The Effects of Lavender and Peppermint Essential Oils on Anxiety-Like Behaviors in Rodents

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Abstract

Lavender essential oil has been used as a treatment for anxiety in humans, while peppermint has been known to improve attention/concentration. There have been published studies showing a correlation between lavender essential oil inhalation and decreased anxiety-like behaviors in rats. However, no such research has been published on the effects of peppermint essential oil on rats. This study examined the effects of peppermint and lavender essential oils on anxiety-like behaviors in rats by using time spent in the closed versus open arms of an elevated plus maze (EPM) as a measure of anxiety-like behaviors (with more time in closed arms indicating anxiety-like aversion to open-arm exploration). Sixteen adult male Long-Evans (outbred) rats were tested in an EPM on three occasions (Baseline 1 [B1], Intervention, Baseline 2 [B2]). In this mixed design study, there was one between-subjects (grouping in Peppermint or Lavender) variable and one within-subjects (three EPM tests) variable. Results indicated no group difference. However, there was a significant difference with regard to the within-subjects variable. Baselines 1 and 2 yielded similar scores; in comparison, the Scent Intervention revealed more time spent in the open arms across both groups. Implications of these findings are that both peppermint and lavender may have had anxiolytic effects.

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Anxiety in Humans

Anxiety, as an emotion, consists of feelings of tension, worried thoughts, and physical changes, such as increased blood pressure, sweating, and breathing rapidly (American Psychological Association [APA], n.d.-a). While occasionally feeling anxiety as an emotion is normal, anxiety disorders are the most common psychiatric disorders with a worldwide prevalence of 3.6%; however, this number is likely an underestimation of the true percentage as a result of underreported cases (APA, n.d-a.; World Health Organization [WHO], 2017). In 2015, 264 million people worldwide reported suffering from anxiety disorders, and anxiety is the sixth largest contributor to global disability as of August, 2020 (WHO, 2017). These disorders are characterized by frequent, intense, excessive and persistent worry, fear, and rumination about everyday situations (APA, n.d.-a). Anxiety disorders include generalized anxiety disorder, social anxiety disorder, specific phobias, separation anxiety disorder, substance-induced anxiety disorder, and panic disorder with or without agoraphobia (Malcolm & Tallian, 2018). Generalized anxiety disorder, specifically, is characterized by persistent and excessive anxiety and worry about activities or events that is hard to control, out of proportion to the actual circumstance, and which negatively alters how the affected person feels physically (Malcolm & Tallian, 2018). The most common treatments for anxiety disorders incorporate both pharmacotherapy and psychotherapy (Lizarraga‐Valderrama, 2020).

Anxiety in Rodents

Research on psychiatric disorders and illness can be challenging to conduct in rodents, as many of the signs and symptoms of these disorders in humans are not observable in rats and mice.

These signs and symptoms include motivations, emotions, and thought processes that cannot be directly assessed in non-human species (Lezak et al., 2017). However, behavior can still be assessed in rodents as indicative of various psychiatric illnesses in humans, such as anxiety disorders. It is important to note that these anxiety-like behaviors in rodents are called such since they mirror anxious behavior in humans. These behaviors are interpreted as anxiety-like, but I take care not to assume that the animal has anxiety because the subject cannot report to me about its affect. Anxiety-like activities in rodents can be characterized by increased vigilance, freezing and/or hypoactivity, elevated heart rate, and suppressed food consumption (Lezak et al., 2017). Additionally, rodents naturally prefer dark and enclosed environments that protect them from predators, as opposed to light and open environments (e.g., Whishaw & Kolb, 2005). One model of anxiety in rats involves the elevated plus maze (EPM); generally, animals spend more time in the dark and enclosed areas compared to the illuminated and open areas (Walf & Frye, 2007). Presumably, time spent in the closed arms of the EPM indicates higher anxiety levels, while time spent in the open arms indicates lower anxiety levels (Walf & Frye, 2007).

Mechanistic Action of Essential Oils on the Central Nervous System

The pharmacology of essential oils (EOs) has been studied to determine the neural pathways involved in their modes of action; this has allowed for insights into both their physiological and psychological effects (Lizarraga‐Valderrama, 2020). The use of animal models has shown the neuropharmacological effects of EOs on the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic nervous system, and in GABAergic, dopaminergic, and serotonergic neurotransmitter systems (Lizarraga‐Valderrama, 2020). According to Chioca et al. (2013-a), the serotonergic system is the source of lavender essential oil's anxiolytic properties. Male Swiss mice were utilized to determine whether the $GABA_A$/benzodiazepine (BDZ) complex or

serotonergic neurotransmission contribute to lavender EO's anxiolytic effect (Chioca et al., 2013-a). When mice were pretreated with a $GABA_A$ receptor antagonist, the anxiolytic-like effects of 5% lavender were not impacted when tested using the marble burying test (Chioca et al., 2013-a). However, when mice were pretreated with a serotonin $5\text{-}HT_{1A}$ receptor antagonist, no anxiolytic-like effect of lavender was observed (Chioca et al., 2013-a). These results support serotonergic neurotransmission, rather than $GABA_A$/BDZ complex neurotransmission, as the mechanism for lavender's anxiolytic effect (Chioca et al., 2013-a). Similarly, Manor et al. (2021) reported that serotonin turnover and olfactory stimulation contributed to lavender's anxiety-reducing potential.

Although Chioca et al. (2013) and Manor et al. (2021) seem to have provided definitive evidence for serotonergic (and not $GABA_A$) mediation in regard to Central Nervous System (CNS) effects of lavender EO, other researchers appear to believe that $GABA_A$ may still be involved (Lizarraga-Valderrama, 2020; Soares et al., 2021; Stea et al., 2014). The proposed GABAergic mechanism is due to linalool, one of the major components of lavender, acting postsynaptically by potentially altering the activity of cyclic adenosine monophosphate (cAMP) (Stea et al., 2014). As a result, linalool has been shown to inhibit $GABA_A$ binding receptor within the CNS in animal models to generate a relaxed state (Stea et al., 2014). However, this inhibition has not been shown in human studies (Stea et al., 2014).

Differing from the proposed CNS mechanisms for lavender, dopamine pathways have been suggested as the source of menthol's, a major component of peppermint, dose-dependent anxiolytic effects (Chumpitazi et al., 2018). Soares et al. (2021) proposed that peppermint inhalation acts as a CNS stimulant, antioxidant, and memory retention agent by binding to the

nicotinic/$GABA_A$ receptor and preventing acetylcholinesterase from hydrolyzing acetylcholine (ACh) into acetic acid and choline.

As a result of the interaction of EOs with this array of CNS receptors, physiological changes are measurable that in turn allow for measurable psychological effects (Lizarraga‐Valderrama, 2020). For example, some of these physiological changes shown in clinical trials as a response to inhalation of EOs include changes in heart rate, blood pressure, respiratory rate, cortisol serum levels, and brain wave composition (Lizarraga‐Valderrama, 2020). Feelings of relaxation, contentment, and alertness were also shown (Lizarraga‐Valderrama, 2020). In order to gain further insight into the physiological and psychological effects of essential oils needed for the development of EO-based drugs, additional clinical research must be conducted to assess the synergetic effects of EOs as well as the complex receptor-EO compound interaction (Lizarraga‐Valderrama, 2020).

Aromatherapy: Lavender and Peppermint Essential Oils

Currently, selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are two commonly used antidepressants, and benzodiazepines are the most frequently prescribed anxiolytic used to treat anxiety disorders (Lizarraga‐Valderrama, 2020). Unfortunately, these treatments bring along various negative side effects that cause suboptimal therapeutic outcomes in some patients (Lizarraga‐Valderrama, 2020). Chronic use of BDZs, for example, cause lethargy, drowsiness, dizziness, vertigo, tolerance, and sedation (Lizarraga‐Valderrama, 2020). Even more concerning, older adults taking BDZs have reported an increased number of falls and exacerbation of cognitive decline (Lizarraga‐Valderrama, 2020).

In an effort to find safe treatment options for anxiety disorders that do not have dependence, withdrawal, or abuse potential, as well as to avoid the side effects associated with

chronic use of synthetic anxiolytic drugs, aromatherapy has been a suggested alternative (Lizarraga‐Valderrama, 2020; Malcolm & Tallian, 2018). Aromatherapy is the use of essential oil inhalation for therapeutic benefit, and an essential oil is a concentrated hydrophobic liquid containing volatile chemical compounds extracted from plants (John Hopkins Medicine, n.d.). Supposedly, only some of the major compounds of EOs, such as linalool, limonene, and pinene, provide significant anxiolytic effects (Lizarraga‐Valderrama, 2020). For this reason, it is expected that EOs that contain high levels of these compounds should provide an anxiolytic effect (Lizarraga‐Valderrama, 2020).

Specifically, lavender EO (*Lavandula angustifolia),* which contains linalool and limonene, has a history of providing an anxiolytic benefit that has been supported by clinical efficacy studies (Malcolm & Tallian, 2018). In addition to lavender, peppermint EO (*Mentha piperita*), which contains limonene, has also been suggested to have anxiety-reducing potential (Akbari et al., 2019). According to Akbari et al. (2019), inhalation of peppermint prior to intravenous catheterization lessened the pain and anxiety caused by the procedure in cardiac patients. Alternatively, peppermint has been suggested to reduce stress as well as improve mental function and attention orientation (U.S. Department, n.d.). According to anecdotal evidence and correlational-based clinical studies, peppermint has been recommended as a possible therapeutic tool to help those with attention-deficit/hyperactivity disorder (ADHD) sustain attention (Göbel et al., 1994). ADHD is a neurodevelopmental disorder characterized by one or all of the following: trouble in paying attention, difficulty in controlling impulsive behaviors, and hyperactivity (Centers for Disease Control and Prevention [CDC], 2020). Treatment for ADHD usually consists of cognitive behavior therapy (CBT) and/or medications, which can be stimulants or nonstimulants (CDC, 2020). Unfortunately, these medications can be accompanied by unwanted

side effects, such as decreased appetite and sleep problems (CDC, 2020). With this being said, peppermint is of specific interest to those suffering from the negative side effects of their ADHD medication, as well as those without ADHD to help orient attention, improve mental function, and lower stress levels (Göbel et al., 1994).

As shown, studies have primarily focused on the use of peppermint EO as an attention-orienting agent in humans (Göbel et al., 1994). The current study did not test peppermint's effect on attention. Instead, I investigated peppermint's effect as a comparative agent relative to lavender and with respect to anxiety-like behaviors in rodents (*Rattus norvegicus*), as there is limited information about the effects of peppermint on rodents. In fact, some of the only information available suggests peppermint as a deterrent with its taste and odor associated with decreased food consumption and gnawing in animals (Orkin, n.d.; Singla et al., 2014). According to Singla et al. (2014), eucalyptus oil odor prevented *Rattus rattus* (house rat) from feeding. Upon daily exposure to 5% and 10% eucalyptus oil for four consecutive days, both male and female rats consumed significantly less food ($p < 0.001$) compared to when the rats had no exposure to eucalyptus oil (Singla et al., 2014). Although eucalyptus is not identical to peppermint, eucalyptus and peppermint oil both contain 1,8 cineole and limonene that contribute to their mint-like smell (Schmidt et al., 2009; Singla et al., 2014). Therefore, Singla et al. (2014)'s study points to peppermint EO's potential role as a rodent deterrent.

To examine the potential effects of lavender and peppermint inhalation as therapeutic substances to alleviate the symptoms of human anxiety disorders, rodents can be first used, as their behaviors can be indicative of anxiety (Chioca et al., 2013-b). Rodents provide a useful model for testing anxiolytic agents (Chioca et al., 2013-b). Additionally, it is important to note that the chemical components of EOs produce their effect synergistically; therefore, the

experimentation for the purposes of this study was conducted using the entire essential oil as opposed to its isolated individual components (Lizarraga‐Valderrama, 2020).

The Current Study

Given the foregoing, a question is whether there are different essential oils that might reduce anxiety in rodents in comparison to lavender. Because peppermint increases focus in humans, it would be logical to conjecture that it might have similar effects in rats. However, there is general lore to indicate otherwise (Orkin, n.d.). So, I proposed to compare lavender and peppermint oil inhalation across two randomly assigned groups of rats and predicted that lavender would be anxiolytic in comparison to peppermint. My measure of anxiety was the relative amount of time a rat spent in closed arms versus open arms of an elevated plus maze.

Methods

Animals and Housing

I conducted a power analysis to indicate the sample size that I would need to detect significance between groups with $\alpha = .05$ and power = .80 (Clincalc, 2021). Estimated group sizes were 4, but I doubled it because there was a within-subjects variable in addition to the grouping variable; thus, there were 8 rats in each group (Lavender v. Peppermint).

Sixteen male Long-Evans (outbred) rats (range: 65.5 – 87 g, average: 76.25 g) were received at postnatal day (P) 27/28 and tested from P65/66 to P107/108. Rats were at P65 or P66 days for Baseline 1, P86 or P87 for Scent Intervention, and P107 or P108 for Baseline 2. At the final test (Baseline 2) the rats' mean weight was 223.50 g (9.89 g standard deviation, 212.5 - 245 g range). Rats were housed individually in polypropylene cages (47.63 cm long x 27.3 cm high x 21.59 cm wide) with hypoallergenic sustainably produced paper bedding. The Long-Evans rat

was chosen due to its accessibility, affordability, and its reputation as a common model in behavioral and psychological research.

Sixteen Long-Evans rats were randomly assigned to two equal groups: a Lavender Group and a Peppermint Group. All animals were their own controls, using an A-B-A within-subjects test of Baseline-Treatment-Baseline. Between-groups tests involved comparisons of the two groups at each stage of testing: initial baseline, treatment, and return-to-baseline.

All animals were housed under controlled lighting on a 12/12 cycle (12 hours dark; 12 hours light). Ambient temperature of the animal housing room was maintained between 18.33 and 23.89 degrees Celsius. Water was given ad libitum; food was provided once daily (after testing) in order to help motivate maze exploration behaviors. Subjects were acclimated to the testing environment and the experimenter throughout their juvenile interval (27/28 through 60 days-of-age) before the behavioral tests began (Stanford, n.d.).

Materials and Procedures

*A full equipment list can be found in the Appendix (p. 31).

Essential Oils

*The following information regarding lavender and peppermint essential oils was obtained from Lizarraga‐Valderrama (2020). The lavender and peppermint EOs utilized in the current study and the gas chromatography-mass spectrometry (GC-MS) results were obtained from PlantTherapy™.

1) Lavender (*Lavandula angustifolia)*

    Using steam distillation, lavender essential oil is extracted from the flowers of the evergreen shrub *Lavandula angustifolia* Mill. GC-MS indicated the concentration of the lavender EO as 32% linalool, 25% linalyl acetate, 6% (Z)-β-ocimene, 4% terpinen-4-ol, 3% (E)-β-ocimene, 3% lavandulyl acetate, 1.4% α-terpineol, 1.4% octan-3-one, 1.1%

1,8-cineole, 1.1% lavandulol, 0.5% limonene, 0.4% β-phellandrene, and 0.2% camphor (PlantTherapy, 2020).

2) Peppermint (*Mentha piperita*)

Peppermint essential oil is extracted from the leaves of *Mentha piperita* by steam distillation. GC-MS indicated the concentration of the peppermint EO as 40.4% menthol, 25.5% menthone, 5.5% 1,8-cineole, 5.1% menthyl acetate, 3.8% isomenthone, 2.7% limonene, 1.6% menthofuran, 0.8% pulegone, 0.1% carvone, and 0.09% total isopulegol (PlantTherapy, 2019).

Elevated Plus Maze (EPM) Equipment

- Apparatus: maze in a plus-shape with two closed and two open arms. See Figure 1 (p. 13) for dimensions, "Construction of the EPM*"* subheading (p. 12) for building instructions, and the Appendix for materials needed to build the maze.
- Camera: had an overhead view of the maze. This provided video of the maze in order for checking of latency measures related to animal movement at the + portion (center) of the maze as well as the closed and open arms.
- Two privacy blinds surrounded EPM that eliminated external room cues.
- Two standing lamps with three white light bulbs each positioned facing the two open arms next to privacy blinds and pointed toward the EPM.
- Baby pool surrounding EPM that contained rats that fell off EPM during testing.
- Oxivir™ disinfectant utilized between trials that eliminated visual and olfactory residue in EPM.

*The EPM materials list above was obtained from Stanford Behavioral and Functional Neuroscience Laboratory's "Elevated Plus Maze Standard Operating Procedure" and modified to

fit the current study's objective (Stanford, n.d.). Oxivir™ was used in place of Virkon disinfectant.

EPM Description

The elevated plus maze is a behavioral assay conducted to test the presence and fluctuation of anxiety-like behaviors in rodents (Lezak et al., 2017). The EPM was utilized in this study as a method for comparison of anxiety-like behaviors before and after the inhalation of lavender and peppermint EOs (Lezak et al., 2017). Structurally, the EPM, as shown in Figures 1 and 2 (pp. 13-14), is a plus sign shaped structure elevated fifty centimeters above the ground made up of two opposing enclosed arms with walls on the sides, two opposing nonenclosed (open) arms, and an open roof that enables video recording from above (Lezak et al., 2017). While the EPM induces anxiety in rodents that are exposed to the open arms, it also tests anxiety levels in rodents by measuring latency to enter, time spent within the open versus the closed arms, as well as the number of entries into the open and closed arms, as these are all methods of measuring anxiety levels (Lezak et al., 2017). As was mentioned, greater amounts of time spent in the open arms is indicative of lower anxiety levels (and more exploration), while less time spent in the open arms is interpreted as higher anxiety levels, as avoidance of the open arms is a proxy for anxiety (Lezak et al., 2017). In this way, the EPM intertwines rodents' preferences for dark spaces and avoidance of illuminated and open areas (Lezak et al., 2017).

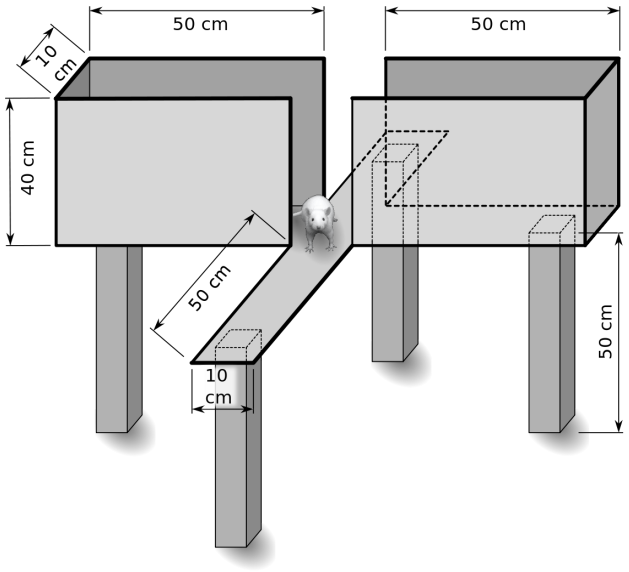
Construction of the EPM

The elevated plus maze shown in Figure 2 was constructed by the researcher, rather than by a manufacturing company, in an effort to lower expenses. The materials needed for EPM construction are listed under the "Elevated Plus Maze (EPM) Equipment" subheading (p. 11) and in the Appendix. The EPM has a small platform in the center with four surrounding arms that are

all 90 degrees from each other. These arms are 50 cm long x 10 cm wide (Maze Engineers, n.d.). Opposing arms match each other, meaning that the open arms are opposite each other and the enclosed arms are opposite each other (Maze Engineers, n.d.). The enclosed arms have approximately 40 cm high walls (Maze Engineers, n.d.). The roofs are open for all of the arms, while the walls are opaque (Maze Engineers, n.d.). The flooring and walls are made of plywood, and the legs are wooden blocks, all of which were purchased at Home Depot. I made sure that the floor did not blend in with the color of the rats being tested to ensure that the test subjects were easily observable when in the maze (Maze Engineers, n.d.). The floor was lined with a plastic sheeting that allowed for ease when cleaning to get rid of the scent from previous trials. The entire EPM apparatus was raised to 50 cm above the ground (Maze Engineers, n.d.). Furthermore, the lighting illumination was consistent throughout the EPM so that the EPM was well lit to avoid shadows in the maze (Maze Engineers, n.d.). The EPM was cleaned both before and after each trial in order to avoid any residual odors from previous experimentation (Maze Engineers, n.d.).

Figure 1

Standard Elevated Plus Maze Apparatus



Note. Figure 1 illustrates a schematic drawing of an EPM with walls surrounding the closed arms. The rat was placed in the center, between the closed and open arms, facing away from the experimenter (Wikimedia Foundation, 2021).

Figure 2

Researcher-Constructed Elevated Plus Maze Apparatus

Note. Shown above is the EPM that the researcher built and used in the current study. All measurements are the same as in Figure 1.

The design for this study was a mixed design with one (3 conditions) within-subjects factor (Baseline 1-Scent Condition-Baseline 2) and one (2 conditions) between-subjects factor (Group 1: Lavender versus Group 2: Peppermint). Sixteen Long-Evans male outbred rats were tested, with eight randomly assigned to each group.

Habituation to Handling and the Scent Chamber

Animals arrived in the animal housing room on 23 August 2021, at 27 or 28 days-of-age and were handled and exposed to a neutral chamber of approximately 24.13 cm wide x 33.02 cm high x 35.56 cm long (used later as a scent-exposure chamber). Animals were handled every other day during the juvenile-to-adulthood interval and exposed to the neutral chamber at the same interval. These events were designed to habituate the animals to human handling and to the

chamber where they were exposed to essential oil (lavender or peppermint). Two identical scent-exposure chambers were used: one for Group 1 (Lavender) and another for Group 2 (Peppermint).

Baseline Testing

Animals were tested in the EPM in random order on testing days (September 30: Baseline 1; October 21: Scent Condition; November 11: Baseline 2). Three weeks were ensued between tests in keeping with Walf and Frye's (2007) recommendation that rodents should not be exposed to an EPM on multiple occasions unless a 21-day period occurred between exposures, as a less than 21-day period between exposures could have caused test decay effects. Test decay effects occur when there are rising differences in EPM behavior when rodents are exposed to the EPM more than once within a 21-day period (Walf & Frye, 2007). An example of a test decay effect is decreased activity on the open arms of the EPM upon second exposure compared to first exposure (Walf & Frye, 2007). Rather than decreased activity on the open arms of the EPM solely due to anxiety induction, the second exposure is a confounding variable (Walf & Frye, 2007). With this knowledge, to avoid a drop in baseline open arm exploration due to second exposure to the EPM within a 21-day period, testing occurred 21 days apart (Walf & Frye, 2007). This allowed for measurement of the unconditioned avoidance response towards the open arms without the effects of previous exposure (Walf & Frye, 2007). A second baseline test (Baseline 2) for each animal occurred on November 11 in order to provide a reliability check for Baseline 1.

Essential Oil Administration and Inhalation

Protocols for essential oil administration followed those of Chioca et al. (2013-b). Distilled water served as a control. The distilled water and essential oils were stored in a polyethylene bottle and amber glass bottles, respectively, until administered. As for the inhalation

procedure, each rat was moved to a scent-exposure chamber. Then, cotton (0.5 grams) was positioned in a small plastic container and soaked with distilled water, lavender EO, or peppermint EO at a fixed volume of 1 mL. Once the cotton ball was soaked, I placed the plastic container with the cotton ball in the corner of the scent chamber. The polyethylene bottle (for distilled water) or glass bottles (for lavender and peppermint EOs) were closed shut. The rats inhaled the distilled water, lavender EO, or peppermint EO for 3 minutes, followed by immediate testing. After each test, the used cotton ball was discarded, and a new cotton ball was prepared for the next animal's test. Exposure to scent in the scent-exposure chamber happened only once, on 21 October 2021, and each animal was tested in the EPM immediately after its scent exposure.

Testing Conditions for the EPM

*Recommendations about timing of testing and handling of animals before testing were adapted from Walf and Frye (2007).

Timing of Testing

Circadian rhythms/light cycle could have influenced rats' behaviors in the elevated plus maze. The rats were housed on a reverse-light cycle with testing always conducted during their dark phase in order to avoid inconsistencies in what phase of the light cycle animals were tested. During their dark phase, rats are most active and have consistent differences in endogenous hormone concentrations, specifically corticosterone, estrogens, progestins, and androgens. Therefore, consistent testing strictly in the animals' dark phase reduced potential for the timing of testing as a confounding variable during behavioral analyses using the EPM.

Handling of Animals before Testing

It was important that no animal had prior handling or stress on the days of testing, as these could have changed the animals' behaviors in the EPM. For this reason, efforts were made to

eliminate inconsistencies relating to the handling of animals and prior stressors immediately before EPM testing. These efforts involved animals being consistently habituated to handling by experimenters, consistency in housing in the vivarium and location of the EPM in the testing room, as well as a consistent transportation procedure from the home cage to the EPM and return to home cage at the end of testing. The housing and behavioral testing sites were in adjacent rooms, so I carried each home cage individually into the testing room during a rat's test, followed by returning the cage to the housing room (vivarium). Therefore, transportation of the animals only took place from the home cage to the scent chamber to the EPM and back to the home cage. Every effort was made to remove stress before testing and maintain consistent experiences across animals.

Elevated Plus Maze Testing

Part 1: Distilled Water/Essential Oil Exposure

1. Selected the rat for testing from the randomized order (below). For baseline measures, no experimental condition was present. However, each rat's scent condition, as determined by a random number generator, was noted in the data collection spreadsheet. The order of testing was the same for Baseline 1, Scent Intervention, and Baseline 2. (Order of testing: 1 = I, 2 = C, 3 = K, 4 = G, 5 = P, 6 = A, 7 = H, 8 = D, 9 = J, 10 = O, 11 = M, 12 = F, 13 = L, 14 = E, 15 = N, 16 = B).
2. Removed the rat from its individual home cage and placed the rat in the scent chamber with a container that held the cotton ball doused with distilled water (1 mL using a syringe) for Baselines 1 and 2 and lavender or peppermint EO for Intervention. The cotton balls were doused for each animal just before the animal was put into the scent chamber. This was because if all the cotton balls were prepared ahead of time, the water or scent

would have evaporated, so each animal would not have been exposed to the same amount of scent. I timed the exposure to the cotton ball (3 minutes). All animals approached the cotton ball during their 3 minutes in the scent chamber.

Part 2: Elevated Plus Maze Testing

3. Cleaned (with Oxivir™ disinfectant) and dried EPM prior to testing.
4. Started the video device. I started the video recording before I placed the rat in the EPM so that no behaviors were missed.
5. Showed the index card for the rat being tested in the camera view before transporting the rat from the scent chamber to the EPM. This was so that the rat's number, letter, and scent condition were recorded.
6. Removed the rat from the scent chamber and placed the rat at the junction of the open and closed arms with the head of the rat facing the open arm opposite to where I was standing. My handling and placement position of the animal into the EPM was consistent for each test subject. I avoided placing rats towards the closed arm and ensured that each rat was placed on the EPM facing the same open arm.
7. Started the 5 minute timer simultaneously with placement of the rat on the EPM. I eliminated unnecessary movement and noise production.
8. Removed rat from EPM at end of 5 minute test and returned rat to its home cage.
9. Cleaned EPM with Oxivir™ disinfectant and dried with paper towels between test subjects.
10. Prepared data collection sheet. The spreadsheet included the subject number of the animal, date, and scent condition (Peppermint or Lavender). Following recording of all EPM tests,

data was collected from the videos and recorded in the data collection sheet. The information and behaviors recorded are listed below.

- Time spent in closed arm
- Time spent in open arm
    - Closed arm time - open arm time indicated relatively more anxiety when a positive score and less when a negative score
- Open arm entries made
- Closed arm entries made
    - Open and closed arm entries were only counted when all four paws of the rat were on the open or closed arm, respectively.
- Total entries made
- Head dips: downward movement of rodent's head towards the floor in the open arm
- Rears: vertical standing of rodent on two hindlegs
    - Supported rearing: rat reared against walls of the EPM (Sturman et al., 2018)
    - Unsupported rearing: rat reared without contacting walls of the EPM (Sturman et al., 2018)
- Stretch-attend posture: body of rodent was stretched forward/toward a stimulus but rodent was motionless; neuraxis was parallel to the floor (rat was on all fours); stretched head and shoulders forward and subsequently retracted to original position (Sestakova et al., 2013)
- Grooming: rodent licked and rubbed its paws on its body (Rousseau et al., 2000)

*The procedure above was adapted from Walf and Frye (2007) with slight modification to fit the current study's objective.

Timing

- Steps 1 and 2: cotton ball preparation and inhalation
    - Each animal was put in the scent chamber for 3 minutes just before it was put into the EPM.
    - 3 minutes per rat x 16 rats = 48 minutes total distilled water or EO inhalation
- Steps 3 and 9: EPM cleaning
    - 3 minutes per rat x 16 rats = 48 minutes total spent preparing (cleaning) the EPM prior to use and between each test
- Steps 4 - 8: EPM testing
    - 5 minutes in the EPM per animal → 80 minutes for 16 animals
- Step 10: data collection
    - I watched each rat's 5 minute test video multiple times in order to collect the different information. This process took about 8 hours total.

Summary of Timing:

- Cleaning EPM (steps 3 and 9) = 3 min per rat x 16 rats = 48 minutes
- 3 minutes scent exposure + 5 minutes in EPM = 8 minutes per rat
    - 8 minutes per rat x 16 rats = 128 minutes (2 hours, 8 minutes) total testing time time
- 128 minutes testing time + 48 minutes cleaning = 176 minutes = 2 hours, 54 minutes needed total for testing days

Responses to Unexpected Circumstances During Testing

Rodents fell off open arms. Rats ran to the edge of the open arms and fell off infrequently (only rat E fell off twice; Walf & Frye, 2007). In keeping with Walf and Frye's (2007) recommendations, under this circumstance, I rapidly picked up rat E and placed him back onto the open arms of the EPM. I recorded this fall on the data sheet but did not exclude rat E's behavioral data from analyses. The 5 minute test was continued with the animal who fell off the open arm in order to ensure that exposure to the EPM was as consistent across animals as possible.

Rodents were immobile/motionless on open arms. Walf and Frye (2007) also provided recommendations for what to do if rats were immobile or motionless on the open arms. However, during the current study's experiments, rats were never motionless on the open arms for an extended period of time in response to noise or movement during testing. If this were to happen, it may have led to the rat spending the majority of the testing time motionless on the open arms of the EPM. If the rat spent more than 30% (100 seconds) of the total testing time on the open arm, this rat was considered to be motionless for an extended period on the open arms. In Walf and Frye's (2007) experiments, this immobility of the rats on the open arms only occurred in less than 1% of the animals that were tested.

In the rare occasion that immobility did occur, but not in response to loud noise or movement, this action should have also been recorded on the data sheet as a deviation from standard behavior and taken into consideration when performing analyses of behavioral results. Alternatively, if immobility occurred as a result of a loud noise or movement, this animal's data was to be excluded from behavioral analyses. Although the data would have been discarded, I would have needed to continue the 5 minute EPM test to keep consistent with the other animals. I reduced potential for immobility of the rats on the open arms by avoiding noise and movements.

While testing, a sign was placed outside of the testing room and vivarium to make others outside the testing room aware of the importance of remaining quiet during testing periods.

Different baseline/open arm activity. It was important to factor in the sex and reproductive age of test subjects for behavioral testing. This was because differing baseline EPM behavioral patterns have been reported by Walf and Frye (2007) depending on sex of experimental animals, age, and stage in the estrous cycle (for females). In order to avoid having the estrous cycle as a potential confounding variable when observing EPM behavior, all male Long-Evans rats were chosen as test subjects. They were 27/28 days-of-age upon arrival in the laboratory. The age was decided on so that the rats were juveniles and to ensure that they were accustomed to the animal handlers before testing began at 60/61 days-of-age (adulthood).

Data Analyses

The experimental manipulations were the inhalations of either distilled water (Baselines 1 and 2) and lavender or peppermint essential oils (grouping variable for the Scent Intervention). In order to determine the effects of EO inhalation on anxiety-like behaviors in rats, I used analysis of variance (ANOVA) for the mixed design. ANOVA was used to determine whether difference scores (time spent in closed - time spent in open arms) were different between groups or across the three times of test (Baseline 1, Scent Intervention, Baseline 2). While raw data were collected regarding additional behaviors (as mentioned above, e.g., entries into closed arms, entries into open arms, rearing, stretch attend postures, etc.), they were not analyzed owing to lack of time and because the primary hypothesis was linked to time spent in closed and open arms. Following the two-way ANOVA, which tested differences between groups and within-subjects, three paired t-tests were performed to determine whether Baseline and Scent Intervention conditions were significantly different from each other.

Results

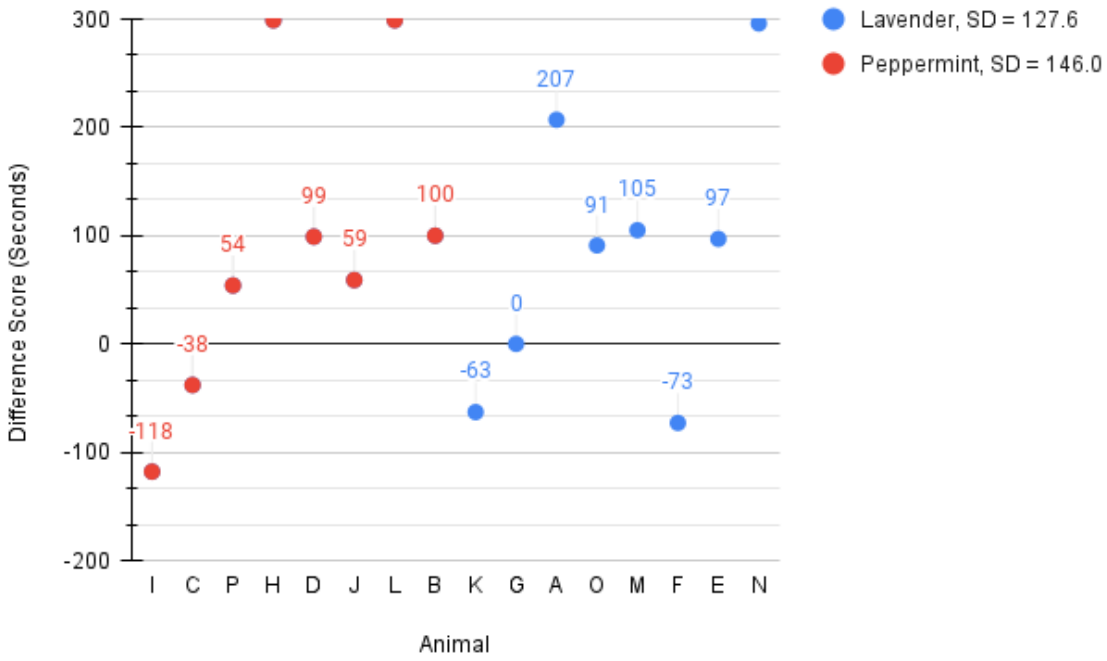
ANOVA for the mixed design yielded no significant effect of the grouping (Lavender versus Peppermint) variable, as shown in Figure 3 (p. 24), $F(1, 14) = 0.002$, $p = 0.96$. However, Figure 4 (p. 24) indicates that there was a significant effect of the within-subjects variable (Baseline 1 - Scent Intervention - Baseline 2), $F(2, 28) = 8.65$, $p < .01$, ($\eta^2_{within(partial)} = .38$). [The interaction effect was not significant, $p > .87$.]

In order to further investigate the significant within-subjects effect, I conducted paired t-tests (post-hoc comparisons). Three paired t-tests were run: Baseline 1 versus Baseline 2, Baseline 1 versus Intervention, and Baseline 2 versus Intervention. The first (post-hoc) t-test revealed that rats spent significantly more time in the closed arms during Baseline 1 ($N = 16$, $M = 194$, $SD = 91.71$) than they did during Intervention ($N = 16$, $M = 88.38$, $SD = 132.64$); $t(15) = -3.57$, $p = .0028$ (*Cohen's d* = .93). The second t-test revealed that rats spent significantly more time in the closed arms during Baseline 2 ($N = 16$, $M = 185.5$, $SD = 103.12$) than they did during Intervention; $t(15) = 3.86$, $p = .0015$ (*Cohen's d* = .82). However, time spent in the closed arms during Baseline 1 and Baseline 2 were not significantly different from each other, as shown in Figures 5 and 6 (pp. 25-26); $t(15) = -.31$, $p = .76$.

I failed to reject the null hypothesis that there was no influence of type of essential oil (Group: Peppermint or Lavender) on time spent in the closed arms of the elevated plus maze. On the other hand, there was an effect of inhaling an essential oil, regardless of whether it was peppermint or lavender, on time spent in the closed arms of the EPM, as demonstrated in Figure 4. A caution is that the sample size was small (i.e., 8 animals per group). Thus, the power may have been exceeded during post-hoc comparisons.

Figure 3

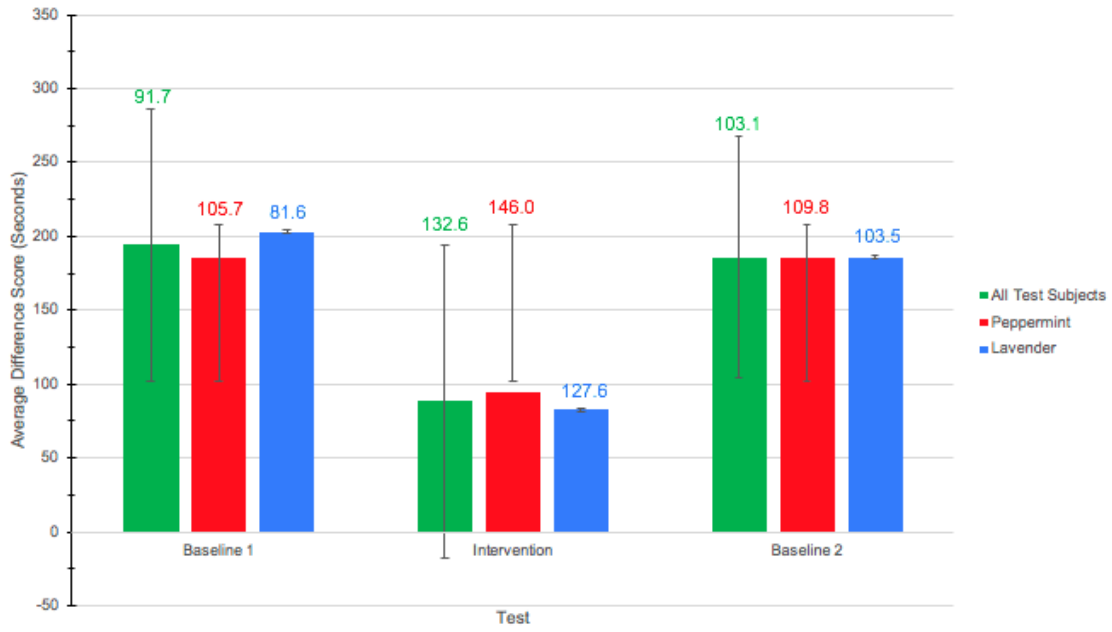
Intervention Difference Score vs. Animal



Note. Difference score is defined as time in the closed arms - time in the open arms in seconds. The difference scores for the Peppermint Group were not significantly different from the Lavender Group, resulting in no grouping difference. The order of the x-axis variables shown above is not indicative of the order of testing. The animals were tested in the same order for Baseline 1, Intervention, and Baseline 2, as listed in the "Elevated Plus Maze Testing" subheading under "Materials and Procedures" (p. 17). The ordering in Figures 3, 5, and 6 groups Lavender animals together and Peppermint animals together for visual purposes only.

Figure 4

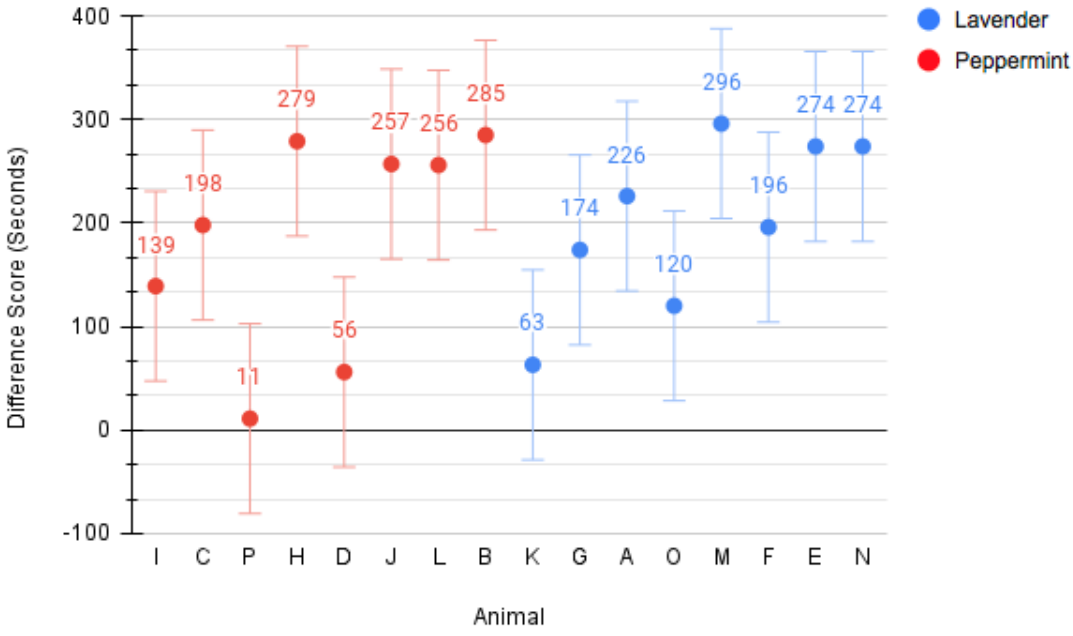
Average Difference Score vs. Test (Baseline 1, Scent Intervention, Baseline 2)



Note. Standard deviations from the mean (seconds) for each group (All Test Subjects, Peppermint, Lavender) and each test (B1, Intervention, B2) are shown above their respective bar. Independent of grouping, the average difference score for Intervention (88.375 s) was significantly lower than for B1 (194 s) and B2 (185.5 s). The average difference scores for B1 and B2 were not significantly different. There is an anxiety-reducing effect of inhaling an essential oil, independent of it being peppermint or lavender, that is illustrated in this figure by the All Test Subjects group spending significantly less time in the closed arms during Intervention than during B1 and B2 (lower average difference score for Intervention for All Test Subjects).

Figure 5

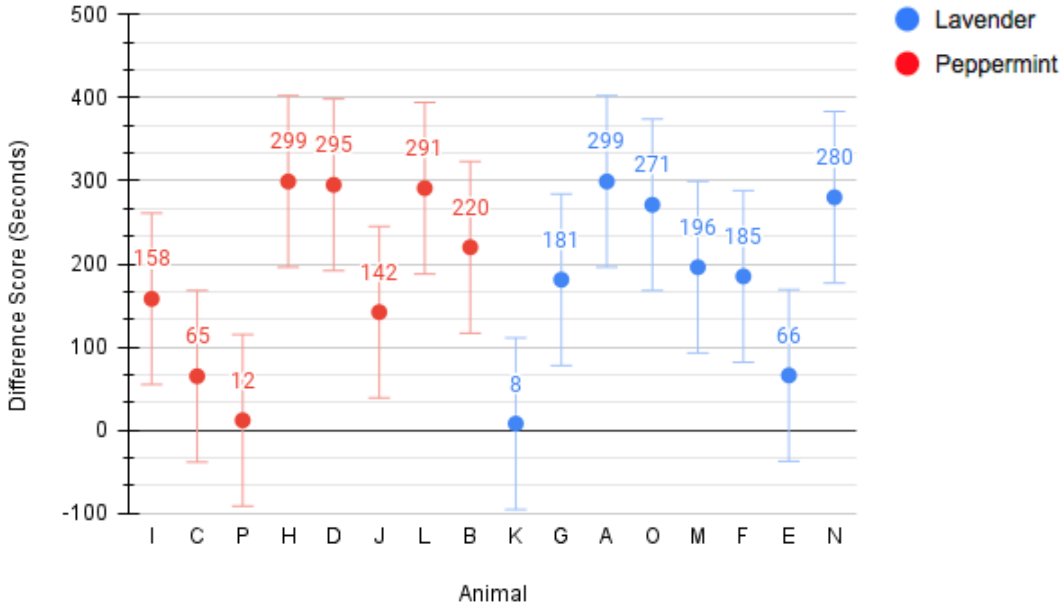
Baseline 1 Difference Score vs. Animal



Note. Standard Deviations from the mean (M = 194) are shown for each animal for Baseline 1. Difference scores for each animal for B1 (shown above) and B2 (Figure 6) were not significantly different.

Figure 6

Baseline 2 Difference Score vs. Animal



Note. Standard deviations from the mean (M = 185.5) are shown for each animal for Baseline 2. Difference scores for each animal for B1 (Figure 5) and B2 (shown above) were not significantly different.

Discussion

As shown by the ANOVA and paired t-tests, there was no significant difference between groups, indicating no difference in the effect of lavender compared to peppermint. While results did not differentiate between the effects of peppermint versus lavender essential oils (Figure 3), results did show that both of these EOs did have anxiolytic effects, as the Scent Intervention times were significantly different than Baseline times (Figure 4). Inhaling something prior to testing lowered anxiety-like behaviors in the EPM as measured by time spent in closed arms - time spent in open arms (Figure 4).

While I predicted, based on published research findings, that lavender would have an anxiety-reducing effect, I was uncertain as to whether peppermint would produce the same effect in rats. As mentioned, in humans, peppermint has been shown to improve concentration, but no such data has been found for rats. The present study did not evaluate different concentration levels of lavender or peppermint inhalation; however, it does show that perhaps peppermint essential oil has anxiety-reducing effects (as does lavender) in rodents.

As mentioned previously, an estimated 3.6% of people around the world have been diagnosed with an anxiety disorder during some point in their life (Lizarraga‐Valderrama, 2020). According to the Anxiety and Depression Association of America (ADAA), “anxiety disorders are the most common mental illness in the U.S., affecting 40 million adults in the United States age 18 or older, or 18.1% of the adult population every year” (ADAA, n.d.-b, Section 1). As for children and teenagers in the U.S., 8% have suffered from the negative impacts of anxiety disorders on their daily lives (National Alliance on Mental Illness [NAMI], n.d.). Beyond the U.S., 7.1% of children worldwide aged 3-17 years have an anxiety disorder (CDC, 2021). Even

more concerning, anxiety rates have been drastically climbing since the rise of the COVID-19 pandemic (APA, n.d.-b). According to APA (n.d.-b), U.S. adults self-reporting symptoms of anxiety per month jumped from 7.4% - 8.6% in January - December 2019 to 28.2% - 37.2% in April 2020 - August 2021.

Although treatments for anxiety disorders are being researched extensively, and variable attainable treatments for these disorders already exist, only 36.9% of people diagnosed are treated (ADAA, n.d.-b). Furthermore, doctor visits are three to five times more likely and hospitalization rates for psychiatric disorders are six times more likely for people diagnosed with an anxiety disorder than people who are not (ADAA, n.d.-b). Along with anxiety disorders, the ADAA (n.d.-b, Section 1) also reports on ADHD, which is another psychiatric disorder that impacts "about 4% of the adult population, or 8 million adults". About 50% of these 8 million adults with ADHD are also diagnosed with an anxiety disorder (ADAA, n.d.-b, Section 3).

The comorbidity between anxiety disorders and ADHD and large portion of adults affected exemplifies the need for effective and affordable treatment options for these psychiatric disorders. While the current study's finding about peppermint and lavender essential oils' potential anxiolytic effects suggests them as possible treatments for anxiety disorders, I am not suggesting that essential oils replace traditional medicine. Rather, peppermint and lavender EOs could be supplemental forms of treatment in addition to traditional medicine used to treat anxiety and ADHD, such as SSRIs or tricyclic antidepressants for anxiety and methylphenidate for ADHD (ADAA, n.d.-b). Treatment of ADHD with stimulants can actually heighten anxiety symptoms in patients diagnosed with both (ADAA, n.d.-b). For this reason, it is imperative to examine alternative treatment options, like aromatherapy, that do not come along with severe negative side effects and are safer and more accessible.

To further my research on peppermint and lavender's aromatherapy potential, analysis of the raw data collected on head dips, rearing, grooming, stretch-attend posture, and number of entries to both the closed and open arms is a next step. Additionally, experimenting with varying concentrations of the EOs would provide insight into the concentration required for peppermint or lavender to have a therapeutic benefit. Different behavioral tests with peppermint could also be run, such as the elevated zero maze (EZM) that assesses anxiety-like behaviors or the 5-Choice Serial Reaction Time Test (5CSRTT) that explores sustained and selective attention. Testing for peppermint's ability to reduce anxiety or improve sustained and/or selective attention would experiment with peppermint as a tool for sustaining attention in addition to reducing anxiety. As previously mentioned, the research of Göbel et al. (1994) displayed peppermint as an attention orienting agent in humans, but the same has not been indicated in rodents. In fact, Orkin (n.d., Para. 1) suggested peppermint as a deterrent for rats, as "in high concentrations, peppermint oil may exhibit some repellency." However, the current study's results showed the opposite. Furthermore, more tests with different protocols are essential to establish the robustness of my findings.

Limitations

As for the limitations of the study, the sample size (16 total test subjects, 8 per group) was small. This decreased statistical power and increased the margin of error (Faber & Fonseca, 2014). However, I did conduct a power analysis before the study, and it indicated that 8 animals per group would be sufficient to detect a group difference and that 16 animals overall should permit detection of a within-subjects (Baselines versus Scent Intervention) effect. Additionally, I only had one semester to complete testing for this study, which left enough time for only three

EPM tests. With added time, I could have conducted more tests, although additional exposures to the EPM might have reduced its validity as a measure of anxiety-like behaviors.

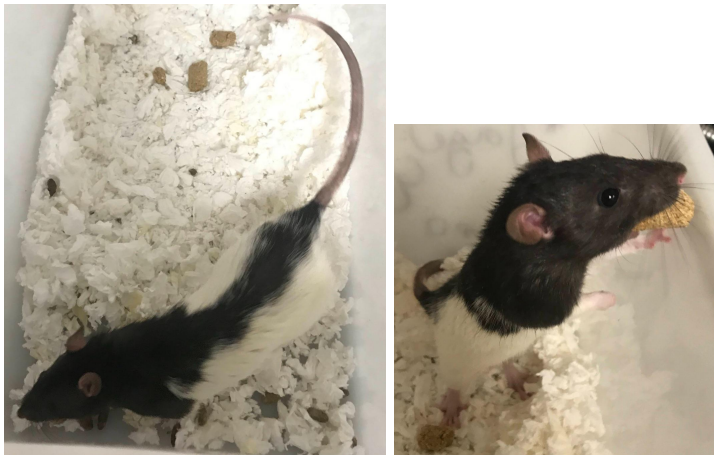
Appendix

Animal and Housing Equipment

- 16 male Long-Evans (outbred) rats received at P27/28

Figure 7

Long-Evans Male Rat



- Nutritive pellet diet and water for rats
- 16 polypropylene tubs with cage-top lids that held water bottles

Figure 8

Housing Arrangement of Rats in Polypropylene Tubs

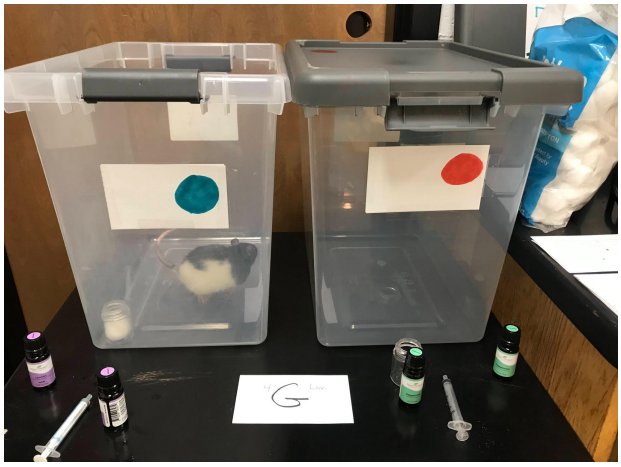
- Hypoallergenic sustainably produced paper bedding

Essential Oil Equipment

- Lavender essential oil in amber bottle
    - 1 mL lavender essential oil per test → (1 ml)(8 test subjects) = 8 mL lavender essential oil needed
- Peppermint essential oil in amber bottle.
    - (1 mL)(8 test subjects) = 8 mL peppermint essential oil needed
- Distilled water in polyethylene bottle
    - (1 mL)(32 trials) = 32 mL distilled water needed total
- Cotton balls
    - 1 cotton ball needed per trial (each cotton ball was soaked in 1 mL distilled water or lavender or peppermint essential oil per trial)
    - (3 testing days)(16 trials per day) = 48 total cotton balls needed
- 2 scent chambers (1 for lavender and 1 for peppermint) approximately 24.13 cm wide x 33.02 cm high x 35.56 cm long
    - I used the neutral scent chamber from B1 as the lavender scent chamber during Intervention. After Intervention, I cleaned the lavender scent chamber with Oxivir™ surface disinfectant and then used it again as the neutral scent chamber for B2.

Figure 9

Lavender (left, blue) and Peppermint (right, red) Scent Chambers

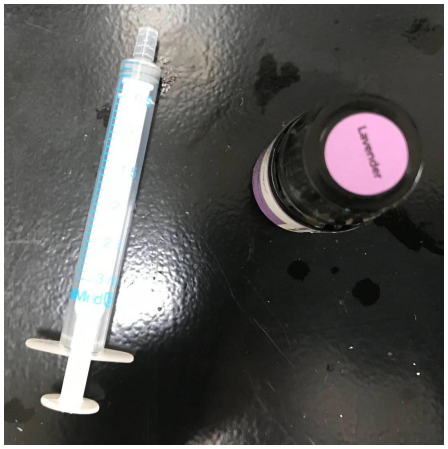


Note. Since rat G was randomly assigned to the Lavender Group, the peppermint scent chamber was closed. This was to ensure that no residual peppermint scent would be inhaled by rat G.

- 3 one mL syringes → used to soak cotton ball

Figure 10

Syringe



- 2 plastic containers → held cotton ball in each scent chamber
- Ionizer → I turned on the ionizer and placed it on the EPM for 5 minutes between each trial to remove scent residual.

Figures 11 and 12

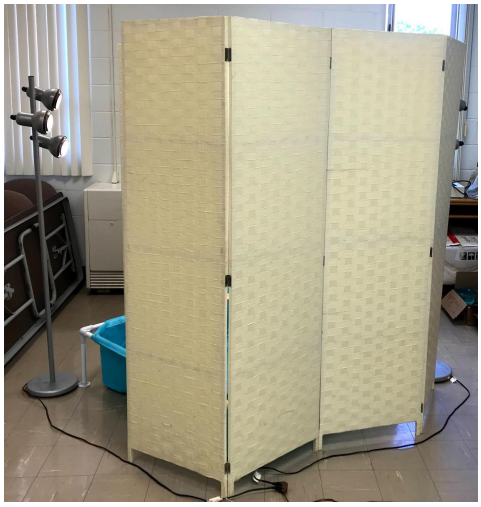
Ionizer

Elevated Plus Maze Equipment

- Camera → I used my phone for all recordings.
- 2 Privacy screens → enclosed EPM from the rest of the room

Figure 13

Privacy Screen



- 2 lamps with 3 light bulbs each (1 lamp facing each open arm)

Figures 14 and 15

Lamps Facing Open Arms of EPM

Note. Lamps were lit for EPM testing.

- Baby pool → surrounded EPM and contained rats that fell of EPM during testing (shown in Figures 2, 11, and 14)
- Timer
- Plywood → built flooring and walls of EPM. Refer to Figure 1 for EPM dimensions.
- Plastic sheeting → lined flooring and walls of EPM
- Wooden blocks → built four legs of EPM
- Drill
- Screws
- Measuring tape
- Saw
- Oxivir™ surface disinfectant cleaner → cleaned EPM between test subjects

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