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THE UNIVERSITY OF NEW HAVEN

RATTUS NORVEGICUS AS A BIOLOGICAL DETECTOR OF CLANDESTINE REMAINS AND THE USE OF ULTRASONIC VOCALIZATIONS AS A LOCATING MECHANISM

A THESIS

Submitted in partial fulfillment

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MASTER OF SCIENCE IN FORENSIC SCIENCE

By:

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West Haven, Connecticut

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RATTUS NORVEGICUS AS A BIOLOGICAL DETECTOR OF CLANDESTINE REMAINS AND THE USE OF ULTRASONIC VOCALIZATIONS AS A LOCATING MECHANISM

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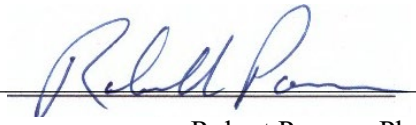

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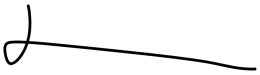

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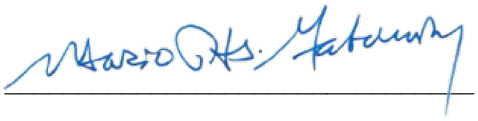

Mario Thomas Gaboury, J.D., Ph.D.

Dean of Henry C. Lee College

Nancy Ortins Savage, Ph.D.

Interim Provost

For Mom and Dad

~

Without you I would not have had this opportunity

I love you both so much

Abstract

In investigations, locating missing persons and clandestine remains are imperative. One way that first responder and police agencies can search for the remains is by using cadaver dogs as biological detectors. Cadaver dogs are typically used due to their olfactory sensitivity and ability to detect low concentrations of volatile organic compounds produced by biological remains. Cadaver dogs are typically chosen for their stamina, agility, and olfactory sensitivity. However, what is not taken into account often is the size of the animal and the expense of maintaining and training the animal. Cadaver dogs are typically large breeds that cannot fit in small, hard to reach places, such as collapsed buildings. Another small animal could be used as a biological detector in addition to cadaver dogs. This research tried to determine if *Rattus norvegicus*, or a brown laboratory rat, could be trained to identify the volatile organic compounds of decomposition and return when called, and if the vocalizations could alert to the location of the clandestine remains as well. Rats were trained in increasingly larger environments using classical conditioning and positive reinforcements. It was determined that while *Rattus norvegicus* could be trained to find the scent of decomposition and could be trained to return when call, *Rattus norvegicus* did not vocalize at the scent of decomposition. Future research projects would need to be done in order to determine the full ability of *Rattus norvegicus* as a biological detector of clandestine remains.

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Chapter 1: Introduction

When searching for human remains, a common practice by police units is the use of cadaver dogs. Cadaver dogs are trained to find the scent of human remains and signal when the location of a scent is detected (Rebmann et al., 2000). Cadaver dogs have been commonly used due to their olfactory sensitivity. Sensitivity to smell is important in the use of working animals to search for scents, and in this case the scent of decomposition.

1.1 Death and Decomposition

Decomposition is the process by which a dead body is broken down by chemical and physical processes. There are many factors that factor into how a body decomposes, such as location of the body, the climate it is in, local flora and fauna, exposure to oxygen and other elements, and time since death (Dent et al., 2004). As the body decomposes it goes through a few varying stages. There are many ways to describe these stages of decomposition. Most processes describe decomposition by the features exhibited on the body. One feature commonly searched for is insects as the stage that an insect is in can help to determine how long the body has been there (Skinner et al., 1988). While there are many ways to define the process, and many different numbers of stages that can occur, one way it can be broken down is into five rough stages: the fresh, the bloat stage, the putrefaction stage, the advance decay stage, and skeletonization (Goff, 2009).

1.1.1 Stages of Decomposition

The first stage of decomposition is called the fresh stage, which occurs from the moment of death until the bloat stage. In this stage the abdomen and skin can become discolored and the body rigid (Goff, 2009). Livor mortis, or the pooling of blood to one side of the body, can be

observed and it can occur within an hour or so after death. Rigor mortis, or body stiffening, as well as algor mortis, body cooling, occur in this stage. In early decomposition, a strong odor is not easily detected (Parks, 2011). Insects are attracted to the body as a place to lay eggs, and in early decomposition, there are possible eggs and larvae activity, but not much of it is affecting the decomposition of the skin.

The next stage of decomposition is called the bloat stage, in which bacteria from the gut begins to digest the tissues of the body; the gas that comes off from this digestion causes the stomach to bloat (Goff, 2009). In this stage the gases produced in the body can cause a green discoloration. As the gases increase, fluids can escape the body and seep into the surrounding surfaces (Goff, 2009). Along with gasses, the skin can discolor and depending on the environment skin slippage may occur (Parks, 2011). Any insect activity present in the body will increase the temperature of the body, which will increase bacteria growth inside the body, increasing bloating, and the insects begin to feed on the body.

The next stage of decomposition is called decay/putrefaction, which begins when the gasses from the abdomen breaks through the skin and escape. In this stage, the body deflates of gasses and odor increases (Goff, 2009). In wet environments, saponification can occur, in which skin turn a grayish color, becomes waxy looking and appears as if it is melting off the body. Larvae activity can be extreme in this stage as there may be large masses of insects and pupae actively feeding on the body.

The next stage of decomposition is called advanced/post decay, where the skin is reduced to skin cartilage and bones (Goff, 2009). The internal gasses have escaped the body and the odor increases exponentially. Larvae will have mostly finished eating the flesh in this stage and will

typically start moving into the soil or ground around the body at this stage. In a warm and dry environment, the skin can begin to mummify (Parks, 2011).

The last stage of decomposition is called skeletonization, when no tissues remain, and only skin and hair are present (Goff, 2009). Typically, there are no larvae left on the body as there is nothing left of the body for the insects to feed on. There is no definitive end to this process as various environments and the passage of time causes individuals to get to each stage differently (Dent et al., 2004; Goff, 2009). Some things that may affect decomposition rates are, how and if a body is buried, if it protected by a coffin, the season and temperature, and the presence of insects or scavenging animals (Goff, 2009).

1.1.2 Volatile Organic Compounds

The smell of decomposition that is diffused into the air are volatile organic compounds (VOCs) (Martin & Verheggen, 2018). VOCs are chemicals that are transmitted through the air due to their evaporative properties. There are many VOCs that emerge from the human body during decomposition. In one study, four main groups of VOCs determined by using 2-dimensional gas chromatography: alcohols, carboxylic acids, aromatics, and sulfides (Stadler et al., 2013). Another study showed that 11 main compounds were detected during the early staged of human decomposition, with many containing sulfurs, such as dimethyl sulfide and dimethyl disulfide (Statheropoulos et al., 2007).

Each of the five general stages of decomposition described earlier give off different concentrations of VOCs. In early decomposition, alcohols and alkenes are common on the first day followed by sulfides and ketones. Then as decay progresses, more sulfides and nitrogen compounds are given off. Alkanes are the last compounds to appear (Ioan et al., 2017). One of

the stronger smells of decomposition is from dimethyl disulfide, which emerges from early human decomposition (Martin & Verheggen, 2018).

1.1.3 Detection of Volatile Organic Compounds

There are many ways to detect VOCs. One way is through instrumentation. Gas Chromatography Mass Spectroscopy (GC-MS) has been used to detect decompositional VOCs in soil up to 7 months past deposition (Perrault et al., 2015). GC-MS has also been used to detect VOCs form the headspace above decomposing animals and humans (Cablk et al., 2012). Using GC-MS in a simulated crime scene showed that when the VOCs were collected from a crime scene, and after 15 days, a detectable change was found in VOC composition from the air of the scene compared to day one (Ueland et al., 2021).

Detection animals can also be used a biological detector of VOCs. Detection animals refers to animals that are trained to use their sense of smell to detect particular odors. Many species of animals have been tested and trained to detect a variety of materials. Olfactory sensitivity of species like humans, rats, mice, dogs, cows and even monkeys have been tested for many types of scents (Laska et al., 2000; Padodara & Jacob, 2014). However, detector dogs are the most used detector animal to find VOCs.

1.2 Detection Dogs

Detection dogs are animals used to detect a specific scent, whether it be biological such as human or animal odors, or non-biological such as illegal drugs, explosives or chemicals (Browne et al., 2006). Detection dogs that search for human remains are known as human remains detection dogs or, more commonly, cadaver dogs (Rebmann et al., 2000).

1.2.1 History of Detector Dogs

The first police dog unit was established in 1899 in Ghent, Belgium, and the first police dog unit in the United States was formed in New York in 1907 (Handy et al., 1961). While police canine units have been around for a while, a cadaver dog that searches for detection of human remains, began in 1974 by the New York State Police Department (Rebmann et al., 2000). In 1977, Connecticut instituted a training program for cadaver dogs and developed a training technique in 1978 that was adapted by all cadaver dog trainers of the time. Today cadaver dogs and specialized canine units are maintained across a number of states and many volunteers train dogs on their own to aid in the search of cadavers (Rebmann et al., 2000).

1.2.2 Pros and Cons of Detector Dogs

The breeds most often used are chosen for their stamina and agility (Martin et al., 2020). However, their size is not taken into account, as most dogs are larger breeds. Most cadaver dogs tend to be medium to large sized dogs. Four of the most common breeds of dogs used as cadaver dogs are Malinois shepherds, German shepherds, English spaniels, and Labrador retrievers (Martin et al., 2020). Besides their size, dogs are typically fast to train from a young age, bond easily with their trainer, and can give clear signals to their trainer (Rebmann et al., 2000). Some limitations with the reliability of successful searches comes from the handler, if the dogs are not trained properly, typically by volunteers not in official police units, can raise concern about reliability (Rebmann et al., 2000).

Another limitation with detector dogs is the expense of maintaining a unit. Starting a police unit to find decomposing remains can be expensive. The cost of training one dog can be

between $12,000-$15,000, which does not include maintenance, housing, veterinarian bills, and the cost of the dog itself (Gilbertson, 2019). This number can increase significantly depending on the number of dogs needed in the unit and the type of training that the dog is given. Many police units do not have the funding for a special dog unit.

1.2.3 Detecting Scents

Many studies have been done on cadaver dogs to determine the accuracy of decomposition odor detection. Mammals smell using olfactory receptors. Olfactory receptors are the one of the hundreds of diverse sensory receptor types present in mammalian noses that contribute to the ability to smell. Olfactory receptors are generally uniform in shape; however, they can vary due to gene type, resulting in many binding sites for different proteins on the olfactory receptors (Fleischer et al., 2009). Due to the variety of gene types in different types of mammals, the functions and abilities of each mammal may be different (Quignon et al., 2005). Humans have around 5 million olfactory receptors while a bloodhound has about 10 million olfactory receptors (Rebmann et al., 2000).

Studies have been done to test and see if cadaver dogs can detect samples of decomposition rather than signaling for false positives (Riezzo et al., 2014). In one study, two dogs were able to find the positive samples 89.19-100% of the time, including low levels of blood, and the dogs were able to discriminate from negative samples 50-100% of the time (Riezzo et al., 2014). While the study showed high accuracy and only evaluated two dogs, studies have shown that cadaver dogs can detect diluted blood samples aged for up to two years (Buis et al., 2019). Like Riezzo, et al. (2014) and Buis, et al. (2019) also only used two dogs in

their study. This could have inaccurately reflected the success rates in both of their testing paradigms.

1.2.4 Other Uses of Detector Dogs

Due to their superior sense of smell, dogs have been used to detect things other than human remains. Drug detection dogs have been trained across a variety of breeds in order to aid police searching for illicit drugs (Jezierski et al., 2014). Scent detection dogs are used for non-biological needs as well such as TNT, land mine detection, accelerant residue detection, and other hazardous chemical conditions. Dogs can also be used in conservation to track and detect rare or endangered animals (Browne et al., 2006). Dogs have also been used to detect the scents of certain types of cancers and human bacterial and viral diseases (Angle et al., 2016; Browne et al., 2006).

Chapter 2: Introduction to Rats

While brown rats in the wild are typically seen as a nuisance to people, when trained properly, domesticated rats can be of great value in research and in the working world. Brown rats are easily domesticated and have been trained for research purposes since the beginning of the 20th century (Small, 1900). Along with being rather inexpensive and easy to breed, brown rats make an excellent and economical training animal.

2.1 *Rattus norvegicus*

The brown rat (*Rattus norvegicus*) is a commonly used animal in laboratory settings. *R. norvegicus* was a very common animal found all over Europe and Asia in the 1700s, and their domestication and use in scientific research began in the 1800s (Sengupta, 2013). *R. norvegicus* have small rounded ears, and their tails and ears are hairless (Armitage, 2004). *R. norvegicus* is born with large incisors that growth continuously throughout its life and chewing keeps their teeth from overgrowing (Kohn & Barthold, 1984).

2.1.1 Strains of *Rattus norvegicus*

There are many strains of brown rat. While there are commonalities between all strains of *R. norvegicus*, there are varying differences between each of the strains as well. There are about 51 different strains of rat used in laboratory experiments (Sengupta, 2013). These different strains can allow for varied choices for scientific experiments. Some commonly used outbred strains of *R. norvegicus* include Wistar, Sprague Dawley, Hairless, and Long-Evans Rats (*Outbred Rats*, n.d.).

2.1.1.1 Wistar Rats

Wistar rats are one of the most commonly used laboratory rats. Wistar rats are an albino rat, solid white colored, and are typically used as multipurpose models, safety testing, and dietary testing (*Outbred Rats*, n.d.). Many studies with Wistar rats are involved with exercise and metabolism testing. One study showed the effect of Vitamin D on endurance training in Wistar rats (Mirghani et al., 2019). Multiple studies have looked at how High Intensity Interval Training affects Wistar rats in regard to tissue damage repair with supplements and gene expression in the hippocampus (Dos Santos et al., 2021; Kerendi et al., 2019).

2.1.1.2 Sprague Dawley Rats

Sprague Dawley rats are another albino strain derived from Wistar rats that are also used as a multipurpose model for dietary, nutritional, and medical related research (*Outbred Rats*, n.d.). Sprague Dawley rats are used in many different types of medical related research. Sprague Dawley rats have been used in studies of traditional medicine such as acupuncture as an investigation to develop treatments for obesity, and to investigate the development of mammary cancer in susceptible rats compared to resistant rats in order to help find possible tumor suppressors (Li et al., 2021; Nishimura et al., 2021).

2.1.1.3 Hairless Rats

Hairless rats were bred from a recessive mutation in typical albino rats and are typically used in studies of wound healing, dermatology, and safety testing (*Outbred Rats*, n.d.). As their skin is unobstructed with fur, many studies focus on experiments that affect the skin. One example on a skin study was focused on healing abrasion. It tested hairless rats to see if

pretreatment of the skin with curcumin and ginger extract helped with wound healing and it showed the abrasions healed faster and resulted in increased collagen levels in the skin (Bhagavathula et al., 2009).

2.1.1.4 Long-Evans Rats

Long-Evans rats are a cross between a Wistar white female and wild gray males (*Outbred Rats*, n.d.). They have a white coat with a black hood and are commonly used for multipurpose models, dietary, and behavioral research. Their coloring makes the rats more distinguishable from one another. One study used Long-Evans to see if stressed rats had a neurobiological response to a probiotic-supplemented diet (Natale et al., 2021). Behavioral research was done in 2021 to see if there was a difference in spatial learning between Long-Evans males and females, which resulted that there was no difference (Bucci et al., 2021). While there have been many behavioral studies done on Long-Evans rats, there have also been many olfactory studies.

Long-Evans males have been studied to see if they could be conditioned into choosing a specific mate based on the scent of the females. The study was successful showing that male Long-Evans neonates that were subjected to citrus-scented bedding chose a mate months later with the same scent on them (Ménard et al., 2020). Behavioral conditioning studies in Long-Evans rats has been going on for a long time. In 1966, a study was done to see if Long-Evans males would be able to be classically conditioned for heart-rate deceleration using partial and continuous reinforcement. The study showed that both partial and continuous conditioning showed a significantly decrease in heart rate compared to than no conditioning at all (Fitzgerald et al., 1966). The study also showed that if the rats were continuously shown the stimulus, then the rats retained the conditioning better, and the conditioned response took longer to extinguish.

2.1.2 Life Cycle of *Rattus norvegicus*

On average *R. norvegicus* lives for between 2.5-3 years, the slight variation depending on the strain, the conditions they live in (Turner & Caspary, 2006). *R. norvegicus* in the laboratory, compared to wild rats, have earlier sexual maturity, no reproductive cycle season, and a shorter lifespan (Sengupta, 2013).

R. norvegicus typically reach sexual maturity around 3 months for males, 4 months for females and are able to mate for about 2 years (Armitage, 2004). *R. norvegicus* is able to produce offspring quickly. Female rats have a gestation period that lasts between 22-24 days. They can produce between 2-14 neonates per pregnancy, with the average being around 9 neonates, and they can also reproduce on average 7 times a year. *R. norvegicus* neonates are taken care of by the females and are typically weaned around 3-4 weeks (Armitage, 2004). Males typically lose their fertility around 16-20 months and females' fertility typically wanes around 19-21 months but is not typically lost until 32 months (Kohn & Barthold, 1984).

2.1.3 Anatomy and Physiology of *Rattus norvegicus*

At birth, *R. norvegicus* weighs ~6.5g and are about 49-52mm in length (Miller, 1911). The average mass of Long Evans rats is around 400g; however, females are typically slightly smaller and weigh between 210-305g after 15 weeks (Armitage, 2004; *Long-Evans Rat*, n.d.). *R. norvegicus* is typically 399mm in length from nose to the tip of their tail (Armitage, 2004). They typically eat about 5g of food per 100g of body weight per day and drink 8-11ml of water per 100g of body weight a day. However, the food intake can vary depending on dietary needs, growth and gestation (Kohn & Barthold, 1984).

Domesticated *R. norvegicus* is typically a docile and easily handled animal. *R. norvegicus* is nocturnal and typically sleeps during the day and is active during the night and early morning (Kohn & Barthold, 1984). Along with their docile behavior, which can be encouraged and improved by frequent human handling, rats naturally search for small openings fit into (Kohn & Barthold, 1984). *R. norvegicus* is able to adapt to new surroundings and can be easily trained on a variety of sensory cues.

2.1.4 Vocalizations of *Rattus norvegicus*

R. norvegicus has the ability to emit ultrasonic vocalizations (USVs), which is used to communicate with other rats, and can change in response to behavior, socialization, and emotional states (Takahashi et al., 2010). Different frequencies of USVs have been shown to correlate to different behaviors or feelings of rats. Takahashi et al., 2010, analyzed 3 frequencies (25 kHz, 40 kHz, and 60 kHz ranges), which corresponded to fighting, feeding, and moving respectively. A study from 2007 found similar ultrasonic frequencies as Takahashi et al., with adult rats emitting a 22 kHz vocalization for an inescapable adverse stimulus and emitting a short 50 kHz chirp for positive conditions. For the 22 kHz vocalizations, the rats are typically tense and frozen in response to an adverse stimulus such as an unknown predator or a loud startling noise (Portfors, 2007). In order to easily record the vocalizations Brudzynski, 2009 used a bat detector, an instrument that is specialized to detect ultrasonic frequencies commonly used to detect USVs of bats, to record the rats. The 22 kHz vocalizations were in response to aversive and dangerous situations while the 50 kHz frequency was emitted during feeding and nonaggressive behaviors (Brudzynski, 2009).

Vocalization responses from rats can be recorded as a spectrogram (Fig.2.1). This visualization can show the speed and frequency of the trills at a 55kHz frequency (Cloutier et al., 2018).

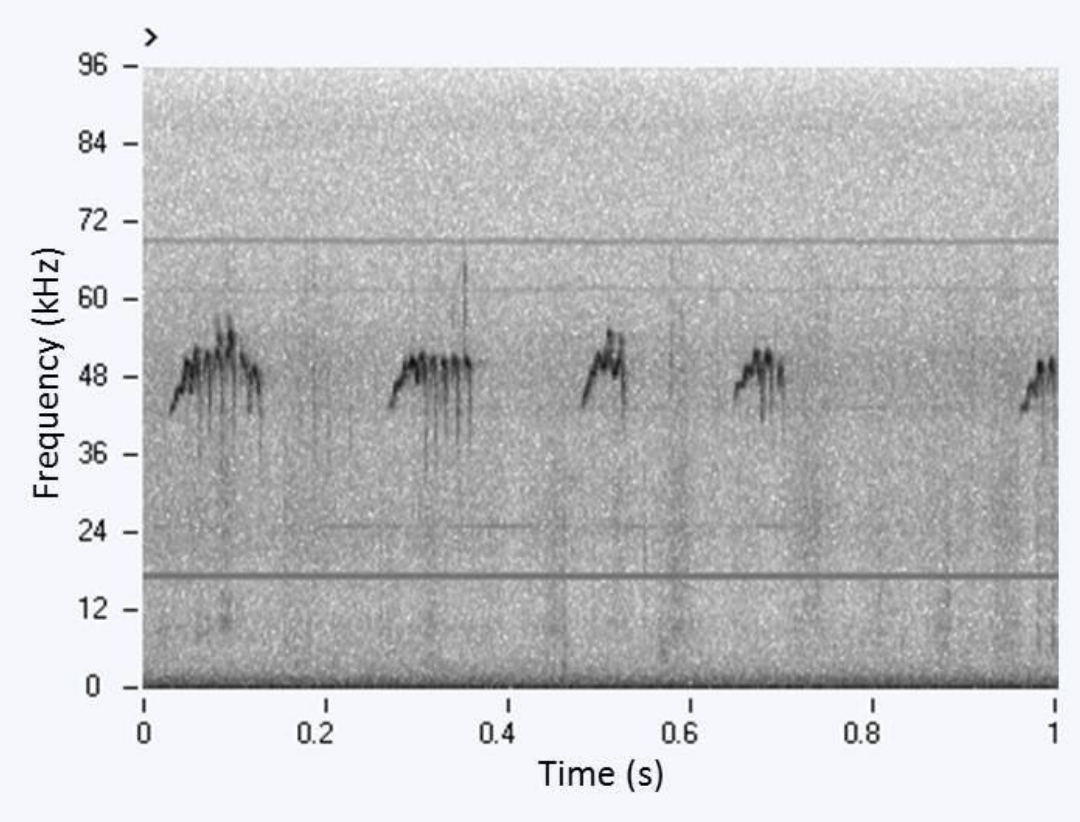

Figure 2.1 A 50 kHz vocalization from rats produced by rat tickling. Image taken from Cloutier et al., 2018

2.1.5 Olfactory Receptors of *Rattus norvegicus*

Both dogs and rats are considered macrosmatic animals, meaning both have highly developed senses of smell (Laska et al., 2000). *R. norvegicus*'s sense of smell is its strongest sensory channel, as it is used to find food and differentiate between other rats in order to socialize (Armitage, 2004). There have been numerous studies done on rats to test their olfactory levels and sensitivity. Olfactory receptor number and strain variation of rats and dogs have been compared using gene size estimations (Quignon et al., 2005). The olfactory receptor genes were

mapped out and translated into the gene sequences of both rats and dogs to identify pseudogenes in order to compare their sensitivity. After comparing the sequencing of the genes, Quignon et al. (2005) found many subclasses of olfactory receptors in both rats and dogs. While there were fewer subfamilies of olfactory receptors in rats than in dogs, the rats' subfamilies were larger and more diverse (Quignon et al., 2005). This shows that there may be differences in olfactory receptor capability and sensitivity of dogs and rats and that the use of their sense of smell could have different capabilities.

Rats have also been tested for their cognition abilities and their reliance on scents in the environment. Slotnick (2001) tested a paradigm that applied a complicated odor system using 28 different two-odor problems in which the rats were tested using odor, visual stimuli and a combination of both stimuli. Throughout the tests, rats showed a high learning ability with odor-paired learning, including using odor only, helping enforce their reliability on smell (Slotnick, 2001).

Similarly, rats have been tested to see if they are reliably trained to primarily use their senses of smell. Rats have been found to learn quickly to train using their senses of smell when their training is broken into stages allowing them to learn at their own pace (Liu et al., 2018). A similar study done on mice also shows that mice are also able to detect lower concentrations of a scent when exposed to that scent over time (Yee & Wysocki, 2001). The scent that Yee & Wysocki (2001) used was androsterone, which is a scent used in many olfactory studies to test olfactory capacities. Even as the concentration of the scent was slowly lowered, the mice learned the scent and became more accustomed to it due to their prolonged exposure to the scent.

2.2 Laboratory Uses of *Rattus norvegicus*

R. norvegicus is considered one of the first mammals used in laboratory research (Modlinska & Pisula, 2020). *R. norvegicus* is used in a lot of medical and genetic research, including research in physiology, immunology, pathology, psychology, cancer, contagious disease and dietary studies (Armitage, 2004).

R. norvegicus is commonly used in laboratory settings and experiments. Rats have been used in experiments from classical conditioning to genome sequencing. In the early 1900s, rats were tested in multiple studies for their cognitive functions to see if they would be able to search through mazes, and progressively learning each time they went through the maze (Small, 1900, 1901). Small tested the rats in classic mazes to determine their ability to learn their environment. Small wrote two books on the subject of rat cognition in 1900 and 1901. This was just the beginning for studies of rat cognition.

2.2.1 Operant vs. Classical Conditioning

There are many different training methods for working animals that can be used. Operant conditioning is the pairing of positive reinforcements or rewards for wanted behaviors and negative reinforcements or punishments for unwanted behaviors. Many training methods for cadaver dogs rely on operant conditioning (Rebmann et al., 2000). However, there has been debate about the ethicality of using negative reinforcement on animals. Many state that the use of negative reinforcement in operant conditioning can be harmful to the animal (Blackwell et al., 2008). A study in the United Kingdom showed that out of 192 dogs trained, the dogs trained with only positive reinforcement showed higher obedience and lesser aggression than dogs trained

with either negative reinforcement or a combination of positive and negative reinforcement (Blackwell et al., 2008).

Positive reinforcement and classical conditioning are more suitable methods of behavioral training due to not only the ethical debates of other types of conditionings but also the success of pairing positive reinforcement with classical training. Classical conditioning is pairing a natural behavior with a chosen stimulus, so over time the conditioned behavior will happen with the stimulus. Classical conditioning can be used to help animals associate an odorant with a conditioned response. Dogs have been tested to see if there would be increased odor sensitivity to odors if classical conditioning was used, meaning there would be more exposure to the particular odor (Hall et al., 2016). It was shown that the sensitivity to a particular odor can be enhanced in dogs using classical conditioning when compared to a control odor. The prolonged exposure—exposure to the scent for more than 10 seconds during initial conditioning—to the same odor also helped to reinforce the scent and learning as well.

There have been many positive reinforcement and classical conditioning studies done with rats. Classical conditioning has been used on rats since the early 1900s. In 1962, newborn rats were classically trained to vibrate a limb when administered a shock in order to show that newborn rats could be classically conditioned (Caldwell & Werboff, 1962). This conditioning showed a physical and easily seen response in the rats. In 1991, rat neonates were tested to see if an odor related response could be conditioned. In the study, neonates were presented a citrus smell and faced towards a door to condition the action of facing the door with the citrus smell (Sullivan et al., 1991). Sullivan et al, (1991) showed that the young rats were not only able to detect odors at a young age, but also responded in the conditioned manner only to the citrus scent.

2.2.2 Male vs. Female Rats

Male and female rats also have some differences when it comes to behavioral training. Studies that show there are no differences in spatial learning between the two sexes (Bucci et al., 2021). This does not mean their behavior is not different. There are some behavioral differences in the male and female rats. As it is with most animals, females tend to be easier to work with and less stubborn than males and are therefore typically easier to train (Rebmann et al., 2000). Females are less stubborn as in they learn faster and will train with less resistance than males. Additionally, studies suggest that female rats retain associations of classical conditioning faster than males do and females also lose the associations slower than males (Dalla & Shors, 2009). This means that training female rats should be faster than male rats and the females should also be able to retain their associations longer. However, the personality of the individual rats can also have an effect on their behavioral training (Dougherty & Guillette, 2018).

2.3 Forensic Relevance

On June 24, 2021, a 12-story condominium in Miami, FL collapsed, resulting in 98 fatalities and the search taking over a month to find all the victims in the rubble (Hauptman & Shammas, 2021). The complex scene of the 12 stories of rubble and debris made it difficult to examine. The area was searched around the clock for over 4 weeks using search teams, detector dog teams, drones, and radar and was slow going due to the hazardous scene (Hauptman & Shammas, 2021). With the search taking weeks to conduct in the Miami summer heat, a smaller animal trained to detect human remains could have helped aid in the search, resulting in faster processing of the scene.

In addition to possibly being used in search and rescue situations, brown rats are already being used to in scent detection in forensics cases as well. In a Rotterdam Police Department, officers are training ten brown rats to smell for gunshot residue, due to their sense of smell, and the fact that rats are easier and cheaper to train than dogs (*Dutch Cops Are Using Brown Rats to Solve Crimes*, 2013). Besides forensics, rats have already successfully been trained as detection animals. APOPO, a nonprofit organization based in Tanzania, is researching and training giant pouched rats to detect the scent of TNT as landmine detecting rats. Training rats to detect landmines in abandoned fields and past warzones so the mines can be safely removed, makes the land safe again for future use. APOPO has also trained the giant pouched trats to detect tuberculosis in people using their senses of smell (APOPO, n.d.). This is done by training the rats to detect the scent of tuberculosis. They are trained to detect any aerosol variants in sputum samples that could be linked to tuberculosis. If it is detected, the rats signal for it, streamlining and speeding up the processing of the samples (APOPO, n.d.).

APOPO trains both pouched rats and dogs in order to search the fields efficiently for landmines and tuberculosis, highlighting the fact that the differences in size does not necessarily affect olfactory ability. The small size of *R. norvegicus* would also be an advantage over larger dog breeds that are commonly used by allowing the rats to search disaster scenes that may be too small for dogs to fit into, such as building rubble or collapsed building debris (La Londe et al., 2015).

2.4 Research Questions

Can *R. norvegicus* be trained to find the scent of decomposition in a field and return to the handler with a high level of success?

Can the ultrasonic vocalizations of *R. norvegicus* be used to alert to clandestine remains?

2.5 Aims and Objectives

The aims of this research are to:

- Test if *R. norvegicus* can be trained to find a known scent of early decomposition and return to the handlers.
- Detect the ultrasonic vocalizations of *R. norvegicus* to see if they will vocalize upon detection of clandestine remains.
- Analyze the ultrasonic vocalizations of *R. norvegicus* to determine if they are unique to individual rats in order to differentiate between multiple rats.
- Access if *R. norvegicus* is a practical biological detector of the scent of decomposition.

The objectives are to:

- Condition the behavior of *R. norvegicus* with positive reinforcements and classical conditioning.
- Record and analyze the ultrasonic vocalizations of *R. norvegicus* during training sessions.
- Monitor the behavior of *R. norvegicus* during training in increasingly more complex environments.

Chapter 3: Methods and Materials

3.1 Materials

While rats require constant monitoring, few materials are required to care for them. Many of the starting materials for this research were found at Dr. O'Brien's Center for Wildlife Forensic Research laboratory. This research was conducted under IACUC approval number 20-03 (Appendix 1). Three female Long-Evans rats were purchased from Charles River Laboratories (*Long-Evans Rat*, n.d.). Long-Evans rats were chosen based on their use in behavioral studies and their coloration. Long-Evans rats have a white coat and black hood that can have a variation in its pattern on the back of the rats. The coloring causes a unique pattern on the back of each of the rats, allowing for identification of each rat by their pattern.

Each rat was numbered with tattoos on their tails–001, 002, 003–a which faded over time and the rats were distinguished from one another by the coloring pattern on their backs. Each rat was named and associated with the pattern on their back for individualization as the tattoos on their tails faded within the first week. Rat 001 had a black patch on the right shoulder and was named Zajac. Rat 002 had a large black patch on the left bottom and was named Simjouw. Rat 003 had a narrow black stripe down the back with no patches and was named Kelly. The rats were referred to individually by these names in data collection and in the results.

3.2 Caretaking Methods

The three rats were housed in suitable living environment. The rats were housed together, as they are social animals who show more positive behaviors when housed in groups (Animal Research Review Panel, 2007). Housing the rats together also allowed them to express a more natural social behavior, as well as develop normally (Council, 2010). The room the rats were

caged in was set to stay between 20-26ºC, which is an optimal temperature for keeping rats (Animal Research Review Panel, 2007).

A double decker, clear plastic research rat cage with two water bottle tops was purchased from Techniplast to house the rats (Fig. 3.1). The floor area of the cage is 1862 cm^2 and 38 cm in height (*Double-Decker Rat*, n.d.). This is plenty of space for three rats to fulfil their basic needs of sleeping, grooming, eating, exercising, and socializing (Council, 2010). The exercising and socializing were enforced by the handlers, however, the two levels in the rat cage allowed for natural rearing and behavior and extra enrichment and space for the rats.

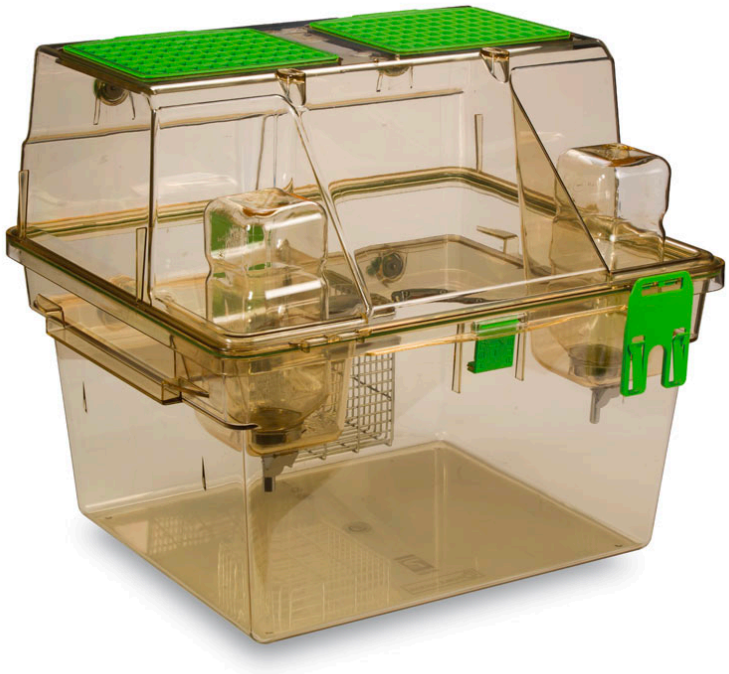

Figure 3.1 GR1800 Double Decker rat cage from Techniplast. Image taken from Techniplast.it

Small Pet Select Aspen Bedding was purchased to cover the floor of the cage for comfort and sanitation. It was a clean, moisture absorbent, dust free, scent free, and chemical free material (Animal Research Review Panel, 2007). The depth of the bedding was around 2 cm

covering the cage floor, to allow for diffing and burrowing, and it was cleaned and replaced as necessary. Animal enrichment toys were available for the rats in their cage to stimulate natural chewing behaviors and activity.

The walls, floor, and ceiling of the cage was also cleaned as necessary. The cage was cleaned and washed with a brush and water, and the bedding was replaced on a schedule of once every five days. Rats typically self-groom and allogroom, so bathing the rats was not necessary. Clean, non-contaminated potable tap water was provided to the rats through drip bottles connected to the cage and refilled as needed.

The rats were given food in the form of *Kalmbach Feeds 23% Rodent Diet Cubes Rat & Mice Food* daily. The rats were allowed ad libitum access to food at all times as they were growing and acclimating, until they reached about 80% of their body weight at which time their total food intake was regulated to ensure they were not overeating. Fruits, such as apples and orange, cut into small pieces, were also feed to the rats during their training sessions to act as a high-value reward for their positive reinforcement during training.

The rats' behavior and health were monitored daily to make sure they were healthy. Rats typically give behavioral cues to show that they are in distress such as aggression, fear, or any abnormal panic responses to touch (Animal Research Review Panel, 2007). None of the rats showed any health issues or signs of distress during the duration of this research.

All handlers (Appendix 2) were properly trained to handle the rats. Each handler was added to the IACUC procedure and passed the CITI (Collaborative Institutional Training Initiative) programs focused on Working with IACUC and Working with Rats in Research Settings. Consistent and systematic handling of rats helped the rats reduce stress and become accustomed to being handled by people (Animal Research Review Panel, 2007). Handling the

rats when they were young helped them become accustomed to being handled. The rats were slowly conditioned to being handled over a month. First the rats became accustomed to seeing the handlers' hands from in their cage and were allowed to sniff it, then after a day, the handler gently stroked the rats if the rats allowed it (Animal Research Review Panel, 2007). This was repeated for short increments daily until the rats allowed the handler to hold them comfortably.

3.2.1 Stress Management

In order to reduce stress, enrichment was left in the cage for the rats to chew on in order to protect their teeth and mentally enrich them. Along with following IACUC and CITI training protocols, handlers were required to complete the Rat Tickling Certification Course from Purdue University.

Another way to help the rats alleviate stress and mimic playing is through heterospecific play or also called rat tickling (LaFollette et al., 2018). Rat tickling is a technique used to habituate rats to the touch of humans that can ultimately reduce handling stress (LaFollette et al., 2017). This was done by stroking the backs of the rats a few times flipping them on their backs and stroking their stomachs (Fig. 3.2).

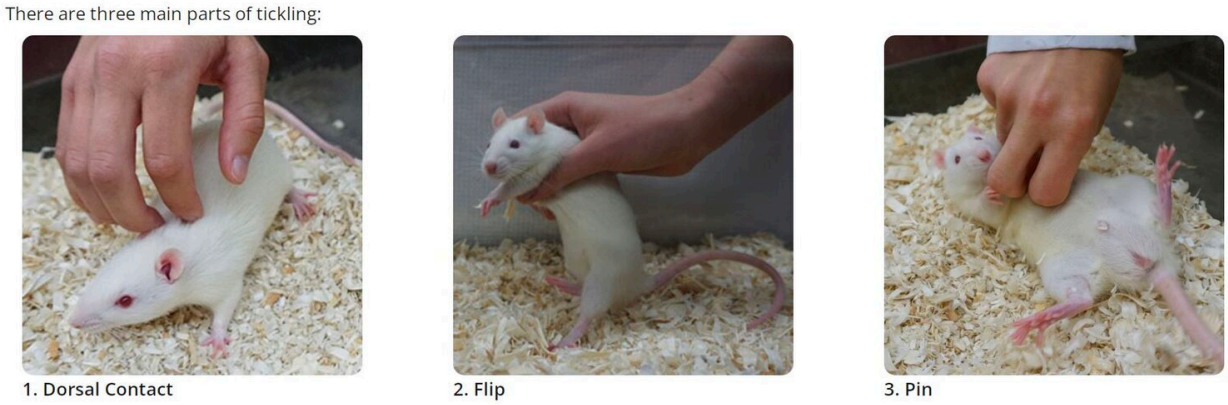

Figure 3.2 The three main parts of rat tickling. Taken from (Campbell, 2021).

Rat tickling is done to mimic how rats play and has been shown to reduce psychological and behavioral stress (LaFollette et al., 2018). The rats were tickled once a day per rat before training began. The rats were tickled in 15 second intervals for 2 minutes in order to destress the rats and prepare them for training (LaFollette et al., 2018).

3.3 Training Methods

Training the rats consisted of two main stages followed by different levels of training (Appendix 3). The Stage 1, the acclamation stage, took a few weeks, as it allowed the rats to adjust to their new environment and learn to be handled by their handlers and allowed them to reach a suitable weight to begin, which was ~80% of their full potential body weight (Gonzalez, 2022). Acclimation allowed the rats to adapt to the new smells, sights, and sounds of their new environment, as well as adapt to their handlers (APOPO, n.d.). As the rats were acclimated to their new living environment, they were played with for two hours a day, in order to get their exercise, socialize, and become acclimated to being handled. During this acclimation phase, the rats were allowed to wander a large tabletop to run around as well as play with animal enrichment toys for mental stimulation. The rats also had enrichment left in their cages in order to chew on for mental stimulation and oral health as well.

The Stage 2 consisted of clicker or scent training and return training. The rat tickling that was used in Stage 1 was carried on daily before each training, as it was a way to reduce stress. Whistles, training clickers, a carrier, and the VOC were used during training the training sessions.

In 2015, La Londe, et. al. tested rats to find people based on training to detect human scent from clothing. As in their study, after the rats were adjusted to their new environment and

training began, there was a food intake adjustment in order to increase responsiveness to food being used as a positive reinforcement during training. The rats were given food during training, enforcing them to each during training, and they were also given pellets overnight to ensure they were well fed, but also motivated to eat during training in the day. The scent training was done to associate the scent of decomposition with a positive reinforcement, while the return-to-handler training was done to associate returning to the handler with a positive reinforcement. The clicker training was done to associate the rats leaving the carrier and starting their search for decomposition with the sound of a clicker. Clicker training was eventually replaced with scent training, which still included the clicker aspect.

Each training, scent and return training, occurred daily, twice a day, with each session lasting no more than an hour (Gonzalez, 2022). Whole food pellets mixed with pieces of chopped fruit was used as the positive reinforcement in each training. Rats training using a positive reinforcement such as treats, respond to scent training better than when no reinforcement is involved (Mahoney et al., 2014). Before each training period, 15 grams of *Kalmbach Feeds 23% Rodent Diet Cubes Rat & Mice Food* and 5 grams of chopped fruit was weighed out per rat and placed into three small plastic containers for the rats to ensure there was more enough food available to the rats throughout the training process and each session.

Both the scent and return trainings were based on previously studied rat training sessions and rat training protocols described in Gonzalez (2022) and the La Londe, et al. (2015) study. All three rats were trained together at once and all training sessions had written records taken, recording the type of session done and the behaviors exhibited and time it took each rat to complete the sessions.

3.3.1 Scent Training

The scent training took place in five stages, using a volatile organic compound (VOC) to mimic the scent of decomposition in the body. VOCs can be used as an alternative when training biological detection animals. While human remains are the best option to use when training, they are not easily accessible. Manufactured VOCs that mimic scents found during human decomposition are the easiest to train animals with (Simon et al., 2020). The Stage 1 started on a ~5ft x 6ft tabletop location in a group setting. The VOC solution was made by Dr. Robert Powers at the University of New Haven and it was a 1:10,000,000 methyl sulfide, methyl disulfide, dimethyl trisulfide concentrated solution. This concentration was used based on previous studies of a concertation detectable by *R. norvegicus* and levels of the scent found in early decomposition (Gonzalez, 2022; Vass et al., 2004). The VOC was dripped onto a sterile swab until the swab was saturated and placed into a sealed urinalysis container with drilled holes in the side so the rats would be able to smell it without having direct access to the swabs. The container with the VOC was kept in a refrigerator (~4ºC) when not in use and the swabs were changed out weekly to keep the scent from dissipating. Food was placed near the container as the positive reinforcement to the smell. The rats were released from the carrier at the sound of a click from a clicker near the food and the smell. The extra sound of the clicker reinforced the food association to the scent training better than with food alone as described in Feng et al. (2016). This extra sound helps to motivate the rats in the future (APOPO, n.d.). Clicker training, scent training without the scent, was done until the scent was manufactured.

The Stage 2 began after two days of Stage 1. In this stage the rats were moved 6 inches away from the food placed near the scent container. The rats were then allowed to leave the carrier at the sound of a clicker and expected to find the food near the VOC. The rats were left to

eat for five minutes, then removed from the food and scent when the time was up and given a five-minute break. This was repeated three times each session. Every two days, 6 more inches of distance is added between the rats and the food.

The Stage 3 took place once the rats could navigate a whole front table. The training was moved to large upstairs room (Appendix 4 and 5). This room had 4 stepped areas which were divided into 3 levels. Level 1 was the smallest blocked off area, which created a small hallway-like area with three lab benches where all exits were blocked off with wooden boards (Fig. 3.3). The same training methods from the second stage was used.

The Stage 4 took place when the rats' training improved and became consistent in Level 1 and consisted of a larger environment (Fig. 3.3). This Level 2 environment contains two steps and 6 lab benches that had to be maneuvered around and all exits were blocked from the rats with wooden boards. The same training from the second stage was used.

The Stage 5 took place when the rats' training improved and became consistent in Level 2 and consisted of an entire room environment (Fig. 3.3). This Level 3 environment contained 4 stepped areas and 7 lab benches. The same training from the second stage was be used.

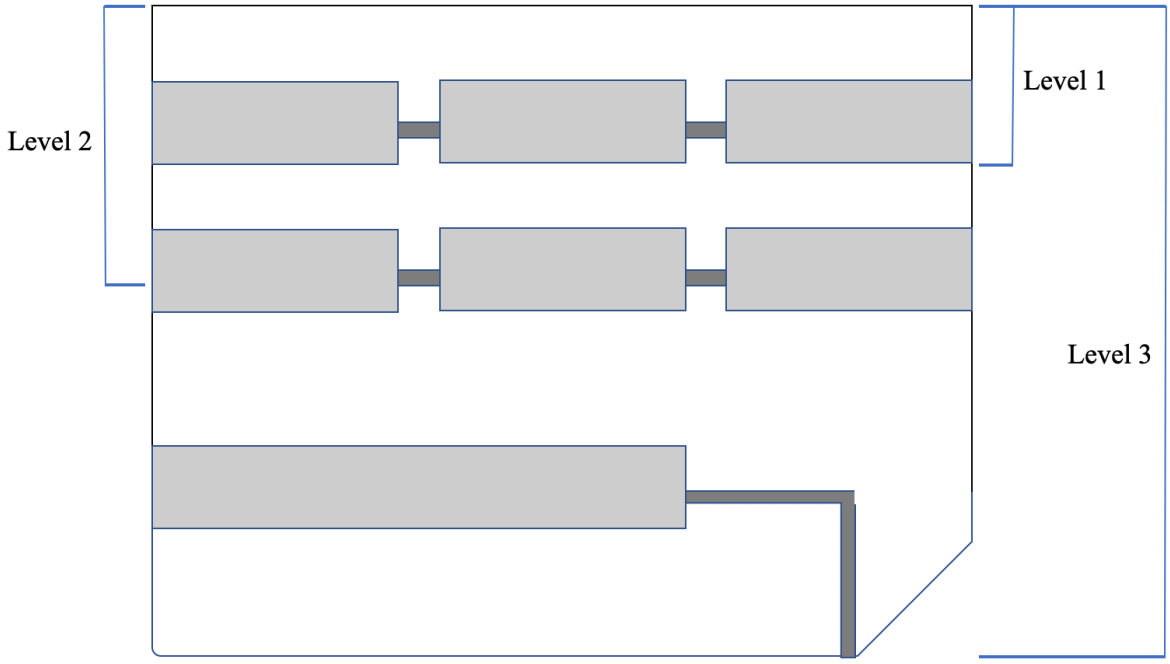

Figure 3.3: A layout of the latter stages of training. Level 1 was an upper level. The dark gray indicates steps that were blocked off per level. The light gray are benches. For each level the area became bigger and the exits were blocked with wooden boards. Figure created by Gabrielle Johnston

3.3.2 Return Training

The return training took place by the handler using a whistle. Like the scent training, the return training took place in five stages. Stage 1 started on a tabletop location in a group setting. Food was placed on the tabletop as the positive reinforcement and the rats were placed on the food. A whistle was blown for two seconds every 30 seconds for the five minutes while the rats were eating. The clicker during scent training helped to associate leaving the carrier to find the scent, while a different noise—a whistle—was used as the sound to call the rats to return to the handler. This tabletop was used to teach the rats the association of the whistle and food. The whistle was the signal that the rats associated with returning to the trainer.

The Stage 2 began after two days of Stage 1. In this stage, the rats were moved 6 inches away from the food near the handler. The whistle was blow and it was expected for the rats to return to the handler with the food. The rats were left to eat for five minutes, then taken away from the food and given a five-minute break. This was repeated three times each session. Every two days, 6 more inches of distance was added between the rats and the food

Stage 3 took place once the rats could navigate the whole front table. The training was moved the same Level 1 as described in the scent training (Fig. 3.3). The same training methods from the second stage was used.

Stage 4 took place when the rats' training improved and became consistent in Level 1 and consisted of a larger environment at Level 2 (Fig. 3.3). The same training from the second stage was used.

Stage 5 took place when the rats' training improved and became consistent in Level 2 and consisted of an entire room environment or Level 3 (Fig. 3.3). The same training from the second stage was be used.

In the later stages of both the scent and return trainings—the third through fifth stage—when the rats have had continuous successes in the training sessions, the rats were tested to see if they were searching for the food or the associated scents and sound. To test if the association would work without a reward, a fourth five-minute session was added to the training with the positive reinforcement taken away to see if the rats reacted the same way to the session as they would with the food there.

3.3.3 Ultrasonic Vocalization Monitoring

Two Ultrasonic Vocalization detectors were purchased. The Titley Scientific© Anabat Walkabout was purchased for handheld use, and the Titley Scientific© Chorus was purchased for continuous sound monitoring. The two monitoring systems were used to record the ultrasonic vocalizations of the rats.

The Anabat Walkabout and the Chorus are both ultrasonic detecting instruments. Designed to detect bats, the instruments were used to detect ultrasonic frequencies of the rats. The Chorus is a detector that record frequencies at a trigger range around the clock during training sessions (Broken-Brow & Thompson, 2021). The trigger range is a frequency range that is programed into the Chorus in order to selectively record a desired range of frequencies. In these sessions the trigger range was set between 10-140kHz. The Chorus is an omnidirectional ultrasonic microphone that can record 10-140kHz frequencies up to 100m away and can record for ~400 hours of battery life. It was set on a tripod in the middle of the room during training to detect any vocalization the rats may have made (Fig.3.4).

Figure 3.4 The Chorus set up on a tripod in the middle of training environment. Image taken by Gabrielle Johnston

The Anabat Walkabout is a detector that is handheld and visually shows frequencies detected in real time on a screen while recording (Broken-Brow & Thompson, 2022). It has a full color LCD touch screen which displays the full spectrum in real time, allowing for real time visualizations of any vocalizations (Fig, 3.5). An assistant stood by the trainer for the duration of

rat training and pointed the ultrasonic microphone at the rats during the training and watched for any vocalizations during training sessions in the mornings.

Figure 3.5 Screen of the Walkabout showing a spectrum from a tickling session. Image taken by Gabrielle Johnston

Both the Anabat Walkabout and the Chorus were used to not only record training sessions but also to record rat tickling sessions for testing the sensitivity and adjusting other software configurations.

3.4 Data Collection

All the training sessions were recorded with written records initialed by the trainer. As the rats were training, a log was kept detailing how long each rat took to complete each type of training session, which rat completed the objective first and how long it took each rat to reach the goal of the session, how long each rat ate, as well as outlying behaviors they exhibited during the session. Along with data logs for training sessions, separate logs were kept each day detailing the type of training session and which handler performed it. A log was also kept to record the rats health, when the cage bedding was changed and cleaned, and when the animals food was changed.

3.5 Previous Testing

Long-Evans rats in the previous study were first trained using operant conditioning, which did not produce favorable results, so the training was switched to classical conditioning; yielding better responses (Gonzalez, 2022). When switching to classical conditioning using positive reinforcement, the rats were not interested in eating any food after training had already started, due to not being hungry. Consequently, the food intake of the rats had to switch from ad lib to being fed during and after trainings. This improved the responsiveness of the rats (Gonzalez, 2022). These initial learnings allowed this project to start with using classical conditioning. In addition, the food intake was adjusted earlier to prevent a lack of motivation for the rats.

The rats in the previous study were also tested to see if they would be able to detect VOCs commonly used for the scent of decomposition using a Skinner Box, and they reacted

positively to being able to detect the VOCs, therefore, the testing will not be duplicated (Gonzalez, 2022).

3.6 Completion of Testing

Testing was completed a year after arrival of the rats. The rats arrived at the Center for Forensic Wildlife Research on 22 February 2022. After the rats were acclimated, training began 7 April 2022. Training completed on 23 February 2023.

3.6.1 Disposition of the Animals

After completion of the study, the rats were then retired then rehomed to an appropriate caregiver. The rats were rehomed together, as the rats had been living together since birth, and separating them could cause them unnecessary stress and anxiety.

3.6.2 Data Storage

All the data collected was transcribed onto Microsoft Excel© spreadsheets. All logs, data collection notebooks, and copies of the Excel spreadsheets were stored at the Center for Forensic Wildlife Research. The data collected from the Anabat Walkabout and the Chorus were downloaded to the laboratory computers at the Center for Forensic Wildlife Research.

3.6.3 Statistical Analysis

The data recorded from the Anabat Walkabout and the Chorus were analyzed using Titley Scientific's© Anabat Insight Software. All the data collected was uploaded to a laboratory

computer analyzed using VSN International GenStat® Ver 22. The data underwent analyses of variance (one-way and two-way ANOVA) and linear regression analyses.

Chapter 4: Results

This chapter shows the results of statistical analysis of this study. It contains the results collected during training and results collected of rat vocalizations.

4.1 Vocalization Results

Vocalization data recorded from scent, clicker, or return training was not recorded on either bat detector. The vocalizations detected and recorded during rat tickling sessions were statistically analyzed for each rat. The recording of the ultrasonic vocalizations of each rat was recorded and analyzed in Titley Scientific's© Anabat Insight Software. The vocalizations were taken during tickling to analyze the frequency range of each rat. The spectrograms created from each session were analyzed to find the overall average vocalization frequency (Fmean), maximum average vocalization frequency (Fmax), and minimum average vocalization frequency (Fmin) for each rat (Fig. 4.1).

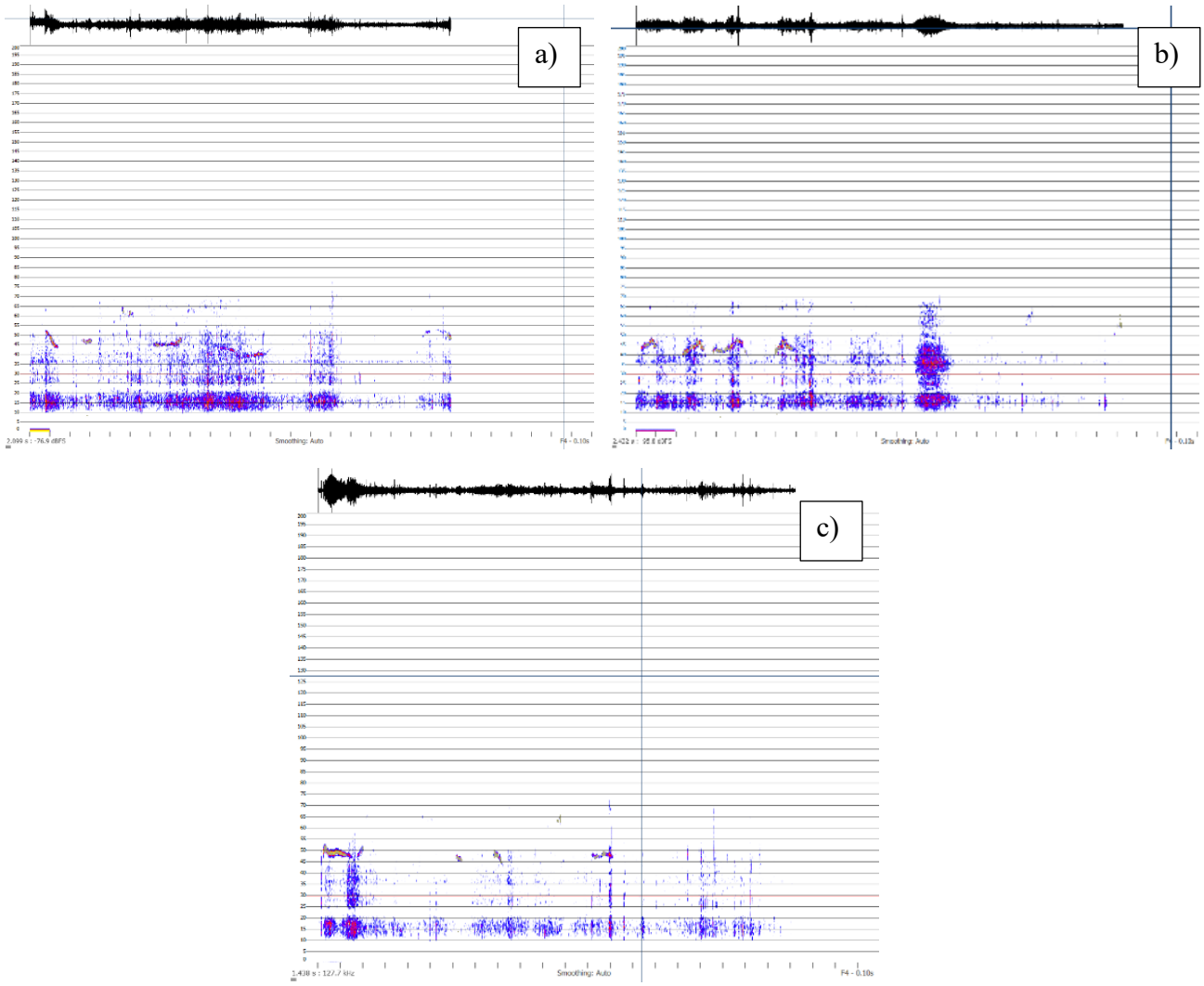

Figure 4.1 Ultrasonic Vocalization Spectrograms created from Anabat Insight a). Spectrogram from Kelly b). Spectrogram from Simjouw c). Spectrogram from Zajac

4.1.1 Fmean Results

There was no difference in Fmean between Simjouw and Zajac (Fig 4.2). There was also no difference in Fmean between Kelly and Zajac. There was a difference in Fmean between Kelly at 54.143 kHz and Simjouw at 48.530 kHz, with Kelly having a higher frequency than Simjouw.

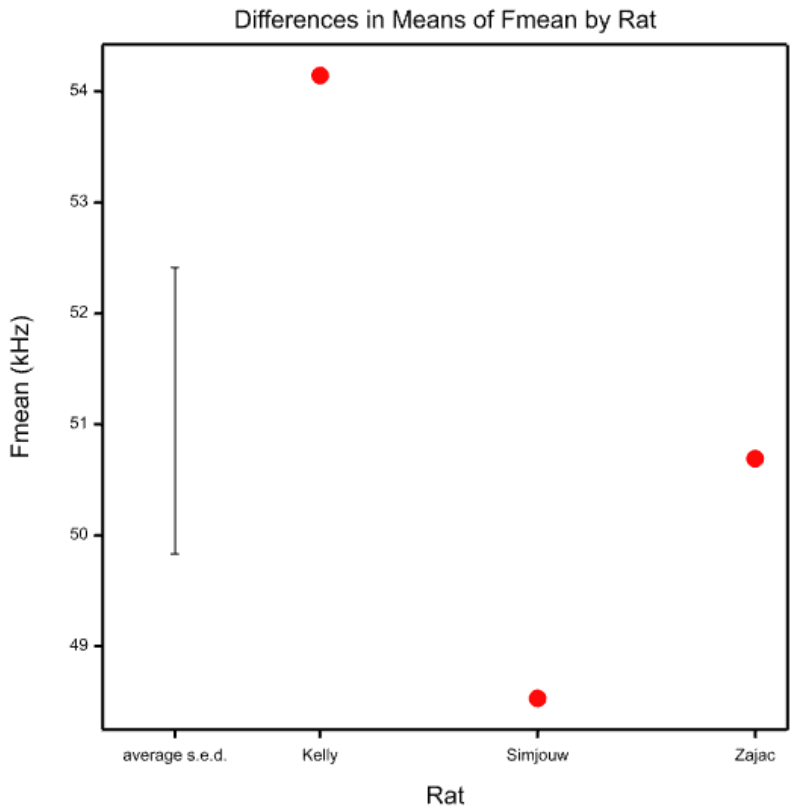

Figure 4.2 Differences in means of the average frequency in kHz of rat vocalizations (ANOVA, $F_{(2,42)} = 3.01$, $p = 0.060$, s.e.d = standard error of differences)

4.1.2 Fmax Results

There was no difference in Fmax between Kelly and Zajac (Fig 4.3). There was also no difference in Fmax between Zajac and Simjouw. Kelly had a higher Fmax frequency at 55.168 kHz than Simjouw at 47.655 kHz.

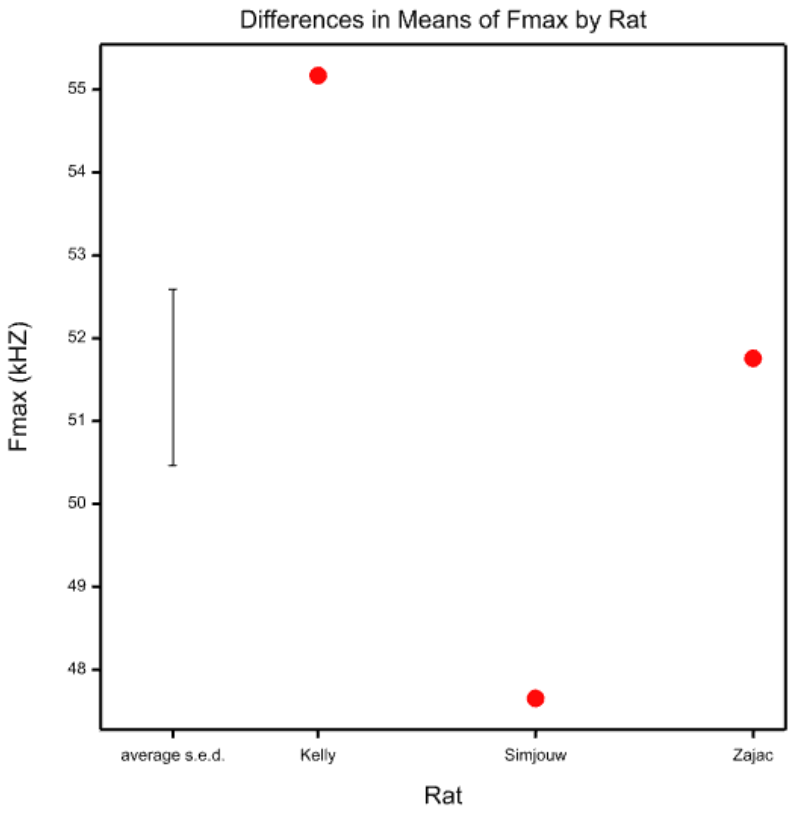

Figure 4.3 Differences in means of the maximum frequency in kHz of rat vocalizations (ANOVA, $F_{(2,42)} = 7.64$, $p < 0.001$, s.e.d = standard error of differences)

4.1.3 Fmin Results

There was no difference of Fmin between Simjouw and Zajac or Zajac and Kelly. (Fig 4.4). There was a difference of Fmin between Simjouw at 45.905 kHz and Kelly at 52.758 kHz.

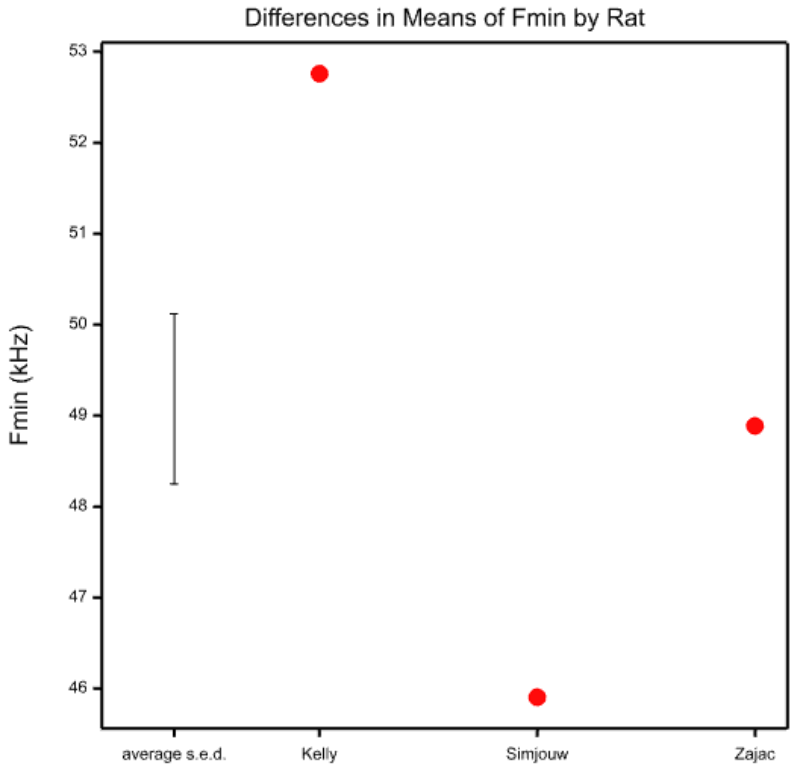

Figure 4.4 Differences in means of the minimum frequency in kHz of rat vocalizations (ANOVA, $F_{(2,42)} = 8.40$, $p < 0.001$, s.e.d = standard error of differences)

Across Fmean, Fmin and Fmax there was no difference between the frequencies of Simjouw and Zajac or the frequencies of Zajac and Kelly. However, there was a difference in frequencies across Fmean, Fmin, and Fmax for Simjouw and Kelly, with Kelly's frequencies being higher.

4.2 Type of Training Results

To interpret how the rats performed in each type of training—scent, return, and clicker—statistical analysis took place. A two-way ANOVA was conducted to evaluate the differences between each type of training and section of training—departure, feeding, resting, and return.

“Departure” refers to the time it took for the rats to leave the carrier once training started in clicker and scent training. “Feeding” refers to the time the rats spent with the trainer or the scent. “Return” refers to the time it took for the rats reach the objectives during training, either to return to the trainer during return training or reach the scent in scent training. “Resting” refers to the amount of time the rats did not spend with the trainer or the scent.

4.2.1 Departure Results

There was no difference in scent training or clicker training departure times for any of the rats (Fig 4.5). There was no difference between Kelly’s sand Simjouw’s scent training departure time. However, there was a difference between Kelly’s scent training departure at 7.497 seconds, and Zajac’s scent training departure at 23.570 seconds, Kelly being faster at departing. There was also a difference between Simjouw’s scent training departure time and Zajac’s, with Simjouw departing much faster on average than Zajac.

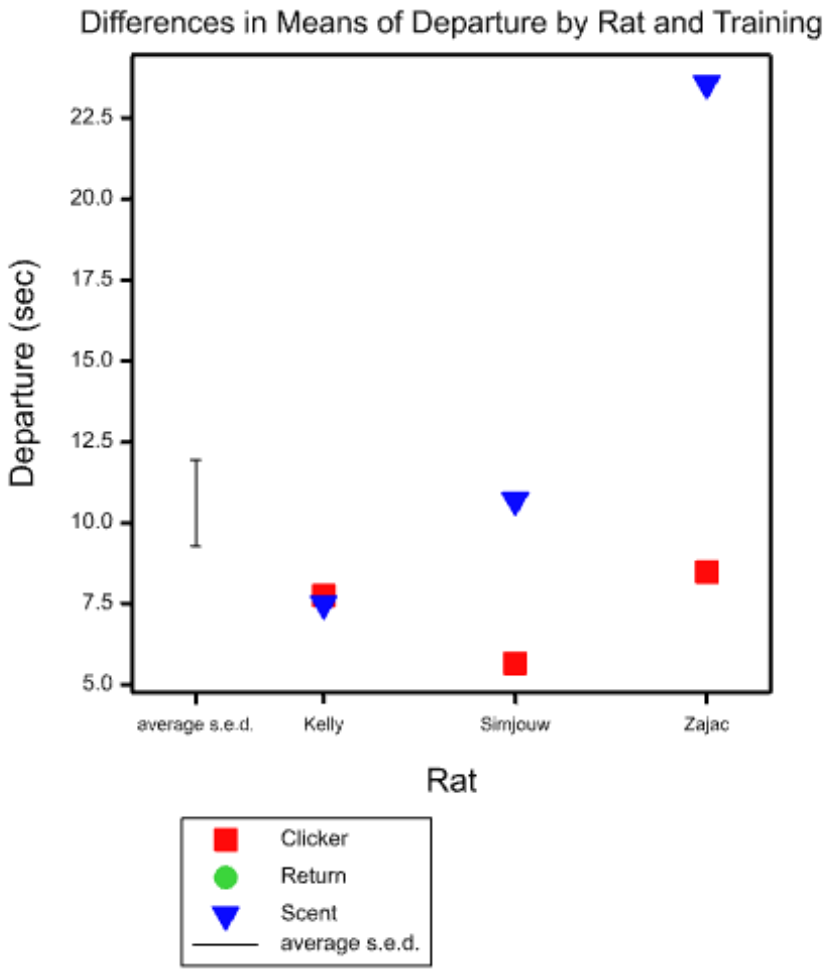

Figure 4.5 Differences in means of departure by rat and training type (ANOVA, $F_{rat(2,2204)} = 35.12$, $p < 0.001$; $F_{training(1,2204)} = 17.73$, $p < 0.001$, s.e.d = standard error of differences)

4.2.2 Feeding Results

There was a difference between Kelly's feeding time for clicker training at 240.768 seconds and scent training, at 204.435 seconds (Fig 4.6). However, there was no difference between Kelly's feeding time for clicker training and return training or between the feeding time for scent training and return training.

For Simjouw, there was no difference between the feeding time for clicker training and return training. However, there was a difference between Simjouw's feeding time for return

training and scent training taking about 30 seconds longer for feeding in return training than scent training. There was also a difference between the time for clicker training, at 268.530 seconds, and scent training at 223.918 seconds for Simjouw.

There was a difference between the time feeding for clicker training and return training. There was a large difference between the time for clicker training at 257.994 seconds and scent training at 201.205 seconds for Zajac, which was almost a minute longer during clicker training. There was also a ~30 second difference between the time for return training and scent training for Zajac.

For clicker training, there was no difference between Kelly's and Zajac's time nor between Simjouw's and Zajac's time. There was a difference between Kelly's time at 240.768 seconds and Simjouw's time at 268.530 seconds.

During scent training, there was no difference between Kelly's time and Zajac's time. Kelly's time was about 20 seconds longer than Simjouw's time feeding during scent training. Simjouw took about 20 seconds longer than Zajac feeding during scent training as well.

For return training, there was a difference between Kelly's time at 222.864 seconds and Simjouw's time at 253.818 seconds, but there was no difference between Kelly's and Zajac's time. There was a difference between Zajac's feeding time at 233.030 seconds and Simjouw's time at 253.818 seconds.

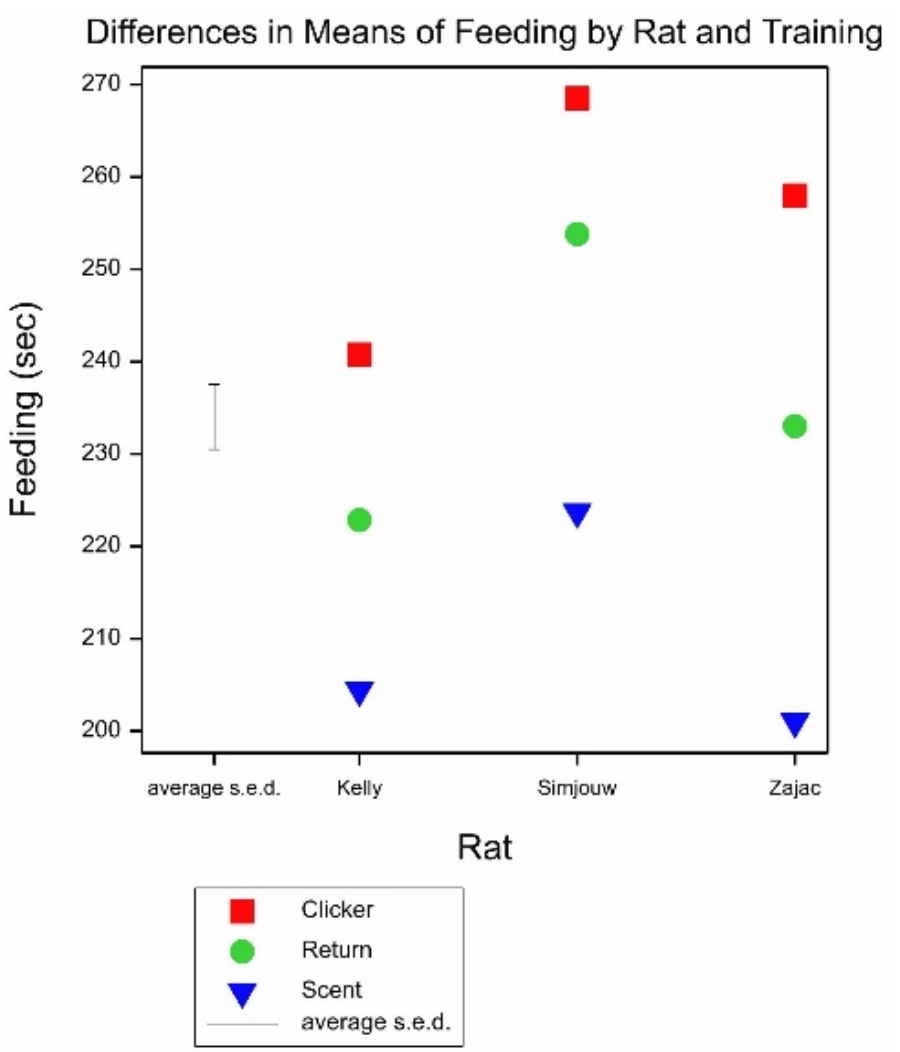

Figure 4.6 Differences in means of feeding by rat and training type (ANOVA, $F_{rat(2,4721)} = 35.00$, $p < 0.001$; $F_{training(2,4721)} = 62.48$, $p < 0.001$, s.e.d = standard error of differences)

4.2.3 Resting Results

For Kelly, there was no difference in time resting between clicker training and return training or between return training and scent training (Fig 4.7). However, Kelly rested for ~35 seconds longer during scent than during clicker training.

There was no difference in time between Simjouw's clicker training and return training. Simjouw did rest for almost 50 seconds longer during scent training than clicker training.

Simjouw rest for almost 40 seconds longer during scent training compared to return training as well.

For Zajac, there was a difference in resting time between clicker training at 41.476 seconds and return training at 66.505 seconds. Zajac also rested for most a minute longer during scent training than clicker training. There was also a ~30 second difference in time between return training at 66.505 seconds and scent training at 99.079 seconds for Zajac.

For clicker training, there was no difference in time between neither Kelly's and Zajac's time nor between Simjouw's and Zajac's time. However, there was a difference between Kelly's time at 58.387 seconds and Simjouw's time at 30.863 seconds.

For return training, there was once again no difference in time between Kelly's time and Zajac's time nor Simjouw's time and Zajac's time but there was a ~30 second difference of resting time between Kelly at 76.747 seconds and Simjouw at 45.923 seconds.

For scent training, there was no difference in time between Kelly's, Simjouw's, or Zajac's time.

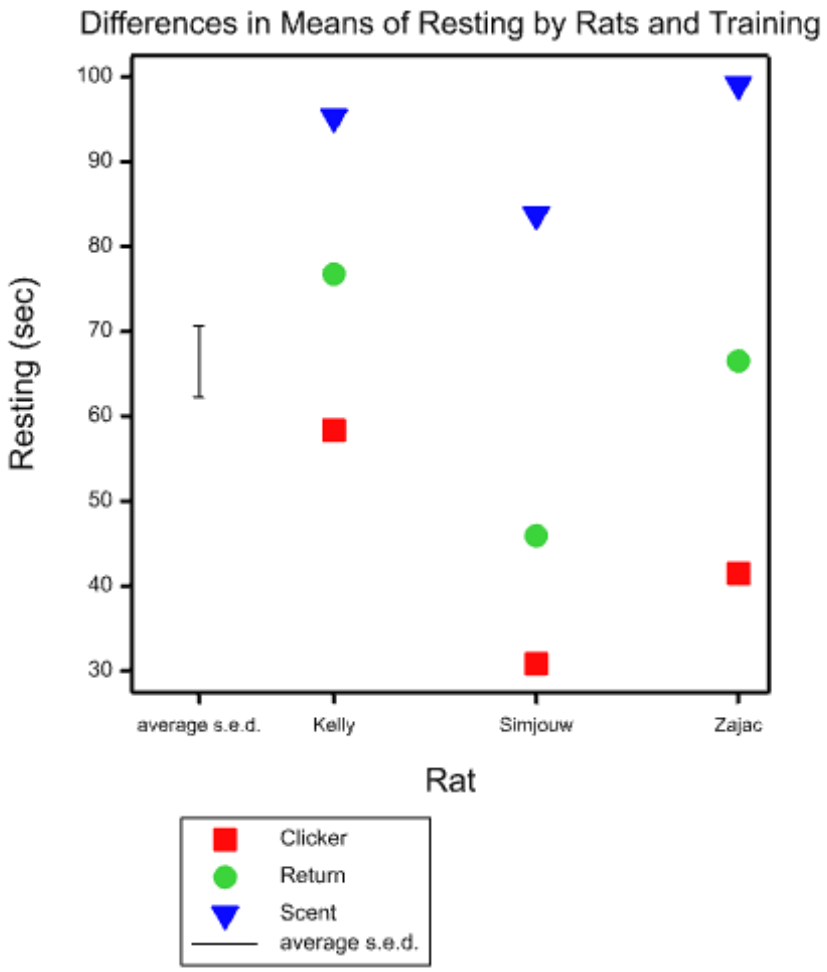

Figure 4.7 Differences in means of resting by rat and training type (ANOVA, $F_{rat(2,4723)} = 19.41$, $p < 0.001$; $F_{training(2,4723)} = 51.99$, $p < 0.001$, s.e.d = standard error of differences)

4.2.4 Return Results

For Kelly, there was no difference in time between any training type (Fig 4.8).

However, for Simjouw, there was a difference in time between return results for clicker training at 156.155 seconds and return training at 130.143 seconds. There was also a ~10 second difference Simjouw's return training at 130.143 seconds and scent training at 149.095 seconds f. There was no difference in time between clicker training and scent training for Simjouw.

Zajac had no difference in return time between neither clicker training and return training nor between clicker training and scent training for Zajac. There was a difference in time between return training at 131.605 seconds and scent training at 153.268 seconds for Zajac.

For clicker training, there was a difference of about 20 seconds between Kelly's time at Simjouw's time, with Simjouw taking about 20 seconds longer to return. There was no difference between Kelly's time and Zajac's time. There was also no difference between Zajac's time and Simjouw's time.

For both return training and scent training, there was no difference between Kelly's time, Simjouw's time, and Zajac's time.

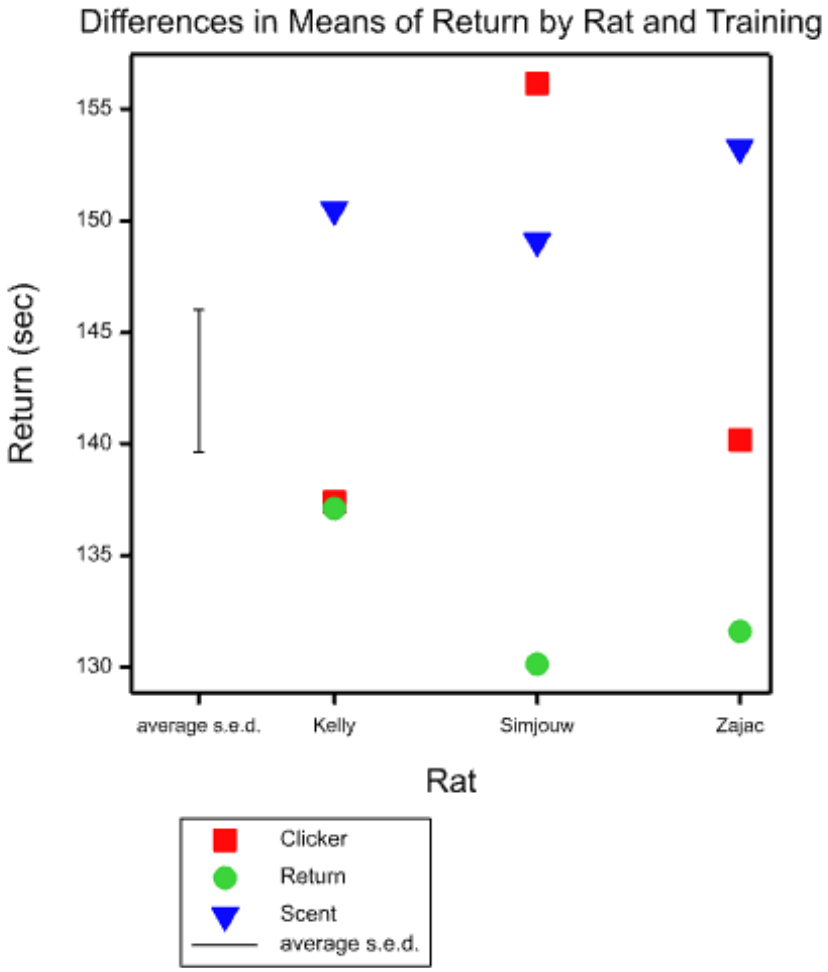

Figure 4.8 Differences in means of return by rat and training type (ANOVA, $F_{rat(2,5279)} = 0.53$, $p = 0.586$; $F_{training(2,5279)} = 24.29$, $p < 0.001$, s.e.d = standard error of differences)

4.3 Training Environment Results

To interpret how the rats did in training in increasingly larger environments, statistical analysis took place. A two-way ANOVA was conducted to evaluate the differences in how each rat did in regard to each type of training in each level of training. The first level was the Front Table (FT), followed by Level 1 (L1), then Level 2 (L2), and then Level 3(L3).

4.3.1 Departure Results

There was no difference in time between FT and L1, L2, or L3 for Kelly's results (Fig 4.9). However, Kelly was 10 seconds faster at departing in L1 than L3. Kelly also departed about 10 seconds faster in L2 than in L3.

For Simjouw, there was no difference in time between FT and L1 or L2. There was a difference in time between FT at 6.600 seconds and L3 at 19.845 seconds for Simjouw. Simjouw was faster at departing in L1 than in L3 by ~15 seconds, and faster at departing in L2 than L3 by about ~15 seconds as well.

There was no difference in Zajac's departure time between FT and L1. However, Zajac did depart faster in FT than L2 and L3. There was a difference in Zajac's time between L1 at 13.648 seconds and L3 at 32.900 seconds for Zajac. There was also a difference in time between L2 at 18.056 seconds and L3 at 32.900 seconds for Zajac.

During the FT stage, there was no difference in time between Kelly, Simjouw and Zajac.

For L1, there was no difference in time between Kelly and Simjouw. However, Kelly departed faster than Zajac by ~10 seconds. Simjouw also departed faster than Zajac during L1.

For L2, there was no difference in time between Kelly and Simjouw. There is a difference in time between Kelly's departure at 3.139 seconds and Zajac's at 18.056 seconds. Simjouw was about ~12 seconds faster at departing than Zajac at this level.

There was no difference in time between Kelly and Simjouw during L3. However, Kelly was faster at departing than Zajac. Simjouw was also faster than Zajac at departing by ~22 seconds.

For all four training levels, there was no time difference between Kelly and Simjouw.

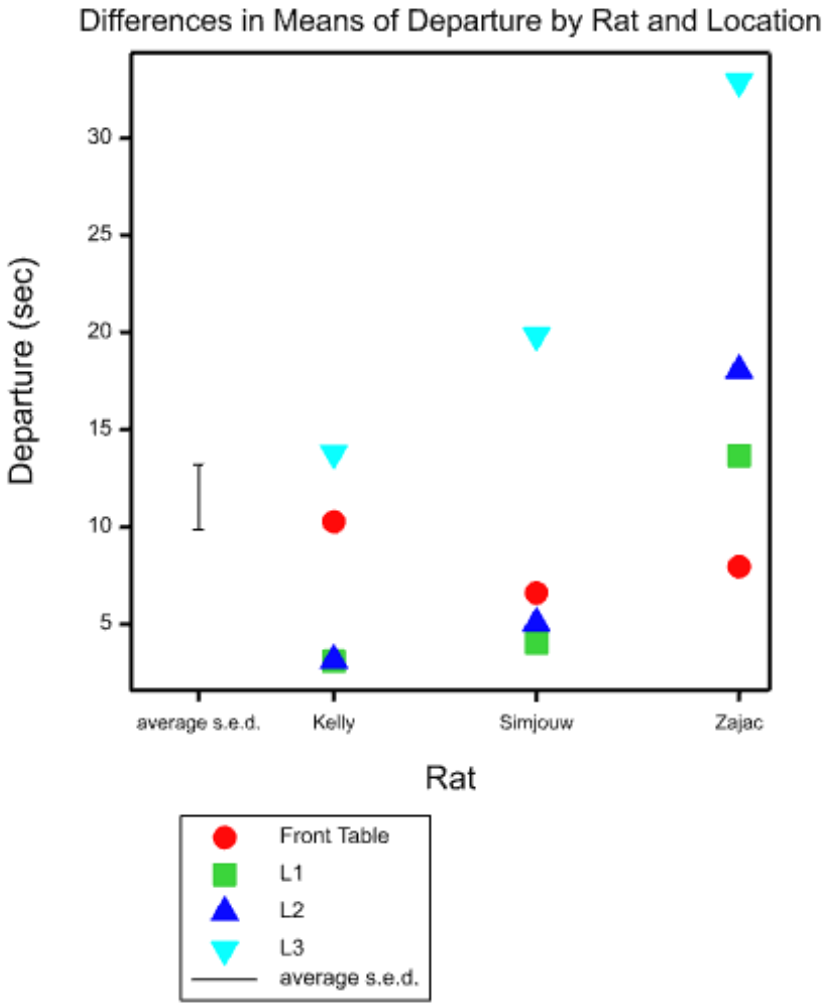

Figure 4.9 Differences in means of departure by rat and training location (ANOVA, $F_{rat(2,2198)} = 36.38$, $p < 0.001$; $F_{location(3,2198)} = 33.12$, $p < 0.001$; s.e.d = standard error of differences)

4.3.2 Feeding Results

Kelly took about 1 minute longer feeding for FT than L1 (Fig 4.10). There was also a difference in time between FT at 262.042 seconds and L2 at 228.255 seconds for Kelly. There was also a difference in time between FT at 262.042 seconds and L3 at 198.415 seconds for Kelly. There was also a difference in time between L1 at 202.955 seconds and L2 at 228.255 seconds for Kelly. There was no difference in time between L1and L3 for Kelly. There was a difference in time between L2 at 228.255 seconds and L3 at 198.415 seconds for Kelly.

For Simjouw, there was no difference in time between FT and L1 or between L1 and L2. Simjouw fed for longer at FT than L2 or L3. There was a difference in time of feeding L1 at 254.802 seconds and L3 at 230.943 seconds for Simjouw. There was no difference in time between L2 and L3 for Simjouw.

Zajac fed for longer at FT than L1, L2, and L3. There was no difference in time between L1 and L2 for Zajac. However, Zajac fed for ~35 seconds longer at L1 than L3. Zajac also fed for ~30 seconds longer at L2 at 230.341 than L3.

For FT and L2, there was no difference in time between Kelly, Zajac, and Simjouw.

Kelly fed for about 50 seconds longer than Simjouw during L1. Kelly also fed for about 30 seconds longer than Zajac. There was a difference in time for feeding between Simjouw at 254.802 seconds and Zajac at 233.037 seconds at L1.

For L3, Kelly fed for about 30 seconds longer than Simjouw at 230.943 seconds, but there was no difference in time between Kelly and Zajac. Zajac fed for about ~30 seconds less than Simjouw during L3.

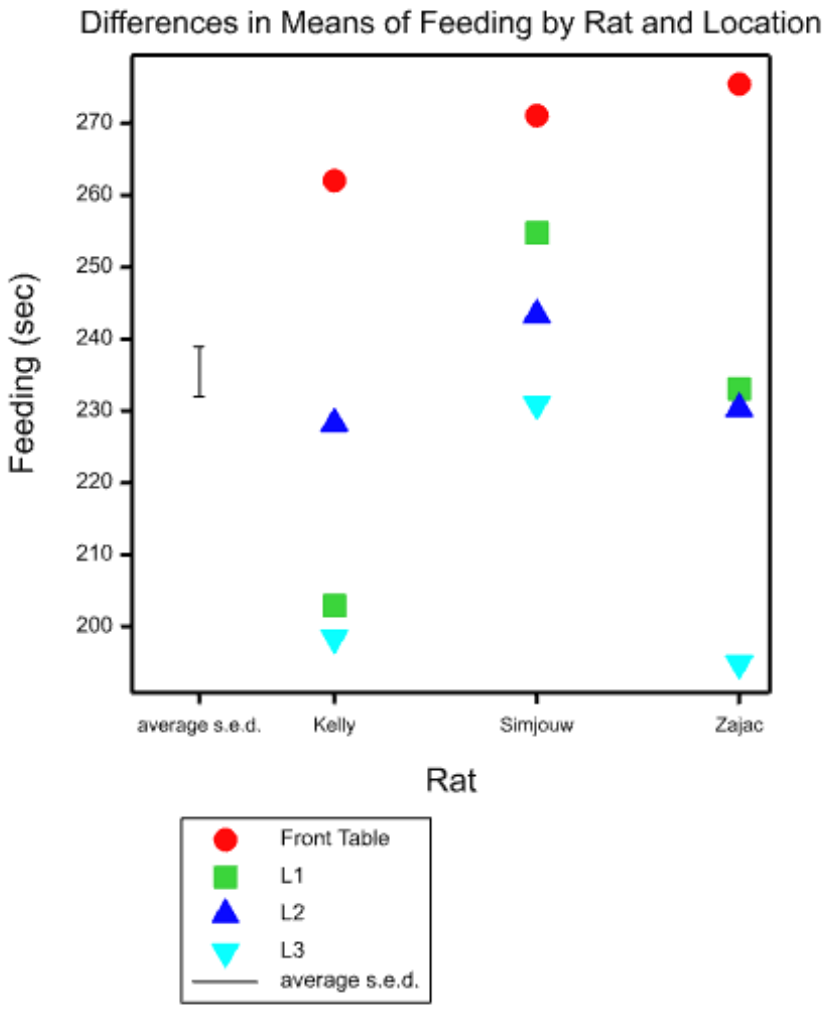

Figure 4.10 Differences in means of feeding by rat and training location (ANOVA, $F_{rat(2,4718)} = 36.21$, $p < 0.001$; $F_{location(3,4718)} = 88.60$, $p < 0.001$, s.e.d = standard error of differences)

4.3.3 Resting Results

Kelly rested longest at FT compared to L1, L2 and L3 (Fig 4.11). There was a difference in time between L1 at 96.255 seconds and L2 at 71.594 seconds for Kelly. There was no difference in time between L1 and L3 for Kelly. There was a difference in time between L2 at 71.594 seconds and L3 at 101.213 seconds for Kelly.

Simjouw had no difference in time between FT and L1 or L2 and L3. Simjouw was resting ~30 seconds longer in L2 than FT. There was also a ~30 second difference in time

between FT at 28.996 seconds and L3 at 68.425 seconds for Simjouw. Simjouw rested fro linger in L2 than L1, and rested longer in L3 than L1

Zajac rested for the shortest amount of time in FT compared to L1, L2, and L3. The greatest difference in time for Zajac was between FT at 24.270 seconds and L3 at 105.364 seconds. There was no difference in time between L1 and L2. However, at L1 Zajac was about 40 seconds longer than at L3. Zajac was also about 30 second longer at L3 than L2.

For FT and L2, there was no difference in time between Kelly, Simjouw and Zajac.

During L1, t Kelly was resting longer than both Simjouw and Zajac. There was also a difference in time between Simjouw at 45.412 seconds and Zajac at 66.177 seconds.

For L3, there was a difference in time between Kelly at 101.213 seconds and Simjouw at 68.425 seconds. Zajac rested about 40 seconds longer than Simjouw at L3. There was no difference in resting time between Kelly and Zajac.

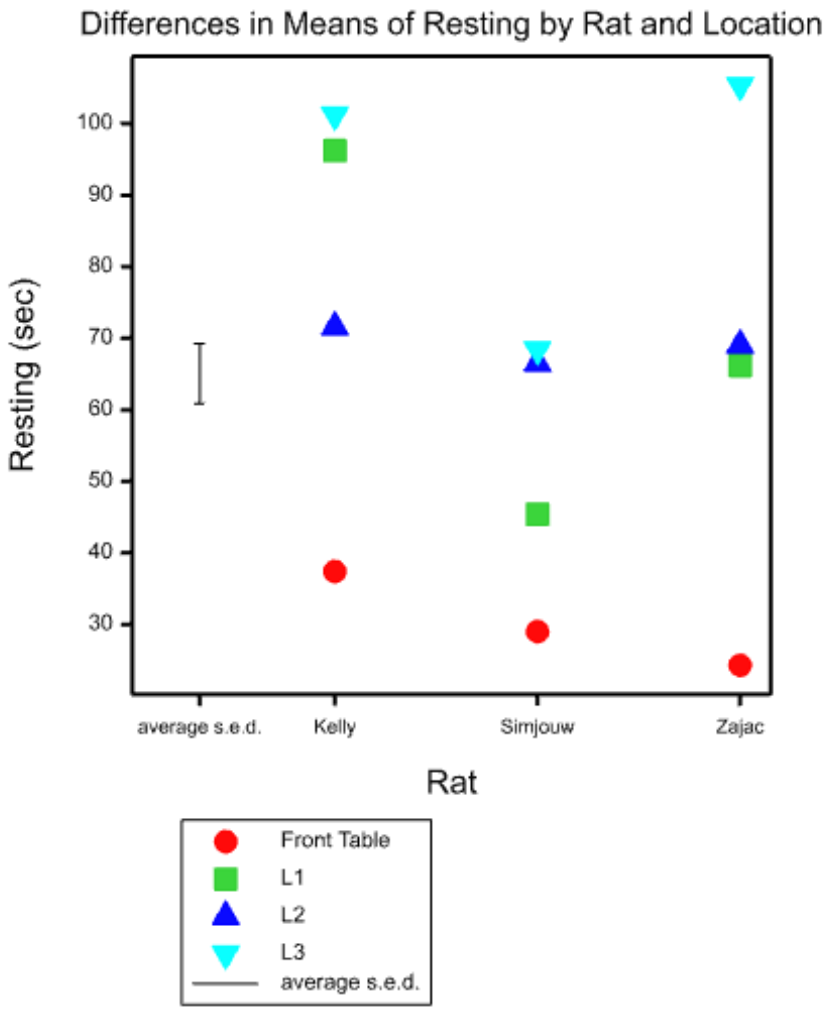

Figure 4.11 Differences in means of resting by rat and training location (ANOVA, $F_{rat(2,4720)} = 19.81$, $p < 0.001$; $F_{location(3,4720)} = 60.72$, $p < 0.001$,, s.e.d = standard error of differences)

4.3.4 Return Results

Kelly returned fastest at FT compared to L1, L2, and L3. at 154.361 seconds (Fig 4.12). There was no difference in time between L1, L2, or L3 for Kelly.

For Simjouw, FT return time took about 70 seconds faster than L1, and ~70 seconds faster than L3. There was an ~80 second difference in return time between FT at 77.299 seconds and L2 at 156.825 seconds for Simjouw. As it was for Kelly, there was no difference in time between L1, L2 or L3 for Simjouw.

Zajac also retuned fastest at FT compared to L1, L2 and L3 seconds for Zajac. There was no difference in time between L1, L2 or L3 for Zajac.

For FT, L1, L2, and L3, there was no difference in time between Kelly, Simjouw and Zajac.

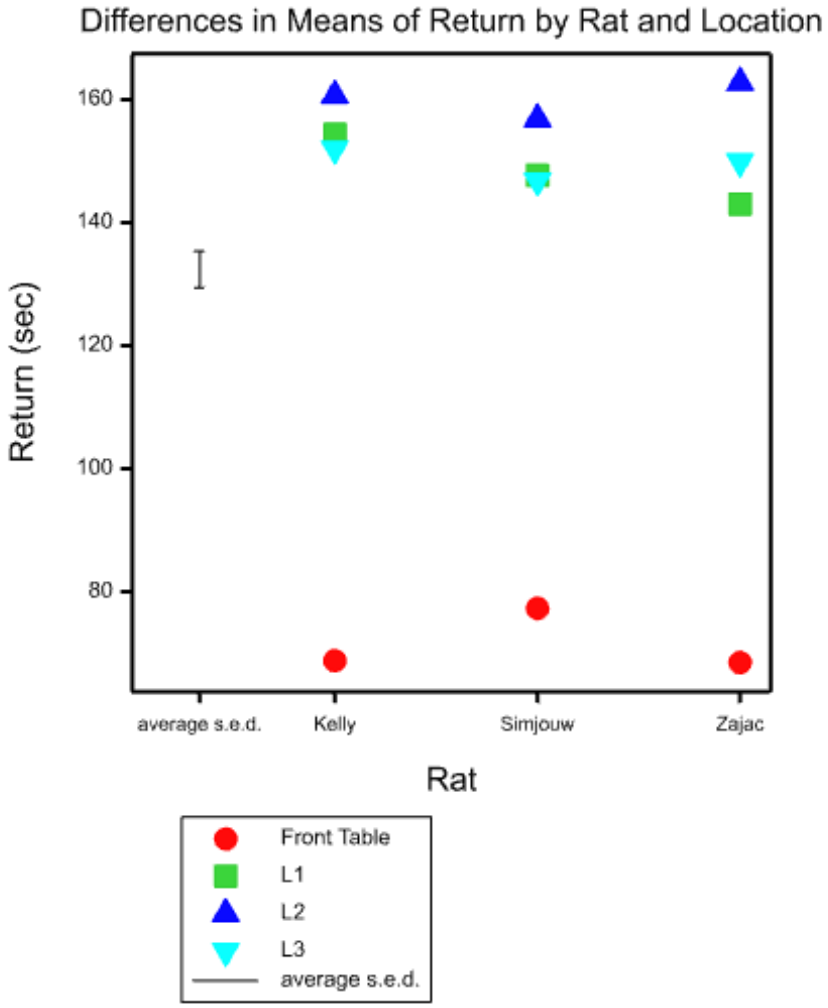

Figure 4.12 Differences in means of return by rat and training location (ANOVA, $F_{rat(2,5276)} = 0.60$, $p = 0.548$; $F_{location(3,5276)} = 244.81$, $p < 0.001$, s.e.d = standard error of differences)

4.4 Training Reward Results

To interpret how well the rats did in sessions with a reward versus without a reward, statistical analysis took place. A two-way ANOVA was conducted to see the differences in how well each rat responded to training with or without reward during each part of training.

4.4.1 Departure Results

For Kelly, there was no difference in time between with a reward and without a reward (Fig 4.13). Simjouw departed faster with a reward at 7.620 seconds and without a reward at 20.972 seconds. Zajac also departed faster with a reward at 18.435 seconds and without a reward at 30.356 seconds.

With a reward, there was no difference between Kelly's and Simjouw's time. However, Kelly departed faster than Zajac with a reward. Simjouw departed faster by ~10 seconds than Zajac.

For without a reward, there was no difference between Kelly's and Simjouw's times. Zajac was slower at departing without a reward than both Kelly and Simjouw.

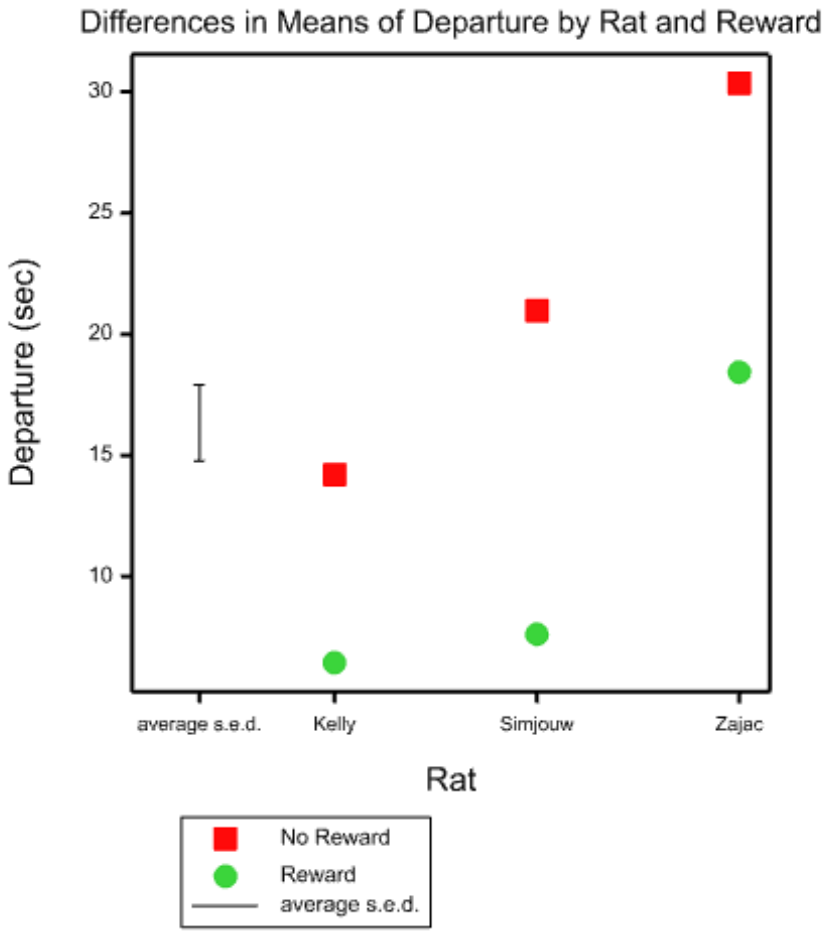

Figure 4.13 Differences in means of departure by rat and reward (ANOVA, $F_{rat(2,2204)} = 35.15$, $p < 0.001$; $F_{reward(1,2204)} = 34.35$, $p < 0.001$, s.e.d = standard error of differences)

4.4.2 Feeding Results

For Kelly, Zajac and Simjouw there was a large difference in time between feeding with a reward and without a reward. (Fig 4.14). Each rat was at the objective for 220 seconds longer when a re reward was present.

When a reward was present there was no difference between Kelly's, Zajac's and Simjouw's times. Without a reward, there was no difference in time between Kelly's, Simjouw's, and Zajac's time.

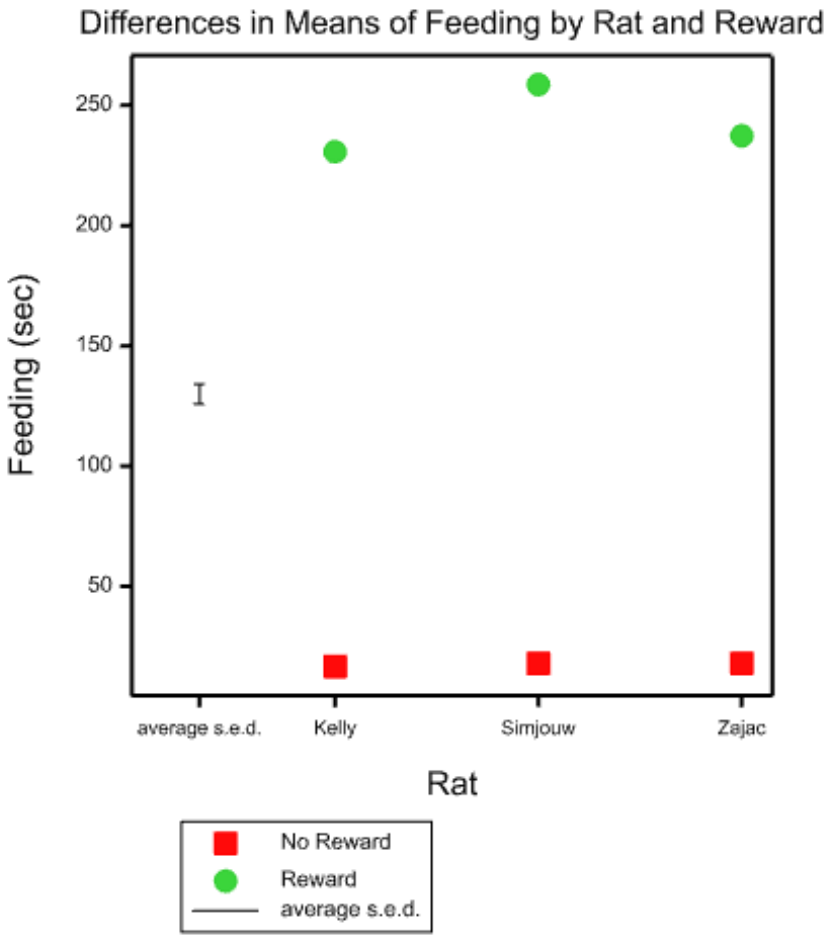

Figure 4.14 Differences in means of feeding by rat and reward (ANOVA, $F_{rat(2,4724)} = 48.66$, $p < 0.001$; $F_{reward(1,4724)} = 2015.68$, $p < 0.001$, s.e.d = standard error of differences)

4.4.3 Resting Results

Kelly, Simjouw, and Zajac rested for much longer when no reward was present than when it was present (Fig 4.15).

With a reward, there was no difference in time between Kelly's, Simjouw's, and Zajac's time. Without a reward, there was no also difference in time between Kelly's, Simjouw's, and Zajac's time.

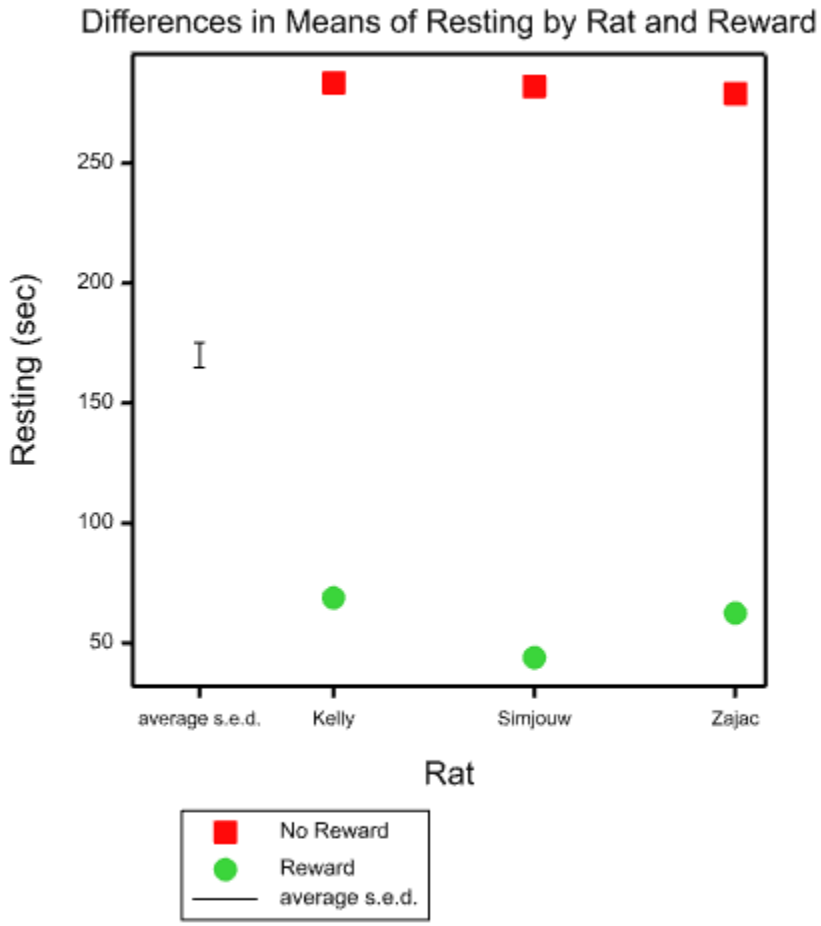

Figure 4.15 Differences in means of resting by rat and reward (ANOVA, $F_{rat(2,4726)} = 23.99$, $p < 0.001$; $F_{reward(1,4726)} = 1243.29$, $p < 0.001$, s.e.d = standard error of differences)

4.4.4 Return Results

For Kelly, there was a difference in time between with a reward at 137.463 seconds and without a reward at 160.706 seconds (Fig 4.16). There was a difference in time between with a reward at 135.421 seconds and without a reward at 154.039 seconds for Simjouw. There was no difference in time of return for Zajac, regardless of a reward being present.

For with a reward, there was no difference in time between Kelly's, Simjouw's and Zajac's time. For without a reward, there was no difference in time between Kelly's, Simjouw's and Zajac's time.

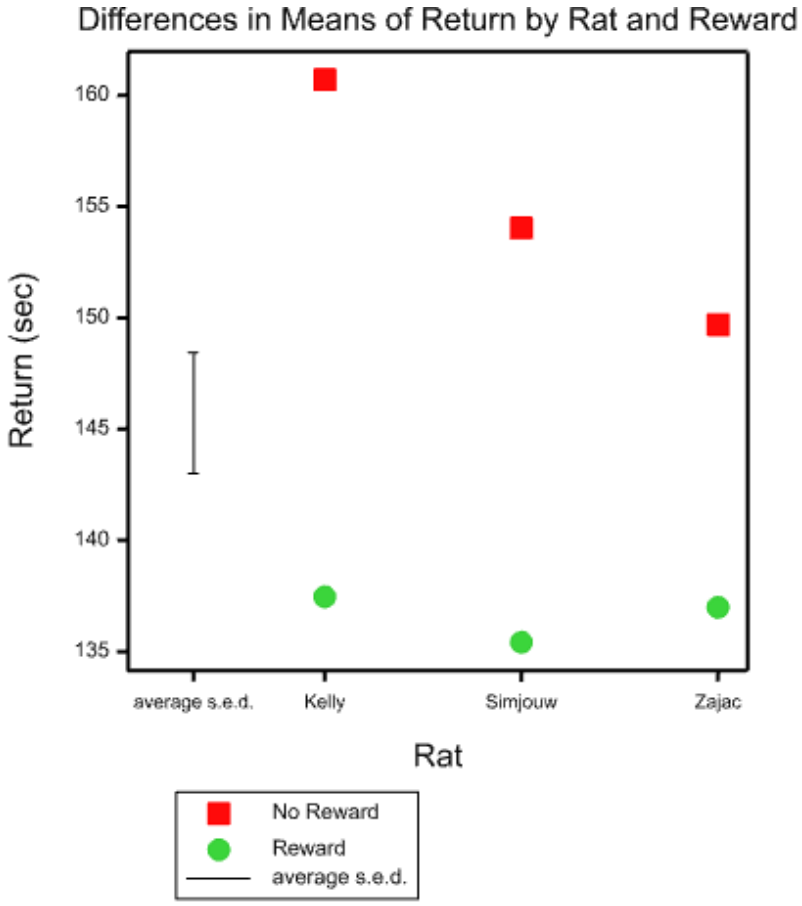

Figure 4.16 Differences in means of return by rat and reward (ANOVA, $F_{rat(2,5282)} = .53$, $p = 0.588$; $F_{reward(1,5282)} = 31.93$, $p < 0.001$, s.e.d = standard error of differences)

4.4 Training Over Time Results

To illicit how the rat's training changed over time, linear regression analysis was used on each part of training to analyze the amount of time during a session the rats were not feeding during training.

4.4.1 Departure Results

This analysis shows that over time, the rats took a little longer to depart from the carrier at the end of training compared to the beginning of training (Fig 4.17).

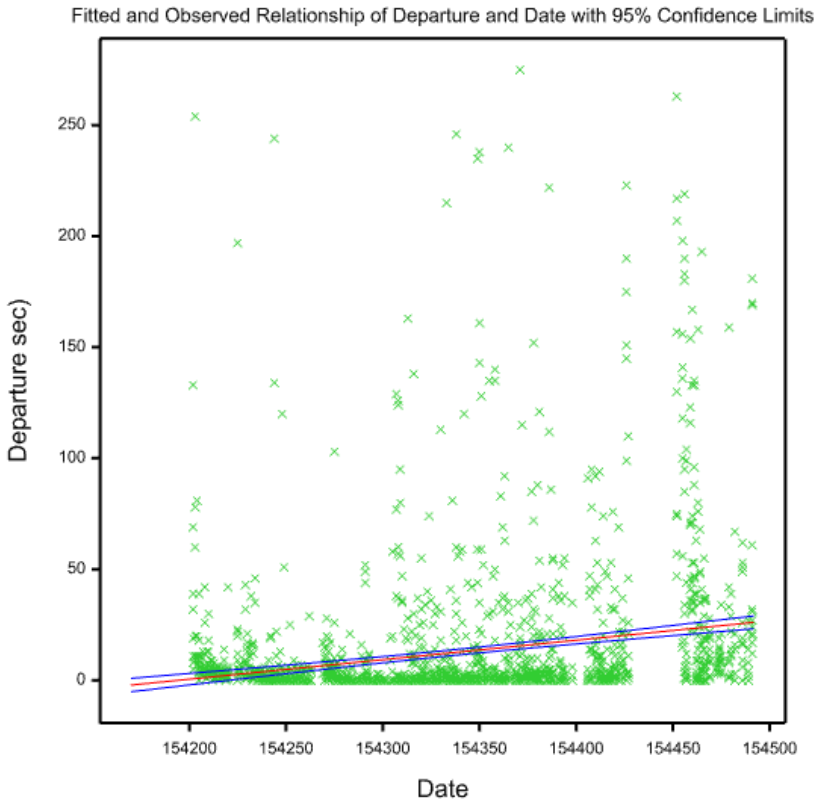

Figure 4.17 The fitted and observed relationship of departure and date with 95% confidence limits (Linear Regression Analysis, $R^2 = 0.046\%$, $F_{(1,2208)} = 107.48$, $p < 0.001$)

4.4.2 Resting Results

This analysis shows that over time, the rats were resting more at the end of training compared to the beginning of training (Fig 4.18).

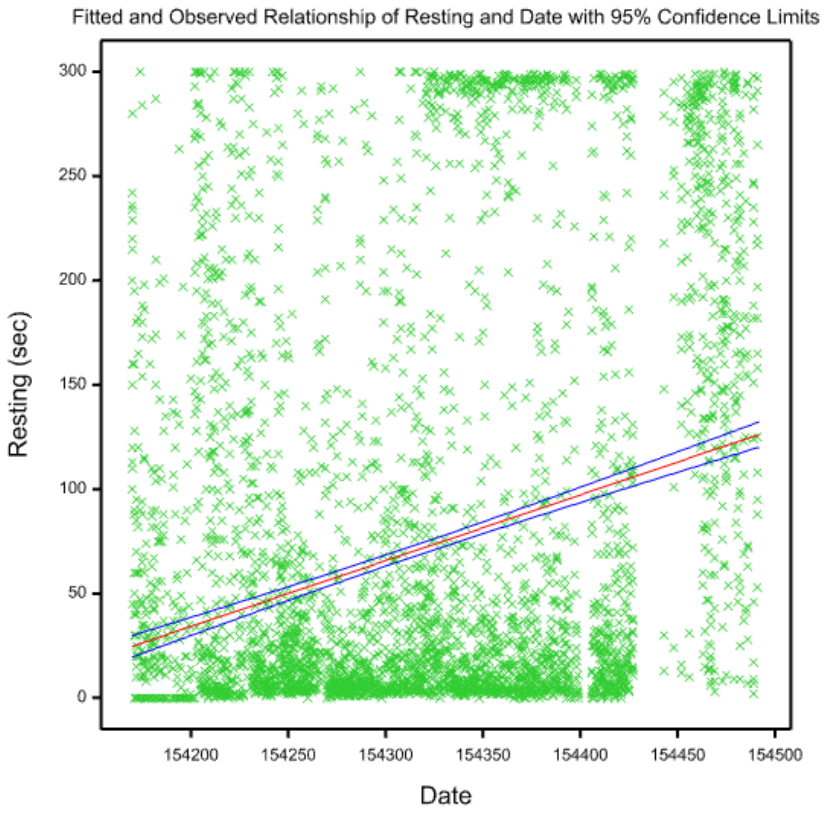

Figure 4.18 The fitted and observed relationship of resting and date with 95% confidence limits (Linear Regression Analysis, $R^2 = 0.079\%$, $F_{(1,4730)} = 406.33$, $p < 0.001$)

4.4.3 Feeding Results

This analysis shows that over time, the rats were feeding for less time at the end of training compared to the beginning of training (Fig 4.19).

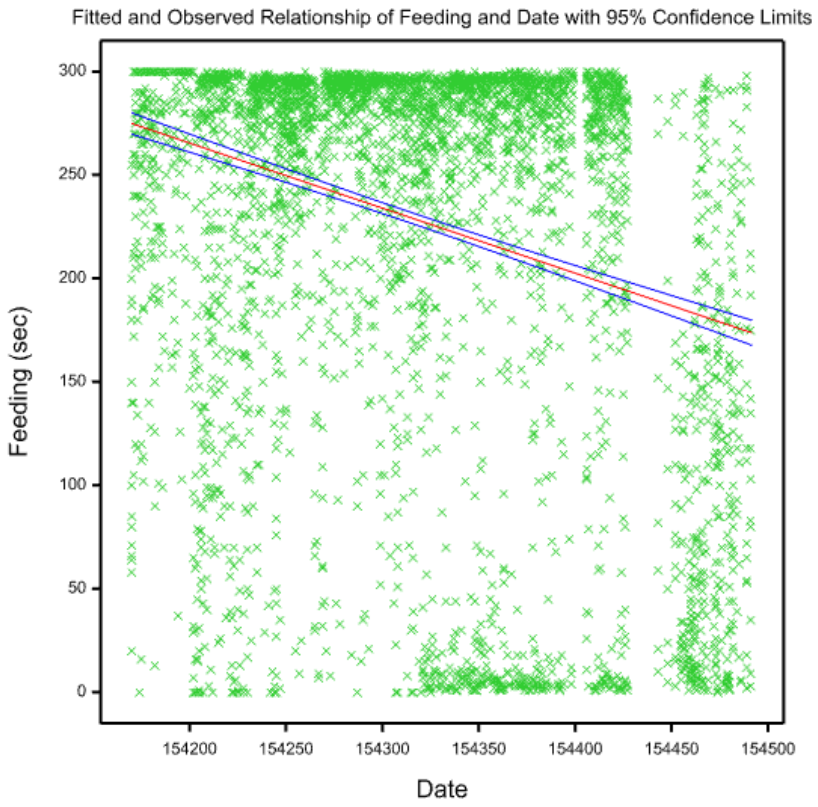

Figure 4.19 The fitted and observed relationship of feeding and date with 95% confidence limits (Linear Regression Analysis, $R^2 = .078\%$, $F_{(1,4728)} = 403.35$, $p < 0.001$)

4.4.4 Return Results

This analysis showed that over time, the length of time the rats spent returning increased over time (Fig 4.20).

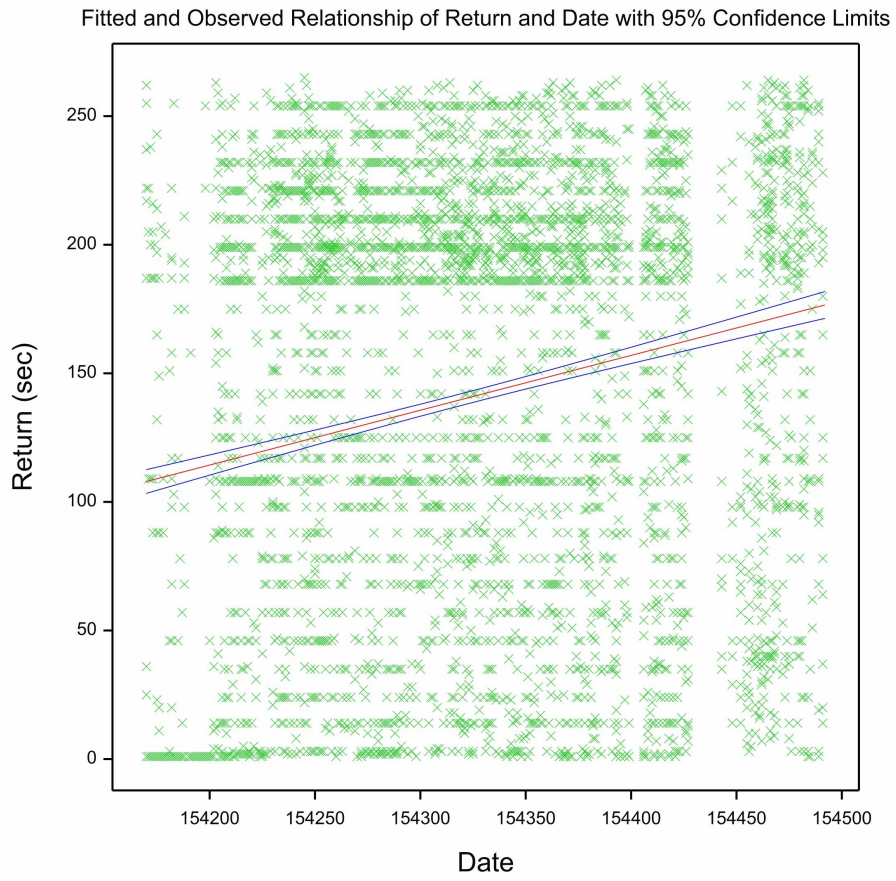

Figure 4.20 The fitted and observed relationship of return and date with 95% confidence limits (Linear Regression Analysis, $R^2 = 0.043\%$, $F_{(1,5286)} = 239.30$, $p < 0.001$)

While all the result over time are statistically significant, in reality the results are not probative. The linear regressions only account for between a 4-7% change in behavior. So, while it appears significant, it only shows a trend across thousands of points of data rather than probative changing data.

Chapter 5: Discussion

5.1 Observed Vocalization Training

While the Anabat Walkabout Bat Detector and Chorus Bat Detector picked up noticeable vocalizations throughout rat-tickling sessions, there were only a few moments noted over many months of return and scent training sessions. The vocalizations recorded during the training sessions did not undergo statistical analysis as there were too few to be of statistical value.

The vocalizations emitted while the rats were around the 55 kHz range, which as described by Portfors, 2007, is within the sound range that was found to be emitted by rats in a positive situation.

In this study, there were some differences in the frequencies of the vocalizations emitted by Simjouw and Kelly, there were not enough difference to individualize between each rat by the frequency range. This difference could be due to having a naturally higher or lower frequency chirp, as all the chirps were in the range that was expected.

5.2 Observed Behavior During Training

During the three types of training, return, clicker, and scent training, each type followed a similar pattern.

5.2.1 Behavior in Return, Scent and Clicker Training

During feeding, or how long each rat was at their objective, the pattern of clicker training having the longest duration, return training having the shortest duration, and scent training between clicker and return was consistent with each rat. During departure, Simjouw fed the longest, while Zajac and Kelly generally fed for the same amount time. The pattern of each rat being consistent for feeding duration across each type of training shows that the trainings did not

differ much except in time, but the rats' behaviors for each training during feeding did not vary much between the rats across the types of training.

During the return portion, or how long it took each rat to complete the sections, the rats followed a similar pattern during return and scent training, but not clicker training. The times for clicker training had a large increase in time spent during the return section. One possible explanation is that clicker training was replaced by scent training before L1 training for clicker took place. This is also highlighted here by the large variation in the data. Since the clicker training was replaced with scent training, as the scent was a critical part in the training, in the early stages of this study before the rats were fully trained, the impact of this data is minimal, especially since there was more emphasis on the scent training aspect than the return training aspect.

5.2.2 Observed Behavior in Training Locations

In general, as each training environment became larger, the time spent searching for the trainer or scent appeared to increase or decrease accordingly for each rat. In departure for each level, Zajac departed from the carrier faster than the other rats. Zajac has always appeared to be the most curious and adventurous rat of the three, so the personality may have been a factor for this. There was a small discrepancy in FT departure time, but that could also be due to the FT also being the stage where the initial association of the clicker and leaving the carrier was formed.

For feeding, the time it took the rats to reach their goal and start to feed decreased with the increase of training complexity. This was expected since the food was further away from the

rats and it took longer for the rats to find and reach the food since the room is larger. Conversely, the resting time increased in each session, as it took longer for each rat to find the food.

For return training, the front table took the shortest amount of time for each rat. However, it took each rat nearly the same amount of time to reach their goal across L1, L2, and L3. On average it took the rats approximately 180 seconds to reach the goal of the session. This time correlates to the time it took in other studies of rats searching for human scents. In La Londe et. al. (2015), the rats found the people they were searching for in under 3 minutes 83% of the time. The time range reported by La Londe et. al. (2015), correlates with the time range analyzed in this study. However, being in this time range across L1, L2, and L3 shows that even as the environment increases in complexity, the rats were able to find the food in around approximately the same amount of time.

5.2.3 Observed Behavior with and without Rewards

All the rats appeared to know when the reward food was present. When the food was present the rats would stay by the trainer and eat the food at their feet or stay by the scent where the food was located and eat the food. After some session, the rat sometimes ran away from the food after presumably getting full.

When no food was present, there was little time spent waiting near the trainer or the scent. There was a large difference in average time across each part between sessions with a reward and without one. This shows that the rats were highly food motivated. This food motivation has been shown in other studies as well. On one study, bananas and other fruits were crushed with food pellets and fed to their pouched rats as a session reinforcer (La Londe et al., 2015).

5.2.4 Training Over Time

Over time there was an increase in the return time, but not a large one. This was expected as this is the time it took the rats to reach the objective. The upward trend corresponds to the increase in length and complexity of the rooms. A similar trend is seen in resting, as this reflects the length of time the rats were not at the objective and the rats took longer to reach the food in the larger training environments. Feeding shows a downward trend which can correlate the rats spending less time eating less or less time at the objective. Once again this shows that the complexity of the environment means it took the rats longer to reach the objective, and that is reflected in the linear expression. It is also possible that this trend shows the rats trying to learn about the larger environment as well, possibly exploring, rather than just taking longer to find the food. However, while these trends show changes over time it does not take into account the stopping time the rats take while searching for the food. If a GPS locating mechanism were put on the rat to locate a body, and the rats stops to rest it may be mistaken as a location. Additional research would need to go into the resting of the rat over time.

5.2.5 Limitations During Training

During return training there were issues when L1 training started, with the rats chasing after the trainer instead of coming after the whistle. So, a door release pully system was used in order to curb the behavior and disassociate the handler from being chased for food (Appendix 6). The rats were released across the room by pulling open the door to the carrier, so that they could not easily reach the trainer. The pully system was terminated after ~3 weeks once the behavior was curbed.

There was also an issue with squirrels in the laboratory. Squirrels were getting into the training area through a hole in the ceiling. When the squirrels were present in the training room, training was not possible, as we did not want the squirrels to attack and stress out the rats. Subsequently, a few training sessions throughout November were cancelled until the squirrels were humanely trapped and removed.

There was also a span of time from December 21, 2022 until January 5, 2023, where the rats could not be trained due to trainer absences. The two-week span can be seen in the data collection in Figures 4.13, 4.14, 4.15, and 4.16 as a blank gap in the data. After this two-week span there was a noticeable change in how the rats trained. The rats refused to leave the carrier for minutes rather than seconds during scent training and they took longer to reach the trainer during return training. This happened far into Level 3 of training, and this prohibited training from progressing onto the next stage. This important observation highlights, in correlation to other studies, that continuous training may be necessary to keep the rats motivated and not have the behavior extinguished (Mahoney et al., 2014).

5.3 Forensic Implications

The Surfside condominium collapse on June 24, 2021 resulted in 98 fatalities and took weeks to find the victims in the rubble of the collapse (Hauptman & Shammas, 2021). Many efforts were taken in order to find the victims. Along with cadaver dogs, radar equipped drones were also used, as areas of the rubble were dangerous for people and rescue crews to stand upon (Leone, 2021).

In the event of another similar building collapse or other disaster, then there may be a need for a smaller detection animal, such as a rat. We found that rats are able to be trained to

detect the smell of early decomposition and can be trained to return to the carrier in a large room environment. If rats can be trained to vocalize at the scent of decomposition, which may be possible using classical conditioning, then rats would be an excellent candidate for another detector animal. The small size of the rats would allow for searching rubble and smaller areas that may be dangerous for larger dogs and people to maneuver. These animals could help assist detector dogs and other technologies to find persons under the rubble. It took over 4 weeks to find all the bodies of the victims of the seaside condo, but with the assistance of trained rats, that number may be able to be decreased.

Rats have been proven effective detector animals for TNT and tuberculosis, but their detecting ability could be further expanded (APOPO, n.d.). Rats could be trained to detect illicit drugs and trade. Rats also could be used more discretely than dogs if necessary, as they are a smaller animal. If they were used to search cars for illicit drugs it would be more discreet than a drug sniffing dog. Detection rats could be kept in the back room of clinics to detect odors for many illnesses, as it is used for tuberculosis by APOPO. While many people have a fear of rats, they could be used behind the scenes as they have been used in a forensic lab in Rotterdam to detect gunshot residue (McCluskey, 2013). Rats could be behind the scenes smelling for disease and for illicit substances. There are so many possibilities for using rats in forensic situations based on their olfactory ability and small size.

5.4 Limitations

There were four main limitations to this research. The first limitation was money. Ideally, this research would use many rats, at least 10 or more, which would require many more research assistants and much more space to house the rats, which is not available. Having that many rats

would also require more cages, food, and other materials, which would require more funding in order to take care of the rats. A second limitation was research assistants. With tight working schedules and COVID-19 still impacting campus activities, more assistants would be needed to allow for more training coverage when both the assistants were unavailable to train. There were days trainings could not commence due to a researcher illness or unavailability. A third limitation is time. This research needed to be completed within a year's time, which means fewer trials and testing would be able to be ran in that amount of time, which limited the number of trials and amount of testing that could be done.

5.5 Future Research

Future research should focus on combining scent and return training earlier in the study to reduce the amount of time spent training. This step would be imperative if this study would be used in the field, as the rats would need to be able to find the scent of decomposition and return to the handler in the same session. The research should also focus on training *R. norvegicus* in more complex environments, such as adding obstacles into the room or using a building complex with multiple rooms. The training should also include other variables such as adding additional trainers as possible distractions and using outdoor environments.

In order to address the rat's tendency to not locate the VOC without a reward present, future researchers may benefit from an altered the protocol that only rewards the rats once the scent has been located instead of having the food laying in front of the scent during the duration of training. Future research could also focus on other factors that may impair *R. norvegicus* from finding the desired VOC. Smoke, other scents, debris, loud noises from many people on a scene,

unknown food, and other obstacles on a scene may make it difficult for *R. norvegicus* to find the scent of decomposition.

Sensitivity of *R. norvegicus* could also be tested using differing concentrations of the VOC. A 1:10,000,000 concentration was used in this study, both other concentrations should be tested as well. Vass, et al. 2004, shows the maximum and minimum concentrations of many compounds in the early middle and late stages of decomposition. Testing different concentrations as well as different odors may be helpful in future research.

Future researchers should also focus on training *R. norvegicus* to vocalize once they detect the scent of decomposition. In this research, the vocalizations of the rats were monitored but the rats were not trained to vocalize. As the vocalizations were noticeable and easily recorded when the rats were tickled, there may be an opportunity to train the rats to vocalize when they detect the desired scent.

Chapter 6: Conclusions

This study focused on evaluating if *Rattus norvegicus* could detect the VOCs of decomposition and return to the handlers at the sound of a whistle. This study also focused on observing if the vocalizations of *R. norvegicus* could be a used as a locating mechanism when they detect the VOCs, and therefore could be used to find clandestine remains.

- *R. norvegicus* found the scent of decomposition during training sessions.
    - *R. norvegicus* reached the scent of decomposition at ~150 seconds during training sessions.
    - *R. norvegicus* stayed at the scent of decomposition for ~200-220 seconds during training sessions.
- *R. norvegicus* returned to the trainer at the sound of a whistle during training sessions.
    - *R. norvegicus* reached the trainer at ~130-135 seconds during training sessions.
    - *R. norvegicus* stayed at the trainer for ~220-250 seconds during training sessions.
- *R. norvegicus* was successful in training when a reward was present during training.
    - *R. norvegicus* could be more successful in sessions without a reward with a modified methodology.
- Ultrasonic Vocalizations of *R. norvegicus* were not useful as a locating mechanism for VOCs of decomposition.
    - Ultrasonic Vocalizations of *R. norvegicus* during tickling sessions showed positive stimulus.

- *R. norvegicus* could not be individualized by their vocalization frequencies.
- With classical conditioning, *R. norvegicus* may be able to be vocalize when VOCs of decomposition are detected.

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Appendices

Appendix 1: IACUC Protocol form

University of New Haven

PROTOCOL FOR ANIMAL USE AND CARE: AMENDMENT & RENEWAL

Please e-mail to: IACUC@newhaven.edu

IACUC USE ONLY

Project No. _____

Protocol Expires:

Project Number: 20-03

Date Originally Approved: 2020

Project Title: *Rattus norvegicus* as a Biological Detector of Clandestine Remains

Principle Investigator: R. Christopher O'Brien, Ph.D.

_____ I request renewal of this expiring Animal Use and Care Protocol with no modifications.

_____ I request renewal of this expiring Animal Use and Care Protocol with the amendments detailed below.

__ X __ I would like to amend this current Animal Use and Care Protocol as detailed below.

AMENDMENTS TO PROTOCOL (if applicable)

Describe proposed changes to animal care and use procedures, including justification for why the change is necessary:

Do proposed amendments change the level of pain expected in the procedure: Yes or No
If yes, attach updated version of Addendum I: CONSIDERATION OF PAINFUL PROCEDURES from application form.

Do proposed amendments require surgery not described in original application: Yes or No
If yes, attach updated version of Addendum II: SURGICAL PROTOCOL from application form.

Additional personnel who will perform animal-related support functions associated with the experimental protocol – for each, please provide:
Full name: Gabrielle Johnston
Affiliation: University of New Haven
Title/degree: Graduate Student, Forensic Science, M.S.
E-mail address: gjohn4@unh.newhaven.edu
Role in animal care/handling: Daily care and training

Description of qualifications: Graduate research student

Full name: Melanie Monetti
Affiliation: University of New Haven
Title/degree: Undergraduate Student, Forensic Science
E-mail address: mmone1@unh.newhaven.edu
Role in animal care/handling: Daily care and training
Description of qualifications: Undergraduate research student

APPROVAL

IACUC members are asked to carefully review this request for an amendment and provide comments**.** If a Full Committee Review is not requested, amendments and annual renewals will be reviewed by the Designated Member Review process detailed in the Standard Operating Procedure.

Signatures indicate acceptance of the amendment or renewal as written.

Designated reviewer(s) – add additional members as necessary:

Carter Takacs	
Name	**Signature**
Assistant Professor, IACUC member	12/6/21
Title	**Date**
Name	**Signature**
Title	**Date**

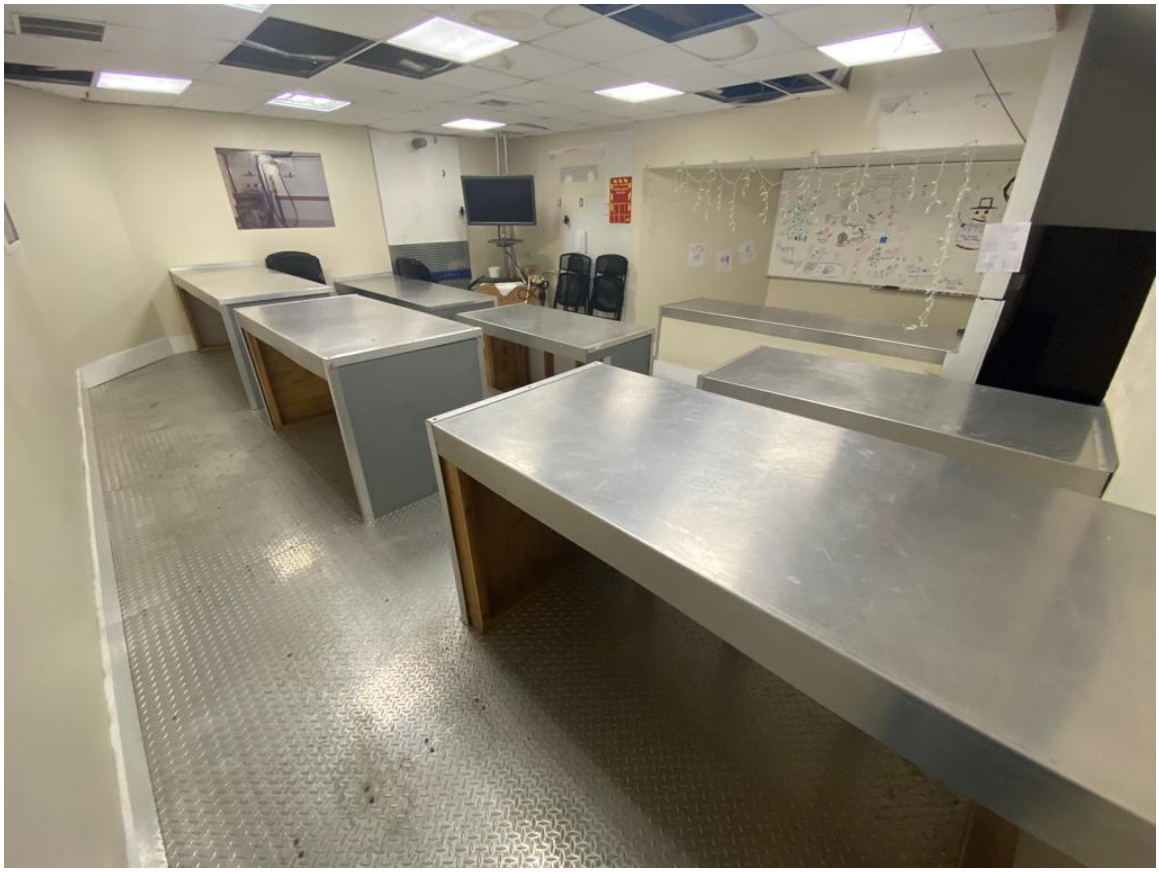
Appendix 2: List of Trainers who Handled the Rats

Trainers of the Rats		
Trainer	Day Training Started	Day Training Ended
Gabrielle Johnston	7 April 2022	23 February 2023
Melanie Monetti	12 April 2022	26 April 2022
Taylor Babcock	28 May 2022	4 June 2022
Sydney Roberts	31 August 2022	23 February 2023

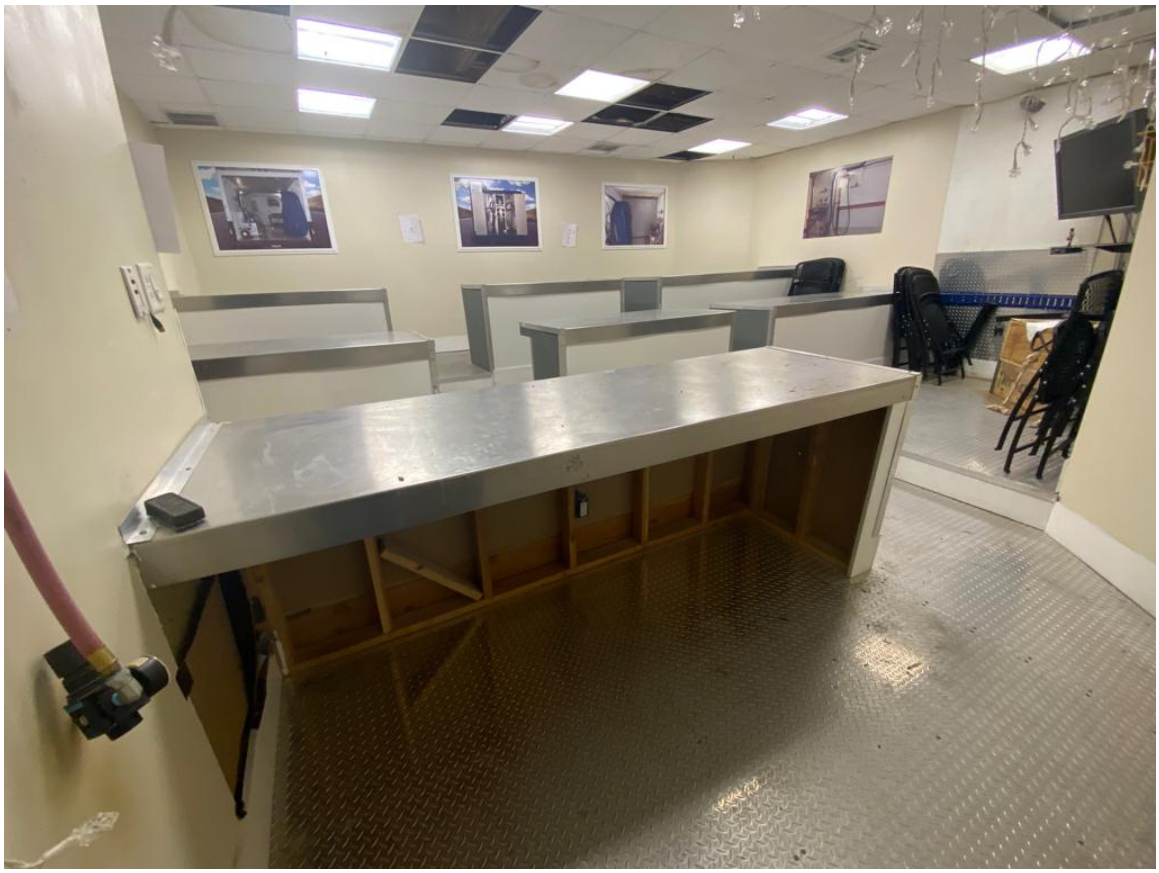
Appendix 3: Table of Training Type Starting and End Dates

Type of Training	Start Date	End Date
Acclimation Stage	February 22, 2022	April 6, 2022
Return Training FT	April 7, 2022	May 9, 2022
Return Training L1	May 10, 2022	June 20, 2022
Return Training L2	June 21, 2022	August 13, 2022
Return Training L3	August 14, 2022	February 23, 2023
Clicker Training FT	May 9, 2022	June 5, 2022
Clicker Training L1	June 6, 2022	July 9, 2022
Scent Training FT	July 15, 2022	August 1, 2022
Scent Training L1	August 2, 2022	August 29, 2022
Scent Training L2	August 30, 2022	October 31, 2022
Scent Training L3	November 1, 2022	February 23, 2023
Vocalization Training Recording	September 21, 2022	November 7, 2022

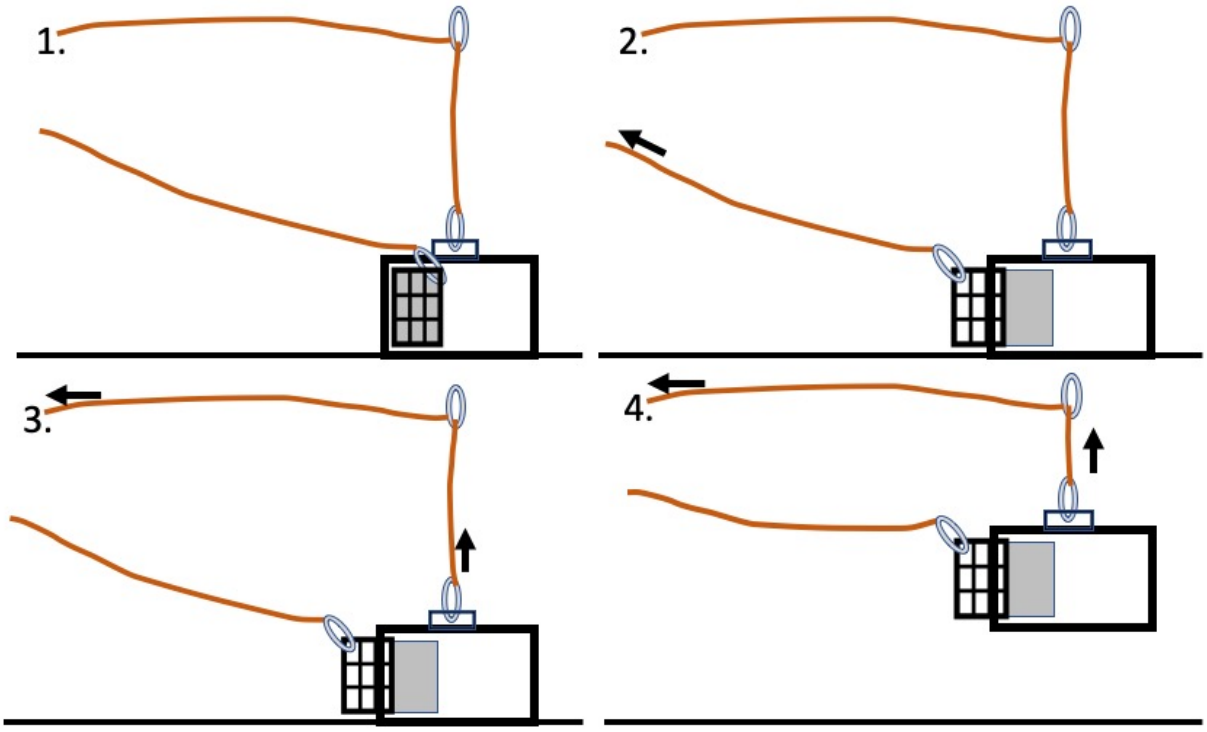
Appendix 4: Photo of the larger environment training area taken from Level 1 area. Photo taken by Gabrielle Johnston



Appendix 5: Photo of the larger environment training area taken from Level 3 area. Photo taken by Gabrielle Johnston



Appendix 6: Diagram illustrating the pully system used in L1 training for return training.



Appendix 6: Diagram illustrating the pully system used in L1 training for return training to curb the rats from chasing the trainer. 1). Two ropes were connected to the carrier with the rats in it. One at the top to lift and one connected to the door to open it. 2). The door was pulled open to allow the rats to leave and go the trainer blowing a whistle. 3). Once all the rats exited the carrier the top rope was pulled. 4). The carrier was pulled into the air and stopped the rats from returning to the carrier for the duration of the session. Image created by Gabrielle Johnston