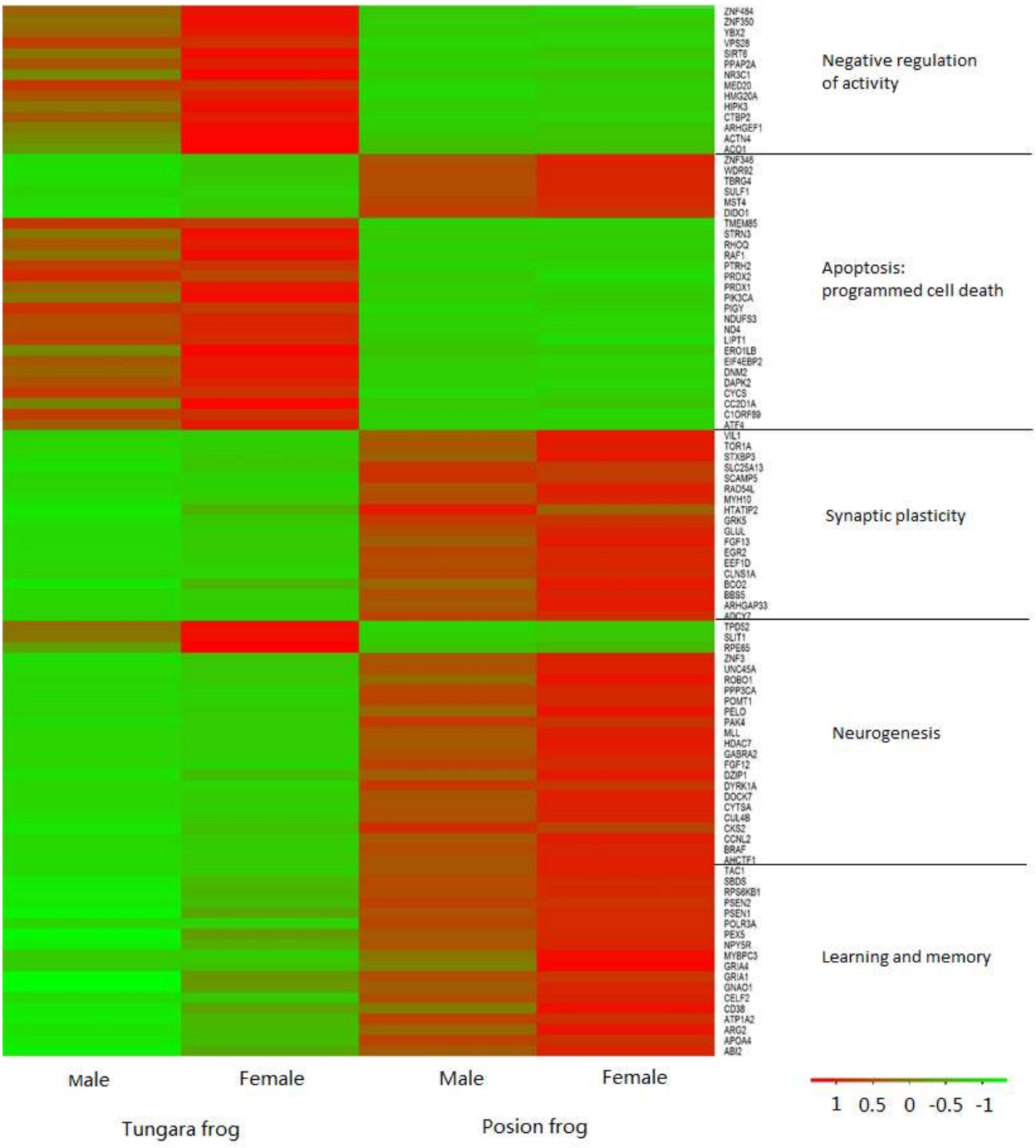

Figure 5.7. Differentially expressed genes which could be categorized into interesting functional categories between túngara frog and poison frog. Green represents downregulation, while red represents upregulation.

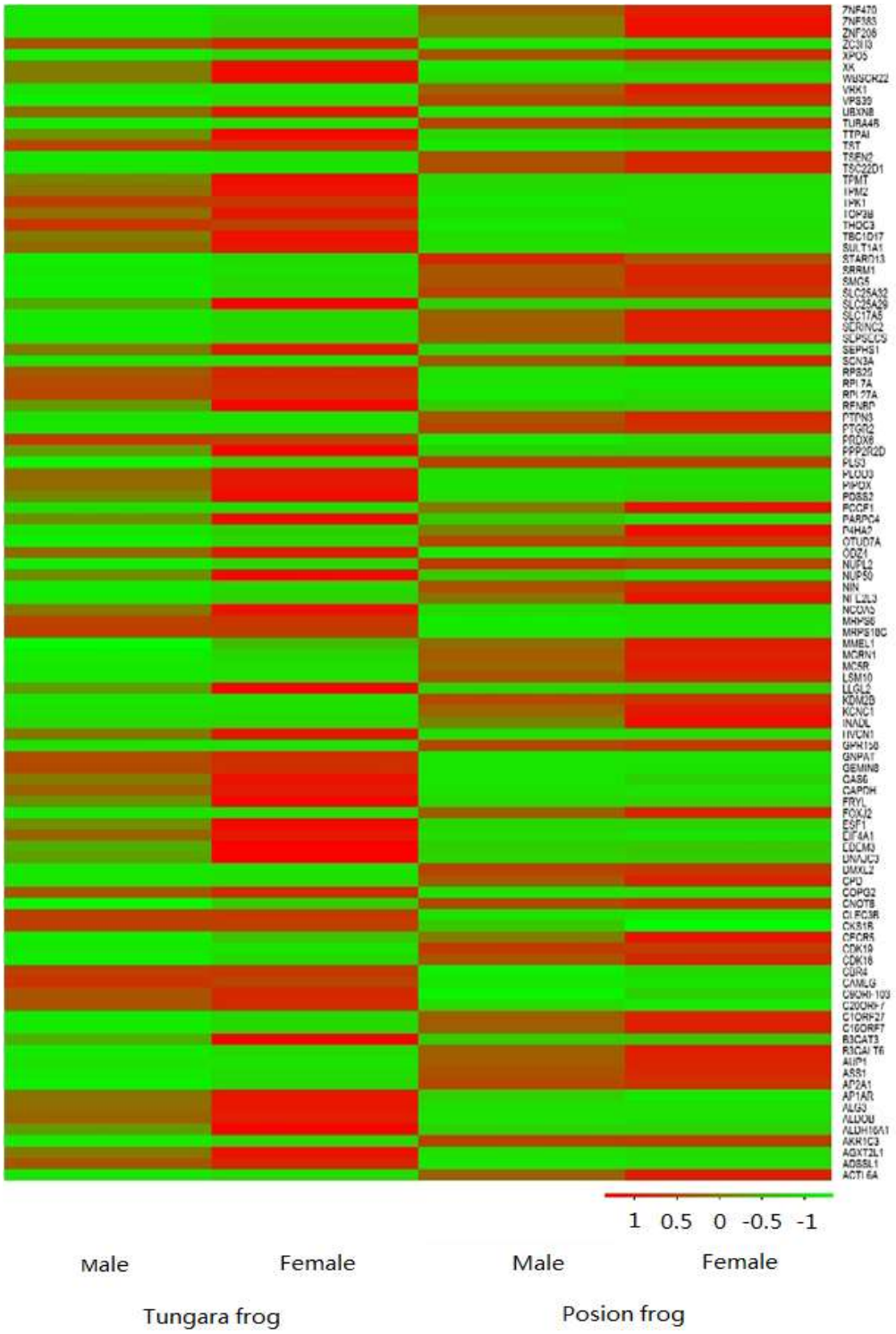

Figure 5.8. Other differentially expressed genes between túngara frog and poison frog. Green represents downregulation, while red represents upregulation.

CHAPTER 6: SYNTHESIS

In the tradition of comparative psychology, cognition can be compared among closely related species to understand questions of adaptation and mechanism, or cognition can be compared among more distantly related species (e.g. different vertebrate clades) to examine the phylogenetic history of cognitive ability (Papini, 2010). In my dissertation work, I found that the túngara frogs showed a sex difference in the ability to use a cue-taxis strategy when solving a two-arm maze, in which females learned this task while males did not. This result is consistent with the natural history of sex differences in the field. Then, I compared two frog species that differ in their natural histories and reproductive behaviors. I found that túngara frogs used a cue-taxis strategy while poison frogs used a landmark strategy to learn the same maze task. Furthermore, poison frogs showed higher behavioral flexibility than túngara frogs by demonstrating reversal learning in a two-arm maze while túngara frogs did not. These results suggest that poison frogs outperform túngara frogs in hippocampally-dependent learning abilities. Hence, I further compared their hippocampal transcriptomes to understand the neurogenomic mechanisms of spatial cognition. I found that poison frogs upregulated expression of genes related to learning and memory, neurogenesis, and synaptic plasticity in the anuran hippocampus and down-regulated expression of genes associated with neural apoptosis and negative regulation of biosynthesis and metabolism compared to túngara frogs. To determine if amphibians have comparable spatial abilities to other vertebrates, I tested poison frogs in serial reversal and Morris water maze tasks. I found that poison frogs were able to use a rule-based learning strategy and a cognitive map to learn serial reversal and the Morris water maze task, and poison frogs showed performance levels that were qualitatively similar to rodents in both tasks. These results suggest that the spatial cognition of frogs is consistent with adaptive hypotheses of spatial cognition, in which sex and species with higher levels of cognitive challenge imposed by the environment should outperform others (Gaulin, 1992; Sherry, Jacobs, & Gaulin, 1992).

One should always be cautious when distinguishing whether animals *did not* learn a particular task or they are *unable to* learn that task. In Chapter 2, female túngara frogs used cue-taxis strategy to learn a two-arm maze task, while males did not. However, this result should not be interpreted as demonstrating that male túngara frogs being unable to use a cue-taxis strategy for place learning. Since I only trained them for nine days which is a relatively short training period comparing to other amphibian species (Daneri, Casanave, & Muzio, 2011; Elepfandt, 1985; Ellins, Cramer, & Martin, 1982), and the success rate may have begun to increase at the end of the acquisition training session (Chapter 2), it is possible that males would learn this task using a cue-taxis strategy if I were to extend the training period. This possibility was confirmed by the experiments reported in Chapter 5 when I trained them for longer time in the second two-arm maze experiment. Although I did not show the results in Chapter 5, males did take longer time and they committed more position errors before they reach the criterion than females. The main sex difference when they were trained in the two-arm maze with local cues is that males were affected more by the left-turn tendency and they were predisposed to the body turn strategy. Therefore, the main point for the sex difference of túngara frogs in place learning is not difference of capability to learn cue-taxis strategy, but in the flexibility to use different types of strategies. It will be interesting to test if male and female túngara frogs differ in their use of the body turn strategy to solve the two-arm maze. This experiment would yield insight into whether learning of a cue-taxis strategy by female túngara frogs is due to their higher flexibility with respect to switching or their inferior ability to use the body turn strategy.

Spatial learning strategies are associated with different types of cues. I distinguished three types of strategies when I trained poison frog and túngara frog in two versions of the two-arm maze (Chapter 2 and 3). In Chapter 2, túngara frogs used a cue-taxis strategy (local cue) and potentially used a body turn strategy (egocentric cue), while poison frogs used a landmark strategy (spatial cue) in Chapter 3. Other amphibians also show an ability to use a body turn strategy and a cue-taxis strategy in place learning (Daneri et al., 2011; Ellins et al., 1982; Lüddecke, 2003; Schmajuk, Segura, & Reboreda, 1980), while I demonstrated for the first time that an amphibian could also use a landmark strategy. However, future

work is needed to determine whether each of the three strategies can be used by poison frogs and túngara frogs.

Natural environments are replete with different types of cues. Hence there is more than one type of learning strategy that animals could use for place learning in nature (Maaswinkel & Whishaw, 1999). Animals typically have a preference when multiple learning strategies are available to solve a tasks (Maaswinkel & Whishaw, 1999). The frogs in these studies showed a tendency to use one strategy rather than another in learning of two-arm maze tasks. The body turn strategy is likely to serve as a default strategy when I trained túngara frogs (Chapter 2). This result is consistent with previous studies in rodents (Maaswinkel & Whishaw, 1999). When both cue-taxis and landmark strategies were available, túngara frogs tended to use a cue-taxis strategy while poison frogs tended to use a landmark strategy (Chapter 5). These results support the hypothesis that species relying more on spatial cognition would be more likely to use spatial cues rather than local cues in place learning (Brodbeck, 1994). Studies on rodents found that there are some hierarchies when they adopted different strategies during place learning (Maaswinkel & Whishaw, 1999). It will be interesting to see if a hierarchy also exists in frogs. Moreover, given that these different type of cues (egocentric, local, and spatial) are coded in different memory systems in the brain (McDonald & White, 1994), studying the neural mechanism underlying strategy preference or hierarchy could aid in understanding of how the memory systems of one modality win when competing with other modalities.

In Chapter 5, I found poison frogs and túngara frogs differ in behavioral flexibility by demonstrating that poison frogs learned the reversal task while túngara frogs did not. However, it is premature to conclude that túngara frog are unable to do reversal learning. Reversal learning has been demonstrated in the place learning of other species of frogs when they were tested using a body turn strategy (Daneri et al., 2011; Schmajuk et al., 1980), a cue-taxis strategy (Daneri et al., 2011), and mechanical cues (Elepfandt, 1985). The conclusion that túngara frogs failed to show reversal learning in this particular experiment was based on the fact that none of them reach the criterion. The criterion was set as 7 successes without error out of 9 successive trials, and the probability to commit an error is much

higher than success if they move randomly (Chapter 3). This criterion is stricter than that used in other studies of other amphibians. Although this work did not resolve whether túngara frogs could achieve reversal learning using visually-based local cues, I did find they could achieve reversal by using an auditory cue (Liu et al. unpublished). Further research is needed to understand modality dependent behavioral flexibility in the túngara frog.

Comparison of patterns of gene expression between hippocampal transcriptomes from poison frogs and túngara frogs showed that genes related to learning and memory, neurogenesis, synaptic plasticity, neural apoptosis, and negative regulation of biosynthesis and metabolism were differentially expressed (Chapter 5). Given their differences in natural history and performance in the mazes, it is possible that their differences in spatial cognition could result, in part, because of differential expression of those genes. However, these results only reveal a correlation between spatial cognition and gene expression of hippocampus, which is not sufficient to demonstrate a causal relationship. Although a landmark strategy and behavioral flexibility have been demonstrated to be associated with hippocampal function in mammals and birds (Rubin, Watson, Duff, & Cohen, 2014; Sherry & Duff, 1996; Wiener, Korshunov, Garcia, & Berthoz, 1995), the functional role of the amphibian hippocampus with respect to spatial cognition has never been tested. Therefore, future research on hippocampal ablation is necessary to confirm its contribution to spatial cognition in amphibians. On the other hand, the hippocampus has been associated with more than one function in mammals (Hölscher, 2003). Therefore, one should not conclude that the differentially expressed genes that I detected are only associated with spatial cognition. It is likely that some of them are associated with other functions of hippocampus. Even for the genes (e.g. *psen1*) that have been demonstrated as causally related to spatial learning in mammals (Reiserer, Harrison, Syverud, & McDonald, 2007), it is still imprudent to conclude they will have the same functions in amphibians. Therefore, gene manipulations (e.g. CRISPR/Cas9 and RNAi) are required to determine their functions with respect to spatial cognition.

Faster, or more accurate, place learning has been associated with bigger volume, higher neural density, and higher neurogenesis rate in hippocampus (Barnea & Nottebohm, 1996; Krebs, Sherry, Healy,

Perry, & Vaccarino, 1989; Van Praag, Shubert, Zhao, & Gage, 2005). The results of hippocampal transcriptome comparison suggest that upregulated neurogenesis genes and downregulated apoptosis genes might result in higher volumes and neurogenesis rates in poison frogs. However, studies on comparative neuroanatomy are needed to verify this possibility. In addition, the morphological traits (i.e. size and neural density) of brain regions depend on neural stem cells. Therefore, a further step to study the mechanism behind spatial cognition of frogs is to compare the gene expression profiles of these neural stem cells between poison frogs and túngara frogs during their development.

I found that poison frogs could learn serial reversal and Morris water maze tasks by using rule-based learning strategy and cognitive map respectively (Chapter 3 and 4). Their rodent-like performance in both experiments demonstrated advanced level of hippocampally-dependent cognitive abilities (Mackintosh, McGonigle, & Holgate, 1968; Morris, 1984). These results open a set of questions. For example, how could the brain, which is much smaller in size and much simpler in structure, code for these complicated behaviors? Are these behaviors in frogs mediated by the same neural mechanisms as in rodents? In terms of understanding the general principles of these advanced cognitive abilities, the simplicity of the frog brain could reveal more parsimonious neural mechanisms.

Both behavioral flexibility and a cognitive map are hippocampally-dependent cognitive abilities. A fundamental aspect of hippocampal function is to associate information concerning where, what, and when together to form episodic memory (Tulving & Markowitsch, 1998). Some dendrobatid frogs have been found to feed their tadpoles with unfertilized eggs (Summers, Weigt, Boag, & Bermingham, 1999), and those tadpoles were not in the same location (Summers & Tumulty, 2013). In order to prevent the situation in which tadpoles in some places are hungry while the other are over fed, I would expect these frogs to have evolved a mechanism to code for time. So far, episodic memory has only been clearly demonstrated in mammals and birds. It will be interesting to see if amphibian also possess it.

Accurate navigation during long-distance migration exists in all vertebrate clades (Aidley, 1981; Alerstam, Hedenström, & Åkesson, 2003; Sinsch, 1990) and homologues of brain regions for spatial cognition are conserved (Butler & Hodos, 2005). These facts suggest that spatial cognition might be

evolutionarily conserved across vertebrates (Broglio et al., 2015). Consistent with this argument, the three spatial strategies (cue-taxis strategy, landmark strategy, and body turn strategy) that I identified in this study are common in all other vertebrates. Yet, the advanced cognitive abilities (i.e. rule-based learning and a cognitive map) that I demonstrated in poison frogs have never before been clearly demonstrated in a non-mammal and non-avian vertebrate. Given that the brain regions for spatial cognition of vertebrates are phylogenetically conserved (Broglio et al., 2015; Butler & Hodos, 2005), but advanced forms of spatial cognition are uncommon, it is reasonable to suggest that the hippocampus of ancestral tetrapods might have possessed a common architecture that was necessary, but not sufficient, for the evolution of these advanced level of cognitive abilities

In conclusion, I distinguished three spatial learning strategies in two species of frogs. The túngara frog showed a sex difference in which females were flexible enough to choose an appropriate strategy to learn a two-arm maze task. The túngara frog and poison frog differed in performance, learning strategy, and behavioral flexibility in learning of two-arm mazes. Poison frogs, with higher navigation demands in their natural history, committed fewer errors, tended to use spatial cues rather than local cues, and showed higher behavioral flexibility when compared with túngara frogs. I also demonstrated the advanced level of cognitive abilities of rule-based learning and a cognitive map, which are comparable to other vertebrates. This work filled some gaps in my understanding of amphibian spatial cognition, and it potentially inspired some efforts to understand the mechanism of spatial cognition from different perspectives (e.g. behavior, neural anatomy, and neurogenomics).

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