2014

White Blood Cell Counts, Parasite Prevalence, and Plasma Cortisol Levels of Dogs in a County Animal Shelter: Changes over Days and Impact of a Program of Repeated Human Interaction

Emily S. Dudley
Wright State University

WHITE BLOOD CELL COUNTS, PARASITE PREVALENCE, AND PLASMA CORTISOL LEVELS OF DOGS IN A COUNTY ANIMAL SHELTER: CHANGES OVER DAYS AND IMPACT OF A PROGRAM OF REPEATED HUMAN INTERACTION

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By

EMILY SUZANNE DUDLEY
D.V.M., The Ohio State University, 1998
B.S., Wright State University, 1994

2014
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

June 16, 2014

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Emily Suzanne Dudley ENTITLED White Blood Cell Counts, Parasite Prevalence, and Plasma Cortisol Levels of Dogs in a County Animal Shelter: Changes over Days and Impact of a Program of Repeated Human Interaction BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

_____

Michael Hennessy, Ph.D.
Thesis Director

_____

Barbara Hull, Ph.D.
Director, Microbiology and Immunology Program

Committee on
Final Examination

_____

Michael Hennessy, Ph.D.
Professor, Department of Psychology

_____

Nancy Bigley, Ph.D.
Professor, Microbiology and Immunology

_____

Cheryl Conley, Ph.D.
Director, Clinical Laboratory Science Program

_____

Robert Fyffe, Ph.D.
Vice President for Research and Dean of the
Graduate School

ABSTRACT

Dudley, Emily Suzanne. M.S. Microbiology and Immunology, Wright State University, 2014. White Blood Cell Counts, Parasite Prevalence, and Plasma Cortisol Levels of Dogs in a County Animal Shelter: Changes over Days and Impact of a Program of Repeated Human Interaction.

Animal shelter housing is highly stressful for a dog, compromising welfare and leading to undesirable behaviors and unknown health consequences. We documented the changes in circulating numbers of white blood cells, plasma cortisol, and fecal parasite shedding of dogs housed for 10 days at a county animal shelter. White blood cell changes were most prominent on Day 10 after arrival to the shelter. Changes included increased total leukocytes, mature neutrophils, and lymphocytes, with less consistent increases in monocytes and neutrophil to lymphocyte ratio (N:L). Fecal parasite shedding was elevated and not affected by day. Cortisol levels of shelter dogs declined over time, and when compared with dogs living in stable home environments were higher on all days measured (1, 3, and 10). Total leukocytes, neutrophils, monocytes, and N:L were also higher in shelter dogs than control dogs. Petting sessions of 30 minutes daily for 10 days reduced the cortisol of shelter dogs, but did not have an effect on white blood cells

or parasite shedding. This study documents high rates of parasitic infection, large and increasing immunological responses, and plasma cortisol elevations of dogs in an animal shelter. Increasing opportunities for daily interaction with caregivers is likely to improve the welfare of shelter dogs, but additional research must be done to identify potential health benefits.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table Page

ACKNOWLEDGEMENT

Acknowledgements: I would like to thank Mitchel Aselage, Jennifer Beasley, Ellen Claiborne, Sean Collins, Feras Deek, Josh Forman, Aaron Fowler, Caroline Foy, Darci Gallimore, Alexis Garybush, Samantha Hagerty, Kalia Haile, Alex Hensley, Casey Hess, Kendra Kerner, Greg Lowery, Sandra Matthews, Colton Metzger, Nikole Ronfeldt, Matt Shiverdecker, Cassandra Tolliver, Samantha Watts, Regina Willen, Elizabeth Wise, and Neena Zwier for their tireless assistance with the dogs. In addition, this work would not have been possible without the support of Mick Sagester and the dedicated employees of the Montgomery County Animal Resource Center, to which I am grateful.

Financial support for this work was derived through Wright State University's Microbiology and Immunology Program, Graduate Student Assembly, and a grant from the Waltham Foundation.

I. INTRODUCTION

An estimated 5-8 million dogs and cats arrive at animal shelters annually in the United States, and stay for variable lengths of time[1]. Of those animals arriving at shelters, approximately 3-4 million are euthanized annually, significant numbers of which have been determined to have behavioral or physical health deficiencies[1,2].

Animals arriving at a shelter are often afflicted with infectious diseases and likely to be exposed to infectious agents while there[3,4]. The prevalence of intestinal parasites among dogs housed in animal shelters has previously been reported as 36-50%, while upper respiratory infections and gastrointestinal parasites are among the most common conditions reported in dogs one week after adoption from a shelter[5,6]. Upper respiratory infections and gastrointestinal diseases may be caused by a variety of bacterial, viral, or parasitic pathogens, and all shelters experience infectious disease outbreaks[4]. On the whole, one might expect the likelihood of infection to increase during the course of a dog's stay in a shelter, though this possibility does not appear to have been examined previously. Shelter management procedures in place to minimize the extent of infection often focus on sanitation and disease communicability; however another important aspect to also consider is the effect of stress on the animal's ability to mount an effective immune response.

While in animal shelters, dogs are exposed to multiple stressors, including confinement, noise, and social instability. Separation from human companions and loss of control in an unpredictable, novel environment also likely contribute to stress. While acute behavioral responses of stress such as barking and whining are readily observable in shelter dogs, long term consequences may also occur, including undesirable behaviors associated with separation anxiety[7]. Human interaction reduces measures of stress while in the shelter environment, and can prevent acute undesirable behaviors and encourage more sociable behavior[8,9]. In a recently published study, shelter dogs receiving daily enrichment and behavioral training spent more time sitting or lying down and less time vocalizing, while dogs that did not receive training and enrichment exhibited increases in jumping, whining, barking, and growling[10]. It is possible that the human interaction contributed to the observed effects in addition to the training itself.

There are two primary physiologic systems activated by stressors, the sympathetic adrenal medullary (SAM) axis and the hypothalamic-pituitary-adrenal (HPA) axis. Stimulation of the SAM axis results in the release of catecholamines, primarily epinephrine, from the adrenal medulla[11-13], while stimulation of the HPA axis causes a cascade of endocrine responses. The HPA axis response is initiated by the secretion of corticotropin releasing hormone (CRH) from the hypothalamus. CRH stimulates the anterior pituitary to secrete adrenocorticotrophic hormone (ACTH), which in turn stimulates the adrenal cortex to secrete glucocorticoids[12,13]. The specific glucocorticoid secreted is species dependent; dogs secrete cortisol primarily[12].

Acute stress, defined as lasting for minutes to hours, is often immunoprotective, enhancing innate and adaptive responses. In contrast, chronic stress can suppress or dysregulate immune function, and is considered immunopathologic[14]. Cortisol suppresses multiple aspects of immune function through effects on cells including natural killer (NK) cells, B lymphocytes, T lymphocytes, and macrophages[15]. The combined actions of epinephrine, norepinephrine, and cortisol also change the relative numbers of circulating white blood cells in preparation for possible injury or infection[14,16]. In dogs, these changes include increased numbers of total leukocytes and mature neutrophils, and decreased numbers of lymphocytes. Monocytes are less consistently elevated[17]. Changes likely occur due to immune cell redistribution and may compromise the immune system's effector and surveillance roles, contributing to some of the immunosuppressive effects of stress[13,16,18-20]. Impaired neutrophil migration into tissues leaves the individual more susceptible to infection[21]. In a study evaluating the effects of 50 minutes of automobile transport stress on dogs, the total leukocytes, neutrophils, and neutrophil to lymphocyte ratio (N:L) were all significantly elevated 3 hours after transport[12]. Another study in dogs evaluating the effects of ground and air transport found significant elevations in neutrophils and N:L, and a significant reduction in lymphocyte counts after each type of transport when compared with baseline values[22]. Although both studies confirmed significant elevations in salivary cortisol at the time of transport, it is unknown if white blood cell changes were associated with altered immune system function.

The health consequences of stress are well documented in humans and laboratory animals, and include impairment of wound healing, increased susceptibility to viral and bacterial infections, modulation of vaccination response, and reactivation of latent Herpesviruses[13,23-28]. Parasites may more readily infect immunocompromised individuals, and if already infected with certain parasites, the immunocompromised are likely to develop more-severe disease[29]. A study in calves found that exposure to respiratory pathogens at the stressful time of weaning and maternal separation significantly increased mortality to levels twice that of calves experiencing weaning and maternal separation two weeks prior to exposure[28].

It is well known that the stress of shelter housing results in elevations of cortisol levels in dogs. Previous studies found highest cortisol levels during the first 3 days after arrival at the shelter; after 10 days the cortisol levels were more comparable to dogs sampled in a home environment[30,31]. However, the immunologic effects of stress in dogs or cats housed in an animal shelter have not been fully evaluated. One study found that 60% of cats developed upper respiratory infections while in a shelter, with infections positively related to behavioral indicators of stress[32]. To the author's knowledge, comparable studies evaluating the stress of shelter housing on immune function in dogs have not been published. In both studies previously cited evaluating transport stress in dogs, the dogs were not shelter dogs, and are presumed to have not been exposed to a pathogen rich environment, as would be found in an animal shelter. The potential exposure to infectious agents while in the shelter environment is a

variable with unknown impact on the function and response of the immune system, particularly when occurring simultaneously with the stress response.

Previous studies in shelter dogs have demonstrated that cortisol elevations can be attenuated through various types of human interaction[8,31,33,34]. In each of these studies, dogs were provided a single human interaction session of 20-45 minutes, with one exception, in which dogs received a 25 minute session on two separate days[8]. It is unknown whether the positive effects of human interaction persist if additional petting sessions are provided during prolonged animal shelter housing, or if dogs become habituated to the effect. Moreover, it is unclear how petting might affect immunological measures in shelter dogs.

The present study was designed to examine measures of infection and immunological activity and their relations to cortisol levels in dogs during their first 10 days in a shelter. In the first experiment, we measured the changes in the numbers of circulating white blood cells which occur after arrival at the shelter, hypothesizing that total leukocytes, neutrophils, and monocytes would increase, and lymphocytes would decrease. In addition we monitored the shedding of parasites in the feces, hypothesizing that fecal shedding of parasites would increase over the 10 day experiment. Finally, we evaluated the relationship of these changes to plasma cortisol levels. In the second experiment, we evaluated whether repeated human interaction sessions over a 10 day period would continue to reduce cortisol levels of dogs and whether human interaction would also reduce the immune and infection measures documented in Experiment 1.

II. MATERIALS & METHODS

Animals. A total of 92 purebred and mixed breed dogs from the Montgomery County Animal Resource Center (MCARC) in Dayton Ohio were enrolled in the study within 30 hours after arrival at the shelter. Dogs were either brought to the shelter as strays, or released by their owners to the shelter. The day of arrival to the shelter was considered day 0, and experimental procedures began the following day. All dogs were between the ages of 6 months and 7 years, as evidenced by lack of deciduous teeth and absence of age related ocular changes (cloudiness of the lens due to lenticular sclerosis). Fifty male and 42 female dogs with body weights ranging from 5.2 kg to 37.8 kg were included. Both intact and gonadectomized dogs were included, although dogs known to be pregnant were not. Dogs were excluded from the study if aggressive or fearful behaviors were displayed or if evidence of imminent owner claim was present. Those with signs of infectious disease including diarrhea or upper respiratory infection were not enrolled, however if symptoms developed while participating in the study, the dog was excluded only when clinical treatment was necessary according to routine shelter procedures. All dogs were single housed in a large intake ward for the duration of the 10 day study. Dogs were housed in one of 75 size-appropriate kennel runs, most measuring 1.5 x 1.2 x 1.8 m. Kennels were arranged on either side of central aisles, with several aisles present within the ward. Individual kennels were constructed of smooth, solid surfaces on three sides, with the front of the kennel constructed of a metal mesh, allowing the dog visual

and auditory contact with others. In addition to the dogs housed at the MCARC, a group of 15 privately owned dogs were evaluated. These dogs were subjected to the same exclusion criteria as the dogs housed at the MCARC.

Blood Sampling Procedure and Laboratory Analysis. Blood samples were collected most often from the cephalic vein of each dog, however depending upon individual dog anatomy and comfort, the lateral saphenous vein or jugular vein were occasionally used as a venipuncture site. Blood samples were collected between 1300 and 1700 hours to control for normal circadian cortisol fluctuations. The time from approach to the cage to sample collection was recorded. The average time required for sample collection was 122 seconds; 95% of samples were collected in less than 4 minutes. Approximately 1 ml of blood was put into a standard clinical collection tube containing the anticoagulant ethylenediaminetetraacetic acid (EDTA), and 1 ml of blood was placed in a collection tube containing 250 IU heparin. All samples were kept on ice until returned to the laboratory for processing. Once in the laboratory, heparinized samples were centrifuged at 2,000 rpm for 20 minutes, and then plasma was removed and frozen until later cortisol analysis. EDTA samples were refrigerated subsequent to complete blood count (CBC) analysis, which occurred within 48 hours of sample collection. Cortisol was measured in duplicate with a standard radioimmunoassay procedure (cortisol Coat-a-Count, Siemens) as in previous studies[34]. Intra and inter-assay coefficients of variation were 7.3% and 19.5% respectively. An automated veterinary hematology unit, the Abaxis HMII or Abaxis HM5, was utilized for CBC analysis. In Experiment 1, the HMII was used and a white blood cell differential manually determined from a Giemsa stained thin

blood smear. Absolute numbers of white blood cells were calculated [e.g. % neutrophils x total white blood cell count = absolute neutrophil count], and the neutrophil to lymphocyte ratio (N:L) determined [absolute neutrophil count/absolute lymphocyte count]. In Experiment 2 the HM5 was used, which automatically differentiates white blood cells and calculates absolute cell counts. N:L was determined as in Experiment 1.

Fecal Analysis. Naturally voided fecal samples were collected from dogs, placed on ice and transported back to the laboratory for analysis. Feces were analyzed for parasite ova using the centrifugal sucrose flotation procedure as previously described[35] . The entire slide was examined using the 10X objective and results recorded. Parasites were identified as roundworm, hookworm, whipworm, tapeworm, or coccidia and the level of fecal shedding quantified by parameters listed in Table 1.

Table 1. Quantification of fecal parasite shedding level.

0	Negative, no ova present.
1	Positive, fewer than 10 ova present on slide. Ova can be from a single or multiple parasite species. Most fields of view on the slide contain no ova.
2	Positive, more than 10 total ova present on slide. Ova can be from a single or multiple parasite species. Most fields of view on the slide contain no ova, but many contain a single ovum and some may contain groupings of up to 10-15 ova.
3	Positive. Ova can be from a single or multiple parasite species. Most fields of view on the slide contain no ova, but many contain groupings of more than 10-15 ova.
4	Positive. Ova can be from a single or multiple parasite species. Most fields of view on the slide contain ova, many with groupings of ova too numerous to count.

Experiment 1. In Experiment 1, we evaluated the effect of day and sex on plasma cortisol, CBC, and fecal shedding of parasites. Forty shelter dogs were enrolled in

Experiment 1. Due to owner claim, 7 of the dogs did not complete the study, leaving a final group size of 33, with 15 privately owned dogs serving as a control group. Shelter dogs were evaluated on Days 1, 3, and 10 after arrival at the shelter. On each of these days, dogs were taken out of their kennels and a blood sample collected for CBC and cortisol analysis. Fecal samples were collected on the same days as blood samples. A single fecal sample and blood sample were collected from each control dog for parasite evaluation, CBC, and cortisol analysis.

Experiment 2. In Experiment 2, we evaluated the effect of human interaction on plasma cortisol, CBC, and fecal shedding of parasites over a 10 day period. Experimental groups included Home Cage, Novel Room, and Petting; dogs were quasi-randomly assigned to experimental groups in the order which they were enrolled in the study. Fifty two dogs were enrolled; however, due to owner claims and a single dog that developed gastrointestinal disease requiring medical intervention, only 47 completed the study. Group size was 16 dogs in the Home Cage group, 16 dogs in the Novel Room group, and 15 dogs in the Petting group.

In the home cage group, dogs were taken from the home kennel and walked in a small enclosed courtyard outside for a 2-3 minute period of time, then returned to their kennel. In the novel room group, dogs were removed from their kennel, walked in the courtyard for 2-3 minutes, then taken to a separate room in the animal shelter and placed in a kennel measuring 1.5 x 1.5 x 1.8 meters with a small blanket placed on the floor. The room was also used by the shelter for storing equipment and supplies; no other dogs were present in this room. Study personnel left the room for 30 minutes

before returning to take the dog back to its original kennel. In the petting group, dogs were removed from their kennel, taken to the courtyard for a 2-3 minute walk, then taken to the novel room and placed in the kennel with a blanket. In addition, a female member of the research team stayed with the dog in the novel environment. This "petter" spoke to the dog in a soothing voice and performed a combination of petting and deep tissue massage around the head, neck, and shoulders of the dog for 30 minutes. At the end of the petting session, the dog was returned to its home kennel. The home cage, novel room, and petting activities occurred daily with the exception of weekends and holidays; each dog received 7-8 experimental sessions over the 10 day study.

Blood samples were collected from all dogs on Days 1 and 10 after arrival at the shelter. On both days, blood samples were collected for cortisol analysis and CBC immediately prior to the experimental session, with an additional blood sample collected immediately after the experimental session for cortisol analysis. Because there was no experimental session for dogs in the Home Cage group, these dogs were returned to their cages for 30 minutes after collecting the first blood sample prior to collection of the second on days 1 and 10. Two fecal samples were collected from each dog, the first on day 1, 2, or 3, and the second on day 10.

All procedures were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Animal Welfare Act and

Animal Welfare Regulations. Wright State University's Institutional Animal Care and Use Committee reviewed and approved all procedures.

Data Analysis. Parametric tests were the preferred means of analysis. When variance was not homogenous as determined by the Levene test, data were transformed logarithmically; if that was ineffective, then square root transformation was performed. For ease of presentation raw data are used in all figures. Nonparametric tests were used for data that were not on an interval scale. Data were analyzed using SPSS, and $p = 0.05$ (2-tailed) was considered statistically significant. Details for individual experiments follow.

In Experiment 1, a 2 (sex) by 3 (day) repeated measure Analysis of Variance (ANOVA) was used to assess white blood cell counts and plasma cortisol. Huynh-Feldt correction was applied when sphericity was significant ($p < 0.01$). Values in shelter dogs on Day 1 were compared with those of privately owned (control) dogs with Student's *t*-test or Mann-Whitney *U* test, as appropriate. When values changed over days for shelter dogs, additional select comparisons between shelter dogs on later days and control dogs were sometimes made. Exploratory analysis of associations of cortisol with fecal and white blood cell results was performed using Spearman Rho correlation coefficients. Visual inspection of fecal results indicated similar patterns for male and female dogs, so these data were combined. The McNemar's test was used to assess changes in parasite incidence over days, while the Fisher test compared parasite incidence in shelter dogs to control dogs. The level of fecal parasite shedding was

evaluated for changes over days with Friedman's two-way ANOVA, while the Mann Whitney *U* test was used to compare these data with those of control dogs.

No significant sex differences were found for any measure in Experiment 1; therefore in Experiment 2, data from male and female dogs were combined. In Experiment 2, a 3 (Condition) X 2 (Day) ANOVA (with day treated as a repeated measure) was used to assess white blood cell counts, while cortisol levels were assessed using a 3 (Condition) X 2 (Day) X 2 (Pre/Post) ANOVA (with day and pre/post treated as repeated measures). Significant interactions were further evaluated using tests for simple main effects[36]. Post-hoc paired comparisons were assessed using the Newman-Keuls test. For fecal measures, changes over days were evaluated with the McNemar test for incidence of shedding, and with Wilcoxon paired comparisons for level of shedding. Comparisons among conditions on each day were made with Chi Square tests for incidence of shedding, and with Kruskal-Wallis for level of shedding.

III. RESULTS

Experiment 1. Total leukocytes $F(1.43, 35.82) = 8.45$, $p < 0.005$, neutrophils $F(1.43, 35.62) = 6.87$, $p < 0.01$, and lymphocytes $F(2, 50) = 7.07$, $p < 0.005$ rose significantly by the end of the 10 day period, however there were no significant changes in monocytes or N:L in shelter dogs (Figures 1a-1e). When compared with control dogs, total leukocytes, neutrophils, monocytes, and N:L were higher in shelter dogs on Day 1 (total leukocytes: $t(52) = 5.00$, $p < 0.001$, neutrophils: $t(52) = 5.86$, $p < 0.001$, monocytes: $t(51) = 2.19$, $p < 0.05$, N:L: $t(52) = 3.43$, $p = 0.001$). There was no difference between control dogs and shelter dogs in lymphocytes on Day 1, or even on Day 10, when lymphocytes were highest in shelter dogs.

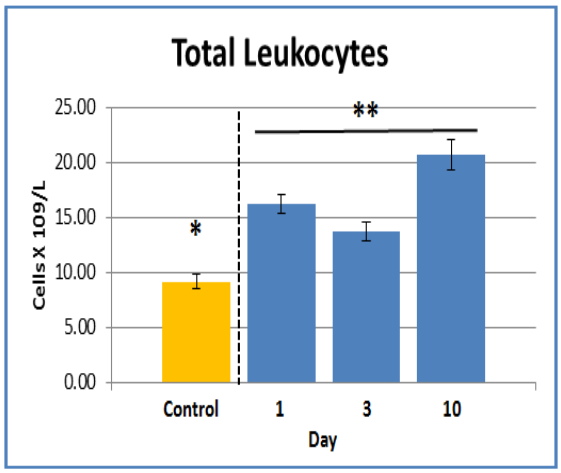

Figure 1a.

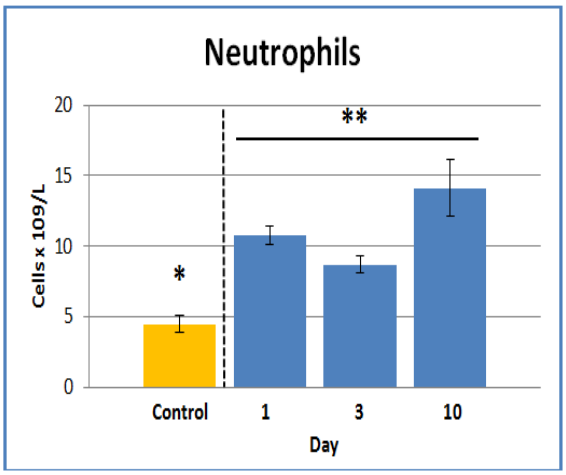

Figure 1b.

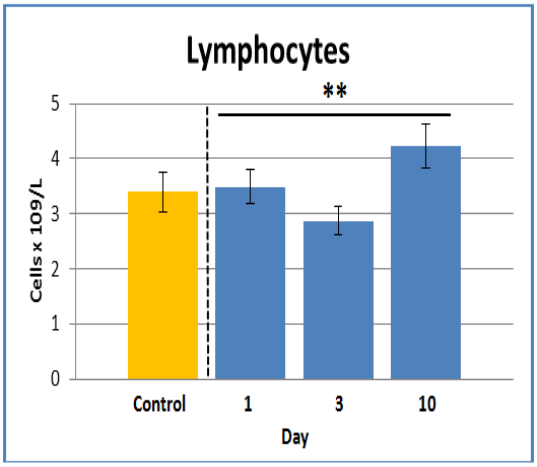

Figure 1c.

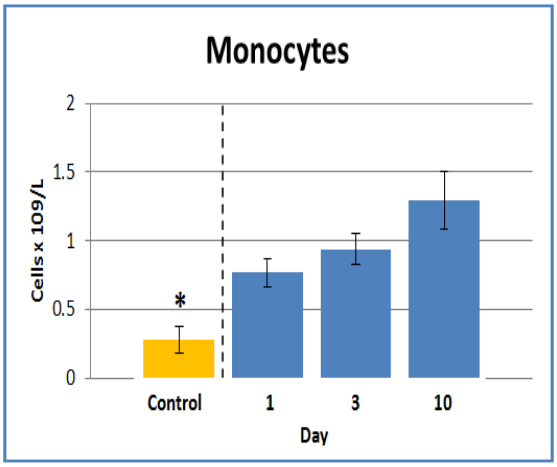

Figure 1d.

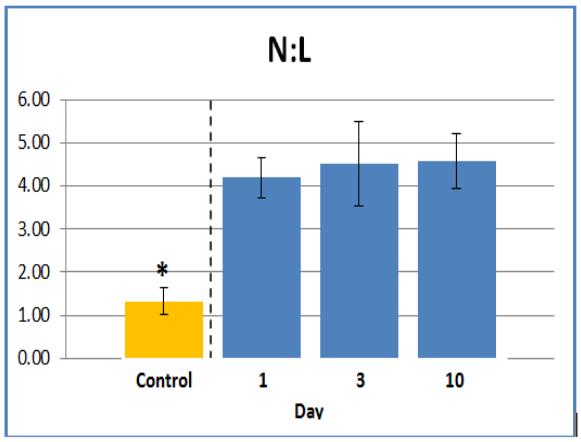

Figure 1e.

Figure 1a-1e. Leukocyte counts (mean ± SE) of control dogs and of shelter dogs on Day 1, 3, and 10 in the shelter. *Significant difference between control dogs and shelter dogs on Day 1. **Significant difference over days in shelter. 1a. Leukocyte counts were lower in control dogs than shelter dogs on day 1 ($p < 0.005$), and changed significantly across days in shelter ($p < 0.001$). 1b. Neutrophil counts were lower in control dogs than shelter dogs on Day 1 ($p < 0.01$) and changed significantly across days in shelter ($p < 0.001$). 1c. Lymphocyte counts changed significantly across days in shelter ($p < 0.005$). 1d. Monocyte counts were lower in control dogs than shelter dogs on Day 1 ($p < 0.05$). 1e. Neutrophil to lymphocyte ratio was lower in control dogs than shelter dogs on Day 1 ($p = 0.001$).

Cortisol concentrations were highest in shelter dogs on Day 1, and declined significantly over the 10 day study $F(2, 52) = 5.35$, $p < 0.01$ (Figure 2). Cortisol concentrations were higher in shelter dogs when compared with control dogs on Day 1, $t(52) = 4.22$, $p < 0.001$. Even on Day 10, when cortisol levels were lowest in shelter dogs, they still were significantly higher than in control dogs $t(45) = 3.70$, $p = 0.001$.

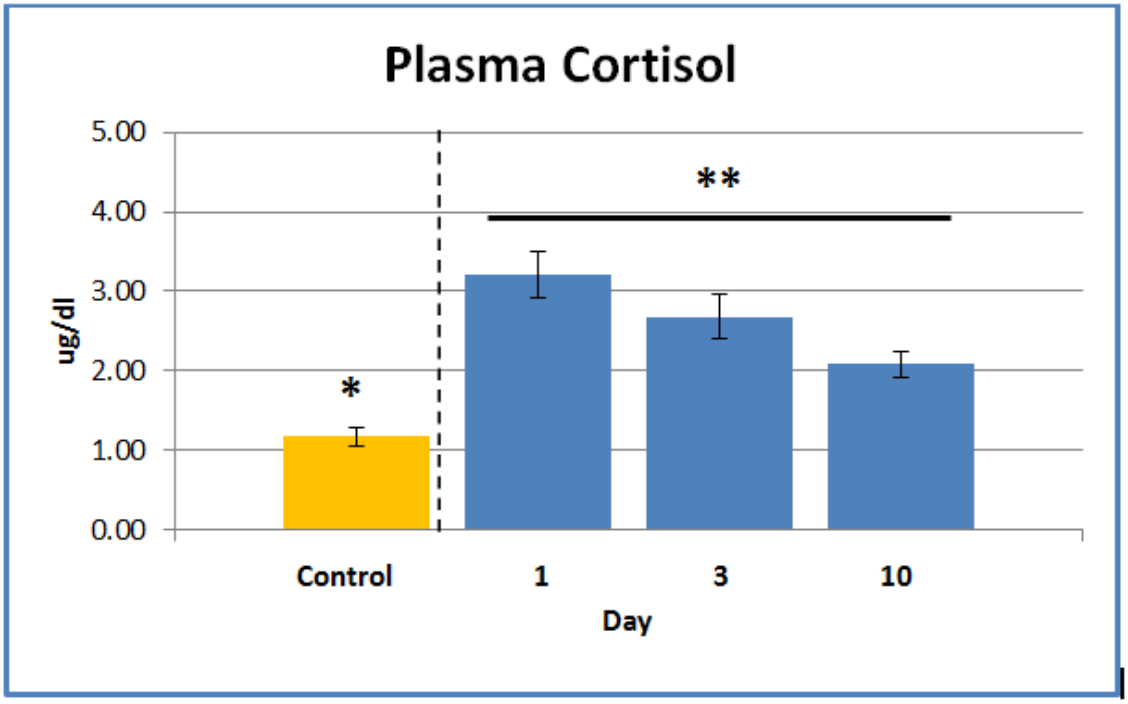

Figure 2. Plasma cortisol levels (mean ± SE) of control dogs and shelter dogs on Days 1, 3, and 10. *Cortisol levels of control dogs differed from those of dogs during their first day at the shelter ($p < 0.001$) and remained lower even than those of dogs during their 10th day at the shelter ($p = 0.001$). **Cortisol levels of shelter dogs were highest on Day 1 and declined significantly over the next 10 days ($p < 0.01$).

Over the course of the 10 day study, 35% to 50% of shelter dogs were shedding parasites in their feces. Of dogs with parasites, 20% were infected with multiple parasites while a single parasite type was identified in the remainder. Parasites present (in order of most to least common) included: whipworms, hookworms, roundworms,

and tapeworms. Day did not have a significant effect on parasite presence in feces (Figure 3), nor did it affect the level of parasite shedding (data not shown). Of dogs with parasite ova present in the feces, 17% were considered heavy shedders (given a score of 4) on Day 1, 33% on Day 3, and 31% on Day 10. The incidence of fecal parasite shedding was significantly higher in shelter dogs when compared with control dogs, not only on Day 1 ($p < 0.05$), but also on the day the fewest shelter dogs were shedding parasites (Day 3) ($p < 0.05$).

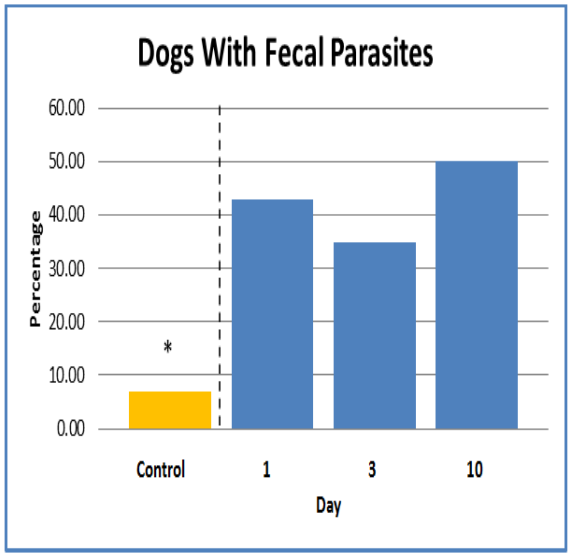

Figure 3. Percentage of shelter dogs on Days 1, 3, and 10 and control dogs that were positive for one or more parasite. * The incidence of fecal parasite shedding was significantly lower in control dogs when compared with shelter dogs, not only on Day 1 ($p < 0.05$), but also on the day the fewest shelter dogs were shedding parasites (Day 3) ($p < 0.05$).

Exploratory correlational analysis of cortisol levels with other measures yielded only 1 significant effect out of 63 comparisons; therefore this effect was considered spurious and not considered further.

Experiment 2 Analysis of white blood cell measures indicated that for most parameters, there was a significant main effect of day, with the highest levels on Day 10 for total leukocytes $F(1, 44) = 76.89$, $p < 0.001$, neutrophils $F(1, 44) = 86.09$, $p < 0.001$, lymphocytes $F(1, 44) = 5.55$, $p < 0.05$, monocytes $F(1, 44) = 16.82$, $p < 0.001$, and N:L $F(1, 44) = 20.72$, $p < 0.001$ (Figures 4a-e). There was a significant main effect of group

on neutrophils $F(2, 44) = 4.44$, $p < 0.05$. Neutrophils were higher in the Home Cage group dogs than the Novel Room or Petting group dogs ($p < 0.05$). There was no evidence that daily petting affected any of the CBC parameters.

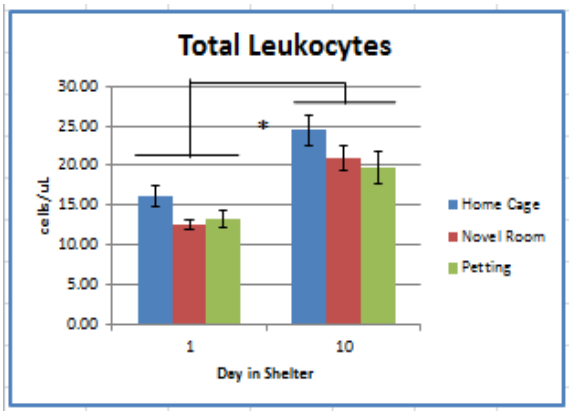

Figure 4a.

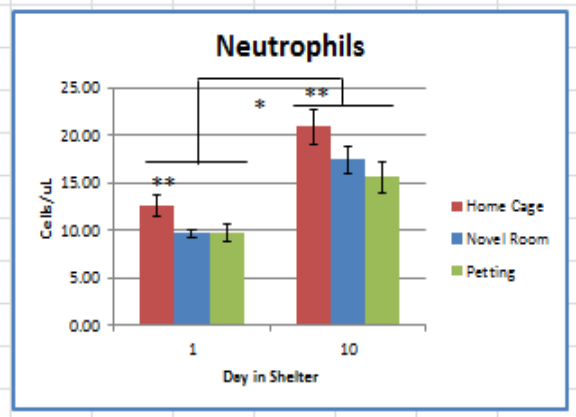

Figure 4b.

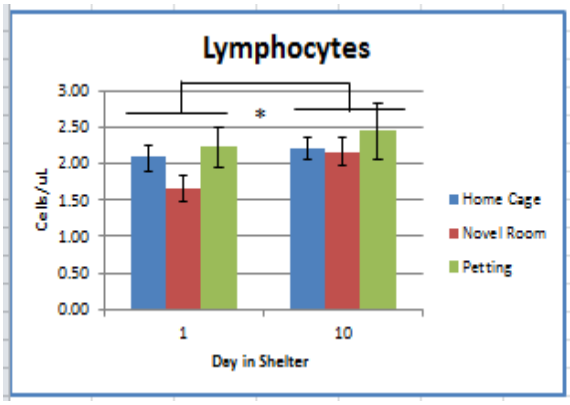

Figure 4c.

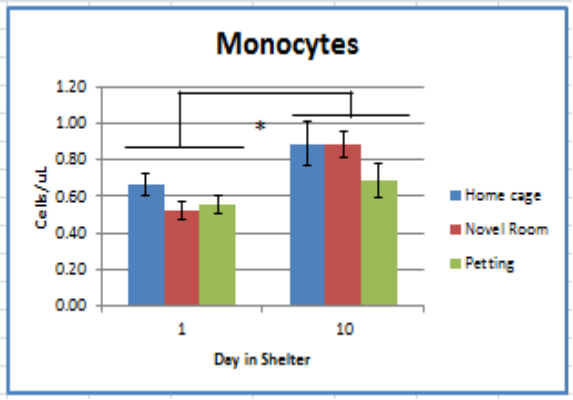

Figure 4d.

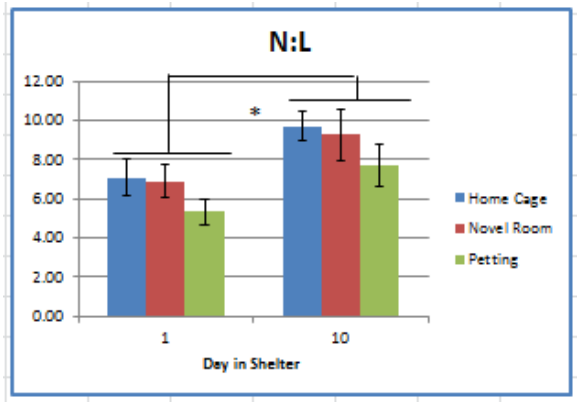

Figure 4e.

Figures 4a-4e. Leukocyte counts (mean ± SE) of dogs on Days 1 and 10 in the shelter. *Significant difference between Day 1 and Day 10. **Significant difference among experimental groups. 4a. Leukocyte counts were higher on Day 10 ($p < 0.001$) 4b. Neutrophil counts were higher on Day 10 ($p < 0.001$) and were higher in the Home Cage group than the Novel Room or Petting groups ($p < 0.05$). 4c. Lymphocyte counts were higher on day 10 ($p < 0.05$). 4d. Monocyte counts were higher on Day 10 ($p < 0.001$). 1e. Neutrophil to lymphocyte ratio was higher on Day 10 ($p = 0.0001$).

ANOVA for cortisol yielded significant main effects for Day, F (1, 44) = 20.68, $p <$ 0.001, and Pre/Post, F(1, 44) = 4.74, $p < 0.05$. These main effects were qualified by significant interactions of Group x Day, F(2, 44) = 4.60, $p < 0.05$, Group x Pre/Post, F(2, 44) = 8.04, $p < 0.001$, and Day x Pre/Post, F(1, 44) = 4.77, $p < 0.05$. Further analysis of the Group X Day interaction with simple main effects yielded a marginally significant effect of Group on Day 1 (Mean ± SE = Home Cage: 3.02 ± 0.60; Novel Room: 4.02 ± 0.60; Petting: 1.79 ± 0.62, $p < 0.1$) and no effect on Day 10. For the Day X Pre/Post interaction, simple main effects showed that across groups, there was a significant decline in cortisol levels from the pretest to the post-test on Day 10 ($p < 0.01$), but not on Day 1 (Day 1: 2.86±0.38 pre, 3.09±0.37 post; Day 10: 1.76±0.12 pre, 1.48±0.17 post). Finally, and most importantly, for the interaction of Group X Pre/Post, simple main effects showed that petting reduced cortisol levels from the pretest to the post-test regardless of day of testing ($p < 0.01$), whereas there was no significant change from pretest to post-test in the Home Cage or Novel Room groups (Figure 5).

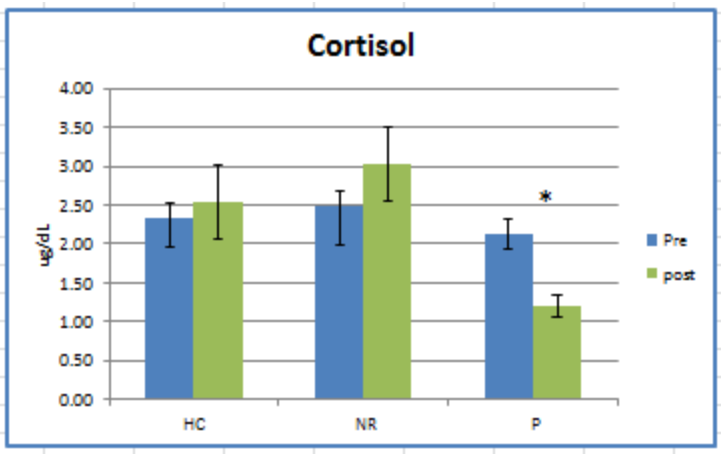

Figure 5. Changes in cortisol level (mean ± SE) from pretest to post-test in each experimental group. The cortisol data are averaged over days for each experimental group. HC = Home Cage, NR = Novel Room, P = Petting. *Significant difference in cortisol level before and after petting ($p < 0.05$).

Similar to Experiment 1, infection with multiple parasites was common. Parasites present (in order of most common to least common) included: whipworms, hookworms, roundworms, and coccidia. There were no differences in the percentage of dogs shedding parasites among experimental groups on Day 1 or Day 10, and there were no differences in percentage of dogs shedding parasites on Day 1 and Day 10 in any experimental group. Overall, results of the first fecal exam (from day 1, 2, or 3) indicated that 41% of dogs were shedding parasites, while on day 10, 37% were shedding. There were no differences in the level of parasite shedding among experimental groups on Day 1 or Day 10. However, when comparing the changes in level of parasite shedding between Days 1 and 10 within each experimental group, a significant difference was found in the Novel Room group (Figure 6). Although the level of parasite shedding among dogs in the Home Cage and Petting Groups did not change from Day 1 to Day 10, dogs in the Novel Room group shed fewer parasites on Day 10 (*p*

< 0.05). Of dogs positive for parasites, 29% were considered heavy shedders at the beginning of the study (5 dogs in the Petting group and 1 dog in the Home Cage group). At the end of the study, only 14% of dogs were considered heavy shedders, including 1 dog in the Petting group and 2 dogs in the Home Cage group. Nonetheless, there was no statistical evidence that daily petting reduced parasite shedding in the shelter dogs.

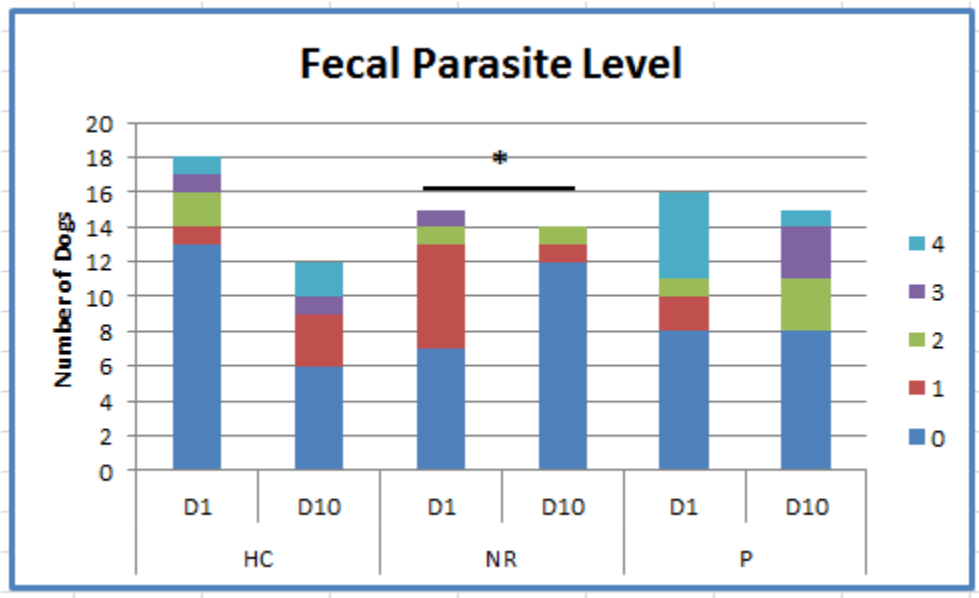

Figure 6. The level of fecal parasite shedding in dogs on Day 1 and Day 10 in each experimental group. HC = Home Cage, NR = Novel Room, P = Petting. Parasite shedding was quantified from 0 (parasites not present) to 4 (ova too numerous to count). *Level of parasite shedding significantly decreased from Day 1 to Day 10 in dogs in the NR experimental group ($p < 0.05$).

IV. DISCUSSION

In general, high circulating numbers of white blood cells were found in shelter dogs, and these cell populations increased further while in the shelter. When compared with control dogs, total leukocytes, neutrophils and monocytes were significantly higher in shelter dogs on Day 1. In both experiments, total leukocytes, neutrophils and lymphocytes were significantly increased on Day 10; in Experiment 2, monocytes were also significantly increased on Day 10. While stress in shelter dogs has been studied for many years, the effect of shelter housing on immune measures has not received prior experimental attention. This study gives the first evidence that numbers of circulating white blood cells are significantly increased as early as the first day of arrival to an animal shelter, and continue to increase over time. These elevations likely occur due to stress, but infection might also contribute to some of the changes.

We found that many dogs in the shelter were affected by infectious agents, most notably parasites. On any day measured, 35% to 50% of shelter dogs were shedding parasites in their feces, and infection with more than one parasite was common. These findings are consistent with previous reports of parasite prevalence in shelter dogs in the Midwestern region of the United States[5]. In Blagburn and Butler's study, a single fecal sample from each shelter dog was evaluated for parasites using the centrifugal

sucrose flotation procedure, and 39% of samples from Midwestern shelters were positive for whipworms, hookworms, or roundworms.

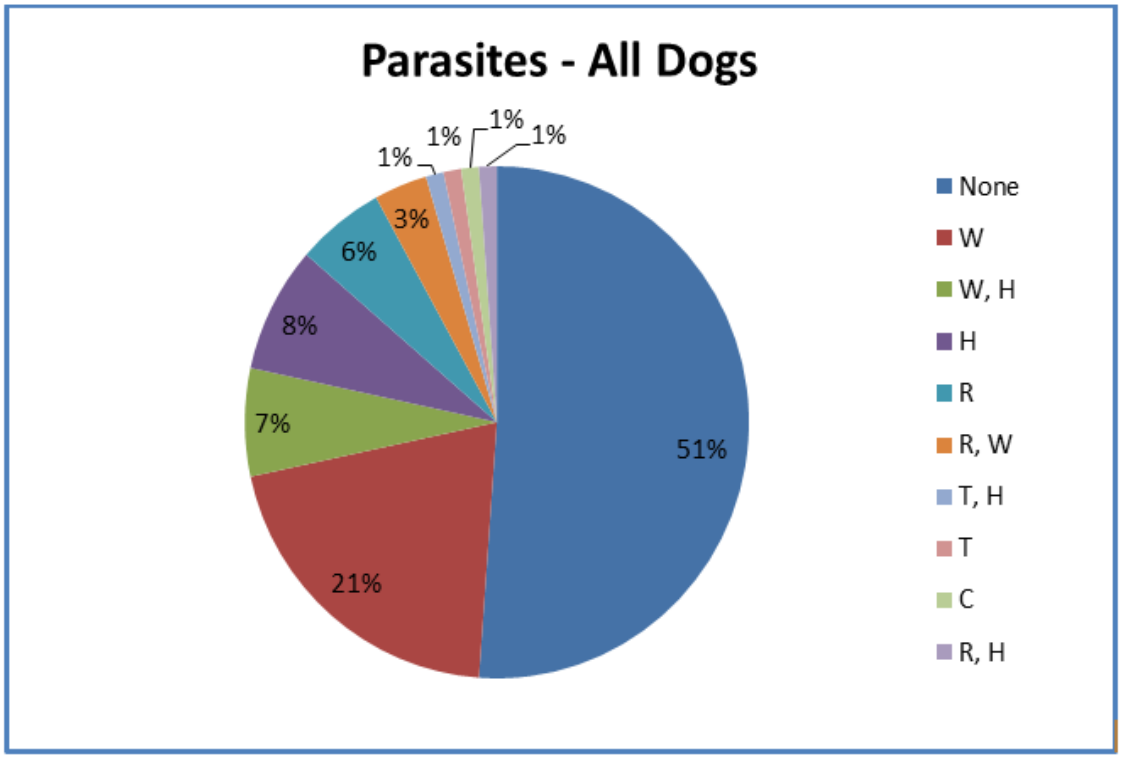

Figure 7. Parasite evaluation of shelter dogs. Up to three fecal samples over a 10 day period were examined; dogs with parasites identified in at least one fecal sample were considered positive for the parasite. W = whipworm, H = hookworm, R = roundworm, T = tapeworm, C = coccidia.

Figure 7 shows results of parasite evaluation of shelter dogs combined from Experiment 1 and Experiment 2. The most common parasites identified included whipworms, hookworms, and roundworms, with 31%, 17%, and 10% prevalence respectively. Comparison of fecal parasite shedding of shelter dogs and control dogs revealed a significantly higher prevalence of parasite shedding in shelter dogs on all days examined. We evaluated multiple fecal samples taken on different days from each dog, and found that 10-20% of dogs were only intermittently shedding parasite ova (data not

shown). This supports recommendations to provide parasite treatment to dogs upon arrival to a shelter[4,37], and treatments should be given universally, not based on the results of a single fecal examination. Canine hookworm and roundworm infections are zoonotic parasites capable of causing sometimes substantial disease in humans,[29] and this also highlights the need for effective hygiene and sanitation protocols while caring for shelter dogs.

Some shelter dogs developed clinical signs of upper respiratory infection during both 10 day experiments. All infections were clinically mild, lacked systemic signs, and no dog met the requirements for intervention per standard animal shelter policy. The etiology of the respiratory infections was not identified and may have been viral, bacterial, or a combination of both. Bacterial infections cause elevated numbers of circulating neutrophils, and indeed, neutrophils significantly increased in number over time in shelter dogs. The analysis reported here includes all dogs sampled in each experiment, as I felt this most accurately represented a typical shelter population. However, analysis was also performed without data from clinically ill dogs, and significant differences reported here remained (data not shown). I do not believe that the parasitic infection or respiratory infection affected the white blood cell parameters reported in this study.

Upon arriving at an animal shelter, a dog experiences multiple psychogenic stressors. These stressors activate both the sympathetic adrenal medullary (SAM) axis and the hypothalamic pituitary adrenal (HPA) axis. Catecholamines (primarily epinephrine) are released when the SAM axis is activated, resulting in elevations in

heart rate[11]. Elevations in heart rate have been demonstrated when a dog experiences transport stress and in response to stimulation with sound, shock and restraint[22,28]. The HPA axis is also responsive to stressors, and I confirmed HPA activation in the shelter dogs of this report through documentation of cortisol elevations. Physiologically, cortisol levels become measurably elevated within 5-10 minutes of a stressor.[39] Ninety-five percent of our samples were collected within 4 minutes of approaching the kennel, avoiding the confounding effect the venipuncture procedure may have on cortisol. Our first sampling in both studies occurred 14 to 30 hours after arrival to the shelter, and we confirmed elevated cortisol levels at that time. As expected, cortisol levels of shelter dogs were higher than those of control dogs, and these levels declined in the shelter dogs over the course of both 10 day studies. These findings were consistent with earlier work done by this laboratory[30]. The novelty of the shelter environment presumably declines over time, which could account for the reduction in cortisol over days. However, other stressors remain. Continued exposure to stressors can lead to a dysregulation of the HPA system (e.g. reduced adrenal sensitivity) and a consequent reduction in cortisol levels[40]. Therefore, the reduction in cortisol levels over days in the shelter may occur due to either a reduction in perceived stress or dysregulation of the HPA axis.

Cortisol induces multiple changes in the number of circulating white blood cells through redistribution between blood and tissues[18]. Changes primarily include an approximate doubling of circulating neutrophils and reduction in lymphocytes, although increases in monocytes are less consistently observed in dogs[17,18]. The number of total

leukocytes increases primarily due to the neutrophilic effect. Calculating the neutrophil to lymphocyte ratio gives an objective measurement which can be used to estimate stress. In normal dogs, this ratio is approximately 3:1[41]. The N:L of shelter dogs ranged from 4-5 on any day measured, more than double the N:L of privately owned dogs, another indicator of the stressful nature of shelter housing.

When compared with control dogs, shelter dogs had higher total leukocytes, neutrophils, and monocytes on Day 1, which is consistent with a stress response. By Day 10 they experienced further increases in total leukocytes, neutrophils, and monocytes (although monocytes only increased in the second experiment). However, changes observed in lymphocyte numbers were unexpected. While cortisol causes reductions in circulating lymphocytes[18], dogs studied here experienced increases in circulating lymphocytes while in the shelter. The numbers of circulating lymphocytes were not significantly different in shelter dogs on Day 1 when compared with control dogs, yet the shelter dogs were obviously stressed. We suspect that the catecholamine response of the SAM axis had an effect on leukocyte distribution as well. Catecholamines lead to elevations in neutrophils, monocytes, and lymphocytes. We believe that the stress of shelter housing stimulated both the SAM and HPA axis, and the white blood cell response is due to the combination of stress hormones. The lack of correlation between cortisol and any of the immune measures supports the possibility of effects from multiple hormones.

These results are consistent with previous studies that evaluated the peripheral leukocyte response to stressors. Three hours after automobile transportation, Beerda et

al. found elevations in total leukocytes, neutrophils, and N:L, while Bergeron et al. found elevations in neutrophils and N:L after automobile transportation and air transportation in dogs.[12,22]

We found that as little as 30 minutes of daily petting caused significant cortisol reductions in shelter dogs, an effect consistent with previous findings showing cortisol reduction following a single petting session shortly after arrival at a shelter.[34] Cortisol reductions after petting occurred on both Days 1 and 10, indicating that the benefits of petting continue beyond the period after arrival to a shelter when cortisol levels are highest. This is the first report that daily petting continues to reduce cortisol levels in dogs beyond the first several days in a shelter.

We hypothesized that daily petting would reduce the stress response of shelter dogs, and therefore attenuate the increase in total leukocytes and neutrophils observed in Experiment 1. As expected, dogs in the home cage group (which did not receive daily petting) had significantly higher neutrophils on Day 10 when compared with other groups; however these dogs also had higher numbers of neutrophils on Day 1 suggesting that this apparent effect of experimental manipulation is likely spurious. The lack of effect of condition suggests that there are other factors in addition to, or instead of, cortisol mediating immune changes.

We evaluated the percentage of dogs positive for one or more parasite, quantified the relative rate of parasite shedding, and did not find any significant difference among conditions on any day. However when comparing the level of parasite shedding between Days 1 and 10 within each group in Experiment 2, a significant

difference was found in the Novel Room group. Dogs in the Novel Room group shed fewer parasites on Day 10. We suspect that this decrease in parasite shedding is incidental; however it may reflect an unknown effect of the novel room. In Experiment 1, the number of dogs considered "heavy shedders" (given a score of "4" when quantifying the number of parasite ova present in the feces) increased over the course of the 10 day study. This was expected, as stress and immunosuppression are likely to exacerbate preexisting parasitic disease.[29] In Experiment 2, the number of heavy shedders decreased substantially in dogs in the petting group (from 5 dogs to 1 dog) and increased in the home cage group (from 1 dog to 2 dogs). Although not statistically significant, this may indicate a beneficial physical impact of daily human interaction, and the effect should be explored further.

There were several limitations which may have affected the outcome of this study. Completing research in an animal shelter environment presented several challenges. Much consideration was given to controlling non-experimental variables, although this was not always possible. Research activities were scheduled around cleaning activities, but at times these coincided. In an effort to minimize variations in treatment of study dogs by shelter staff, an overview of the research procedures and goals was provided. The dogs evaluated came from unknown backgrounds, and despite their outwardly healthy appearance, undetected underlying health disorders may have existed. Prior to arriving at the shelter, some dogs may have been given medications which could affect cortisol or immune measures. While the majority of dogs studied were found as strays, many dogs were surrendered by owners for various reasons. Dogs

accustomed to living in a home environment with regular human companionship may suffer in the shelter from greater stress and anxiety than dogs found as strays, and may also respond differently to human interaction. Experimental variability is overcome through increasing sample size, and the sample size used in experiments may have been too small for some analyses (such as fecal analysis).

The only hormone measured in this study was cortisol. Blood samples had to be transported back to the laboratory for analysis and although samples were chilled and cortisol is extremely stable, the plasma was not separated and frozen until 14-16 hours after collection. Additional analysis of catecholamine levels would have been helpful in interpreting the unexpected immune measure findings.

A final limitation of the study was its duration. While 10 days may seem like a long time for the dog awaiting adoption, it is a relatively short time to study infection and immune measures. However 10 days was all that was practical at the shelter due to space requirements, beliefs that undesirable behavior changes might occur in dogs held longer in the intake ward of the shelter, and infectious disease outbreaks. Animal shelter design and management techniques can vary between facilities, and applicability of our findings to other shelters is unknown.

In conclusion, this study finds that significant physiologic changes are evident after a dog arrives to a shelter. Within the first 24 hours, robust increases in total leukocytes, neutrophils, monocytes, and N:L are apparent in shelter dogs relative to dogs living in stable home environments. By Day 10, the circulating white blood cell populations increase even further. We documented a high prevalence of parasitic infections in

shelter dogs and observed outbreaks of infectious upper respiratory disease. Cortisol levels of shelter dogs were markedly high, and remained significantly higher than control dogs throughout the 10 day experiment. While it is possible that some elevations in white blood cells could be due to infection, we suspect the majority of changes occurred due to stress. It is likely that the stress of shelter housing stimulated both the HPA and SAM axes, and the observed changes in immune measures are due to a combination of cortisol and catecholamines. Cortisol levels of shelter dogs are reduced by petting. This effect occurs on Day 1, and petting continues to reduce cortisol levels even on Day 10.

This study provides valuable insight into the depth of physiologic effect that shelter housing has on a newly arriving dog. Cortisol measurements combined with behavioral assessments have been proposed as a tool for assessing welfare; therefore efforts should be made to attenuate the cortisol elevation which occurs while in a shelter environment[38,42,43]. In addition to reducing cortisol, human interaction improves behavior and temperament test scores, increasing desirable behaviors such as sociability in shelter dogs[8,9]. By minimizing the stress experienced by dogs in the shelter environment, we can prevent many of the known deleterious effects of chronic stress and improve the shelter management's ability to offer the public a healthy and behaviorally sound dog.

Future efforts in assessing the effects of stress on the health of shelter dogs should include assays focusing on a specific function of the immune system. Mitogen stimulated lymphocyte proliferation assays would be useful to determine effects on the

adaptive, or cell mediated, immune system. Effects on the innate immune system could be demonstrated through neutrophil function tests such as the nitro blue tetrazolium assay. Cytokine analysis would prove useful as well. Studies in people have suggested that chronic stress may cause a shift from TH-1 to TH-2 cytokine production.[13,44,45] Specific efforts might focus on measurement of TH-1 cytokines such as IL-2, which promotes clonal expansion of activated T cells, and IFNγ, responsible for macrophage activation. TH-2 cytokines include pro-inflammatory IL-6, which is known to be elevated by stress and associated with many diseases in humans[44]. While these assays would undoubtedly give valuable information on the effects of stress on a molecular level, those responsible for managing animal shelters would likely be interested in tangible evidence such as vaccine response trials, serial health assessments, and client satisfaction surveys. Lengthening the study period beyond 10 days would likely be necessary to demonstrate some of these effects.

V. REFERENCES

1. **American Humane Association** accessed on 10/24/13 at http://www.americanhumane.org/assets/pdfs/pets-fact-sheet.pdf

2. **Assilomar Accords** accessed on 10/24/13 at http://asilomaraccords.org

3. **Steneroden, K.K, Hill, A.E., & Salman, M.D.** (2011). A needs-assessment and demographic survey of infection-control and disease awareness in western US animal shelters. Prev Vet Med, 98:52-57.

4. **Miller, L., Zawistowski, S. (Eds).** (2013). Shelter Medicine for Veterinarians and Staff (2nd edition). Ames, Iowa:Wiley-Blackwell.

5. **Blagburn, B., Lindsay, D., Vaughan, J., Rippey, N., Wright, J., Lynn, R., et al.** (1996). Prevalence of canine parasites based on fecal flotation. Comp Cont Ed Pract, 18(5):483-509.

6. **Lord, L., Reider, L., Herron, M., & Graszak, K.** (2008). Health and behavior problems in dogs and cats one week and one month after adoption from animal shelters. J Am Vet Med Assoc, 233(11), 1715-1722.

7. **Voith VL, Borchelt PL.** (1985). Separation anxiety in dogs. Comp Cont Educ Pract, 42(7):42-52.

8. **Menor-Campos, D.J., Molleda-Carbonell, J.M., Lopez-Rodriguez, R.** (2011). Effects of exercise and human contact on animal welfare in a dog shelter. Vet Rec, doi: 10.1136/vr.d4757

9. **Bergamasco, L., Osella, M.C., Savarino, P., Larosa, G., Ozella, L., Manassero, M. Badino, P., Odore, R., Barbero, R., Re, G.** (2010). Heart rate variability and saliva cortisol assessment in shelter dog: Human-animal interaction effects. Appl Anim Behav Sci, 125:56-68.

10. **Herron, M.E., Kirby-Madden, T.M., Lord, L.K.** (2014). Effects of environmental enrichment on the behavior of shelter dogs. J Am Vet Med Assoc, 244(6):687-692.

11. **Goldstein D.S. (2001).** The Autonomic Nervous System in Health and Disease. New York, NY: Marcel Dekker, Inc.

12. **Beerda, B., Schilder, M.B.H., van Hooff, J.A.R.A.M., De Vries, H.W., Mol, J.A.** (1997). Manifestations of chronic and acute stress in dogs. Appl Anim Behav Sci, 52:307-319.

13. **Padgett, D.A., Glaser, R.** (2003). How stress influences the immune response. Trends Immunol, 24(8):444-448.

14. **Dhabhar FS.** (2009). Enhancing versus suppressive effects of stress on immune function: Implications for immunoprotection and immunopathology. Neuroimmunomodulat, 16:300-317.

15. **Lovallo, W.** (2005). Stress & Health: Biological and Psychological Interactions. Thousand Oaks, California: Sage Publications.

16. **Dhabhar, F.S., Malarkey, W.B., Neri, E., McEwen, B.S.** (2012). Stress-induced redistribution of immune cells – from barracks to boulevards to battlefields: a tale of three hormones – Curt Richter Award winner. Psychoneuroendocrino, 37:1345-1368.

17. **Thrall M.A.** (2004). Veterinary hematology and clinical chemistry. Philadelphia, PA: Lippincott Williams & Wilkins.

18. **Dhabhar FS, Miller, AH, McEwen BS, Spencer RL.** (1995). Effects of stress on immune cell distribution – dynamics and hormonal mechanisms. J Immunol, 154:5511-5527.

19. **Glaser, R., Rice, J., Stout, J.C., Speicher, C.E., Kiecolt-Glaser, J.K.** (1986).Stress depresses interferon production by leukocytes concomitant with a decrease in natural killer cell activity. Behav Neurosci, 100(3):675-678.

20. **Ghoneum, M., Gill, G., Assanah, P., Stevens, W.** (1987). Susceptibility of natural killer cells activity of old rats to stress. Immunology, 60:461-465.

21. **Ettinger, S., & Feldman, E. (Eds.).** (1995). Textbook of Veterinary Internal Medicine (4th ed., Vol. 2). Philadelphia, PA: W.B. Saunders.

22. **Bergeron, R., Scott, S.L., Émond, J.P., Mercier, F., Cook, N.J., Schaefer, A.L.** (2002). Physiology and behavior of dogs during air transport. Can J Vet Res, 66:211-216.

23. **Cohen, S.** (2005). Keynote presentation at the eighth international congress of behavioral medicine. Int J Behav Med, 12(3):123-131.

24. **Rozlog, L, Kiecolt-Glaser, J., Marucha, P., Sheridan, J., & Glaser, R.** (1999). Stress and immunity: implications for viral disease and wound healing. J Periodontol, 70(7):786-792.

25. **Webster Marketon, JI; Glaser R.** (2008). Stress hormones and immune function. Cell Immunol, 252:16-26.

26. **Rojas, I., Padgett, D., Sheridan, J., & Marucha, P.** (2002), Stress-induced susceptibility to bacterial infection during cutaneous wound healing. Brain Behav Immun, 16(1):74-84.

27. **Hunzeker, J., Padgett, D.A., Sheridan, P.A., Dhabhar, F.S., Sheridan, J.F.** (2004). Modulation of natural killer cell activity by restraint stress during an influenza A/PR8 infection in mice. Brain Behav Immun,(18):526-535.

28. **Hodgson PD, Aich P, Stookey J, Popowych Y, Potter A, Babiuk L, Griebel PJ.** (2012). Stress significantly increases mortality following a secondary bacterial respiratory infection. Vet Res, 43(1):21 doi: 10.1186/1297-9716-43-21.

29. **Markell, E.K., Voge, M., John, D.T.** (1992). Medical Parasitology. Philadelphia, PA: W.B. Saunders.

30. **Hennessy, M., Davis, H., Williams, M., Mellot, C., & Douglas, C.** (1997). Plasma cortisol levels of dogs at a county animal shelter. Physiol Behav, 62(3):485-490.

31. **Coppola, C., Grandin, T., & Enns, R.** (2006). Human interaction and cortisol: can human contact reduce stress for shelter dogs? Physiol Behav, 87: 537-541.

32. **Tanaka A, D. W.** (2012). Associations among weight loss, stress, and upper respiratory tract infection in shelter cats. J Am Vet Med Assoc, 240(5):570-576.

33. **Hennessy, M., Williams, M., Miller, D., Douglas, C., & Voith, V.** (1998). Influence of male and female petters on plasma cortisol and behaviour: Can human interaction reduce the stress of dogs in a public animal shelter? Appl Anim Behav Sci, 61:63-77.

34. **Shiverdecker, M.D., Schiml, P.A., Hennessy, M.B.** (2013). Human interaction moderates plasma cortisol and behavioral responses of dogs to shelter housing. Physiol Behav, 109:75-79.

35. **Blagburn BL, Butler JM**. (2006). Optimize intestinal parasite detection with centrifugal fecal flotation. Vet Med-US, 101(7):455-464.

36. **Winer B.J.** (1971). Statistical principles in experimental design. New York: McGraw-Hill.

37. **Newbury, S., Blinn, M.K., Bushby, P.A., Barker Cox, C., Dinnage, J.D., Griffin, B., Hurley, K.F., Isaza, N., Jones, W., Miller, L., O'Quin, J., Patronek, G.J., Smith-Blackmore, M., Spindel, M.** (2010). Guidelines for Standards of Care in Animal Shelters. The Association of Shelter Veterinarians. Accessed on 4/18/14 at

http://sheltervet.org/wp-content/uploads/2012/08/Shelter-Standards-Oct2011-wForward.pdf

38. **Beerda, B., Schilder, M.B.H., van Hooff, J.A.R.A.M., De Vries, H.W., Mol, J.A.** (1998). Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs. Appl Anim Behav Sci, 58:365-381.

39. **Sapolsky, R.M., Romero, L.M., Munck, A.U.,** 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev, 21:55-89.

40. **Hennessy M.B.** (2013). Using hypothalamic–pituitary–adrenal measures for assessing and reducing the stress of dogs in shelters: A review. Appl Anim Behav Sci, 149:1-12.

41. **Feldman, B.F., Thomason, K.J.** (1989). Useful indexes, formulas, and ratios in veterinary laboratory diagnostics. Comp Cont Educ Pract, 11(2):169-180.

42. **Kirschbaum, C., & Hellhammer, D.** (1989). Salivary cortisol in psychobiological research: an overview. Neuropsychobiology, 22, 150-169.

43. **Beerda, B., Schilder, M.B.H., Van Hooff, J.A.R.A.M., De Vries, H.W., Mol, J.A.** (2000). Behavioural and hormonal indicators of enduring environmental stress in dogs. Anim Welfare, 9:49-62.

44. **Kiecolt-Glaser, J.K., Preacher, K.J., MacCallum, R.C., Atkinson, C., Malarkey, W. B, Glaser, R.** (2003). Chronic stress and age-related increases in the proinflammatory cytokine IL-6. Proc Natl Acad Sci USA, 100(15):9090-9095.

45. **Glaser, R., MacCallum, R.C., Laskowski, B.F., Malarkey, W.B., Sheridan, J.F., Kiecolt-Glaser, J.K.** (2001). Evidence for a shift in the Th-1 to Th-2 cytokine response associated with chronic stress and aging. J Gerontol A Biol Sci Med Sci, 56(8):M477-M482.