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# 1. INTRODUCTION

**1.1 Common Stressors in Horses**

*Equus caballus* L. is the present day result of a fifty-million year evolutionary story. Horses are natural born prey animals that are consistently looking out for danger. While this innate survival characteristic may be masked in modern-day horses through training and trust, it is still an innate reaction. It is a common goal among veterinarians, owners, and riders to reduce stress levels in horses in order to optimize wellbeing and health.

Competition horses endure a variety of stressors including travel, new environments, unfamiliar noises, high performance or athletic demands, and limited turnout. It has been previously demonstrated that trailering a horse can initiate a stress response. Fazio et al. (2008) found there were statistically significant differences in cortisol, beta-endorphin, and adrenocorticotropic hormones circulating in the blood before and after trailer rides at various distances. These results can be caused by a combination of factors including limited space in the trailer for the animal, an unstable surface, unfamiliar noises, and a warm environment.

If a horse is not yet adjusted to the high levels of stress experienced, or if a horse may experience a greater degree of symptoms related to stress at international competitions, there are limited legal medications available based on the controlled and prohibited medications list from the Federation Equestre International (2017). Illegal medications include, among others, the sedatives acepromazine, detomidine, hydroxyzine, injectable magnesium sulphate, and xylazine; the muscle relaxants methocarbamol, and dantrolene; and the parasympathetic receptors aminorex, bethanechol, N-butyl scopolamine, carbachol, muscarine, and scopolamine. With limited selection of medications remaining, horses become more vulnerable to effects of high stress. According to the United States Equestrian Federation (2017), lavender, when inhaled through the air, is not an illegal substance. The Federation Equestre Internationale stresses the importance of checking all supplemental ingredients of the essential oil with a veterinarian, as it may contain inactive ingredients harmful to the animal (United States Equestrian Federation, 2017).

Ferguson et al. (2013) examined the effects of lavender aromatherapy in horses, and found no statistical difference in heart rate and respiratory rate between the control horses and treatment horses after they received the stressor. In this study, I examined the effects of lavender aromatherapy has on the stress response of horses. My hypothesis is that lavender aromatherapy has positive effects on helping horses cope with stress. I predict that these effects can be measured in lowered heart rate, cortisol, and norepinephrine levels in horses that have been subjected to lavender aromatherapy during a stressor.

**1.2 Essential Oils**

There are over one hundred different varieties of lavender cultivars. Lavender or *Lavandula angustifolia* Mill. is a purple, herbaceous perennial flower shrub native to the Mediterranean region with a pleasant fragment used to attract pollinating insects (Cornell University).

An essential oil (EO) is a secondary metabolite formed from aromatic plants. These metabolites have a strong, which may serve to attract pollinators, but also may protect plants from infection because they possess antibacterial, antiviral, and antifungal properties (Bakkali, Averbeck, Averbeck, & Idaomar, 2008).

The chemical compositions of EOs are complex. A single EO can contain up sixty different components in a variety of different concentrations. These components are characterized by a low molecular weight, and typically fall into one of two families: terpenes/terpenoids, and aromatic/aliphatic. Two or three of these components are expressed at higher concentrations depending on the EO. Examples of such components include carvacrol, thymol, vanillin, linalool, camphor, limonene, sabinene, farnesol, cinnamaldehyde, eugenol, and menthol (Bakkali et al., 2008).

Differences in chemical compositions and concentrations can be attributed to genetics and the environment in which the plants are grown. One study conducted by Lu H. et al. (2010) identified China-grown *Lavender angustifolia* EO as having the following components: 1,5-Dimethyl-1-vinyl-4-hexenyl but yrate (43.73%), 1,3,7-Octatriene, 3,7-dimethyl- (25.10%), Eucalyptol (7.32%), and Camphor (3.79%). In contrast, Yuanyuan et al. (2008) discovered over seventeen compounds found in *Lavandula angustifolia* EO from China, with linalool (44.54%), geraniol (11.02%), lavandul acetate (10.78%), 3, 7-dimethyl-2, 6-octadien-1-ol (10.35%), and isoterpineol (6.75%) being the most prominent (Saadatian et al., 2013). Plants produce an essential oil with varying components and concentrations making each essential oil unique. The chemistry of the EO gives rise to the properties of the oil, and thus perceived sent (Lu et al., 2010).

The EO extraction process should not be overlooked, as it can alter the chemical composition and concentration of the EO molecules, which could lead to alterations in perceived scent. The most commonly used process of EO extraction is through steam distillation (SD). This process involves heating the plant with steam in a distillation pot. The EOs are vaporized from the plant into a condensing pot where the EOs condense to a liquid. Because water can have a higher boiling point than the EOs, the combined mixture will boil at a temperature slightly less than the normal boiling point of water, and this allows the oil to vaporize under mild conditions. Once condensed back into a liquid, the EO is dried over an anhydrous solution.

A variation of the traditional SD process exits as Steam Distillation-Solvent Extraction (SDS), which uses a solvent to extract the oil after the EO and water condense back into liquid form. The separation of water and oil must occur to obtain the pure EO. A separatory funnel and a nonpolar solvent such as diethyl ether can be used to separate the oil from the water. Once multiple separations have been carried out, it may be optimal to evaporate the remaining diethyl ether in order to obtain a more-pure EO sample.

 One study compared the chemical composition of oriental arborvitae or *Platycladus orientalis* (L.) Franco EO using SD and SDS techniques. While percentage compositions did differ among the two distillation techniques it was not statistically different. The highest statistical difference observed was in oxygenated monoterpene components of the EO. These volatile components were expressed in lower percentages in SD (3.67%) than in SDS (6.87%) due to their tendency to be more soluble in water, and thus, more diluted (Asili et al., 2007).

 Supercritical Fluid Extraction (SFE) is yet another method by which EOs can be extracted from plants. A supercritical fluid is a chemical compound that exists past its critical point, which allows it to dissolve readily into solids and liquids, and can be altered at different pressures and temperatures in order to do so. CO2 is a common supercritical solvent that is used for EO extractions. CO2 is heated in a compression vessel where it will enter into a chamber where the plant is located. The CO2 will diffuse into the plant, dissolve and separate EOs, and return to an extraction vessel, upon which it will cool, return to atmospheric pressure, and diffuse out of the compression system, leaving behind an EO. When the extraction of EO was performed using SFE technique, the only difference in composition was within the oxygenated monoterpene compounds (Pournortazavi & Hajimirsadeghi, 2007). It is becoming increasingly more frequent to find EOs labeled as “aromatherapy grade” or “therapeutic grade” to indicate that the oils have been extracted to a certain standard. It is important to note that the Food and Drug Administration (FDA) does not regulate the quality of EOs (United States Food and Drug Administration, 2016).

Aromatherapy has been used throughout history. It is believed to have been used by the Egyptians for embalmment, by the Greeks for fragrance, and during the renaissance period for wound healing. The term “aromatherapy” was coined in 1937 by chemist Rene-Maurice Gattefosse, and since then has been applied liberally to serve multiple uses (Aromatherapy, 2010). Because the study of aromatherapy can cover a wide variety of topics such as wound healing, infection, and sedation, and the word itself implies that it has “therapeutic” effect, it is often misused and mislabeled. Essential oil aromacology is the study of how certain EOs affect the physiology and psychological state of an organism. The focus of this paper will examine the physiological aspect of EOs, and their use in repressing equine stress factors.

**1.3 Olfaction in Horses**

Horses have flexible nostrils that are able to open and close to alter airflow. This trait is not only an important aspect of olfaction, but it is also noteworthy because horses are obligate nasal breathers. In regards to EOs, once inhaled through each nostril separated by a septum composed mainly of hyaline cartilage, the chemical components of the EO are carried through the nasal cavity, which is supported by thin turbinate bone allowing for increased surface area for olfactory receptors (OR) to reside. The chemicals bind to a single OR proteins expressed on single mucus coated, ciliated, epithelium cells leading to the activation of a G-protein coupled cascade. This indicates that a single OR neuron will express a single gene encoding for a specific OR protein (Briggs, 2013).

In 2004 the Nobel Prize in physiology and medicine was awarded to Richard Axel and Linda B. Buck for their work in the discovery of over 1,000 olfactory receptor genes. Since then, more knowledge has been gained in this field of study. Odorant receptor proteins belong to the G-protein coupled-receptor family A, leading to the activation of adenylyl cyclase or phospholipase C. Protein members in this family share similar features such as having seven hydrophobic transmembrane helices, and three intracellular and extracellular loops. In horses and humans, along with other vertebrate species, it is believed that a single odorant receptor gene was forced to evolve and expand due to selective pressures, and now OR proteins are clustered in groups throughout most of the organism’s chromosomes (Lancet, 2006).

The binding of the chemical to the olfactory receptor protein will cause a G-protein coupled cell-signaling cascade through the cell. The activated G-protein will activate the protein enzyme adenylate cyclase, which is responsible for cleaving ATP into cAMP. cAMP opens cAMP ion channels, allowing for sodium and calcium to enter the cell. This influx of sodium and calcium is the generating potential. An additional mechanism GPCR can generate an influx of sodium and calcium by activating phospholipase C to produce inositol triphosphate. The intracellular calcium will open the chlorine ion channel, allowing for chlorine to exit the cell. This further depolarizes the cell. If this depolarization due to the chlorine reaches threshold, it will cause the voltage-gated sodium channels to open, allowing sodium to enter the cell. This even greater influx of charge stimulates the action potential. The action potential is transmitted down the axon of the specific afferent olfactory receptor neuron, which goes through the cribriform plate, and enters the olfactory bulb of the central nervous system (CNS) (Kaupp, 2010).

The glomerulus is a specialized structure located in the olfactory bulb. It consists of two regions, one of which is dedicated to olfactory receptor cells. This region is believed to receive signals from individual OR cells with the same or similar receptors. Within the glomerulus, the dendrites of the incoming OR cells synapse with mitral cells and tufted cells (Kaupp, 2010). While there is not enough evidence to fully understand the detailed mechanism of the mitral cells, there are a handful of hypotheses. One hypothesis suggests that the mitral cells report information regarding odor strength depends on the timing of the mitral and ORC’s action potential firing. The mitral cells will transmit action potentials to various regions of the brain including the frontal lobes, and components of the limbic system. The limbic system is composed of the amygdala, hippocampus, thalamus, hypothalamus, and basal ganglia. Its main purpose is to process primary emotions including fear and stress (Gire et al., 2012).

Norwood and Leslie (2011) propose a potential mechanism by which EOs could reduce stress. When rats were treated with lavender aromatherapy and chlordiazepoxide they displayed a decrease c-fos expression in the paraventricular nucleas and in the dorsomedial hypothalamic nucleus, suggesting that lavender can have anesthetic-like effects. Additionally Chioca et al. (2013) showed that the inhalation of lavender oil does not suppress GABAa neurotransmission, but does suppress serotonin 5-HT1A neurotransmission. While additional research is needed to show how this particular pathway operates, literature suggests that the inhalation of lavender suppresses neuronal activity in the brain.

**1.4 Entry Routes**

Components of the EOs not only bind to the olfactory receptors, but they also permeate through the mucosal membrane of the lungs into the bloodstream. The EO is transported through the nasal passage, through the trachea, and into the lungs. Once in the lungs, the chemicals travel through the bronchi, bronchioles, alveolar ducts, and alveoli. The alveoli are surrounded by simple squamous epithelial tissue, which the chemical must diffuse across. After, the chemicals bypass the basal lamina, and endothelial cells, and enter into the bloodstream (Medinsky et al., 1993). It has been reported that linalool, one of the main components of lavender oil, has been effective when administered topically or into the blood in reducing blood pressure and heart rate due to the vasodilative effects it has on tissues.

A more recent study conducted by An and Liggett (2017) identified olfactory receptors in human airway smooth muscle cells located in the lungs and trachea. This is a unique finding because chemoreceptors had been thought to localize in the olfactory bulb. The ORs identified through rt-PCR included OR51E2, OR152, OR10Q1, OR2A1, OR2W3, OR1J4, OR2A7, OR1Q1, OR6A2, and OR1J1. This study approached this topic by analyzing the possible interaction of pharmaceuticals on asthma-based cells. The study also gives rise to the possibility that other ORs could have an effect on the smooth muscle, and vasodialative efforts of the lungs (Anjos, 2013; Heuberger et al., 2004; Santos et al., 2011). This finding may give rise to an additional mechanism by which EOs could alter the normal physiological state of an animal.

**1.4 Cortisol**

When a horse encounters a stressful stimulation, the horse will interpret the stressor through vision, olfaction, audition, somatosensation, and gustation (Smith, 2009). Once one or more of these signals is processed in the brain as a stressor, the brain will react by propagating signals through the limbic system. The neurosecretory cells of the hypothalamic periventricular nucleus will secrete neurotransmitters and neuropeptides at the median eminence. In specific to a stress response, norepinephrine and glutamate will be secreted. Norepinephrine will bind to the alpha-adrenergic receptors, and glutamate will bind to the NMDA and GluR5 receptors on the CRH neuron leading to an increase in calcium, and neuronal excitability. In addition to neuronal excitability, cell signaling transduction cascades are initiated causing for the release of prepackaged corticotropin-releasing hormone (CRH) into the bloodstream, which is followed by transcription of the CRH gene through cyclic AMP/protein kinase A/phospho-CREB pathways (Aguilera & Liu, 2011).

CRH and an additional neuropeptide called urocortin, which acts to fine-tune the signal cascade, bind to one of two seven transmembrane domain receptors on corticotropic cells in the anterior pituitary gland: CRH receptor 1 (CRH-R1) and CRH receptor 2 (CRH-R2). The binding stimulates a GPCR cascade leading to the synthesis and release of POMC via the cAMP-protein kinase A pathway (Majzoub, 2006). Pre-pro-opiomelanocortin is cleaved into proopiomelanocortin (POMC), which gives rise to multiple polypeptides through various protein modifications (Radioimmunoassay of Human Plasma ACTH, 1964). One of these polypeptides, called adrenocorticotrophic hormone (ACTH), is secreted from the corticotropic cell and diffuses into the portal vein of the anterior pituitary. ACTH travels through the superior and medial suprarenal arteries of the adrenal gland where it diffuses to the adrenal cortex.

In the adrenal cortex of the kidney, ATCH binds to the melanocortin-2 receptor and in the presence the accessory protein MRAP1, induces a G-protein coupled cascade. This cascade induces adenylate cyclase to cleave ATP into cAMP. cAMP activates protein kinase A (PKA). PKA will go to the drial membrane, where it phosphorylates the steroidogenic acute regulatory protein (StAR). Once activated, StAR transports cholesterol into the mitochondria.  Once in the mitochondrial, P450ss enzyme will cleave ester the side chain from cholesterol. Following, 17-alpha hydroxylase will oxidize the steroid at C17, followed by the action3-beta-hydroxysteroid dehydrogenase***.*** This gives rise to 17-alpha-hydroxyprogesterone. The new molecule is transported out of the mitochondria to the endoplasmic reticulum where the enzyme, 21 beta hydroxylase, adds a hydroxy group into the 21st carbon giving rise to the molecule 11-deoxycortisol. 11-deoxycortisol is transported back to the mitochondria where 11-beta-hydroxylase oxidizes C11. This results in cortisol – a stress hormone. Cortisol then leaves the mitochondria, and diffuses across the cell membrane into the bloodstream (Radioimmunoassay of Human Plasma ACTH, 1964; AN & C, 2016).

Cortisol is involved in a wide variety of cellular functions, including immunological, metabolic, muscular, bone development, and blood pressure. Cortisol’s main function during a stress response is to redirect energy to organ systems that will allow the organism to survive. Almost all cells express the glucocorticoid receptor (GCR), which is a cytoplasmic receptor to which cortisol, among other glucocorticoids bind. Cortisol will freely permeate across the cell membrane, into the cytoplasm of the cell. Once cortisol binds to GCR, it will cause GCR to alter its shape, and the two supporting heat shock proteins that reside on the inactive GCR are released. Then, GCR + cortisol are able to act directly on DNA as a transcription factor. An alternative route upon activation of the GCR by cortisol includes transrepression, or inhibiting or silencing transcription factors through direct interaction. Some of these transcription factors are NF kappa B and Activator protein 1  (AN & C., 2016).

In skeletal muscle cells, cortisol's main function is to increase the levels of blood glucose, which will be redirected as fuel in the brain. Cortisol regulates glucose by repressing insulin and glycogen synthase activity. In skeletal muscle cells, there are 173 genes that GCR + cortisol potentially target. Nine of these genes work to inhibit insulin's action. An additional mechanism by which cortisol targets glycogen synthase is through protein synthesis. Increased levels of cortisol decrease the rate of protein synthesis, and increase the rate of muscle proteolysis, especially the proteolysis of leucine, isoleucine, and phenylalanine amino acids. Treating rats via intravenous injection of GCR’s resulted in significant muscle cell atrophy. This is due to the utilization of proteins in the skeletal muscle, which are used to in hepatic gluconeogenesis. Gluconeogenesis results in increased levels of glucose, which is then used as energy for the brain (Kuo, et al., 2013). Cortisol also targets adipose tissue to release fatty acids, which is used to replace glucose as the cell’s major energy source.

Too much circulating cortisol for prolonged periods of time can have a negative impact on the wellbeing of the animal. It can increase blood glucose levels, inhibit insulin production, vasoconstrict arteries, and modulate the immune response by promoting anti-inflammatory molecules. Issues such as cardiovascular and autoimmune diseases, and gastrointestinal, fertility, and sleep disorders can result in prolonged, chronic stress, therefore, it is important to reduce unnecessary chronic stress levels in horses (Aronson, 2009).

**1.6 Circadian Rhythm**

The circadian rhythm is a 24-hour cycle that allows for an individual to adapt to bodily needs by affecting individual cells at the transcriptional level. The circadian rhythm is responsible for sleeping, waking, hunger, migration, and shedding. The circadian rhythm is not always the same from day to day, as it must be able to adjust to changes in the environment. Such changes could include stress, daylight, and food availability. A region of the hypothalamus called the suprachiasmatic nucleus processes the environmental changes and activates the major gene responsible for controlling the circadian rhythm, Circadian Locomotor Output Cycles Kaput (CLOCK) gene (Chan & Debono, 2010). The hypothalamus at this time will also stimulate the production of cortisol. GCR + cortisol will stimulate *Per1, Per2,* and *E4bp4.* These genes are responsible for cellular circadian rhythm cycles (Mavroudis et al., 2012). Therefore, it is important to take into consideration the date and time of day at which cortisol samples are collected due to its fluxuation throughout the year and day.

**1.7 The Autonomic Nervous System**

The autonomic nervous system is responsible for unconscious body functions, and includes three branches: sympathetic, parasympathetic, and enteric. The sympathetic nervous system is responsible for an organism fight or flight response when a certain danger is present or a stress. The parasympathetic nervous system is responsible for the organism’s rest and digest response, which is unregulated in relaxed states. The enteric nervous system is referred to as the second brain, as it appears it may work independently of the autonomic nervous system. The enteric nervous system is responsible for the digestive system. Both the parasympathetic and sympathetic nervous systems are controlled by the central nervous system and can be altered based on communication between the nerves in the spinal Sympathetic nerves typically sprout from the thoracic and lumbar portion of the spinal cord; parasympathetic nerves typically sprout from the sacral and cervical portion of the spinal cord. Sympathetic preganglia are found close to the spinal cord allowing for signals to be transmitted quickly to a variety of organs; parasympathetic preganglia are longer and found farther away from the spinal cord. Acetylcholine will be released from sympathetic preganglionic neurons, stimulating the postganglionic cells. Postganglionic neurons are associated with a variety of effector organs and cause the release of neurotransmitters or hormones, which induce a specific response. The autonomic nervous system is responsible for controlling cortisol, norepinephrine, and other stress-related biomolecules in stress response. Understanding how it operates is an important aspect to the stress response.

**1.8 Norepinephrine**

An additional stress-related hormone called norepinephrine (NE) is also secreted during a stress event. Norepinephrine is both a hormone and neurotransmitter, depending on the environment in which it is released, and acts on blood vessels and muscles to raise blood pressure and increase HR. NE is synthesized in a cell through various steps before it can be released. It is synthesized from tyrosine, which undergoes hydroxylation catalyzed by tyrosine hydroxylase, resulting in dihydroxyphenylalanine. The new molecule undergoes a decarboxylation step by pyridoxal phosphate and dihydroxyphenylalanine decarboxylase and results in dopamine. Dopamine is oxidized into NE by the enzyme dopamine beta hydroxylase and ascorbate, which acts as a cofactor. The majority of these steps occur in the cytoplasm, but some do occur while the neurotransmitters are in vesicles (Dienel & Cruz, 2016).

NE can be secreted from various parts in the body, including the brain, sympathetic nervous system, and the kidneys. In the brain, NE can work specifically in the pons through a nucleus called locus coeruleus. NE can be secreted from these specific neurons, and act on surrounding neurons in various areas of the brain, including the cerebral cortex, basal forebrain, limbic system, spinal cord, cerebellum, and thalamus. Here, NE can bind to alpha-1 or alpha-2 receptors, which are facilitated by GPCR, and cause neuronal excitability; ultimately these results in enhancement of alertness, arousal, vigilance, increase in sympathetic activity, and decrease in parasympathetic activity (Samuels & Szabadi, 2008).

In the sympathetic nervous system, presynaptic ganglion neurons will stimulate postsynaptic ganglion. The postsynaptic ganglion will release NE directly onto the effector organ. Some of the effector organs include eyes, striated muscle, lungs, and kidneys, in which NE will bind to receptors. An additional mechanism by which NE can be secreted is from the kidneys into the blood. This typically involves the stimulation of chromaffin cells located in the innermost region of the adrenal gland called the adrenal medulla. Once NE is secreted either into the blood, or by a neuron, it will bind to a specific receptor on an effector organ (de Diego et al., 2007; Periman & Chalfie, 1977).

The family of receptors NE binds to belong to the adrenergic receptor family, and there are two different types (alpha or beta) that NE binds to, depending on the type of response. To inhibit an effector organ such as the gastrointestinal tract, and specific components of the immune system, NE will generally bind to the alpha-2 adrenergic receptor. To excite an effector organ such as skeletal and striated muscles, the radial muscle in eye, sweat and salivary glands, glycogenolysis, and kidney secretion NE can bind to beta and alpha-1 receptors. The binding of NE is processed through a GPCR. The specific binding of NE to its targeted receptor insures that NE selectively impacts those cells that will help the organism complete a fight or flight response. (de Diego et al., 2007; Periman & Chalfie, 1977).

Competition horses are susceptable to a variety of unnecessary stressors. These stress-related biomolecules can have a negative impact on a horse’s immune system, metabolism, and performance; therefore, it should be a shared goal to reduce these unnecessary stressors in order to optimize the quality of life for the animal. Lavender aromatherapy has the potential to reduce stress levels based on previous studies. While little is known on how lavender aromatherapy can supress equine stress, there is supportive evidence in other species. The hypothesis of this study is that lavender aromatherapy has positive effects on helping horses cope with stress. I predict that these effects can be measured in lowered HR, cortisol, and NE levels in horses that have been subjected to lavender aromatherapy during a stressor.

# 2. METHODS

Seven castrated male horses, and one female horse were used from the Nancy G. Held Equestrian Center of Albion College located in Albion, Michigan, in this cross-over study, in which each horse was exposed to both control and experimental treatments. The horses ranged in age from eight to twenty-one years old and had previous experience in trailering. The breeds of the horses included thoroughbreds, warmbloods, quarter horses, Arabians, and Morgan-cross horses.

On November 12, 2016, baseline heart rates (HR) and blood samples were taken on each of the eight horses prior to loading in a 1.95m x 2.88m two horse Sundowner 777 Sunlite bumper pull horse trailer. Heart rates were taken from behind the left elbow using a stethoscope, and blood samples were taken through the jugular vein utilizing vacutainer ® brand collection needles, and BD vacutainer serum separator tubes. A licensed and practicing small animal veterinarian with over thirty years of experience collected blood samples, and a handler took heart rates. Eight horses were loaded in pairs into the trailer. Horses #1-4 were randomly assigned as the treatment and received a 20% lavender oil, 80% distilled water aromatherapy treatment using lavender (product #3575) from Young Living Essential Oil administered by a diffuser (product #058321) from Spa Room, and horses #5-8 were used as the control group and received a distilled water aromatherapy treatment in the same diffuser. Once the paired horses were loaded in the trailer, the diffuser was activated. The control horses were trailered first to prevent any possible responses to leftover scents. All air vents, and windows remained closed to insulate the scent. The eight horses were trailered in groups of two for 15 minutes of driving 5.5 for miles over even to slightly rolling terrain; each drive included five turns (three right-handed turns, two left-handed turns). The trailer ride served as the stressor allowing cortisol levels and HRs to increase. Immediately after the ride, the second collection of HRs and blood samples was made. The third collection of HRs and blood samples was made 50 minutes after the trailer ride. Blood samples were centrifuged in a Hamilton Bell VanGuard V6500 centrifuge at 3,437 rotations per minute (rpm) between 5-7 minutes at room temperature (RT) allowing for the separation and isolation of serum, after which, the serum was frozen at -32º C until further quantifications were made.

On December 3, 2016, the same eight horses were trailered in the same pairs as before, following the same route previously described, in the same horse trailer. Horses #1-4 were used as the control and received a distilled water aromatherapy treatment, and horses #5-8 were used as the treatment group and received 20% lavender oil, 80% distilled water aromatherapy treatment.

A cortisol ELISA kit (ADI-900-071) purchased from Enzo Life Sciences was used to quantify cortisol levels. Sample preparation involved three separate 200uL diethyl ether extractions followed by drying under nitrogen. The first two serum samples for each horse were plated in duplicates, and the third serum sample for each horse was plated in singlets due to limited number of wells. The cortisol ELISA kit from Enzo life sciences had previously been used to quantify equine cortisol levels in a published article (Lemasson et al., 2015). In addition, five serum samples were sent to the Diagnostic Center for Population and Animal Health at Michigan State University to quantify cortisol samples to ensure correct application of the kit from Enzo Life Sciences. The third stress marker that was being measured was Norepinephrine. An ELISA kit (ka1891) purchased from Abnova was used to quantify Norepinephrine levels in the serum samples.

All data analyses were performed using Excel, and included calibrating averages, standard errors, one-tailed paired t-tests, and one-tailed homoscedastic t-tests. T-tests performed on the averaged raw data were one-tailed homoscedastic t-tests, while those performed on the *stressed minus baseline* values utilized the one-tail paired t-test. All significance values were calculated at the five percent level.

# 3. RESULTS

A total of three blood samples and three heart rates were taken during an individual testing of each horse: before loading into the horse trailer--baseline, immediately after the trailer ride—stressed, and 50 minutes after the initial trailer ride—recovery. Heart rates, cortisol, and norepinephrine levels were quantified and compared amoung the tree conditions. The data below include the results using averaged raw data for heart rate and cortisol, as well as results based on a more in-depth statistical analysis.

**3.1 Raw Heart Rate Results**

The average baseline HR for the control horses was 37.9 b/m (+/- 3.6 SE), which increased to 48.5 b/m (+/- 3.9 SE) after the trailer ride stressor (Figure 1). Average baseline HR was significantly lower than the average stressed HR for the control (t =1.8, p =0.03, df =14). Average HR returned to 36.25 b/m (+/- 1.3 SE) fifty minutes after the initial trailer ride. Average stressed HR was significantly higher than the average recovery HR for the control (t =1.8, p =0.005, df =14).

The average baseline HR for the treatment horses was 35.5 (+/-1.7 SE), which elevated to 44.8 b/m (+/- 3.5 SE) after the stressor (Figure 1). Average baseline HR was significantly lower than the average stressed HR for the treatment (t =1.8 , p =0.02, df =14). Average HR returned to 35.3 b/m (+/- 2.0 SE) fifty minutes after the initial trailer ride. Average stressed HR was significantly higher than the average recovery HR for the treatment (t =1.8, p =0.016, df =14). There was no statistical difference between the average baseline HR for the control and treatment horses.

**3.2 Raw Cortisol Results**

The average baseline cortisol level for the control horses was 539.4 pg/uL (+/- 156.7 SE), which increased to 4286.6 pg/uL (+/- 969.8 SE) after stressor (Figure 2). Average baseline cortisol was significantly lower than the average stressed cortisol for the controls (t =1.8 , p =0.0009, df =14). Average cortisol levels returned to 1,220.0 pg/uL (+/- 394.3 SE) fifty minutes after the initial trailer ride. Average stressed cortisol was significantly higher than the average recovery cortisol for the controls (t =1.8, p =0.005, df =14).

The average of baseline cortisol levels for the treatment horses was 785.8 pg/uL (+/- 254.7 SE), which elevated to 3297.6 pg/uL (+/- 1191.8 SE) after the stressor (Figure 2). Average baseline cortisol was significantly lower than the average stressed cortisol for the treatments (t =1.8, p =0.03, df =14). Average cortisol levels returned to 1,335.6 pg/uL (+/- 703.8 SE) fifty minutes after the initial trailer ride. Average stressed cortisol did not differ from the average recovery cortisol for the treatments (t =1.8, p =0.09, df =14). There was no statistical difference between the average baseline cortisol for the control and treatment horses.

**3.3 Raw Norepinephrine Results**

Norepinephrine results have not been calculated. However, preliminary results from the plate reader are documented in Table 1.

**Figure 1*.*** Average heart rate for control and treatment horses as baseline, stressed, and recovery. Error bars represent standard errors. Statistical significance was generated using a one-tailed homoscedastic t-test (\* =p <0.05).

Figure 2. Average cortisol for control and treatment horses at baseline, stressed, and recovery. Error bars represent standard errors. Statistical significance was generated using a one-tailed homoscedastic t-test (\* =p <0.05).

Table 1. Optical density values of the preliminary norepinephrine values.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| 1.23 | 2.24 | 2.21 | 2.44 | 2.25 | 2.39 | 2.20 | 2.23 | 2.17 | 2.16 | 2.13 | 2.20 |
| 1.21 | 1.82 | 2.11 | 2.07 | 2.27 | 2.06 | 2.25 | 2.29 | 2.48 | 2.17 | 2.05 | 2.15 |
| 1.28 | 1.93 | 2.14 | 2.19 | 2.25 | 2.12 | 2.06 | 1.99 | 1.84 | 2.11 | 1.97 | 1.94 |
| 1.25 | 1.43 | 2.23 | 2.11 | 2.10 | 2.08 | 2.11 | 1.93 | 1.92 | 2.23 | 2.08 | 2.01 |
| 1.07 | 1.45 | 2.42 | 2.48 | 2.36 | 2.30 | 2.31 | 2.41 | 2.24 | 2.45 | 2.08 | 2.17 |
| 0.87 | 0.72 | 2.16 | 2.18 | 2.10 | 2.00 | 2.18 | 2.07 | 1.97 | 2.04 | 1.9 | 2.08 |
| 0.94 | 0.66 | 2.04 | 2.12 | 2.07 | 2.04 | 2.24 | 1.91 | 1.88 | 2.04 | 1.96 | 2.02 |
| 0.93 | 0.50 | 2.30 | 2.09 | 2.13 | 2.15 | 2.21 | 2.05 | 2.56 | 2.05 | 2.06 | 1.77 |

**3.4 Statistical Analysis**

Individual baseline values were subtracted from individual stressed values for both control and treatment horses, as baseline values varied from individual horses between November 19, and December 3. The average increased HR for the control horses was 10.6 b/m (+/- 2.6 SE); the average increase HR for the treatment horses was 9.3 b/m (+/- 2.6 SE) (Figure 3). These values did not differ significantly (t =1.9, p = 0.37, df =7).

The average increase in cortisol for the control horses (3,747.2 pg/uL +/- 864.2 SE) was significantly greater than the average increase in cortisol for the treatment horses (2,511.8 pg/uL +/- 1009.9 SE) (t =1.9, p =0.038, df =7) (Figure 4)

**Figure 3.** *Stressed minus baseline* heart rates for individual control and treatment horses. Statistical significance was generated utilizing a one-tail paired t-test. (p-value= 0.37)

**Figure 4.** *Stressed minus baseline* heart rates for individual control and treatment horses. Statistical significance was generated utilizing a one-tail paired t-test (p-value=0.038).

# 4. DISCUSSION

Both the control and treatment horses showed significant increases in average HR and serum cortisol levels when trailered, indicating that trailering is a form of stress on the animals. This finding was expected based on previous literature (Fazio et al. 2008). In addition average stressed HR were significantly higher than average recovery HR for both the control and treatment groups, indicating that HR decreased once the horse was removed from the stressor. With regards to cortisol, control horses showed significantly higher levels when stressed than then in recovery but there was no significant difference found among treatment horses between stressed and recovery states. Last, there was no statistical difference between the baseline HRs or cortisol levels for both the control and treatment groups, indicating that the horses HRs and cortisol levels were not different on November 19 and December 3.

 The *stressed minus baseline* values show a statistically significant difference between average heart rates for the control and treatment groups meaning that the lavender aromatherapy did not cause a significant difference in heart rate (p = 0.37). This is what was expected based on a previous study (Ferguson et al., 2013). However, results of cortisol levels suggest that such levels were suppressed in horses when they received the lavender aromatherapy. Up to this point, no previous reports have studied cortisol levels in horses treated with any form of essential oil.

 Though there is limited research in this field as it relates to horses, it is appropriate to discuss findings in other species. Previous reports support that oregano essential oil, and major chemical components of lauraceae EO can decrease blood cortisol levels in pig following transportation stress, and fish when subjected to stress (Garlet, et al., 2015; Zou, et al., 2016). When administering lavender to groups of nervous and calm sheep Hawken and Blache (2012) found that lavender significantly reduced cortisol levels throughout a thirty-minute time interval in calm sheep, but increased cortisol in the group of nervous sheep. The lavender oil reduced movement and vocalization frequency in calm sheep, but only affected cortisol when concentrations were already low. In the treatment group of nervous sheep which received lavender, movement, vocalization frequency, and cortisol concentrations increased after thirty minutes compared to the control group of nervous sheep. These results suggest genetic differences in the effects lavender can have on an animal therefore suggesting the need to evaluate the emotional state of the animal prior to using lavender to treat stress-related (Hawken et al., 2012).

In addition to cortisol repression by lavender aromatherapy, there is evidence of heart rate repression correlated with lavender aromatherapy. Using a combination of light therapy and three different scents of aromatherapy (lemon, peppermint, and lavender) on humans, Doung and Jacob (2016) found that heart rate and blood pressure (BP) decreased in patients, though the greatest decrease in the HR and BP occurred when lemon aromatherapy was used in conjunction with light therapy.

Follow-up studies should include a more in-depth analysis of stress related serum molecules including glucose and beta-endorphin. In addition, different essential oils at varying concentrations could be tested to determine their effectiveness in reducing stress including lavender, chamomile, and peppermint. It may be beneficial to examine how aromatherapy can affect baseline values without a stressor.

Overall, my results show that cortisol levels were suppressed in stressed horses that received lavender aromatherapy. These conclusions partially support the original hypothesis that lavender aromatherapy has positive effects on horses during a stressful situation. Cortisol was the only parameter that was lowered in horses that were subjected to lavender aromatherapy during a stressor.

# 6. ACKNOWLEDGEMENTS

 This research would not have been possible without Dr. Eric Heitman, who donated his time, skill, and supplies to perform all the blood draws, and trailer the horses. Dressage4Kids Incorporated: *Lendon Gray Scholarship*, and The Foundation for Undergraduate Research, Scholarship, and Creative Activity at Albion College provided funds to carry out this research. I acknowledge the Albion College Biology department including Mr. Kurt Hellman and Mr.David Carey for their assistance; the Albion College Equestrian facility and staff for use of the horses. Lastly, I would like to thank my thesis committee advisor and members for their support, guidance, and patience: Dr. Rabquer, Dr. Kennedy, Dr. Streu, and Dr. Skean.

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