PHEROMONES AND INTEROMONES CHANGE HEART RATE AND BEHAVIOR OF ANXIOUS DOGS

By

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A Thesis

In

ANIMAL SCIENCE

Submitted to the Graduate Faculty

of Texas Tech University in

Partial Fulfillment of

the Requirements for

the Degree of

MASTER OF SCIENCE

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May, 2013

ACKNOWLEDGEMENTS

Since being here at Texas Tech University I have had the great opportunity to work with some really great people. I would like to thank Stanley and Edward for giving me my first start working with pigs. I will always remember washing out rooms and then going to throw hay in the winter time. I never thought I would ever work with pigs but that just goes to tell you that you have to be prepared for anything. I would like to thank my fellow graduate students Brittany Backus and Avi Sapkota. These two people helped me more than most teaching me lab and field techniques, and treated me like a little brother taking me under their wing. We have had a lot of fun times doing research together and I wish these two the very best. I would also like to thank Dr. Brooks and Dr. Norman for taking the time to serve on my committee and share their ideas with me. Thanks to ACS members Amber and Mark for keeping on me about keeping the animal care facilities clean and aiding me anyway I could possibly need.

I greatly appreciate Dr. John McGlone, my committee chair, for first hiring me as an undergraduate research assistant and giving me my start in animal research. Without your help and guidance I don't know where I would be today. I will always be grateful for the opportunity that you gave me, by taking a gamble on someone you know for only a short amount of time. You have showed me that I can do more than I ever thought possible and I hope to show you that your investment in me was worth it.

Lastly I would like to thank my friends and fellow graduate students Matt, Tyler, and Cameron for all the "encouragement" they have given me. Also to my family that supported me for so many years in school and let me choose my own path.

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ABSTRACT

The objective of the following studies was to evaluate the efficacy of different pheromones/interomones in anxious dogs. Pheromones have been used in the past as alternative methods in behavior modification. Two studies were designed to test the differences in pheromone collars that are now sold currently and to develop new pheromone collars that may aid in behavior modification in the future.

The methods for the following studies differed from previous studies on pheromone collars in that our model had a highly controlled environment. Previous studies had surveys at the end of home trials in which owners answered a set of questions about their dogs' behavior before and after different treatments. We employed a Latin Square experimental design that exposed each subject to each treatment in random order with appropriate wash-out period between treatment applications. Data was summarized in two phases (baseline and startle) in which a trained individual recorded behaviors and heart rate and was unaware of different treatment groups. Additionally dogs were diagnosed as having anxiety by a behavior-boarded veterinarian. The results of these studies aided in the understanding of how anxious dogs react to different pheromones. The data showed that not only did a conspecific pheromone (DAP) create change in behavior and heart rate compared to placebo, but heterospecific pheromones (RP) also created change. This is important because the data supports the idea that anxious dogs do not respond uniformly to each

pheromone/interomone, and different pheromones from other species could be used to treat anxiety in dogs.

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CHAPTER I

INTRODUCTION

Each year thousands of dogs are relinquished to animal shelters because pet owners are unable to control unwanted behaviors (Patronek et al., 1996). Salman et al., (2000) found that behaviors such as biting, aggressiveness, escaping, destruction of property, and disobedience were the most common. However, a closer examination of why dogs are relinquished by their owner showed that 30.4 % of animals are given up due to housing situations, and 27.1% were given up due to human health and personal issues (Scarlet and Salman 1999). Many owners have tried alternative therapies such as shock collars, obedience school, and alternative training techniques. Shock collars were found to be stressful (Schalke 2007) and dogs that did not receive obedience training and were treated anthropomorphicly had more behavioral problems compared to dogs that had obedience training and were treated non-anthropomorphicly (Voith et al., 1992). Alternative training techniques included kneeing the dog in the chest (Koehler 1996) and stepping on a leash to prevent the animal from jumping (Bridwell, 2007) have been used with less efficacy to aide in training a hyperactive dog.

Another alternative therapy that is continuously being explored is the use of pheromones. Pheromones can be defined as "substances secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction" (Karlson and Lushcher 1959). Some pheromones can change the way an animal behaves as well as their physiology. For example, Watson

and Randford (1960) found that a boar pheromone induced immobility in sows during estrus.

Some chemical signals operate across species and can either benefit or harm either the receiver or the sender. For example, a kairomone is a chemical produced and released by one species, benefiting another species, and often harms the emitter (Wyatt, 2003). Mostly kairmones can be thought of as a predator and prey scenario (cats and mice). The term interomone is defined as a chemical that operates in a given species but will have a very different effect on the receiver of a different species (McGlone, 2012 unpublished data). The key concept is that an interomone does not have to benefit or negatively impact the sending or receiving species. Some pheromones such as Dog Appeasing Pheromone (DAP, CEVA Sante Animale, France) have been shown to aid in comfort and support attachment of the puppy to the mother (Pageat, 1999). This study focuses on dogs that were clinically diagnosed as being anxious. Our objective was to compare several different types of pheromones collars and the affects that they had on HR and behavior.

CHAPTER II

LITERATURE REVIEW

Introduction of the Olfaction System

Dogs have been known to have a very sophisticated olfactory system. Dogs have been used for many years to sniff out and find many items from missing persons to bombs. Dogs can be trained to sniff out certain items because they have sensitive olfactory systems. What sets dogs apart from other species is the differences in: (1) dogs have a larger surface of olfactory epithelium, suggesting that the number of olfactory neurons and the density of olfactory receptors are higher than those of humans; (2) the brain structures involved in the olfactory function such as the olfactory bulb are larger relative to the rest of the brain; (3) the number of functional olfactory receptor (OR) genes is higher, as in the case of mouse (at least three times), and consequently the number of specific subtypes/subfamilies is also higher, permitting probably a more fine tuning to chemical cues (Rouquier and Giorgi, 2007). There has been lab work that shows that the dogs' OR repertoire contains approximately 1300 OR genes, and these genes are composed of a pseudogene fraction ranging from 12-18% (Olender et al., 2004; Quignon et al., 2003).

The Olfactory System of Dogs

For a long time there appeared to be two main regions that make the olfactory system of a dogs: the vomeronasal organ (VNO) and the main olfactory bulb. The VNO is a bilateral symmetric structure that lies along the ventrorostral aspect of the nasal septum (Adams and Wiekamp, 1984), and was thought to be the mechanism

that can detect pheromones. The main olfactory bulb was thought to be responsible for recognizing the volatile odorant molecules (Tirindelli et al., 2009) of smells found commonly in the environment. Recent studies have shown that when these systems work together, with additional help from another olfactory organ the accessory olfactory system. Pheromones can be detected regardless of their nature or molecular weight of the compound (Baxi et al., 2006). Evidence has also been shown that an animal will change its behavior based of which system detects the pheromone, or if a combination of the two working together made the detection.

The Vomeronasal Organ

As previously stated, the VNO is responsible for detecting certain pheromones but only if they are in a non-volatile state. These pheromones can either be interspecific or intraspecific (Tirindelli et al., 2009). The VNO sends its' neuronal signals to the accessory olfactory bulb (AOB) and then to the amygdala, and then ultimately to the hypothalamus. The VNO is made up of a blind-ended mucus-filled tube in cross section, and has a thick pseudostratified sensory epithelium medially and thinner ciliated-on-sensory epithelium laterally (Brennan, 2001; Halpern, 1987).

With the detection of molecules in the VNO certain species show a physically noticeable behavior display defined as the flehmen response in males and females including: pigs (Martys, 1977), cats (Beaver, 1992), horses (Crowell-Davis and Houpt, 1985) and cattle (Reinhardt, 1983). In goats, D'Hospital and Hart (1985) showed that fluid passed from the mouth to the vomeronasal cavity during flehmen.

Vomeronasal Organ Structure and Receptors

The VNO lumen is made up of three main cell types: (1) supporting cells, which are found superficial layer of the sensory epithelium; (2) the basal cells are located on the basement membrane near both the sensory and non sensory epithelium (Giacobini et al., 2000); (3) lastly the receptor cells/sensory neurons, which can be found in the vomeronasal epithelium and can be broken further down into two different classes termed Vomeronasal Receptor 1 (V1R) and Vomeronasal Receptor 2 (V2R) (Brennan, 2001; Connor and Lynds, 1977; Conzelmann et al., 2002; Crish et al., 2003). These V1R class receptors are expressed by the VRNs located more superficially near the lumen of the VNO, whereas the V2Rs are found in the VRNs of the basal region (Brennan, 2001; Conzelmann et al., 2002). Not only are these two classes of pheromone receptors located in different areas of the VNO, but have little homology between each other. V1Rs are shown to differ in their transmembrane domains which are composed of different ligand binding site (Døving and Trotier, 1998). Also studies have shown that V1Rs express the G protein $G_{i\alpha 2}$ and these signals are projected to the anterior part of the AOB. On the other hand, V2Rs have a high degree of variability in a large extracellular N-terminal domain (Brennan, 2001), which respond to different compounds compared to that or V1Rs. Additional studies found that V2Rs express G_0 and project to the posterior part of the AOB (Clancy et al., 1984). These subdivisions between the of receptors can be visualized using antibodies to cell surface proteins such as olfactory cell adhesion molecule (OCAM) (von Campenhausen et al., 1997) and had been previously identified with monoclonal

antibodies (Mori et al., 1987; Schwarting et al., 1994) and lectin binding patterns (Halpern et al., 1995; Takami et al., 1992).

Main Olfactory System and Accessory Systems

The main olfactory system (MOS) is compiled of the main olfactory epithelium (MOE), main olfactory bulb (MOB), some associated nerves, glands and tracts. There is compeling evidence that show that some pheromones are detected by the MOE, but the general use of this structure is to sense volatile molecules, usually everyday smells that are found in the environment. The MOE is similar to the VNE because it is mainly composed of three main cell classes: olfactory sensory neurons, supporting cells, and basal cells. Here in the mucosal surface of the MOE, cilia are the site of primary transduction for odorants and pheromones.

Odorant Receptors and Trace Amine-Associated Receptors

In 1991 odorant receptor genes were discovered by Buck and Axel. Each odorant receptor is found in one of four partially overlapping zones of the olfactory epithelium (Miyamichi et al., 2005; Ressler et al., 1993). How these receptors work is very unique, in that these receptors are able to sense several different types of odorant molecules. For instance, if a single odorant molecule can be responsible for binding to several different odorant receptor sites, which allows the olfactory system to recognize a enormous number of odorants found in the environment (Buck, 2000; Mombaerts, 2004). The role of these ORs is not clearly understood in how they play a role in pheromone recognition. However, they can be found in the VNO and therefore

are thought to be conveying information both of the quality of an odorant and the individual that has emitted it (Kobayakawa et al., 2007).

Trace Amine-Associated Receptors (TAAR) were discovered in 2006 (Liberles and Buck, 2006) and reported as an second set of chemosensory receptors. These receptors are not related to ORs but do share some of the same biogenic amines. They are not thought to have much to do with pheromone recognition but recent studies have shown that isoamylamine and trimethlamine bind to two of the different classes of TAARs. This is important because these compounds are found in the urine in female mice at the onset of puberty (Nishimura et al., 2005). Therefore TAARs could potentially be used to discriminate differece in gender and social status of individual animals.

Anatomical Organization and Target Projections of the Main and Accessory Olfactory Bulbs

The main olfactory bulb is the target region of an olfactory neuron, and the vomeronasal organ sensory neurons target the accessory olfactory bulb. When an action potential takes place, the synaptic vesicles empty their neurotransmitters at the synaptic cleft with the mitral/tufted cells. In most animals the level of synapses between the glomeruli and mitral cells differs between the MOB and AOB. The glomeruli in MOB are anatomically separated, encapsulated by periglomerular cells, and uniform in size at approximately 50μm diameter in mice (Tirindelli et al., 2009). While in the AOB synaptic structures are diffusely organized and are surrounded by a

small number of periglomerular cells, and variable in size (10-30μm diameter in mice).

The mitral cell action potential propagates throughout the axon and the synaptic cells empty the contents vesicles to the third level of synapse, the granule cells (Mombaerts et al., 1996). In both the MOB and the AOB, the activation of the mitral/tufted cells causes activation of the granule cells. These granule cells will then produce negative feedback on to their neighboring mitral/tufted cells. Also dendrites of the principal cells, which are found in the external plexiform layer, also receive inhibitory input from the granule cells.

The Olfactory Bulbs signals to other parts of the Brain

As previously stated in section 2.7, the MOB mitral/tufted cells directly transmit signals from glomeruli to pyramidal neurons in the olfactory cortex. The primary olfactory cortex is composed of several anatomically distinct areas: the piriform cortex, olfactory tubercle, anterior olfactory nucleus, cortical amygdale, and entorhinal cortex. These regions of the brain receive direct input from the olfactory bulb. Most of these cortical regions project back to the main olfactory bulb and to other areas of the brain including: the thalamus, hypothalamus, hippocampus, frontal cortex, and orbitofrontal cortex.

The AOB on the other hand has mitral cells that project to areas of the limbic system: the vomeronasal amygdale, accessory olfactory tract, and the bed nucleus of the stria terminalis (Newman and Winans, 1980; Scalia and Winans, 1975). These signals are then directed to the ventomedial and the medial preoptic areas. These

areas have been identified as being involved in reproduction and social behaviors (Mohedano-Moriano et al., 2008; Mohedano-Moriano et al., 2007).

More importantly the medial amygdala receives direct input from both the AOB and MOB, which is important for social recognition among certain species. Pheromones as well as environmental odorants, from both the VNO and MOE, are processed together and one is not more important than the other as far as we know at this time.

The main olfactory system and accessory olfactory system are complementary

As previously stated, the MOE was thought to only be able to detect volatile molecules and that the VNO could only detect non volatile, low-volatility molecules, and pheromones. However multiple studies support the hypotheses that these two systems may complement each other rather than working separately from one another. Baxi et al., (2006) found that low molecular weight molecules, normally volatile and detected by the MOE can bind to protein carriers and be transported to the VNO (Trinh and Storm, 2003).

The complete loss of the MOE-mediated olfaction in type-3 adenylyl cyclase knockout mice can detect certain volatile odorants via the VNO (Trinh and Storm 2003). In pigs, The accessory olfactory epithelium is involved in social interactions, reproduction, and interactions between sows and piglets through pheromones (Doty, 1986). However, Dorries et al., (1997) showed that blocking the VNO of sows did not stop them from responding to the boar pheromone 5-α-androst-16-en3-one. This

phenomenon is significant because it shows that if the VNO is blocked there is still pheromone receptors located in the MOE. Similar studies showed that a functional VNO was not necessary for male mice to show a response to fresh urine of female mice or to mate (Sipos et al., 1995; Yoon et al., 2005).

Imaging data in mice showed a response of the VNO receptor neurons to general odorants but not from the MOE receptors (Sam et al., 2001). Meredith and O'Connel (1979) showed that electrical activity in single units from the AOB showed that AOB neurons responded to typical environmental odor molecules as well as pheromonal molecules.

Hamster mounting behavior is prevented by a non-functional VNO in naive males (Meredith, 1991). However, mounting behavior was not affected in experienced males with a non-functional VNO (Baxi et al., 2006). This study shows that there is a learning process associated with some behaviors and pheromones/odors. This study reiterates the point that some behaviors may be "learned" or induced if the VNO and MOS are activated.

Pheromone History

Pheromones are an important precursor to a variety of different behaviors initiated in most species. These air-borne chemical cues can be found in feces, urine, or secreted from cutaneous glands (Rekwot et al., 2001). In order for a substance to be classified into a pheromone, the substance must be released by an individual that elicits a behavior or physiological change in other individuals of the same species (Rekwot et al., 2001). Pheromones act on different areas of the sensory system such

as the vomeronasal organ and main olfactory system. As stated previously, pheromones are intraspecific and fall into two distinct classes, releasers or primers. Among these two classes these can be further divided into aggregation, alarm, epideictic (a pheromone used in insects to deter conspecifics from landing near their food source), territorial, trail, and sex pheromones (Mostafa et al., 2012).

However, Wyatt (2010) and others have found that some chemical cues can be detected interspecifically, meaning that the receiving species does not have to be of the same class as the producing species. These types of chemicals are known as allelochemicals and can be further dissected into three subclasses: allomones, kairomones, and synomons. These subclasses are all similar because they all involve an individual producing a substance (producer) that attracts a recipient. Dicke and Sabelis (1988) reported allomones differ by only benefiting the producer organism (a defense mechanism for prey), kairomones benefit only the recipient (a prey pheromone detected by a predator), and synomons benefit both producer and recipient (plants that attract insect pollinators).

Anxiety and Separation Anxiety in Dogs

Until recently many problems associated with dog behavior was termed as anxiety. Anxiety is the displeasing and sometimes overwhelming feeling of fear and concern. The term separation anxiety defines activities such as destruction, vocalization, and house soiling by dogs, while the owner was away from the house or the dog was left unattended in an area without supervision (Borchelt and Voith, 1982; Bradshaw et al., 2002). These two terms are not exclusive and therefore one term may

be used in place of the other. However, recent studies have shown that there are different levels of separation anxiety (McCrave, 1991). Once a dog is diagnosed with separation anxiety many will include the behavior associated with the problem such as: housing soiling and urine marking or destruction and puppy chewing. There have been many suggested treatments for separation anxiety including: behavior training, medication, and pheromone collars.

Behavior modification training has proven methods to see a dogs' behavior become more beneficial to the owner. However, in most cases the owners did not reinforce positive habits that are encouraged during training or owners became aggressive toward their dog and no positive behavior was produced (Chandler, 2001). Medication such as an antidepressant, clomipramine, has been used to treat separation anxiety, obsessive compulsive disorders, and noise phobias in dogs. This medication has proven effectiveness compared to placebo; dogs receiving a standard dose were improved three times faster for signs of destruction, defecation and urination (King et al., 2000). Although many pet owners do not feel comfortable giving these medication themselves, the cost to take the animal to clinics becomes expensive so affected dogs may be relinquished to pet shelters.

Pheromones and their uses in dog behavior

Many dog pheromone studies looked at how the VNO was activated (Quignon et al., 2003) or how different pheromones can be used to relieve anxiety (Gaultier et al., 2008; Kim et al., 2010; Mills et al., 2006; Taylor and Mills, 2007; Tod et al., 2005). These studies reported behavioral effects of a pheromone called dog-appeasing

pheromone (DAP; Ceva Sante Animale, Libourne, France). DAP is secreted from the sebaceous glands between the mammary chains of lactating bitches directly after parturition. Pageat and Gaultier (2003) reported that this pheromone can be detected by the VNO, and has calming effects in both young and adult dogs under stressful situations such as: separation-related behavior problems, phobias, and hyper-attachment (Mills, 2005).DAP has also been reported to reduce separation-induced anxiety (Gaultier et al., 2005), stress associated with transportation (Estellés and Mills, 2006), and in puppies in a new environment (Taylor and Mills, 2005). DAP can also reduce the level of anxiety seen in aggressive dogs (Mills and Hargrave, 2004), dogs in public shelters (Tod et al., 2005), police dogs (Schroll et al., 2005), and puppies in learning and socialization (Denenberg et al., 2005).

Effects of Rabbit Pheromone and its use in dogs

Rabbit Pheromone (2-methylbut-2-enal – RP) is a chemical secreted from around the nipple of lactating mother rabbits shortly after birth. These chemicals carry an attractive property that attracts young kits to the teat where milk is being produced. It has been shown to function as a cognitive organizer, that promotes early learning of relevant environmental cues (Coureaud et al., 2006). How DAP and RP are secreted in nature, chemical composition, their calming effects on newborns, and how pheromones can be interspecific, is the association between RP an DAP that interested us in seeing if a similar effect could be achieve in dogs.

CHAPTER III

PHEROMONES AND INTEROMONES THAT CHANGE HEART RATE AND BEHAVIOR OF ANXIOUS DOGS: TESTING THE COMBINATION OF TWO PHEROMONES IN THE SAME COLLAR.

Abstract

Pheromones are species-specific odors used in communication. Interomones are pheromones in one species, but have diverse effects on other species. The objective of this study was to assess efficacy of pheromones/interomones to modulate heart rate and behavior in adult anxious dogs (trembling, cowering, shy). The dogs (n = 4; average weight 8.1±0.18 kg; estimated 5-12 yr of age intact males) were obtained from a local research facility. Body weights and feed intake were recorded daily. Each dog was housed in a separately-ventilated room with a minimum of 12 m² of floor space. Heart rate (HR) and surface temperature was measured using a telemetry system (Data Science International, St. Paul, MN USA). Behavior was recorded on a DVR and later reviewed by a trained individual. A scan sample was used with a recording interval of 5 min over 24 h. At the end of 24 h with a given pheromone collar, each dog was startled with a 110 db fog horn 12 cm from the dog's head while behavior and heart rate were recorded. Each dog received each treatment in a Latin square design with repeated measures over time. This model allowed evaluation of effects of treatment, dog, treatment by dog, time, treatment by time and dog by time.

Treatments were given in the form of a collar containing each pheromone/interomone and included Placebo, Sergeant's (SERG), 2-methylbut-2-enal -- Rabbit Pheromone (RP), or a SERG and RP combined collar. Placebo/application and startle HR did not differ among treatments but Rp+Serg during placebo/application phase did show a slight trend at ($P = 0.09$ Placebo 101.3 ± 14.3 vs Rp + Serg 99.0 ± 14.3). However, there were several treatment by dog interactions ($P<0.05$) indicating certain dogs were more responsive than others. Dogs with RP collars spent more time lying down (63.19 ± 5 % vs. 87.39 ± 5.69 % of time, $P = 0.04$) and less time pacing (3.18 ± 0.17 % vs. 2.99 ± 0.02 % of time, $P < 0.05$) than placebo-treated dogs. Pace/walk changed differentially among treatments and dogs (dog by RP ($P = 0.01$) and SERG ($P = 0.02$). In summary, SERG+RP showed a trend to lower HR while SERG and RP did not change dog HR. However, pheromone/interomone treatments had differential effects on individual dog behavior and HR. Pheromones/interomones can cause meaningful changes in dog behavior and HR among certain anxious dogs. This is the first report of pheromone efficacy using clinically-diagnosed anxious dogs in a highly controlled environment.

Materials and Methods

General

The study was performed in a animal laboratory setting at Texas Tech University. The institution is AAALAC International accredited and the work was approved by the Institutional Animal Care and Use Committee prior to its conduct. Dogs were obtained from a research facility where they had been used as test subjects

primarily in flea and tick research. In that facility, the animals were kept in concrete-floored kennels with chain link fencing. They were then transported to the Texas Tech University facility, bathed, given a physical exam by a veterinarian and rested for more than a week before testing. Dogs were initially selected because the investigators considered the subjects anxious. A board certified veterinary behaviorist (Dr. Valarie Tynes) examined each dog and confirmed that they were extremely fearful and/or anxious. All dogs displayed hyper-vigilance, avoided novel people and showed minimal interest in socializing with any but a few very well-known individuals (their primary caretakers). They startled easily when presented with novel stimuli and demonstrated avoidance behaviors, including shaking and cowering with tails tucked and ears lowered. Data are presented as least square means with associated standard errors. Statistical significance was declared at $P < 0.05$, and a trend toward significance was declared at $P < 0.01$.

Four dogs were mixed breeds and estimated to be 5 to 6 yr of age. The dogs weighed 8.1kg ± 0.18 kg at the beginning of the study. Both weight and feed intake were measured throughout the study and dogs did not change significantly over the course of the study.

Dogs were housed in separate rooms. Each room had 100% fresh air intake and exhaust. Dogs were fed twice (am and pm) per day and water was available ad libitum. Rooms provided a minimum of 12 m^2 of floor space (well in excess of the space required by USDA). The rooms were cleaned while the dogs were exercised daily.

Placebo and pheromone collars were manufactured by Sergeant's (Ohmaha, NE). The placebo collar was the same material (Co-Polymer) as the other collars, but with no active ingredient. The Sergeant's (SERG) collar contained the 6 % of Sergeant's pheromone that is currently marketed (its contents are proprietary). The Rabbit Pheromone (RP) collar contained 0.02 % of 2-methylbut-2-enal embedded in the plastic. The SERG+RP collar contained both SERG and RP compounds.

Treatments and Statistics

Heart rate was measured by use of a telemetry system (Data Science International, St. Paul, MN USA). Leads were placed on the dogs' shaved skin while sensors measured heart rate. A specially-fit jacket held the transmitter. A computer collected HR data continuously by telemetry with data points recorded each 5 seconds. HR data were averaged each hour. The lab technician was blind to treatment days and scanned and eliminated any misleading entry points (as happens when the leads become loose).

Video cameras were mounted overhead in the rooms. The dogs' behaviors were captured on video media at a sampling rate of 30 frames/second. Data were played back while observers, blind to treatments, recorded behaviors. Behaviors that were recorded are defined in Table 1. A scan sampling method (Altman, 1974) was used to record dogs' behaviors every 5 minutes. Data were summarized by each hour. Behaviors were mutually exclusive in the ethogram (Table 1). Observers were trained to evaluate the dogs overall behavior at the 5 minute scan sample data point. For

example, if a dog demonstrated lying behavior for a 5 minute period but then stood at the point of data recording, then perceived to lye back down; then the observer would record lying because that was a more accurate summary of the dogs' behavior.

Each dog received each treatment in a Latin Square design with repeated measures over time (24 h per day). This model allowed evaluation of effects of treatment, dog, dog by treatment, time, treatment by time and dog by time. The treatment and dog effects were tested using the dog by treatment effects. The remaining independent variables were tested using the residual error term. Data are presented using Least Squares means generated from the General Linear Models procedure within the SAS software (Statistical Analysis Systems, 2009). Standard errors were produced by SAS using the appropriate error term. Percentage data were transformed (because percentage data are not normally distributed) by square root arcsin transformation before analysis to approximate normality.

The study was conducted in three phases: Baseline, Pheromone/Placebo application, and Startle Test. Baseline can be described as the period of time when no collar was applied. After the baseline period, treatments were administered in random order. Pheromone/Placebo application is the period of 24 h when the dogs received each treatment via a collar. A lab technician would enter the rooms in the same order and place the collar on the dog. HR data was then analyzed from the point each collar was placed on each individual dog. Lastly, startle refers to the 2-hour time period after the 24 h of collar application when dogs were startled with an air horn. The air horn produced a noise at 110 db (measured by a sound level meter from Radio

Shack) and was placed by a technician approximately 7-10 inches away from the dogs face. Each dog received a blast from the air horn in the same order that the collars were placed on the dogs. The startle period was measured 2 h after the startle test (sound of the air horn for each dog), then all collars where removed. Behavior and HR data were measured for all the phases previously mentioned. Dogs were allowed at least a 24 h recovery period in which no pheromone or data measurements took place between treatments. Heart rate data were collected each 5 s for 24 h and each second during the startle phase for at least the first 10 min after startle.

The different phases of the study were analyzed independently from one another, but the same model was kept constant throughout. This allowed for direct comparisons among treatment groups for that particular phase.

Additional data was collected on HR and behavior at times when the dogs would come into contact with the technician collecting HR data (to fix a malfunction or check the contact of leads on the skin) and the interaction with facility cleaning members. HR data was taken out for these periods of time and behavior data was omitted. Cleaning personnel would take the dogs (out of view) outside the rooms while the rooms were cleaned. This amount of time was approximately around 5 minutes. Therefore we would still collect the behavior data at that time point as out of view and still have our twelve scan samples per hour.

In addition to the General Linear Models analysis as described above, regression equations were calculated to describe HR over time after startle. We

wished to document if HR increased, did not change or decreased over time after startle with use of pheromones/interomones by calculation of the regression equation and R^2 for each treatment group. These regression lines are purely descriptive.

Results

Baseline behavioral and HR effects

Treatment and interaction P-values are presented in Table 2 for HR and behavioral measures. Dog HR did not differ among treatments or interactions among treatments ($P > 0.05$; average ± SE, 114.2 ± 14.2 bpm).

Many behavioral effects were observed while the collars were applied compared to the placebo collars (P-values are in Table 2). The interaction between SERG and RP ($P = 0.02$) showed that SERG, RP and SERG+RP collars all reduced walking compared with dogs wearing the placebo/control collars (Figure 1). Furthermore, the RP reduced dog walking more than dogs that wore a SERG collar (Figure 1).

The main effect of RP collars significantly changed dog pacing, lying and walking (Table 3). Dogs wearing RP collars spent less ($P = 0.007$) time pacing and walking than dogs wearing a Placebo collar. Conversely, dogs wearing the RP-impregnated collar spent more ($P = 0.04$) time lying down than dogs with control collars.

Individual dogs varied in their responses to the pheromone/interomone collars; the SERG by dog and RP by dog, interactions were significant ($P < 0.05$) for

pacing and walking behaviors (Table 4). Dog 1 increased pacing and walking with a SERG collar, while RP decreased pacing and walking compared with the same dogs wearing a placebo collar. The pacing behaviors of dogs 2 and 3 were not changed by use of the SERG collar, however, dog 2 showed reduced pacing and walking with the RP collar. Dog 2 showed reduced walking with SERG collar. Dog 4 showed reduced walking and pacing with a SERG collar, however, dog 4 showed increased pacing and walking when wearing a RP collar.

The interaction between dog, SERG and RP was significant for lying (P = 0.0005) and sitting (P = .0001) behaviors (Fig 2). For dog 1, all collars increased lying (they were less active). The RP collar caused more lying alone or in combination with SERG for dogs 3 and 4; however, dog 2's lying behavior was not changed by any collar treatment. Dogs 1 and 3's sitting behavior did not differ among any collar treatment. However, for dog 2, SERG collar increased sitting behavior while for dog 4, percentage time spent sitting was decreased with use of SERG or RP, but not the combined SERG and RP collar (Fig 2).

The RP pheromone/interomone collar by time interaction was highly significant (P = 0.004; Fig 3). Dogs with the RP collar spent more time lying down early on when treatment was applied. During times of day when people were in the facility (but not in the dog's rooms except briefly to clean and feed), dog lying behavior was increased among dogs wearing the RP collar (and inversely generally activity was reduced) compared with the same dogs wearing the control collars.

The interaction between both main effects (RP and SERG) and time was not significant ($P > 0.20$) for lying and sitting behaviors. However, this interaction was significant ($P < 0.05$) for pacing, standing and walking (Figures 4 and 5). Control dogs seemed to pace ($P = 0.04$) and walk ($P = 0.04$) significantly more approximately the eighth hour compared to all other treatments. For unknown reasons, both pacing and walking showed a spike in activity between the sixth and thirteenth hour (this was true on average therefore over many days).

The dogs also expressed a similar spike in standing (Figure 6, $P = 0.01$) activity during the eighth and sixteenth hours, which was expected as dogs were active during these time points. However, Placebo-collared dogs stood the most at the beginning of this period of increased activity.

Behavior and HR after dogs were startled

After dogs were startled, there were no significant effects on mean HR (114.7 ± 21.5 bpm; Tables 5, 6 and Figure 7); however, HR changed over time in placebo vs. collar treatments (Figure 7). When wearing the Placebo collar, dog HR increased after startle (slope = 22.9 bpm/hour; $R^2 = 0.70$) – this is to be expected since the dogs were startled and general activity was also increased (see below). Dogs wearing the SERG collar also increased HR over time, but less so (slope = 6.25 bpm/hour; $R^2 = 0.40$). Dogs wearing the RP collar had no meaningful change in the 2 hours after startle (slope = 1.85 bpm; $R^2 = 0.09$). Dogs wearing the SERG+RP had a decline in HR over time after startle (slope = -7.05 bpm; $R^2 = 0.36$). The decline or lack of rise in HR

was associated with changes in and sitting behavior (see below). Also, HR was analyzed for the first 10 minutes after startle. One minute averages were taken and although no data was significant (P = 0.4; Placebo = 112.8 ± 21.1 bpm, RP = 86.9 ± 25.0 bpm) RP was still able to lower HR.

Treatments had many effects on dog behaviors after startle. RP collars increased dog pacing, sitting and decreased lying down compared with dogs wearing control collars (Table 6). Basically, the startle caused Placebo- and SERG-treated dogs to lay down, while dogs with collars containing RP paced and sat down (Table 6).

Following startle, both SERG and RP increased sitting behaviors compared to placebo, but the interaction between SERG and RP was also significant (Figure 7). The combined SERG and RP collars increased sitting more than other treatment groups especially immediately after startle.

The dog by SERG by RP effect was significant (P = 0.0007) for walking behaviors. Dogs 2, 3 and 4 walked a similar amount after startle regardless of pheromone/interomone treatment. However, after startle, dog 1 increased walking if it wore a RP or SERG+RP collar. Because SERG alone did not increase walking, this effect could be due to the RP alone.

Discussion

Selection and deployment of the model

Conducting research with domestic dogs is challenging for the Institutional Animal Care and Use Committee (IACUC) due to public sensitivities and federal regulations. Consequently, the Texas Tech University IACUC (and others) apply the 3 R's, originally proposed by Russell and Birch (1959) to conduct research with dogs. Two of the Rs include reduction in numbers of animals used in the research and refinement of technique.

We employed a powerful experimental design in an attempt to use the least numbers of dogs that could be used in this study. The Latin Square experimental design exposed each subject to each treatment in random order with an appropriate wash-out period between treatment applications. With each animal experiencing placebo and experimental pheromone-impregnated collars, we executed a highly-controlled experimental model. Dogs were housed in separately-ventilated, temperature and light-controlled rooms with an amount of space several times larger than the minimum required by law. Furthermore, dogs were selected from a research facility based on symptoms of being nervous and anxious. Their anxious diagnosis was confirmed by a behavior-boarded veterinarian. Thus, we used a highly uniform experimental setting with subjects presenting a uniformly-diagnosed behavioral problem. Pheromones were administered in collar form because there was data to support that dogs behave differently when a collar is applied vs no collar (Mcglone

2011, unpublished). Therefore we wanted to mimic regular practices seen by most pet owners.

One alternative to use of the highly-controlled laboratory setting is to conduct in-home studies (Gaultier et al., 2008, Sheppard and Mills, 2003). This approach would require a great deal more subjects due to the variation among environments and subjects. We do know from product reviews and personal communication that the response of dogs to pheromones is highly variable (Petsmart, 2012) and that not all dogs respond the same to pheromones. Knowing about this inter-dog variation, it was important to be able to use an experimental design that could detect a significant dog by treatment interaction should they be present. By collecting data over time (a complete 24 hour day), we were able to test the dog by treatment interaction and we were able to detect variations in response within times of day. Indeed, this statistical design and model generated critical information about treatment effects, variable responses among subjects, and responses that may vary with time of day.

A complete understanding of pheromone effects should employ both the highly controlled experimental procedures and in-home or field studies to demonstrate efficacy. Each level of experimentation provides useful information that has the potential to improve dog welfare.

Main treatment effects

We recognize the variable reports of pheromone efficacy in the field. For this reason, overall main effects of pheromones were unlikely to be significant or meaningful if the dog by treatment effect was significant. So when the pheromone/interomone significantly changed behavior, we can certainly assume that that molecule is having an effect on the dog's olfactory receptors and its brain (dogs were housed individually and they could not lick or ingest the collar). In this report, the rabbit pheromone (2-methylbut-2-enal) significantly changed dog behavior. The main effect of RP (more than other treatments) significantly impacted pacing, walking and lying down (Tables 2 and 3). The RP reduced pacing and walking and increase the time dogs spent lying down. Pacing is common among anxious dogs and is a form of stereotyped behavior that becomes an aberrant stereotypie in some animals. This is the first report that a pheromone/interomone can reduce sterotyped pacing and increase the time anxious dogs spent resting.

None of the main effects of the SERG pheromone collar reached statistical significance. However, the interaction between the SERG and RP collars was significant for walking (Table 2 and Fig 1). The SERG collar reduced walking compared with the control collar, but RP treatment group was even lower. The combined SERG+RP collar caused an intermediate amount of walking. Clearly, the RP had a more powerful effect on dog behavior than the SERG pheromone collar.

Variation among treatments and dogs

Recognizing the variation in responses in the field from pheromones (Petsmart, 2012), the experimental design was set up to detect potential dog by pheromone interactions – and there were many (Table 2). The most complex to explain are the significant dog interactions with both the SERG and RP pheromones/interomones. If the goal is to increase the time dogs spent lying down (and not pacing), then we should carefully examine the data in Figure 2 and Table 4. Some dogs responded differentially to the pheromone/interomone treatments. This means that some dogs benefited (or at least were impacted behaviorally) by certain pheromones/interomones. While the RP had the greatest overall effects, some dogs responded better to the SERG or the SERG+RP collars.

Where pheromones have been examined in the past for behavioral problems, the sample has not been dogs diagnosed as anxious, rather the interpretation of anxious was left up to owners (Mills et al., 2006). Others such as Ley et al., (2010) used dogs that were diagnosed as anxious for their study on the effectiveness of DAP, but the results were still left up to the owners discretion. Thus, this is the first report, in a highly-controlled setting that demonstrated efficacy of pheromones/interomones in addressing some behavioral problems of professionally-diagnosed anxious dogs.

Behavior and HR after Startle

Some dogs may have noise phobia that are not clinically anxious. That is, an otherwise non-anxious dog may be fearful of fireworks or loud noises. Likewise, clinically anxious dogs may or may not have noise phobia. The model we report used the startle of a loud noise to assess behavior and physiology of anxious dogs. Examining the data from control dogs, one can see that the response of anxious dogs to the startle with a placebo collar was to increase HR over time (Fig 7) and to decrease sitting and become more active (walking and pacing, but not lying down). The RP was especially effective at changing the anxious dogs' post-startle behavior. The RP-treated dogs sat down after startle instead of walking. Their HR decreased over time rather than increasing over time as did the control-treated dogs (Figure 7).

Our highly-controlled startle model differs from the clinical trial to assess DAP among dogs fearful of fireworks. Sheppard et al., (2003) asked dog owners to score dog behavioral responses to fireworks with and without DAP delivered by air diffuser. In their work, owners scored dog behavior. In our study, investigators (blind to treatments) scored the dog behaviors objectively over a 24 hour period and then during a 2-hour post-startle period. While Sheppard et al., (2003) studied DAP and we studied SERG and RP, it is important to compare the two models. Ours is highly controlled laboratory study with clinically-diagnosed anxious dogs. Sheppard et al., (2003) used client dogs in a home environment without 24-hour behavior assessments. Both models are valuable, but ours provides a more precise estimate of

pheromone/efficacy among anxious dogs because environmental variables were controlled.

Tables

Table 3.1. Definitions of mutually-exclusive behaviors that the observers used when classifying an animal's behavior while blind to treatment groups.

Behavior	Definition
Pacing	Locomotion in a specific repeated pattern back and forth between 2 positions.
Walking	Locomotion in any pattern from point A to B but not repeating the path
Stand	Supported by limbs, not moving/walking/pacing
Sit	Posterior on ground while front feet support the animal
Lay down	Dog's body not supported by any limb
Lick self	Tongue touching any body part
Eat	Head in dog bowl
Drink	Head in water bowl
Urinate	Urine stream observed
Defecate	Defecation observed
Activity	Obtained by calculation; all behaviors other than lying down; also, the inverse of lying down

Table 3.2. Pheromone/Placebo application phase treatment P-values. The * indicates an interaction effect. SERG = Sergeant's pheromone collar; RP = Rabbit Pheromone collar. Time represents 24, 1 hour periods (a 24 hour day).

Model effect	HR	Pacing	Stand	Sit	Lying	Walking
SERG	0.74	0.47	0.91	0.62	0.69	0.95
RP	0.66	0.05	0.29	0.51	0.04	0.007
SERG*RP	0.09	0.16	0.34	0.85	0.87	0.02
Dog	0.79	0.04	0.08	0.33	0.37	0.005
Dog*SERG	0.15	0.02	0.38	0.88	0.62	0.004
Dog*RP	0.22	0.01	0.67	0.64	0.66	0.003
Dog*SERG*RP	0.50	0.90	0.19	0.0001	0.0005	0.98
Time	0.90	0.0001	0.002	0.0001	0.99	0.0001
Time*SERG	1.00	0.20	0.98	0.62	0.99	0.17
Time*RP	1.00	0.18	0.74	0.53	0.004	0.23
Time*RP*SERG	1.00	0.04	0.01	0.34	0.98	0.04

Table 3.3. Pheromone/Placebo application phase treatment P-values and Least Squares means for the main effect of Placebo/control vs. RP on behavior and heart rate.

Item	Control		Rabbit Pheromone		P-values
	Mean	**SE**	**Mean**	**SE**	**Rabbit Pheromone**
Heart rate, bpm	129.37	12.85	107.92	11.41	0.30
Pacing	3.18	0.17	2.99	0.19	0.05
Stand	1.32	0.411	2.11	0.47	0.29
Sit	6.93	1.82	4.88	2.07	0.51
Lying	63.19	5.00	87.39	5.69	0.04
Walking	7.79	0.11	2.81	0.11	0.007

Table 3.4. Main effects of SERG and RP as they interact with each dog's pacing and walking behaviors. The interaction of dog and SERG and dog and RP were significant (P < 0.05) for pacing and walking behaviors.

	Pacing, % time				Walking, % time			
Dog	Control	SERG	Control	RP	Control	SERG	Control	RP
1	2.5±0.34	4.5±0.39*	4.8±0.34	2.3±0.39*	2.4±0.20	4.5±0.22*	4.8±0.20	2.2±0.22*
2	3.8±0.34	2.4±0.40	4.8±0.35	1.4±0.39*	3.8±0.20	2.4±0.23*	4.8±0.20	1.4±0.22*
3	2.0±0.35	3.0±0.39	1.8±0.34	3.1±0.40	1.2±0.20	3.0±0.22	1.8±0.20	2.4±0.23
4	5.7±0.34	3.3±0.39*	3.8±0.34	5.2±0.39*	5.7±0.20	3.3±0.22*	3.8±0.20	5.2±0.22*

***Control and treatment least square means differ P < 0.05.**

Table 3.5. Startle test treatment P-values for HR and behaviors during the 2-hour period after startle.

Model effect	HR	Pacing	Stand	Sit	Lying	Walking
SERG	0.72	0.70	0.09	0.006	0.70	0.93
RP	0.37	0.04	0.38	0.002	0.04	0.77
SERG*RP	0.70	0.29	0.07	0.03	0.29	0.29
Dog	0.48	0.14	0.13	0.2119	0.14	0.56
Dog*SERG	0.33	0.82	0.08	0.1439	0.82	0.90
Dog*RP	0.43	0.29	0.35	0.0926	0.29	0.70
Dog*SERG*RP	0.50	0.63	0.64	0.890	0.63	0.0007
Time	0.90	0.67	0.37	0.028	0.67	0.22
Time*SERG	0.93	0.90	0.74	0.420	0.90	0.45
Time*RP	0.83	0.90	0.95	0.139	0.90	0.80
Time*RP*SERG	0.94	0.74	0.30	0.03	0.74	0.50

Table 3.6. Startle test Least Squares means for HR and dog behaviors from Placebo/control and Rabbit pheromone-collars.

	Control		Rabbit Pheromone		P-values
Item	Mean	SE	Mean	SE	Rabbit Pheromone
Heart rate, bpm	125.8	16.76	101.28	16.76	0.37
Pacing	2.08	1.69	7.64	1.69	0.04
Stand	3.13	1.45	5.21	1.45	0.383
Sit	4.51	0.74	13.89	0.74	0.002
Lying	77.08	10.90	72.22	10.90	0.04
Walking	2.08	1.17	7.64	1.17	0.773

Figures

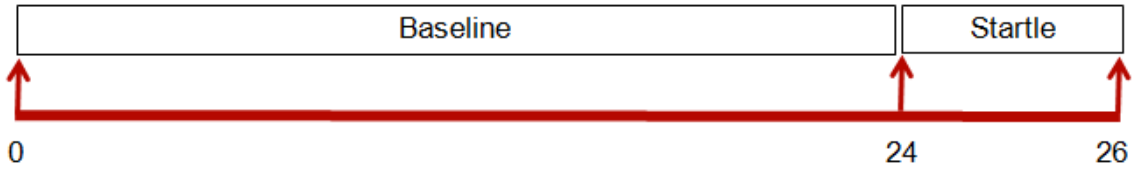

Figure I. Showing the different phases for the study. After the 26th hour dogs would then begin their washout period of no pheromones collars for 24 hours.

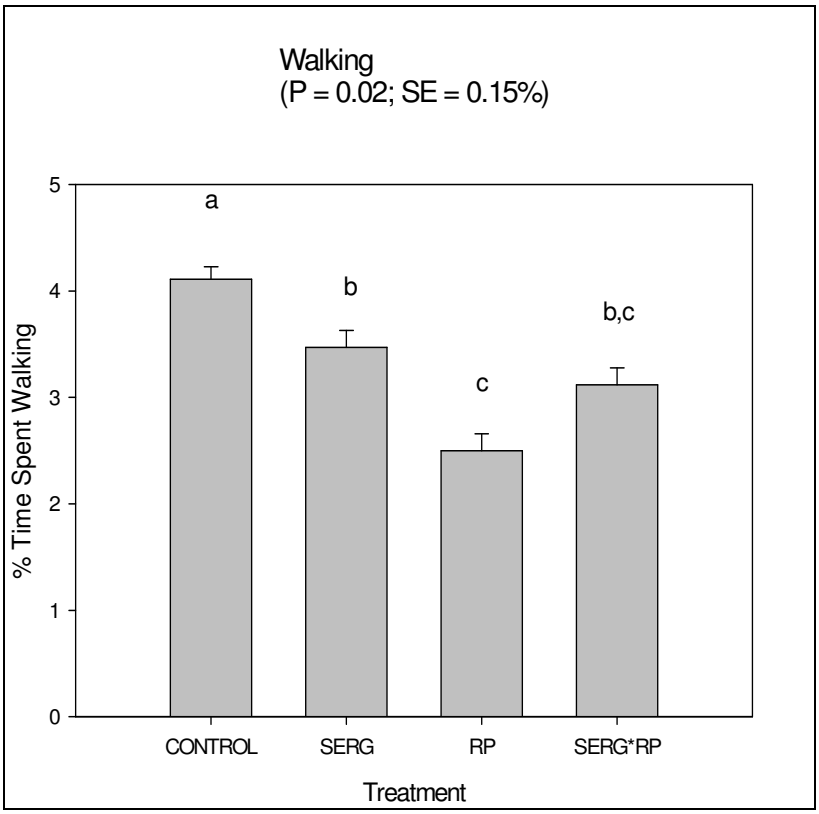

Figure 3.1. Pheromone/Placebo application showing the interaction of SERG and RP for percent time spent walking. Least Squares means with different superscripts indicate Least Squares means differ (P < 0.05). Each treatment reduced walking compared with the Placebo/control. RP was lower than SERG collared dogs' behavior while the combined SERG+RP was intermediate in walking behavior.

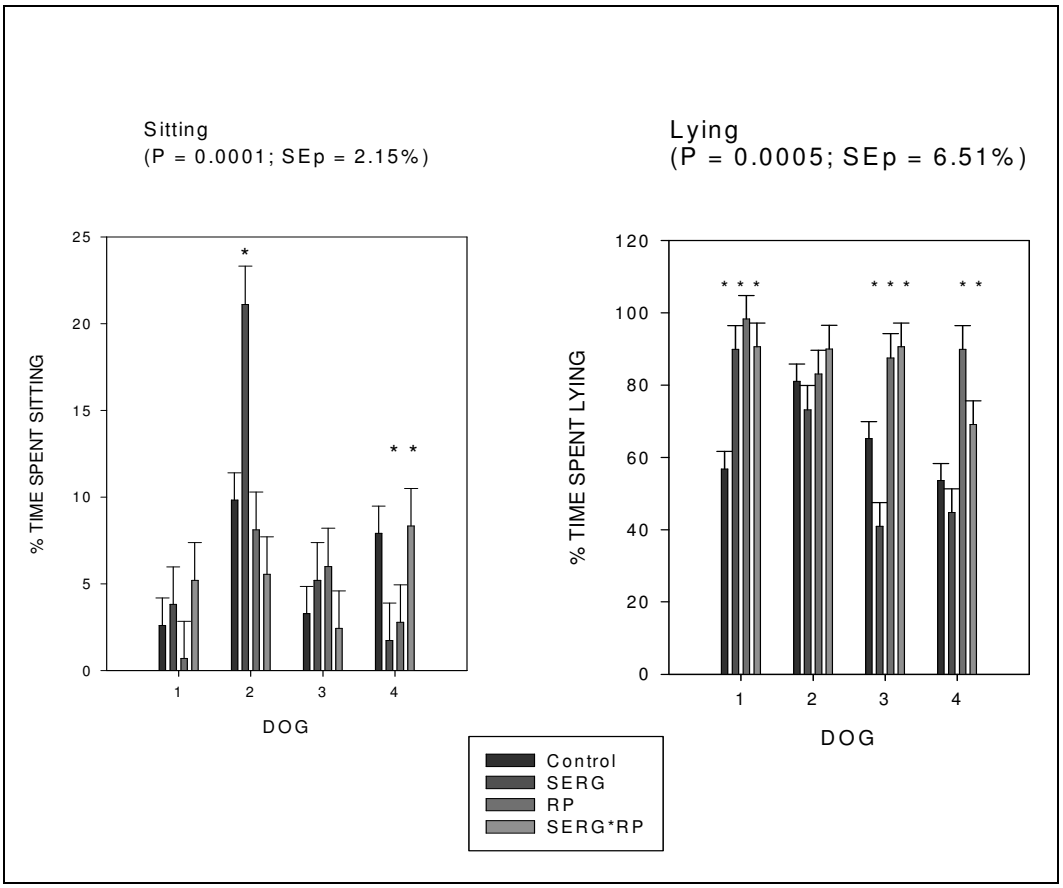

Figure 3.2. Pheromone/Placebo application showing the interaction of dog by SERG and RP for percent time spent sitting and lying.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

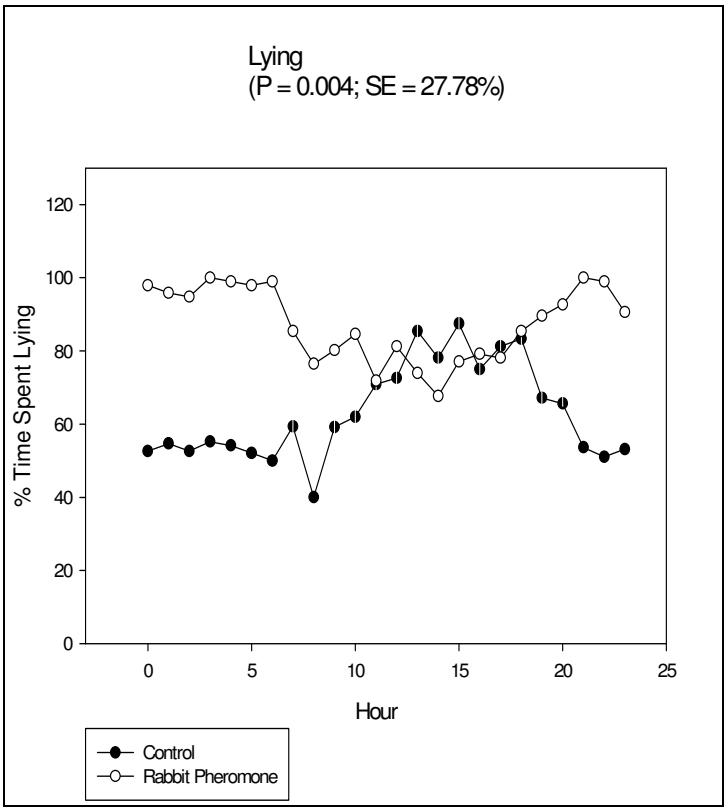

Figure 3.3. Pheromone/Placebo application showing the hour by RP interaction (P = 0.004) for percent time spent lying down.

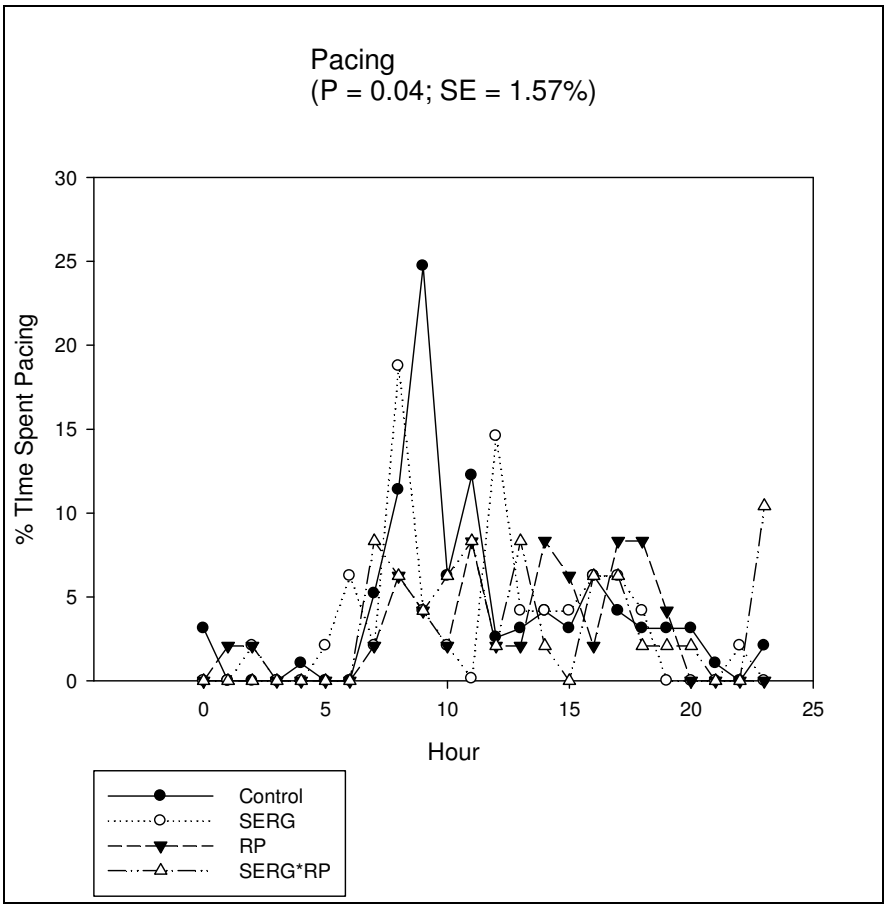

Figure 3.4. Pheromone/Placebo application showing Hour by SERG by RP and the amount of time spent pacing.

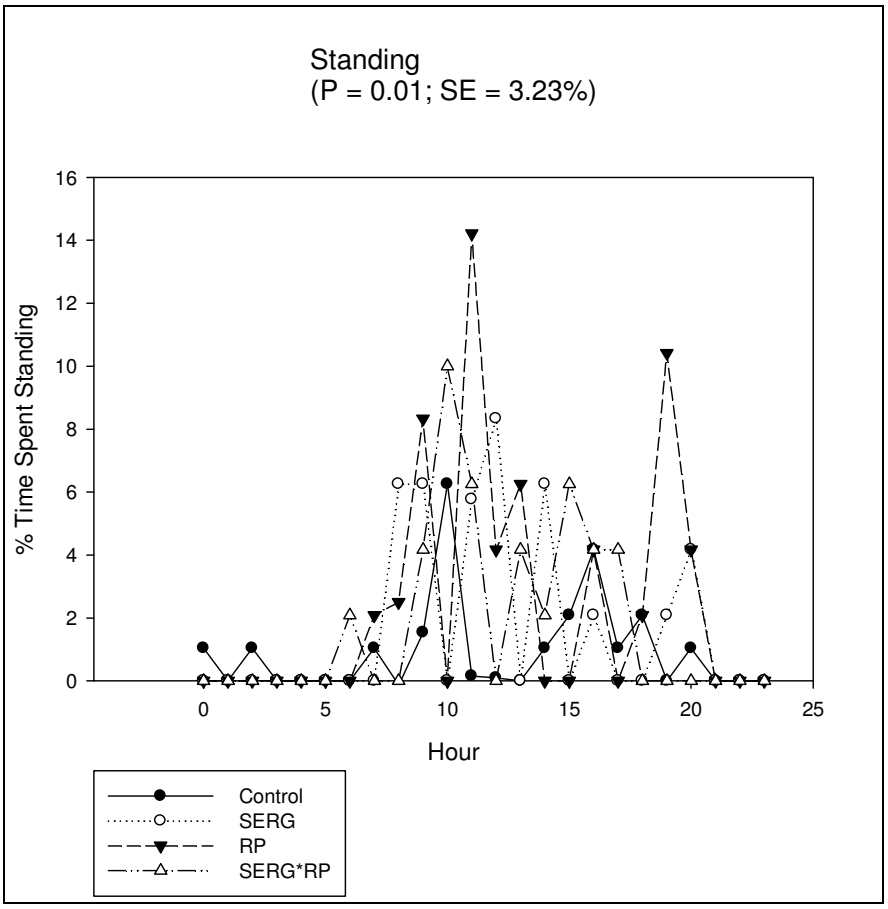

Figure 3.5. Pheromone/Placebo application showing hour by SERG by RP interaction (P = 0.01) and the amount of time spent standing.

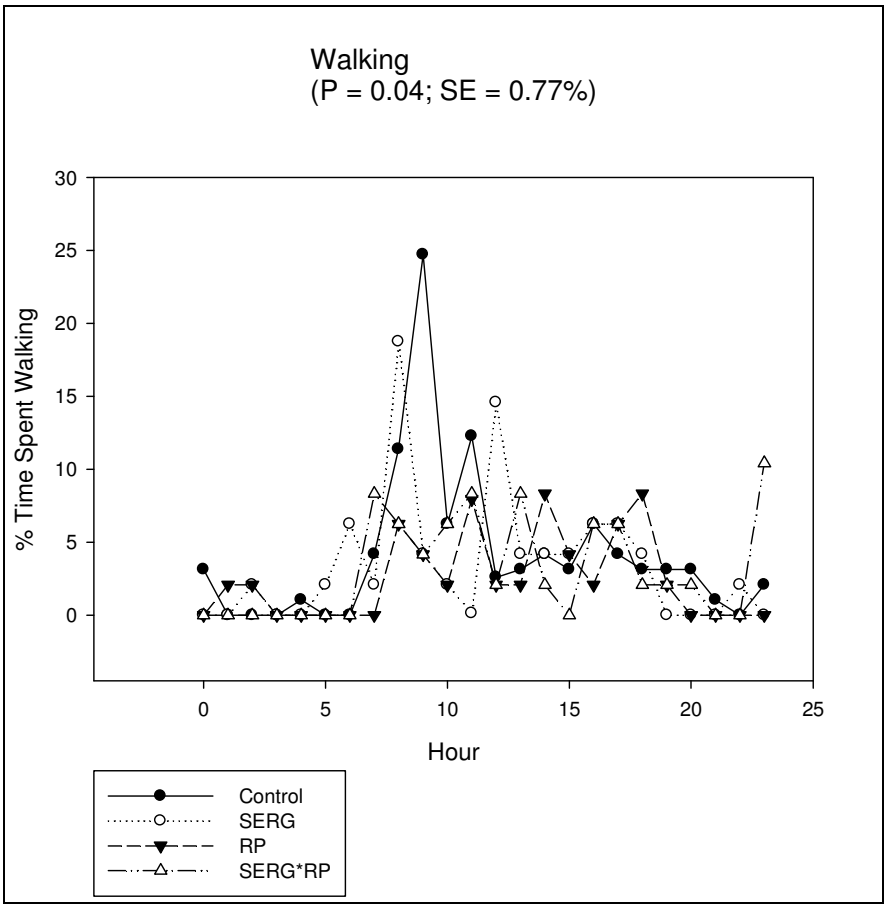

Figure 3.6. Pheromone/Placebo application showing hour by SERG by RP (P = 0.04) and the percent of time spent walking.

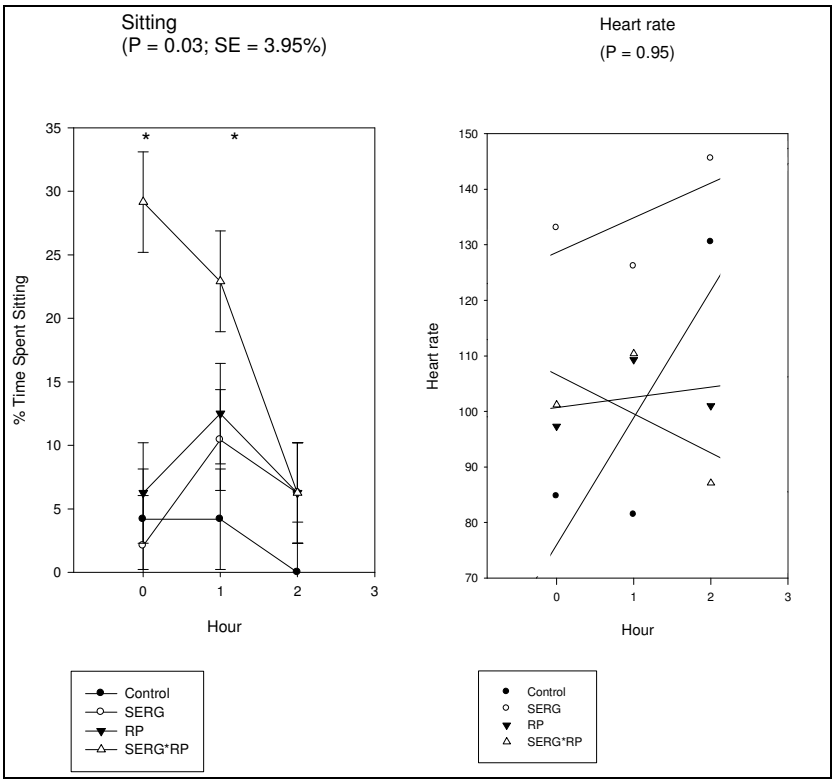

Figure 3.7. Startle test showing the interaction of hour by SERG and RP for percent time spent sitting and heart rate regression for time after startle. See Results for the slopes of HR over time after startle.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

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CHAPTER IV

PHEROMONES AND AN INTEROMONE THAT CHANGES THE PHYSIOLOGY AND BEHAVIOR OF ANXIOUS DOGS. TESTING THE DIFFERENCES IN FOUR PHEROMONE COLLARS

Abstract

The objective of this study was to assess the efficacy of pheromones/interomones to modulate heart rate (HR) and behavior in adult anxious dogs (trembling, cowering, shy). Four dogs (10.2±4.2 kg; estimated 5-10 yr old, intact males) were professionally diagnosed as anxious. Treatments were given in the form of a collar containing each pheromone/interomone and included Placebo, Sergeant's Formula H (SERG), 2-methylbut-2-enal -- Rabbit Pheromone (RP), or Dog Appeasing Pheromone (DAP) collar. During baseline 24 h, DAP increased HR in 1 dog and decreased HR in 2 dogs; SERG increased HR in 2 dogs and decreased HR in 1 dog and RP increased HR in 2 dogs and decreased HR in 2 dogs (dog by trt, $P < 0.0001$). Dog lying behavior changed with SERG and RP but not DAP. After startle, each treatment changed dog HR in at least one dog (dog by trt, $P = 0.002$). Treatments caused one dog to increase while another decreased lying (dog by trt, $P < 0.001$) after startle. Individual dogs changed behavior and HR differentially in response to the pheromones/interomones evaluated. The pheromones/interomones tested clearly changed dog heart rate and

behavior. However, anxious dogs did not respond uniformly to each pheromone/interomone tested.

Materials and Methods

General

The study was performed in a animal laboratory setting at Texas Tech University. The institution is AAALAC International accredited and the work was approved by the Institutional Animal Care and Use Committee prior to its conduct. Dogs were obtained from a research facility where they had been used as test subjects primarily in flea and tick research. In that facility, the animals were kept in concrete-floored kennels with chain link fencing. They were then transported to the Texas Tech University facility, bathed, given a physical exam by a veterinarian and rested for more than a week before testing. Dogs were initially selected because the investigators considered the subjects anxious. A board certified animal behavior veterinarian (Dr. Valarie Tynes) examined each dog and confirmed that they were clinically anxious. This is the first report of pheromone efficacy using clinically-diagnosed anxious dogs in a highly controlled environment.

All dogs were mixed breeds and estimated to be 5 to 10 yr of age. The dogs weighed 10.2 kg ± 4.2 kg at the beginning of the study. Placebo and pheromone collars were manufactured by Sergeant's (Ohmaha, NE). The placebo was the same plastic material as the other collars, but with no active ingredient. The Sergeant's (SERG) pheromone collar contained 6% of the pheromone and is currently marketed. The Rabbit Pheromone (RP) collar contained 0.02% of 2-methylbut-2-enal embedded

in the plastic. The Dog Appeasing Pheromone (DAP) that is currently marketed by CEVA (5% DAP; Ceva Sante Animale, Libourne, France).

Dogs were housed in separate rooms. Each room had 100% fresh are intake and exhaust. Dogs were fed twice (am and pm) per day and water was available ad libitum. Rooms provided a minimum of 12 m^2 of floor space (well in excess of the space required by USDA). The rooms were cleaned while the dogs were exercised daily.

Placebo and pheromone collars were manufactured by Sergeant's (Ohmaha, NE). The placebo was the same plastic material as the other collars, but with no active ingredient. The Sergeant's (SERG) collar contained the Sergeant's pheromone that is currently marketed (its contents are proprietary). The Rabbit Pheromone (RP) collar contained 2-methylbut-2-enal embedded in the plastic.

Treatments and Statistics

Heart rate was measured by use of a telemetry system (Data Science International, St. Paul, MN USA). Leads were placed on the dogs' shaved skin while sensors measured heart rate. A specially-fit jacket held the transmitter. A computer collected HR data continuously by telemetry with data points recorded each 5 seconds. HR data were averaged each hour. The lab technician was blind to treatment days and scanned and eliminated any misleading entry points (as happens when the leads become loose).

Video cameras were mounted overhead in the rooms. The dogs' behaviors were captured on video media at a sampling rate of 30 frames/second. Data were played back while observers, blind to treatments, recorded behaviors. Data were summarized by hour. Behaviors that were recorded are defined in Table 1. A scan sampling method (Altman, 1974) was used to record dogs' behaviors every 5 minutes. Behaviors were mutually exclusive in the ethogram (Table 1).

Each dog received each treatment in a Latin Square design with repeated measures over time (24 h per day). This model allowed evaluation of effects of treatment, dog, dog by treatment, time, treatment by time and dog by time. The treatment and dog effects were tested using the dog by treatment effects. The remaining independent variables were tested using the residual error term. Data are presented using Least Squares means generated from the General Linear Models procedure within the SAS software (Statistical Analysis Systems, 2009). Standard errors were produced by SAS using the appropriate error term. Percentage data were transformed (because percentage data are not normally distributed) by square root arcsin transformation before analysis to approximate normality.

The study was conducted in three phases: Baseline, Pheromone/Placebo application, and Startle Test. Baseline was the period when no collar was applied. After the baseline period, treatments were administered in random order. Pheromone/Placebo application is the period of 24 h when the dogs received each treatment via a collar. Lastly, startle refers to the 2-hour time period after the 24 h of collar application when dogs were startled with an air horn that produced a fog horn

noise at 110 db. The startle period was measured 2 h after the startle test, then all collars where removed (See Figure I). Behavior and HR data were measured for all the phases previously mentioned. Dogs were allowed at least a 24 h recovery period in which no pheromone or data measurements took place between treatments. Heart rate data were collected each 5 s for 24 h and each second during the startle phase for at least the first 10 min after startle.

In addition to the General Linear Models analysis as described above, regression equations were calculated to describe HR over time after startle. We documented if HR increased, did not change or decreased over time after startle with use of pheromones/interomones by calculation of the regression equation and R^2 for each treatment group. These regression lines are descriptive.

Results

Baseline behavior and HR effects

Treatment and interaction P-values are presented in Table 2.1 for HR and behavioral measures. The dog by treatment and was significant (P = 0.0001) for baseline HR. Dog 1 showed a decrease in HR when treated with DAP; while an increase was seen when treated with RP as seen in Figure 2.1. Dogs 2 and 4 had similar results as the RP collar showed a decrease in HR compared to placebo. However, the SERG and DAP pheromone collars had opposite effects as Dog 2 showed an increase in HR with the SERG and an decrease in HR with the DAP; while Dog 4 showed a decrease in HR with the SERG and an increase in HR with the DAP

compared to placebo treated dogs, (Figure 2.1). Dog 3 showed an increase in HR when treated with SERG and RP pheromone collars compared to placebo.

Several behavioral effects were observed when collars were applied compared to placebo collars (P-values are in Table 2). The dog by treatment interaction for standing, sitting, and lying were significant ($P < 0.05$). Dog 3 saw the greatest change in standing behavior. When treated with DAP the dog stood less, and when treated with SERG it stood more compared to the placebo collar (Figure 2.2). RP also increased standing behavior but was not significantly different that placebo. Dogs 1, 2, and 4 standing behavior was unchanged by any treatment. Dog 1 sat ($P = 0.0001$) less when treated with all treatments compared to placebo; however, dog 2 responded in an opposite manner and sat more with every treatment compared to placebo. Dog 2 sat the most when treated with SERG and RP, and both were significantly higher compared to dogs with DAP collars (Figure 2.3). Dogs 3 and 4 behavior were not changed for sitting, however these dogs sat for less than ten percent of the time during this 24h period. This makes sense because their lying behavior was not changed and these dogs performed this behavior around eighty percent of the time (Figure 2.4). However, dog 1 actually increased lying when treated with all the treatments compared to placebo although only SERG was significantly different. Conversely dog 2 saw a decrease in lying behavior (increased activity) when treated with all treatments compared to placebo. Only SERG and RP were significantly lower in activity than placebo but dog 2 did not become active because it sat more often (see results above).

Startle behavior and HR effects

After dogs were startled, the dog by treatment interaction was significant for HR (Table 2.2; Figure 2.5; P = 0.002). Dog 1 saw a decrease in HR when treated with the DAP pheromone collar. Dog 3 only showed an increase in HR when treated with RP. Dog 4's HR decreased when they were treated with both the SERG and RP pheromone collars. The only dog to not have their HR significantly affected by any pheromone collar was Dog 2, but there was a trend at P = 0.10 when treated with the RP pheromone collar compared with placebo.

Only the dog by treatment effects were significant for behavior after startle (Table 2.2). The SERG and RP were the only treatments that affected standing behavior in dog 3, while all other dogs' standing behavior did not change (Figure 2.6). Dog 1 showed a decrease in sitting (P = 0.0001) behavior when treated with all treatments compared to placebo (Figure 2.7). Dog 1's lying (P = 0.0002) behavior was increased when exposed to all treatments compared to placebo. Although dog 2 had different reaction for sitting and lying as they increased sitting (P = 0.0001) and decreased lying (P = 0.0002) for SERG and RP treatments compared to placebo (Figure 2.8). Dog 4 was the only dog that did not see any behavioral changes after startle.

Discussion

Selection of the model

The Institutional Animal Care and Use Committee (IACUC) at Texas Tech University (and others) have to follow strict guidelines when performing research with domestic dogs. Therefore this research project was conducted using a reduced number of animals as proposed by Russell and Birch (1959). To accommodate reducing the numbers of animals, we utilized a powerful experimental design that would be sufficient for the number of animals we were using. The Latin Square experimental design exposed each subject to each treatment in random order with an appropriate wash-out period between treatment applications. By having each dog serve as their own control in random order, we executed a highly-controlled experimental model. Additionally, dogs were selected from a research facility based on symptoms of being nervous and anxious which was confirmed by a behavior-boarded veterinarian. Thus, we used a highly controlled and uniformed experimental design by controlling our setting and selecting our subjects.

One alternative to using a highly-controlled laboratory setting would be to conduct in-home studies (Gaultier et al., 2008, Sheppard and Mills, 2003). This approach would be valuable because it would provide more information on how the pheromone collars performed on a larger sample number of dogs. However, there would have been significant variation among environments and subjects. Also, we know that not all dogs respond the same to pheromone collars (Chapter VII). Knowing about this inter-dog variation, it was important to be able to use an

experimental design that could detect a significant dog by treatment interaction should they be present. By collecting data over time (a complete 24 hour day), we were able to test the dog by treatment interaction and we were able to detect variations in response within times of day. Indeed, this statistical design and model generated critical information about treatment effects, variable responses among subjects, and responses that may vary with time of day.

To be able to completely understand how pheromones may affect animals both the highly controlled experimental procedures and in-home or field studies to demonstrate efficacy. Each level of experimentation provides useful information that has the potential to improve dog welfare.

Main treatment effects

The results from this study are significant because the data show that different pheromones/interomones were able to change both the behavior and HR of certain individual dogs. This report shows that rabbit pheromone (2-methylbut-2-enal) significantly increased the HR in two dogs and decreased HR in two dogs (Figure 2.1) compared to placebo. However, the dogs did not become more active because standing (Figure 2.2) was not affected. Also, pacing and walking were not significantly changed by any treatments that were applied. Instead the dogs became less active because they sat longer or increased their lying behavior (Figure 2.3 and 2.4). Furthermore, RP was the only treatment that changed the HR in all dogs.

The SERG pheromone collar had very different affects on HR. Dogs 2 and 3 were similar (increased) when treated with SERG pheromone collar, but it had very different results for dogs 1 and 4. When the SERG collar lowered HR, dogs spent more time lying down. However, the SERG treatment caused increased HR dog 2 it sat more compared to placebo treatment. Dog 3 saw the opposite effect and became more active by standing more. This is important because it shows that even if this collar elevates HR the dog does not always become more active in the same manner.

The DAP collar caused changes in some dogs HR and behavior. HR was decreased in dog 2 and increased it dog 1 compared to placebo. As HR was decreased in dog 2 the behavior was changed as the dog sat significantly more than placebo (Figure 2.3). Dog 4 showed that DAP increased their HR to approximately 190 bpm, but the behavior of this animal did not change compared to placebo. This could be explained by an unknown illness at the time of treatment. The dogs' heart rate and immune system were elevated trying to fight off infection and therefore his behavior was inactive.

Behavior and Heart rate after Startle

By examining the data collected from these anxious dogs, one can see that dogs did respond to the loud noise and with changes in HR or behavior in some dogs. This is important because it was thought that some dogs may or may not have an issue in coping with loud noises even if they have not been diagnosed as clinically anxious. This phase of the study shows that when dogs were given a pheromone

collar and then startled with a loud noise as suggested in the model, they will not necessarily become more active. Certain dogs with a pheromone collar sat or laid down for longer periods. The RP treatment was the only pheromone collar that changed either the HR or the behavior (or both) of every dog. More importantly we can see that if a dog stood, sat, lied, or had a different HR compared to placebo before startle, they consistently kept that relationship post startle.

This study was successful in producing both physiological and behavioral changes in anxious dogs. The data showed that both RP and SERG could change the HR and behavior of dogs individually compared to the combination as seen in Chapter III. The study further emphasizes the point that anxious dogs do not respond uniformly to each pheromone/interomone tested.

Tables

4.1. Pheromone/Placebo application phase treatment P-values for HR and behavior.

Model effect	HR	Pace-Walk	Stand	Sit	Lying	Walking
TRT	0.646	0.430	0.749	0.745	0.602	0.447
DOG	0.537	0.0003	0.024	0.004	0.019	0.0003
TRT*DOG	0.0001	0.859	0.002	0.0001	0.0001	0.871
TIME	0.0001	0.024	0.0001	0.0001	0.0001	0.027
TIME*TRT	0.9911	0.203	0.874	0.5371	0.615	0.182

4.2 . Startle test phase treatment P-values for HR and behaviors during the 2-hour period after startle.

Model effect	HR	Pace-Walk	Stand	Sit	Lying	Walking
TRT	0.722	0.462	0.406	0.799	0.763	0.462
DOG	0.354	0.865	0.183	0.111	0.352	0.865
TRT*DOG	0.02	0.221	0.020	0.0001	0.0002	0.221
Time	0.240	0.643	0.902	0.717	0.910	0.644
TIME*TRT	0.735	0.898	0.754	0.343	0.816	0.880

Figures

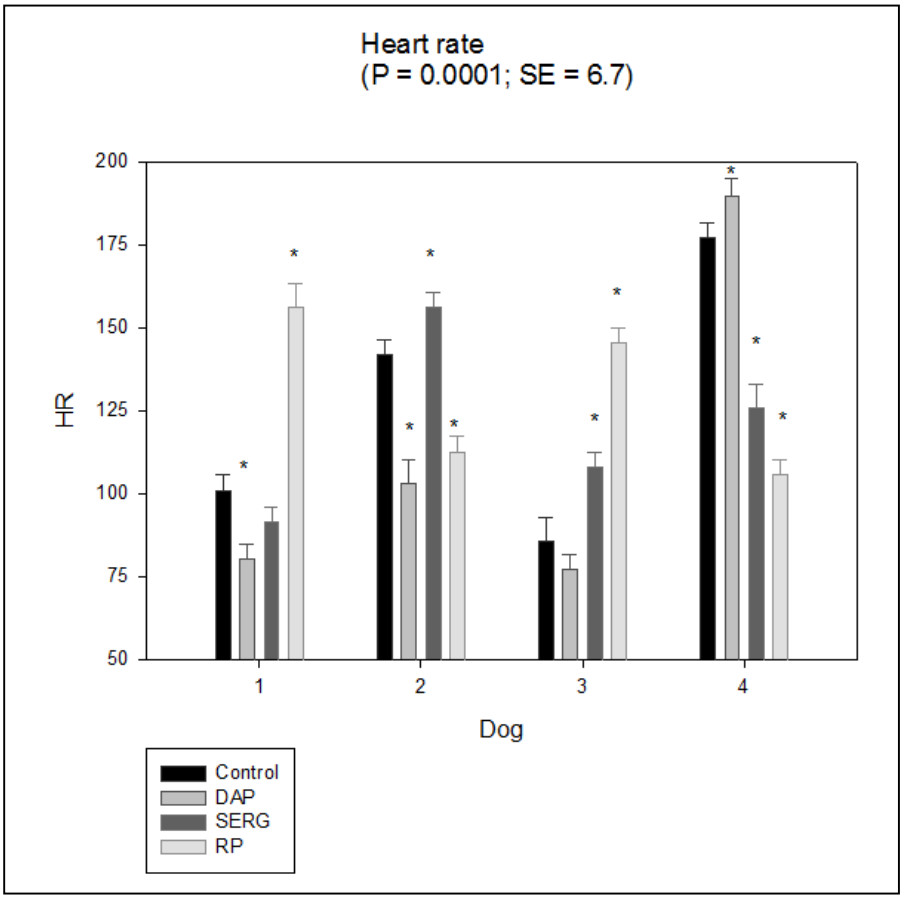

Figure 4.1. Pheromone/Placebo application showing the interaction of dog by treatment for HR.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

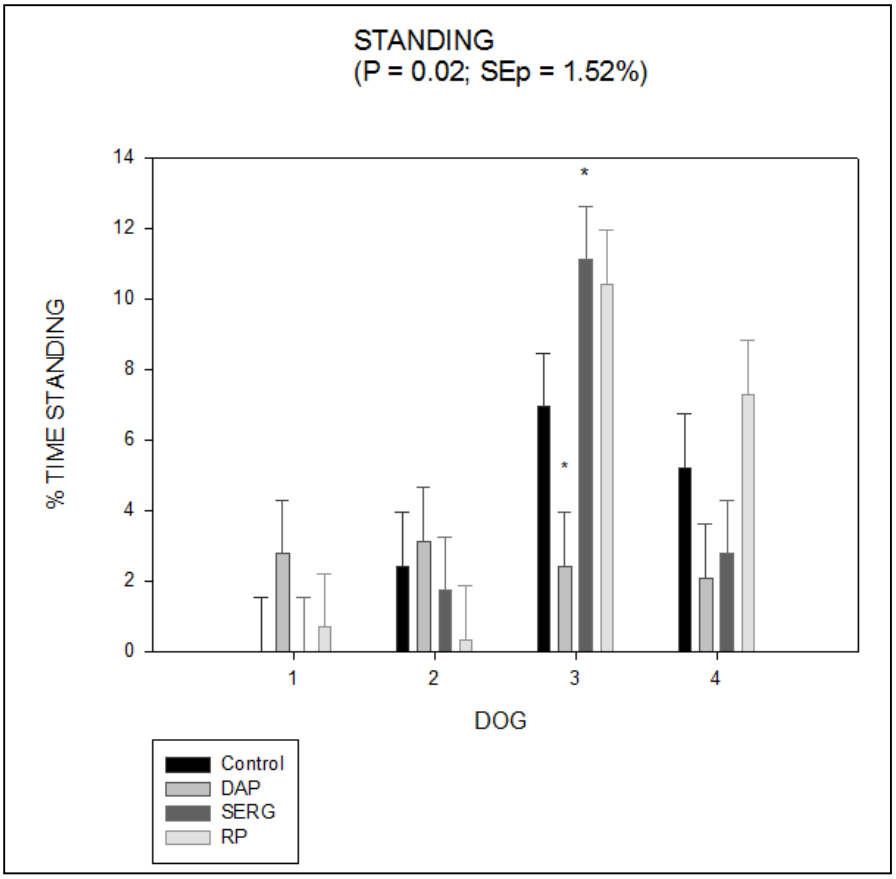

Figure 4.2. Pheromone/Placebo application showing the interaction of dog by treatment for percent time spent standing.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

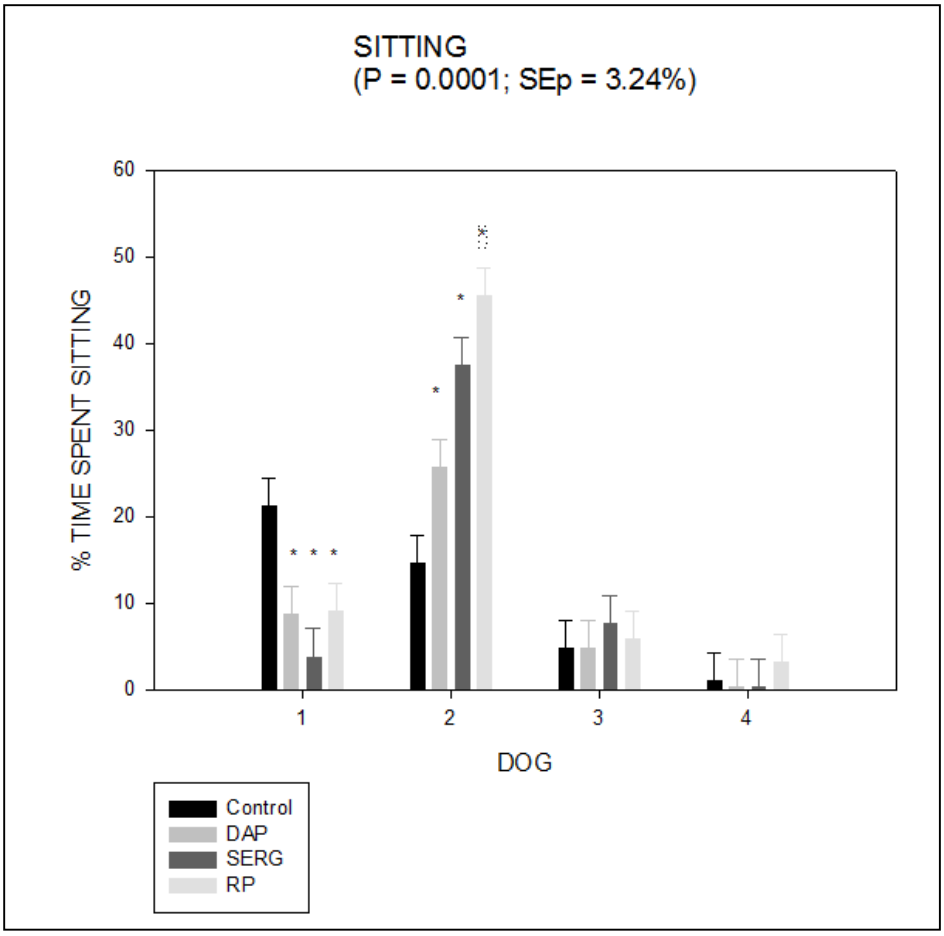

Figure 4.3. Pheromone/Placebo application showing the interaction of dog by treatment for percent time spent sitting.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

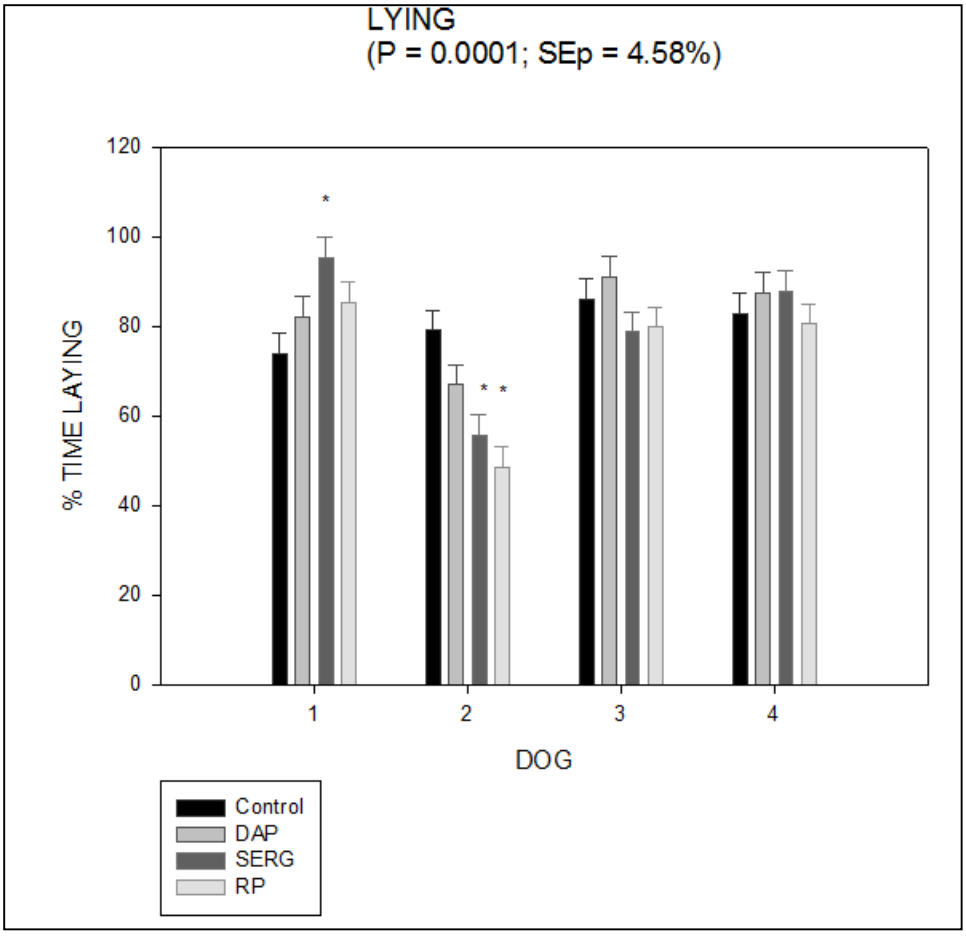

Figure 4.4. Pheromone/Placebo application showing the interaction of dog by treatment for percent time spent lying.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

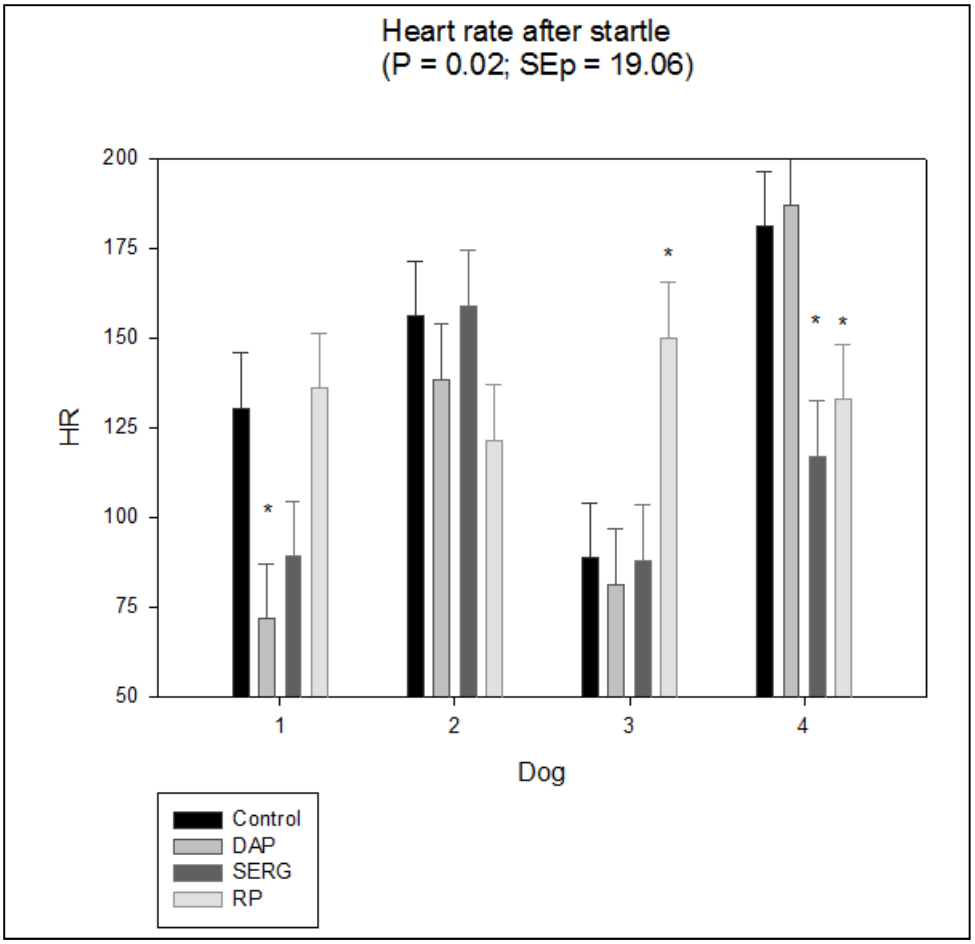

Figure 4.5. After startle test showing the interaction of dog by treatment for HR.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

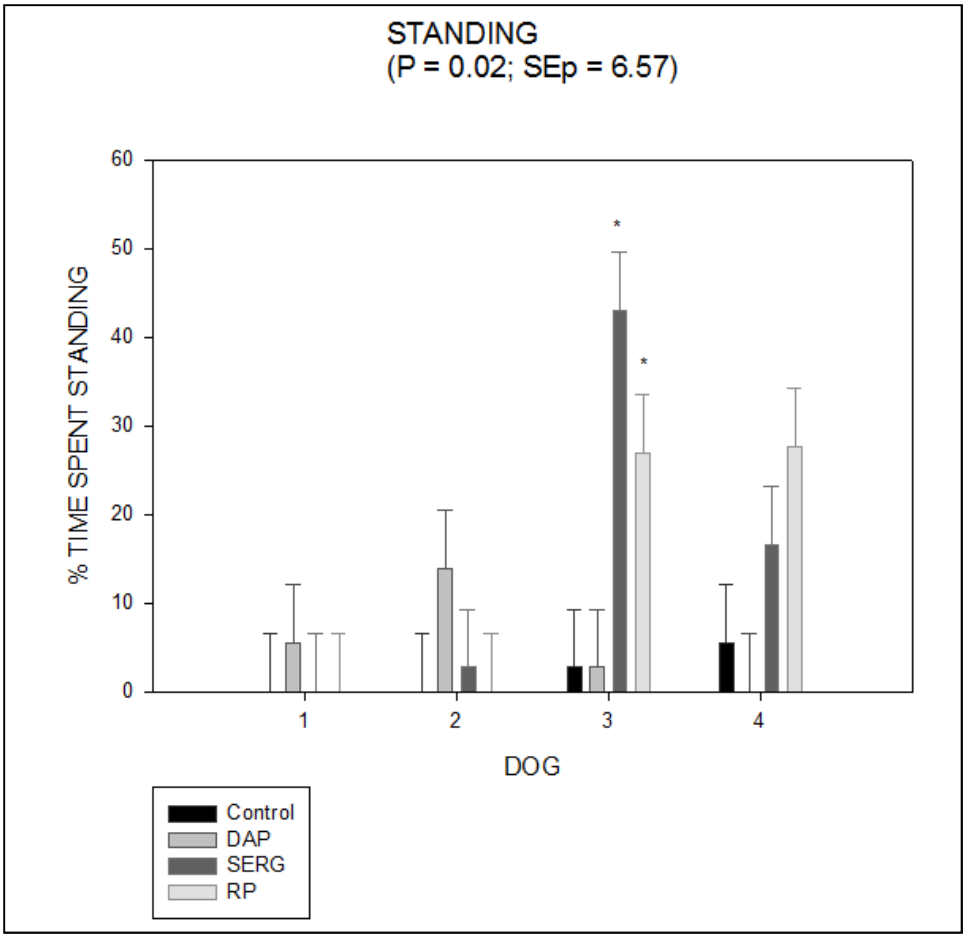

Figure 4.6. After startle test showing the interaction of dog by treatment for percent time spent standing.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

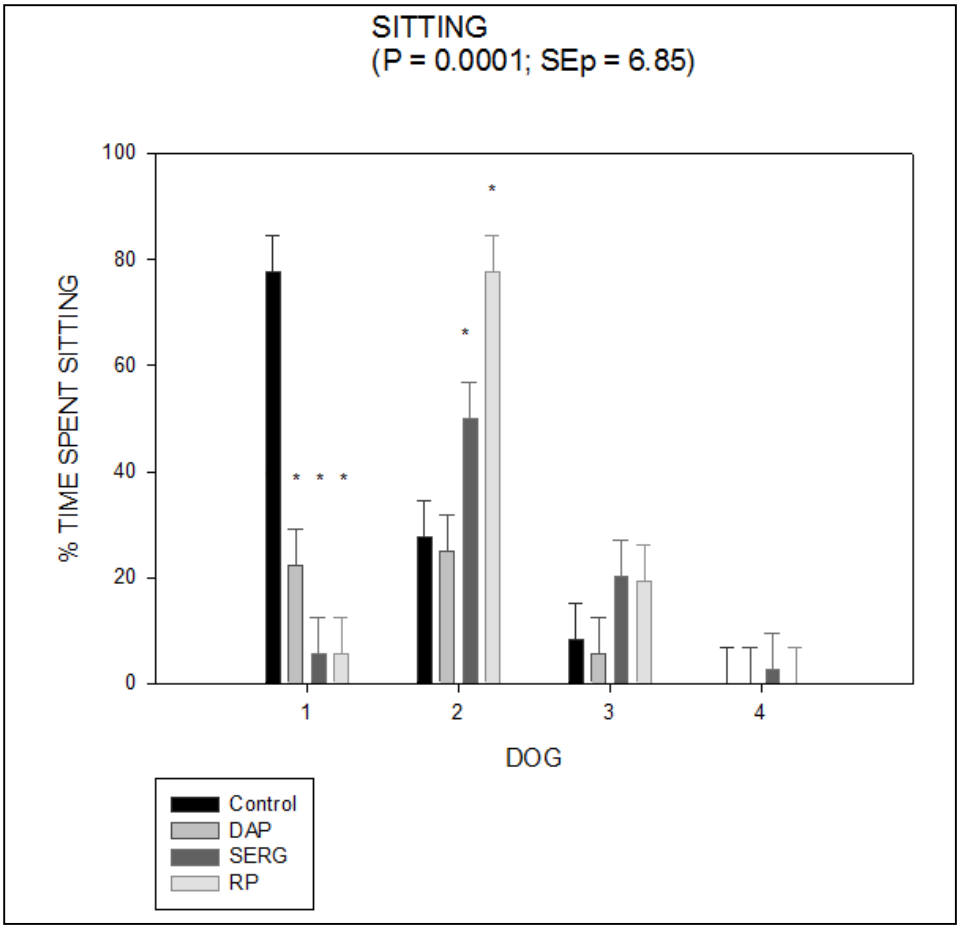

Figure 4.7. After startle test showing the interaction of dog by treatment for percent time spent sitting.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

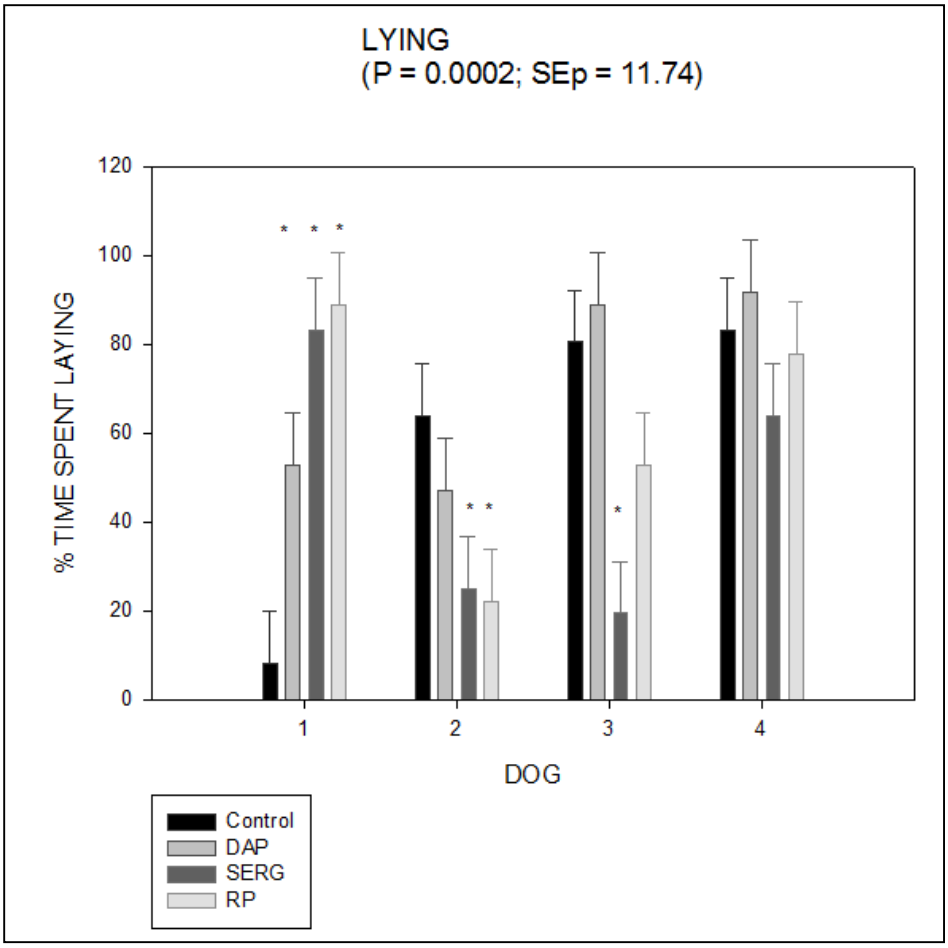

Figure 4.8. After startle test showing the interaction of dog by treatment for percent time spent lying.

***Indicates that treatment Least Squares means differ (P<0.05) compared to control.**

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CHAPTER V

CONCLUSION

The Interomone concept

The Interomone concept is a new concept for olfactory communication. The idea stemmed from a progression of thinking about chemical communication in animals. First, the dog appeasing pheromone (DAP) is a collection of fatty acids that have reported pheromonal effects. The cat appeasing pheromone is very similar in composition to the dog appeasing pheromone (the same can be said for horse, pig and other appeasing pheromones). Pheromones are, by definition, species specific. If similar molecules operate in different species, then it is likely that the same molecules would operate, in varying degrees in other species. This may particularly be true for pheromones that operate at close range such as in the maternal-neonatal environment. Thus, if a variation of the DAP might have efficacy in another mammal other than a dog (say a cat), then the RP maternal-neonatal pheromone may operate in species others than the rabbit. By definition, the RP, as a pheromone, must be species specific. Rather than throwing out the useful concept of pheromone (species specific molecules), the interomone concept can be used to explain how a pheromone in one species may change the behavior or physiology of another species.

The rabbit pheromone is a highly effective, single organic molecule that is a different class of organic molecule than the fatty acids in the DAP. Neonatal rabbits that are exposed to the RP orient towards the odor in a reliable manner (Schaal et al., 2003 and Coureaud et al., 2004). The dog may use the RP exact molecule or a

different but similar molecule during maternal-neonatal interactions. Our hypothesis was that if one class of maternal-neonatal pheromone (DAP) would have behavioral effects on adult dogs, then another class (RP) might have similar effects among adult dogs. However, the pheromone concept does not allow for inter-species chemical communication. Thus, there is a need for another term to describe such effects.

A pheromone is a molecule (or collection of molecules) that are found in a species that change the behavior or physiology of another animal of the same species (Karlson and Luscher, 1959). Other terms such as Kairomones and Synomones describe inter-species chemical communication that benefits the sending or receiving species (such as predator-prey odors, or feeding/aggregation odors). An interomone is a molecule or collection of molecules that act as a pheromone in one species, but has a different effect on another species without the need to benefit or harm the sending or receiving species and potentially having other behavioral effects (other than nipple orienting in the case of RP). The molecule 2-methylbut-2-enal is clearly a pheromone in the rabbit (Schaal et al., 2003) and an interomone in the dog (and perhaps other species). The demonstration that a pheromone may act as an interomone opens up the possibility of many biologically-relevant and clinically-important molecules that have the potential to be identified.

We document here that the Latin Square design sampled over time provides a statically-powerful tool to evaluate pheromone/interomone efficacy in an ethically responsible manner. This experimental design is often used to evaluate animal metabolism with expensive drugs or food additives in a highly-controlled

experimental setting. We demonstrate that this experimental design can be applied to research with domesticated pet species to answer difficult questions about behavior and physiology. Furthermore, the Latin Square design provided enough experimental control that behavioral differences – usually a highly variable biological measure -- among treatments could be statistically detected.

Due to the recent research conducted that questions the efficacy of pheromones and their use in dogs (Frank et al., 2010), we demonstrated in a highly-controlled experimental model, that pheromones/interomones can change the behavior of anxious dogs. By using this scientific model, we can conclude that Rabbit Pheromone collars had the highest rate of success at either changing the heart rate or the behavior of anxious dogs but the SERG and DAP collars also changed the physiology or behavior of some dogs. Futhermore, this is the first study that shows how pheromone collars can change a dogs' heart rate and behavior before and after startle.

We confirmed here that significant variation in response among dogs is found in pheromone/interomone efficacy in a controlled setting. In our highly-controlled setting, our data support the hypothesis that the pheromones/interomones tested will change behavior of some dogs and not others

Future Areas of Study

Further research is needed to look at the behavior of dogs that are deemed as anxious or that have separation anxiety. We still do not know if the use of pheromone

collars is more beneficial than the other alternative treatments available. As long as dogs are considered anxious there is going to be a welfare problem. Most pet owners are not trained in the correct methods of behavior modification. Also there are many different environmental factors that need to be looked at such as: amount of time played with, children in the house, time of day fed, and others. Also because the dogs were all intact males, difference in sex and castrates needs to be looked at as a possible explanation for HR and behavior differences. Factors such as these are always changing and need to be measured if at all possible.

Heart Rate and Behavior

The data shows that pheromone collars can make a dog less active by lowering heart rate and changing their behavior. However, no study has shown that lowering heart rate is beneficial toward the dog. On the other hand, no study has shown that elevating heart rate is not beneficial either. Therefore, more research needs to be conducted to give researchers a better idea to what is going on both behaviorally and physiologically.

Pheromones

The studies within this thesis suggest that there are benefits to using pheromones to change heart rate and behavior of anxious dogs. Since the data supports the idea that a rabbit pheromone can produce results with dogs (a heterospecific) other pheromones from other species may work as well. Different pheromones from different species could have attractive or aversive effects on dog

behavior. This would be important for wanting to attract an animal to certain area or keeping them away.

Genetic relationships

We know that behavior problems have been identified in family lines of different dog breeds from work done by Overall (2005). More research needs to be done looking at the genetic similarities of dogs that are clinically anxious. By being able to target non-anxious animals in different breeds, breeders can start to select for animals that will not show behavior problems as those previously mentioned.

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