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Associative and Non-associative Learning in the Monodelphis Domestica

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ASSOCIATIVE AND NON-ASSOCIATIVE LEARNING
IN THE MONODELPHIS DOMESTICA

A Thesis

by

ANA C. RAMIREZ

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

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Major Subject: Experimental Psychology

ASSOCIATIVE AND NON-ASSOCIATIVE LEARNING
IN THE MONODELPHIS DOMESTICA

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December 2018

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ABSTRACT

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Learning encompasses multiple important processes that vary within organisms and that are affected by different factors. Two of the most important types of learning are associative and non-associative learning. Even though past research has studied animal learning in multiple species, there is limited research on learning in the *Monodelphis domestica*. It is imperative to further the knowledge on this species since multiple fields use it as an animal model for research. The current study examined both non-associative and associative learning in the *Monodelphis*, as well as sex differences. Non-associative learning was explored by using a habituation-dishabituation olfactory paradigm, while associative learning was examined using the conditioned place preference (CPP) paradigm.

DEDICATION

To those who inspired it but will probably never read it; especially Dr. Mark H. Winkel.

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CHAPTER I

LEARNING AND THE MONODELPHIS DOMESTICA

Learning encompasses multiple important processes that vary within organisms and that are affected by different factors. Since learning is a very broad and extensive topic, its definition often varies. Powell, Honey, and Symbaluk (2013) define learning as a relatively permanent change in behavior that results from experience; they also suggest that change in behavior does not have to happen immediately, and that in some circumstances it might not become evident until much later. Similarly, Okano, Hirano and Balaban (2000) state that memory is an essential component of learning, defining memory as change in behavior caused by an experience, and defining learning as a process for acquiring memory. However, De Houwer, Barnes-Holmes, and Moors (2013) argue that learning is not only limited to changes in behavior, but more of an ontogenetic adaptation. When defining learning they also consider the regularity in the environment of an organism, and the causal relation between the regularity in the environment and the changes in behavior of the organism (De Houwer, Barnes-Holmes, & Moors 2013). While there may be different arguing definitions of learning, there is no debate about the importance it plays in psychology and many other related fields.

Like previously mentioned, learning encompasses many different processes. The assumption that there are different and multiple types of learning is not a novel idea. In fact, Tolman (1949) proposed that there are different kinds of learning, and that each includes drive

discriminations, learning of motor patterns, and formation of cognitive maps. He also suggested that each type of learning had different laws of acquisition and retention (Tolman, 1949). By the same token, it has been proposed that learning might be adaptively specialized, meaning that each type of learning serves a different purpose (Rozin & Kalat, 1971). Notably, Churchland (1986) listed “habituation, sensitization, classical conditioning, operant conditioning, imprinting, habit formation, post tetanic potentiation, imitation, song learning, one-shot learning to avoid nausea-producing foods, and cognitive mapping” as some of the most commonly recognized learning processes (p. 152).

Similarly, many of these learning processes fall into learning types such as non-associative and associative learning. Non-associative learning refers to learning that takes place without any associations between stimuli. Behavior sometimes changes even when there is an absence of a reinforcing or punishing stimulus. In other words, learning occurs without the need of consequences following a particular behavior. On the other hand, there is associative learning. Associative learning refers to the change in behavior due to the formation of associations between stimuli and responses (De Houwer, Hughes, & Barnes-Holmes, 2016).

The high interest in learning is well deserved since it is vital to function in everyday life. Some degree of memory, experience, and learning is required to perform even the simplest tasks or to develop basic skills, reason why learning research is of utmost importance. One approach to further investigate learning processes is through the use of animals. Animal use for research purposes is a longstanding practice that dates back to ancient Greece (Ericsson, Crim, & Craig, 2013) and has proved to be essential for fields like biomedicine and psychology (Barré-Sinoussi & Montagutelli, 2015). To emphasize, according to the American Psychological Association

(APA), animal research has been the major contributor to the current understanding of basic learning and motivational systems.

There are many different animal models in science ranging from bees to primates, with mice being the “powerhouse in biomedical research” (Ericsson et al., 2013, p. 2). Even though rodents have become the hallmark and stereotype of animal research, new animal models are now being used. Such new models include the *Monodelphis domestica* (Figure 1), a marsupial that has increasingly gained popularity as a laboratory animal (Keyte & Smith, 2008). According to Saunders, Adam, Reader, and Mollgard (1989) this species “is one of the very few marsupial species and the only *Didelphis* to have been bred under laboratory conditions in



Figure 1: Monodelphis domestica

sufficient numbers to be useful for experimental purposes” (p. 227) The *Monodelphis domestica* (Didelphidae, Marsupialia) is the most commonly used laboratory marsupial, and with good reason. There are multiple factors that make the *Monodelphis* a rather convenient animal subject, such as small size, ease of care,

non-seasonal breeding, and the fact that it is the first marsupial whose genome has been sequenced (Keyte & Smith, 2008). Breeding colonies currently exist at academic and research institutions all over the world (VandeBerg & Robinson, 1997) with animals breeding throughout the year with an estrous cycle of 28 days and a gestation period of 14 days (Sanders et al., 1989). This “small, rodent-like pouchless marsupial” is native to Brazil and some neighboring regions (Saunders et al., 1989, p. 227).

The next pages will focus on non-associative and associative learning in the *Monodelphis*. Even though past research has explored the learning abilities of other animals, there is very limited research on learning in the *Monodelphis*. It is imperative to further the

knowledge on this species because multiple fields use it as an animal model for research. Exploring both non-associative and associative learning tasks within the Monodelphis could help better understand the effects medical and psychological disorders have on learning in humans. The purpose of the present study is to extend previous research by examining and comparing the performance of the Monodelphis in non-associative and associative learning tasks, examining sex differences, and therefore providing new learning and behavioral research routes that could significantly impact the understanding of learning in humans.

CHAPTER II

NON-ASSOCIATIVE LEARNING

Like mentioned in previous pages, non-associative learning refers to learning that occurs without the formation of associations between stimuli. Two primary forms of non-associative learning are habituation and dishabituation. In behavioral terms, habituation is often defined as a decrease in response strength or intensity after repeated exposure to a stimulus (Thompson & Spencer, 1966). Habituation is considered a form of non-associative learning because exposure to a stimulus is not accompanied by any consequence, either appetitive or aversive (Leussis & Bolivar, 2006). A practical example of habituation can be getting used to the feeling of a new watch on your wrist. After wearing it a couple of days (repeated exposure) you are no longer constantly aware of its presence on your wrist (response decrement). As opposed to response decrements due to aging, trauma, and growth, habituation is reversible (Humphrey, 1933). The process in which habituation is reversed is called dishabituation. Dishabituation refers to the process in which a response resurfaces after undergoing habituation, usually after the presentation of an irrelevant novel stimulus (Powell, Honey, & Symbaluk, 2013). If you stop wearing your watch for a couple of weeks and then decide to wear it again, you will once again experience the same awareness and reactivity as when you wore it for the first time. It is worth mentioning that habituation and dishabituation are interrelated processes.

Even though habituation is often described as the most primitive and simplest form of learning (Thorpe, 1956), this process is comprised of several characteristics (Rankin et al., 2009; Thompson & Spencer, 1966). First and foremost, after repeated exposure to a stimulus a response decrement can be observed in some behavioral dimension of the response. This decrement may be reflected in the frequency, intensity, or duration of a behavior (Rankin et al., 2009). The frequency of stimulation plays an important role, being that the more frequent the stimulation the faster habituation will occur. Consequently, the absence of stimulation affects the habituation process. After a period of non-exposure, the previously observed response tends to recover, often referred to as spontaneous recovery. Furthermore, the intensity of the stimulus also affects responding. If a stimulus is “weak” or has low intensity the decrease in responding will take place at a faster pace; if a stimulus is “strong” or has high intensity, sensitization may take place instead (Thompson & Spencer, 1966). For example, one might easily get used to or “habituate” to T.V. noise in the background of a doctor’s office. On the other hand, combat soldiers do not habituate to the noise of bombs, gunfire, etc. Instead, soldiers become sensitized and their reaction to noise is stronger. Moreover, habituation to a certain stimulus may result in generalization to other similar stimuli. This suggests that habituation is stimulus specific (Rankin et al., 2009). It is important to note that the decrement in responding observed in habituation is not a result of fatigue or sensory adaptation. Habituation has been distinguished from sensory adaptation and fatigue by the process of dishabituation (Rankin et al., 2009) It is also important to notice that dishabituation and sensitization are not interchangeable terms. Sensitization consists of an increase in the strength of a response while dishabituation refers to the reappearance of a previously habituated response (Powell, Honey, & Symbaluk, 2013).

In experimental conditions habituation can be examined in two different ways: intrasession (within-session), and intersession (between-session). In the latter, the level of responding is compared between sessions with the expectation that the organism retained information from the previous session, resulting in a decrease in responding due to recognizing the environment. Intrasession observations focus on habituation within the time period of a single exposure (Leussis & Bolivar, 2006). It has been suggested that intrasession habituation measures adaptivity, whereas intersession habituation also suggests memory of the prior sessions (Mueller et al., 1994). Intersession habituation, in which an organism must recognize a novel stimulus and then retain information and use it in subsequent trials is often considered a better indicator of learning and memory (Fraley & Springer, 1981).

Non-associative learning paradigms have been used in the past to examine learning in multiple animal species and within different areas of interest. Platel and Porsolt (1982) used a habituation paradigm to examine exploratory behavior in rodents and to compare the effects of learning enhancing and impairing drugs. In their study mice were placed in novel photo-cell activity cages and were free to explore. Retention or learning was defined as a decrease in exploratory behavior (i.e. habituation). After being exposed to the cage once, the animals were injected with a drug to either enhance or impair learning. Those animals injected with a learning enhancing drug completed the exploration task again 7 days after their initial exposure to the cage; those injected with a learning impairing drug were exposed to the cage 3 days later. Results suggested that the animals that received the enhancing drug showed a decrease in exploratory activity (they habituated) as opposed to the control group. By contrary, those animals that received an impairing drug did not show a decrease in exploratory behavior.

Furthermore, habituation and dishabituation paradigms have been used to observe reflexes. Sherrington (1906) examined the effects of repeatedly exposing monkeys to electrical stimulation, and observed a decrease in scratch reflex and flexion reflex. Best et al. (2008) studied habituation of the acoustically generated startle response (ASR) in zebrafish larvae. The study consisted of placing the larvae in a plate and then exposing them to auditory cues of 200 Hz. The ASR was quantified by measuring the distance each larva moved in response to the auditory stimulus. The results indicated that the distance traveled by the larvae in the first exposure to the auditory cue was always greater than in subsequent exposures. Additionally, Wiel and Weeks (1996) demonstrated habituation and dishabituation of proleg withdrawal reflex in caterpillars. In their study, repeated deflection of 1 or 5 mechanosensory hairs on a proleg resulted in a decrease in evoked force of proleg withdrawal reflex, while habituated responses recovered after cessation of stimulation or pinch of the body wall (dishabituation).

Moreover, habituation and dishabituation have also been explored using olfactory tests. Olfactory tasks mainly focus on testing odor discrimination. In the past, odor discrimination testing was primarily conducted using operant conditioning techniques (Gregg & Thiessen, 1980). While using these techniques might be an effective way to clearly show olfactory discrimination, the use of them also clouds and confounds the actual discrimination of the odors due to the motivational states needed in order for operant conditioning to be successful. To address this issue Gregg and Thiessen (1980) utilized a habituation olfactory test, predicting that the animal would initially respond vigorously to a novel odor, that the response rate would decrease as the animal became familiar with the odor (habituation), and that it would notably increase once a different odor was introduced (dishabituation). To test this, they exposed gerbils to three different social odors consisting of different urine samples. The test consisted of four 6-

minute sessions; the first 6-minute interval served as a baseline and the three subsequent intervals presented a different urine sample. Results indicated that the gerbils habituated to urine samples during the 6-minute exposures, and that the frequency of rearing increased once a novel sample was introduced. Similarly, Sundberg et al. (1982) also examined habituation of social odors by exposing sexually inexperienced rats to the odors of female rats' urine. Results suggested that all of the rats stopped reacting to the odor after repeated exposure, and regain interest once a new urine sample was released through the air stream of the experimental apparatus.

In addition, non-social odors have also been used in the past to conduct olfactory tasks. Wren, Harris, Saavedra, and Crawley (2003) conducted an olfactory habituation-dishabituation test in mice utilizing non-social scents. The test consisted of multiple presentations of three different odors (water, banana extract, and almond extract). Each odor was presented to the mice three times for 3 minutes each time. The order of the presentation of stimuli was water, banana extract, and almond extract. Results suggested that the animals spent less time sniffing the stimulus as the number of presentations of that same stimulus increased, and that once a new stimulus was introduced time spent sniffing the odor increased again.

A major objective of the present study is to replicate Wren et al. (2003) non-social olfactory test. It has been suggested that non-associative learning in the form of habituation and dishabituation is present across animal species. The purpose of the current experiment is to extend past research by shedding light on the *Monodelphis domestica*, while also looking at sex differences. There is no research available on habituation/dishabituation paradigms in this particular species. It is expected that some form of habituation and dishabituation will be

observed in all of the subjects in this experiment, supporting the hypothesis that some degree of non-associative learning takes place across species.

Methods

Subjects

Ten gray short-tailed opossums ($n = 10$, 5 males and 5 females) were used for this experiment. The average age of the animals was 142 days. All of the animals were housed in a climate-controlled room in plastic cages. Some of the animals were single-housed while others had cage mates. Animals with cage mates shared a cage that was divided by a plastic wall, having their own small quarters within the cage. Food and water was freely available to the animals in their cages. This study was approved by the IACUC.

Materials and Apparatus

Three different non-social odors were used as olfactory stimuli; water, lime extract, and almond extract. Distilled water was used to dilute the lime and almond extract (1:100 dilution). Filter paper and microscope slides were used to present the odors to the animals. Plastic vials and



Figure 2: Habituation Dishabituation Olfactory Apparatus

a pipette were used to dispense odors onto the filter paper. A Habituation Dishabituation Olfactory apparatus (Figure 2) was created for this experiment, which consisted of an aquarium glass tank (20 x 10 x 12 in) with a designated section to place the odor samples.

Alconox and ethanol were used to clean the equipment in between sessions. All trials were recorded and quantified through ANY-maze software.

Procedure

Three different odors were selected as the olfactory stimuli to be presented to the animals. The stimuli were water, lime extract (diluted in water), and almond extract (diluted in water). These odors were chosen based on Wren et al. (2003) study, replacing the banana odorant with a lime odorant due to availability. Experimenters presented the stimuli by applying the odorant to a small piece of filter paper. The piece of filter paper was then put in a microscope slide, and then placed inside the apparatus. Velcro was used to secure the microscope slide in place in one of walls of the apparatus. Before exposing the animals to the olfactory stimuli they were allowed to freely explore the apparatus for 3 minutes in order to eliminate novelty prior to starting the non-associative

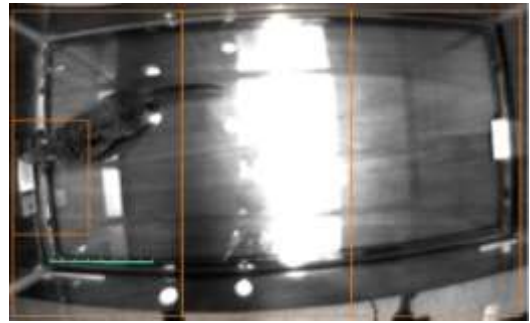


Figure 3: Subject in Apparatus While Testing

learning task. After allowing the animals to get used to the equipment, each stimulus was presented three times for a total of 9 trials, with each trial lasting 3 minutes. Odorant slides were replaced after each trial. The order of the presentation of the stimuli was as follows: three trials of water, three trials of lime, and three trials of almond. Habituation and dishabituation were examined by comparing performance between trials (intersession). Habituation was defined as a progressive decrease in duration of olfactory investigation (sniffing) after the repeated presentation of the same odor, while dishabituation was defined as a reinstatement of sniffing when a novel odor was presented (Yang & Crawley, 2009). Sniffing was measured in terms of frequency and duration. Sniffing was defined as orienting nose toward and within 5 mm of the microscope slide or physically contacting the muzzle to the microscope slide. The experimenters used ANY-maze to quantify habituation and dishabituation.

Statistical Analysis

A one-way repeated-measures ANOVA followed by Tukey's post hoc test was used to identify changes in olfactory investigation across the nine 3-minute trials of each animal, as well as to examine sex differences. Statistical significance was defined as $p \leq 0.05$. IBM SPSS version 25 was used to analyze all data.

Results

Figure 4 and 5 show the habituation and dishabituation data for the duration and frequency of sniffing. For frequency of sniffing there was a significant main effect of stimulus presentation $F(2.280, 18.244) = 6.533$ ($p < .006$), however there was no statistically significant difference in performance between the sex of the animals ($p < .648$). Duration of sniffing also showed a significant effect, $F(1.432, 11.446) = 6.700$ ($p < .01$), while no significant sex differences were observed ($p < .614$).

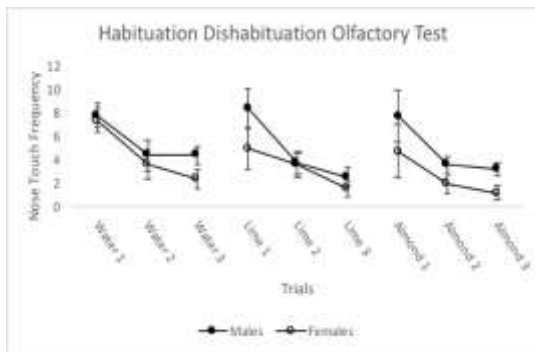


Figure 5: Habituation Dishabituation Frequency Data

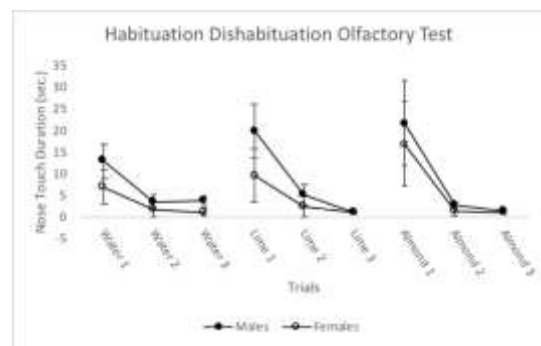


Figure 4: Habituation Dishabituation Duration Data

Discussion

Data results show that there was a decrease in frequency and duration of olfactory investigation with each presentation of the same odor. Both male and female subjects spent less time engaging in sniffing behavior after the repeated introduction of the same odor. This suggests that the animals recognized the stimulus and retained that information. By the same token, all animals effectively detected changes in the environment once a new odor was introduced. Once a new odor was introduced the animals attended to the stimulus and the time spent investigating the novel stimulus increased compared to subsequent trials in which repeated exposure of the same stimulus was occurring. This reinstatement of responding indicates that the animals were aware of something being different or amiss. It can be argued that learning took place because the animals' behavior changed as a product of experience.

The successful demonstration of habituation and dishabituation in all of the participants of this experiment further supports the notion that non-associative learning is one of the simplest forms of learning, ergo present across species. Even though the sample size used was small, the habituation and dishabituation effects were so strong that statistical analysis was able to detect them.

CHAPTER III

ASSOCIATIVE LEARNING

Associative learning is usually defined as learning that takes place through the formation of associations between stimuli. The first associative learning theory applied to animals was proposed by Thorndike (Pearce & Bouton, 2001). He argued that learning consists of the formation of associations between stimuli and responses, and that these associations are formed when responses are followed by a reward (Thorndike, 1898). Thorndike's work paved the way into new associative learning theories which mainly shared the assumption that learning is based on stimulus-response associations (Hull, 1943).

Even though stimulus-response theories were regarded as important in learning, focus shifted to stimulus-stimulus theories of associative learning. These theories suggested that learning could occur through the connection of two stimuli, such as in the work of Pavlov. In his famous classical conditioning studies, Pavlov (1927) paired an unconditioned stimulus (US) with a neutral stimulus (NS), and after several presentations of both the US and the NS together, the NS elicited the same response as the US, becoming a conditioned stimulus (CS) and the response a conditioned response (CR). Rescorla and Wagner (1972) expanded on classical conditioning by proposing that a particular US can support only so much of conditioning, and that this amount of conditioning should be distributed between the CSs that are present.

Another important form of associative learning is operant conditioning. Like previously mentioned, Thorndike paved the way for associative learning theories, but he was also a pioneer in operant conditioning studies by being the first to introduce a stimulus-response theory. According to Thorndike (1898), behaviors leading to a satisfying state are strengthened, while behaviors leading to an unsatisfying or annoying state are weakened; he called this the law of effect. Skinner, however, believed that behavior should be explained in less mentalistic terms. Skinner's (1953) definition of operant conditioning differed from Thorndike's in that he focused on the effect consequences had on behavior, not on whether these consequences were pleasant or aversive. Today, operant conditioning is usually defined as learning in which the future probability of a behavior is influenced by its consequences (Powell, Honey, & Symbaluk, 2013). In other words, consequences affect behavior and they can either increase or decrease its frequency. Consequences that increase the future probability of a behavior are called reinforcers, and consequences that decrease behavior are called punishers. Reinforcers and punishers are considered so by the effect they have on behavior, not by whether they are appetitive or aversive. It is important to distinguish that reinforcement and reinforcer, and punisher and punishment are not the same. Reinforcers and punishers are the consequences that influence future behavior, while reinforcement and punishment are the procedures in which consequences change behavior. Thus, giving praise to students for participating in class in order to increase class participation is an example of reinforcement, while praise itself is the reinforcer. Similarly, a child touching a hot stove and feeling pain is an example of punishment, while pain is the punisher. Operant conditioning is considered a form of associative learning due to the formation of associations between behavior and consequences.

Classical and operant conditioning paradigms are commonly used to observe animal learning. Although there is no research on classical and operant conditioning conducted with the gray short-tailed opossum, both procedures have been used with the Virginia opossum. James (1984) conducted an avoidance conditioning task in which the opossum was confined and restrained in an apparatus. Avoidance conditioning consists of teaching a response in order to avoid the delivery of an aversive stimulus. For this task, a shock intense enough to cause a leg movement was delivered at the same time as a door buzzer. It was expected that the opossums would exhibit a leg flexion when they heard the door buzzer. Results indicated that after multiple trials, the opossums did not exhibit the desired leg flexion, but instead a whole bodily reaction was observed. James (1984) argued that the absence of the desired behavior did not imply a lack of learning, but rather that the opossum's nervous system could not confine action to the one leg segment.

Moreover, to test operant conditioning procedures in this particular species, Angermeier, McLean, Minvielle, and Grue (1987) used an operant box and positive reinforcement in the form of food to establish and increase lever pressing behavior. Data showed that all the animals in the study learned the required behavior. Comparatively, James (1955) replicated the work of Guthrie and Horton, creating his own Guthrie-Horton box. In this task, the opossum was placed in the back of the apparatus and had to move a pole located in the middle of the box in order to open a door that provided access to food. Results showed that in a small number of trials, the animals successfully performed the behavior that allowed access to food, and quickly became proficient at it. Maze learning was also used to observe animal learning (James, 1959). In this task, the opossums were required to find the correct path in order to get food. Results yielded that opossums not only learned how to get the food, but also easily eliminated non-reinforced alleys.

Another interesting and important associative learning task is discrimination training. Discrimination training involves the reinforcement of responding in the presence of a specific stimulus and not another stimulus. For example, if your boss usually allows you to leave early when he is in a good mood and does not let you leave early when he is in a bad mood, you learn to only ask to leave early when he is in a good mood. Multiple variations of discrimination training procedures have been used with the Virginia opossum, testing the animals' ability to discriminate colors, symbols, objects, etc. For color discrimination training, Friedman (1967) wanted to investigate the presence or absence of color vision in the Virginia opossum. The animals were presented with a choice of two stimuli, and they had to select a stimulus by touching it with their nose. If the correct selection was made, the animals were provided a food pellet. Both animals in the study learned to successfully discriminate each color, suggesting they are physiologically capable of distinguishing colors. James and Turner (1963) conducted an experiment with a similar procedure. Instead of using colors, they used vertically and horizontally striped cards, and black and white cards. Results suggested that the animals could differentiate the two signals, consecutively selecting the correct signal. For object discrimination, James and McFarland (1966) examined the opossum's ability to discriminate between different shapes and sizes, and black and white colors. With this purpose, the animals were placed in operant boxes with two levers. The animals could get food by nosing or pushing one of the two levers; each lever had a shape mounted on top varying in form, size, and color. Results indicated that the opossums discriminated between the black and white colors, and several shapes. However, it was noted that the animals had difficulty differentiating shapes that were similar in form and size (e.g. square and rectangle; small and medium).

While all of the associative learning tasks discussed above offer great insight regarding animal learning and memory, this paper will focus on the conditioned place preference (CPP) paradigm. The CPP paradigm is a behavioral model most commonly used to study the rewarding and aversive effects of drugs; although food, sexual behavior, and other rewarding stimuli have also been used (Prus, James, & Rosecrans, 2009). The main purpose of this task is to create an association between a particular environment and a rewarding stimulus, followed by the association of a different environment with the absence of the rewarding stimulus. The ability of a stimulus to produce a preference for the associated environment is generally considered a product of classical conditioning. Thus, repeatedly pairing food (US) with a neutral environment (NS) would be expected to result in extending the rewarding effects to the neutral environment (CS) (Prus, James, & Rosecrans, 2009) In simpler and more general terms, conditioned place preference occurs when a subject prefers one place more than another because the preferred location has been previously paired with rewarding events (Huston et al., 2013).

Procedures using the CPP paradigm vary depending on the effects and stimuli of interest. Generally speaking, CPP consists of having an animal experience two or more distinct neutral environments that are eventually paired with either a rewarding or non-rewarding stimulus. After pairing, the animal is given the chance to explore either environment freely (Koob, Arends, & Le Moal, 2014). If the animal spends more time in the environment that was previously paired with a rewarding stimulus it can be assumed that place preference occurred.

To facilitate the assessment of place preference, apparatuses with multiple compartments are used to represent different environments, with each compartment serving as a neutral environment. CPP experiments usually start with pre-test trials, which simply involve letting the animal explore the entire apparatus (all chambers). Pre-test trials serve not only to allow the

animal to habituate to the apparatus, but to establish a baseline of time spent in each chamber. After pretesting, a chamber in the apparatus is assigned to be the one paired with a rewarding stimulus. Once the reward chamber is assigned, conditioning takes place for several sessions.

Conditioning consists of exposing the animal to a rewarding stimulus only in the chamber that



Figure 6: CPP Two-Chamber Apparatus



Figure 7: CPP Three-Chamber Apparatus

has been assigned as the reward chamber. During conditioning trials the animal is also exposed to a non-reinforced, or “no reward”, chamber. In this chamber the animal is not exposed to any stimulus. Following conditioning trials post-testing is performed. Post-testing is exactly the same as pre-testing in that the animal is allowed to freely explore the entire apparatus without the exposure to any external stimulus while time spent in each chamber is measured. Preference is determined by recording the duration of time spent in each chamber; the higher duration, the higher the preference. To assess whether place preference changed, duration data from pre-testing is compared to post-testing data. If the animal’s preference changed due to the exposure to a rewarding stimulus, an increase in time spent in the reward chamber should be observed. There are several apparatuses used to study this particular model, with the two-chamber (Figure 6) and three-chamber (Figure 7) boxes being the most common. The two-chamber CPP box is usually referred to as a “forced choice” apparatus since the animal has to choose a chamber to be in. The three-chamber box is regarded as an “unforced choice” apparatus because it counts with a third compartment that mainly serves as a connection or pass way between the two conditioning chambers and it is used as a “start area” for pre and post testing (Tzschentke, 1998).

Although the two-chamber box is widely used, some concern exists suggesting that the use of a forced choice procedure could pose potential bias for the compartment the animal was placed in during initial test sessions (Prus, James, & Rosecrans, 2009). To address potential bias, the design of the study needs important consideration. The assignment of conditioning chambers can be done using a “biased” or “unbiased” research design. These research designs are used to take into account the fact that subjects may have an initial preference for a particular compartment of the apparatus, even before any conditioning has taken place. It may be the case that during pretesting trials subjects spend more time in chamber 1, while others spend significantly more time in chamber 2. In an unbiased design, the assignment of a chamber paired with a rewarding stimulus is determined at random, regardless of the preference of each subject prior to conditioning. In a biased design however, the preference of each individual subject is taken into consideration. The least preferred chamber (where the animal spent less time during pre-testing) for each subject is then assigned to be the conditioning compartment.

Like previously discussed, the conditioned place preference paradigm is usually utilized to study the rewarding and aversive effects of drugs. A large number of studies have confirmed the CPP-inducing effects of stimulant drugs such as amphetamine and cocaine (Tzschentke, 1998). The induction of CPP by cocaine and amphetamine is regarded as so reliable that its induction has been selected as a positive control for the patency of an IV catheter construction in mice (Kelley et al., 1997). Just like stimulant drugs, opiates have also shown significant CPP-inducing effects. Bardo, Rowlett and Harris (1995) conducted a meta-analysis using the data of 55 drug-induced CPP studies, primarily focusing on the effects of morphine, heroin, amphetamine and cocaine. From those 55 articles they got 208 separate groups, with each group representing an independent sample within an article. In total they assessed 76 groups

using morphine, 39 using heroin, 45 using amphetamines, and 48 using cocaine. They encountered that when all of the data was included for each drug there was no significant correlation between effect size and the drugs. Once groups were excluded on account of dosage and effects, a significant correlation was observed. Their findings showed that drug-dosage affected CPP when conditioning with morphine, heroin, and amphetamines, but did not with cocaine. The relevance of these studies is that findings can be used to better understand and treat addiction and drug dependency in humans.

The CPP paradigm can be used in a variety of ways and to explore different processes. For instance, Itzhak and Martin (2002) used the paradigm not only to establish place preference, but to investigate the extinction and reinstatement of place-conditioned responses using cocaine in Swiss Webster mice. To do this they first induced place preference using a two-chamber box, with one compartment painted white with a textured floor and the other painted black with a smooth floor. Mice were injected with cocaine in their assigned compartment and with saline solution in their non-assigned compartment. After CPP was established, mice were injected with saline solution in both the cocaine and saline-paired compartments for 8 days to extinguish the previously attained place preference. Following the extinction of CPP, preference was reinstated by challenging the animals with either cocaine, METH, MDP, or PCP. Results suggested that there was a significant preference for cocaine-paired compartments, that extinction abolished CPP effects, and that after reinstatement the mice once again showed a preference for the previously cocaine-paired chamber.

Even though CPP is mainly used in pharmacological studies, research has also focused on non-drug reinforcers. An advantage of this, is that reinforcers can be tested for motivational properties. CPP studies assessing non-drug stimuli have used socially mediated reinforcers such

as social interaction, sexual behavior, and play. For instance Gil et al. (2013) used the CPP paradigm to evaluate the rewarding effects of social interaction in male Syrian hamsters. Findings suggested that the male hamsters not only found social interaction rewarding, but that social dominance increased the reward value of the interaction. Similarly, Tenk et al. (2009) assessed sexual reward in male rats, focusing on whether ejaculation and intromissions differ in rewarding value, while also considering prior sexual experience. Their results indicated there is a hierarchy of rewarding sexual behavior, with ejaculation being the most rewarding component, and that the rewarding value of other components of sexual behavior such as intromission is dependent on prior sexual experience. In addition, Calcagnetti and Schechter (1992) examined the rewarding value of social play in dominant juvenile rats. Results showed that the dominant rats increased the amount of time spent in the chambers associated with a playing partner, suggesting that they found the opportunity to engage in play as rewarding.

Another important non-drug stimulus that has been used in the past to induce CPP is food. Rubinow, Hagerbaumer, and Juraska, (2009) studied the effects of food reward in male and female rats with the purpose of finding sex and age difference in performance. To do this, a three-chamber box was used. The larger chambers offered sensorial cues to help the animals identify each chamber easily; the floor of one compartment was covered with a novel corn cob bedding, and the other was paired with a cinnamon oil scent. Prior to the experiment the animals were food deprived. During conditioning they used Froot Loops as a rewarding stimulus. Data suggested that adolescents failed to learn the task after a period of training sufficient for adult learning, that performance was unaffected by sex, and that there was an interaction between age and performance.

In a similar fashion, Lepore et al. (1995) used food reward to achieve place preference

while also comparing its effects to drug reward effects in rats. The rewarding properties of THC, cocaine, morphine, and Froot Loops were studied using a three-chamber apparatus. Results indicated that significant food place preference occurred in both deprived and non-deprived animals, and that just like with food, stimulants, and opiates, THC produced rewarding effects.

The present study will use the CPP paradigm as an associative learning task to study the associative learning abilities of the *Monodelphis domestica*. Just like with non-associative learning, there is no research that focuses on associative learning in the *Monodelphis domestica*. The purpose of the current study is to examine learning in the *Monodelphis domestica* through the CPP paradigm using food reward. It is expected that the animals will develop place preference for conditioned environments.

Methods

Subjects

Six gray short-tailed opossums (n=6, 3 males and 3 females) were used in this experiment; two were used as controls and four as experimental animals. The average age of the animals at the time of the experiment was 367 days. All of the animals were housed in a climate-controlled room in plastic cages. Some of the animals were single-housed while others had cage mates. Animals with cage mates shared a cage that was divided by a plastic wall, having their own small quarters within the cage. Food and water was freely available to the animals in their cages (not deprived prior to the study). This study was approved by the IACUC.

Materials and Apparatus

To induce place preference, Froot Loops were used as the rewarding stimulus. Past studies using Froot Loops as CPP food reward have used 50 Froot Loops. Since most research using Froot Loops as reward studied rats, and rats are bigger in size than the Monodelphis, 10 Froot Loops (5g) were used in this study. A three-chamber conditioning box was used (Figure 8). Of those three chambers two were identical in size (40cm x 40cm x 35cm) The additional



Figure 8: CPP Apparatus Used

compartment served as a connection between the two large chambers and also as a removable holding cell. The CPP apparatus was made entirely of black Plexiglas with the exception of the floor, which was a stainless-steel base. Similar to Gil et al. (2013), the two larger chambers had textured mats; one of the compartments had black fiberglass flooring while the other had a white no-slip rug pad flooring. The two different textured mats served as contextual cues to help the animal recognize each chamber. Each animal had its own two mats that were used throughout the experiment, adding up to 12 textured mats in total (6 black and 6 white). Due to the color of the mats, the chamber with black flooring will be referred to as “black chamber” and the chamber with white flooring will be referred to as “white chamber”. Alconox and ethanol were used to clean the equipment in between sessions. All trials were recorded and quantified through ANY-maze software.

Procedure

The conditioning procedure consisted of ten days. During the first two days pretesting was conducted. Two pre-tests were conducted in order to avoid error due to novelty, and to allow animals to habituate to the equipment. Each pre-test consisted of allowing the animal to explore

the entire apparatus for 15 minutes. Prior to starting each pre-test, the animal was placed into the small holding box for 30 seconds before being released into the apparatus. ANY-maze was used to record the amount of time spent in each chamber. The third day no experimentation took place. Days 4-8 were used for conditioning with one conditioning trial per day. All animals were randomly assigned to either the black or white chamber (Unbiased design) using an online random number generator. Those animals in the experimental condition received 10 Froot Loops in their assigned conditioning chamber, while the control group did not receive any stimulation. Each conditioning trial consisted of 30 minutes in the assigned chamber and 10 minutes in the non-assigned chamber, adding up to 40 minutes per conditioning session per animal. During conditioning trials the animal had access to only one chamber at the time. During days 9-10 post-tests were conducted. Post-tests were carried out following the same procedure as pre-tests.



Figure 9: Subject Eating the Food Reward

Statistical Analysis

To determine whether food reward induced place preference, a preference score was calculated. Preference scores were calculated by dividing time spent in the conditioned compartment (C) over the sum of time spent in the neutral compartment (N) and time spent in the conditioned compartment ($C/N+C$) (Gil et al., 2013). Preference scores were calculated for both experimental and control animals. It should be noted that to examine whether CPP took place preference scores and duration scores were obtained by using averages from pre-tests and post-tests. A one-way repeated-measures ANOVA followed by Tukey's post hoc test was used to

assess place preference as well as to examine the sex interactions. Statistical significance was defined as $P \leq 0.05$. IBM SPSS version 25 was used to analyze all data.

Results

During pre-testing, there was no significant difference in preference between pre-test 1 and pre-test 2. Even though there was no statistically significant difference between pre-test 1 and pre-test 2, Figure 10 shows a comparison between male and female subjects' initial preference. It can be observed that males did not show a preference for either the black or white chamber, while females spent more time in the white chamber in both pre-test 1 and 2. Although preference scores were calculated to analyze the data, they were not able to pick up the general effect and duration data was used instead. For CPP, there was no significant effect in the control group (Figure 11). In the experimental group there was no effect (Figure 12), but a decreasing trend can be observed ($p < .08$). However, there was a statistically significant sex interaction ($p < .04$).

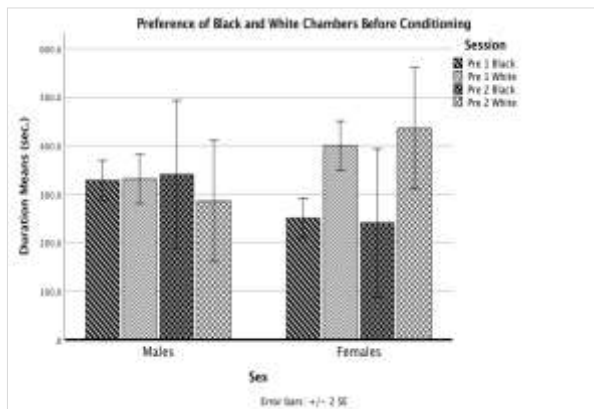


Figure 10: CPP Initial Preference Comparison

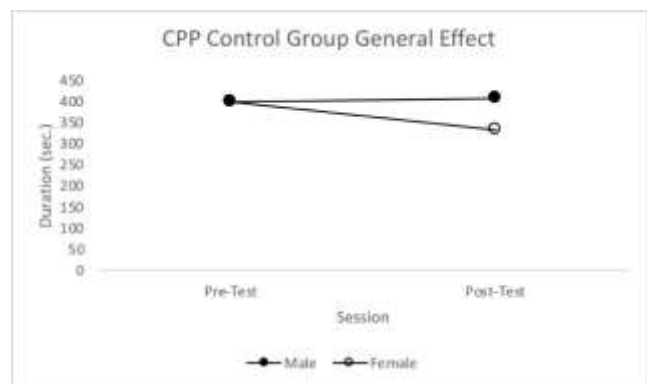


Figure 11: CPP Control Group Results

Discussion

Data from the study suggests that place preference did not take place to statistically significant levels, but there was a significant sex interaction. At a glance, Figure 12 shows that even though not statistically significant, there was a decrease in time spent in the assigned chamber during post-testing within the experimental group. However, Figure 13 depicts the difference in behavior between the males and females of this group. For males, there appears to be a very slight increase (or no change) in time spent in their conditioned chamber, while females' time decreased. It can be said that females are driving the general effect since there is little to no change in males.

There are multiple factors that could have influenced the results of the study. First of

all, the animals were not food deprived before starting the conditioning trials. This presents an issue because if animals were satiated while running conditioning trials, the Froot Loops would lose reinforcing value. Some studies have shown that deprivation does not impact the significance of results in CPP, but it is important to consider that the Monodelphis is an understudied species and findings in other animals do not necessarily apply to them. Secondly, some of the animals might have been reluctant to eat the Froot Loops due to neophobia. To avoid this, future studies can perform food habituation prior to testing. Although Froot Loops are commonly used as food reward for rodents in CPP experiments, it might also be the case that the

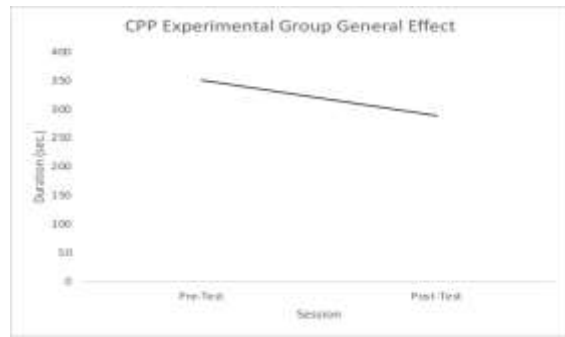


Figure 12: CPP Experimental Group Results

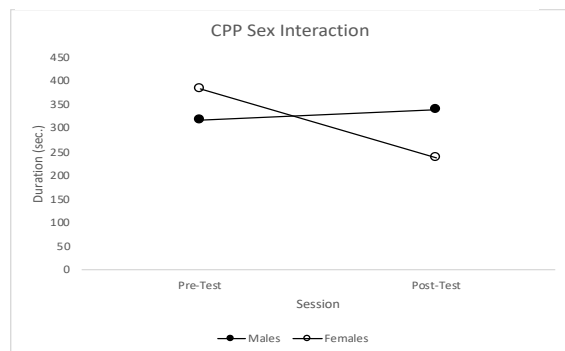


Figure 13: CPP Sex Interaction

opossums simply did not find the Froot Loops rewarding compared to other animals. Another critical aspect is that the sample size used in this experiment was very small.

With the exception of sample size, it is hard to address the issues discussed above because there is no learning or behavioral research on the *Monodelphis domestica* to use as a guide. Once procedure logistics are established, the rewarding properties of new stimuli should be studied. Sex differences should also be taken into consideration. It is difficult to make assumptions about why an animal behaves a certain way, especially with so little research on this particular species. Data on sex differences from this study may open new behavioral research routes such as examining anxiety and avoidant behavior in *Monodelphis* females, and novelty seeking behavior in males.

CHAPTER IV

TRANSLATIONAL VALUE OF ANIMAL MODELS

AND GENERAL DISCUSSION

Animal research is a long standing practice that has proved to be of tremendous value across a variety of fields. The impact of laboratory animals in science has been so great that by the beginning of the 20th century 94 out of 106 Nobel Prizes awarded in Physiology and Medicine were dependent on animal research (Pasquali, 2018). But even though animal research has provided important knowledge that has shaped the current understanding of human health and behavior, it is not uncommon for some members of society to criticize and condemn it as cruel and as a violation of animal rights. While a valid concern, most members of the public are not aware of the level of care animals receive in a laboratory setting, the role of on-call veterinarians, the considerable amount of training required to handle animals, how all animal-related research is submitted for extensive and thorough ethical review to protect the wellbeing of animals, and how animal research is only done when alternatives would not yield the same information (Bennett & Ringach, 2016).

The continuous journey of expanding and improving the knowledge of processes and underlying mechanisms that affect humans is one that still depends a great deal on the use of animals. Using laboratory animals for research can help expand the current knowledge on the

interaction between humans and the environment, improve the detection of illness, and aid in the development of treatments (Pasquali, 2018). Although it might seem preferable to study humans directly, doing so might be unethical. Animal models offer researchers the ability to circumvent issues such as ethical concerns related to human experimentation, allowing them to study a wide range of possibilities that might not even be feasible in humans (Hooijmans & Ritskes-Hoitinga, 2013). For instance, animal models in pain research offer advantages in respect to standardization, safety, and economy. Also, even though DNA can be easily extracted from human subjects, animals offer the possibility of obtaining mRNA from pain-relevant tissues (Mogil, Davis, & Derbyshire, 2010). Furthermore, the use of animals for pain research not only helps provide a better understanding of the physiology of pain, but also examine analgesics that could be of use for humans.

Similarly, animal models have been used to study addiction. The use of laboratory animals offers the opportunity to assess effects of specific neural manipulations on processes that mediate addictive behaviors (Belin et al., 2016). Addiction research in animals focuses on consumption and aspects such as compulsion, craving, and relapse, while also attempting to identify specific neurobiological mechanisms (Lamontagne & Olmstead, 2018). In sum, addiction animal studies provide the tools necessary to understand the relationship between behavioral and biological processes, and how these are influenced by developmental and environmental changes (Lamontagne & Olmstead, 2018).

Moreover, laboratory animals have also been utilized to study mental illness and developmental disabilities. For example, animal models for autism have been established to examine different components of Autism Spectrum Disorders (ASD), such as repetitive behaviors, communication, socialization, molecular, cellular and pathomorphological processes

involved. Since it is hard to encompass all of the characteristics of ASD in one specific animal, animal models usually focus on one particular aspect of autism (Sharma, Jamwal, & Bansal, 2017).

Similar to the areas of study previously mentioned, research on learning in animals offers a great amount of information. Animal learning has always been an important area of focus in the field of psychology, where learning processes have been dissected with the main goal of defining underlying cognitive mechanisms (Papaj, Snell-Rood, & Davis, 2008). Through the use of animal models, it is now known that non-associative learning processes such as habituation and dishabituation are vital in order to detect change in the environment. This is relevant because as a means to save energy and resources, an organism's priority is to detect changes, not constant states. In other words, by habituating to a certain stimulus one can allocate attention to other more relevant stimuli at that moment in time. Habituation helps reduce energy-wasteful, time-consuming responses to meaningless stimuli (Papaj, 2009), while dishabituation allows an organism to refocus on a stimulus if needed. By the same token, animal models have contributed to the body of knowledge on learning that takes place between stimulus-stimulus and stimulus-response associations, also known as associative learning. The study of associative learning is important because this type of learning is the one that enables organisms to gain important correlative information about their environment and how to adopt certain behaviors that take advantage of that information (Papaj, Snell-Rood, & Davis, 2008).

With both the importance of animal models and learning research established, it is important to mention the need to gain more knowledge about understudied animal models such as the *Monodelphis domestica*. The two previously discussed experiments serve as preliminary studies that can open future research directions. Even though learning has been thoroughly

studied in multiple other animal species, each species has individual characteristics. For instance, the opossums in the CPP study did not ingest a significant amount of Froot Loops, which could have influenced the results; while other animal models have used this same stimulus as food reward with positive effects.

Even with a small sample size in the CPP experiment, sex differences were detected. It is necessary to further look into behavioral sex differences within the *Monodelphis* to better understand the function of their behavior. Future studies with larger sample sizes are important in order to increase statistical power. Since the main purpose of conducting animal research is to gain valuable knowledge that can be applied to humans, it is important to direct further research looking at sex differences. Sex differences are greatly overlooked in many fields, especially in the medical and pharmacological industry. Just as an example, Platel and Porsolt (1982) failed to include female subjects in their screening test for memory enhancing drugs. Although their results were significant, they are not representative of both sexes. Issues like these skew the understanding of learning, potentially affecting areas of human research regarding biased drug dosage. Learning and behavioral research about the *Monodelphis* is also relevant because the use of this model keeps increasing, mainly in biomedical research. Establishing a baseline of learning abilities and behavior patterns in this species could potentially allow researchers and clinicians to use this animal model to study the development and decline of learning and memory in individuals affected by mental illness, neurodegenerative disorders, and neurodevelopmental disabilities.

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