THE BIOBEHAVIORAL EFFECTS OF STRESS RELATED TO FEAR AND ANXIETY IN DOMESTIC CANINES

A Thesis in
Biobehavioral Health

by
Nancy A. Dreschel

© 2007 Nancy A. Dreschel

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

August 2007
The thesis of Nancy A. Dreschel was reviewed and approved* by the following:

Douglas A. Granger
Professor, Biobehavioral Health
Thesis Advisor
Chair of Committee

Elizabeth J. Susman
Professor, Biobehavioral Health

David R. Johnson
Professor, Sociology and Human Development and Family Studies

Laura Cousino Klein
Associate Professor, Biobehavioral Health

Collins O. Airhihenbuwa
Professor, Biobehavioral Health
Head of the Department of Biobehavioral Health

*Signatures are on file in the Graduate School
ABSTRACT

Fear and anxiety in dogs are common causes of a variety of behavioral disorders that affect the lives of dogs themselves, as well as those who live and work with them. This dissertation explores the biobehavioral stress response of fear and anxiety in the domestic dog. While it is known that these problems have profound effects on dogs’ interactions with their human companions, the effect on their physical well-being is less well studied. In all species, a physiological stress response occurs following exposure to a fear or anxiety-provoking stimulus. This stress response is thought to have both short and long-term effects on health and lifespan of the individual.

Salivary cortisol as a measure of the stress response has been used in humans and other species, but factors related to collection methods, ability to collect saliva in diverse environments and under different situations, and the use of other salivary measures has not been well described for the canine species. A series of studies show that saliva can be easily collected in the home, kennel, and veterinary environments by pet owners, veterinary assistants, veterinarians, and behavior researchers. The materials and techniques used in the collection of saliva do influence the amount of saliva that can be retrieved as well as the final cortisol measurement. Cotton rope and hydrocellulose sorbettes can both be used with limited effect on cortisol concentration, and citric acid in small amounts is unlikely to have an effect on cortisol measurement, while increasing the volume of saliva collected. However, the use of beef-flavoring to enhance saliva collection in dogs is shown to introduce unpredictable error in salivary cortisol measurement.

The physiological and behavioral effects of fear in dogs are described in a study of dogs and their owners’ reactions to a thunderstorm recording. It is shown that dogs have significant and long-lasting adrenal stimulation, as measured by salivary cortisol, following exposure to a known stimulus. These effects are compared to their behavioral response as well as to the behavioral and physiological response of their owners. Interestingly, owners’ responses to their dogs’ stress are not related to the amount of change seen in their dogs’ biobehavioral responses.
An epidemiological survey of previous dog owners examines the long-term health and lifespan consequences of being a fearful or anxious dog. It is shown that certain fears and anxieties are prevalent in the domestic canine population and that there are prominent breed predispositions to particular behaviors that may have genetic components. These tendencies towards fear and anxiety are associated with specific health and lifespan consequences, including an increased severity and frequency of skin disorders in dogs with non-social fear and separation anxiety and a shortened lifespan in those dogs that are more fearful of strangers.

Suggestions for further research into the measurement and role of fear, anxiety and other behavioral disorders in dogs are offered. This research has implications for both pet animals and working dogs, and offers a model for human fear and anxiety.
# TABLE OF CONTENTS

LIST OF FIGURES.........................................................................................x

LIST OF TABLES..........................................................................................xii

ACKNOWLEDGEMENTS................................................................................xiv

CHAPTER 1- INTRODUCTION......................................................................1

Overview........................................................................................................1

Background Information................................................................................3

- Canine Fear and Anxiety...........................................................................3
- The Role of Dogs in Human Society.........................................................4
- The Canine Stress Response......................................................................6
  - Overview of the Stress Response..........................................................6
  - Physiology of Stress..............................................................................7
  - Behavioral Concomitants of Stress.......................................................9

Measurement of the Canine Stress Response...........................................10

- Hypothalamic-Pituitary Adrenal (HPA) Axis.........................................10
  - Cortisol and other hormones.............................................................10
  - Limitations of HPA axis measurements............................................12
- Sympathetic Nervous System (SNS).......................................................17
  - Catecholamines.................................................................................17
  - Heart rate, blood pressure...............................................................18
  - Salivary amylase, mucins and other proteins.....................................18
  - Limitations of SNS measurement.....................................................20
- Immune System.......................................................................................21
  - White blood cell numbers, salivary IgA, antibody response, cytokines.................................................................21
  - Limitations of immune measures.....................................................22
- Behavior..................................................................................................23
  - Owner-reported scales.........................................................................23
Observation…………………………………………………………24
Limitations of observation and coding……………………………26
Conclusion and Recommendations………………………………27
Individual Differences…………………………………………………27
Breed and genetics……………………………………………………27
Sex differences…………………………………………………………28
Health influences……………………………………………………….29
Physical and Social Environment……………………………………31
The Effects of Stress and Anxiety on Health…………………………32
Hypotheses………………………………………………………………36

CHAPTER 2- MEASUREMENT OF SALIVARY BIOMARKERS IN CANINE SALIVA.

Introduction………………………………………………………………38
Study 1: Measurement of Alpha-amylase in Canine Saliva……………40
  Introduction………………………………………………………………40
  Methods…………………………………………………………………40
  Results……………………………………………………………………41
  Conclusion………………………………………………………………41
Study 2: Collection of Salivary Cortisol in a Clinical Veterinary Setting……42
  Introduction………………………………………………………………42
  Methods…………………………………………………………………42
  Results……………………………………………………………………44
  Conclusion………………………………………………………………44
Study 3: In Vitro Determination of the Effect of Method Collection on Canine
  Salivary Cortisol Measurement………………………………………46
  Introduction………………………………………………………………46
  Methods…………………………………………………………………48
  Results……………………………………………………………………50
REFERENCES.......................................................................................................................116

Appendix A- Veterinary Clinic Saliva Collection Protocols and Data Collection Forms.................................................................................................................................128

Appendix B- Subject Recruitment for the Behavior and Health Survey..................131

Appendix C- Dog Behavior and Health Survey...............................................................132
LIST OF FIGURES

Figure 1-1: Schematic model of Interactions between Fear and Anxiety, Genetics, Social Environment and Stress Response in Relation to Canine Health .................................1

Figure 2-1: Absorption and retrieval of saliva using beef-flavored cotton rope results in increased cortisol measurement compared with cotton or hydrocellulose. (Error bars represent SEM)...............................................................................................................................50

Figure 2-2: The addition of citric acid to canine saliva causes a decrease in salivary pH at concentrations of .001g/ml in vitro. (Error bars represent SEM).................................51

Figure 2-3: The addition of citric acid to canine saliva at concentrations of .01g/ml or greater results in elevated cortisol measurement in vitro. (Error bars represent SEM)...52

Figure 2-4: There is no statistically significant difference in cortisol measurement in canine saliva collected with beef-flavored ropes or hydrocellulose swabs or when salivation is stimulated with citric acid in vivo.................................................................56

Figure 2-5: There are no significant differences in cortisol concentration in 2 samples collected 20 minutes apart for any of the collection methods tested. (Error bars represent SEM)...............................................................................................................................59

Figure 3-1: The mean (+/- SD) canine (A) and owner (B) cortisol levels at baseline, 20 and 40 min post-recording on the “thunderstorm” day (solid line) and control day (hatched line). Error bars represent standard error of the mean. Canine cortisol increased significantly from baseline to 20 min on the “storm day” and was still elevated at 40 minutes. Canine cortisol on the control day and human cortisol on both days did not show a significant change from baseline.................................................................78
Figure 3-2: Mean (+/- SD) salivary cortisol levels at baseline, 20 and 40 min post-stressor on the “thunderstorm” day. Dogs living without other dogs (solid line) had significantly more change from baseline to 40 minutes post-stressor than dogs living in multi-dog households (hatched line).................................................................79

Figure 4-1: Breeds occurring in the sample population classified by genetic relatedness as described by Parker, Kim, and colleagues (2004). Four distinct clusters of closely related subpopulations are highlighted by the red, yellow, blue and green groups. Some breeds that have characteristics of more than one subpopulation are shown in overlapping circles. (Based on Figure 3, Parker and Ostrander, 2005)..............................................88

Figure 4-2: Mean (+SEM) specific behavior scores for dogs classified into genetically-related breed groupings. The “modern” hunting dog (red) group breeds have significantly higher body sensitivity and non-social fear than the rest of the subjects......................98

Figure 4-3: Mean (+SEM) specific behavior scores for dogs classified into AKC breed groupings. The herding breeds have significantly higher non-social fear and thunderstorm fear than the rest of the subjects. Border collies and Golden Retrievers had higher thunderstorm fear than other breeds.................................................................99
LIST OF TABLES

Table 2-1: Concordance coefficients for saliva collection methods. Hydrocellulose has the most consistent concordance between samples in all studies. Salivary stimulation with citric acid at low levels also has high concordance between samples. Beef-flavored rope yields very unreliable cortisol measurements.

Table 3-1: Intrinsic behavioral characteristics of dogs based on the Canine Behavioral Assessment and Research Questionnaire (Hsu and Serpell, 2003). Nonsocial fear, separation-related anxiety and excitability were used in this study.

Table 3-2: Coding scheme used to code videotaped human and canine behaviors during exposure to the thunderstorm recording.

Table 4-1: Classification of dog breeds included in the breed analysis based on AKC classification system (AKC, 2006).

Table 4-2: Average anxiety scales and standard error of the mean for specific breed categories. The modern-hunting type dogs scored higher on body sensitivity and nonsocial fear compared to the rest of the population. Note- breed numbers do not add up to total due to mixed breeds and breeds not included in the genetic groupings.

Table 4-3: Regression models and corresponding beta coefficients examining the effects of non-behavioral factors on lifespan. Weight, neutering status, and death by accident have independent and significant effects on longevity. Death by euthanasia and classification in the modern hunting dog group also have independent significant effects when weight and neutering status are controlled.
Table 4-4: Regression models and corresponding beta coefficients examining the effects of behavioral factors on lifespan. How “well-behaved” the owner considered the dog to be and the level of stranger-directed fear (SDF) both have independent and significant effects on longevity when weight, neutering status and death caused by accidents are controlled for.

Table 4-5: Regression models and corresponding beta coefficients examining the effects of behavioral factors on disease. Body sensitivity scores had a significant independent predictive value of arthritis when weight was controlled for. Both non-social fear and separation anxiety positively predict the incidence and severity of skin disorders.
ACKNOWLEDGEMENTS

This thesis is a culmination of a number of years of graduate work, but reflects many more years of preparation through practice, previous study, and family nurturing. It is an accomplishment that I share with all those that have helped me along my life course. Specifically I would like to thank my advisor, Doug Granger, for believing and respecting my clinical intuition and encouraging and guiding my development as a researcher. I also thank the members of my committee, Liz Susman, Laura Klein and David Johnson, for their guidance and having patience while I worked through this. In addition, I would like to thank all the faculty and staff in the Biobehavioral Health Department, many of whom have no idea how much a statement they may have made in a class or a suggestion given, a technique demonstrated, or merely a kind word spoken, influenced the course of my thoughts and career. I also thank and remember all my fellow graduate students who made everything bearable and offered friendship, support, and intelligent discussion through the years. Thank you to the staff and friends at Salimetrics, who provided me with test kits, advice and help as I ran cortisol assays in dog saliva over many years. Thank you to the veterinarians and staff at Centre Animal Hospital for all their help in collecting data, recruiting subjects and for their long-time friendship. Thank you also to the faculty and friends in the Department of Dairy and Animal Science who have given me a home and freedom to pursue my interests in a meaningful way.

I could not have accomplished what I have without the support and upbringing of my parents, Barbara and Jack Erickson, who taught me to always try my best, whatever the given circumstances, and have loved me through it all. I have also depended on the support of my brother, Jim, and sister, Connie, as well as my in-laws, who have all set paths of excellence in their own lives which serve as a role model for my own.

My life has been enriched by contact with many animals who have shared both my working hours and my home. From cattle and horses to dogs, cats, and mice, they have all had an impact on my views and goals. I am especially indebted to and honor the memories of Moses, Tigre-Fleur, and countless hamsters, mice and fish. Mishka
continues to serve as a loving companion as well as an inspiration to decode the processes of canine fear and Kitty reminds me that there are some animals we will never understand. I especially thank Merlin, who has been with me since the start of my career 18 years ago and has always been there for a purr and a cuddle.

My greatest appreciation and thanks go to my family- to my husband, life-partner and best friend, Bill, who has supported me and kept me sane, and to Ben and Nate, the best sons a mother could ever hope for, who bring joy to my life and who gladden my heart.
Overview

Behavior problems related to fear and anxiety are common in the domestic dog. While it is known that these problems have profound effects on dogs’ interactions with their human companions, the effect on their physical well-being is less well studied. In all species, a physiological stress response occurs following exposure to a fear or anxiety-provoking stimulus. This stress response is thought to have both short and long-term effects on health and lifespan of the individual. This dissertation explores the measurement and health outcome effects of fear and anxiety in the domestic dog. A working model of the interactions between exposure to a stimulus, the stress response, environmental, genetic and health factors is shown in Figure 1-1.

Figure 1-1: Schematic model of Interactions between Fear and Anxiety, Genetics, Social Environment and Stress Response in Relation to Canine Health

An exposure to a fear or anxiety-inducing stimulus results in a physiological stress response (Letter A in Figure 1-1). This multi-faceted response is characterized by
the activation of the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic nervous system (SNS) and the immune system, and is influenced by a number of factors, including the social environment of the animal, the breed or genetics of the animal and the health of the individual. A number of methods are available for measuring each arm of the physiological stress response. The strength of the fear response (Letter B in Figure 1-1) will depend on the severity of the stimulus as well as the dog’s underlying predisposition to fear or anxiety and the social environment in which the exposure occurs (Letter C in Figure 1-1). The stress response has been shown to have effects on the health and lifespan of the individual animal as well (Letter D in Figure 1-1). These effects include but are not limited to: accelerated aging; decreased longevity; increased immune-mediated diseases; predisposition to neoplasia, infectious diseases, and endocrine disorders; and cognitive disorders. It is also known that genetics, social and physical environment, and many other factors are important in determining overall health of the animal.

Examination of several specific aspects of the psychobiological stress response secondary to fear and anxiety in domestic dogs is the focus of this dissertation. Background information on specific issues related to canine fear and anxiety, an overview of the role of dogs in human society and a description of what is known about the canine stress response and its measurement is provided in the Introduction. Chapters Two, Three and Four describe specific studies focused on the measurement of the stress response in dogs, the relationship between physiological and behavioral responses in dogs and the effect of social environment on these responses, and the long-term effects of the stress response on the health and lifespan of companion dogs. Chapter Five provides a summary and conclusion including suggestions for further research development in this area.
Background Information

Canine Fear and Anxiety

Domestic dogs suffer from a variety of anxiety-related disorders including stimulus-specific fears and phobias (e.g. noise, thunderstorm, vacuum cleaners, people, places, other animals, etc.), fear-related aggression, separation anxiety, and obsessive-compulsive disorders. Although there are few good studies on the prevalence of fear and anxiety disorders in the general canine population, fear of separation and fear of loud noises are some of the most common presenting complaints among dogs with behavioral problems (Tuber, Hothersall et al. 1982). Shull-Selcer and Stagg (1991) reported that approximately one-third of the patients presented to a behavior clinic at a university veterinary teaching hospital had fear-related behavior problems. Dog walkers surveyed in Southern England, indicated that 13% of their dogs exhibited separation-related anxiety behaviors and an additional 11% had exhibited these behaviors at some time in the past (Blackwell et al. 2002).

As a species, dogs have overcome the inherent fear that most wild animals have of human beings and our environment in order to co-exist with us, filling an ecological niche that was established as the domestication process began. However, living with a fearful or anxious animal can have tremendous impact on the relationship between the dog and the human. Fearful dogs can be aggressive, destructive, and difficult to live with.

Aggression towards humans (particularly owners or those close to them) is an extreme but all too common example of this bond “gone wrong” and is the most common behavior problem seen in clinical veterinary practice (Borchelt and Voith 1996). There are a number of causes of canine aggression, and fear and anxiety play important roles in many of them. Aside from the obvious “fear-aggression” which occurs when an animal feels they cannot escape a threatening situation and must take responsive, protective action; impulse control disorders (e.g. “dominance aggression”) can occur when animals
with underlying anxiety in their relationship with a human take a proactive aggressive role (Overall 2001).

Fearful dogs can cause tremendous damage to homes and to themselves. Thunderstorm-phobic dogs have been known to jump out of second-story windows, run into the path of oncoming vehicles, and destroy doors, windows and carpeting. Dogs with severe separation anxiety can destroy a home within minutes, chew through metal crates and eat through wall-board and solid wooden doors. Besides the financial implications of this, severe personal injury is often incurred by the animal itself. Inappropriate defecation and/or urination also frequently occur and are common signs of generalized anxiety in the dog (Blackwell, Casey et al. 2006). Many animals with severe fear or anxiety problems are eventually removed from the household or euthanized. Behavior problems, including those that may be fear or anxiety related, are the number one cause of relinquishment to shelters (Salman, Hutchison et al. 2000). To prevent this disruption of the human- dog relationship, and to improve the use of dogs in their multiple societal roles, it is important that we understand canine fear-related problems and develop ways of measuring, preventing and treating them as they arise.

The Role of Dogs in Human Society

Dogs fill a variety of roles in human society beyond companionship. Although the number of dogs used in biomedical research has decreased over the past twenty years, dogs are still used in a number of important studies both as models of human disease and in research related to canine disease and nutrition (FBR 2006). It is important from a scientific and a welfare point of view to decrease both fear and anxiety that could result from participating as subjects in these studies. Ethically, researchers are bound to provide for the welfare of their subjects. Scientifically, it is important that the physiological and behavioral responses to anxiety or stress do not alter the data. Because pain, illness, and anxiety are often manifested in similar ways behaviorally, it is important that researchers be able to differentiate and measure these responses.
Increasing numbers of dogs are also used in service and therapy throughout the world. Dogs are used to guide and assist individuals with visual impairment, hearing disorders, and other physical disabilities. It has been shown that dogs serving disabled individuals decrease personal care hours by 68% (Allen and Blascovich 1996). Some dogs are able to predict seizures in humans and warn them so they take necessary steps to prevent the seizure onset or are safe when it occurs. Dogs that serve the disabled are granted access to all business premises where customers are generally allowed under the Americans with Disabilities Act (1990). These dogs are therefore exposed to a variety of stimuli that could be considered fear-inducing and must work without distraction in these situations.

Increasing numbers of dogs are also used by police forces, the military, customs and border protection, and many other human protection agencies. Dogs are used to sniff out drugs, explosives, land mines, and contraband. Working dogs are under tremendous stress and understanding how this is manifested and could be prevented is extremely important to the life and career of the animal. In a study of behavioral complaints in military working dogs, inappropriate aggression, repetitive behaviors and arousal and attempt to escape or avoid a particular situation or stimulus made up over 60% of the problems (Bughardt 2003). “Burn-out” from stress is also a common cause of the shortening of these animals’ useful careers. Preventing the development of, and screening for fears and anxieties in service and working animals is of great interest to those breeding and raising them (Serpell and Hsu 2001; Bughardt 2003). Genetic, social and environmental influences on canine personality are being investigated (Saetre et al. 2006; Svartberg 2005; Appleby, Bradshaw et al. 2002).

Dogs have also been proposed as an important animal model of anxiety disorders in humans. Due to the number of naturally occurring anxiety disorders recognized in dogs, the veterinary behavior literature is rich with information on the treatment, and classification of anxiety-related disease. Also, because of the controlled breeding and common line and inbreeding that occurs in purebred dogs, the incidence of anxiety within lines and families can be (and has been) mapped over generations, lending information on
the potential role of genetics on the manifestation of signs. (Reese 1979; Shekhar, McCann et al. 2001; Overall 2000)

The Canine Stress Response

Overview of the Stress Response

The stress response is a multi-faceted reaction based on the interaction of multiple physiological and behavioral systems (Chrousos and Gold 1992; Sapolsky, Romero et al. 2000; Casey 2002; Selye, 1976). The basic stress response is an evolutionarily adaptive one (Canon 1932). Organisms function by maintaining homeostasis in all systems. When a living system is disturbed in some way (either physically or psychologically), the stress response acts to bring these homeostatic mechanisms into play and to re-establish the balance of life. Although the stress response is protective, it can also be damaging depending on the degree to which it is enacted, the length of time that the organism is under stress and the organism’s further response to the stress response itself. A variety of individual differences, such as genes, experience, physical and social environment, will influence the physiological and behavioral response of a particular animal to a particular stress (McEwen 1998). If the stressor is a short acting one and the response is able to occur rapidly and to be closely regulated by feedback mechanisms, then the response will likely be functional. If, however, the stress is of prolonged duration and the organism achieves a new steady state of functioning in which the original adaptive response is prolonged, a pathophysiological state may occur. (Chrousos and Gold 1992; Sapolsky, Romero et al. 2000; McEwen 2005)

Although the focus of this dissertation is the stress response to fear and anxiety, it must be remembered that stress occurs in response to many different psychological, social and physical stimuli. Disease, hunger, thirst, and pain are basic physiological stressors that can result in a host of both physiological and behavioral changes.
Environmental stressors might include weather, temperature, and availability of shelter or food. As social animals, typical stressors in dogs might also include interactions between conspecifics (establishment of dominance hierarchy, overcrowding, aggression) as well as interactions with other species (humans, cats, other companion animals). In humans, both marriage and divorce are considered to be potential stressors. Despite the variety of triggers, the basic physiological and behavioral manifestations of the stress response are similar in most mammalian species.

**Physiology of Stress**

There are two primary physiological components of the stress response, the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). In the HPA axis, corticotropin releasing factor (CRF), oxytocin and vasopressin are released from the hypothalamus in the presence of a stressor. This leads to release from the anterior pituitary of adrenocorticotrophic hormone (ACTH) which then stimulates the release of corticosteroids from the adrenal cortex into the circulation. The glucocorticoids have a host of physiological and behavioral effects that are commonly thought of as the “stress response”.

At the same time, the sympathetic nervous system (SNS) is activated by direct neural activation of the adrenal medulla which leads to the release of catecholamines (norepinephrine and epinephrine) with their concomitant physiological and behavioral effects (e.g. increased heart rate, blood pressure, etc.). Timing of the physiological stress response is related to the type of stressor and the aspect of the stress response that is activated. While it may take 20-30 minutes for the HPA axis to become fully stimulated and for one to see peak glucocorticoid levels in the blood stream, the SNS response is almost immediate. Therefore, the sympathetic nervous system effects usually have a very rapid onset but dissipate quickly when the stressor is gone. In contrast, the HPA endocrine effects have a much longer lasting effect on the body.
The immune system is also highly integrated into the stress response. The immune response consists of both an innate or natural immunity consisting of phagocytic cells (e.g. macrophages, neutrophils, eosinophils) and natural killer cells; and an acquired, specific immunity generated by lymphocytes derived from the thymus and bone marrow. Cytokines are small protein molecules that act as intra- and inter-cellular messengers between immune cells. (Apanius 1998)

The neuro-endocrine and the immune networks work together in complex ways that affect all branches of all systems. For example, both corticosteroids and epinephrine have effects on circulating white blood cells. Epinephrine causes a shift of neutrophil white blood cells from the marginal pool to the circulating neutrophil pool, which results in a dramatic increase in neutrophils in the blood stream. Epinephrine can also cause an increase of circulating lymphocytes. Glucocorticoids, such as cortisol, cause an increased release of mature neutrophils into the blood stream and decreased migration of neutrophils into tissue, with a concomitant decrease in numbers and percentages of lymphocytes, resulting in lymphopenia (Weiser 2004). Flow cytometry has shown that the B cell, NK cell, and monocyte numbers decrease more than T cells in the circulatory system following mild acute stress (Dhabhar et al. 1995). These numbers rapidly return to normal when the stress is over.

The stress response also has widespread immune effects which vary depending on the type, length and severity of the particular stressor. Sapolsky and colleagues (2000) offer a review of the many conflicting effects of glucocorticoids on immune system function and competence. Corticotrophin releasing hormone (CRH) decreases cytotoxic immune effects, but enhances B cell proliferation, lymphocyte response and numbers of cytokine receptors. Infectious agents activate the immune system long before cortisol is affected, and psychological stressors cause cytokine release which precedes a glucocorticoid response. Glucocorticoids have been shown to have both immunosuppressive and anti-inflammatory effects as well as immune-enhancement effects. The field of psychoneuroimmunology has evolved to examine these relationships. The one thing that is clear from the literature is that stress has reliable effects on the immune system.
Behavioral Concomitants of Stress

Because dogs do not communicate verbally with humans, and because there is scientific controversy surrounding their emotional and cognitive ability, all of our clues to their mental state must be interpreted through their behavior. Written over 130 years ago, Charles Darwin’s description of a dog in “extreme terror” is still applicable today:

A dog under extreme terror will throw himself down, howl and void his excretions, but the hair, I believe, does not become erect unless some anger is felt. I have seen a dog much terrified at a band of musicians who were playing loudly outside the house, with every muscle of his body trembling, with his heart palpitating so quickly that the beats could hardly be counted, and panting for breath with widely open mouth, in the same manner as a terrified man does. Yet this dog had not exerted himself: he had wandered slowly and restlessly about the room, and the day was cold.

-Charles Darwin, 1872 (in Tuber, Hothersall et al. 1982)

Visual communication, particularly posture, tail carriage, ear carriage, and lip position, is very important in all canine species (Overall 1997). Early signs of uneasiness or conflict might include yawning and licking of the lips (Voith and Borchelt 1996). When dogs are presented with a threatening stimulus, the ears flatten against the head, the tail is down and often tucked between the legs, and the animal crouches away from the threat. In addition to these signs, a fearful dog may either exhibit a submissive behavior (lying down on its side, lifting a hind leg, displaying the abdominal and inguinal area, and sometimes urinating) or an aggressive posture (raised fur on its shoulders and over the rump and lips retracted in a snarl) (Houpt and Wolski 1982). Some dogs will exhibit multiple conflicting signs at once. The terrified small dog huddled in a corner with its belly and all of its teeth showing is a familiar sight to anyone who works with this species.

In addition, fearful dogs are often disinterested in food, play, or other interactions. Other anxiety-associated behaviors include those related to autonomic arousal (panting, drooling, urination and defecation), motor restlessness (pacing, digging, destructiveness, excessive licking) as well as hiding, shedding, and whining (Overall 1997). These signs may occur singly or in combination, and there is a great deal of individual variation in
their expression. Behavioral responses are often stimulus specific. If there is no perceived escape from a threatening situation, aggression (barking, growling, snapping, biting) is common. Destructive behaviors during separation anxiety or thunderstorm phobic behaviors are escape behaviors and are often directed towards windows, doors, and other points of exit (Voith and Borchelt 1996). It is interesting that even when the dog manages to “escape” from this situation, it is often found nearby, pacing and panting on the roof or close to the home. On the other hand, some animals run in terror from the home in storm-situations and are lost or killed on the road.

**Measurement of the Canine Stress Response**

**Hypothalamic-Pituitary-Adrenal (HPA) Axis**

*Cortisol and other hormones*

Cortisol has been extensively used as a measure of HPA axis activity. It can be measured in the plasma, saliva, feces and urine of dogs and other species (Vincent and Michell 1992; Beerda, Schilder et al. 1998; Beerda, Schilder et al. 1999b; Schatz and Palme 2001; Kobelt, Hemsworth, et al. 2003; Stephen and Ledger 2006). In many species, cortisol has strong diurnal, circadian and seasonal rhythms of secretion (Kirschbaum, Read et al. 1992; DePew, Thompson et al. 1994; deJong, Pelle et al. 2000). It is important that researchers understand the timing of these normally occurring patterns so that they can control for them in the experimental design. For example, in humans, cortisol secretion has a strong diurnal rhythm with the highest levels occurring in the morning and then falling to a plateau later in the day (Kirschbaum, Read et al. 1992). It is therefore important to collect all subject samples at similar times of the day and stage of the reproductive cycle and to preferably have control baselines at similar times on other days so that differences in levels would not be attributed to inter-
individual behavioral differences. In dogs, plasma cortisol falls within a wide range of values (.5-6.0 µl/dl) and normal dogs often show fluctuations throughout the day based on the episodic secretion of both cortisol and ACTH (Feldman and Nelson 2004). However, the presence or absence of a predictable diurnal cycle of cortisol secretion in dogs is controversial. Plasma cortisol has not been shown to have a diurnal rhythm in a number of studies (Johnston and Mather 1978; Chen, Gelatt et al. 1980; Gordon and Lavie 1985). However, while some researchers failed to show a change in salivary cortisol over time (Koyama, Omata et al. 2003), Beerda and colleagues (1999b) recorded significantly higher mean salivary cortisol levels in their canine subjects in the morning than during the rest of the day. They noted that this daily fluctuation was more pronounced when dogs were socially and spatially restricted. It is possible that these variations have more to do with the specific stressors the dogs were experiencing, than with a physiologically based diurnal cycle.

Likewise, patterns of secretion may be related to other behaviors such as exercise, sleep and eating. Ingestion of a protein-rich meal has been shown to increase salivary cortisol levels in humans (Gibson, Checkley et al. 1999). This response has not been tested in dogs to our knowledge. Exercise has been shown to increase plasma cortisol concentrations in dogs (Raekallio, Kuusela et al. 2005).

While resting baseline measurements of plasma cortisol are less useful, tests of plasma cortisol response to dexamethasone or ACTH injection are commonly used in the diagnosis of hyperadrenocorticism in dogs. Dogs are usually hospitalized during this time and trained veterinary professionals draw the blood samples. In this situation, pronounced responses to suppression or stimulation are expected (Feldman and Nelson 2004). Because the drawing of a blood sample in a laboratory or veterinary setting can be potentially stressful, noninvasive measures of cortisol response are particularly important in research situations.

Urinary and fecal cortisol measurement have been increasingly examined as noninvasive measures of stress in domestic and wild animal species (Creel, Fox et al. 2002; Millsbaugh, Washburn et al. 2002; Young, Walker et al. 2004). Dogs have been found to excrete only a small portion of cortisol metabolites in their feces, but excrete
larger amounts in urine (Schatz and Palme 2001). It takes approximately one day to see peak cortisol metabolites in the feces following injection of ACTH. Urinary cortisol is compared to creatinine to make up for differences in urine output. Fecal collection is the least invasive collection method, and is useful in kennel situations with dogs that can not be handled or in naturalistic settings with wild or feral dogs. Both urinary and fecal cortisol have been recommended as measures of stress in shelter dogs (Stephen and Ledger 2006).

In dogs, salivary cortisol has been shown to be highly correlated with plasma cortisol with an approximately 20 minute temporal lag period in the time that cortisol increases in plasma are reflected in saliva (Vincent and Michell 1992). As compared to fecal or urinary cortisol, salivary cortisol is temporally more closely related to circulatory levels. In addition, as compared to plasma, saliva can be collected at convenient and meaningful times of the day in more naturalistic settings. It is not technically challenging and it is thought that most people can be easily trained to collect the samples. Although handling of the animal is required, saliva collection is not a particularly noxious stimulus and seems to be tolerated well by most dogs. The materials used to collect saliva are not complicated and do not require special storage. Once collected, saliva must be frozen and transported frozen, but it does not require further manipulation from the sampler.

While other steroid hormones (e.g. estrogen, progesterone, testosterone) also likely play important roles in the stress response and have been extensively studied in the human literature, little attention has been given to them in the veterinary behavioral field. The majority of companion dogs in the U.S. have been gonadectomized so the levels of circulating sex hormones are minimal.

**Limitations of HPA axis measurements**

A number of limitations exist in the collection and interpretation of cortisol levels by the various methods discussed previously. Each of these can impact assay validity, complicate statistical analyses and compromise the interpretation of bio-behavioral
relationships. Whether measured in plasma, saliva, feces or urine, cortisol levels exhibit a high degree of intra-individual variation (Beerda, Schilder et al. 1998; Schatz and Palme 2001; Kobelt, Hemsworth et al. 2003; Stephen and Ledger 2006). Therefore, measurement and comparison of single samples across individuals has little meaning. In experimental designs, baseline measurements should be included and individuals should serve as their own controls.

An important issue of sample timing involves the “lag period” between the time in which a biomarker is secreted and the concentration changes in the blood stream and when the levels change in saliva. For example, although the HPA axis is rapidly activated by a stressor, it takes several minutes for the cascade to result in increased cortisol in the blood stream and saliva. Cortisol finds its way into saliva via passive diffusion (Kirschbaum, Read et al. 1992). Therefore it takes time for a change in bloodstream concentration of these hormones to be reflected in a change in salivary concentration. It is important to understand the timing of the response for a particular biomarker. If one were to search for biomarkers in saliva at a time when they would not yet have appeared, or would already have disappeared, one could mistakenly fail to find a relationship. Most biomarkers are episodically secreted with varying lengths of time in which they can be found in the saliva. This is not a unique problem to salivary testing and the same issues also hold for serum sampling. In fact, because saliva sampling is non-invasive, it allows for more frequent sampling over a longer period of time to be able to see these peaks.

A similar but longer lag period exists for cortisol changes in urine and fecal samples. Response to a stressor will not be evident in fecal cortisol for at least 24 hours. If one isn’t observing the animal during that entire time, other stressors may come into play that could result in unexpected fecal cortisol spikes (Schatz and Palme 2001). The delay between stressor and ability to measure an increase in cortisol in urine is of unknown length, although the kidneys begin secreting cortisol precursors within 30 minutes of an induced shock (Beerda, Schilder et al. 1996).

The most obvious limitation of plasma cortisol collection is that it is an invasive procedure, requiring skilled technical capabilities; a compliant subject; and sample collection, processing and storage capabilities. Handling and venipuncture have been
shown to increase canine blood cortisol levels 20 minutes later (Hennessy, Williams et al. 1998). In behavioral or biobehavioral research, one is often interested in subtle changes in cortisol levels over a period of time. The response to the venipuncture can cause spurious increases in cortisol levels that may be more related to the stress of the sampling than the response to the stimulus of interest.

Collection of urine can also be noninvasive, but can sometimes be difficult to accomplish. If dogs are kenneled, they may urinate in their cage before being walked outside. Dogs can be kenneled in cages that allow for the collection of urine, but the potential exists for contamination with feces. Urine may also be obtained through catheterization or cystocentesis but these are much more invasive procedures. Because urinary cortisol is not an immediate reflection of plasma cortisol, it is difficult to use it to measure a specific response to an acute stressor.

Although collection of saliva is much easier and less-invasive than blood collection, there are limitations associated with particular collection methods. A significant limitation with the collection of canine samples is the ability to collect an adequate volume of sample. This is also a concern for researchers of infant biobehavioral interactions. Although many of the new assays require very little sample, there is still some difficulty in obtaining even this small amount from small canines. The number of tests that can be run is limited by the sample available, limiting the ability to run tests in duplicate for validity. Recent research also indicates that low volume samples result in a disproportionate percentage of cortisol retrieval from absorbent materials, resulting in the potential for considerable error variance in measurement (Granger, Harmon, et al, 2006).

While salivary stimulants (e.g. citric acid crystals, powdered drink mix) have been frequently used in both human and canine research to obtain greater quantities of saliva for testing (Beerda, Schilder et al. 1998; Bergeron, Scott et al. 2002; Coppola, Grandin et al. 2005), there is evidence that these can artificially increase the levels of both cortisol and testosterone in humans (Schwartz, Granger et al. 1998; Granger, Shirtcliff et al. 2004). This is likely related to an increase in sample acidity. Whether this is a problem in canine sample collection has not been tested.
Research has shown that cotton-based sample collection methods can interfere with a variety of salivary biomarkers, including testosterone, DHEA, progesterone, estradiol and sIgA (Shirtcliff, Granger et al. 2001). These alterations in measured levels introduce unsystematic error so that the correlations between serum and salivary levels are no longer strong. This challenges the validity of the test and could make the interpretation of biobehavioral relationships meaningless. Cortisol does not seem to be affected by cotton-based collection. The results of cotton-based samples can therefore be interpreted with confidence, as long as the sample volume is adequate. As new materials and methods of collection are used, however, they must be tested for interference. Researchers must be aware of the limitations of the assay and biomarker with which they are working.

Although saliva collection requires much less technical ability than plasma collection, proper storage of samples before analysis is very important. While most steroid biomarkers commonly measured seem to be quite stable when stored at low temperatures (-80 °) over long periods of time, there is evidence to indicate that some hormones, such as testosterone, are sensitive to repeated freeze-thaw cycles and storage at higher temperatures (4 °, -20 °, -40 °). The reasons for these are not known, however several hypotheses have been advanced (Granger, Shirtcliff et al. 2004). One theory is that there is a high bacterial load in saliva, as compared to blood, and bioproducts of this activity may cross-react with the testosterone assay, causing spurious high levels. Lower temperatures (-20 °, -40°) and freeze-thaw cycles may be related to the natural degradation of testosterone expected over a period of time. It is likely that this also occurs in frozen serum samples. However, serum (unless taken from a bacteremic individual) is usually sterile and without the presence of free-living bacteria. Regardless of the cause, if researchers are unaware that these conditions may be causing invalid levels of measured biomarkers, they can draw invalid or inconclusive results.

Another limitation of saliva sampling is the potential for contamination of samples. This can occur with food particles remaining in the mouth during sampling or blood leakage into the mouth. Hormones and hormone-like substances in breast milk or infant formula can cause spurious elevations in measured salivary hormones in infants.
Food particles of animal or plant origin may also contain products that can cross-react with the antibodies used in salivary immunoassays (Magnano, Diamond et al. 1989). In humans, these limitations are easily worked around if the researcher instructs the individuals collecting saliva to rinse their mouth prior to collection or to refrain from eating for a certain period of time before collecting. These issues are very important to animal researchers. Many dogs chew on bones, rawhide chews and other plant and animal-based products on a regular basis throughout the day. Many dogs are fed ad libitum or “free choice”. Because animals do not frequently have their teeth cleaned, there is also a greater potential for food products to remain in the mouth for longer periods of time. Again, researchers can avoid some of these potential problems by having owners or those collecting the samples refrain from giving their dog a treat, allowing them to chew on rawhide or other animal-based products and allowing them to eat in the time preceding sample collection.

Blood leakage is another potential source of contamination of saliva samples. This can occur as a result of microinjury (burns, abrasions, tooth-brushing) or more serious damage (dental cleaning, gingivitis, chewing related injuries). Because the levels of many biomarkers are 10-100 times higher in the serum than in the saliva, the presence of blood in the saliva has great potential to increase levels of these markers. It is likely that small amounts of leakage would be diluted so much that they would not cause problems, while there is likely a threshold over which the salivary levels would be elevated. Some hormone measures are more highly affected by mild-moderate blood leakage. For example, cortisol levels seem to be little affected by microinjury as experimentally produced by toothbrushing, while measurement of testosterone is more sensitive to these injuries (Kivlighan, Granger et al. 2003). Testing of samples for blood content through visual examination at the least or measurement of transferrin levels can help researchers to determine if blood leakage will be a problem for their samples or not. Again, it is important to recognize the differences between biomarkers.

Because hormones enter saliva through passive diffusion, only the unbound (“free”) molecule is present in saliva. Because of this, anything that affects the protein-binding of these hormones or levels of binding proteins in the serum has the potential to
affect the level that will be measured in the saliva. For example, influences that cause very sharp peaks in serum cortisol release can lead to much higher increases in saliva due to an ‘overloading’ of the available cortisol binding globulin (CBG) (Kirschbaum, Read et al. 1992). The relation between serum and salivary cortisol levels in pregnant animals is unclear. Because there are changes in many of the steroid hormones at these times, there is also thought to be an increase in serum CBG. However, hormones such as progesterone and cortisol compete for the binding sites on CBG and the free level of hormone available to diffuse into the saliva is unclear. Researchers studying individual differences in cortisol responses in relation to behavioral effects need to be aware of and control for this limitation in their study design.

While there are limitations to the use of salivary cortisol, many of these limitations may be overcome by planning, careful instruction to those collecting samples, and research design. Because salivary samples may be collected in the absence of the researcher, adherence to collection methods and times takes on a new importance. The researcher is dependent on those collecting samples to adhere to times and procedures that have been specified. It is important for the researchers to be aware of these limitations so that their results can be properly interpreted.

**Sympathetic Nervous System (SNS)**

**Catecholamines**

In order to measure the sympathetic nervous system response, one can either measure the results of catecholamine response or the levels of catecholamines themselves. Epinephrine and norepinephrine are released into the blood stream from the adrenal medulla following direct neural stimulation. This response has been shown to reliably occur in dogs following exposure to a noise stressor (Engeland, et al 1990). Blood samples can then be analyzed to measure concentrations of these substances in the plasma and serum. Samples must be taken quickly following a potential stressor because
of the rapid autonomic response. In Engeland’s (1990) study, catecholamine levels remain at increased levels for approximately four minutes following the acute stressor. An indwelling venous catheter is frequently placed in order to collect blood samples quickly and with minimal restraint and anxiety.

Heart rate, blood pressure

Another common way to measure the autonomic nervous system response is to measure the physiological changes that occur in response to the release of epinephrine and norepinephrine. In humans, blood pressure and heart rate are frequently measured. While these responses can also measured in dogs, they often require more invasive methodology. Heart rate can be measured easily through the pulse on the femoral artery in awake animals or on the tongue in anesthetized animals. ECG or Doppler heart rate measurement by way of a Holter cardiac monitoring harness is used to record heart rate and variability in dogs over an extended period of time (Beerda, Schilder et al. 1998). Due to their small peripheral blood vessels, blood pressure is less easily measured in dogs. Although doppler blood pressure measurement is useful for diagnosis and monitoring of hypertensive disease states, there is a great deal of intraindividual variation based on animal and researcher technique (Tilley and Smith 2004). Central venous pressure and mean arterial pressures are measured in anesthetized dogs to measure the physiological effects of medications or painful procedures. Ultrasonic imaging of the heart can also be performed but does require minimal restraint in most dogs.

Salivary amylase, mucins and other proteins

In humans, salivary amylase levels have been shown to change rapidly as a direct response to sympathetic nervous system stimulation. The literature suggests that salivary alpha-amylase is a surrogate marker of adrenergic activity under conditions of stress. Alpha amylase levels increase under a variety of physically (i.e., exercise, heat and cold)
and psychologically (i.e. written examinations) stressful conditions in human subjects (Gilman, Thornton et al., 1979; Chatterton, Vogelsong et al. 1996). Most importantly, salivary alpha-amylase concentrations are predictive of plasma catecholamine levels, particularly norepinephrine (NE), and are highly correlated with NE changes in response to stress (Chatterton, Vogelsong et al. 1996). Stress-related increases in salivary alpha-amylase can be inhibited by the adrenergic blocker propranolol, and beta-adrenergic agonists are capable of stimulating alpha-amylase release without increasing salivary flow (Speirs, Herring et al. 1974; Gallacher and Petersen 1983). The link between salivary alpha-amylase and plasma catecholamines suggests that the same stimuli that increase concentrations of catecholamines in the blood may activate sympathetic input to the salivary glands. In summary, studies show that blood levels of NE, associated with the stress response of the locus ceruleus/autonomic (sympathetic) nervous system, can be estimated by the concentrations of alpha-amylase in whole saliva specimens, and that salivary alpha-amylase measurements may be employed as a non-invasive measure of plasma NE concentrations in human subjects.

Whether amylase exists in dog saliva is unclear from the literature. Animal physiology texts commonly state that carnivores do not have salivary amylase (Reece, 1994), while experimental studies have found modest amylase activity in the saliva of the domestic dog (Scannapieco, Solomon et al. 1994). It is proposed that amylase in dogs does not come from the parotid gland but may enter the mouth from circular fluid or is synthesized by minor glands (Scannapieco, Solomon et al. 1994). Whether measurable quantities of salivary amylase exist in canine saliva and show an autonomic response to stress has not been studied, to my knowledge.

In dogs, it has been shown that other salivary proteins increase in response to sympathetic stimulation. Fractionation of these proteins indicates that mucin secretion in particular is regulated by the sympathetic nervous system (Kinjo, Nishikawa et al. 1983). Further studies show that particular cell types in the salivary glands are under regulation of both the parasympathetic and sympathetic divisions of the autonomic nervous system (Uddin 1991). No published studies exist to my knowledge that investigated these effects in response to stress situations.
Limitations of SNS measurement

As described above, measurement of the sympathetic nervous system is often invasive and technically difficult. Catecholamine measurement requires specific equipment (refrigerated centrifuge), freezing, and collection techniques that may not be readily available in many laboratories. Any sampling technique that involves intensive handling and or pain in the subject will activate the sympathetic nervous response itself and potentially interfere with data collection. In addition, the rapid and short-lived response of the autonomic nervous system makes the timing of measurement imperative if one expects to see changes relative to what is being measured. If the sampling takes too long, one will not only potentially induce changes, but possibly miss any changes that are related to the stressor. Some of these limitations can be avoided by placing an indwelling venous catheter before sampling begins.

In humans, “white-coat” syndrome is a recognized cause of hypertension in patients whose blood pressure is normal when measured at home. While the relationship of this syndrome to health risk is controversial, it is known that it occurs and is a common cause of difficulty in measuring blood pressure in clinical settings. (Celis, Den Hond et al. 2005). A similar situation exists in dogs. It is currently difficult to measure blood pressure and heart rate in dogs without handling them. Because any type of handling can cause them to be either excited or frightened, the autonomic nervous system will likely be activated. The animal must therefore be habituated to any equipment, handling techniques, and human researchers before valid data can be collected.

Although epinephrine and norepinephrine can be measured in urine and saliva, they have not been shown to be correlated with plasma epinephrine and norepinephrine levels and are therefore not useful as a measurement of stress response (Beerda, Schilder et al. 1996; Beerda, Schilder et al. 1999a; Beerda, Schilder et al. 1999b).

Many of the limitations already discussed of the measurement of salivary biomarkers for the HPA axis, also potentially exist for the measurement of salivary amylase or proteins in dog saliva. It is likely that food particles or proximity to feeding would have a large effect on salivary amylase production. Handling of the samples will
also dictate whether one can measure proteins and mucins in the saliva. Freezing and centrifuging is specifically performed to break down salivary mucins prior to measurement of salivary hormones. In addition, it is unknown whether salivary amylase increases in dog’s saliva following sympathetic nervous system stimulation.

**Immune System**

**White blood cell numbers, salivary IgA, antibody response, cytokines**

While the focus of this thesis is primarily on the non-invasive measurement of the HPA axis and behavior in dogs, it is important to keep in mind that the immune system is tied closely to the autonomic nervous system and the HPA axis. The effects of stress on the immune system are many but are not completely understood. These effects may be related to outcome variables of disease and decreased lifespan in dogs under stress so although they were not specifically measured in these studies, it is important to understand how they might be measured in future investigations of disease in stressed animals.

Complete blood counts on whole blood give both a relative proportion and absolute number of red and white blood cells in the circulatory system. Specifically, lymphocyte subset counts (e.g. CD4+ and CD8+ T-cells, Natural Killer cells) are frequently used as measures of immune efficacy (Vedhara, Fox et al. 1999). Because specific changes occur in many lymphocyte subpopulations following acute stress, these counts can be useful, although they may not reflect functional changes.

Antibody assays are commonly performed using ELISAs. High levels of circulating antibodies indicate at least a functioning humoral arm of the immune system. Often specific antibodies to latent viruses such as herpes simplex virus-1 and Epstein Barr virus in humans are used because some level of antibodies to these viruses are often circulating in the host and change in response to stress. (Vedhara, Fox et al. 1999) Antibody response to specific vaccines has also been used to measure the humoral
immune competence of subjects (Vedhara, Cox et al. 1999; Glaser, Robles et al. 2003). In order to decrease variability due to previous exposure to a particular vaccine or virus, some researchers have examined the immune response to a completely novel antigen (e.g. keyhole limpet hemocyanin) in the context of a stressor (Smith, Vollmer-Conna et al. 2004).

Salivary IgA has been shown in humans to decrease during times of chronic stress and to transiently increase in response to acute stress (Hucklebridge, Clow et al. 1998). This release is highly correlated with cortisol release, and exhibits a similar diurnal cycle. Kikkawa, Uchida and colleagues (2003) showed that dogs exhibited a decreased salivary IgA following a noise stressor and exhibited a consistent diurnal pattern of secretion, unlike cortisol secretion.

Cytokine levels have also been measured using ELISAs (Vedhara, Fox et al. 1999). The absence of a particular cytokine may be the cause of observed clinical disease. High levels of a particular cytokine may indicate an active immune system. Changes in cytokine types in response to a particular antigen are probably most indicative of the occurrence of an immune response. Measurements of cytokines in dogs are most commonly performed in studies of diseases such as rheumatoid arthritis, atopic dermatitis, and inflammatory bowel disease (Cave 2003; Hegemann, Wondimu et al. 2005; Marsella, Olivry et al. 2006).

**Limitations of immune measures**

While white blood cell counts on whole blood are easily performed, circulating white blood cells account for only 5% of the total pool of lymphocytes and give little information as to what might be going on in the tissues themselves (Robinson, Mathews et al. 2002). In fact, low numbers of circulating white blood cells may be accompanied by high numbers in the tissues. Trafficking of white blood cells between the circulatory and tissue compartments is highly affected by both cortisol and epinephrine. Absolute and relative counts of lymphocytes and lymphocyte subsets also give no information on the actual efficacy of the immune system. Measures of functionality include cytotoxicity and
lymphocyte proliferation assays which have limitations of their own (Vedhara, Fox et al. 1999).

Antibody levels vary greatly depending on exposure to particular viruses and vaccines. Puppies obtain antibodies via passive transfer from their dam in utero and in colostrum. These can last for 3-16 weeks depending on the particular infectious agent and the levels of antibodies in the mother. Exposure to the specific disease agent itself or to a vaccine will result in varying levels depending on the antigenic load, the type of vaccine and the presence of other factors besides stress levels.

As with many other biomarkers, salivary IgA exhibits a large amount of individual variation in both baseline levels and response to stress. Salivary IgA appears to be under the influence of many hormones and neurotransmitters (Hucklebridge, Clow et al. 1998). Although a fairly non-invasive measure, collection of saliva may cause changes in the concentration related to sampling as opposed to the stressor of interest. There is also a consistent diurnal pattern of secretion in dogs, so that the best sampling time is in the morning. (Kikkawa, Uchida et al. 2003)

Although much research has been directed towards cytokine measurement in humans over the past 15-20 years, little is still known about the interactions of cytokines and stress related disorders. The biggest limitation to cytokine measurement is that by definition, cytokines exist in very small amounts in the circulation of normal individuals and are therefore difficult to measure. (Vedhara, Fox et al. 1999). The magnitude of the change of particular cytokines is most useful.

Behavior

Owner-reported scales

Collection of behavioral data occurs in a variety of ways. For humans, many self-report scales and questionnaires have been developed and validated. Spielberger’s State Trait Anxiety Index (STAI) (Spielberger, Gorsuch et al. 1970), the Profile of Mood States
(POMS) (McNair, Lorr et al. 1971), and the Pet Attitude Scale (Templer, Salter et al. 1981) are just a few examples of validated scales developed to measure anxiety, depression, and the relationship between humans and pets. Often multiple scales are included in human studies to determine the effect of stress on an individual.

Although dogs are unable to “self-report”, similar questionnaires have been developed to collect behavioral information from owners of companion dogs. The C-BARQ was developed by Serpell and colleagues at the University of Pennsylvania and validated as a scale to measure behavioral characteristics and problems in guide dogs (Serpell and Hsu 2001). This scale has since been used in several other studies to measure behavior and temperament traits in pets dogs and to characterize behavioral problems of dogs relinquished to animal shelters (Hsu and Serpell 2003; Segurson, Serpell et al. 2005). Other questionnaires such as the Storm Phobia Assessment (SPA), a Likert scale questionnaire, have been developed and utilized to determine the presence and intensity of specific behaviors (Crowell-Davis, Seibert et al. 2003). Scales must be validated before using, however. A number of standardized temperament tests have been developed as predictive tests of how puppies, dogs of breeding age, and unknown adult dogs in shelters may respond in different situations (e.g. American Temperament Test Society, Inc., ASPCA Meet Your Match Canine-ality Program). These tests, although commonly used as a basis for placement, breeding and euthanasia, have little scientific proof of validity.

**Observation**

Diaries or commentaries by owners or those observing animals can provide useful behavioral data. This data can then be analyzed in a more qualitative fashion and often include more of the owner’s perspective, as well as the background of the animal. Voith and Borchelt (Voith and Borchelt 1996) comment that owners are “usually accurate” when describing their fearful pet. Owners are aware of the individual pet’s personality, its
history, and the circumstances surrounding the fear response and are aware of both the situations that produce the fear and the specific behavior pattern their pet exhibits.

Observation and description or coding of an animal’s behavior is the classic tool of the animal behaviorist and ethologist (Martin and Bateson 1986) and can be utilized by dog behaviorists in a number of experimental and naturalistic settings. In this case, the observer records the animal’s behavior through note taking, behavior coding or computer entry. The frequency with which a behavior occurs, latency from a specific event to the onset of a behavioral sequence, and duration and intensity of a specific behavior can all be scored. In addition, the movement of animals within their environment can be tracked by radio-telemetry both in experimental and naturalistic settings. This quantitative data can then be analyzed statistically. Reliability of data must be established through either intra-rater reliability scores (if one individual is coding) or inter-rater reliability tests (in the case of more than one rater).

Videotaping of behavior can be a valuable tool if the animal is acclimated to the videocamera. Timers can be set to videotape at different times during the day or during different events. Veterinarians and applied behaviorists have found videotaping to be especially helpful in the diagnosis of problems such as separation anxiety when behaviors occur when the owner is not around, or in aggression or obsessive-compulsive disorders where specific signs can be subtle and not noticed or misinterpreted by the owners in their descriptions of the event.

New computer software (e.g. Observer by Noldus) has greatly enhanced the behavior measurement process and allows for coding of multiple behaviors, as well as integration of physiological and behavioral data. Etho Vision (Noldus) has been used to track movement speed and frequency as well as total distance traveled in studies of pain following ovariohysterectomy in dogs (Hansen 2003). Because pacing and/or hiding are common anxiety responses in dogs, there may be a role for this type of monitoring in more general stress response situations.
Limitations of behavior observation and coding

Self-report questionnaires can be time-consuming for participants to complete. Robinson, et al. (Robinson, Mathews et al. 2002) caution against overburdening participants with too many psychometric questionnaires to fill out and recommend eliminating highly correlated measures. Questionnaires must also be carefully worded to avoid confusion, bias, and to minimize the role of social desirability (Graham 2001).

Diaries or commentaries may be particularly biased. While owners may be good at determining a general concept such as “fear” in their dogs, they may also interpret their dog’s behavior. Humans have a propensity to anthropomorphize their animals’ behaviors and assign characteristics such as “spite”, “anger”, “boredom” and “sadness” to behaviors that may be anxiety or fear related. The owner also brings their own expectations of what is and is not “acceptable” behavior on board when answering questions about their own animal’s behavior. For example, in one study, owners of storm-phobic dogs interpreted that their dogs did not improve following treatment for storm phobia because their dogs still did not want to go outside by themselves to go to the bathroom in the rain, while their overall storm phobia assessment scores (which included information on amount of panting, pacing, destruction, etc.) actually improved greatly (Crowell-Davis, Seibert et al. 2003). In contrast, in the same study, the researchers found that the caregivers’ assessment of dogs’ behavior during real storms was more valuable compared to the dog’s videotaped response to an audio recording in a clinical setting.

While videotaping may be useful for obtaining data to be analyzed at a later time, it may be difficult to assess subtle behavioral changes on tape (e.g. trembling, drooling, low volume whining). Unless one uses multiple cameras, it may be difficult to capture the entire behavior on film. The animal may also be facing the wrong way to be able to adequately assess what it is doing.

Direct observation is particularly time consuming, labor intensive, and sometimes inefficient. If the desired observed behavior is rare, there may be long periods when nothing of interest is happening. The researcher must also be careful not to interpret behavior as it is occurring instead of objectively recording the events. Observers
sometimes focus on specific behaviors and fail to recognize that other behaviors are occurring. Most importantly, direct observation has the potential to alter the animal’s behavior, particularly if the observer is an unfamiliar person. This may be a subtle effect but should be minimized (Martin and Bateson 1986).

**Conclusion and Recommendations**

It is generally best to use multiple variables in measuring the stress response. As discussed, it is obvious that any one particular measure has limitations and carries the potential to miss important information. By using both behavioral and physiological measurements, a more complete picture of the animal’s response can be obtained. It is also important to understand the limitations of each measure. Timing, sample collection and a consideration of the impact of the measure itself on the situation must be accounted for so that valid and reliable measurements can be made.

**Individual Differences**

**Breed and genetics**

It is clear that some animals have a genetic predisposition to showing anxiety or fearful behavioral responses. Prey species, such as antelope, rodents, etc. are more likely to respond to a particular stressful situation with fearful behavior (Casey 2002). While dogs are genetically similar to wolves, a predatory species, they have been under intense selection pressure for centuries to live with humans.

It is thought that dogs were domesticated 12-16,000 years ago (Coppinger and Coppinger 2001). The relationship between dogs and their owners or handlers is key to all aspects of their development. Unlike most other domestic animals, dogs have been selected for specific behaviors as well as physical conformation and production capabilities. Because of this, dog behavior has been shaped by human intervention and is
likely to have genetic and breed specific components. This is quite evident in many of the
breeds that exist today. Even within “types” of dogs, there are specific behavioral
characteristics that are expected from individuals of that breed. For example, as herding
dogs, Australian Blue Heelers chase after and bite at the heels of their charges, while
border collies stalk and “give eye” to the sheep they want to move to a new location. Other “sheep dogs” such as those of the Great Pyrenees and Kuvasz breeds are socialized
within the flock and serve to guard and protect their charges from predators, but do little
in terms of “moving” the sheep around. Herding behaviors are complex and highly
heritable predatory behaviors that become evident in puppies as young as 10 weeks of
age (Coppinger and Coppinger 2001). It should be no surprise then, that traits such as
propensity to fear and anxiety may also have genetic components. Overall (1997) notes
that there will be variation around specific behaviors that have been bred for. For
example, in herding dogs, some dogs may be too fierce (ravage the sheep) and some may
be too shy (are afraid of the sheep) to accomplish the task effectively. As a breeder
selects for the desired behavior, the distribution of that trait shifts and the overall
population can end up with a higher percentage of dogs at either end of the behavioral
spectrum (i.e. they are either too shy or too fierce), depending on the selection pressure
applied.

**Sex differences**

A number of studies show an increased incidence of anxiety disorders in female
humans as compared to boys or men. Shekhar and colleagues (2001) postulate that
gender likely impacts multiple points in the development of anxiety-related disorders in
humans, including genetic, developmental, hormonal, neurochemical, and psychosocial
processes. However, surprisingly little research has considered gender in animal models
of stress and anxiety.

There is evidence to suggest that, for humans and other animals, both behavioral
and physiological stress responses vary for males vs. females (Taylor, Klein et al. 2000).
While the basic neuroendocrine core of the stress response doesn’t vary substantially
from male to female, the role of other steroid hormones in the body may mediate the stress response by interacting with the stress molecules released in the HPA axis. For example, more oxytocin is normally released in females than males. In addition, testosterone, which is found in much higher quantities in males than females, interferes with the release of oxytocin (Klein and Corwin 2002). The effects of oxytocin are also strongly modulated by estrogen. Since oxytocin appears to reduce the effects of the stress response, one might expect a lower HPA response in females.

Some studies have suggested that female dogs may show an exaggerated behavioral response to stress (Beerda, Schilder et al. 1999a). Other studies have failed to show any cortisol differences between male and female dogs (Stephen and Ledger 2006; Tuber, Hennessy, et al. 1996). Taylor and colleagues (2000) propose that female animals show a different behavioral response to stress based on an attachment-caregiving system. Because the characteristics of the exaggerated behavioral response Beerda and colleagues are describing is unclear, it may be that female dogs show a need for a ‘tend-and-befriend’ type of response that they are unable to express in the laboratory setting. It would seem for females that this pattern of seeking others would help to prevent the long-term effects of stress that could be seen, as mentioned earlier. Because of this, females may actually be more resilient to the detrimental effects of stress over long periods of time given the opportunity for social interaction.

Sex differences related to hormonal influences are likely altered in domestic dogs that are castrated or ovariectomized. Social environment also likely plays a role in the development of sex differences. Many dogs are kept in single homes or with just one other dog which may or may not be of the same sex. After the age of 8-10 weeks, many singly-housed dogs do not see other dogs of either the same or opposite sex.

**Health Influences**

Because of the integrated relationship among all aspects of the animal’s physiology, it is obvious that the overall health of the animal will also affect its stress response. Stress is a cumulative phenomena such that multiple stressors may combine
together to cause more of a response than if any of them had occurred by itself. In practice, it is often difficult to distinguish whether disease results in a stress response or is a result of stress itself (Landsberg, Hunthausen et al. 2003).

Endocrine disorders can directly affect the physiologic stress response. For example, two diseases of domestic dogs affect the HPA axis directly and interfere with the individual’s ability to respond to a stressful situation. Hyperadrenocorticism (Cushing’s disease) is one of the most common endocrine diseases in dogs. Eighty five percent of dogs with this disease have a functional tumor in the pituitary gland, which causes excessive production of ACTH which then leads to uncontrolled secretion of cortisol from the adrenal medulla. The other 15% of dogs with the disease have functional adrenal tumors. All affected individuals have constantly high levels of circulating cortisol which are unresponsive to intrinsic feedback control systems. The opposite problem is seen in dogs with hypoadrenocorticism (Addison’s disease). These dogs have atrophy of the adrenal glands, usually as a result of an autoimmune disorder or from excessive treatment for Cushing’s disease. They fail to produce both mineralocorticoids and glucocorticoids and are unable to mount a stress response when needed (Bruyette 2005).

Diabetes mellitus also impacts the animal’s ability to respond to stressors, because of interactions with the HPA axis and effects on energy metabolism. Exogenous administration of corticosteroids and other hormones also impact the animal’s stress response. High doses of corticosteroids, commonly used for inflammatory diseases, suppress the immune system, cause the decrease of endogenous cortisol production and impact energy metabolism. Long-term use can also cause iatrogenic Cushing’s disease (Bruyette 2005). Hypothyroidism, another common disease of domestic dogs, has also been associated with anxiety and irritation and has been linked, at least anecdotally to a number of behavior disorders (Landsberg, Hunthausen et al. 2003; Overall 2003; Feldman and Nelson 2004). While many of these reports have not been tested, there is some evidence that thyroid hormone may be linked to the serotonin-dopamine pathway in the central nervous system (Feldman and Nelson 2004).
Disease and pain serve as intrinsic stressors. Immune stimulation in disease processes causes the release of cytokines that have a number of both physiological and behavioral effects. Cytokines increase HPA activation, affect central neurotransmitter and neuroendocrine response and have been linked to depression, anxiety and other psychopathologies (Granger and Dreschel 2006). Pain is also a physiological stressor and activates both the HPA and autonomic nervous system. Many of the behavioral symptoms of pain are similar to those of anxiety- panting, whining, barking, and restlessness. Much research is being pursued in the veterinary and laboratory animal fields to identify both behavioral and physiological measures of pain (Firth and Haldane 1999; Holton, Reid et al. 2001; Morton, Reid et al. 2005).

Physical and Social Environment

Social relationships have been shown to affect stress response in a number of different species (Koolhaus, Meerlo et al. 1997). Domestic canines are highly social animals. They form strong social relationships with members of both their own and different species, including but not limited to humans. The quality of the relationship between conspecifics is an integral part of dogs’ social environments. The author is unaware of any studies that examine the effect of other dogs in the household on either the frequency of behavior problems (other than inter-dog aggression) or on the behavior of dogs with phobias or anxiety-disorders.

Social influences are particularly important during early development. The amount of caregiving that a mouse or rat pup receives, as well as the times during development that this occurs has lasting effects on that animal’s hippocampal development, behavior and HPA axis reactivity (Anisman, Zaharia et al. 1998; Liu, Diorio et al. 2000). Many children who have been abused develop profound psychopathologies and neuroendocrine disorders (Cicchetti 2003). Abuse and neglect of companion animals is a common problem. What effect this might have on HPA reactivity has not been specifically studied.
Not surprisingly, current research in canine welfare is particularly directed towards the physical and social worlds of dogs, including the role of human contact, conspecific interaction, environmental enrichment and housing (Hubrecht 1995; Clark, Rager et al. 1997; Beerda, Schilder et al. 1999a,b; Hennessy, Voith et al. 2002). Hennessy and colleagues (2002) demonstrated that petting dogs in a public animal shelter attenuated their cortisol reactivity to an acutely stressful situation. Odendaal and Meintjes (2003) found that dogs had significant responses in blood pressure, beta-endorphin, oxytocin, phenyl acetic acid and dopamine following a positive affiliative experience with a human.

Only a handful of studies, however, have attempted to link the prevalence of anxiety disorders and aspects of owner behavior or owner-dog interactions. Jagoe and Serpell (1996) showed that dogs who belong to first time owners exhibited higher rates of fear, including fear of loud noises, and higher prevalence of general overexcitement than dogs belonging to experienced owners. Serpell (1996) also found evidence to suggest a causal relationship between aspects of an animal’s behavior and its owner’s level of attachment. Dog owners who reported weaker attachments for their pets were less satisfied with most aspects of their dogs’ behavior compared with those who reported stronger attachments. Clark and Boyer (1993) showed that increased training and time spent with their owners improved both obedience and the owner’s perception of the quality of the dog-owner relationship.

The Effects of Stress and Anxiety on Health

One feature of the stress response is that a single acute stressor can lead to long-term neurochemical changes (Koolhaus, Meerlo et al. 1997). Little information currently exists as to how long after a stressor the effects are felt on the body. It is thought that different stressors activate different parts of the stress response system (Stroud, Salovey et al. 2002). Due to the temporal dynamics of the HPA system vs. the SNS, one would expect those stressors that specifically activate the SNS to be shorter-lasting and to have
less of an effect on the health and well-being of the individual. On the other hand, a stressor that activates the HPA axis, particularly if it occurs over a long period of time or without time for recovery in between, could have detrimental effects on the individual.

If anxious animals produce a stress response to many day-to-day stimuli, it follows that they may live in a state of chronic physiological stress. For example, a dog with thunderstorm phobia living in a storm-prone area, would mount a stress response every time a storm is experienced. A dog suffering from separation anxiety would react every time she was left alone. Over a lifetime, these animals would suffer chronic repeated and uncontrolled stressful episodes. Chronic stress associated with high levels of cortisol in the blood stream has been related to a number of human disorders including obesity, insulin resistance, cardiovascular disease, immune disturbances, altered endocrine responses and nervous system disorders (McEwen 2005).

One of the most well known and complex effects of chronic stress is immune dysregulation. As discussed previously, the immune, endocrine and central nervous systems are highly integrated (Glaser, Robles et al. 2003). Acute stress enhances immune function through trafficking of white blood cells and release of cytokines, while chronic stress tends to have more of an immunosuppressive effect (McEwen 1998; McEwen 2005). High cortisol levels cause a shift from a Th1 lymphocyte response to a Th2 lymphocyte response while low levels of corticosteroids shift towards a Th1 response. A Th2 shift results in susceptibility to intracellular pathogens and cancer cells, while a Th1 shift leads to autoimmune and inflammatory disorders (Pajer, Rabin et al. 2002). The immune response of acute stressors can be explained through adaptive mechanisms, but long-term, chronic or overpowering stress ceases to be adaptive and becomes dysregulated within the neuroendocrine-immune system (Apanius 1998).

It is well known that stress exacerbates a number of skin related disorders. While acute stress promotes wound healing by increased local inflammation and white blood cell mobilization, chronic stress suppresses delayed type hypersensitivity and interferes with wound healing. Because of the immune involvement previously discussed, a Th2 shift can lead to deterioration of some immune-mediated skin diseases such as pemphigus and atopic eczema (Tausk and Nousari 2001). Increased stress has also been correlated
with disruption of the epidermal barrier function, which may precipitate inflammatory skin disorders independently of the immune-mediated functions (Garg, Chren et al. 2001).

One function of stress related hormones is the mobilization of energy stores as an adaptive response to provide fuel for an animal in distress. A consequence of chronic stress, therefore, is high energy stores, increased appetite, and food seeking-behavior. Because the body may not burn off the energy that has been mobilized by the stress hormones, long term effects of stress can include obesity, insulin resistance and diabetes, and cardiovascular disease (McEwen 2005). Atherosclerosis and hypertension, two predisposing factors of cardiovascular disease are also recognized sequelae of prolonged stress (McEwen 1998).

Nervous system effects of chronic stress are particularly apparent in the brain. In rodents, humans and other primates, glucocorticoids have been shown to have adverse effects on the hippocampus, which is important to learning and memory. Short term stress may enhance the ability to store information, but it also leaves the brain susceptible to injury (Sapolsky 1996; Casey 2002). Long term stress results in hippocampal neuron loss resulting in memory loss, and over a long period of time, to cognitive defects (Casey 2002). Increased calcium currents in the brain may be related to the hippocampal dysfunction (McEwen 1998). Besides the actual loss or atrophy of neurons, a reorganization of nerve cells takes place (McEwen 2001). Learning and memory problems, as well as depression and anxiety have therefore all been linked to prolonged HPA axis activation.

All of the above factors could lead to decreased longevity in an individual under chronic stress. In fact, it has been shown that rats with neophobia characterized by a larger HPA response than their non-fearful littersmates die sooner than their cohorts (Cavigelli and McClintock 2003). Although the pathologies related to death are similar (e.g. tumors), the neophilic animals seem to be able to handle a larger disease load. Similar results have been seen in studies of human caregivers of disabled spouses experiencing mental or emotional strain. A study by Schulz and Beach (1999) showed
that these individuals had increased mortality compared to non-caregiver individuals, even when other factors affecting disease and mortality were taken into account.

While there are a number of reasons why chronic stress would lead to shortened lifespan on a systemic level, the cellular mechanisms for death in these individuals is less well understood. A particularly interesting discovery is that emotional or physical stress is associated with higher oxidative stress, a lower telomerase activity and shorter telomere length. This leads to earlier cell death and aging (Epel, Blackburn et al. 2004). This may explain the cellular mechanism by which some of the cardiovascular, endocrine and immunologic changes occur with chronic stress.
Hypotheses

Three specific hypotheses related to the model illustrated in Figure 1-1 were proposed and tested through the answering of specific research questions as described below.

**Hypothesis 1:** The physiological stress response can be measured non-invasively in domestic canines using salivary stress hormones. (See letter A in Figure 1-1)

Research questions related to this hypothesis include:

a) Can saliva for the testing of stress hormones be collected in non-laboratory settings?

b) Are factors related to ease of collection, including stress or excitement of the dog, related to the quantity of saliva collected?

c) Do collection methods (including collection material or the use of salivary stimulants) interfere with salivary hormone measurements in dogs?

This hypothesis is primarily addressed in Chapter Two. Several studies of methodology and collection techniques for salivary cortisol in the dog are presented.

**Hypothesis 2:** Fear in the presence of a known stimulus leads to HPA activation and the initiation of the stress response in canines. (See letter B in Figure 1-1) This response may be mediated by the social environment of the animal. (See letter C in Figure 1-1)

Research questions related to this hypothesis include:

a) What is the strength and duration of this response in dogs?
b) Is the strength of this response related to the interaction with the owner or other dogs?

c) Is the owner’s behavioral or physiological response related to the dog’s response?

The study reported in Chapter 3 addresses this hypothesis and related questions, as well as lending support for the first hypothesis. The physiological and behavioral effects of playing a thunderstorm recording on thunderstorm phobic dogs and their owners are described. In addition to examining the practical aspects of saliva collection in a home environment, the social environmental effects of owners’ responses and living with other dogs are also investigated.

**Hypothesis 3: Stress caused by living with anxiety or fearfulness has deleterious effects on health and life-span in canines. (See letter D on Figure 1-1)**

The study reported in Chapter 4 examines the long-term effects of anxiety on health outcomes as predicted in this hypothesis. A retrospective survey of owners of deceased dogs was completed to examine the interactions between behavior (specifically anxiety and fear-related behaviors) and long-term health and lifespan.
CHAPTER 2-
MEASUREMENT OF SALIVARY BIOMARKERS IN CANINE SALIVA

Introduction

Non-invasive testing of physiological measures, such as salivary cortisol, has been increasingly used in animal behavior and welfare studies (Bergeron, Scott et al. 2002; Millspaugh, Washburn et al. 2002; Coppola, Grandin et al. 2005; deJong, Prelle et al. 2000; Moons, Laughlin et al. 2005). In dogs, salivary cortisol values are highly correlated with plasma cortisol values (Vincent and Michell 1992). Salivary alpha-amylase has also found increasing use in reflection of sympathetic nervous system activity in humans (Chatterton, Vogelsong et al. 1996). However, the applicability of canine salivary alpha-amylase in stress measurement has not been investigated.

Although saliva has a number of advantages for sampling in biobehavioral studies, it also has a number of limitations that researchers in the field must be aware of. Most importantly, measurement of markers in saliva from dogs depends on acquiring an adequate sample. Various methods of saliva collection have been used in different species. The volume of sample collected is often the limiting step in the ability to assay salivary cortisol in dogs. Although good-natured dogs are generally compliant with sample taking, it is sometimes difficult to obtain a large enough sample to run the assay. Limited volumes of saliva obtained from collection media have been shown to interfere with salivary cortisol measurement (Granger, Harmon et al. in review). The percentage of saliva retrieved from different materials was shown to have an effect on salivary cortisol measurement. The determination of a collection method that allows for adequate sample volume while minimizing collection stress to the subject, or interference with testing, is imperative to the continuation of this line of research.
This chapter investigates a number of the questions and concerns in measuring salivary biomarkers in the dog. A series of small studies designed to specifically evaluate saliva collection methods and use for further research are described. In the first study, the usefulness of salivary alpha-amylase in the reflection of autonomic nervous system activity in dogs is investigated. Second, a study of the ease of collection of saliva in a clinical veterinary setting is described. Third, a series of three studies designed to evaluate alternative saliva collection techniques are described in order to make recommendations for their use in salivary cortisol determination.
**Study 1: Measurement of Alpha-amylase in Canine Saliva**

**Introduction**

Salivary alpha-amylase (SAA) has found increasing use as a reflection of autonomic nervous system activity in humans (Chatterton, Vogelsong et al. 1997; Chatterton, Vogelsong et al. 1996). Stress-related increases in human SAA can be inhibited by the adrenergic blocker propanolol, and beta-adrenergic agonists are capable of stimulating alpha-amylase release without increasing salivary flow (Speirs, Herring et al, 1974, Gallacher and Peterson 1983). The link between SAA and plasma catecholamines suggests that the same stimuli that increase concentrations of catecholamines in the blood may activate sympathetic input to the salivary glands in humans.

While it would be very useful to have a salivary marker of sympathetic activity in canine saliva, it is unclear that salivary alpha-amylase is present in the dog. Dogs generally swallow large boluses of food at a time and digestion in the canine species is thought to begin in the stomach, with very little pre-swallowing enzymatic activity occurring in the mouth (Case 2000). Some papers have reported the presence of SAA activity in dogs (Scannapieco, Solomon et al. 1994), although most veterinary and canine nutritional texts state that alpha-amylase is not found in canine saliva (Reece 1994). Due to the potential benefits of having an additional and noninvasive measure of sympathetic nervous activity in dogs, canine saliva was tested for measurable levels of alpha-amylase.

**Methods**

Saliva samples from six neutered dogs ranging in age from 1-13 years were collected by their owners by holding a 3” cotton dental rope in the dog’s mouth for approximately one minute. The saliva-saturated end of the rope was cut off and placed in
a 5 ml syringe and compressed to extract the saliva into a 2 ml cryogenic vial. The samples were then frozen and processed for salivary cortisol measurement as part of another study involving thunderstorm fear in dogs (Dreschel and Granger 2005). The samples were later thawed and tested for salivary alpha-amylase using a commercial enzymatic SAA assay (Salimetrics, State College, PA). This kinetic activity test measures the change in a chromagenic substrate which is proportional to the activity of alpha-amylase present in the sample. This is converted to a measure of alpha-amylase in U/ml. In accordance with the human measurement protocol, samples were tested at a 200X dilution. Samples were then tested at a 10X dilution and in undiluted saliva. All procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

Results

No measurable salivary alpha-amylase activity was detected in any of the samples, diluted or undiluted.

Conclusion

No absorbance change was seen in any of the samples, even at the highest (undiluted) concentration. While amylase is an important pancreatic enzyme and is likely produced by some extrapancreatic tissues in dogs (Mocharla, Mocharla et al. 1990), there is not a measurable amount of amylase in canine saliva. Salivary alpha-amylase is therefore unlikely to be a useful surrogate of sympathetic nervous system activity in dogs. Future research into non-invasive measurements of autonomic activity in dogs is warranted.
Study 2: Collection of Salivary Cortisol in a Clinical Veterinary Setting

Introduction

The collection of saliva in a clinical setting has potential diagnostic and research applications. However, the success with which this is achieved could depend on many variables, including the skill of the person collecting the sample as well as the personality and cooperation of the patient. Veterinarians and veterinary technicians are trained professionals whose education and day to day experience involve hands-on manipulation of animals’ mouths for physical examination and dental care, while pet owners may have limited training and experience in handling their animals’ mouths. Dogs presenting to veterinary clinics for wellness exams, disease diagnosis, and treatment may be excited, anxious or non-reactive. For saliva collection to be used as a research or diagnostic tool, it is important to understand the technical aspects and limitations of collection in a variety of settings.

The purpose of the current study was threefold. First, a quantity of canine saliva was desired for further in vitro testing of collection methods. Second, the ease with which skilled professionals were able to obtain saliva samples in a clinical setting was examined. Third, the effects of perceived subject “stress” or “excitement” on the ease of collection and volume of sample collected was investigated. The clinical veterinary setting was chosen because of the large number of patients presented, the already established handling and technical skills of the veterinarians and technicians, the range of expected temperaments of dogs presenting to the hospital, and the potential future application of this technique in this setting.

Methods

Three veterinarians and three veterinary technicians/assistants working at an active small animal hospital were recruited to collect saliva samples over a four month
period from healthy, adult dogs weighing over 20 pounds. Previous trials have shown that it is difficult to collect adequate saliva volume for testing in smaller dogs. Training of saliva sampling from dogs consisted of showing one veterinarian, one technician and one veterinary assistant how to collect samples. Those individuals then trained the others who participated. Written protocols and explanations about the study purpose were also posted on cabinets and bulletin boards near the sample collection materials (see Appendix A). The primary investigator (ND) was present approximately 16% of the time that the hospital was open for regular hours during that time frame but sample collection was not restricted to this time.

Boxes with cotton ropes, salivettes, and instructions were placed at two different sites within the hospital. Simple charts to record data at the time of sample collection were posted nearby (See Appendix A). Saliva samples were obtained from 23 dogs presenting to the hospital over the course of four months. After owners gave permission to obtain a sample from their dog, samples were collected by allowing the subject to chew on a 3” cotton dental rope. The saturated end of the rope was then cut off, placed in a salivette, and the samples were frozen in the clinic freezer until transport on ice to the Behavioral Endocrinology Laboratory where they were stored at -40°C. The individual collecting the sample recorded the time of collection, and rated each dog on a scale of 1-5 on the following perceived characteristics: ease of handling (1 - “very easy” to 5 - “very hard”), excitement of the patient (1- “very mellow”, 5- “very excited”), and stress of dog (1- “not stressed”, 5- “majorly stressed”). The samples were then thawed and spun in a centrifuge at 3000 rpm for 15 minutes. An approximate volume was estimated for each sample based on comparison to a graduated tube of the same size. Five samples with large volumes were selected for use in a subsequent study. All procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

Descriptive statistics were computed on the quantity of saliva obtained, ease of collection and characteristics of the subjects. Correlational analyses were performed to determine if there was a relationship between the volume of saliva obtained, the ease of collection and the relative excitement or stress of the dog. A 2 x 2 (collector x characteristic) ANOVA was performed to determine if there were differences in amount
collected or ease of collection if the sampler were a veterinarian or a veterinary assistant/technician.

Results

The volume of saliva collected ranged from 0-1500 µl, (M = 351.1 µl, SE = .48). The mean “ease” of collection recorded was 1.6 (SE = .52), the mean “stress” of the dogs was 2.2 (SE = .52), and the mean “excitement” of the subjects was 2.9 (SE = .54).

There were no correlations between perceived ease of sampling, stress of dog, excitement of dog and the amount of sample collected. There were also no significant differences in amounts collected or ease of collection if the collector were a veterinarian or veterinary technician/assistant.

Conclusion

Considering the length of time that samples were collected and that approximately 20 dogs were seen per day at this veterinary hospital, relatively few samples were collected. Subjects were primarily healthy dogs visiting for yearly checkups and vaccinations. Presenting the study to the entire hospital staff at one time, perhaps at a staff meeting, may have yielded higher numbers. This was a very busy hospital and collection of samples was not a high priority.

The mean volume of saliva collected was fairly high. All but one sample yielded enough to perform a salivary cortisol assay. Because of an inherent selection bias for good-natured, easily-handled dogs from which to obtain samples, it is no surprise that the mean “ease” of sample collection was relatively low (“easy”). It is interesting to note that there was no correlation between perceived “stress” and perceived “excitement” in dogs, and that both of these characteristics fell in the middle range. Many dogs presenting to veterinary clinics are perceived to be either “stressed” or “excited” and are sometimes both. None of these factors influenced the volume of saliva collected. Veterinarians and
veterinary assistants/technicians were equally adept at collecting adequate saliva samples from this population. This study indicates that with relatively little additional training, veterinarians, veterinary technicians and veterinary assistants are able to collect adequate quantities of saliva to measure salivary biomarkers in a clinical setting.
Study 3: *In Vitro* Determination of the Effect of Method Collection on Canine Salivary Cortisol Measurement

**Introduction**

Previous studies in dogs have used salivary stimulants, such as citric acid, a food preservative and flavoring used in many foods and drinks, to ensure adequate sample size. Techniques that have been described to induce salivation in dogs include sprinkling a “few pellets” (Beerda, Schilder et al. 1998) or a “number of crystals” (Bergeron, Scott et al. 2002) of citric acid on a dog’s tongue, and wiping the dog’s tongue and palette with a citric acid soaked cotton ball (Kobelt, Hemsworth et al. 2003). However, citric acid has been shown to artificially increase the levels of both cortisol and testosterone measured in human saliva, likely related to an increase in sample acidity (Schwartz, Granger et al. 1998; Granger, Shirtcliff et al. 2004). The effect of citric acid stimulation on a dog’s salivary biomarker levels has not been investigated.

Typical collection of saliva in dogs involves the subject chewing on or keeping a cotton dental rope in its mouth for one to two minutes. It was suggested by participants in previous studies (Dreschel and Granger 2005) that flavoring the cotton rope would aid in collection. However, food particles in the mouth, including breast milk in samples from human infants, lead to spurious results in the measurement of many hormones, including cortisol, in human samples (Magnano, Diamond et al. 1989). Animal or plant origin food particles may also contain products that cross-react with the antibodies used in salivary immunoassays (Shirtcliff, Granger et al. 2001). Therefore, it is unknown if a flavored collection rope would give valid results.

Collection materials may also interfere with hormone testing in saliva. Cotton-based sample collection methods interfere with a variety of salivary biomarkers in humans, including testosterone, DHEA, progesterone, estradiol and sIgA (Shirtcliff, Granger et al. 2001). These alterations in measured levels introduce unsystematic error so
that the correlations between serum and salivary levels are no longer strong, challenging the validity of the test. Salivary cortisol levels seem to be unaffected by cotton-based collection, so that the results of cotton-based samples can be interpreted with confidence. However, it can be difficult to obtain a good percentage yield of saliva from a cotton rope. Because cotton is such an efficient absorptive material, the lower volume of sample results in an even lower percentage yield. Surgical micro-sponges made from hydrocellulose are effective at absorbing saliva and give a higher percentage yield when spun down than cotton (Granger, Harmon et al. in review). Hydrocellulose has not been previously tested as a collection material for canine saliva.

The purpose of this study was to determine, in vitro, if citric acid stimulation interferes with cortisol measurement in canine saliva, and, if so, to determine at what concentration of citric acid this effect is seen. In addition, hydrocellulose and beef-flavored cotton ropes as collection materials were tested for interference with cortisol measurement. To determine this, saliva samples were subjected to different methods of collection and the amount of agreement between the cortisol measurements in corresponding samples was calculated.

Several statistical methods can be used to measure agreement between two tests. Correlation between data points is commonly used, as is determining if a statistical difference occurs between the different measures. With a small sample size, the likelihood of a single outlier causing a significant difference is increased, so non-parametric tests, such as the Wilcoxon signed-rank test are used. However, in tests of method comparison, showing that there is no significant difference does not necessarily show that the measures are equivalent (Christley and Reid, 2003). Although correlation between measures can be computed, as Christley and Reid (2003) point out, it would be likely that any two methods measuring the same thing would show a significant relationship. Correlation only shows that they are changing in the same direction, not that they are providing equivalent data. It is important, therefore to determine that the measures are both accurate and precise.
The concordance correlation coefficient was developed by Lin (1992) to evaluate agreement for two responses on a continuous scale. The degree of concordance between two variables, X and Y is defined as

\[ \rho_c = \frac{2 \sigma_{xy}}{\sigma_{xx} + \sigma_{yy} + (\mu_x - \mu_y)^2} \]

where \( \sigma_{xx} = \text{variance (X)} \), \( \sigma_{yy} = \text{variance (Y)} \) and \( \sigma_{xy} = \text{covariance (X, Y)} \) (King and Chinchilli, 2001). The concordance correlation coefficient will have a value of 0 to 1.0 with 1.0 indicating perfect concordance between the two variables.

**Methods**

Samples from six individual dogs were collected from patients at a private veterinary hospital as part of the previously described study. A 3” cotton dental rope was held in the dog’s mouth for approximately 1-2 minutes, placed in a salivette device (Sarstedt, NC), and immediately frozen after collection. After all the samples were collected, they were thawed, centrifuged at 3500 rpm for 5 minutes, aliquoted into six microcentrifuge tubes and refrozen at -40°C before testing.

All samples were assayed for salivary cortisol using a highly-sensitive enzyme immunoassay kit (Salimetrics, State College, PA). The test uses 25 µl of saliva (for singlet determinations), has a range of sensitivity from .007 to 1.8 μg/dl, and average intra- and inter-assay coefficients of variation of less than 10% and 15% respectively. Method accuracy, determined by spike recovery, and linearity, determined by serial dilution are 105% and 95%. All samples were performed in duplicate and those with coefficients of variation greater than 15 were repeated.

Control samples containing 80 µl of saliva from each subject were tested for cortisol and for pH using visual pH strips (colorpHast Indicator strips pH 0-14, EM Science, Gibbstown, NJ).
To determine the effect of citric acid on cortisol measurement, .01 g (10 mg) of food grade citric acid crystals were added to 100 µl of saliva from each subject to create a .1g/ml solution. Serial dilution then yielded two additional concentrations of .01 g/ml and .001 g/ml citric acid/saliva. The pH of each concentration was measured using visual pH strips. Cortisol levels were measured at each concentration of citric acid.

To examine the effect of collection material on cortisol concentration, 120 µl of saliva from each subject was absorbed with a BD hydrocellulose eye sponge. The handle of the sponge was cut short and the sponge was placed handle down in a microcentrifuge tube and re-spun. Saliva was harvested and tested for cortisol concentration.

Beef-flavored dental rope was prepared by soaking a cotton dental rope in a solution prepared by mixing one food grade beef-bouillon cube with 1 cup of boiling water as per instructions on the bouillon label. The cotton rope was then dried by baking in an oven at 140°F for 2 hours. The flavored dental rope was cut into 7½ cm lengths and the ends were discarded. Two hundred µl of saliva from each subject was absorbed into the dental rope and re-extracted by pressing out of a 5 ml BD syringe. Cortisol was measured in each sample.

Examination of the cortisol data revealed that the distributions were positively skewed. Natural log transformation was performed to establish normal distributions prior to analysis. As appropriate, analyses were conducted using the transformed values, but for ease of interpretation, the values in the text and figures are raw scores. Paired sample t-tests were performed to compare the control procedure with the experimental procedure. For ease of comparison with the follow-up studies, non-parametric Wilcoxon signed-rank tests were performed on the skewed data. The SPSS statistical software package (SPSS, Inc., Chicago, IL) was used for all analyses. A concordance correlation coefficient to determine the reproducibility of scores using each method was calculated for all pairs (Lin 1989).
Results

The mean salivary cortisol for the control samples was .17 µg/dl (SE = .06). The mean cortisol with the beef flavored rope was .20 µg/dl (SE = .06) and the mean cortisol from the samples passed through the hydrocellulose was .15 µg/dl (SE = .05). There was a significant difference between the control and the beef-flavored rope values (p<.01). There was no significant difference between the mean values of the control and the hydrocellulose (See Figure 2-1). The concordance coefficient for the beef flavored rope collection method was .92 and the concordance coefficient for the hydrocellulose method was 1.0.

![Effect of collection method on cortisol measurement](image_url)

Figure 2-1: Absorption and retrieval of saliva using beef-flavored cotton rope results in increased cortisol measurement compared with cotton or hydrocellulose. (Error bars represent SEM)
The addition of citric acid decreased salivary pH from a mean of 8.4 at control to 1.8 at .1 g/ml, 3.2 at .01 g/ml and 5.5 at .001 g/ml (Figure 2-2).

Figure 2-2: The addition of citric acid to canine saliva causes a decrease in salivary pH at concentrations of .001g/ml in vitro. (Error bars represent SEM)

Cortisol at the .1 g/ml level could not be measured, as it was off the scale (>1.8 µg/dl). The mean cortisol at the .01 g/ml dilution was .85 µg/dl (SE = .37) and at the .001 g/ml citric acid dilution the mean was .18 µg/dl (SE = .05). The difference between cortisol measurement at .01 g/ml and at control was significant (p<.01), but there was not a significant difference between the .001g/ml level and the control (Figure 2-3). The concordance coefficient for the .01 g/ml citric acid concentration was .16 and the concordance coefficient for the .001 g/ml citric acid concentration was 1.0.
Conclusion

This study clearly shows the potential limitations of using citric acid as a stimulant for saliva collection for cortisol measurement. As little as .01g/ml of citric acid caused a significant drop in pH and a corresponding significant increase in measured salivary cortisol concentration. Thirty individual crystals of citric acid weigh approximately .01 g. Because the quantity of saliva typically collected from a dog’s mouth is in the .1-.5 ml range, there is easily the possibility of achieving a .01 g/ml concentration with a “pinch” of citric acid placed in a dog’s mouth. Lower concentrations (.001 g/ml, approximately 1-4 crystals of citric acid per ml) did not significantly lower the pH concentration and did not have an effect on salivary cortisol measurement in vitro.

This study did show a high concordance between measurements taken before and after passing the saliva through a beef-flavored cotton rope. However, there was a

Figure 2-3: The addition of citric acid to canine saliva at concentrations of .01g/ml or greater results in elevated cortisol measurement in vitro. (Error bars represent SEM)
significant difference between the measurements with a higher cortisol concentration measured in the beef-flavoring condition than in the control condition.

Hydrocellulose appeared in vitro to be a useful collection medium. There was not a significant difference between the salivary cortisol concentration measured before and after the saliva was passed through a hydrocellulose sponge. The concordance coefficient was very high for this measure, also.
Study 4: In Vivo Determination of the Effect of Collection Methods on Salivary Cortisol Measurement

Introduction

Based on the findings of the previous study, an in vivo challenge was designed to determine if salivary stimulation with citric acid or sampling with beef-flavored cotton ropes or hydrocellulose swabs would have an effect on cortisol measurement in the live animal setting. This study sought to determine if there are mechanisms within the live animal system to counteract the negative consequences of using citric acid stimulation. The best-described and most controlled citric acid stimulation technique in the literature involves swabbing the inside of a dog’s mouth and tongue with a citric acid-soaked cottonball and then collecting saliva with a cotton rope (Kobelt, Hemsworth et al. 2003). It is known that dog saliva has some buffering capabilities to protect the individual against ingestion of more acidic substances (Reece 1994). Because of the potential limitations of the various techniques highlighted in the previous study, it was believed that further study in the live animal was warranted. In addition, empiric descriptions of the dogs’ acceptance of different methods of saliva collection were obtained.

Methods

Saliva samples were collected from 24 dogs (18 females, 6 males representing 6 sporting-dog breeds) by the researcher at a commercial breeding and training kennel. Eight subjects were enrolled in each experimental condition (citric acid, hydrocellulose, beef-flavoring). Control salivary samples were collected from all dogs using a cotton dental rope as previously described. For each dog the control sample was collected first, followed by the experimental sample, to minimize the elapsed time between samples and to avoid contamination between the experimental procedures and the control. All samples
were immediately put on ice and frozen. One subject in the beef-flavoring condition did not have an adequate volume of sample to measure control cortisol.

To determine the effect of citric acid on cortisol measurement in the field, the inside of the dog’s mouth and gums was swabbed using a citric acid coated cotton ball as described by Kobelt and colleagues (2003). The cotton balls were prepared by adding 1.25 ml of a 5% citric acid solution to ½ a cotton wool ball and drying in an oven at 60°C for 3 hours. Immediately following the swabbing of the dog’s mouth, saliva was collected using a plain cotton rope, placed in a salivette, and frozen.

To examine the effect of collection material on cortisol measurement, saliva was collected using a hydrocellulose eye sponge (BD). The sponge was held by the handle and remained in the dog’s mouth for approximately 30-60 seconds. The handle of the sponge was then cut so that it could be placed handle side down in a microcentrifuge tube and frozen. Beef flavored dental ropes prepared as described above were held in the subject’s mouth for 1-2 minutes. They were then placed in salivettes and frozen. All procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

Salivary cortisol was measured in all samples and the pH was measured in the control and citric acid samples as described previously in “in vitro determination of the effect of collection methods on salivary cortisol measurement”. Concordance correlation coefficients were calculated for each pair of measurements. Because the data was skewed and could not be normalized through transformation, differences between the control and experimental samples were examined with the nonparametric Wilcoxon signed rank test.

**Results**

The mean salivary cortisol values in the control and experimental samples are shown in Figure 2-4. There are no significant differences between the controls and any of the experimental conditions in the Wilcoxon signed rank test. The concordance coefficient for hydrocellulose and control in the kennel dogs is .53. The concordance coefficient for the measurements following swabbing with citric acid and the control is
.63. The concordance coefficient for the measurement from the beef flavored rope and the control is .3. Graphs of the mean salivary cortisol in the experimental and control samples are seen in Figure 2-4. There was not a significant difference between the control pH (M = 8.5, SD = .53) and the experimental pH (M = 8.7, SD = .92) for those dogs whose mouths were swabbed with the citric acid cotton ball.

Empirically, the dogs resisted having their mouths swabbed with the citric acid flavored cotton ball before saliva collection, but this did not interfere with the researcher’s ability to collect saliva. The dogs readily chewed on the beef-flavored cotton ropes and also chewed on the unflavored cotton ropes, although without as much enthusiasm. The hydrocellulose swabs were particularly easy to use, as the handles fit within the interdental space in the dogs’ mouths and they were able to close their mouths without chewing on the swab or stick.
Conclusion

The *in vivo* experiment sheds light on the use of citric acid for saliva stimulation in dogs. Using the technique of wiping the inside of the dog’s mouth with a citric acid soaked cotton ball did not cause a decrease in pH of the dog’s saliva. In fact, in several cases, the pH increased following the procedure, indicating a buffering response of the dogs’ saliva. However, despite the absence of a significant pH change, there was a very low concordance in measurement between the control condition and the experimental condition. Dogs resisted having their mouths wiped with the cotton swab but it did increase saliva flow and allow for a second sample to be collected. There was no difference in the volumes collected before and after swabbing the dogs’ mouths.

Non-parametric testing did not show significant differences in cortisol concentration measured in unflavored cotton rope vs. beef-flavored rope or hydrocellulose, but there was little correlation between the measurements taken with the various methods. There was also a very low concordance coefficient and a large standard error in the measurements between the control and the beef-flavored rope conditions as well as between the hydrocellulose and cotton rope conditions. Qualitatively, some of the dogs did seem to enjoy chewing on the beef-flavored rope more than the plain cotton rope and the hydrocellulose sorbettes were quite easy to manipulate in the dogs’ mouths.
Study 5: Consistency in Cortisol Measurement Across Time Using Various Methods of Saliva Collection

Introduction

Because of the lack of concordance between different measures which was not reflected by significant differences between the measures, a third study was performed. It was postulated that because the samples were taken within minutes of each other in the previous study, the collection of the first sample may have interfered with the collection of the subsequent samples. The final study looked specifically at the reliability of test measures at two collection times spaced at a 20 minute interval.

Methods

Saliva was collected from six privately owned dogs by their owners in their own homes. The dogs, 5 neutered males and 1 spayed female of primarily mixed-breed origins, ranged in age from 1.8-9 years (M= 4.4 years). Samples were collected using three different methods at six distinct time points. Each method was repeated two times, with a period of 20 minutes occurring between collection times. The hydrocellulose sorbette, beef flavored rope, and citric acid stimulation methods were used as described previously under “in vivo determination of the effect of collection methods on salivary cortisol measurement”. Owners were instructed to choose a time of day when their animal was at rest and not stimulated by other activity and to not allow their animals to eat or chew on plant or animal based products in the 20 minute interim between sample collections. At least 1 hour elapsed between different collections using different methods. All procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.
Samples were frozen and stored at -40\(^\circ\) C until testing. Samples were assayed for salivary cortisol using an ELISA test (Salimetrics, State College, PA) as previously described. Data from samples previously collected using cotton rope at 20 minute intervals on a control day for a previous study were also used (Dreschel and Granger, 2005). This data was analyzed and compared to the other collection techniques.

Concordance coefficients were calculated for each pair of measurements. The Wilcoxon signed rank non-parametric test was used to detect differences in cortisol concentration between the time points within each experimental condition.

**Results**

There were no significant differences using the Wilcoxon signed rank test between cortisol concentration at the 20 minute time points for any of the samples. The concordance coefficient over time for hydrocellulose was .88, for citric acid it was .49 and for the cotton rope alone, it was .36. The concordance coefficient for the beef flavored rope was 0. The means and standard errors at the different time points are shown in figure 2-5.

![Mean salivary cortisol](image)

Figure 2-5: There are no significant differences in cortisol concentration in 2 samples collected 20 minutes apart for any of the collection methods tested. (Error bars represent SEM)
Conclusion

From previous research (Dreschel and Granger 2005), it was known that there was no significant difference in samples taken with cotton rope at 20 minute intervals on the non-experimental day in a pet’s own home. In this study, we also found no significant differences in measurements taken at 20 minute intervals using any of the collection techniques. We did, however, find a very low concordance for beef-flavored rope using this technique. Although it appears from Figure 2-5 that the hydrocellulose technique had wide variance, the concordance coefficient for this technique was quite high. The variance evident in the graph is likely due to a single outlier which had little effect on the concordance between the other samples.
Conclusion and Recommendations for Canine Salivary Collection for Cortisol Determination

Previous research has shown that there are potential limitations in the use of various collection methods and media. In order to produce meaningful and comparable results, researchers must be aware of and control for these limitations. The evidence from the studies described in this chapter support specific recommendations for the collection of saliva for cortisol determination. It is clear that there is a great deal of inter and intra-individual variation in salivary cortisol levels. For a comparison of the concordance coefficients for the various methods of collection, see Table 2-1.

Table 2-1: Concordance coefficients for saliva collection methods. Hydrocellulose has the most consistent concordance between samples in all studies. Salivary stimulation with citric acid at low levels also has high concordance between samples. Beef-flavored rope yields very unreliable cortisol measurements.

<table>
<thead>
<tr>
<th>Collection method</th>
<th>( \rho_c ) (in vitro)</th>
<th>( \rho_c ) (in vivo)</th>
<th>( \rho_c ) over time (20 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric Acid (.01 µg/dl)</td>
<td>.16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citric Acid (.001µg/dl)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citric Acid (cotton ball swab)</td>
<td>-</td>
<td>.63</td>
<td>.49</td>
</tr>
<tr>
<td>Beef flavored rope</td>
<td>.92</td>
<td>.3</td>
<td>0</td>
</tr>
<tr>
<td>Hydrocellulose sorbette</td>
<td>1.0</td>
<td>.53</td>
<td>.88</td>
</tr>
<tr>
<td>Cotton alone</td>
<td></td>
<td></td>
<td>.36</td>
</tr>
</tbody>
</table>
Beef-flavored ropes, though well accepted by canine subjects, introduce unpredictable variability to cortisol concentration measurement. This is consistent with previous research findings that food particles can interfere with cortisol measurement. Ropes flavored by this method are not reliable for saliva collection for cortisol determination. It is also important to note that many dogs chew on bones or other animal based products on a regular basis throughout the day or are fed free choice and allowed to eat as they want. Because animals do not frequently have their teeth cleaned, there is a great potential for food products to remain in the mouth for longer periods of time. Researchers can avoid some of these potential problems by not allowing dogs to have treats, chew on rawhide or other animal-based products or eat in the time preceding sample collection.

Citric acid in small amounts does not appear to affect salivary cortisol measurement to a significant degree in the dog. Although the in vitro study illustrates the potential effects of high levels of citric acid, the dog’s mouth in vivo appears to have a buffering capability that counteracts the acidity of this salivary stimulant. Anecdotally, dogs resisted having their mouths swabbed with the citric acid cottonball and significantly more saliva was not obtained with the citric acid than with cotton rope alone. However, in cases where larger volumes would be useful, citric acid stimulation is unlikely to affect cortisol concentration variability.

Hydrocellulose as a collection media appears to be very effective for canine saliva collection. The concordance coefficient in all the studies was quite high, exceeding that of cotton rope alone in the third study. One concern with this technique is obtaining adequate sample volume with which to run the assays. It is recommended that researchers use two or more swabs at the same time and reinforce the importance to those collecting the sample to leave the swabs in the dog’s mouth for an adequate length of time.

In addition, there is evidence that collecting two consecutive samples immediately following each other may affect the measured cortisol levels between the samples. This is most likely due to a decreased yield from the collection material on the second sample. There does not seem to be a large variation between samples taken at 20 minute intervals, if no other stressor is present during that time.
I hypothesized that the physiological stress response can be measured non-invasively in domestic canines using salivary stress hormones. Although salivary alpha-amylase is not useful as a stress marker in dogs, salivary cortisol can be reliably measured if consistent saliva collection techniques are used. Salivary collection for cortisol concentration measurement is a viable and relatively easily performed procedure. Both professionally trained individuals (veterinarians and veterinary technicians) and dog owners were able to perform this procedure both at home and in a clinical setting. It does not require a great deal of training and there are several methods available that should yield adequate quantities of saliva for testing.
CHAPTER 3- PHYSIOLOGICAL AND BEHAVIORAL REACTIVITY TO STRESS 
IN THUNDERSTORM-PHOBIC DOGS AND THEIR CAREGIVERS

Introduction

It is hypothesized that exposure to a fear inducing stimulus in a dog’s own home will result in a stress response measurable by both behavioral and physiological means. It is also hypothesized that the way in which an owner responds and/or the relationship quality between owner and dog will affect this response. Specifically, in this study, we examine the interactions between hypothalamic-pituitary-adrenal (HPA) axis activation, relationship quality, and behavioral response in thunderstorm-phobic dogs and their owners.

Thunderstorm phobia is an excessive fear response to storms that is disproportionate to the danger presented and commonly increases with increasing intensity of the storm. It is often associated with other anxiety disorders, particularly separation anxiety (Overall, Dunham et al. 2001). Caregivers of thunderstorm-phobic dogs face a variety of specialized challenges, including loss of sleep, destruction of household items and furnishings, and worry about their dog’s physical and mental health. While the health benefits of companion animals have been studied now for decades, very little research has explored the potentially negative health consequences of caring for an animal with severe behavior problems. We anticipate that caregivers with high quality relationships with their dogs will have more pronounced stress responses when their dogs encounter a thunderstorm than caregivers with poor or lower quality relationships.

Domestic dogs are highly social animals and the quality of the relationship between conspecifics is an integral part of dogs’ social environments. Not surprisingly, current research in canine welfare is particularly directed towards the physical and social worlds including the role of human contact, inter-dog interaction, environmental


A number of studies have used physiological measures such as plasma cortisol and heart rate monitoring to index the effects of stressful situations on domestic canines (Clark, Rager et al. 1997; Hennessy, Williams et al. 1998; Hennessy, Voith et al. 2001). Non-invasive testing of physiological measures, such as salivary cortisol, have recently been used in wild and domestic animals as a measure of stress (Beerda, Schilder et al. 1996; Beerda, Schilder et al. 1997; Beerda, Schilder et al. 1998; Bergeron, Scott et al. 2002; Millsbaugh, Washburn et al. 2002). In dogs, salivary cortisol values are highly correlated with plasma cortisol values (Vincent and Michell 1992). Besides the reduction in sampling-imposed stress hormone levels, salivary cortisol collection is easily performed in non-laboratory settings.

We hypothesized that phobic dogs’ cortisol levels would increase in response to a simulated thunderstorm. More importantly, we expected that individual differences in this response should be related to (a) the severity of the dog’s behavioral response, (b) the dog’s intrinsic behavioral profile such that dogs rating higher in excitability and nonsocial fear would show a greater cortisol response and dogs rating higher in attachment/attention-seeking would be more likely to elicit comfort from their owners, (c) the owner’s baseline levels of anxiety, and (d) features of the social environment, including the quality of the dog-owner relationship, the supportiveness of their owners and the presence of other dogs in the household. With respect to the latter, we expected that dogs with owners who were stressed by the thunderstorm experience and were least responsive to their pets would have more pronounced salivary cortisol responses to the simulated storm. We also anticipated that the owner’s cortisol levels and negative affect would increase in response to experiencing their companion confront a simulated thunderstorm. Individual differences in the owner’s response should be related to (a) the severity of the dog’s behavioral reaction, (b) the quality of their relationship and attachment to the dog, and (c) the degree to which the dog elicited their comforting behavior and attention. To explore these hypotheses we drew on a sample of dog-owner
pairs, selected because the dog was diagnosed with thunderstorm phobia. Saliva samples were collected from dogs and owners before and after the pair experienced a simulated thunderstorm, and behavior observations were made.

**Materials and Methods**

**Participants**

Owners of adult dogs exhibiting fear of thunderstorms were recruited by word of mouth, local TV announcements, and flyers posted at veterinary hospitals, pet stores and community bulletin boards in a small northeastern city in the United States. Forty-seven owners were screened by phone to exclude animals that were highly aggressive toward owners, less than 15 lbs, under medical care for a chronic endocrine or immune-related health problem, or currently taking prescription medication. To be eligible, owners had to report their dog consistently showed behavior changes (e.g., trembling, hiding, pacing, destructiveness, vocalizing) during thunderstorms. All participating owners reported that their dogs exhibited these signs 100% of the time when there was a storm. Dog-owner dyads were also excluded if owners had a chronic disease, or were on anti-inflammatory, anti-depressant, or hormonal replacement medication.

Nineteen owner-dog dyads met these strict criteria and participated in data collection. The human participants’ (16 females, 3 males) ages ranged from 21-78 years (x = 44.5, SD = 12.2). The dogs (10 females, 9 males) ranged in age from 1-13 years (x = 6.8, SD = 3.1) and were all gonadectomized. A variety of breeds were represented, nine pure-bred, including 5 pure-bred golden retrievers, and 10 mixed-breed dogs. Five of the subjects were pure or mixed-breed herding-type dogs. Ten dogs lived with at least one other dog in the household (range 1-7 other dogs). All protocols were approved by the Pennsylvania State University Institutional Animal Care and Use Committee and the Institutional Review Board. Owners were compensated for their participation with a copy of the thunderstorm recording and behavioral consultation on thunderstorm phobia.
including desensitization therapy. If warranted, they were referred to their regular veterinarian for pharmacological treatment.

**Experimental design**

The cortisol component of the study employed a 2 (stress condition) by 3 (sample collection time) by 2 (subject- owner vs. dog) factorial design. Stress condition (simulated storm vs. control no-storm day) was a within subject variable with order counterbalanced between subjects. To simulate a storm, a commercially available compact disc recording of a thunderstorm was played on a large portable stereo system (see below). Saliva samples were collected from each member of the dyad before, 20 and 40 minutes (“sample time”) after the simulated thunderstorm as well as at matching times on a control (“non-storm”) day.

The observed behavioral component of the study compared coded videotape observations of the owner and dog responses during the audio playing of the “storm” on the experimental day. The intrinsic behavioral dispositions of the dogs and relationship quality between dog and owner were determined by questionnaires filled out by the owners prior to the experiment.

Interactions between the cortisol, behavioral and relationship quality components of the study were then examined.

**Procedure**

To minimize the influence of unfamiliar settings and strangers on the dyads’ behavior, all testing took place in the dogs’ own homes and was performed by their primary caregiver, sometimes with the help of other family members. The principal investigator (NAD) visited the home at least 4 hours (usually > 24 hours) prior to testing to demonstrate and explain the testing procedure and to set up the videotaping and sound equipment.
To simulate a storm, a compact disc recording of a thunderstorm (Suburban Thunder, F7 sound and vision, Tampa, FL) was played on a large portable stereo system. The same system was used for all exposures. The 5 minute stimulus, played as a load volume, included the beginning of a rainstorm that quickly built to a full thunderstorm with loud thunderclaps starting approximately 30 seconds into the recording and continued with high intensity wind, rain and approximately 19 different instances of thunder for the remainder of the five minutes. Playing a high quality recording of a thunderstorm has been shown to elicit a fear response in many dogs that are thunderstorm anxious (Crowell-Davis, Seibert et al. 2003). Recordings of this type are often used for desensitization therapy to thunderstorm noise. During the playing of the recording, the owners were instructed to treat their animal as they normally would during a storm.

The dog and the owner’s response to the thunderstorm recording were video-recorded with a Handycam Video 8 mm camera (Model CCD-TR83, Sony Electronics, Park Ridge, NJ) mounted on a tripod. The camera was placed in a corner of the room so that most of the room was visible. The owner was instructed to aim the camera towards the dog at the beginning of testing but not to follow the dog with the camera or force the dog to stand in front of the camera after testing began.

Saliva samples were collected for cortisol measurement from both the owner and dog at baseline (before any testing occurred), and 20 and 40 minutes after the thunderstorm recording ended. This timing was to take into account the lag that may occur in the expected increase in salivary cortisol following a stressor in both canines and humans, and the expected return to baseline within 40 minutes (Vincent and Michell 1992; Dickerson and Kemeny 2004). All testing occurred between 14:00 and 18:00 to correspond with a circadian plateau in human cortisol levels (Dickerson and Kemeny 2004). Saliva samples were also collected from the same participants on a control day at the same times as on the “storm” day. No recording was played on the control day and owners were told to interact as they normally would with their pets on any other day except for the collection of saliva samples.
Biological assessments

Owners were instructed not to feed their dogs or allow them to chew on rawhide or other animal based products immediately before saliva collection. Owners collected saliva samples from the dogs by holding a 3” cotton dental rope in the dog’s mouth for approximately 1 minute. They were encouraged to give their dog a treat immediately following collection, with the prospect of the offered treat often serving to stimulate saliva flow during collection. The saliva-saturated end of the rope was cut off and placed in a 5 ml syringe and compressed to extract the saliva into a 2 ml cryogenic vial. Approximately 80 µl of saliva was obtained for each time point. Human saliva samples were collected using a 2 inch cotton pledget held in the mouth for two minutes and then placed in a Salivette device (Sarstedt, NC).

All samples were frozen in each participant’s freezer and then transported on ice to the Penn State Behavioral Endocrinology Laboratory and stored frozen at -40 C until assayed for cortisol. On the day of testing, all samples were centrifuged at 3000 rpm for 15 minutes to remove mucins. Samples were measured in duplicate unless the volume of saliva collected prevented this, and their values were averaged for use in analyses. All samples from each dyad were run in the same assay on a single plate. Cortisol readings were examined for outliers and all baseline values exceeding 3 SD were excluded from analysis.

All samples were assayed for salivary cortisol using a highly-sensitive enzyme immunoassay kit (Salimetrics, State College, PA). The test uses 25 µl of saliva (for singlet determinations), has a range of sensitivity from .007 to 1.8 µg/dl, and average intra- and inter-assay coefficients of variation were less than 10% and 15% respectively. Method accuracy, determined by spike recovery, and linearity, determined by serial dilution are 105% and 95%. Examination of the cortisol data revealed that the distributions were positively skewed, therefore the data were log transformed to establish approximately normal distributions prior to analysis. As appropriate, analyses were conducted using the transformed values, but for ease of interpretation, the values in the text and figures are raw scores.
Behavioral/personality assessments

To assess underlying behavioral characteristics of the participating canines, the Canine Behavioral Assessment and Research Questionnaire (C-BARQ) (Hsu and Serpell 2003), consisting of 103 behavioral rating questions, was filled out by each owner and used to establish a baseline behavioral profile of each dog. The questionnaire consists of 8 sections which are used to determine scores for 11 subscales (see Table 3-1). We used the scales related to nonsocial fear, separation-related anxiety, excitability, attachment/attention seeking behavior and canine rivalry in analysis. Three questions in the nonsocial fear scale relate specifically to reactions to thunderstorm, wind and noises.

Table 3-1: Intrinsic behavioral characteristics of dogs based on the Canine Behavioral Assessment and Research Questionnaire (Hsu and Serpell, 2003). Nonsocial fear, separation-related anxiety and excitability were used in this study.

- Stranger-directed aggression
- Owner-directed aggression
- Dog-directed aggression
- Canine rivalry
- Chasing
- Stranger-directed fear
- Nonsocial fear
- Separation-related anxiety
- Body sensitivity
- Excitability
- Attachment/attention-seeking behavior

a Subscales used in this study
To measure the quality of the relationship between the owners and the dogs in this study, the Companion Animal Bonding scale (CAB) (Poresky, Hendrix et al. 1987) was used. This validated scale, an 8 item instrument, asks owners to answer questions such as “How often do you hold, stroke, or pet your dog?” and “How often does your dog sleep in your room?” on a 5 point scale ranging from “always” to “never”.

To evaluate the mood of the owners during the thunderstorm recording, the Profile of Mood States (POMS) was used. This 65 question scale measures 6 constructs including tension-anxiety, depression-dejection, anger-hostility, vigor, fatigue, and confusion-bewilderment (McNair, Lorr et al. 1971). This questionnaire was completed by the owners in the 20 minutes following the recording to describe how they felt during the time of the recording.

**Behavior observation**

All videotapes were coded for the presence or absence of clinical signs of canine anxiety as described in the literature (Overall, Dunham et al. 2001; Crowell-Davis, Seibert et al. 2003) (see Table 3-2). Signs including salivating, vocalizing, hiding, pacing, panting, remaining near the owner, and trembling were rated on a scale of 1-5 based on severity or amount seen during the playing of the recording (1- small amount/not severe; to 5- extensive amount, very severe). Yawning, destructiveness and elimination were recorded as events due to their infrequent occurrence. A dog behavior score was computed from the sum of the scores for whining, hiding, pacing, panting and remaining near the owner. The owners’ behaviors including how much they petted the dog and talked to the dog were rated on a scale of 1-5 (See Table 3-2) and then summed into one score. The amount of time that the dog and owner were in physical contact was recorded in seconds. An estimate was made of the percentage of time that the owner initiated this contact and that the dog initiated the contact. A measure of the amount of owner-initiated contact time and dog-initiated contact time was made using the percentage of the total time spent in contact. A trained observer, blinded to the cortisol results, made a
subjective evaluation on a scale of 1-5 (1= no reaction, 5= extreme fear as evidence by shaking, pacing, panting, whining, etc.) of the overall fear reaction of the dog.
Table 3-2: Coding scheme used to code videotaped human and canine behaviors during exposure to the thunderstorm recording.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dogs’ behaviors (rated 1-5)</strong></td>
<td></td>
</tr>
<tr>
<td>Excessive salivation</td>
<td>Licks lips excessively, swallows a lot, dampness seen</td>
</tr>
<tr>
<td>Vocalizing</td>
<td>Barks, whines, whimpers</td>
</tr>
<tr>
<td>Hiding</td>
<td>Leaves room/goes under or behind furniture, cabinets, curtains</td>
</tr>
<tr>
<td>Pacing</td>
<td>Walks back and forth, doesn’t remain in one place</td>
</tr>
<tr>
<td>Remains by owner</td>
<td>Remains near owner (within touching distance)</td>
</tr>
<tr>
<td>Panting</td>
<td>Visual open-mouthed panting</td>
</tr>
<tr>
<td>Trembling</td>
<td>Quivers, skin and muscles visibly moving, tag on collar moving without panting</td>
</tr>
<tr>
<td><strong>Dogs’ behaviors (events)</strong></td>
<td></td>
</tr>
<tr>
<td>Yawning</td>
<td></td>
</tr>
<tr>
<td>Destructiveness</td>
<td>Scratches/chews at windows, doors, carpeting, home furnishings</td>
</tr>
<tr>
<td>Elimination</td>
<td>Urinates or has bowel movement</td>
</tr>
<tr>
<td><strong>Owners’ behaviors</strong></td>
<td></td>
</tr>
<tr>
<td>Pet dog</td>
<td>Contacts dog in “comforting” manner (pet, hold on lap, hug, rub with feet)</td>
</tr>
<tr>
<td>Talk to dog</td>
<td>Owner speaks directly to dog</td>
</tr>
<tr>
<td><strong>Dyadic behavior</strong></td>
<td></td>
</tr>
<tr>
<td>Contact</td>
<td>Direct physical contact between owner and dog, involving any part of either subject’s body (measured in seconds)</td>
</tr>
<tr>
<td>Contact initiated by owner</td>
<td>Owner approaches dog and makes contact or owner calls dog to him/her and dog walks over immediately and physical contact occurs (estimated as percentage of total contact time)</td>
</tr>
<tr>
<td>Contact initiated by dog</td>
<td>Dog walks to owner and makes direct physical contact without owner calling him/her over (estimated as percentage of total contact time)</td>
</tr>
</tbody>
</table>
One researcher performed all the videotaped behavioral coding. Intra-rater reliability was determined by repeated coding of four trials at a later time and the concordance coefficient was calculated. \( \rho_c \) (dog behaviors) = .87, \( \rho_c \) (human behaviors) = .69, \( \rho_c \) (contact time) = 1.0, \( \rho_c \) (% contact time initiated by owner) = .92, \( \rho_c \) (overall fear reaction) = 1.0).

To confirm that dogs responded to the “storm”, owners provided a short written description of how their pet behaved during the recording and whether it responded as it would during a real storm. A response score of 1-3 was determined based on the owner’s view of whether the dog responded less, the same, or more to the recording than to a real storm.

For two trials the dogs’ full response was not captured on videotape because they left the room or view of the camera during the recording. One trial was not videotaped due to equipment failure. For these subjects, the owners’ description of what their dog did was used to establish that they responded but their storm response behavioral data was otherwise not used in analysis.

**Analytical strategy**

First, the behavioral responses of dogs and owners to the stress manipulation are described. Then, the main analyses employed a repeated measures 2 (stress) x 3 (sampling time) x 2 (dog/owner dyad) ANOVA with salivary cortisol as the dependent variable. Sampling time (baseline, 20 minute and 40 minute post), stress (control vs. "storm" day), and dyad (dog vs. owner) were within-subject factors. Percentage change scores between time points were calculated and paired sample t-tests were used to compare the changes between time points. Next, we examined sources of individual differences in behavioral and cortisol responsiveness. To test effects of the presence of other dogs in the home, an ANOVA with sample times on the "storm" day as within-subject factors and the presence of other dogs in the household as a between subject factor was computed. Bivariate correlations were used to explore the relationships
between canine and human cortisol levels and change scores and intrinsic canine behavior profiles (based on C-BARQ), canine behavior responses (overall fear, sum of response scores, percentage of time spent in contact with owner), human behavior responses (talking to dog and petting dog) and human mood profiles (POMS subscales). Partial correlations were used to take into account relationship quality when human and dog responses were correlated. Differences in baseline and changes in cortisol based on gender and order of presentation of “storm” vs. control days were examined by paired T-tests. Associations between cortisol and age were investigated through correlational analysis. The SPSS statistical software package (SPSS, Inc., Chicago, IL) was used for all analyses.

Results

Behavioral response

Dogs

Of the 19 dogs that participated in the study, all but one showed a behavioral response to the recording. This was defined as showing a score of 3 or greater on at least one fear sign other than “remain near owner”. The data from the excluded subject (who fell asleep on the couch) was not included in the analyses. According to their owners, eight of the other dogs responded as they would to a real storm, eight responded less than they would to a real storm and two responded more severely than they would to a real storm.

Behavioral responses varied for the dogs responding to the thunderstorm recording but some common themes were evident. “Panting” and “remaining near the owner” were the most common signs, both occurring in 15 dogs. Eleven dogs exhibited “pacing”, 10 showed a “hiding” response and 7 showed some “vocalization” (whining or “barking”). Videotape quality was sufficient to determine the absence/presence of
trembling for six subjects but was difficult to determine for the others. One dog defecated and showed destructive behavior towards the windows and doors. Dogs spent 40.3% of the 300 s recording time in direct contact with their owner (M = 120.8 seconds, SD=112.7). The mean amount of contact time initiated by the owner was 39.4 s (SD=48.4) and the mean amount of contact time initiated by the dog was 74.0 s (SD=100.4). The mean subjective “fear” reaction coded from videotape was 3.8 (SD=1.1) out of 5. In summary, the audio recording was effective in eliciting behavioral signs of anxiety in the majority of the dogs.

Owners

Owners also responded in different ways to their dogs. Eight had little or no contact with their dogs, while the rest talked to and petted their dogs, or made other physical contact with them (e.g. rubbing the dog with their feet if it was sitting near them on the floor). Several owners tried to hold their dogs back or to get them to sit near them when they tried to hide or escape. There seemed to be conflict between the dog wanting to get away and the owner wanting the dog to stay with them in six of the cases.

There were a number of significant correlations between the owner’s mood during the thunderstorm recording as reflected by their POMS scores and their behavior towards their dog. Owners scoring higher on the anger/hostility subscale were less likely to interact with their dog (r = -.67, P=.005). Owners were also less likely to interact with their dog if they scored higher on the depression/dejection subscale (r= -.50, P<.05) and on the fatigue subscale (r = -.58, P<.05).

HPA activation

A three-way interaction between dog/owner dyad, stressor (presence of storm recording) and time was seen, F(2,28)=10.50, P <.001.
For dogs, there were significant main effects on salivary cortisol levels for both time, $F(2,30) = 3.76, P < .05$, and treatment, $F(1,15) = 8.15, P < .05$ and a significant time by treatment interaction, $F(2,30) = 10.40, P < .001$. Paired sample t-tests confirm there were no differences between treatment conditions at baseline (Figure 3-1 A). Within the “storm” condition, cortisol levels increased over baseline at 20 minutes, $t(17) = -3.71, P < .005$. At follow-up 40 minutes after the recording, cortisol levels had declined from their peak at the 20 minute post mark, but were still higher than baseline, $t(17) = -2.51, P < .05$. The mean cortisol change from baseline to 20 minutes post-recording for responding dogs on the thunderstorm-recording day was substantial, a 206.6% increase. At 40 minutes post, cortisol levels remained 150% over baseline. By contrast, on the control day, cortisol levels at 20 and 40 minutes did not significantly differ from baseline.

Owners’ cortisol levels did not increase significantly on the thunderstorm-recording day. In fact, there was a decrease in cortisol levels on both the control and the “storm” days over the course of the trial $F(2,15) = 13.84, P < .001$ (Figure 3-1 B).
A. Canine

Figure 3-1: The mean (± SD) canine (A) and owner (B) cortisol levels at baseline, 20 and 40 min post-recording on the “thunderstorm” day (solid line) and control day (hatched line). Error bars represent standard error of the mean. Canine cortisol increased significantly from baseline to 20 min on the “storm day” and was still elevated at 40 minutes. Canine cortisol on the control day and human cortisol on both days did not show a significant change from baseline.
**Individual differences in behavioral and HPA responses to stress**

Dogs

There were no significant correlations between canine cortisol baseline or change scores and the nonsocial fear, separation-related anxiety, excitability, or attachment/attention seeking behavior subscales from the C-BARQ questionnaires. There were also no significant correlations between cortisol change and behavior response as measured by the coded videotape composite score and subjective fear rating.

![Cortisol response of single dogs vs. dogs in multi-dog households](image)

Figure 3-2: Mean (+/- SD) salivary cortisol levels at baseline, 20 and 40 min post-stressor on the “thunderstorm” day. Dogs living without other dogs (solid line) had significantly more change from baseline to 40 minutes post-stressor than dogs living in multi-dog households (hatched line).

There were no significant differences in mean cortisol levels between dogs living in multi-dog households and dogs living in single dog households at 20 minutes or 40 minutes post-stressor, although there was a trend for a higher baseline cortisol in the multi-dog households t(15)= -1.9, P= .08. Paired sample t-tests showed dogs living with other dogs had a significantly lower overall percentage change in cortisol from baseline.
to 40 minutes post recording, $t(15)=2.24$, $P<.05$ (Figure 3-2). A trend towards a greater initial increase in cortisol following the stressor in those dogs living in single-dog households than in multi-dog households was also seen, $t(15)=1.96$, $P=.07$.

Owners

There were no significant correlations between the owner’s cortisol change and behavior towards his/her dog. There was also no association between the total time of contact between owner and dog and the owner’s cortisol change. There were no significant correlations between the conflict seen between owners and their dogs and any other biological or psychometric measures.

Discussion

As hypothesized, the subjects in this study displayed profound behavioral and physical stress responses following exposure to a recording of a thunderstorm at a loud volume. Most dogs exhibited classic signs of fear including pacing, whining, trembling, and either hiding or wanting to be near their owner. The average increase in cortisol following the recording was substantial (207%). Surprisingly, there were few effects of the owners on the dogs’ behavior or HPA reactivity. However, the presence of other canines may be a mediator in the recovery of cortisol response. There are also a number of significant effects of the dogs’ responses on their owners’ behavioral and physiological response.

We expected that there would be a relationship between canine behavioral profiles and HPA activity, however, underlying behavioral characteristics as measured with the C-BARQ did not appear to play a role in the response of these dogs. Although we predicted that more fearful or excitable dogs would have an exaggerated response, this was not seen. We speculate that exposure to the thunderstorm recording was a
specific and overpowering stress-stimulus for these phobic dogs so that any effects of underlying characteristics may have been hidden.

There were no individual variations in the dogs’ responses related to their owners’ stress levels, attachment, or behavioral responses to the storm. Although much has been discussed about owners causing their dogs’ anxiety and the dogs perceiving their owners’ moods and responding in turn, there was no evidence that the dogs in this study were differentially affected by their owners’ moods or behavior. This does not discount the fact that some of the dogs’ behaviors could be conditioned responses to the owner, or that a specific behavior towards an individual dog would make a difference in its response. Further controlled studies could shed light on those questions. The owners who participated in this study were highly motivated to contribute and may have been more bonded to their animals than the average pet owner. Their companion animal bonding scale mean of 33.2 is higher than the 28.6 mean of the sample used for validation of the scale (Poresky, Hendrix et al. 1987).

It is of interest that dogs from multi-dog households had significantly less overall change in salivary cortisol from baseline (time 0) to 40-minutes post recording and a trend towards a lesser initial increase in salivary cortisol compared to dogs living in single-dog households. This trend and decreased overall change corresponds to a less extreme reaction and a more complete return to baseline in the 40 minutes following the stressor. However, there is also a trend towards a higher baseline cortisol in the multi-dog households which could indicate that dogs living with other dogs are under more stress. (Beerda, Schilder et al. 1999b) showed that the urinary cortisol/creatinine ratio in dogs individually housed in a laboratory setting for 6 weeks was greater than when they were in group-housed situations. However, contrary to the findings of our study, they found that individually housed dogs did not have as high a salivary cortisol response to a sudden sound blast. They attribute these findings to a HPA hyporesponsiveness following chronic stress. We would not expect our “single-housed” dogs to suffer this chronic stress since although they lacked canine companionship, they lived in homes and were not socially isolated. Further studies investigating the effect of living with other dogs on cortisol reactivity are indicated.
It is interesting to note in our study that the dogs living in multi-dog households did not interact with the other dogs during the storm recording. Although the other dogs were often visible, walking through the field of view on the tape, they did not contact or show any evidence of providing visible support to the fearful dog. Some owners had removed the other dogs in the household from the testing area but the findings did not depend on the other dog being present at the time of the storm. There were also no differences in the behavioral responses of the dogs living in multi-dog households. On both the composite score of fear-related responses and the coder’s subjective rating of fear, the dogs that lived in multi-dog households showed the same amount of behavioral response as those living alone. Only their cortisol changes showed differences, indicating that living with another dog may be a physiologically protective factor in dealing with stressors such as this one. If HPA stress response is related to the presence of other dogs in the social environment, it is likely that the relationship quality between con-specifics would affect this response. The development of other measures of canine relationship quality in multi-dog households would be useful.

It has been suggested that females may show an HPA hyperreactivity to stressors (Beerda, Schilder et al. 1999b) but there were no sex effects in this study. It has been hypothesized that differential gender effects of stress are related to androgen-inhibited and estrogen-enhanced oxytocin effects (Taylor, Klein et al. 2000). As all our canine subjects are gonadectomized, the differential effects of these hormones are negligible and may explain the absence of this effect in our sample.

We hypothesized that owners would respond to their dogs’ anxiety during the storm with a concomitant increase in cortisol. We also hypothesized that owners who were more attached to their dogs would have a greater increase in cortisol. However, the owners’ cortisol levels fell over the course of the collection time as they did on the control day. In this artificial situation, though the owners may have felt anxious for their dogs, they knew that the recording was only 5 minutes long. Whether the same pattern would occur during a real storm is unknown. The dogs’ responses were unrelated to the owners’ responses. Most of the owners reported that their dogs responded as they would
to a real storm, so it is unlikely that the dogs perceived from their owners’ behavior that this was not a real storm.

While there was little relation between dogs’ cortisol levels and behaviors and their owners, there were relations between owners’ behaviors towards their dogs and owners’ mood scores. Owners that were more tense or anxious, depressed, or fatigued during the recording were less likely to interact with (talk to or contact) their dogs.

Although the owners were instructed to treat their dogs as they normally would during a storm, some of the owners did hold their dogs back and try to comfort them in one place in view of the camera. It is not known whether this was to insure that the dog’s full reaction would be recorded or if this is how they normally comfort their dog. It appeared that for some owners, this was the normal way that they would comfort their dogs in a storm situation. There did not appear to be a relationship between conflict seen between owners and dogs and the dogs’ cortisol levels or behavior.

Research has shown that humans’ blood pressure, heart rate, cortisol and other measures of stress response decrease following positive interactions with animals in the face of laboratory stressors (Friedmann and Thomas 1995; Allen, Blascovich et al. 2002; Odendaal and Meintjes 2003), but little research has examined the effect of animals being the cause of stress. Although we tried to examine this in this study, we are limited in that the response of our owners to their dogs was likely affected by a number of factors including doing what they needed to for their anxious dogs and doing what they needed to carry out the study. It is also likely that the presence of the video camera affected some of the participants. Due to sample size limitations, we also had limited power in examining the number of relationships that we were interested in.

Much has been written and discovered on the effects of stress on health, including the immune system, the cardiovascular system, and the neuroendocrine system (Chrousos and Gold 1992). Animals with elevated cortisol levels that do not return quickly to baseline are thought to be more at risk of these physiological dangers. Although thunderstorm phobia is a problem for a number of individual dogs, many dogs also exhibit the same behavioral signs for other types of fear or anxiety (e.g. separation anxiety, fear of novel stimuli, visits to the veterinarian or groomer, etc.) (Voith and
Borchelt 1996; Overall, Dunham et al. 2001). If elevations in cortisol are similar for anxious dogs under those situations, it could possibly affect the mounting of immune response to vaccines and the health status of dogs in kennel or shelter situations.

Salivary cortisol measurement appears to be an effective means of measuring canine and human physiological stress responses in laboratory as well as non-laboratory and clinical settings. Although many owners remarked that it was difficult to collect saliva from their dogs, cortisol did not increase on the control day, indicating that saliva collection is not stressful enough to cause an HPA response in dogs. Some owners reported difficulty in collecting enough saliva and others reported that their dogs resisted collection. However, the owners were able to collect adequate samples to run complete analyses for each time point. The only inadequate sample in the study was from a small breed dog. The assay used in this study requires only 25 µl of sample. Further investigations of sample collection techniques and the use of salivary stimulants are warranted. A reliable technique that does not interfere with the cortisol assay would be very useful and allow sampling from smaller dogs.

This study was based on a small sample, making it difficult to evaluate all the complex relationships between the variables that were measured. It is possible that we did not have adequate power to fully elucidate the interactions between the variables. However, the primary results were strongly significant.

The study took place in a naturalistic setting, unencumbered by a researcher’s presence, but was controlled as to time of day that it took place, timing of sample collection and length of the recording. The setting was artificial in that a recording was used instead of a true thunderstorm, and that the videotape equipment may have been distracting or anxiety-inducing to the pets or owners. No specific effort was made to measure owners’ compliance with timing of samples, but the time of sampling was recorded on a sheet and these times corresponded with the protocol as described. The importance of taking the samples in the afternoon was emphasized to all participants.

The results suggest future studies could look at dogs’ responses to naturally occurring thunderstorms and other stressors. One would expect that other acute stressors in dogs’ lives would elicit similar physiological responses. The effect of stress-reducing
environmental or pharmacological interventions on physiological measures as well as behavior measures should be examined. Our results also indicate a need to examine the role of other pets in the environment on canine responses to stress. Although humans are often considered to be important members of their dogs’ social group, this research may indicate that other dogs may play a greater role on dogs’ responses than their human companions. If the presence of other dogs is a stress-modifying variable, this could have important implications on canine welfare and housing in research and humane shelter facilities. The role of canines on their owners’ stress responses may also be more complex than previously examined.

Conclusion

I hypothesized that fear in the presence of a known stimulus leads to HPA activation and the initiation of the stress response. Listening to a recording of a thunderstorm at a loud volume elicited profound behavioral and/or physiological responses in nearly all of the thunderstorm-phobic canine subjects but not their owners. I also hypothesized that this response would be mediated by the social environment of the animal. This hypothesis was partly supported in that dogs’ responses did seem to be related to the presence of other dogs in the household. However, the dogs’ behavioral and physiological responses were not affected by their owners’ behavioral or physiological responses in this study. Salivary cortisol can be collected by pet owners in a home situation. Multiple measures of stress (e.g. behavioral and physiological) are needed to investigate the complex interactions between humans, companion animals, and their environment.
CHAPTER 4-
HEALTH OUTCOME EFFECTS RELATED TO FEAR AND ANXIETY IN DOGS

Introduction

Stress responses in animals and humans are related to a number of changes in HPA axis modulation and immune function. Fear, anxiety and phobias are common behavioral characteristics of individual dogs. Separation anxiety, noise phobias, and fear of strangers are common reasons for which dogs may be presented to animal behaviorists or veterinarians. Beerda and colleagues (1998), showed that a number of different stimuli, including loud sound and sudden movement, elicited responses in previously unexposed dogs. If dogs with preexisting anxiety have dramatic cortisol responses to a fear-eliciting stimulus, it is likely that this type of response occurs many times throughout the course of an anxious or fearful dog’s lifetime.

Because many of the inciting causes for these responses are part of the everyday life experiences of most dogs and are unavoidable, dogs with these fears and anxieties are likely to have chronic HPA and sympatho-adrenal stimulation, possibly leading to disease and decreased longevity. Cavigelli and McClintock (2003) showed that neophobic rats were identifiable within 20-24 days after birth, and that this phobic trait affected their HPA response to stress, persisted throughout their lifetime, and had eventual detrimental effects on their health and longevity.

Because of the multi-directional effects of the immune, endocrine, and nervous systems, it is also likely that animals with disease processes will exhibit behaviors related to inflammation and cytokine release. Inflammatory responses including cytokine release from activated lymphocytes have been shown to alter central nervous system activity, hormone levels, and neurotransmitter levels, which in turn have profound effects on organism behavior (Ader, 1996). Specifically, these adaptive “sickness behaviors” relate to changes in appetite, activity and state of consciousness (Hart, 1987).
The purpose of this study is to determine if dogs with fear and anxiety characteristics have shorter natural life-spans and a higher incidence of disease than those not exhibiting these behaviors. I hypothesize that stress caused by living with anxiety or fearfulness has deleterious effects on the health and life-span of domestic canines. (See D on Figure 1-1.) Because of known effects of chronic HPA stimulation on the endocrine and immune systems, I hypothesize that more fearful dogs will have higher incidences of immune-mediated disorders, neoplasia, allergies, infection and skin diseases. This is likely a “dose-dependent” relationship and environmental factors that relate to the amount of exposure to fear-eliciting stimuli will affect the amount of disease seen.

The third major component of this thesis is a retrospective epidemiological survey examining the relationship between behavioral characteristics (specifically fear and anxiety), breed, and health outcomes in dogs. Factors in the animal’s social environment (e.g. the presence of other dogs, animals and humans) that may play a mediating role in the expression of these diseases will also be examined.

There is evidence that some breeds may be predisposed to certain types of fears or anxiety. Herding breeds, in particular, were over-represented in an internet study of thunderstorm phobia (McCobb, Brown et al. 2001). Anecdotal evidence and participant response in a study of thunderstorm fear in dogs suggests an over-representation of the Golden Retriever breed. Because of the large number of individual breeds within the canine species, it is necessary to use a classification system that categorizes the breeds into a limited number to be studied. Parker and colleagues (2004) utilize microsatellite genetic markers to classify 85 breeds based on cluster analysis into four subpopulations with some breeds falling into more than one cluster (See Figure 4-1). One cluster (designated “yellow”) encompasses dogs of more primitive origin and contains wolves, as well as many breeds of Asian and African origin. A second cluster (designated “blue”) is characterized by large, mastiff-type dogs. The third cluster (designated “green”) contains breeds that are “sight hounds” (older breeds originally bred for hunting by sight, instead of by odor) and herding dogs, while the fourth cluster (designated “red”) contains more modern hunting dogs, including scent hounds, terriers and retrievers. It is interesting to note that some breeds that seem functionally and phenotypically similar,
such as the Labrador Retriever and Golden Retriever, are not as closely related genetically. In addition, some dogs that were developed as herding dogs, such as the Border Collie and Old English Sheepdog, are classified in the more modern group instead of with the herding dogs and sight hounds.

Figure 4-1: Breeds occurring in the sample population classified by genetic relatedness as described by Parker, Kim, and colleagues (2004). Four distinct clusters of closely related subpopulations are highlighted by the red, yellow, blue and green groups. Some breeds that have characteristics of more than one subpopulation are shown in overlapping circles. (Based on Figure 3, Parker and Ostrander, 2005)
The American Kennel Club classifies breeds based both on functional type and relatedness (AKC 2006, see Table 4-1). Although some of the breeds in the AKC classification scheme are historically or genetically related, others are functionally related but are not thought to be highly genetically related. For example, sighthounds, retrievers, pointers and setters were all bred for hunting but have very different historical backgrounds and exhibit different behaviors while hunting. Breed differences in behavior are likely related to both genetics and environmental influences.

<table>
<thead>
<tr>
<th>Breed category (Hunting, Retrieving)</th>
<th>Included breeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporting</td>
<td>Water spaniel, Chesapeake Bay retriever, Clumber spaniel, American cocker spaniel, English cocker spaniel, Flatcoat retriever, German shorthair pointer, Golden retriever, Labrador retriever, Pointer, English setter, Irish setter, Welsh springer spaniel</td>
</tr>
<tr>
<td>Herding</td>
<td>Australian shepherd, Belgian shepherd, Belgian tervuren, Border collie, Collie, German shepherd, Old English sheepdog, Shetland sheepdog, Welsh corgi</td>
</tr>
</tbody>
</table>

A pilot pen and paper based survey seeking to determine the prevalence of thunderstorm fear in several populations attracted primarily only those owners whose dogs suffered from thunderstorm fear as subjects. As a result, an on-line survey, targeted at previous dog owners of all types, instead of identifying a particular behavioral issue, was proposed to be more likely to be successful. A retrospective methodology with on-line data collection was chosen for the ability to collect a fairly large sample size in a relatively short period of time. A large sample size should enable the researcher to elucidate breed predilections for fear and anxiety disorders, particularly when breeds are grouped by genetic relatedness.
An important consideration in using an online survey is that web-based survey respondents have been shown to have higher levels of education and to enjoy higher socio-economic status than the general population. Recently, however, it was shown that this is changing as web-use becomes more available to all (Im, Chee et al. 2006). Snowball sampling, particularly through web-based means, is useful in providing a sample population with very specific characteristics. In this case, it was important to identify and recruit individuals who had owned dogs that had died within the past five years.

**Methods**

**Participants**

Participants over the age of 18 whose dogs had passed away within five years were recruited to fill out an on-line survey about their deceased pet. All procedures were approved by the Pennsylvania State University Institutional Review Board for Human Subject Participation. Participants were instructed to fill out the survey while considering the last dog they had that had died.

Seven hundred forty-one participants from the U.S. and around the world logged on to the specific survey website. Of these, 721 completed the survey and included the age at which their dog died. Of the twenty that did not complete the survey, ten did not complete any information, eight indicated that their dog was not deceased and two attempted to enter information for more than one dog. Only those who included the dog’s age at death, or the years of both his birth and death, were included in the analysis of lifespan and behavior. Four randomly chosen participants received $20.00 money orders for participating.
**Sampling**

Recruitment for the study was targeted towards obtaining dogs of all breeds and temperaments. The survey was advertised as a canine health and behavior survey to avoid selection bias for dogs with particular behavioral, personality or health problems. Sampling was primarily through the “snowball method”. Flyers were posted at veterinary offices, in public places (e.g. grocery stores, restaurants, pet-related organizations) and on a University campus. Participants were also recruited by “word-of-mouth”. The survey web-link appeared on a number of pet-loss related websites and list-servs, and in a national magazine which ran a short article on a related study. Participants and other interested parties were encouraged to send the link to friends, relatives, colleagues and others who might qualify. A call for participants through a veterinary behavior list-serv elicited a large numbers of replies and distribution to other websites and list-servs. In particular, the web-link was distributed to a number of breed-specific organizations, training groups and veterinary professional groups. See Appendix B for a list of places and websites where the link was known to be advertised.

**Questionnaire**

The survey included questions about the dog’s breed, social environment, geographic location, and health history (see Appendix C). The age of death and cause of death were also included. Questions making up the subscales related to stranger-directed fear, non-social fear, separation-related anxiety and body sensitivity from the C-BARQ questionnaire (Hsu and Serpell 2003), a validated behavior instrument, were included. The survey was reviewed by three individuals with veterinary behavior backgrounds and their suggestions were considered and incorporated. Several “non-professional” dog owners also reviewed the survey before posting.

The survey was hosted on a web-site served by PsychData (www.psychdata.com, State College, PA), a social science survey research company. Potential participants were directed to this website where they entered a specific survey number to access the survey.
from 10/28/05 through 4/17/06. All responses were anonymous and no identifying information was obtained from participants as part of the full survey. Participants were able to enter a drawing for a prize by completing an additional page with their identifying data, but these two instruments were unlinked in the final data form.

When the data collection was complete, the survey was inactivated and the data was downloaded to a text file. The data was purged from the PsychData server two weeks after the survey was inactivated. SPSS statistical software was used to analyze all data.

**Analytical strategy**

Descriptive statistics were computed on the physical, social, and behavioral characteristics of the dogs. Those variables that were positively or negatively skewed underwent log transformation for further statistical analysis. For ease of understanding, non-transformed data is presented in tables and figures. Because variables such as the size of the dog and neutering status have been shown previously to be related to lifespan, correlations between these variables and lifespan were computed and these variables were controlled in further analyses. Prevalence statistics of specific diseases and behavioral qualities were computed.

If the owner did not specify an “age at death”, a value was calculated by subtracting the year of birth from the year of death. Categories for the cause of death were constructed based on responses given to the open-ended question “If your dog died from a disease or diseases, please specify what they were”. Using the most frequent responses, categories were constructed including “unknown”, “immune” (including autoimmune hemolytic anemia, allergies, immune-mediated thrombocytopenia, and granulomatous meningoencephalomyelitis), “infection”, and “cancer”. An “other” category included other organ system diseases not falling into the above categories (neurological, cardiovascular, endocrine, renal, hepatic, pulmonary, and musculoskeletal diseases), as well as death due to “old age”, bloat, surgical complications and other rarer diseases. A continuous score from a Likert scale of 0-5 based on severity and frequency
of disease was calculated for diseases that affected the dog during adulthood, but did not necessarily cause the death of the individual.

Scores were calculated for the stranger-directed fear, nonsocial fear, separation-related anxiety and body sensitivity subscales of the C-BARQ (Hsu and Serpell 2003). Scores for thunderstorm fear were based on a single question of the survey. To determine scores indicative of severe fears and anxieties, calculations based on mean scores plus two standard deviations were computed. These scores were then used to determine the prevalence of specific extreme fears and anxieties in the general population.

Breeds were grouped according to genetic relatedness as described by Parker and colleagues (2004, 2005) (See Figure 4-1). Only those dogs falling within a single cluster were used for this analysis. For ease of comparison to other studies, breed groupings according to the American Kennel Club designations of “sporting dogs” and “herding dogs” were also included (see Table 4-1). One-way MANOVAs looking at the overall relationship of breed according to the genetic and AKC classifications and the fear and anxiety related behaviors were performed. Differential anxiety scores for each breed grouping were determined by comparing specific breed groupings to the general population (remaining breeds) using t-tests.

In order to test the effect of fear and anxiety on long-term health and lifespan, while controlling for those variables already know to affect lifespan, a series of regression analyses with weight (as an indication of overall size), neutering status, death due to accident, and behavior scales as predictor variables and lifespan (in years) as the dependent variable were performed. Further analysis investigated the relationship between behavior scores, specific diseases affecting dogs during adulthood, and causes of death. To investigate the situational effect of separation anxiety, 2x2 anovas (high and low anxiety, high and low amount of time the dog was left alone) with specific diseases and age at death as dependent variables were performed.
Results

Descriptive statistics

Physical characteristics

Of the 721 respondents included in analysis, 77% indicated only one breed for their dog, while 23% indicated that their dog was a mix of two or more breeds. The dogs ranged in weight from 3-200 pounds (M = 59.6, SD = 30.7). The majority of dogs (92%) in this study were neutered by the time of death, 54% were female and 46% were male. Nearly half of the dogs (48%) fell into the “red” breed category (“modern” hunting dogs), 22% in the “blue” breed category (mastiff-type dogs), 15% in the “green” breed category (herding and sight hounds) and 10% in the “yellow” breed category (Asian and African origin). Sixty three percent of the dogs were vaccinated yearly as adults, and another 25% were vaccinated every 2-3 years.

Social environment

Most of the owners (94%) indicated that they had obtained their dog for companionship. Other reasons noted for obtaining dogs included protection, hunting, breeding, showing and competition, herding, service, and police work. The majority of the dogs had lived with other animals during their lifetimes. Eighty two percent had lived with other dogs and 52% had lived with cats. Other species that the dogs had lived and interacted with included birds, horses, cattle, sheep, goats, llamas, pigs, small mammals, and reptiles. Nearly one quarter (24%) of the dogs had lived in households with children less than 12 years old, 25% had lived with teenagers and young adults, and 10% had lived with seniors over 65 years old.
Behavioral characteristics

The descriptive statistics for the anxiety scales (range 1-5) for all the subjects and the various breeds can be seen in Table 4-2. The overall prevalence of extreme body sensitivity, non-social fear, and separation anxiety based on those scores more than 2 standard deviations above the mean were less than 5%. The percent of those dogs with extreme stranger directed fear was 8% and the overall percentage of those dogs with extreme thunderstorm fear (greater than 4.0 on a 1-5 scale) was 20%.

<table>
<thead>
<tr>
<th>Breed type</th>
<th>Valid N</th>
<th>Mean body sensitivity score (SEM)</th>
<th>Mean stranger-directed fear score (SEM)</th>
<th>Mean non-social fear score (SEM)</th>
<th>Mean separation anxiety score(SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects</td>
<td>555</td>
<td>1.78 (.03)</td>
<td>1.66 (.04)</td>
<td>1.92 (.03)</td>
<td>1.38 (.02)</td>
</tr>
<tr>
<td>Asian/African Origin (Yellow cluster)</td>
<td>39</td>
<td>1.82 (.10)</td>
<td>1.83 (.14)</td>
<td>1.99 (.12)</td>
<td>1.48 (.10)</td>
</tr>
<tr>
<td>Herding and Sight Hounds (Green cluster)</td>
<td>87</td>
<td>1.67 (.07)</td>
<td>1.63 (.10)</td>
<td>1.97 (.08)</td>
<td>1.31 (.05)</td>
</tr>
<tr>
<td>“Modern” Hunting Dogs (Red cluster)</td>
<td>145</td>
<td>1.93 (.06)**</td>
<td>1.60 (.07)</td>
<td>2.02 (.06)*</td>
<td>1.38 (.04)</td>
</tr>
<tr>
<td>Mastiff type (Blue cluster)</td>
<td>85</td>
<td>1.72 (.08)</td>
<td>1.72 (.10)</td>
<td>1.89 (.08)</td>
<td>1.38 (.05)</td>
</tr>
</tbody>
</table>

*, **significantly different from those not in that breed category (*p<.05, **p<.005)

Table 4-2: Average anxiety scales and standard error of the mean for specific breed categories. The modern-hunting type dogs scored higher on body sensitivity and non-social fear compared to the rest of the population. Note- breed numbers do not add up to total due to mixed breeds and other breeds not included in the genetic groupings.

Only 11% of dogs had not received any formal training at home or at a school. Forty six percent had received basic group training lessons and 28% had received advanced group lesson training. Most owners felt that their dogs were “very well trained
and responded quickly to commands” (M = 4.1, SD = .9 on a 1-5 Likert scale) and were “very well behaved and never caused any problems” (M = 4.1, SD = 1.0 on 1-5 Likert scale).

Lifespan and disease

The average age at death was 11.6 years (SD = 3.6), with a range of 4 months to 22 years. Cancer was the most common cause of death, affecting 38% of dogs in this study. Most dogs (79%) were euthanized while 21% were reported to have died a natural and unassisted death.

Differential Effects

Differential effects of non-behavioral factors on lifespan

Regression coefficients for weight, neutering status, accidental death, and breed are presented in Table 4-3. These non-behavioral individual factors account for 17% of the variability in lifespan for this population. Weight and neutering status were shown to have significant effects on lifespan with smaller dogs living longer than larger dogs and neutered dogs having significantly longer lifespans (M = 11.9 years, SD = 3.4) than unneutered dogs (M = 9.6 years, SD = 4.8), t (73.2) = 3.7, p < .001. No significant difference in lifespan for sex of the animal was noted.
Lifespan was shortened for dogs that had died in accidents. Accidents are a common cause of death for younger animals. Belonging to the red breed grouping ("modern" hunting dog types) positively predicted lifespan, but none of the other breed groupings had significant effects on lifespan. Euthanasia positively predicts lifespan, with older animals more likely to be euthanized than younger animals. Whether dogs had lived with other dogs during their life did not predict lifespan.

Breed differences in behavioral scores

The multivariate analysis showed a significant effect of genetically classified breed on body sensitivity score, F(4)= 2.75, p<.05. Further analyses showed no significant differences from the general population for any of the anxiety scales for the yellow, green, or blue breed groupings (see Figure 4-2). However, dogs classified in the red breed groupings ("modern" hunting dogs) were found to have significantly higher body sensitivity, t (630) = -2.97, p<.005, and non-social fear, t (654) = - 2.23, p<.05, scores than those dogs not in the red group.

<table>
<thead>
<tr>
<th>Model</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>-.33***</td>
<td>-.33***</td>
<td>-.31***</td>
</tr>
<tr>
<td>Neuter</td>
<td>.17***</td>
<td>.16***</td>
<td>.16***</td>
</tr>
<tr>
<td>Accident</td>
<td>-.11***</td>
<td></td>
<td>-.11***</td>
</tr>
<tr>
<td>Euthanasia</td>
<td></td>
<td>.14***</td>
<td></td>
</tr>
<tr>
<td>Breed (Red)</td>
<td></td>
<td></td>
<td>.14***</td>
</tr>
<tr>
<td>R²</td>
<td>.15***</td>
<td>.16***</td>
<td>.17***</td>
</tr>
</tbody>
</table>

***p<.005
When classified according to the American Kennel Club designation, a significant effect was seen on non-social fear, \( F(2) = 3.421, \ p < .05 \). Further analyses showed the herding breed group had significantly more non-social fear, \( t(654) = -2.27, \ p < .05 \), and thunderstorm fear, \( t(702) = -3.21, \ p < .005 \), than those dogs not classified as “herding” dogs (see Figure 4-3). There were no significant differences in the fear scales of the sporting dog group according to the AKC classification system, but a follow-up t-test showed Golden retrievers had significantly higher thunderstorm fear scores \( t(702) = -2.71, \ p < .05 \) than non-Golden retrievers.
Results of the regression model testing for behavior scores and lifespan are shown in Table 4-4. In each model lifespan is the dependent variable and each behavior score is the independent variable. Weight, neutering status and accidental death were controlled for, as these have significant and independent effects on lifespan. A series of regression analyses were run to investigate the effects for each behavior subscale on longevity of the dogs.

The first model shows how “well behaved” the owner considered the dog to be had a significant positive effect on the lifespan of the dog. Owners’ ratings of how well their dogs were trained and how well they behaved were highly correlated, $r (724) = .31$, $p<.001$, but training did not independently predict lifespan, while how well the dog behaved did positively predict the dog’s lifespan.
Stranger directed fear (SDF) significantly predicted decreased lifespan when weight, neutering status and accidental death were controlled for. Further investigation into causes of death failed to show significant differences in any disease categories at death and SDF. There was a significant difference between the stranger-directed fear scores and whether dogs were euthanized or not, with euthanized dogs having significantly lower stranger directed fear scores than those that died a natural death.

Table 4-4: Regression models and corresponding beta coefficients examining the effects of behavioral factors on lifespan. How “well-behaved” the owner considered the dog to be and the level of stranger-directed fear (SDF) both have independent and significant effects on longevity when weight, neutering status and death caused by accidents are controlled for.

<table>
<thead>
<tr>
<th>Model</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>-.33***</td>
<td>-.35***</td>
<td>-.33***</td>
<td>-.33***</td>
<td>-.34***</td>
</tr>
<tr>
<td>Neuter</td>
<td>.18***</td>
<td>.17***</td>
<td>.17***</td>
<td>.17***</td>
<td>.17***</td>
</tr>
<tr>
<td>Accident</td>
<td>-.10**</td>
<td>-.10**</td>
<td>-.11**</td>
<td>-.11***</td>
<td>-.17***</td>
</tr>
<tr>
<td>“Well-behaved”</td>
<td>.18***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stranger-directed fear (SDF)</td>
<td></td>
<td>-.10**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-social fear (NSF)</td>
<td></td>
<td></td>
<td>-.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Sensitivity</td>
<td></td>
<td></td>
<td></td>
<td>.02</td>
<td></td>
</tr>
<tr>
<td>Separation Anxiety</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-.06</td>
</tr>
<tr>
<td>$R^2$</td>
<td>.18***</td>
<td>.16***</td>
<td>.14***</td>
<td>.15***</td>
<td>.15***</td>
</tr>
</tbody>
</table>

***p<.005, **p<.001

Stranger directed fear (SDF) significantly predicted decreased lifespan when weight, neutering status and accidental death were controlled for. Further investigation into causes of death failed to show significant differences in any disease categories at death and SDF. There was a significant difference between the stranger-directed fear scores and whether dogs were euthanized or not, with euthanized dogs having significantly lower stranger directed fear scores than those that died a natural death,
\( t(203.2) = 2.5, p<.05 \). Other behavioral subscales, including non-social fear (NSF), body sensitivity, and separation anxiety failed to predict lifespan. Further regression analyses examined the relationship between anxiety scores and diseases that dogs suffered from as adults (see Table 4-5). Non-social fear and separation anxiety both positively predicted the severity and incidence of skin problems in the dogs as adults. Weight and body sensitivity predicted arthritis signs in adult dogs. None of the behavioral scores were shown to be predictive of the incidence and severity of immune mediated diseases, endocrine disorders, or cancer in the dogs as adults. To investigate the interaction of more severe separation anxiety and social surroundings on various health conditions, a 2 x 2 Anova (high and low separation anxiety, high and low amount of time the dog was left alone) was performed. No significant effects on specific diseases or age at death were noted.

Table 4-5: Regression models and corresponding beta coefficients examining the effects of behavioral factors on disease. Body sensitivity scores had a significant independent predictive value of arthritis when weight was controlled for. Both non-social fear and separation anxiety positively predict the incidence and severity of skin disorders.

<table>
<thead>
<tr>
<th></th>
<th>Skin disorders</th>
<th>Arthritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td></td>
<td>.11*</td>
</tr>
<tr>
<td>Body sensitivity</td>
<td></td>
<td>.08***</td>
</tr>
<tr>
<td>Non-social fear</td>
<td>.12**</td>
<td></td>
</tr>
<tr>
<td>Separation anxiety</td>
<td>.11*</td>
<td></td>
</tr>
<tr>
<td>R²</td>
<td>.03***</td>
<td>.03***</td>
</tr>
</tbody>
</table>

*p<.01, **p<.005, ***p<.001
Discussion

The average life-span of dogs in this study is similar to that previously reported (Michell et al 1998). This study also supports previous findings that cancer is one of the leading causes of death in the canine population (Bronson 1982; Ogilvie and Marks 2000). Not surprisingly, dying in an accident (e.g. hit by a car or other trauma) was predictive of a shorter lifespan.

As expected, higher weight was highly predictive of shorter life span. Larger breeds of dogs by both weight and height have been shown to live shorter lives than smaller breeds (Greer et al. 2007). Although obesity is known to decrease life-span, it is important to keep in mind that in this study, weight was representative of overall breed related size instead of individual obesity. Another finding supporting the previous research of Michell and others (1998), is that neutered dogs had significantly longer lifespans, living an average of 2.3 years longer than their un-neutered counterparts. Because weight, neutering status and dying in an accident accounted for 15% of the variability in lifespan, these factors were controlled for in further analysis of lifespan and behavior.

Behavior significantly impacts lifespan in dogs. Behavior problems are recognized as being a leading cause of death in pet dogs due to euthanasia. In this sample, how much the owner perceived the dog to be “well-behaved” was significantly predictive of their length of life. An obvious reason for this would be premature euthanasia of pets that were aggressive towards their owners or others. It is important to remember that this study did not measure aggressive behaviors which are more likely to play a role in earlier euthanasia or surrender to a new home. In fact, death by euthanasia was related to longer lifespan in this population. This indicates that dogs were older when they were euthanized. Well-behaved dogs may live longer because they may be under less stress living in a more harmonious household.

I hypothesized that stress caused by living with anxiety or fearfulness has a deleterious effect on health and life span in canines. This study shows that stranger directed fear independently and significantly predicts decreased lifespan when other
variables related to shortened lifespan are controlled for. Extreme stranger-directed fear (a rating of 5 on the stranger-directed fear scale) corresponds to a one-half (.5) year shorter life span than a dog with no stranger directed fear. While the mechanism of this relationship is unknown, it supports the hypothesis that stress secondary to fear or anxiety may lead to deleterious effects on health and lifespan. Interestingly, neither stranger directed fear nor any of the other fear or anxiety scales were related to specific disease causes of death. Based on what is known of the effects of stress on the living system, one might expect an increase in deaths due to immune disorders (autoimmune diseases, cancer, infection) or endocrinopathies. However, this research supports that of Cavigelli and McClintock (2003) who found that although neophobic rats had decreased life-spans, there were no differences in the specific diseases causing their deaths. There is evidence that stress may be more related to accelerated aging of cells at the molecular level, thereby increasing the speed at which the system succumbs to natural disease processes, instead of introducing new disease processes. A lifetime of stress resulting from interacting with strangers may take its toll on the body at a molecular level, causing accelerated aging in cells and earlier death from any cause.

Dogs suffering from stranger-directed fear could also be less likely to improve in a hospital situation where they are exposed to and handled by strangers. The stress associated with medical care and hospitalization may diminish their ability to physically respond to therapy. Dogs dying in accidents also had higher stranger-directed fear scores. The fear of being handled by strangers at a veterinary hospital following an accident may have had negative consequences on their physical ability to handle the acute effects of stress and the survival from the accident. However, there were no similar effects seen for dogs that had non-social fear. Perhaps loose dogs afraid of strangers were more likely to flee from those trying to help them and were therefore more likely to be involved in accidents.

Although fear and anxiety measures were not correlated with the presence or absence of disease, several scores did predict the frequency and severity of specific diseases during the dogs’ adult lives. Specifically, stranger-directed fear and separation anxiety were predictive of increased skin problems. The effect of psychological stress in
a number of human skin diseases has been recognized for many years (Tausk, 2001). In particular, psychological stress in humans has been found to disturb the epidermal barrier function (Garg, et al, 2001). A similar disturbance could lead to increased susceptibility to fungal and bacterial infection as well as an increase in atopic or allergic reactions in dogs. Also, considering the common embryological neural crest origin of cells in the epidermis, endocrine systems and the central nervous system, genetic or molecular variation in the early germ cells could affect all of these systems later in life.

Other significant findings include the prediction of body sensitivity on arthritis. When examining relationships between physical disorders and behavioral scales, it is important to note that these are correlational relationships. Particularly in the case of body sensitivity, it is possible that these physical disorders are the cause, rather than the result of behavioral signs. Animals with arthritis are likely in pain and are often protective of themselves. It would be expected that a dog in discomfort or pain would resist grooming and having his feet manipulated for toweling dry or nail trimming. Dogs with arthritis may show aggression when bumped into or stepped upon. Arthritis in dogs is frequently secondary to skeletal malformation (e.g. hip or elbow dysplasia) or intervertebral disc extrusion, versus an autoimmune disorder such as rheumatoid arthritis. It is still a highly inflammatory condition, however, and interactions of cytokines with the peripheral and central nervous system are likely. Body sensitivity in these individuals may also be a “sickness behavior” that is activated by the inflammatory response.

Breed was predictive of life span if the dog was in a breed that fell into the “red” category. The “red” breed grouping contains both the greatest number of breeds, as well as the greatest percentage of individual dogs from the sample. Although described as the “modern” hunting dog group, this breed grouping is varied, containing many of the terrier and sporting breeds, as well as most of those dogs that have been specifically bred as “pets” instead of as “working” dogs. Being a member of the “red” breed grouping corresponded to a longer lifespan than not being a member of that group, when weight, neutering status and whether the dog had died an accidental death were controlled for. There was no effect (positive or negative) of being a member of any of the other breed groups. Selection for longevity has likely played a role in the breeding of animals that are
chosen as “pets”. An interesting finding of this study is the difference in fear and anxiety scores seen between the breed groups. When compared to dogs that did not fall into this breed, the dogs in the red grouping were found to have significantly higher body sensitivity and non-social fear scores.

The findings of this study support a previous study (McCobb, Brown et al. 2001) that found that dogs with thunderstorm phobia were more likely to fall into the AKC herding breed group. Dogs in the herding breed group of this study population showed significantly more non-social fear (i.e. fear related to the environment rather than to other dogs or humans) and fear of thunderstorms (which is a component of non-social fear). Although the dogs in the herding group span the genetically based classification system with member breeds falling into the green, red and blue groups, no individual breed in this grouping appeared to have a greater incidence of thunderstorm fear than the others. At first it would seem counter-intuitive that dogs bred to spend all their time in the environment, exposed to weather and noises would develop a fear of thunderstorms, however, it makes sense that these highly intelligent breeds could be hyper-vigilant, always looking for cues in their environment and being prepared for danger. They may be more anxious in their environment because they are more aware of it.

The sporting breeds were also bred to spend their lives in the outdoors and are also alert to their surroundings. It is interesting to note, therefore, that as a group, they do not show any differences in nonsocial fear or anxiety compared to other groups. Perhaps their task is to note changes in the environment, focus on it, and then either approach or alert their handler. They do not have to take responsibility for a herd of sheep or cattle when danger strikes as herding dogs do. A notable exception to this group is the Golden retriever which shows significantly higher thunderstorm fear scores than any of the other groups or breeds. In fact, there was a 33% prevalence of extreme thunderstorm fear (based on the overall mean score plus 2 standard deviations) in Golden retrievers in this study. Why this occurs is open to speculation. The Golden retriever has been one of the most popular breeds in the U.S. for years, resulting in significant breeding pressure. Perhaps over-breeding or in-breeding for other desired physical or behavioral traits has led to an increase in this specific behavioral characteristic.
A previous study indicated that dogs that lived with other dogs may not mount as high a cortisol response and may recover more quickly after a stressor than those dogs living alone (Dreschel and Granger 2005). The majority of dogs in this study had lived with other dogs during at least some part of their lives. However, no significant difference in lifespan between these dogs and those living without canine companions was seen. How the presence of conspecifics affects stress and behavior in dogs is an interesting question that merits further research.

Overall, this sample appears to be representative of a well-cared-for companion dog population. A broad distribution of dogs across breed, gender, and weight range is seen. Nearly all the owners (94%) had obtained their dog for companionship. It is interesting to note that such a high percentage of dogs were neutered by the time of their death and that most had received some training, either at home or with professional trainers. Also, nearly 90% of the dogs were vaccinated at least every 2-3 years as adults. While it is recommended that dogs visit the veterinarian 1-2 times per year, the vaccination schedule varies and vaccinations every 2-3 years are becoming standard. The high percentage of vaccinated, neutered, and trained dogs in this sample indicates a responsible pet-owning survey population. This may reflect a selection bias to be expected from an online survey which might attract more educated respondents with more resources. It is also likely that respondents motivated to complete this survey may have been more closely attached to and responsible for their pets than the general population. Although these could be confounding factors in this study, how they might affect the basic hypotheses of relationships between behavior, anxiety and health outcome are not apparent.

There was a low prevalence of extreme non-social fear, separation anxiety and body sensitivity in this sample. Stranger-directed fear affected a higher percentage of dogs and thunderstorm phobia affected one out of five dogs in this sample. It is possible that the thunderstorm fear prevalence may be artificially inflated because the survey was advertised in a sidebar to an article on thunderstorm fear in a popular magazine. Those who had read the article may have been more likely to have dogs that suffered from thunderstorm phobia and were more motivated to respond to the survey. However, the
survey was also advertised in many other media outlets that were unrelated to behavioral problems. The high prevalence of thunderstorm fear reflects what is commonly seen in clinical practice and spoken about anecdotally.

There are a number of inherent limitations in a study of this type. With a cross-sectional design, all results must be correlational. Although a prospective longitudinal study would enable us to make determinations of cause and effect, one that includes lifespan as an outcome variable would require 15-20 years to complete. As a result, using this design, we can hypothesize the mechanisms of interaction and the treatments that might be effective, but until we have longitudinal data, it will be difficult to determine cause and effect of particular diseases and personality traits. It is likely that these are complex relationships related to genetics, early experience, environment and physical health.

Another limitation of this particular design is the effect of historical factors. Although many of these dogs lived during some of the same time, their lives span a 20-25 year period. Much has changed in pet care over that time period, including health and vaccine status, parasite control, dog food and training methods. It is likely that dogs that lived to be 18 or 20 years old and passed away four or five years ago, would have experienced different social and physical environments as puppies, than those that lived to be eight or 10 years old and passed away a year previously.

Likewise, this study was also highly dependent on the respondents’ abilities to recall information that may have spanned up to 20 years time. While some owners knew exactly how old their animals were and reported their exact dates of birth and death, others were less clear, giving a range of 1-2 years. Some of the dogs were rescued animals with unknown birthdates and their ages were likely estimated when they were initially adopted. The cause of death, particularly for older animals with a number of disease processes, is often unknown. Because euthanasia is a common, accepted and often encouraged cause of death in dogs, lifespan analysis in pet dogs is limited. At what point an owner decides to euthanize his or her dog will be highly dependent on many factors, including, but not limited to, the age of the dog, the ability of the owner to care for the dog, the dog’s role in the household and ability to maintain that role, and the
attachment between the animal and the owner. Although owners may state that they elect euthanasia because the dog has severe arthritis and is in pain, the dog’s size and ability to be transported outside to urinate and defecate likely play an important role in this decision. Likewise, an owner may be less likely to pursue treatment for a dog that has become aggressive towards family members. The concern of premature euthanasia as a confounding factor in this study, however, is somewhat lessened by the results that showed that dogs that were euthanized actually had longer life spans than those that weren’t.

An additional limitation of this survey was a lack of power to fully investigate the interactions of breed and behavior. Although a fairly large number of respondents participated, there were relatively few in each of the specific breeds. Consolidating the breeds into categories increased the sample size, although the breed categories were rather restrictive, particularly when only those purebred dogs that fell into a single breed category were used. The failure to find more significant interactions could be related to a lack of power, limiting the statistically conclusive effects. It is important that future studies ensure that there will be adequate power to test the hypotheses proposed.

Despite the limitations of the study, a number of conclusions can be made. In particular, estimations of breed and population prevalence of anxiety and fear disorders were determined. This may lead to earlier detection, screening and prevention of behavioral problems in working and companion dogs.

I hypothesized that stress caused by living with anxiety or fearfulness has deleterious effects on health and life-span in canines. I found that fear, specifically the fear of strangers, is related to shortened lifespan. While the presence of fearful or anxious behaviors was not related to death by any particular disease, I did find a significant relationship between skin disorders and anxiety that is intriguing and merits further attention. Further investigation into the cellular mechanisms surrounding skin disorders and their relationships with the nervous system is warranted.

The physical and social environment of the domestic dog is a complex one, with many variables that are difficult to control in experimental research. Further research into these interactions promises to be rich and applicable to many species.
Chapter 5- Conclusion

Like many other species, domestic dogs suffer from fears, anxiety and stress. This dissertation has shown that stress secondary to fear and anxiety has behavioral, HPA axis and health effects on pet dogs. Because we live closely with our canine companions and use them for a variety of tasks, their stress responses also influence human lives. The effects of fear and anxiety in dogs can be measured using a variety of methods and evaluated with a number of different methodologies.

As hypothesized, this thesis shows that physiological stress can be measured non-invasively in domestic canines using salivary stress hormones in a variety of non-laboratory environments. Saliva can be collected in a clinic, kennel and home situation by both animal professionals and by dogs’ owners. It has also been clarified that dogs do not produce adequate amounts of salivary alpha amylase for this to be a useful tool in stress measurement.

Salivary cortisol was shown to increase in response to a stressful stimulus. However, a number of variables do have the potential to interfere with saliva collection for cortisol measurement and must be taken into account when designing studies. This dissertation examined a number of these variables and provides practical guidelines for future studies, including the avoidance of food-based additives in collection materials and assurance of adequate sample quantity. The potential danger of citric acid salivary stimulation was highlighted but the buffering capability of canine saliva was found to diminish this concern. In addition, the usefulness of newer collection materials, specifically hydrocellulose sponges, was demonstrated. While salivary cortisol is a useful tool for stress measurement it does have its limitations in applicability and interpretation. The combination of multiple stress measurement methods is important for the evaluation of canine well-being. Cortisol has been shown to increase in exercise and pleasurable states as well, so is inappropriate as a sole measure of animal welfare. However, it plays a valuable role when used in combination with other behavioral and physiological measurements.
It is known that dogs experience environmental and social stressors in their everyday lives. These vary based on the dog’s use or role within a household. There are, however, a number of fears which are common to many dogs regardless of their specified use, and which affect their daily lives and interactions with their owners and handlers. Fear of thunderstorms is one example and can serve as a model for dogs’ responses to other fear or anxiety inducing stimuli. It was hypothesized that fear in the presence of a known stimulus leads to HPA activation and the initiation of the stress response in canines. The third chapter of this dissertation clearly shows that dogs that suffer from severe thunderstorm fear experience a significant physiological stress response that is long-lasting relative to the stimulus. It is also shown that, at least in this artificial situation, the owners’ response had little effect on the dogs’ physiological or behavioral response. In addition, although one might expect owners to exhibit a stress response to seeing their dog suffer in this way, owners’ HPA axis responses actually decreased following the stimulus. An interesting and unexpected finding of this study was that dogs that lived with other dogs had lower overall cortisol responses. As social animals, it is expected that social environment will affect dogs’ stress responses. The mechanism and direction of these effects on the stress response of dogs is still unclear.

Because previous studies have shown an effect on lifespan and disease processes of stress and fear, it was hypothesized that stress caused by living with anxiety or fearfulness has deleterious effects on health and life-span in dogs. The fourth chapter of this dissertation tests this hypothesis. Although fear and anxiety measurements did not predict incidence of disease, decreased life span was predicted by fear of strangers. In addition, the severity and frequency of skin disorders in individuals was highly predicted by both non-social fear and separation anxiety. Arthritis was also shown to be related to body sensitivity, however, it is likely that body sensitivity is a direct behavioral effect of the pain or discomfort associated with arthritis versus a cause of arthritis in these dogs.

A number of intriguing breed predispositions for fear and anxiety were also uncovered which leads to a hypothesis of further genetic influences on these characteristics. It is difficult to determine prevalence of behavioral characteristics in the general dog population, but these numbers are important in predicting and preventing
escalation of fears into phobias or behavioral problems that interfere with a dog’s functioning in a particular job or as a member of a household.

**Future Research**

The field of applied animal behavior and animal welfare is growing rapidly, particularly as society places increased importance on animals both as pets and as service providers. In addition, dogs provide unique opportunities as research models into specific disease processes. They are social creatures, as humans are, and the role of their social environment (including the presence of humans, other species, and other dogs) on their social and mental development has not been adequately explored. Through years of controlled breeding, they also provide a natural model for behavioral genetics. With the recognition that some of the behaviors are breed specific, or at least occur with increasing frequency in some breeds, and with the decoding of the canine genome, a great opportunity exists for pinpointing the genetic basis of some of these very common fears.

**Measurement Issues**

Exploring basic methodologies for research into the canine stress response will allow for further research into the short and long-term effects of stress. As animal welfare concerns become increasingly important in both research and everyday use and living conditions of domestic animals, it becomes increasingly important to have solid metrics with which to gauge animal well-being. In order to measure the stress response, however, it is important to have valid and accurate scales. Having common research methodology will allow for comparisons among studies.

While salivary cortisol is only one method of measurement, it is useful in studies of canine stress response. Continued investigation into non-invasive measurement of cortisol both in dogs and other species is warranted. Particular attention should be directed towards reliability of measurements and pre- and post-test sampling. Because of
the inter- and intra-individual variability in cortisol, researchers must not place undue value on single measurements. It is hoped that other measures of the physiological stress response will continue to be discovered and utilized in conjunction with mental or behavioral measures.

Further scales must also be developed to measure behavioral response in dogs. The C-BARQ has been very useful in many studies and the predictive value of this scale is being examined by other researchers. Objectively measuring behavior in domestic dogs and refusing to anthropomorphize or draw too many conclusions from their wild relatives is essential. Dogs have evolved over 15,000 years to live in close contact with humans. While studies of wolf behavior have their role in understanding canine behavior in general terms, different selection pressures have separated the species dramatically. Studies of normal domestic dog behavior are warranted to understand abnormal or inappropriate behaviors.

In addition, much could be learned by developing more predictive human-animal interaction scales. Despite the increasing recognition of the “human-animal bond” over the past 20-25 years, this field still struggles with producing sound scientific research. Good research has shown that living with dogs has been shown to have many benefits for human beings. On the other hand, there is much evidence that dogs can cause stress in human lives. How this animal-related stress affects human life and health is a largely unexplored area. Not all stress is bad. I believe that interacting with dogs as living, slightly unpredictable but generally non-judgmental and supportive creatures, is overall beneficial to human beings. Perhaps some of their benefits come from the fact that they do create variability in our lives. Measurement scales to capture this would be enlightening and would allow for non-biased results.

An important component in the future research of these topics will be the inclusion of adequate sample sizes to elucidate differences in these complex relationships. The completion of power analyses before research is initiated is important to determine adequate sample size. The field will benefit greatly from well-designed research with carefully planned analyses.
Fear Specific Issues

In order to further investigate behavioral issues or problems, it becomes increasingly important to distinctly define what is meant by specific terms. In human psychiatric research and medicine, the Diagnostic and Statistical Manual (DSM) was developed to provide consistent diagnoses of mental health illnesses and an objective terminology for researchers to use in correspondence and publishing. No such scale exists for non-human species. As a result, terms such as anxiety and fear, which have been shown to have distinct neuro-physiological bases, are often used interchangeably, adding confusion to the published research literature.

An area of great potential for further discovery is the causation and, inversely, the prevention of fear development. Fear is both an innate and a learned response. How animals are bred, socialized and incorporated into our lives affects the fear responses that will result. While prevention of fear responses would make for better canine companions, it is of utmost importance for dogs that are raised for specific tasks. Police, military, service, and guide dogs require a great deal of training to be able to perform their duties well. A sizeable investment of time and money is put into each individual. Fear related to a single stimulus can render these dogs incapable of performing their required tasks. A service dog with thunderstorm phobia that won’t leave the house three hours before a storm or a police dog with separation anxiety that can’t be left in a squad car without tearing the upholstery out will soon be retired. Preventing these problems in companion animals is also important, as many dogs become destructive and/or aggressive when frightened and then find themselves euthanized or in need of a different home.

The role of human owners and handlers on the development and expression of fear should be examined. There is a great deal of anecdotal information that many people accept as scientific proof. The impact of the dog’s social environment- whether it lives inside or outside, how many other dogs it has contact with, whether living with other species has any effect, has not been investigated. Specific effective socialization techniques and training techniques must be developed. In addition, it would be ideal to be
able to identify animals that may be at risk genetically for these fears, and to intervene with effective techniques if possible.

It is also important to develop reliable and effective treatment modalities to care for dogs that show signs of fear that are disabling to themselves or disruptive to their living situation. A number of pharmacological agents have been used in dogs with separation anxiety and thunderstorm phobia, as well as many other behavioral problems. These treatments have been found to be most effective in combination with behavioral modification. They require expense and time to owners and are not 100% effective. Further treatments, such as pheromone therapy, aromatherapy and nutraceuticals such as melatonin have also been shown to have positive effects on fears and anxieties. It is hoped that new therapies with limited side effects and increased ease of implementation will be developed.

Effects of Fear, Anxiety and Stress on Health and Lifespan

There is great opportunity for the increased investigation of the effects of fear, anxiety and stress on physical health and lifespan. Specifically, the interaction of the skin, endocrine and nervous systems is a very interesting topic of further research. Skin problems are very common reasons for dog owners to seek medical care for their pets. It has also been observed in practice that some of the dogs with the most severe anxiety-related disorders also have skin problems. Whether this is a cause, an effect or an unrelated coincidence is unknown. Perhaps there are common physiological or developmental linkages.

Decreased life-span has been shown to be related to fear of strangers. The specific mechanism of this is unknown. Frequent and uncontrolled stress may work at a molecular level causing accelerated aging of cells and a resulting shortened lifespan. Dogs in a variety of situations often encounter fear-inducing and stressful stimuli. How the stress associated with putting dogs in these situations (working dogs, therapy dogs, military dogs, etc.) affects them is important. Alternatively, the observed effect may be a correlational relationship that is related to another unmeasured factor.
Final Notes

Central to this thesis has been the practical aspect of measurement and applicability of research methods to problems faced by dogs and their owners every day. Evaluation of animal welfare has practical applications in all aspects of animal use. Whether we are examining the welfare of dogs in pet food research facilities, long-term non-euthanasia or traditional rescue facilities, disability assistance programs, animal-assisted therapy or just as pets living in our homes, how our activities affect dogs becomes increasingly important.

We must know these things because it is our responsibility to know them. If we are going to use dogs and put potential stressors on them and keep them in environments different from those in which they’ve evolved, we need to know how these things affect them. There is an increasing public mandate to evaluate the ways in which the stressors animals are under affect their well-being. Only well designed scientific measures and studies will be useful in our current culture of anthropomorphism. Whether animals feel “emotion” as we do is a moot point- stress does affect them physiologically and can have long-term effects on their physical health and well-being. As we use animals as research models for human diseases, as service providers or as companions, we must take into account the role of stress on their lives.
References


Appendix A

Veterinary Clinic Saliva Collection Protocols and Data Collection Forms

Dr. Dreschel's saliva testing in dogs

What is going on with the saliva testing?

Saliva will be collected from pet dogs using a plastic collection pipette or a cotton dental rope. This saliva will then be used in vitro (in a lab at Penn State) to determine if different collection techniques (for example the use of meat flavoring or fruit drink crystals to stimulate salivation) interfere with the determination of salivary hormones. Only the samples will be used for these studies, all the saliva will be collected with an unflavored cotton rope or plastic pipette.

Blood that is already being collected for other diagnostic purposes (e.g. Heartworm testing, complete blood count) from some of the same dogs will be used in in vitro experiments to see if blood contamination in saliva affects hormone measurement in saliva.

Why are we doing it?

There is an increasing use of noninvasive testing of animals for stress using salivary hormones. These tests require very little saliva, but the use of flavorings or fruit drink crystals increases the flow of saliva and the dog's cooperation in giving a sample. However, there is evidence from the human literature that various methods of collection and blood contamination from oral disease or injury may interfere with hormone determination in saliva. This study will elucidate what methods can be used in collecting saliva from dogs without concern for interference with hormone measurement. The ability to measure stress in animals non-invasively has great implications for assessing animal welfare in research, veterinary practice, humane shelters, training, and other situations.
Collection of Saliva Samples

1) Ask the owner for permission to take a saliva sample for a Penn State Research project on hormones in saliva.

2) Don’t collect samples immediately after the dog has eaten a meal or had a treat.

3) Holding one end of the cotton rope, encourage the dog to chew on the other end, trying to moisten the rope as much as possible with the dog’s saliva

   Holding it under the dog’s tongue is also useful if possible.

4) You can encourage the dog to produce saliva by offering him a treat but not giving it to him until the sample collection is complete.

5) Keep the rope in the dog’s mouth for approximately 1-2 minutes, as he/she will allow.

6) Give the dog a treat after collection.

7) Place the cotton rope in the top part of the salivette.

8) Write down the sample number and the patient’s id number on the list.

9) Place the samples in the bag in the freezer.
### Saliva Collection Data Sheet

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Date</th>
<th>Patient id Or full name</th>
<th>Time collected</th>
<th>Ease of Collection 1-easy 5-very hard</th>
<th>Excitement of Dog 1-very mellow 5-very excited</th>
<th>“Stress” of Dog 1-not stressed 5-majorly stressed</th>
<th>Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B

Subject Recruitment for the Behavior and Health Survey

Posters distributed:
Weis supermarkets- State College, PA
Pennsylvania State University Campus, University Park, PA
Tudek Dog Park, State College, PA
Veterinary Hospitals in State College, PA
Pet Supply Stores in State College, PA

Known Forums/groups/listservs print and on-line:

www.petloss.com (2 message boards)
www.lightning-strike.com (pet loss forum)
www.animalforum.com (5 message boards)
Salisbury Maryland Kennel club
Akita-l@apple.ease.lsoft.com (national group)
Belgian Tervuren list-serv
Certified Pet Dog Trainers group
World canine freestyle organization
Homeschooling lists in AZ and IN
Akita club of America
American Veterinary Society Animal Behaviorists listserv and newsletter
News section of the Pennsylvania State University Department of Dairy and Animal Science website (companion animals)
Research studies informational
Vermont Veterinary Medical Association and listserv
DesMoines Obedience Training Club (DMOTCNewsletter@yahoogroups.com)
The Bark Magazine (article appearing 1/06)
Nittany Greyhounds
Daniel Mills (veterinary behaviourist in UK)
Appendix C

Dog Behavior and Health Survey

Hello- Thank you for your interest in our dog health and behavior survey.

There are many factors that contribute to the longevity and health of all animals. We are particularly interested in learning how dogs’ social environments, behavior and personality might affect their long-term health and lifespan. This on-line questionnaire is part of a research project, “The Effect of Canine Anxiety on Canine Health Outcomes”, being conducted through the Biobehavioral Health and Dairy and Animal Science Departments at Penn State University. This information will be useful to veterinarians and others who treat pets and for researchers studying the effects of anxiety on physical health.

To participate you must have owned a dog that has died or been put to sleep within the last 5 years and you must be 18 years or older.

There are 85 multiple choice questions about your dog’s general background, personality, social history, general health and cause of death. You will select your choices by clicking on the answer you desire. It is estimated that the survey will require approximately 30-40 minutes to complete.

Your help in completing this questionnaire is completely voluntary and all responses will be confidential. Your confidentiality will be kept to the degree permitted by the technology used. No guarantees can be made regarding the interception of data sent via the Internet by any third parties. This website and server are designed specifically to provide confidentiality and safety of all data. All responses are automatically encrypted and the survey will not reside in the cache or on this computer when you have completed it. Your responses will not be linked to your name or other identifying information about you. The Office for Research Protections may review records related to this research.
We understand that remembering and answering questions about a deceased pet can sometimes elicit painful memories of your loss. These feelings should not be beyond what a person would encounter in normal daily living. You may withdraw at any time and you may decline to answer any specific questions that you choose. We ask you to share this information so that strides can be made in the understanding of the effects of anxiety on animal health.

If you have any questions about this study, please contact Dr. Nancy Dreschel, DVM at nad5@psu.edu or at (814) 863-4197 or Dr. Douglas Granger at dag11@psu.edu or (814) 863-8402. If you have questions about your rights as a research participant, contact Penn State’s Office for Research Protections at (814) 865-1775.

Thank you very much for your help.

This informed consent form was reviewed and approved by the Office for Research Protections (IRB# 21518) at The Pennsylvania State University on 09-12-2005. It will expire on 09-08-2006. (DWM)

Please print this page for future reference by clicking on the Printer button at the top left of the screen.

Your voluntary completion and submission of the questionnaire imply your informed consent to participate in this research.

If you have read and understand the above statements, please click on the “Continue” button below.

-CONTINUE-
Dog Behavior and Health Survey

Think of the most recent dog you owned that died or was put to sleep.

General Information
What was your dog’s name?
What was your dog’s approximate date of birth? (select-Month/Yr)
How old was your dog when you got it? (select from list)
What sex was your dog? M/F
Was your dog neutered/spayed (“fixed?”) Y/N
At approximately what age was your dog spayed/neutered? (Select from list)
  Less than 5 months old
  5-12 mos.
  1-3 years
  Greater than 3 years old
What was your dog’s breed?
  (Select from List- if mixed, check all that you think apply)
What geographic areas did your dog live in? (select from list- check all that apply)
What year did your dog die? (select from list)
Did you obtain or use your dog for any of the following reasons? (please check all that apply)
  Companionship
  Protection
  Hunting
  Breeding
  Show
  Herding
  Service (e.g. guide dog, hearing dog, etc.)
  Police work
  Military work
Other (please specify)

How much did your dog weigh (approximately)?
(Pick from list of wt. categories)

General Social History
How many people lived in the same home as this dog? (select from list)

What were the age ranges of the people who lived in the home with this dog during the dog’s life? (check all that apply- for example, if a child grew from 1-12 years with the dog, include all ages up to 12 years, also include the parent(s) ages)
0-2 years
3-4 years
5-12 years
13-18 years
19-25 years
26-45 years
46-65 years
Over 65 years

How many other animals lived in the same home as this dog during the dog’s life?
Dogs (0, 1-3, 4-6, more than 6)
Cats (0, 1-3, 4-6, more than 6)
Birds(0, 1-3, 4-6, more than 6)
Other?________

Approximately what percentage of each day did your dog spend outside? (select from list)

Approximately how many hours per day was the dog alone during the day?
When your dog was in his/her “prime” adult life, approximately how much active exercise did he/she get per day (on at least 5 days/week)?

- Less than 15 minutes
- 15-30 minutes
- 30-60 minutes
- Over 1 hour

General behavior history

What was your dog’s obedience school history? (check all that apply)

- No formal training at home or school
- Trained at home by owner
- Puppy kindergarten
- Basic group lessons
- Advanced group lessons
- Private trainer at house
- Private trainer- sent to trainer

On a scale of 1-5, how well do you feel your dog was trained? (1- not well trained, was not responsive to commands, 3- knew some commands, responded sometimes, 5- very well trained, responded quickly to commands)
On a scale of 1-5, how well do you feel your dog behaved? (1- had serious behavior problems, 3- had some problems but nothing serious, 5- was very well behaved, never caused any problems)

General Fearfulness

Dogs sometimes show signs of anxiety or fear when exposed to particular sounds, objects, persons, or situations. At the mild to moderate end of the spectrum, typical signs of fear include: avoiding eye contact, avoidance of the feared object; crouching or cringing with tail lowered or tucked between legs; whimpering or whining, freezing, and shaking or trembling. Extreme fear is characterized by exaggerated cowering, and/or vigorous attempts to escape, retreat or hide from the feared object, person or situation.

Using the following scale (0=No fear, 4= Extreme fear), please indicate your previous dog’s general tendency to display fearful behavior in each of the following circumstances:

When approached directly by an unfamiliar adult while away from your home

When approached directly by an unfamiliar child while away from your home.

In response to sudden or loud noises (e.g. vacuum cleaner, car backfire, road drills, objects being dropped, etc.)

When unfamiliar persons visited your home.

When an unfamiliar person tried to touch or pet the dog.

In heavy traffic (walking or in car).
In response to strange or unfamiliar objects on or near the sidewalk (e.g. plastic trash bags, leaves, litter, flags flapping, etc.)

When examined/treated by a veterinarian.

During thunderstorms.

When approached directly by an unfamiliar dog of the same or larger size.

When approached directly by an unfamiliar dog of a smaller size.

When first exposed to unfamiliar situations (e.g. first car trip, first time in elevator, first visit to veterinarian, etc.)

In response to wind or wind-blown objects.

When having claws clipped by a household member.

When groomed or bathed by a household member.

When stepped over by a member of the household.

When having his/her feet towed by a member of the household.

When unfamiliar dogs visited your home.

When barked, growled, or lunged at by an unfamiliar dog.
Dogs sometimes show signs of anxiety or abnormal behavior when left alone even for relatively short periods of time. Thinking back over the past, how often did you find evidence of or did your dog show each of the following signs of separation-related behavior when left, or about to be left, on its own (never, seldom, sometimes, usually, always)

Shaking, shivering, or trembling

Excessive salivation.

Restlessness, agitation, pacing.

Whining

Barking

Howling

Chewing/scratching at doors, floor, windows, curtains, etc.

Loss of appetite.

General Health History
- What kind of food did your dog eat? (check all that apply)
  Store bought
  Table scraps
  Home cooked
  Other__________
-How often was your dog vaccinated (got “shots”) as an adult? (every year, every 2-3 years, every 4-6 years, less than every 6 years or never)

-Did your dog have any of the following problems as a puppy (less than 1 year of age)? Please rate each problem on a scale from 0-5 (0- did not have the problem, 1- not severe or frequent, 5-very severe or frequent)
  o Allergies (environmental or food)
  o Skin problems
  o Arthritis
  o Muscle/skeletal problems
  o Bacterial Infections (e.g. skin, urinary tract, etc.)
  o Viral infections (e.g. distemper, parvovirus)
  o Heart problems
  o Lung problems
  o Internal Parasites (e.g. Worms)
  o External Parasites (e.g. Fleas, ticks, mange)
  o Colitis
  o Chronic diarrhea
  o Seizures/epilepsy
  o Immune-mediated diseases (e.g. Thrombocytopenia, hemolytic anemia)
  o Endocrine problems- Hypothyroid, diabetes, Cushing’s disease, Addison’s disease
  o Cancer
  o Other _____________________

-Did your dog have any of the following problems as an adult (more than 1 year of age)? Please rate each problem on a scale of severity from 0-5 (0- did not have the problem, 1- not severe or frequent, 5-very severe or frequent)
  o Allergies (environmental or food)
o Skin problems
o Arthritis
o Muscle/skeletal problems
o Bacterial Infections (e.g. skin, urinary tract, etc.)
o Viral infections (e.g. distemper, parvovirus)
o Heart problems
o Lung problems
o Internal Parasites (e.g. Worms)
o External Parasites (e.g. Fleas, ticks, mange)
o Colitis
o Chronic diarrhea
o Seizures/ epilepsy
o Immune-mediated diseases (e.g. Thrombocytopenia, hemolytic anemia)
o Endocrine problems- Hypothyroid, diabetes, Cushing’s disease, Addison’s disease
o Cancer
o Other _____________________

- Was your dog on any medications long-term? (y/n- check all that apply)
  o Flea, tick preventative (e.g. Advantage, Frontline)
  o Heartworm preventative (e.g. Interceptor, Heartguard)
  o Anti-inflammatory- e.g. Aspirin, Rimadyl, Carprofen, Etopesic
  o Glucosamine, Adequan
  o Anti-seizure (e.g. Phenobarbital)
  o Corticosteroids (e.g. prednisone, prednisolone)
  o Thyroid supplementation
  o DES (diethylstilbestrol) (e.g. for urinary incontinence)
  o Phenylpropanolamine (e.g. for urinary incontinence)
  o Anti-anxiety medications (e.g. Clomicalm, amitryptilline, fluoxetine)
  o Tranquilizer (e.g. Acepromazine)
  o Other
Cause of death

How did your dog die?

Natural Death due to:

Disease(s) (please specify if known) ________________________

Accident/ Trauma

“Old Age”

Unknown

Other (please specify)

“Put to sleep”/ Euthanized due to:

Disease(s) (please specify if known) ________________________

Injuries from accident/trauma

Old age

Behavior Problems (please specify)

Other (please specify)

Thank you very much for your participation. If you would like to be entered in a drawing to win one of four $20.00 prizes, please enter your name, address, telephone number and e-mail address. This information will not be used for any other purpose and will not be connected in any way with your survey answers.

Name-
Address-
Phone number-
e-mail address-Start here
VITA

Nancy A. Dreschel

Department of Dairy and Animal Science
324 Henning Building
The Pennsylvania State University
University Park, PA 16802
(814) 863-4197; nad5@psu.edu

Personal Data
Home: 207 Morningside Circle
       State College PA, 16803
       (814) 867-9070
Birthdate: January 9, 1964
       Ithaca, NY

Educational History
Cornell University, College of Agriculture and Life Science
B.S. 1986
Major: Animal Science

Cornell University, New York State College of Veterinary Medicine
D.V.M. 1989
Honors: Expanding Horizon Fellowship-Institut Agronomique et Veterinaire- Rabat, Morocco
Senior Seminar Title: The epidemiology of white muscle disease in Morocco.

Pennsylvania State University
PhD 2007
Major: Biobehavioral Health
Thesis: The Psychobiological Effects of Fear and Anxiety in Domestic Canines
Advisor: Douglas Granger, PhD
Honors: University Graduate Fellowship 2001
         2003-2004 Mary Boyle Weaver and Rebecca Boyle Sutherland Scholarship
         2004-2005 Ruth W. Ayres-Givens Scholarship

Professional Experience
Penn State University Department of Dairy and Animal Science November 2004-Present
University Park, PA
Instructor, Small Animal Science

Penn State University Department of Biobehavioral Health September 1999-November 2004
University Park, PA
Research Assistant, Teaching Assistant, Project manager

Centre Animal Hospital- State College, PA
Animal Medical Hospital- State College, PA
Suburban South Veterinary Hospital- Depew, NY
Hermitage/Conneaut Lake Veterinary Clinics- Hermitage and Conneaut Lake, PA
Private veterinary practitioner