SPATIAL LEARNING IN RED SWAMP CRAYFISH (*Procambarus clarkii*)

A Thesis

by

MARIE PENDLETON

BS, Texas A&M University-Corpus Christi, 2016

Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in

BIOLOGY

Texas A&M University-Corpus Christi
Corpus Christi, Texas

December 2022

December 2022

SPATIAL LEARNING IN RED SWAMP CRAYFISH (*Procambarus clarkii*)

A Thesis

by

MARIE PENDLETON

This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

Kim Withers, PhD
Chair

Riccardo Mozzachiodi, PhD
Committee Member

Jenifer Pollack, PhD
Committee Member

December 2022

ABSTRACT

Spatial learning, or the process by which animals gather and use information within their environment to navigate and remember the location of stimuli, is of significant ecological importance. Spatial learning is commonly studied in vertebrates using various types of mazes. In this experiment two types of mazes (multiple-turn maze, consecutive T-maze) were used to determine if aquatic arthropods exhibit spatial learning. Three cohorts of red swamp crayfish (*Procambarus clarkii*) were given the opportunity to learn two mazes using food as a motivator over two consecutive six-week periods. The crayfish were exposed to the multiple turn maze first and then to the consecutive T-maze. The prediction was that exposure to the multiple turn maze would improve crayfish performance in the consecutive T-maze. Time to completion was measured and the number of wrong turns was counted for each individual in the conditioned group in each maze type; a control or unconditioned crayfish was placed in a maze start gate so that all crayfish were handled in the same way but was not allowed to try to learn the maze. After the 4-week conditioning period, all crayfish underwent a 1-week latency period and conditioned and unconditioned crayfish were then tested in the maze without the motivator to determine if spatial learning had taken place. Crayfish then were conditioned in the T-maze in the same fashion. Although there was a great deal of variability, conditioned crayfish showed some improvement in completion time in both the multiple- turn maze as well as the consecutive T-maze, and the number of wrong turns decreased slightly, but they did not show improvement when compared to the control group. The starting mean completion time for the multiple turn maze conditioned group was 1745.81 sec and by week 6 was 1653.29 sec compared to the unconditioned mean of 1082.41 sec. The starting mean for the consecutive T-maze conditioned

group was 831.62 sec and by week 6 was 397.44 sec compared to the unconditioned 834 sec. The starting mean number of wrong turns for the multiple turn maze conditioned group was 2.5 and by week 6 was 1.9 compared to the unconditioned mean of 1.76. The starting mean for the consecutive T-maze conditioned group was 1.62 and by week 6 was 1.67 compared to the unconditioned 2.25. There were also no notable differences in the mean completion time 1653.29 sec vs 1082.41 sec (df = 38.527, t=1.497, p=0.143) between the conditioned and unconditioned crayfish in the multiple-turn maze. A repeated-measures ANOVA with all cohorts combined showed that there was a difference between weeks in mean completion time (df = 4, F = 2.806, p = 0.031), with mean times in week 3, 1548.89 sec, being lower than those in week 2, 2202.82 sec, (mean difference= -1048.7; p = 0.032). Although the mean number of wrong turns for all cohorts combined showed a slight downward trend, there was no statistical difference between weeks (df = 4; F = 0.099; p = 0.983). The mean number of wrong turns, was similar, 1.9 vs 1.76 (df = 33.644, t=0.281, p=0.780) between the conditioned and control groups. In the T-maze for completion times, a repeated-measures ANOVA, with Greenhouse-Geisser correction, showed similar completion times between weeks with mean completion times from week 1 through 6 being, 831.62 sec, 1052.86 sec, 601.5 sec, 917 sec, and 397.44 sec (df = 4, F = 1.115, p = 0.353). After the latency period, the mean completion time of the conditioned group dropped to about half that of the unconditioned individuals, 397.44 sec vs 834 sec, but there was no statistical difference (df = 26.853, t=-1.674, p=0.106). The number of wrong turns was similar between weeks for the T-bar maze with mean number of wrong turns from week 1 to week 6 being, 1.62, 2.14, 1.6, 2, and 1.67 (df = 4, F = 1.611; p = 0.195). Although the number of wrong turns in conditioned individuals, 1.67, was slightly less than that of the control group, 2.25, there was no statistical difference (df = 21.180, t=-1.146, p=0.265). This experiment was marked by a great

deal of variability in the results due factors such as high mortality, molting, and aspects of the experimental set up, such as training crayfish on mazes in bright light. Additional research is warranted using larger sample sizes to evaluate responses with greater statistical power. This experiment failed to provide evidence for spatial learning in *Procambarus clarkii*, but did not eliminate the possibility for spatial learning to occur in this species. Crayfish are model organisms for neurological studies due to their large easily accessible neurons, further studies are needed to determine if spatial learning can be found in this species.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my professor and chair Dr. Kim Withers, without her guidance and patience I would not have been able to perform this research. I would also like to thank the members of my committee Dr. Jennifer Pollack and Dr. Riccardo Mozzachiodi who provided knowledge and expertise. Additionally, I would like to thank Captain Jay Tarkington for constructing the mazes for this research. Special thanks to my husband Edward Elizondo for his support during my time in the program. I would also like to thank my dad Joseph Pendleton who supported me financially during my last few semesters. Lastly, I would like to thank my friends Erica Andleson and Alandra Johnston for staying up with me in the lab during my late nights.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

PAGE

CHAPTER I: BACKGROUND AND RELEVANCE

Learning is defined as a relatively permanent change in behavior as a result of experience (Nordell and Valone 2021). Simple learning such as habituation (i.e., decreased response to repeated stimuli) and sensitization (i.e., amplification of a response following repeated stimuli) has been observed in many vertebrates and invertebrates (Perry et al., 2013). However spatial learning, a more complex type of learning that plays an important role in adaptation to changing environmental conditions (Mery, 2013), has mostly been studied in vertebrates (i.e., small mammals) with some studies on invertebrates such as octopuses (e.g., Boal et al., 2000) and insects (e.g., Collett, 2009). Spatial learning relies heavily on visual cues such as landmarks (Heijiningen, 2022) and is the process by which animals gather information about their environment and use it to navigate and remember the locations of stimuli (Floresco, 2014). Alterations of behavior attributed to spatial learning are often used to measure memory formation (Kastner et al., 2022). The goal of this research was to better understand spatial learning and memory in crustaceans using mazes as the method and red swamp crayfish (*Procambarus clarkii*) as the model organism.

Spatial Learning

Cognition is the process of learning, remembering, and using knowledge and is a vital part of developing behaviors necessary for survival (Cherry, 2022). Cognitive ability varies between species as well as between individuals within a population (Prentice et al., 2022) and is likely heritable and subject to natural selection. Spatial learning occurs through a series of cognitive processes in which animals acquire, process, store, and use information gathered from the environment (Shettleworth, 2010). Arthropods, such as crustaceans and insects, live in dynamic habitats in a multitude of complex environments and systems. They play important

roles as scavengers, predators, and prey organisms, and some are even considered habitat modifiers (van der Zee et al., 2016), organisms which can alter their abiotic environment through actions such as bioturbation. These organisms navigate different environments within their habitats and as they move about gain information on landmarks, obstructions, and points of interest. This spatial information is then presumably incorporated into the organism's memory and the stored information can then be recalled for later use.

In the brains of mammals, each temporal lobe contains a hippocampus which is one of the most important regions of the brain associated with spatial learning (Savage & Ma, 2014). The hippocampus is strongly associated with formation and storage of memories and is where spatial learning is mediated (Kosaki et al., 2014). The rear part of the hippocampus is thought to be where spatial memories are processed (Guy-Evans, 2021). Although invertebrates do not have the same brain structures as vertebrates, they are able to form and store memories (Sanchez, 2014). In invertebrate brains the learning centers which are responsible for the storage of memory are different than those of mammals; for example, in insects it is the mushroom body, in octopi the vertical lobes, and in terrestrial slugs the precerebral lobes (Menzel & Benjamin, 2013).

In humans, spatial learning is a form of declarative memory, i.e., consciously recalled information based on facts and events (Burgess et al., 2002). In animals it can be difficult to test spatial learning using declarative memory due to their inability to follow verbal direction or to respond verbally (Stark, 2010). However, there are methods in which declarative memory can be tested in animals that do not require a verbal response. Rodents, typically rats and mice, are often used to study memory with the use of mazes such as the Morris water maze (Vorhees & Williams, 2014). The Morris water maze consists of a pool of opaque colored water with an

escape platform hidden just below the surface. Animals cannot rely on scent or sight to find the escape route and therefore must learn and remember where it is over repeated trials (Nunez, 2008).

Invertebrates like crustaceans can also be excellent subjects for research on learning and memory due to their less complex nervous system (Schnaitmann, 2010). They can be tested using methods similar to those used in vertebrate studies, such as mazes, and, because they are relatively small, fewer resources are needed to conduct the research or for their maintenance.

Overview: Learning in Invertebrates

Ninety-five percent of known species are invertebrates (McConnell, 1966) making them valuable subjects in learning research. Invertebrates are less structurally complex (e.g., have fewer neurons) than mammals which can make it easier to study learning behavior. Most invertebrate nervous systems contain only a few thousand cells making them significantly smaller and easier to study when compared to the several billion cells of the vertebrate nervous system (Kesner et al. 1990). For example, the central nervous system of insects is small and highly compartmentalized with obvious separations between neurons which makes them ideal organisms for studying learning and memory (Hammer & Menzel, 1995).

Bees are one of the most common invertebrate animal models for studies of learning. Because bees feed on the nectar and pollen of flowering plants and are important pollinators, studies of how they perceive their world could have a significant impact on food production (Kevan & Menzel, 2012). Most learning studies on bees are appetitive with a neutral stimulus paired with a reward, but there have been some studies using simple mazes (Nouvian & Galizia, 2019). There have been many studies of how bees navigate and how they find food. Do they fly aimlessly hoping to find flowers, or do they learn what to look for when searching? Bees exhibit

two types learning: associative, which involves encoding of relationships between stimuli, and latent, which is the retention of information without reinforcement, (i.e., observatory). While both types play a role in spatial learning, latent learning is the more important of the two since bees often learn from observing one another (Menzel, 1993). Honey bees learn that specific scents, colors, and geometrical patterns are associated with food (Heinrich, 1984). Bees also use and remember landmarks to navigate and find their way to and from food sources (Cartwright & Collett, 1983). Not only are bees capable of seeing the colors of flowers and picking up on their scents, they can also remember and distinguish one flower from another nearby flower and visit only those that offer enough nectar and pollen to be worth visiting (Menzel & Erber, 1978).

Another invertebrate favored for the study of learning and memory is the sea hare (*Aplysia* spp.) because the obvious defensive withdrawal of the mantle organs can be readily elicited and quantified (Rankin & Carew, 1987). *Aplysia* have some of the largest neurons in the animal kingdom (up to 1.1 mm in diameter) and are model organisms for study of the role of neurons in learning and behavior (Moroz, 2011). Studies of learning in *Aplysia* consist of aversive classical conditioning experiments where stimuli are used to elicit head and siphon withdrawal, inking, or escape locomotion (Walters et al., 1981). The large neurons in *Aplysia* can also be directly manipulated. Withdrawal behaviors can be elicited by physically or chemically stimulating part of the body, but the large neurons of *Aplysia* can be isolated and stimulated directly. These responses can be quantified allowing researchers to locate the specific neuron(s) that controls a response (Argranoff et al., 1999).

The withdrawal reflexes of *Aplysia* can not only be used to study learning to associate stimuli with danger or with food, but also whether or not these processes are affected by aging. In a study by Kempsell and Fieber (2015), *Aplysia californica* were subjected to a series of

behavioral and electrophysiological experiments where a reflex was triggered and measured. This was done during two periods in their lives, once when they were mature at 7-8 months old and again at 12-13 months old nearing the end of their lifespan. Reflex performance was significantly decreased in the older animals compared to the mature animals indicating that the neurons responsible for governing nonassociative learning were compromised with age.

One of the more charismatic invertebrates appearing in studies of learning and memory are cephalopods such as squid and octopus. While many invertebrates are small and go unseen, the larger cephalopods with their relatively large brain and interesting behaviors have captured the attention of non-scientists as well as scientists seeking to understand consciousness in non-human animals (Mather, 2020). Cephalopods are interesting creatures for studying learning in invertebrates because, unlike *Aplysia* which have few large neurons, the cephalopod nervous system comprises half a billion fairly small neurons and is larger than most other invertebrate nervous systems as well as the nervous systems of some primitive vertebrates (Turchetti-Maia et al., 2019). While the larger number of neurons in comparison to other invertebrates makes them interesting subjects, it does make it more difficult to pinpoint where memory is stored and which neurons might be associated with a specific behavior. There are some studies on the chambered nautilus (*Nautilus pompilius*), which has a less complex brain than more modern cephalopods like octopuses (Crook & Basil, 2013). Studies using classical conditioning as well as modified mazes showed that capacity for learning and long-term memory retention in chambered nautilus was similar to that of their soft-bodied relatives (Basil & Crook, 2021).

Octopus and mammal memory systems share functional and structural similarities despite different brain organization (Darmaillacq et al., 2014). Vertebrate brains consist of a cerebral cortex, thalamus, basal ganglia, midbrain, cerebellum, hypothalamus, brain stem, and spinal cord

(Shigeno et al., 2018). The cephalopod brain has a ganglia-like structure that is typical of invertebrates with densely packed neural cell bodies in the outer layer and branched processes and synapses in the neuropil (Deryckere & Seuntjens, 2018). Cephalopods have well-developed eyes and olfactory organs which make them excellent candidates for studies of spatial learning. The cuttlefish (*Sepia officinalis*) exhibited spatial learning during modified maze experiments (Karson et al., 2003). Spatial learning in cephalopods has also been explored using detour experiments. In these experiments, the subject must attempt to reach a stimulus that is blocked from direct view. Spatial learning is demonstrated when the subject is able to maintain its position relative to the stimulus even after its view has been obstructed (Alves et al., 2007). During an experiment with an octopus, the octopus needed to either maintain contact with the wall obstructing its view, or if it could not touch the wall, it had to train its sight on the wall. While the octopus did not exhibit evidence of spatial representation (i.e., knowledge/memory of the position of the out-of-sight but desired stimulus) the experiment did show that octopuses have the capacity to develop strategies for solving spatial tasks.

Studies on learning and memory in crustaceans are becoming more common partially due to the number of introduced or invasive crustaceans that are being found in freshwater and marine systems (Weis, 2010). The ability to learn may contribute to success of species invasions and/or expansions because introduced or invasive species may be able to adapt better and more quickly to changing environmental conditions and/or competition than native species. Crustaceans have small, simple nervous system making them good model organisms for neurophysiological research (Bukowski-Thall, 2020). The nervous system of most crustaceans consists of a supraesophageal ganglion, connected to a ventral nerve cord of ganglia or to nerve centers (Gordon & Green, 2022).

Crabs in the genus *Chasmagnathus* are commonly used model organisms in neurobiology studies, particularly context-signal memory experiments where a stimulus is presented eliciting an escape response and memory of the stimulus is studied over time and exposure (Tomsic & Romano, 2013). When *Chasmagnathus* crabs were repeatedly exposed to a fear stimulus, such as a potential predator, the fear response decreased over time and the memory of the reduced response was retained (Maldonado, 2002). In another study on *Gelasimus dampieri* by Donohue et al., visual cues were used to study how fiddler crabs made escape decisions. During this experiment crabs were placed on specialized treadmills surrounded by four computer monitors which could depict a threat coming toward them at varying speeds, sizes and angles. Using these methods, they were able to determine that crabs can accurately gage angular size and speed of potential threats and modify their escape responses to those and other factors such as distance from shelter accordingly (Donohue et al.).

Studies of the homing behavior of fiddler crabs have also shed light on spatial learning and navigation in crustaceans. Fiddler crabs (*Uca rapax*), aligned the transverse axis of their body with their burrow entrance when foraging and were able to return to the burrow even when they were displaced or if the entrance to the burrow was covered (Layne et al., 2003). Experiments to determine the methods of path integration used by fiddler crabs consisted of placing them on rotating disks to reorient them as well as having them run over a slippery surface which affected their running velocity. Results from these spatial experiments showed that when there running velocity was reduced, crabs stopped short of their burrows suggesting that path integration was determined either by leg proprioceptors or by efferent commands (Layne et al., 2003).

Spatial Learning in Crayfish

There are over 500 species of crayfish in the world with 400 species in North America and around 353 species inhabiting the waters of the United States (Helfrich & DiStefano, 2020). Crayfish are economically important with ~80,000 tons valued at over $200 million USD farmed or caught worldwide each year (Helfrich & DiStefano, 2020). With so many species, crayfish are in the unique position of being both keystone species and invasive species (Bittel, 2019). Studies of learning in crayfish have shown that one of the key factors in a species becoming invasive is the ability to retain information longer after exposure to stimuli. For example, in Italy the invasive red swamp crayfish (*Procambarus clarkii*) retained learned association of certain olfactory cues to food and predation longer than the native white-clawed crayfish (*Austropatmobius pallipes*) (Hazlett et al., 2002).

Decapod crustaceans such as crayfish rely heavily on visual cues and landmarks for navigation and other activities. Rusty crayfish (*Orconectes rusticus*) were able to navigate a maze using only egocentric (directional) response cues but had less success when using only external (landmark) place cues; crayfish had the most success when the two cues were used together (Tierney et al., 2018). Crayfish have been used as a model organism for the study of specific neural circuitry (University of Maryland, 2010). They make good subjects in learning studies because they have a few large and accessible neurons in a modularly organized nervous system (Jackson & van Staaden, 2019). Crayfish have movable stalked compound eyes giving them a broad field of view and increased binocular spread (Cronin, 1986). On top of good vision, crayfish also possess an impressive sense of smell (Wood & Moore, 2020).

The Australian crayfish (*Cherax destructor*), uses visual cues to recognize and remember individuals that have been encountered during fights (Van der Velden et al., 2008). Olfactory

cues may also be useful especially if visual cues are obscured or absent, such as in murky water. Odorants are dispersed via currents and provide alternate or complementary (i.e., combined with visual cues) ways of finding food or avoiding predators. Spatial learning in crayfish appears to rely on both olfaction and sight. Without olfactory cues to point them in the right direction, crayfish were unable to locate food and other stimuli as quickly as when olfactory cues were present (Michaelis et al., 2020).

Using Mazes in Studies of Spatial Learning

Spatial learning and memory acquisition can be assessed using a variety of mazes (Dean, 2019). Mazes serve as an artificial environment in a controlled setting where spatial information can be manipulated, and spatial learning can be observed. Thorndike (1999) described the process of learning as consisting of motivation, random responses, elimination of unsuccessful responses, and fixation of successful responses which satisfy a motive. Although these features of learning are mostly used to describe learning in humans, they also apply to studying learning and memory in non-human animals including invertebrates like crayfish. Mazes used in a variety of animal studies fulfill all of Thorndike's factors for learning. Within the maze there is motivation, the reward for solving the maze. While navigating a maze animals will exhibit random responses as they attempt to find their way through and over time, if learning is taking place, they should begin to eliminate wrong turns or unsuccessful paths. Finally, if learning is taking place, the animal should fixate on the promised reward at the end of the maze, improving their performance over time. Maze studies allow acquisition of spatial learning to be quantified (Davies et al., 2019) because organisms can be tested in the same way over time. The expectation is that if spatial learning is occurring then the time needed to complete the maze would decrease and/or the accuracy of performance of the maze would improve.

One of the most famous mazes used in the study of spatial learning is the Morris water maze (D'Hooge & De Deyn, 2001) named after Richard Morris who invented it and patented it in 1984 (Nunez, 2008). Rodents are placed in an open swimming arena with a submerged platform which would allow them to escape. Spatial learning is measured over the course of multiple trials in which the amount of time it takes the rodent to swim to the escape platform is measured along with the rodent's preferred route (Vorhees & Williams, 2006). Other common mazes used to assess spatial learning include the radial arm maze, multiple-turn maze, and one-decision or multiple-decision T- mazes or Y-mazes; these mazes are "baited," often with a food reward.

Spatial learning has not been well studied in aquatic arthropods (Davies et al., 2019) such as crayfish. Crustaceans, in general, have demonstrated a range of navigational behaviors to find food and shelter (Boles & Lohmann, 2003), but studies on spatial learning and memory have been limited to simple one-decision Y- or T- mazes. In these mazes there is only one choice to be made before finding the reward. In the research reported herein, two kinds of mazes, a multiple-turn maze and a consecutive T-maze, which both require several decisions before the reward is found and the maze can be considered completed. Developing a better appreciation of memory and spatial learning in the red swamp crayfish, which is invasive in some parts of the world, will further our understanding of their habitat use and resource exploitation.

CHAPTER II: OBJECTIVES AND APPROACH

The purpose of this study was to develop a better understanding of spatial learning and memory in the red swamp crayfish using two types of mazes. Davies et al. (2019) demonstrated that, after a four-week conditioning period and a latency period of one-week green shore crabs (*Carcinus maenas*), which are considered invasive throughout much of the world, completed a multiple-turn maze faster and with fewer wrong turns when compared to the control group. They suggested use of a T-maze in future studies as the next step in understanding spatial learning in these animals.

Crayfish were chosen for this study because they are easily obtained (i.e., can be purchased) and make good subjects for research on memory and learning since their neurobiology has been studied for over fifty years (Edwards et. al., 1999). In addition, they have good eyesight and a good sense of smell (Wheeler, 2022) so use of maze completion times and a count of wrong turns to study spatial learning is appropriate.

CHAPTER III: MATERIALS AND METHODS

For this experiment four waterproof mazes (2 multiple-turn mazes and 2 multiple (consecutive) T-mazes) were constructed of plastic lumber and galvanized hardware cloth. Mazes were sized to fit within a 50cm x 76cm x 13cm wading pool that was filled with dechlorinated water. The multiple turn maze was constructed to have the same design and structure as a maze from a recent, similar spatial learning experiment on *Carcinus maenas* (Davies et al., 2019). The authors of that study suggested doing the experiment again using several other methods including a consecutive T-maze. The consecutive T-maze (Figure 2) designed for the current study was structured differently but consisted of the same number of possible right and wrong turns.

Crayfish were divided into an experimental (or conditioned) group and a control (or unconditioned) group. The conditioned group was first trained on a multiple-turn maze (Figure 1) and then exposed to a consecutive T-maze (Figure 2) to determine if training on the multiple-turn maze facilitated learning the T-maze. Control individuals were left in the maze "starting gate" while conditioned individuals were allowed to learn the maze to ensure that handling stress for control individuals was similar to that of conditioned individuals. The times/success of control individuals when allowed into a maze was then compared to conditioned individuals after a period of latency and when exposed to an unbaited maze. These comparisons were used to determine if spatial learning had occurred.

A small group of test subjects was purchased to ensure that housing, water quality, and food/feeding were adequate to keep the animals in good condition throughout the experiment. Crayfish were housed individually in small plastic aquaria ("critter keepers") filled with 3-4 L of dechlorinated water and aerated. Individuals were weighed and their carapace length was

measured prior to being housed. Ammonia was monitored twice weekly using test strips and water exchanges were done once a week or more as needed. Crayfish were fed 2-3 times a week a combination of algae wafers, omnivore pellets, and shrimp pellets and their wet weights were measured weekly to ensure they were not gaining or losing too much weight. The initial group was kept under observation in the laboratory for two weeks.

Multiple-Turn Maze Experiment

Three separate crayfish cohorts were conditioned in the multiple-turn maze. Each cohort consisted of 24 small crayfish (less than 7.5 cm in length) purchased from Carolina Biological Supply. Within 2 hours of delivery, individuals were removed from packing material, housed in the manner described above, and acclimated to laboratory conditions for 7 days. Each cohort was divided in half: 12 were assigned to the control group and 12 to the conditioned group.

Prior to beginning conditioning in the maze, crayfish in both groups (control and conditioned) were fasted for three days to ensure that they would be interested in a food reward. Maze conditioning was conducted between 5 pm and 1 am. Mazes were set up submerged in the wading pool with a food reward placed at the end. All test subjects, including the control group, were placed in the start chamber for 60 seconds to acclimate to the maze setting. After acclimation the conditioned group was allowed to leave the start chamber and explore the maze whereas the control group was not; they were confined to the starting gate. The control group was subjected to the same handling processes as the experimental group except that they were not allowed to explore the maze. Control animals were fed after each round of conditioning when they were returned to their aquarium.

Individuals in the conditioned group were allotted a maximum of 3600 seconds (1 hour) to complete the maze; individuals that did not complete the maze within the allotted time were

recorded as 3600 seconds. In addition to the time taken to complete the maze, the numbers of wrong turns were counted. Incidents where a subject backtracked and took a previous wrong turn for a second time were included in the wrong turn count.

Each cohort ran the maze (or sat in the starting gate) once a week for four weeks after which there was a one-week hiatus or latency period during which neither the conditioned group nor the control group were exposed to the maze or the starting gate. During this time, crayfish were weighed, fed, and the water was changed as usual. The mazes were thoroughly cleaned with warm water and allowed to sit dry for the entire week to remove any lingering food scent. At the end of the latency period both conditioned and unconditioned (control) crayfish were allowed to run the maze, but with no food reward at the end. Completion time and number of wrong turns were recorded for both conditioned and unconditioned individuals.

Multiple (Consecutive) T-Maze Experiment

The goal of this experiment was to determine if exposure to/learning the multiple-turn maze would affect the rapidity/accuracy of learning. After the latency period, the cohort of crayfish that had just finished the multiple-turn maze experiment moved on to the consecutive T-maze experiment. Experimental conditions, protocols, and procedures remained the same except that the multiple-turn maze was replaced by the consecutive T-maze. Although a total of 21 conditioned and 29 unconditioned crayfish went on to the consecutive T-maze experiment, only 9 conditioned and 21 unconditioned completed all 6 weeks of testing due to significant mortality.

Data Analysis

To test for differences in completion time and number of wrong turns between weeks, a one-way, repeated measures ANOVA was used. A Greenhouse-Geisser correction of the F-test was utilized when needed due to violation of the assumption of sphericity (i.e., if Mauchly's test

of sphericity was significant). When sphericity assumption is satisfied, the F-test in a standard analysis of variance is accurate, but when the sphericity assumption is violated, the F-test in a standard analysis of variance will be positively biased which could cause a rejection of the null hypothesis even when it should not be (Berman, 2022). Post-hoc pairwise comparisons were conducted using a Bonferroni adjustment. A two-sample t-test was used to test for differences in completion times and wrong turns between conditioned and control crayfish after the latency period. All statistical analyses were conducted using SPSS Statistics (William, 2022).

CHAPTER IV: RESULTS

Wet weight (by week), sex (when determined) and mortality (if relevant) of each individual in each cohort is shown in Appendix 1. 8 out of 12 of Cohort 2 conditioned crayfish and 5 out of 12 of unconditioned crayfish perished by the end of the multiple-turn maze experiment. Few specimens were lost in Cohort 1 until Week 5 by which time 6 of the 12 individuals in the conditioned group perished; Cohort 3 survival was greater than 80% to the end of the experiment. Among all cohorts, of the 21 conditioned and 29 unconditioned crayfish that went on to the consecutive T-maze, only 9 conditioned and 21 unconditioned survived until the end of the experiment. Wet weights remained relatively constant, (8.33 g for Cohort 1, 3.58 g for Cohort 2, and 7.58 g for Cohort 3) regardless of whether they were assigned to conditioned or control experimental groups. Crayfish in Cohort 1 were not sexed. The sex ratio (male:female) in Cohort 2 was 9:15 and in Cohort 3 was 16:8. Among all cohorts, of the crayfish that went on to the consecutive T-maze, there were 15 males and 6 unknowns in the conditioned group and 5 males, 12 females, and 12 unknowns in the control group. The sex ratio (male:female:unknown) of crayfish that died during the experiment was 5:6:6 in the conditioned group and 2:5:0 in the control group.

Multiple-Turn Maze

A repeated-measures ANOVA with all cohorts combined showed that there was a difference between weeks in mean completion time ($df = 4$, $F = 2.806$, $p = 0.031$) (Figure 3). The source of the difference was between weeks 2 and 3, with mean times in week 3, 1548.89 sec, being lower than those in week 2, 2202.82 sec (mean difference= -1048.7; $p = 0.032$). Completion times for week 6, 1653.29 sec, after the latency period, were not significantly different than any other week. Even though the mean completion time of the control group,

1082.41 sec, was less than the mean completion time of the conditioned group, 1653.29 sec, after the latency period and there were no significant differences in the mean completion times (df = 38.527, t=1.497, p=0.143) between the conditioned and unconditioned crayfish for all cohorts combined.

The starting mean number of wrong turns for the conditioned group was 2.5 and by week 6 was 1.9 compared to the unconditioned mean of 1.76. The mean number of wrong turns for all cohorts combined showed a slight downward trend (Figure 4) but a repeated-measures ANOVA did not reveal any significant differences between weeks (df = 4; F = 0.099; p = 0.983) and week 6 after the latency period was not significantly different than any other week. The mean number of wrong turns made by conditioned, 1.9, and unconditioned, 1.76, individuals was virtually identical and there was no significant difference (df = 33.644, t=0.281, p=0.780) between the groups.

T-maze

In the T-maze experiment there were no significant differences in either the mean completion times (Figure 5) or the mean numbers of wrong turns (Figure 6). The starting completion time mean for the conditioned group was 831.62 sec and by week 6 was 397.44 sec compared to the unconditioned 834 sec; the starting wrong turn mean for the conditioned group was 1.62 and by week 6 was 1.67 compared to the unconditioned 2.25. For completion times, a repeated-measures ANOVA, with Greenhouse-Geisser correction, showed no significant differences between weeks (df = 4, F = 1.115, p = 0.353). After the latency period, the mean completion time of the conditioned group, 397.44 sec, dropped substantially, and was about half that of the unconditioned individuals, 834 sec, but there was no significant difference between the conditioned and control groups however (df = 26.853, t=-1.674, p=0.106).

The repeated-measures ANOVA also showed no significant differences between weeks in the number of wrong turns ($df = 4$, $F = 1.611$; $p = 0.195$). The number of wrong turns in conditioned individuals, 1.67, was slightly less than that of the control group, 2.25, but there was no significant difference ($df = 21.180$, $t=-1.146$, $p=0.265$).

CHAPTER V: DISCUSSION

There was some marginal difference in completion times with the most obvious being between weeks 2 and 3 with mean times in week 3 being significantly lower than those in week 2. Overall, it was not enough to indicate retention over time. Mean numbers of wrong turns in the multiple-turn maze experiment did trend down slightly over time, but the mean number of wrong turns made by control (unconditioned) crayfish was slightly, if not significantly, less. The unconditioned crayfish also outperformed the conditioned crayfish in mean completion times in the multiple-turn maze although there was no significant difference between the groups.

There was no notable change in completion time or number of wrong turns from week to week in the consecutive T-maze experiment, however, after the latency period the mean completion times of the conditioned crayfish were less than during the preceding weeks and mean completion time for the control crayfish was more than twice that of the conditioned group. The mean numbers of wrong turns made by the control crayfish was slightly more than, but not significantly different from, those made by the conditioned crayfish.

In the Australian crayfish, individuals with lesions on their antennae did not respond to spatial changes within their environment (Basil & Sandeman, 2001). In the present study many of the crayfish, particularly in those in Cohort 2 which had very poor survival in both treatment groups, arrived with wounds to their carapaces, legs, and antennae. The condition of some of the animals may have contributed greatly to the observed variability and lack of clear evidence of spatial learning.

Red swamp crayfish exhibit two common behaviors associated with movement, a wandering phase consisting of short bursts of high-speed movement and a stationary phase where they remain in their burrow venturing out only after dark to forage; they are also primarily

nocturnal (Gherardi et al., 2000). Australian crayfish often exhibited wall-hugging behavior during exploration. Wall-hugging behavior was also observed during this study, with individuals taking wrong turns which fell along the wall they were following. Many of the crayfish in this study seemed to prefer whichever side of the maze cast a shadow, even crossing over from one wall to another if the other wall cast a shadow. Wall hugging is seen in other decapod crustaceans and is associated with thigmotaxis or predator avoidance behavior during which an animal would seek cover or shelter rather than expose itself to an open area (Burrows et al., 1999). Wall hugging produced at least two wrong turns every time a crayfish ran the maze in this experiment. The mean number of wrong turns was fairly constant (~2) regardless of maze type but ranged as high as 4 or 5. The wrong turns produced by wall-hugging behavior may be at least partially responsible for the variability of mean completion time since wrong turns would be expected to increase completion times.

Crayfish in this study also had a tendency to backtrack as they worked their way through the maze. In some cases, they would get nearly to the end where a food reward was waiting only to turn around and retrace their steps all the way to the beginning and start again. While little is known about backtracking, studies have shown that it is an important behavior in navigation and spatial orientation (Javadi et al., 2019). Other arthropods such as ants, when moved from one location to an unfamiliar location, backtrack to reorient themselves (Wystrach et al., 2013). Crayfish, which can be easily displaced by currents or other water movements could have a similar strategy for getting their bearings.

There were other observations which could have played a role in maze performance. During weeks when crayfish molted, their performance in mazes typically suffered. In some cases, molted individuals would not even leave the start chamber within the hour they were

allotted to complete the maze. Wild crayfish usually hide after molting until the new exoskeleton hardens; molting also requires a lot of energy (Su et al., 2021). Even though crayfish wet weights remained relatively constant over the experimental period, crayfish may have performed poorly until the shell hardened, which takes 1-4 days. Over the course of the experiment almost every crayfish in every cohort molted at least once.

It is also possible that practice runs were spaced too far apart for long term memory to have been able to form. Long term memory can last anywhere from hours to years to a life time (Markowitsch, 2013). Studies on the Australian crayfish, *Cherax destructor*, indicate that they maintain memory of other members of their species once introduced for at least 24 hours, with their behavior still being influenced for up to two weeks (Van der Velden, 2008). It is possible that even if some long-term memory formation had taken place during the learning period of the mazes, that a significant portion of what was learned could have been forgotten before the next trial.

One key difference between the crayfish used in this research and other similar studies was that the crayfish used were purchased instead of wild caught. Wild-caught crayfish live in an ever-changing environment where they must fend for themselves, avoid predation, and find their own food. Farmed crayfish live in stable pond environments and although supplemental feeds are not typically provided (Fletcher, 2022) submerged vegetation to provide the basis of a food web is encouraged, predators are of less concern, and the brood stock may be more genetically homogenous. Environmental predictability is thought to play a role in whether or not the ability to learn confers greater fitness. Learning may be costly and without clear benefits in an environment where conditions are very predictable (Hollis & Guillette, 2015). A study of fruit flies (*Drosophilia*) demonstrated that if the reliability of environmental cues is greater than the

reliability of a fixed response, learning can emerge over successive generations (Dunlap & Stephens, 2009). On the opposite end of the spectrum, it was also demonstrated that when a fixed pattern becomes more reliable, the capacity for learning is lost (Dunlap & Stephens, 2009). The lack of clear evidence of spatial learning in this study of non-wild crayfish may be because after generations of captive breeding in a highly predictable environment the ability to learn and/or the benefit of learning is much less than the "cost" and does not confer greater fitness.

Recommendations for Future Studies

- Crayfish should be kept on a 12:12 day:night light cycle while being housed in the lab.
- Crayfish should be sexed.
- Initial condition of crayfish, whether purchased or caught, should be assessed in more detail and wounded or damaged crayfish should be excluded from experiments.
- This study was conducted in a laboratory setting under artificial white light. Using a red light or covering the mazes with an opaque cloth could provide a more natural environment and might reduce wall-hugging behavior.
- Since molting is unpredictable it would not be practical to completely remove molting/recently molted individuals from the experiment. However, ensuring that sufficient nutrients (e.g., calcium) for shell hardening are always available, could reduce variability in performance in mazes that might be due to molting. In addition, recently molted crayfish could be skipped during the week they molted and return to the experiment the next week.
- Conducting experiments comparing farmed and wild-caught crayfish could determine if there is evidence of a difference in learning capacity.

- After crayfish complete the T-maze and have them run the multiple turn maze once more to see if there is evidence of long-term memory.

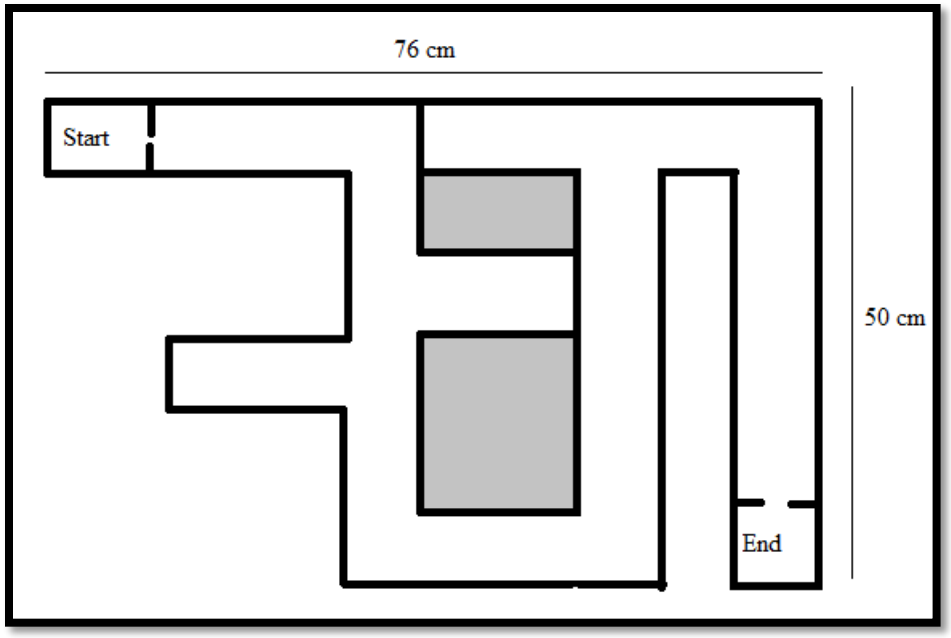

Figure 1. Multiple-turn maze after Davies et al., (2019).

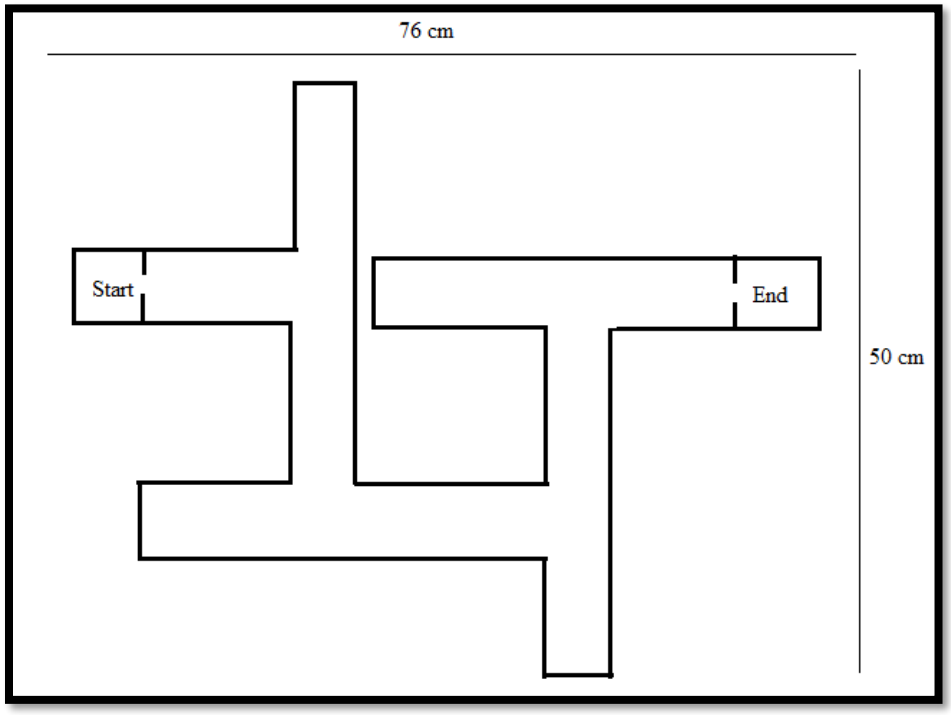

Figure 2. Multiple (consecutive) T-maze.

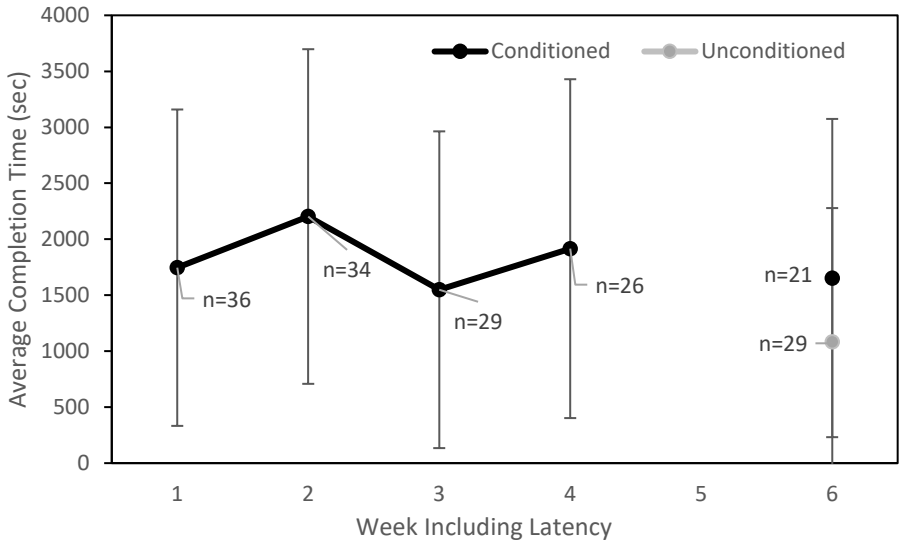

Figure 3. Mean completion times with standard deviation of the combined cohorts in the multiple-turn maze experiment by week. Week 5 is the latency period for the conditioned crayfish when the maze was not run. During Week 6 the unconditioned crayfish were also allowed to run the maze.

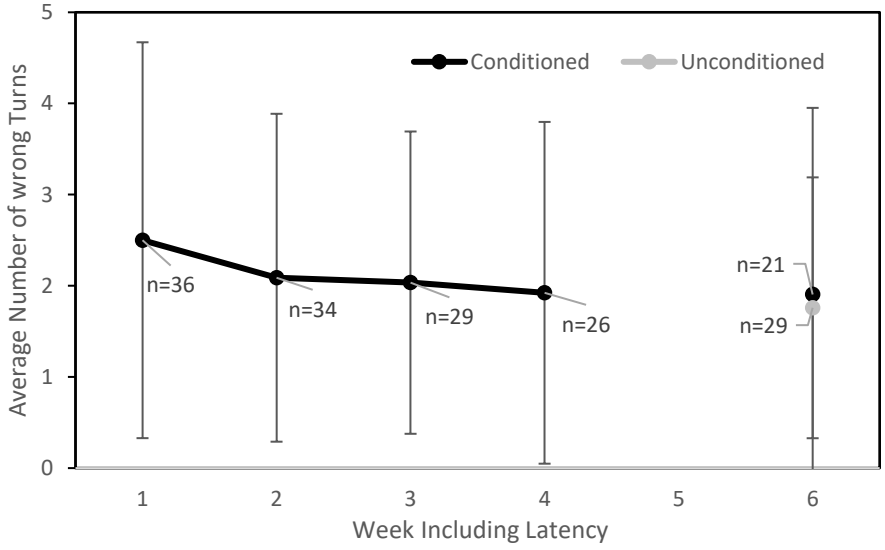

Figure 4. Mean numbers of wrong turns with standard deviation of the combined cohorts in the multiple-turn maze experiment by week. Week 5 is the latency period for the conditioned crayfish when the maze was not run. During Week 6, the unconditioned crayfish were also allowed to run the maze.

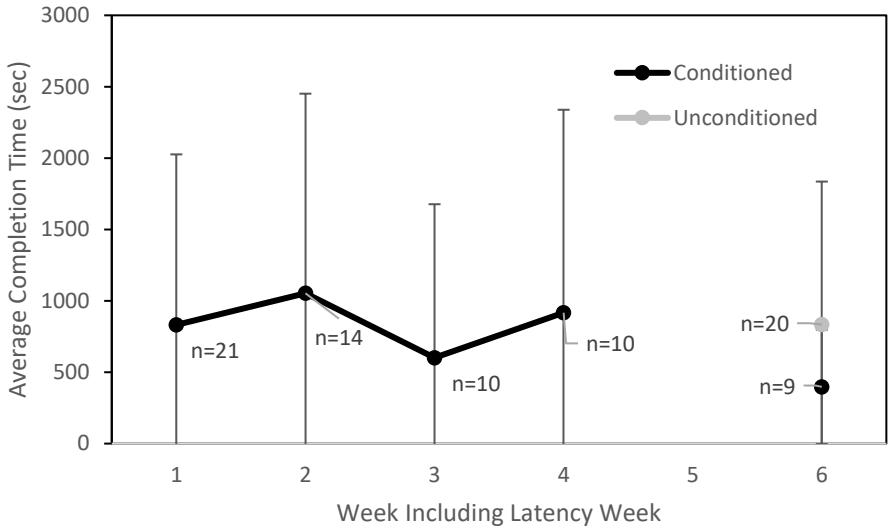

Figure 5. Mean completion times with standard by week of crayfish that survived the multiple-turn maze experiment and went on to the consecutive T-maze experiment. Week 5 was the latency period for the conditioned crayfish when the maze was not run. During Week 6 the unconditioned crayfish were also allowed to run the maze.

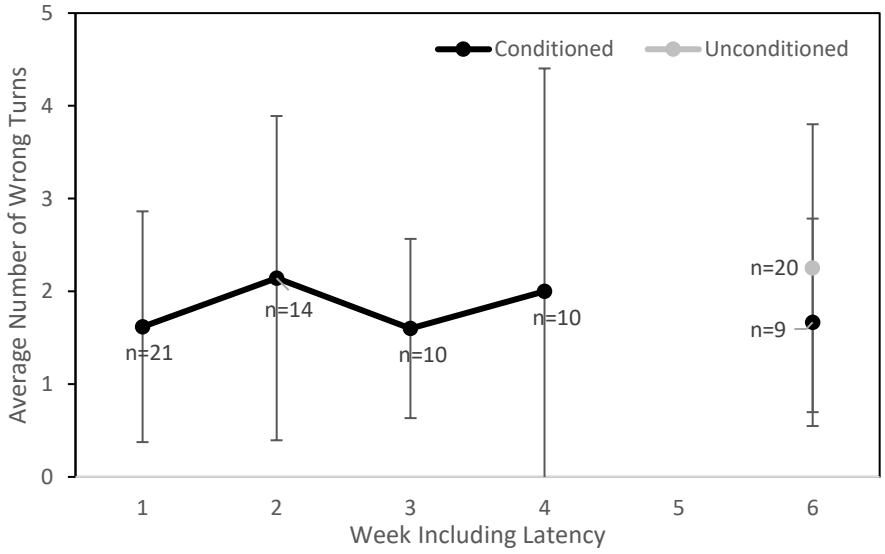

Figure 6. Mean numbers of wrong turns with standard deviation by week of crayfish that survived the multiple-turn maze experiment and went on to the consecutive T-maze experiment. Week 5 was the latency period for the conditioned crayfish when the maze was not run. During Week 6, the unconditioned crayfish were also allowed to run the maze.

REFERENCES

Agranoff, B. W., Cotman, C. W., & Uhler, M. D. (1999). *Invertebrate learning and memory - basic neurochemistry - NCBI bookshelf*. National Library of Medicine. Retrieved August 3, 2022, from https://www.ncbi.nlm.nih.gov/books/NBK28212/

Alves, C., Boal, J. G., & Dickel, L. (2007). Short-distance navigation in cephalopods: A review and synthesis. Cognitive Processing, 9(4), 239–247. https://doi.org/10.1007/s10339-007-0192-9

Basil, J., & Crook, R. (2021). Learning and memory in the living fossil, Chambered Nautilus. Physiology of Molluscs, 103–136. https://doi.org/10.1201/9781315207117-4

Basil, J., & Sandeman, D. (2001). Crayfish (*Cherax destructor*) use tactile cues to detect and learn topographical changes in their environment. Ethology, 106(3), 247–259. https://doi.org/10.1046/j.1439-0310.2000.00524.x

Berman, H. (2022). Sphericity and Repeated Measures ANOVA. Sphericity in ANOVA. Retrieved November 21, 2022, from https://stattrek.com/anova/repeated-measures/sphericity

Bittel, J. (2019, July 11). The unassuming crayfish-and its path of devastation. NRDC. Retrieved August 4, 2022, from https://www.nrdc.org/onearth/unassuming-crayfish-and-its-path-devastation

Boal J. G., Dunham A. W., Williams K. T., & Hanlon R. T. (2000). Experimental evidence for spatial learning in octopuses (*Octopus bimaculoides*). *J. Comp. Psychol. 114, 246–252*. (doi:10.1037/0735-7036.114.3.246)

Boles LC, & Lohmann KJ. (2003). True navigation and magnetic map in spiny lobsters. *Nature* 421, 60–63. (doi:10.1038/nature01333.1)

Bukowski-Thall, G. (2020, September 21). Crabs come out of their shells in the research world. Future Frogmen. Retrieved August 3, 2022, from https://www.futurefrogmen.org/blog/2020/9/21/crabs-come-out-of-their-shells-in-the-research-world

Burgess, N., Maguire, E. A., & O'Keefe, J. (2002). The human hippocampus and spatial and episodic memory. *Neuron, 35*(4), 625–641. https://doi.org/10.1016/s0896-6273(02)00830-9

Burrows MT, & Kawai K, Hughes RN. 1999 Foraging by mobile predators on a rocky shore: underwater TV observations of movements of blennies *Lipophrys pholis* and crabs *Carcinus maenas*. Mar. Ecol. Prog. Ser. 187, 237–250. (doi:10.3354/meps187237)

Cartwright, B.A., & Collett, T.S. Landmark learning in bees. *J. Comp. Physiol.* **151,** 521–543 (1983). https://doi.org/10.1007/BF00605469

Cherry, K. (2022). What is cognition? Verywell Mind. Retrieved November 16, 2022, from https://www.verywellmind.com/what-is-cognition-2794982

Collett M. (2009). Spatial memories in insects. Curr. *Biol.* 19, 1103–1108. (doi:10.1016/j.cub.2009.10.004)

Cronin, T. W. (1986). Optical Design and Evolutionary Adaptation in Crustacean Compound Eyes. Journal of Crustacean Biology, 6(1), 1–23. https://doi.org/10.2307/1547926

Crook, R.J. and Basil, J.A. (2013), Flexible Spatial Orientation and Navigational Strategies in Chambered Nautilus. Ethology, 119: 77-85. https://doi.org/10.1111/eth.12040

Darmaillacq, A.-S., Dickel, L., & Mather, J. A. (2014). *Cephalopod cognition*. Cambridge University Press.

Davies, R., Gagen, M. H., Bull, J. C., & Pope, E. C. (2019) Maze learning and memory in a decapod crustacean. *Biology Letters*, 15(10), 20190407. doi: 10.1098/rsbl.2019.0407

Dean Delphine JoVE Science Education Database. (2019). Behavioral science. Spatial memory testing using mazes. JoVE, Cambridge, MA.

Deryckere, A., & Seuntjens, E. (2018). The cephalopod large brain enigma: Are conserved mechanisms of stem cell expansion the key? Frontiers in Physiology, 9. https://doi.org/10.3389/fphys.2018.01160

D'Hooge, R., & De Deyn, P. P. (2001). Applications of the Morris Water Maze in the study of learning and memory. Brain Research Reviews, 36(1), 60–90. https://doi.org/10.1016/s0165-0173(01)00067-4

Donald H. Edwards, William J. Heitler & Franklin B. Krasne. (1999). Fifty years of a command neuron: the neurobiology of escape behavior in the crayfish. *Trends Neurosci*. (1999) 22, 153–161.

Donohue et al. (2022). Fiddler crabs are unique in timing their escape responses based on speed-dependent visual cues, Current Biology, https://doi.org/10.1016/j.cub.2022.10.013

Dunlap, A., & Stephens, D. W. (2009). Components of change in the evolution of learning and unlearned preference. Proceedings of the Royal Society B, 276, 3201-3208. doi:10.1098/rspb.2009.0602

Fletcher R. (2022). Crawfish Biology. The Fish Site Retrieved November 16, 2022, from https://thefishsite.com/articles/crawfish-biology

Floresco S. B. (2014). Spatial learning in animals. In I. Stolerman, Price L. (Eds), *Encyclopedia of psychopharmacology* (pp. 310-332). Berlin: Springer.

Gherardi, F., Barbaresi, S., & Salvi, G. (2000). Spatial and temporal patterns in the movement of *procambarus clarkii*, an invasive crayfish. Aquatic Sciences, 62(2), 179–193. https://doi.org/10.1007/pl00001330

Gordon, I. & Green,. James (2022). crustacean. Encyclopedia Britannica. https://www.britannica.com/animal/crustacean

Guy-Evans, O. (2021). *Hippocampus function and location*. Hippocampus Function and Location | Simply Psychology. Retrieved June 25, 2022, from https://www.simplypsychology.org/hippocampus.html#:~:text=The%20rear%20part%20of%20the%20hippocampus%20is%20believed,containing%20information%20on%20relative%20locations%20in%20specific%20environments.

Hammer, M., & Menzel, R. (1995). Learning and memory in the Honeybee. *The Journal of Neuroscience*, *15*(3), 1617–1630. https://doi.org/10.1523/jneurosci.15-03-01617.1995

Harvard Health. (2022). Retrieved June 25, 2022, from https://www.health.harvard.edu/topics/memory

Hazlett, B. A., Acquistapace, P., & Gherardi, F. (2002). Differences in Memory Capabilities in Invasive and Native Crayfish. Journal of Crustacean Biology, 22(2), 439–448. http://www.jstor.org/stable/1549968

Heijningen, S. van. (2022). *5 proven ways to measure spatial learning in rodents*. Noldus. Retrieved June 25, 2022, from https://www.noldus.com/blog/5-ways-to-measure-spatial-learning-rodents#:~:text=Spatial%20learning%20basically%20refers%20to%20the%20association%20or,cues%20and%2For%20landmarks%2C%20whether%20in%20humans%20or%20animals.

Heinrich, B. (1984). Learning in invertebrates. *The Biology of Learning*, 135–147. https://doi.org/10.1007/978-3-642-70094-1_7

Helfrich, L. A. & DiStefano, R. J. (2020, March 24). Sustaining America's aquatic biodiversity - crayfish biodiversity and conservation. VCE Publications | Virginia Tech. Retrieved August 4, 2022, from https://www.pubs.ext.vt.edu/420/420-524/420-524.html

Hollis, K. L. & Guillette, L. M. (2015). What associative learning in insects tells us about the evolution of learned and fixed behavior. International Journal of Comparative Psychology, 28. https://doi.org/10.46867/ijcp.2015.28.01.07

Jackson, C., & van Staaden, M. (2019). Characterization of locomotor response to psychostimulants in the parthenogenetic marbled crayfish (*Procambarus fallax forma virginalis*): A promising model for studying the neural and molecular mechanisms of drug addiction. Behavioural brain research, 361, 131–138. https://doi.org/10.1016/j.bbr.2018.12.024

Javadi, A.-H., Patai, E. Z., Marin-Garcia, E., Margois, A., Tan, H.-R. M., Kumaran, D., Nardini, M., Penny, W., Duzel, E., Dayan, P., & Spiers, H. J. (2019). Backtracking during navigation is correlated with enhanced anterior cingulate activity and suppression of alpha oscillations and the 'default-mode' network. Proceedings of the Royal Society B: Biological Sciences, 286(1908), 20191016. https://doi.org/10.1098/rspb.2019.1016

Karson, M. A., Boal, J. G., & Hanlon, R. T. (2003). Experimental evidence for spatial learning in cuttlefish *(Sepia officinalis*). Journal of Comparative Psychology, 117(2), 149–155. https://doi.org/10.1037/0735-7036.117.2.149

Kastner, D. B., Miller, E. A., Yang, Z., Roumis, D. K., Liu, D. F., Frank, L. M., & Dayan, P. (2022). Spatial preferences account for inter-animal variability during the continual

learning of a dynamic cognitive task. *Cell Reports*, *39*(3), 110708. https://doi.org/10.1016/j.celrep.2022.110708

Kempsell AT, Fieber LA (2015) Aging in Sensory and Motor Neurons Results in Learning Failure in *Aplysia californica*. PLoS ONE 10(5): e0127056. https://doi.org/10.1371/journal.pone.0127056

Kenning, M., Lehmann, P., Lindström, M., & Harzsch, S. (2015). Heading which way? Y-maze chemical assays: Not all crustaceans are alike. Helgoland Marine Research, 69(3), 305–311. https://doi.org/10.1007/s10152-015-0435-6

Kesner, R. P., Olton, D. S., & Byrne, J. H. (1990). Learning and Memory in *Aplysia* and Other Invertebrates. In *Neurobiology of Comparative Cognition* (1st ed.). essay, L. Erlbaum Associates.

Kevan, P. G., & Menzel, R. (2012). The plight of pollination and the interface of neurobiology, ecology and food security. *The Environmentalist*, *32*(3), 300–310. https://doi.org/10.1007/s10669-012-9394-5

Kimble G. A., Hilgard E. R, & Marquis D. G. (1961). The definition of learning (chapter 1) In: Kimble G. A. (Ed.). *Hilgard and Marquis' conditioning and learning*. Appleton-Century-Crofts, New York, pp. (1–3).

Kosaki, Y., Lin, T.-C. E., Horne, M. R., Pearce, J. M., & Gilroy, K. E. (2014). The role of the hippocampus in passive and active spatial learning. *Hippocampus*, *24*(12), 1633–1652. https://doi.org/10.1002/hipo.22343

Layne, J. E., Barnes, W. J., & Duncan, L. M. (2003). Mechanisms of homing in the fiddler crab *Uca rapax* 1. spatial and temporal characteristics of a system of small-scale

navigation. Journal of Experimental Biology, 206(24), 4413–4423. https://doi.org/10.1242/jeb.00660

Layne, J. E., Barnes, W. J., & Duncan, L. M. (2003). Mechanisms of homing in the fiddler crab *Uca rapax* 2. information sources and frame of reference for a path integration system. Journal of Experimental Biology, 206(24), 4425–4442. https://doi.org/10.1242/jeb.00661

Maldonado, H. (2002). Crustaceans as models to investigate memory illustrated by extensive behavioral and physiological studies in *Chasmagnathus*. The Crustacean Nervous System, 314–327. https://doi.org/10.1007/978-3-662-04843-6_24

Markowitsch, H. J. (2013). Memory and self–neuroscientific landscapes. ISRN Neuroscience, 2013, 1–26. https://doi.org/10.1155/2013/176027

Mather, J. (2020). Why are octopuses going to be the 'poster child' for invertebrate welfare? *Journal of Applied Animal Welfare Science*, *25*(1), 31–40. https://doi.org/10.1080/10888705.2020.1829488

McConnell, J. V. (1966). Comparative physiology: Learning in Invertebrates. *Annual Review of Physiology*, *28*(1), 107–136. https://doi.org/10.1146/annurev.ph.28.030166.000543

Menzel, R., & Benjamin, P. R. (2013). Beyond the cellular alphabet of learning and memory in invertebrates. Invertebrate Learning and Memory, 3–5. https://doi.org/10.1016/b978-0-12-415823-8.00001-0

Menzel, R., & Erber, J. (1978). Learning and Memory in Bees. *Scientific American*, *239*(1), 102–111. http://www.jstor.org/stable/24955780

Menzel, R. (1993). Associative learning in Honey Bees. *Apidologie*, *24*(3), 157–168. https://doi.org/10.1051/apido:19930301

Mery, F. (2013). Natural variation in learning and memory. Current Opinion in Neurobiology, 23(1), 52–56. https://doi.org/10.1016/j.conb.2012.09.001

Michaelis, B. T., Leathers, K. W., Bobkov, Y. V., Ache, B. W., Principe, J. C., Baharloo, R., Park, I. M., & Reidenbach, M. A. (2020). Odor tracking in aquatic organisms: the importance of temporal and spatial intermittency of the turbulent plume. Scientific reports, 10(1), 7961. https://doi.org/10.1038/s41598-020-64766-y

Moroz LL. (2011). *Aplysia*. Curr Biol;21(2):R60-1. doi: 10.1016/j.cub.2010.11.028. PMID: 21256433; PMCID: PMC4024469.

Muth, F. (2013). Lost ants backtrack their steps. Scientific American Blog Network. Retrieved July 25, 2022, from https://blogs.scientificamerican.com/not-bad-science/lost-ants-backtrack-their-steps/

Nouvian, M., & Galizia, C. G. (2019). Aversive training of honey bees in an automated Y-Maze. *Frontiers in Physiology*, *10*. https://doi.org/10.3389/fphys.2019.00678

Nunez J. (2008). Morris Water Maze Experiment. Journal of visualized experiments : JoVE, (19), 897. https://doi.org/10.3791/897

Prentice, P. M., Mnatzaganian, C., Houslay, T. M., Thornton, A., & Wilson, A. J. (2022). Individual differences in spatial learning are correlated across tasks but not with stress response behaviour in guppies. *Animal Behaviour*, *188*, 133–146. https://doi.org/10.1016/j.anbehav.2022.04.009

Perry C. J., Barron A. B., & Cheng K. (2013) Invertebrate learning and cognition: relating phenomena to neural substrate. *WIREs Cogn. Sci*. 4, 561–582. (doi:10.1002/wcs.1248)

Rankin, C. H., & Carew, T. J. (1987). Development of learning and memory in *Aplysia*. II. habituation and dishabituation. *The Journal of Neuroscience*, *7*(1), 133–143. https://doi.org/10.1523/jneurosci.07-01-00133.1987

Savage, S., & Ma, D. (2014). III. animal behaviour testing: Memory. *British Journal of Anaesthesia*, *113*(1), 6–9. https://doi.org/10.1093/bja/aeu014

Schnaitmann, C. (2010). Appetitive and aversive visual learning in freely moving *drosophila*. *Frontiers in Behavioral Neuroscience*, *4*. https://doi.org/10.3389/fnbeh.2010.00010

Shigeno, S., Andrews, P. L., Ponte, G., & Fiorito, G. (2018). Cephalopod Brains: An overview of current knowledge to facilitate comparison with vertebrates. Frontiers in Physiology, 9. https://doi.org/10.3389/fphys.2018.00952

Shettleworth, S. J. (2010). Cognition, evolution, and behavior (2nd ed.). Oxford University Press https://books.google.com/books/about/Cognition_Evolution_and_ Behavior.html?id¼-Qs1qGys0AwC

Stark, C. (2010). Declarative memory. *Encyclopedia of Behavioral Neuroscience*, 370–375. https://doi.org/10.1016/b978-0-08-045396-5.00133-0

Su, S., Munganga, B. P., Tian, C., Li, J. L., Yu, F., Li, H., Wang, M., He, X., & Tang, Y. (2021). Comparative analysis of the Intermolt and Postmolt hepatopancreas transcriptomes provides insight into the mechanisms of *Procambarus clarkii* molting process. Life, 11(6), 480. https://doi.org/10.3390/life11060480

Thorndike, E. L. (1999). *Education psychology briefer course*. Routledge.

Tierney, A. J., Baker, A., Forward, J., Slight, C., & Yilma, H. (2018). Response and place learning in crayfish spatial behavior. Learning & Behavior, 47(1), 80–90. https://doi.org/10.3758/s13420-018-0345-y

Tomsic, D., & Romano, A. (2013). A multidisciplinary approach to learning and memory in the Crab *Neohelice* (*Chasmagnathus*) *granulata*. Invertebrate Learning and Memory, 337–355. https://doi.org/10.1016/b978-0-12-415823-8.00026-5

Turchetti-Maia, Ana, Tal Shomrat, & Binyamin Hochner, (2019). 'The Vertical Lobe of Cephalopods: A Brain Structure Ideal for Exploring the Mechanisms of Complex Forms of Learning and Memory', in John H. Byrne (ed.), The Oxford Handbook of Invertebrate Neurobiology, Oxford Handbooks https://doi.org/10.1093/oxfordhb/9780190456757.013.29, accessed 2 Aug. 2022.

Sanchez Raymond University of Arizona. (2014). Of bugs and brains: Striking similarities in brain structures across invertebrates. ScienceDaily. Retrieved October 11, 2022 from www.sciencedaily.com/releases/2014/12/141218131935.htm

University of Maryland. (2010). Crayfish brain may offer rare insight into human decision making. ScienceDaily. Retrieved August 1, 2022 from www.sciencedaily.com/releases/2010/06/100615191751.htm

Van der Velden, J., Zheng, Y., Patullo, B. W., & Macmillan, D. L. (2008). Crayfish recognize the faces of fight opponents. PloS one, 3(2), e1695. https://doi.org/10.1371/journal.pone.0001695

Van der Zee, E. M., Angelini, C., Govers, L. L., Christianen, M. J., Altieri, A. H., van der Reijden, K. J., Silliman, B. R., van de Koppel, J., van der Geest, M., van Gils, J. A., van der Veer, H. W., Piersma, T., de Ruiter, P. C., Olff, H., & van der Heide, T. (2016). How habitat-modifying organisms structure the food web of two coastal ecosystems. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1826), 20152326. https://doi.org/10.1098/rspb.2015.2326

Vannini M, Cannicci S. (1995). Homing behaviour and possible cognitive maps in crustacean decapods. J. Exp. Mar. Bio. Ecol. 193, 67–91. (doi:10.1016/0022-0981(95)00111-5)

Vorhees, C. V., & Williams, M. T. (2014). Assessing spatial learning and memory in rodents. ILAR journal, 55(2), 310–332. https://doi.org/10.1093/ilar/ilu013

Vorhees, C. V., & Williams, M. T. (2006). Morris water maze: Procedures for assessing spatial and related forms of learning and memory. *Nature Protocols*, *1*(2), 848–858. https://doi.org/10.1038/nprot.2006.116

Walters, E. T., Carew, T. J., & Kandel, E. R. (1981). Associative learning in *Aplysia* : Evidence for conditioned fear in an invertebrate. *Science*, *211*(4481), 504–506. https://doi.org/10.1126/science.7192881

Weis, J. S. (2010). The role of behavior in the success of invasive crustaceans. Marine and Freshwater Behaviour and Physiology, 43(2), 83–98. https://doi.org/10.1080/10236244.2010.480838

Wheeler, C. (n.d.). Take online courses. earn college credit. Research Schools, Degrees & Careers. Study.com | Take Online Courses. Earn College Credit. Research Schools, Degrees & Careers. Retrieved November 16, 2022, from https://study.com/learn/lesson/crayfish-anatomy-habitat-characteristics.html

William, K. (2022,). What is SPSS? definition, features, types, and use cases. SurveySparrow. Retrieved November 21, 2022, from https://surveysparrow.com/blog/what-is-spss/#:~:text=SPSS%20is%20popular%20because%20of,it%20for%20analyzing%20survey%20data.

Wood, T. C. & Moore, P. A. (2020). Big and bad: how relative predator size and dietary

information influence rusty crayfish (*Faxonius rusticus*) behavior and resource-use decisions. Can. J. Zool. 98, 62-72. https://doi.org/10.1139/cjz-2019-0089

Wystrach, A., Schwarz, S., Baniel, A., & Cheng, K. (2013). Backtracking behaviour in lost ants: An additional strategy in their navigational toolkit. Proceedings of the Royal Society B: Biological Sciences, 280(1769), 20131677. https://doi.org/10.1098/rspb.2013.1677

APPENDIX 1

CRAYFISH CONDITION TABLES

Appendix 1.1. Condition over time of crayfish in Cohort 1 of the multiple-turn maze. Conditioned crayfish are numbered 1M1-1M12; control crayfish are numbered 1C1-1C12. Mortality of an individual is indicated by a "0" in the wet weight column. Week 5 is the latency week. Sexes were not determined in Cohort 1. Carapace length was measured only at the beginning of the experiment. Whether or not the individual went on to the T-maze experiment is indicated by "yes" or "no" in the T-Maze column. Individuals that went on to the T-maze retained their original number.

Crayfish	Sex	Carapace Length (cm)	Wet Weight (g)							T-Maze?
			Initial	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
1M1	nd	2.5	8	8	0	0	0	0	0	No
1M2	nd	2	5	5	6	6	6	6	6	Yes
1M3	nd	2.3	6	6	7	7	7	6	7	Yes
1M4	nd	2.3	6	6	6	6	7	7	0	No
1M5	nd	2.1	6	6	6	6	7	7	7	Yes
1M6	nd	2.7	9	9	10	9	10	10	10	Yes
1M7	nd	2	4	4	6	6	6	0	0	No
1M8	nd	2.5	8	8	7	9	9	9	9	Yes
1M9	nd	2.8	14	14	15	0	0	0	0	No
1M10	nd	1.9	5	5	5	6	6	6	0	No
1M11	nd	2.4	7	7	9	8	8	9	9	Yes
1M12	nd	1.7	3	3	4	5	4	4	0	No
1C1	nd	2.2	6	6	7	6	7	7	7	Yes
1C2	nd	2.8	13	13	14	13	14	17	19	Yes
1C3	nd	2.5	9	9	13	14	13	14	14	Yes
1C4	nd	1.8	7	7	11	10	11	11	11	Yes
1C5	nd	2.4	11	11	12	15	15	15	15	Yes
1C6	nd	1.9	6	6	7	9	9	9	8	Yes
1C7	nd	2.7	11	11	12	14	16	15	16	Yes
1C8	nd	2.1	6	6	11	11	11	11	11	Yes
1C9	nd	2.4	10	10	11	14	15	15	14	Yes
1C10	nd	2.5	10	10	11	11	14	14	14	Yes
1C11	nd	2.1	6	9	9	11	11	12	12	Yes
1C12	nd	2.4	6	10	10	11	10	10	11	Yes

Appendix 1.2. Condition over time of crayfish in Cohort 2 of the multiple-turn maze. Conditioned crayfish are numbered 2M1-2M12; control crayfish are numbered 2C1-2C12. Mortality of an individual is indicated by a "0" in the wet weight column. Week 5 is the latency week. Carapace length was measured only at the beginning of the experiment. Whether or not the individual went on to the T-maze experiment is indicated by "yes" or "no" in the T-Maze column. Individuals that went on to the T-maze retained their original number.

Crayfish	Sex	Carapace Length (cm)	Injuries	Wet Weight (g)							T-Maze?
				Initial	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
2M1	M	2.5	Carapace wound, antennae lesion	9	9	9	10	11	10	11	Yes
2M2	M	2.4	Carapace wound	10	10	11	11	11	0	0	No
2M3	M	2.3		7	7	8	9	9	9	9	Yes
2M4	M	2.1		8	8	0	0	0	0	0	No
2M5	F	2.3		8	8	9	0	0	0	0	No
2M6	F	2.3		9	9	10	0	0	0	0	No
2M7	F	2.2		7	7	7	0	0	0	0	No
2M8	M	2.1	Carapace wound	7	7	8	8	9	9	9	Yes
2M9	F	2.1	Missing leg	7	7	8	8	0	0	0	No
2M10	F	2		6	6	8	0	0	0	0	No
2M11	F	2.2		7	7	8	7	8	0	0	No
2M12	M	2.2		8	8	9	8	9	8	9	Yes
2C1	M	2.4		8	8	10	10	10	10	11	Yes
2C2	F	2		4	4	6	7	7	7	6	Yes
2C3	F	2.3		8	8	0	0	0	0	0	No
2C4	M	2.5	Carapace wound	9	9	10	10	10	0	0	No
2C5	F	2		6	6	7	0	0	0	0	No
2C6	F	2		6	6	6	6	7	0	0	No
2C7	F	2		5	5	6	6	7	7	6	Yes
2C8	F	2		5	5	5	6	6	6	6	Yes
2C9	F	2		6	6	6	0	0	0	0	No
2C10	F	2.1		6	6	7	7	7	7	8	Yes
2C11	F	2		4	4	6	6	5	6	6	Yes
2C12	M	2		3	3	4	4	5	5	5	Yes

Appendix 1.3. Condition over time of crayfish in Cohort 3 of the multiple-turn maze. Conditioned crayfish are numbered 3M1-3M12; control crayfish are numbered 3C1-3C12. Mortality of an individual is indicated by a "0" in the wet weight column. Week 5 is the latency week. Carapace length was measured only at the beginning of the experiment. Whether or not the individual went on to the T-maze experiment is indicated by "yes" or "no" in the T-Maze column. Individuals that went on to the T-maze retained their original number.

Crayfish	Sex	Carapace Length (cm)	Wet Weight (g)							T-Maze?
			Initial	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
3M1	M	2	4	4	5	5	6	6	6	Yes
3M2	M	1.9	4	4	6	7	10	10	10	Yes
3M3	M	2.1	5	5	8	9	9	10	10	Yes
3M4	M	2.2	7	7	9	11	11	11	14	Yes
3M5	M	1.6	3	3	5	5	6	7	0	No
3M6	M	2	4	4	7	6	7	7	7	Yes
3M7	M	1.9	5	5	7	8	8	8	9	Yes
3M8	M	1.9	5	5	6	7	7	7	7	Yes
3M9	M	1.9	4	4	7	5	6	6	6	Yes
3M10	M	2	5	5	7	8	8	8	7	Yes
3M11	M	2	4	4	5	8	8	10	10	Yes
3M12	M	1.9	4	4	6	7	9	9	9	Yes
3C1	M	1.9	4	4	0	0	0	0	0	No
3C2	M	2	4	4	6	7	7	9	9	Yes
3C3	M	1.8	3	3	5	5	8	8	8	Yes
3C4	M	1.6	3	3	5	6	7	7	7	Yes
3C5	F	1.7	3	3	6	7	6	9	9	Yes
3C6	F	2	4	4	5	6	7	9	9	Yes
3C7	F	1.9	3	3	5	5	6	7	8	Yes
3C8	F	1.9	3	3	5	6	6	8	8	Yes
3C9	F	1.8	4	4	5	6	0	0	0	No
3C10	F	1.8	4	4	7	7	7	9	9	Yes
3C11	F	2	4	4	7	7	7	9	9	Yes
3C12	F	2.1	6	6	7	8	9	9	11	Yes

Appendix 1.4. Condition over time of crayfish in T-maze experiment. Crayfish retained their number from the multiple maze experiment and condition all crayfish individuals that went on to the T-maze experiment are summarized in this table. Mortality of an individual is indicated by a "0" in the wet weight column. Week 5 is the latency week.

Crayfish	Sex	Wet Weight (g)						
		Initial	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
1M2	nd	6	6	6	6	6	4	4
1M3	nd	7	7	7	7	7	6	6
1M5	nd	7	7	6	7	6	7	7
1M6	nd	10	10	10	0	0	0	0
1M8	nd	9	9	9	0	0	0	0
1M11	nd	9	9	9	0	0	0	0
2M1	M	11	11	11	10	11	11	11
2M3	M	9	9	9	9	9	0	0
2M8	M	9	9	9	9	9	8	8
2M12	M	9	9	9	0	0	0	0
3M1	M	6	6	0	0	0	0	0
3M2	M	10	10	0	0	0	0	0
3M3	M	10	10	0	0	0	0	0
3M4	M	14	14	0	0	0	0	0
3M6	M	7	7	0	0	0	0	0
3M7	M	9	9	9	9	9	9	9
3M8	M	7	7	0	0	0	0	0
3M9	M	6	6	0	0	0	0	0
3M10	M	7	7	8	8	7	8	8
3M11	M	10	10	10	10	10	10	10
3M12	M	9	9	9	9	9	9	9
1C1	nd	7	7	7	0	0	0	0
1C2	nd	19	19	19	0	0	0	0
1C3	nd	14	14	14	14	14	14	14
1C4	nd	11	11	10	10	12	11	11
1C5	nd	15	15	13	15	15	14	14
1C6	nd	8	8	9	9	9	9	9
1C7	nd	16	16	15	16	0	0	0
1C8	nd	11	11	11	11	10	11	11
1C9	nd	14	14	14	15	15	15	15
1C10	nd	14	14	14	0	0	0	0
1C11	nd	12	12	12	12	12	16	16
1C12	nd	11	11	10	10	10	10	10
2C1	M	11	11	11	0	0	0	0
2C2	F	6	6	7	8	8	8	8
2C7	F	6	6	7	6	8	8	8
2C8	F	6	6	0	0	0	0	0
2C10	F	8	8	9	9	10	10	10

Table 1.4. Continued.

Crayfish	Sex	Wet Weight (g)						
		Initial	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2C11	F	6	6	6	6	6	6	6
2C12	F	5	5	5	5	5	5	5
3C2	M	9	9	10	9	9	9	9
3C3	M	8	8	8	8	8	8	8
3C4	M	7	7	7	7	7	7	7
3C5	M	9	9	9	9	9	9	9
3C6	F	9	9	9	9	9	9	9
3C7	F	8	8	8	7	7	7	7
3C8	F	8	8	0	0	0	0	0
3C10	F	9	9	10	9	9	9	9
3C11	F	9	9	0	0	0	0	0
3C12	F	11	11	0	0	0	0	0

APPENDIX 2

GRAPHS: COMPLETION TIMES, NUMBER OF WRONG TURNS, COHORT BY WEEK

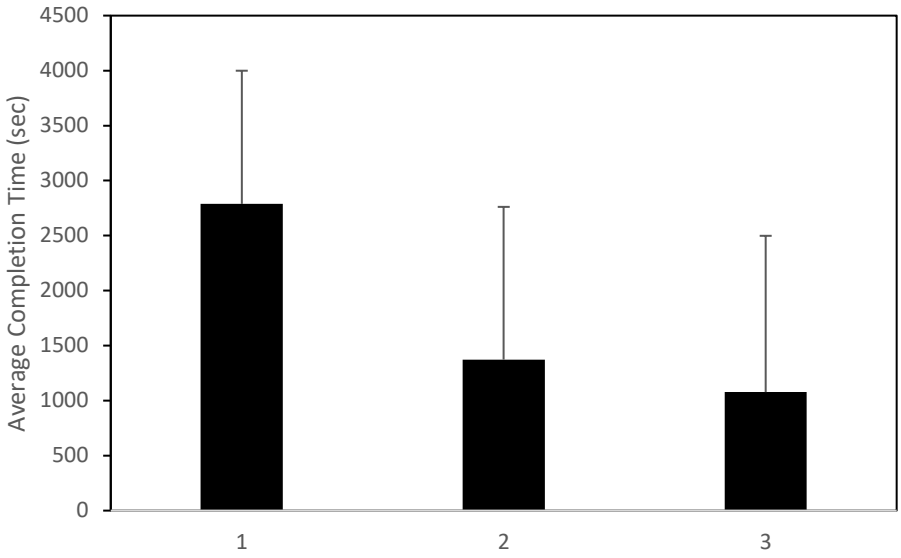

Figure 2.1. Week 1 average completion times for the multiple-turn maze with standard deviation by cohort.

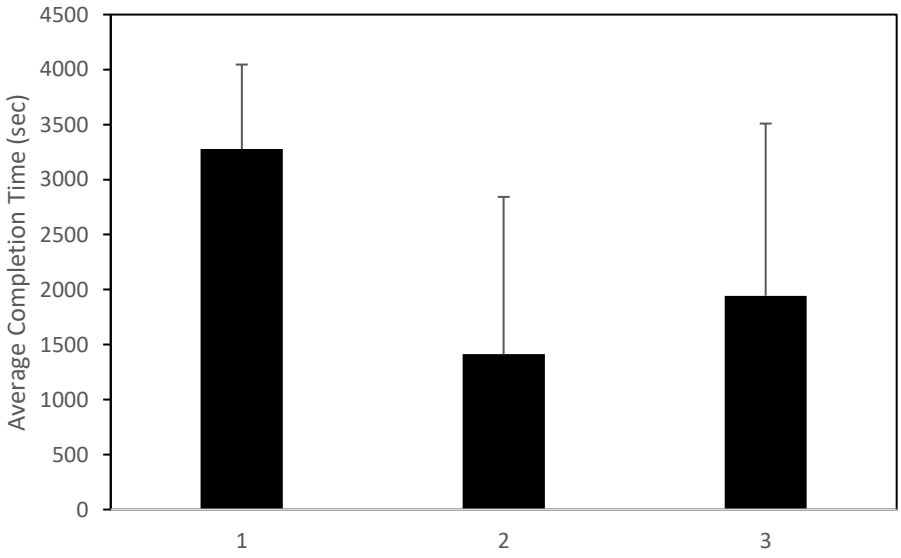

Figure 2.2. Week 2 average completion times for the multiple-turn maze with standard deviation by cohort.

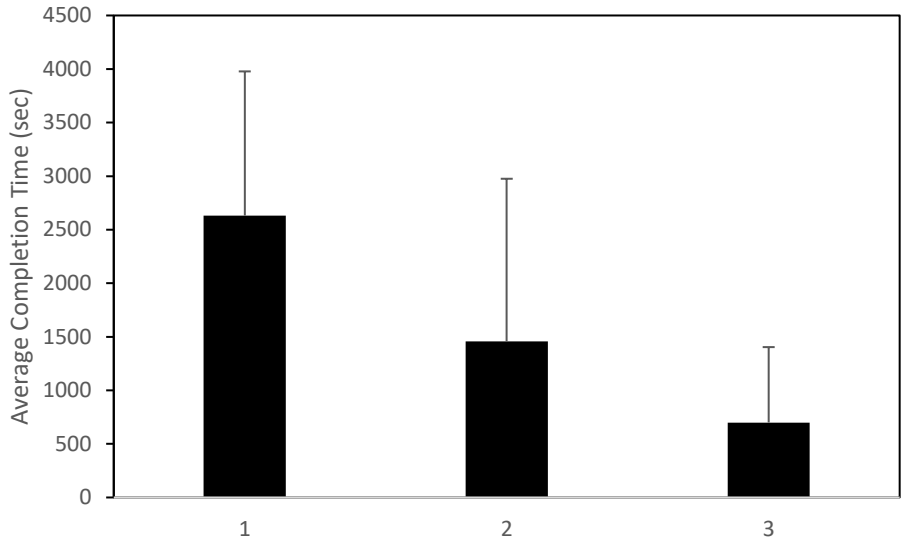

Figure 2.3. Week 3 average completion times for the multiple-turn maze with standard deviation by cohort.

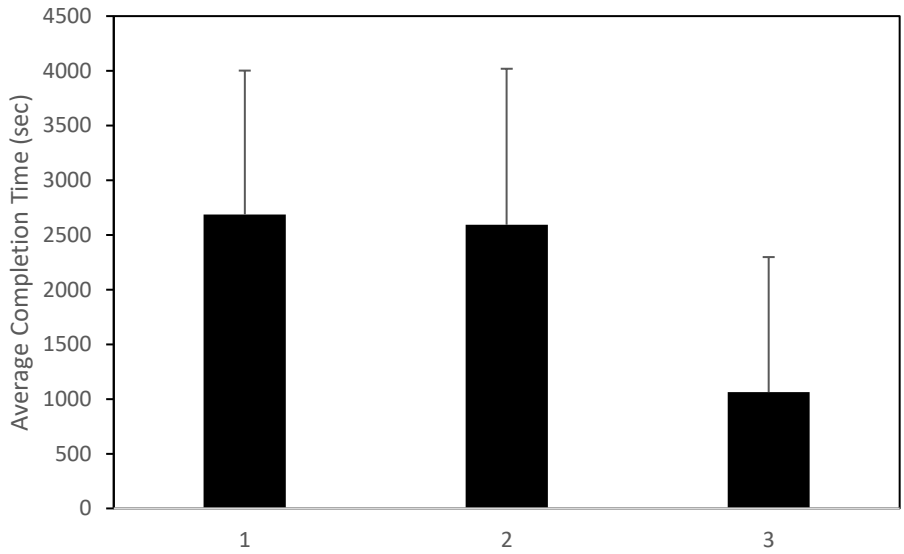

Figure 2.4. Week 4 average completion times for the multiple-turn maze with standard deviation by cohort.

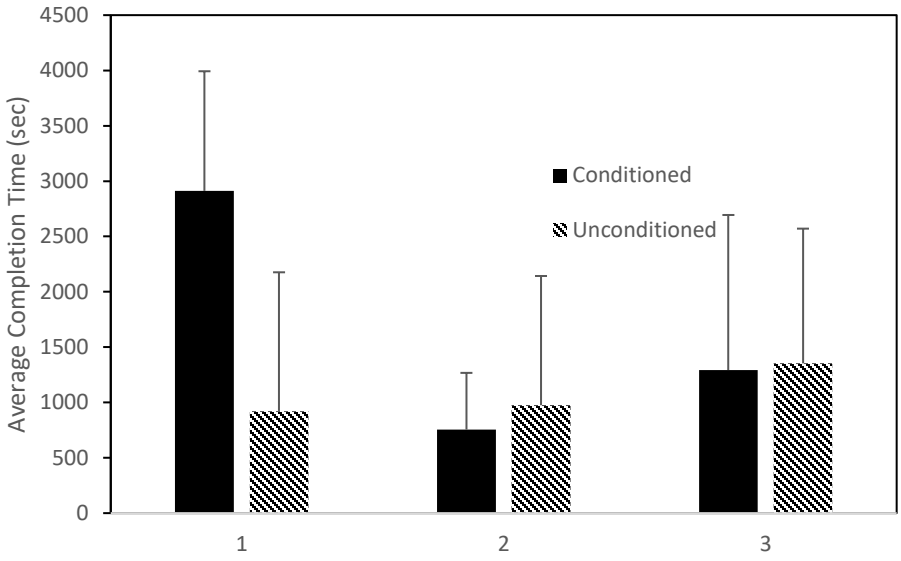

Figure 2.5. Completion times for the multiple-turn maze with standard deviation after the latency period (Week 5) by cohort and experimental group.

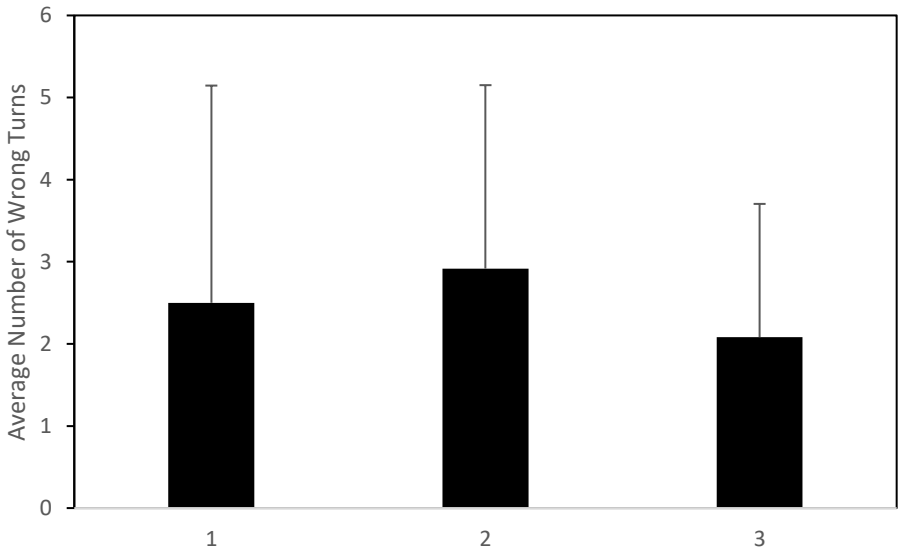

Figure 2.6. Week 1 average wrong turns for the multiple-turn maze with standard deviation by cohort.

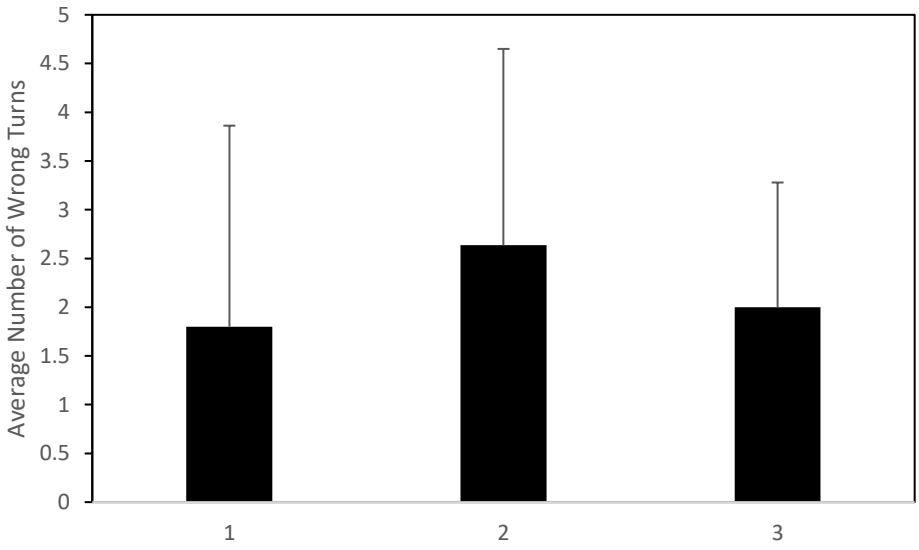

Figure 2.7. Week 2 average wrong turns for the multiple-turn maze with standard deviation by cohort.

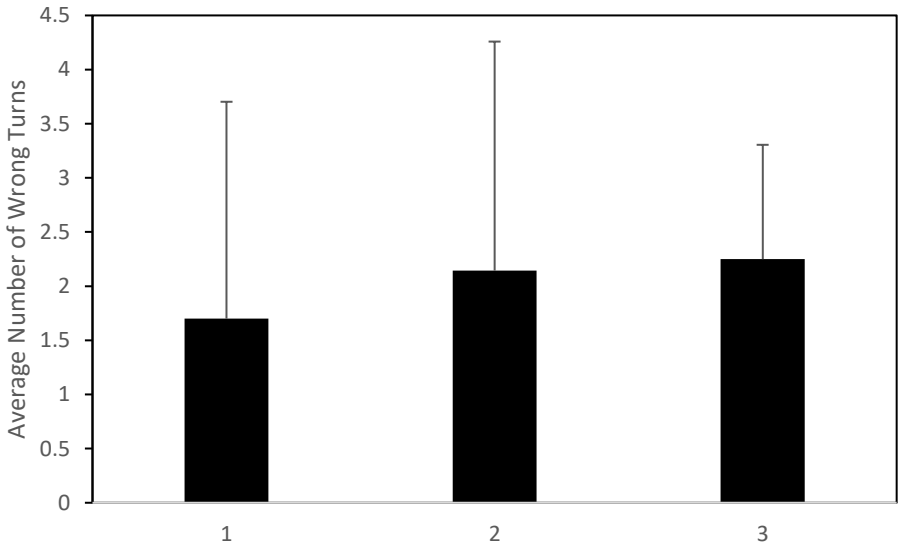

Figure 2.8. Week 3 average wrong turns for the multiple-turn maze with standard deviation by cohort.

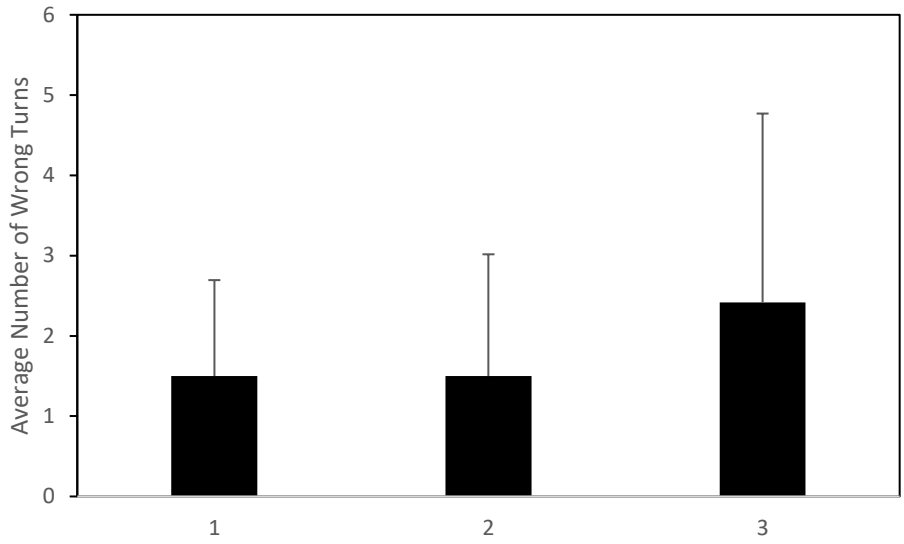

Figure 2.9. Week 4 average wrong turns for the multiple-turn maze with standard deviation by cohort.

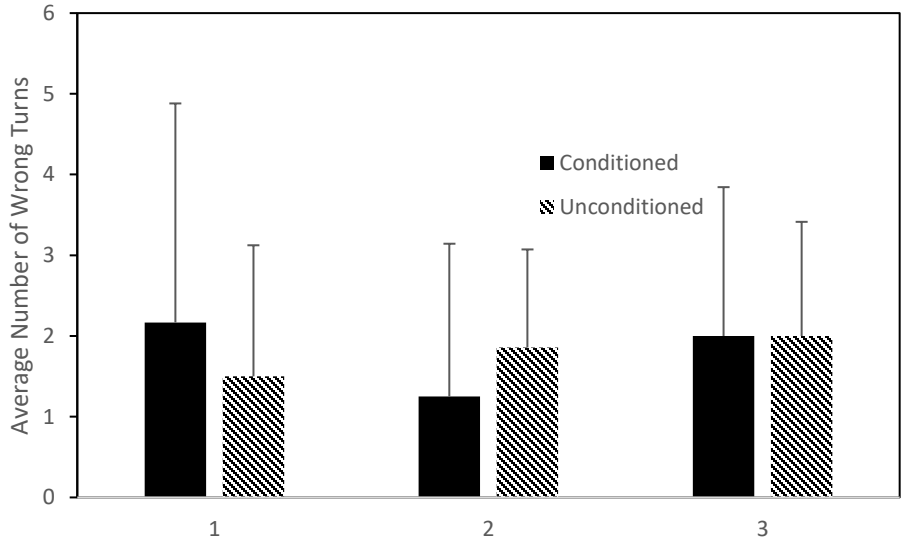

Figure 2.10. Average number of wrong turns for the multiple-turn maze with standard deviation after the latency period (Week 5) by cohort and experimental group.