Wagging more, barking less: Glucocorticoid and behavioral responses of shelter dogs to human interaction

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Abstract

Dogs are the most common household pet in the United States [1], yet millions of dogs are relinquished to animal shelters each year [2]. Even in a well-run and attentive shelter, dogs are introduced to a variety of psychological stressors and experience a substantial increase in glucocorticoid levels [3]. Exercise, play, and human contact are simple and cost-effective means that shelters can adopt to optimize the welfare of their dogs [4] by promoting positive experiences within the kennel environment [3]. The present study examined whether interacting with a consistent volunteer or with rotating volunteers more effectively reduced physiological and behavioral indicators of stress in shelter dogs. Over the course of several days, shelter dogs were subjected to working with a consistent volunteer or with rotating volunteers. Urine and fecal samples were assayed for glucocorticoid metabolites (GCM) and behavior was coded using thirty-second scan sampling. Though there was no significant difference in any of the variables between the two treatment groups, approach latency and the percentage of time spent resting decreased considerably over time for both groups of dogs. Additionally, urine GCM/creatinine levels and frequency of behaviors associated with poor welfare generally decreased over time. These results suggest that mere human interaction rather than the development of the dog-human bond appears to be effective at reducing stress in kenneled dogs. However, due to the small sample size and experimental limitations, using human contact to reduce stress in shelter dogs warrants further investigation.
Introduction

Stress and Animal Welfare

Stress, defined as a response to aversive stimuli [5], is an ambiguous term yet a universal phenomenon to all living beings [6,7]. Pertinent to a variety of organisms and situations [8], stress can be discussed in the biological context of resource availability, mating opportunities, physical confrontations, and predator-prey interactions. Stress can also be applied to more psychological circumstances, such as coping with major life events, trauma, and even our daily burdens [7]. Be it a physiological or psychological stressor, the body will respond to the challenge at hand. And, as the neurological circuitry regulating emotional and social behavior is fundamentally conserved between mammalian and non-mammalian vertebrates, the impact of stress on the body and mind has important implications for understanding coping strategies of humans and non-human animals [9].

Psychological and physical stress acts to modulate behavioral, emotional, and physiological parameters, including cardiovascular, digestive, immune, and nervous functioning [10-12]. These stress-related changes are mediated by heightened activity of the hypothalamic-pituitary-adrenal (HPA) axis and result in increased production and release of cortisol, the primary glucocorticoid in mammals [13]. Many mammal species exhibit increased cortisol levels that may or may not be sustained in response to temporary aversive stimuli [13]. Such short-term changes in glucocorticoid production initiate physiological and behavioral coping mechanisms in attempt to revert the body back to homeostasis. Under normal circumstances, elevated glucocorticoid levels are maintained until the animal has successfully coped with the threat, after which glucocorticoid production is decreased, metabolites are excreted from the body, and hormonal levels return to baseline. Conversely, persistent aversive stimulation induces prolonged
disruptions to normal bodily operations [13, 14]. Elevated glucocorticoid levels may progressively desensitize the pituitary to the adrenocorticotropic hormone (ACTH) releasing hormones vasopressin and corticotropin-releasing hormone (CRH), thus disturbing the negative feedback system that regulates glucocorticoid secretion and release [13]. Moreover, chronic stress compromises immunocompetence and can cause lymphopenia, eosinopenia, and increased susceptibility to disease [13]. Consequently, long-term exposure to negative stimuli can be detrimental to an animal’s health, welfare, and ability to respond to future events.

Animal welfare, which “comprises the state of an animal’s body and mind, and the extent to which its nature is satisfied” [15], is influenced by both physiological and behavioral factors. The physical health of animals has received much emphasis in the study of animal welfare, yet fulfilling an animal’s physical requirements such as supplying ample food, water, and exercise is only a constituent component of quality care [16]. Though the mental health of animals is frequently disregarded, emotional needs, including social companionship and mental stimulation, are equally important to an animal’s well-being [17]. Therefore, understanding animal mental health is of paramount importance to maximize enjoyment of and enrich life experiences in animals [17].

Relinquishment to an Animal Shelter

Dogs (*Canis lupus familiaris*) are the most common household pet in the United States [1], yet millions of dogs are relinquished to animal shelters each year [2]. Shelters house and care for stray, abused, injured, and released animals and present the opportunity for their adoption [18]. However, even in a well-run and attentive shelter, dogs are introduced to a variety of psychological stressors: they are exposed to novelty and noise; their environment is
unpredictable and uncontrollable; there is a lack of social interaction with humans and other dogs; and some animals have been separated from previous attachment figures [19-24].

Entry into a shelter is an acute stressor for any dog. Previous studies incorporating physiological and behavioral measures have revealed that immediately upon admittance into rescue centers, dogs experience a substantial increase in glucocorticoid levels; indeed, kennel-kept dogs have elevated urinary cortisol levels in comparison to their home-kept counterparts [3]. Because the typical shelter dog has not experienced a great deal of distress prior to arriving at the shelter, the shelter dog will likely display behavioral symptoms of acute stress such as pacing, excessive drinking, repetitive licking, and superfluous self-grooming [25]. Although most shelter dogs are provisionally exposed to kennel stressors, some dogs have experienced more grave circumstances. For example, puppy mill rescue dogs have endured the harrowing environment of puppy mills, large-scale commercial breeding facilities that mass-produce dogs who are housed in overcrowded and squalid conditions. The mills place profit above animal well-being and fail to apply proper husbandry practices: the dogs are bereft of adequate food, water, socialization, positive human interaction, and veterinary care, all of which inevitably give rise to illness, congenital disease, and crippling psychological problems [26]. Puppy mill dogs commonly develop extreme phobias and stereotypies [27]; neglect, abuse, infection, and disease are puppy mill hallmarks, and lasting physiological and behavioral abnormalities go hand in hand with the industry.

The Dog-Human Bond

For tens of thousands of years, dogs have been selected for dependency on and attachment to humans [28,29]. Wolf domestication is thought to have occurred 14,000 years ago
when a group of less-fearful wolves were attracted to nomadic encampments to scavenge kills, after which humans exploited these animals as human invader and predator sentinels [30]. As a product of their domestication, dogs display an innate responsiveness towards humans that is neither influenced by feeding nor diminished by punishment [31].

Similar to their wolf ancestors who live cooperatively in hunting packs, dogs are social animals equipped with an advanced ability to form complex relationships; they also possess sophisticated interspecies socio-cognitive capabilities [32]. Dogs are extremely skillful at interpreting human behavior, even more so than our closest primate relatives, and do so from the first trial with little to no learning effects irrespective of age and rearing history [32-34]. In contemporary Western society, dog owners often harbor an emotional intimacy for their pets and consider them as sources of comfort, support, relief, and love [33,35]. With regard to the dog-human bond, dogs give preferential attention to their owner [36] and display a strong attachment to their owner or a familiar person that is analogous to human attachment behaviors [35]. Additionally, both dogs and their owners experience an increase in oxytocin, a neuropeptide associated with social bond formation [37], and a decrease in cortisol levels after interacting with each other [38].

Experimental and observational studies have shown that positive human contact and long-standing relationships are pleasurable and reinforcing for dogs [17]. As such, exercise, play, and human contact are simple and cost-effective means that shelters and rescues can adopt to optimize the welfare of their animals [4] by both minimizing negative and promoting positive experiences within the kennel environment [39]. For example, Shiverdecker et al. (2013) demonstrated that a 25-minute session of exercise and human contact reduces cortisol levels and behavioral indicators of stress in shelter dogs [4,40]. However, no study has yet evaluated
whether this reduction in physiological and behavioral signs of stress is attributable to mere human interaction or the development of the dog-human bond.

**Hypothesis and Predictions**

To definitively test the relationship between the dog-human bond and stress, the present study examined whether repeated interactions with the same volunteer or interactions with rotating volunteers more effectively reduced physiological and behavioral measures of stress in shelter dogs. I hypothesized that working with a steadfast volunteer would more effectively reduce stress in and improve the welfare of these dogs relative to working with multiple volunteers. I expected that all dogs would exhibit elevated cortisol metabolite levels and behaviors associated with poor welfare preceding volunteer introduction but would exhibit a reduction in these variables succeeding several human interaction sessions. Moreover, I predicted that dogs working with a consistent volunteer would exhibit a more robust reduction in physiological and behavioral signs of stress relative to dogs working with rotating volunteers.

**Methods**

**Ethical Note**

All experimental procedures and analyses were approved by Franklin & Marshall College’s Institutional Animal Care and Use Committee (IACUC #R_10DGA48jeUSaZCM). Handling of and interactions with the subjects during the study were conducted in accordance with IACUC provisions.
Subjects

Twenty-six shelter dogs participated in this study. The shelter dogs were housed at the Humane League of Lancaster County (Lancaster, Pennsylvania) and all dogs were cared for in adherence to the shelter’s regular protocols. The study had originally intended to include puppy mill rescue dogs but due to unforeseen circumstances, only shelter dogs from the HLLC were used. A minimum of one week of data collection was originally required for each dog with the intent to remove dogs claimed by an owner or adopted out before one week’s worth of samples had been collected from all analyses. However, as a result of the rapid turnover of dogs at the HLLC, usable data was only obtained on eight dogs, four per treatment (consistent volunteer and rotating volunteer); these dogs are hereafter referred to as three-day dogs (Table 1). Because only two urine and/or fecal samples were collected for a majority of the three-day dogs, dogs that volunteers only interacted with for two days, hereafter referred to as two-day dogs, were included in hormonal analyses but not behavioral analyses.
Table 1. Summary of the three-day dogs used in the study.

<table>
<thead>
<tr>
<th>Dog Name</th>
<th>Treatment</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Breed</th>
<th>Weight (lbs)</th>
<th>Fixed</th>
<th>Acquisition</th>
<th>Health Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frank</td>
<td>Consistent</td>
<td>4</td>
<td>M(^2)</td>
<td>Boxer Pit Bull</td>
<td>68.7</td>
<td>No</td>
<td>Stray(^3)</td>
<td>Growth on front paw, thinning hair on back, hair loss on feet</td>
</tr>
<tr>
<td>Bella Blue</td>
<td>Consistent</td>
<td>4</td>
<td>F(^3)</td>
<td>American Pit Bull Terrier</td>
<td>56.8</td>
<td>n/a(^4)</td>
<td>Surrendered(^6)</td>
<td>Skin issues, spayed between second and third interaction day</td>
</tr>
<tr>
<td>Rosie</td>
<td>Consistent</td>
<td>0.25</td>
<td>F</td>
<td>Mix</td>
<td>14.8</td>
<td>n/a</td>
<td>Surrendered</td>
<td>Spayed between second and third interaction day</td>
</tr>
<tr>
<td>Rico</td>
<td>Consistent</td>
<td>5</td>
<td>M</td>
<td>Pit Bull</td>
<td>98.9</td>
<td>Yes</td>
<td>Surrendered</td>
<td>Overweight</td>
</tr>
<tr>
<td>Rosie</td>
<td>Consistent</td>
<td>0.25</td>
<td>F</td>
<td>Mix</td>
<td>14.8</td>
<td>n/a</td>
<td>Surrendered</td>
<td>Spayed between second and third interaction day</td>
</tr>
<tr>
<td>Reba</td>
<td>Rotating</td>
<td>Adult(^1)</td>
<td>F</td>
<td>Toy Poodle Mix</td>
<td>5.9</td>
<td>Yes</td>
<td>Stray</td>
<td></td>
</tr>
<tr>
<td>Bella Beagle</td>
<td>Rotating</td>
<td>0.16</td>
<td>F</td>
<td>Beagle</td>
<td>5.52</td>
<td>n/a</td>
<td>Surrendered</td>
<td>Spayed between second and third interaction day</td>
</tr>
<tr>
<td>Holly</td>
<td>Rotating</td>
<td>6</td>
<td>F</td>
<td>Beagle Mix</td>
<td>20.6</td>
<td>No</td>
<td>Stray</td>
<td>Dirty ears, vulva puss</td>
</tr>
</tbody>
</table>

\(^1\) Exact age unknown

\(^2\) Male

\(^3\) Female

\(^4\) Fixed during the study

\(^5\) Stray: dog brought to the shelter after being found homeless

\(^6\) Surrendered: dog relinquished by the owner to the shelter

Treatment Assignment
History does not seem to significantly affect urinary cortisol/creatinine levels in kenneled dogs upon initial admittance into a shelter or rescue facility [12,41]. Therefore, every shelter dog available on the experimental start date was randomly assigned to an experimental condition: interaction with a consistent volunteer or with rotating volunteers. F&M Unleashed, an established student-run club at Franklin & Marshall College (Lancaster, Pennsylvania) whose members volunteer at local animal shelters, provided the human
volunteers for this study. All volunteers, who assumed liability for interacting with the shelter dogs, were randomly assigned to act as either a consistent volunteer or a rotating volunteer and were oriented to kennel decorum by the HLLC prior to the experimental start date. During the course of the study, volunteers traveled to the shelter every other day at 10:00 AM, three times a week, for a minimum of one week and a maximum of two weeks per dog.

Interaction Session

Prior to the interaction session, dogs were videotaped using iPhones (©Apple, Inc.) or cameras (Flip UltraHD U32120) for thirty-five minutes, after which volunteers timed the latency of their dog to make contact with them upon opening of the kennel door. The leash and data collection equipment were kept out of the dog’s sight until the dog made contact with the volunteer; had the dog not made contact with the volunteer after ninety seconds, the approach latency was labeled as such. After the approach latency was timed, the volunteer took the dog out of his/her kennel according to shelter protocol and proceeded to take the dog on a fifteen-minute walk, during which urine and fecal samples were collected. The volunteer then played with the dog for fifteen minutes; play activities consisted of tossing a toy or ball, basic positive reinforcement training, and petting. Though volunteer behavior was consistent in that all volunteers spent fifteen minutes walking and fifteen minutes playing with their dog, how the volunteer acted during the session was at his/her discretion in response to the dog’s behavior. After the thirty-minute interaction session ended, the dog was returned to his/her kennel.
Hormonal Sampling and Analysis

Urine and fecal collection

Whereas cats mainly excrete cortisol metabolites in their feces, dogs excrete the highest concentration of cortisol metabolites in their urine (following an injection of radio-labeled cortisol, 77% of total recovered radioactivity appeared in urine as compared to 23% in feces) [42]. Urinary glucocorticoid metabolite (GCM)/creatinine ratios prove to be a valuable indicator of welfare status in dogs, as samples can be noninvasively collected and, if collected in the early morning, represent the cortisol production rate over twenty-four hours [12]. Additionally, although interactions between stress physiology and behavior are convoluted, pairing urine GCM/creatinine levels with behavior can provide a more robust gauge on welfare, for behavior is easily observable and discloses information about an animal’s demands, preferences, and internal state [12].

Prior to the interaction session, urinary collection tubes and fecal collection cups were labeled with the animal’s name, time, and date of collection. To obtain urine samples, volunteers caught the urine midstream during the fifteen-minute walk; if not enough urine (1-4 mL) was caught the first time, urine was collected again on the same walk. In addition, volunteers collected any available feces the dog produced on the walk. After the interaction session ended and the dog was returned to his/her kennel, volunteers transferred 1-4 mL of urine into the labeled urinary collection tube using a sterile syringe. Both the urine and fecal samples were placed in a cooler and then stored at -20 °C with a maximum acceptable time between urination/defecation and freezing of three hours.
Hormone assay validation

Prior to hormonal analysis, a dog validation study was completed to validate the specific commercial assay kit to be used in the shelter dog cortisol metabolite analysis. Friends (n=6) of the experimenter who owned a dog that had a scheduled vet visit between November 2016 and May 2017 were asked to collect first morning urine and fecal samples from their dogs pre- and post-vet visit. Having owners, who regularly interact with and walk their companion animals, collect samples from their own dogs should have minimized any stress associated with excretion collection whilst permitting GCM levels to be accurately measured before and after a stressful event i.e. a trip to the vet. Samples were transferred to the lab on ice and stored at -20 °C. Urine GCM were assayed directly whereas fecal GCM were extracted from each sample using the protocol below, and the diluted extracts were assayed.

Because the exact chemical structures and relative prevalence of GCM can vary as a function of species, sex, and diet, specific assays must be validated for detection of GCM in the target population [42]. The urine and fecal samples were tested in both monoclonal cortisol and polyclonal corticosterone enzyme-immunoassay kits (Arbor Assays, Ann Arbor, Michigan) to determine which kit most reliably detected increased GCM levels in post-vet samples (Fig. 1). The percent change between pre- and post-vet visit GCM levels for both urine samples and fecal extracts was highest in the corticosterone assay, thus the corticosterone assay was used to measure cortisol metabolites in the shelter dogs.
Fig. 1. Chemical structures of (A) cortisol, (B) corticosterone, and (C) creatinine. Urine and fecal cortisol metabolite levels were determined using a corticosterone and creatinine assay.

Extraction and metabolite determination

5 mL of 80% methanol were added to 0.5 ± 0.005 g of dried homogenized fecal samples. The samples were placed on a rotator (The Belly Button Shaker® BBUAUVIS, IBI Scientific) and after shaking (13.5 hours) and centrifugation (1000g; 15 minutes), the supernatant was decanted into clean 7-mL vials and stored at -20 °C until analysis.

Corticosterone standards (serial dilution of 100,000 pg/mL in a stabilizing solution), urine samples (1:50), and fecal extracts (1:130) were diluted in assay buffer and pipetted into wells of a clear plastic 96-well microtiter plate coated with donkey anti-sheep IgG. A corticosterone-peroxidase conjugate and a corticosterone-specific sheep polyclonal antibody were added to each well, and the plate was incubated in the dark for one hour at 25 °C on a shaker (Professional 1000MP Incubating Microplate Shaker® 980181, Talboys). The plate was washed four times with wash buffer, after which tetramethylbenzidine (TMB) substrate was
added to each well (Fig. 2A). After a thirty-minute incubation time in the dark at 25 °C, the optical density of the reaction was read at 450 nm (ELx808 Absorbance Microplate Reader® 212739, BioTek Instruments; Fig. 3).

To standardize for variations in urine volume and concentration, metabolite excretion rate, and body weight, absolute urine GCM values were expressed as a ratio with creatinine (GCM/creatinine), a by-product of muscle breakdown that is produced and excreted at a constant rate [12]. In the creatinine assay, creatinine standards (serial dilution of 100 mg/dL creatinine solution) and urine (1:20) were diluted in milliQ water and pipetted into wells of a clear plastic 96-well microtiter plate. Creatinine reagent was added to each well and after a thirty-minute incubation time in the dark at 25 °C, the optical density of the reaction was read at 490 nm (ELx808 Absorbance Microplate Reader® 212739, BioTek Instruments; Fig. 2B). The GCM/creatinine ratio for each sample and the percent change in GCM/creatinine between the first and last interaction day were calculated.
tetramethylbenzidine (TMB) substrate with peroxidase to produce a blue color, after which the reaction is stopped with acid to generate a yellow color. (B) The Jaffe reaction in which creatinine reacts with picric acid in an alkaline medium to produce a red-orange chromogen complex [43]. This reaction is clinically used to estimate urine and serum creatinine and can be utilized to assess kidney function [44].
Fig. 3. Schematic representation of the corticosterone enzyme-immunoassay. Standards of known corticosterone concentration and urine/fecal samples are pipetted into a 96-well microtiter plate. A secondary antibody that is conjugated to both corticosterone and peroxidase is added to the plate wells, and this complex competes with the GCM present within the samples to bind the immobilized capturing antibody. The plate is sufficiently washed with buffer to remove any unbound molecules, after which TMB substrate is added to the plate wells. The substrate is oxidized by the bound corticosterone-peroxidase conjugate to produce a blue color, the reaction is stopped with acid to generate a yellow color, and the optical density is read at 450 nm. The intensity of the generated yellow color is inversely proportional to the GCM concentration of the urine/fecal sample (Adapted from [45]).

Cortisol metabolite analysis

In attempt to increase the sample size, the difference in urine and fecal cortisol metabolite concentration from the first to last interaction day was calculated for two- and three-day dogs. A one-way ANCOVA, using the number of days between the first and last interaction day as a covariate, was run to determine if there was an overall decrease in cortisol metabolite levels in urine and feces over time. Analyses were performed on the combination of two- and three-day dogs and exclusively on the three-day dogs.
Behavioral Sampling and Analysis

Approach latency

The approach latencies for each three-day dog per experimental condition were averaged to produce an overall mean latency time for each interaction day per treatment. As the data did not meet the assumption of normality (Kolmogorov-Smirnov test: \( p=0.036 \)), the latencies were \( \ln \)-transformed. A mixed-model repeated-measures ANOVA was run to assess the predicted relationship between decreased approach latency and increased strength of the dog-human bond.

Behavioral analysis

An ethogram of nine behaviors associated with poor and good welfare in dogs, derived from previous studies \([12,41,46]\), was developed (Table 2). The videotapes of each three-day subject were randomized and examined, with the first five minutes of the video disregarded, and the defined behaviors observed were recorded using thirty-second scan sampling and analyzed as a frequency percentage. Pacing and circling were not observed in any of the videos; therefore, these behaviors were excluded from all analyses. Additionally, two videos were missing and during one interaction day, the back half of the kennel was inaccessible to one of the dogs; as such, the corresponding behaviors for these dogs on these days were mean-replaced. Because the assumption of normality was not met, a Friedman’s test as well as a mixed-model repeated measures ANOVA (a test robust to violations of the normality assumption \([47]\)) were run to determine if there was an overall decrease in behavioral indicators of stress over time.
All statistical analyses were conducted using SPSS Statistics 22.0 (©SPSS Inc.). Significance was assumed below the 5% level but due to the small sample size, results below the 10% level were considered and are discussed.
Results

Fecal GCM and Urine GCM/Creatinine

The overall difference in fecal GCM levels from the first to last interaction day did not significantly differ for dogs that interacted with consistent and rotating volunteers (One-way ANCOVA: $F_{(2,6)}=0.468$, $p=0.655$; Fig. 4). There was neither a significant change over time (One-way ANCOVA: $F_{(1,6)}=0.383$, $p=0.580$; Fig. 4) nor difference between the two treatment groups (One-way ANCOVA: $F_{(1,6)}=0.774$, $p=0.444$; Fig. 4). The amount of days in between the first and last interaction day did not appear to affect fecal GCM levels (One-way ANCOVA: $F_{(1,6)}=0.044$, $p=0.848$).

Fig. 4. Fecal GCM (ng/g) of two- and three-day dogs interacting with either a consistent volunteer (n=2) or with rotating volunteers (n=4). The consistent treatment group is represented by filled-in circles with a solid trendline; the rotating treatment group is represented by open diamonds with a dotted trendline. There was no overall significant difference in fecal GCM levels over time and between treatment groups (One-way ANCOVA: $F_{(2,6)}=0.468$, $p=0.655$).
The overall difference in urine GCM/creatinine levels from the first to last interaction day for two- and three-day dogs did not significantly differ for dogs that interacted with consistent and rotating volunteers (One-way ANCOVA: $F_{(2,8)}=0.373$, $p=0.706$; Fig. 5A, 5B, and 5C). There was neither a significant change over time (One-way ANCOVA: $F_{(1,8)}=1.861$, $p=0.231$; Fig. 5A, 5B, and 5C) nor difference between the two treatment groups (One-way ANCOVA: $F_{(1,8)}=0.023$, $p=0.884$; Fig. 5A, 5B, and 5C). The amount of days in between the first and last interaction day did not appear to affect urine GCM/creatinine levels (One-way ANCOVA: $F_{(1,8)}=0.730$, $p=0.432$).

The overall difference in urine GCM/creatinine levels from the first to last interaction day for solely the three-day dogs did not significantly differ for dogs that worked with consistent and rotating volunteers (One-way ANCOVA: $F_{(2,5)}=1.865$, $p=0.337$; Fig. 5A, 5B, and 5D). There was neither a significant change over time (One-way ANCOVA: $F_{(1,5)}=4.505$, $p=0.168$; Fig. 5A, 5B, and 5D) nor difference between the two treatment groups (One-way ANCOVA: $F_{(1,5)}=2.195$, $p=0.277$; Fig. 5A, 5B, and 5D). The amount of days in between the first and last interaction day did not appear to affect urine GCM/creatinine levels (One-way ANCOVA: $F_{(1,5)}=0.982$, $p=0.186$).
Fig. 5. Urine GCM/creatinine (pg/mg) of (A) two- and three-day dogs interacting with either a consistent volunteer (n=4) or with rotating volunteers (n=4) and (B) the relative percent difference of interaction day 1 values in urine GCM/creatinine over time. The consistent treatment group is represented by filled-in circles with a solid trendline; the rotating treatment group is represented by open diamonds with a dotted trendline. (C) and (D) depict the mean urine GCM/creatinine difference (pg/mg, ±SEM) from the first to last interaction day for the two- and three-day dogs and solely the three-day dogs, respectively. There was no overall significant difference in urine GCM/creatinine over time and between the two treatment groups.
for the combination of two- and three-day dogs (One-way ANCOVA: $F_{(2,8)}=0.373, p=0.706$) and for solely the three-day dogs (One-way ANCOVA: $F_{(2,5)}=1.965, p=0.337$).

**Approach Latency and Behavior**

**Approach latency**

Latency to make contact with a human volunteer upon opening of the kennel door did not significantly differ between the two treatment groups for each time point (ln-transformed; Repeated-measures ANOVA: $F_{(1,6)}=0.272, p=0.621$; Fig. 6) nor over the course of three interaction days (ln-transformed; Repeated-measures ANOVA: $F_{(2,12)}=0.063, p=0.939$; Fig. 6). However, approach latency was markedly reduced for both treatment groups over time (ln-transformed; Repeated-measures ANOVA: $F_{(2,12)}=6.068, p=0.015$; Fig. 6), with the greatest reduction between interaction day 1 and day 3 (Bonferroni test: $p=0.025$).

![Image](image.png)

**Fig. 6.** Approach latency (ln-transformed, ±SEM) of three-day dogs interacting with either a consistent volunteer ($n=4$) or with rotating volunteers ($n=4$) over three interaction days. The consistent treatment group is represented by filled-in circles with a solid trendline; the rotating treatment group is represented by open diamonds with a dotted trendline. Approach latency did
not significantly differ between the two treatments for each time point (Repeated-measures ANOVA: $F_{(1,6)}=0.272, p=0.621$). Although approach latency did not significantly differ between the two treatments overall (Repeated-measures ANOVA: $F_{(2,12)}=0.063, p=0.939$), it markedly decreased over time for both groups of dogs (Repeated-measures ANOVA: $F_{(2,12)}=6.068, p=0.015$), with a pronounced decrease from interaction day 1 to day 3 (Bonferroni test: $p=0.025$).

Behavior

The frequency of all behaviors did not significantly differ between the two treatment groups for each time point and over the course of three interaction days (Fig. 7; Table 3). Of the nine defined behaviors, only the proportion of time spent resting markedly decreased over time for both groups of dogs (Friedman’s test: $\chi^2(2)=7.00, p=0.030$), with a notable reduction from interaction day 2 to day 3 (Wilcoxon signed-rank test: $Z=-2.521, p=0.012$; Fig. 8A). Although there was no overall significant difference in the other behaviors, the percentage of time spent self-grooming exhibited a general decrease (Friedman’s test: $\chi^2(2)=0.750, p=0.687$; Fig. 8C) whereas the percentage of time spent exploring exhibited a general increase (Friedman’s test: $\chi^2(2)=0.839, p=0.657$; Fig. 8D) for both treatment groups. Additionally, whilst not significantly different, the percentage of time spent in the back half of the kennel strikingly increased over time for both treatment groups (Friedman’s test: $\chi^2(2)=1.750, p=0.417$; Fig. 8B).

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Within Subjects</th>
<th>Between Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\chi^2$</td>
<td>$p$</td>
</tr>
<tr>
<td>Back half</td>
<td>1.750</td>
<td>0.417</td>
</tr>
<tr>
<td>Chew</td>
<td>2.667</td>
<td>0.264</td>
</tr>
<tr>
<td>Explore</td>
<td>0.839</td>
<td>0.657</td>
</tr>
<tr>
<td>Lick</td>
<td>0.348</td>
<td>0.840</td>
</tr>
<tr>
<td>Object manipulation</td>
<td>2.600</td>
<td>0.273</td>
</tr>
<tr>
<td>Other</td>
<td>1.750</td>
<td>0.417</td>
</tr>
<tr>
<td>Rest</td>
<td>7.000</td>
<td>0.03**</td>
</tr>
<tr>
<td>Self-groom</td>
<td>0.750</td>
<td>0.687</td>
</tr>
</tbody>
</table>

Table 3. A within-subjects Friedman’s test and between-subjects repeated-measures ANOVA examining the frequency of seven behaviors exhibited by three-day dogs interacting with a consistent volunteer (n=4) or with rotating volunteers (n=4) over three interaction days ($p<0.05**$, $p<0.10*$).
Fig. 7. Distribution of percentage of behaviors exhibited by three-day dogs interacting with a consistent volunteer (n=4) or with rotating volunteers (n=4) over three interaction days. An ethogram of nine behaviors associated with poor and good welfare in dogs was developed (refer to Table 2) and the defined behaviors were recorded using thirty-second scan sampling. Pacing and circling were not observed and therefore these behaviors were excluded from all analyses. Any missing behavioral data were mean-replaced.
Fig. 8. Percentage of time spent (A) resting, (B) in the back half of the kennel, (C) self-grooming, and (D) exploring (±SEM) exhibited by three-day dogs interacting with either a consistent volunteer (n=4) or with rotating volunteers (n=4) over three interaction days. The consistent treatment group is represented by filled-in circles with a solid trendline; the rotating treatment group is represented by open diamonds with a dotted trendline. Although there was no significant change over time in the percentage of time spent in the back half of the kennel (Friedman’s test: $\chi^2(2)=1.750, p=0.417$), self-grooming (Friedman’s test: $\chi^2(2)=0.750, p=0.687$), and exploring (Friedman’s test: $\chi^2(2)=0.839, p=0.657$), there was a notable decrease in time spent resting (Friedman’s test: $\chi^2(2)=7.00, p=0.030$), with a pronounced reduction from interaction day 2 to day 3 (Wilcoxon signed-rank test: $Z=-2.521, p=0.012$).
Discussion

The purpose of this study was to definitively test the relationship between the dog-human bond and stress. This was accomplished by examining whether repeated interactions with a consistent volunteer or interactions with rotating volunteers more effectively reduced physiological and behavioral signs of stress in shelter dogs.

First used to describe the bond between an infant and his/her caregiver, the construct of attachment can also be applied to the dog-human relationship, which is relatively similar to the asymmetrical and dependency-based child-parent relationship [48]. Topál et al. (1998) demonstrated that when in the presence of their owner, dogs tended to play more, explore more, and displayed higher levels of contact seeking toward their owner compared to when in the presence of a stranger [49]. These dogs had been in their owner’s possession ranging from one to ten years, giving ample time for a long-term bond to form between the dog and his/her owner. In the case of the present study, three days may not have conferred sufficient time for a meaningful bond to form between the shelter dog and the human volunteer. Moreover, shelter dogs are often kept in their kennels for extended periods of time accompanied by sporadic walks with kennel attendants. It is thus conceivable that shelter dogs prefer more social stimulation and human interaction in lieu of steadfast relationships by virtue of their habitual isolation. Nonetheless, because companion dogs do display a distinct preference for their owner, more frequent interaction with the human volunteers or allowing more time for shelter dogs to form a bond with a human volunteer could reflect this partiality.

Approach latency was intended to function as a measure of the development of the dog-human bond. The results were not congruous with my prediction that approach latency for the consistent dogs would exhibit a more substantial decrease over time relative to that for the
rotating dogs. Interestingly, both groups of dogs exhibited an overall latency reduction over time, which suggests that the dogs were eager to interact with humans, regardless of whether or not a bond with the human volunteer had been formed (Fig. 6). Other possible explanations for the latency reduction in both treatment groups include an association with the human volunteers and a dog-driven pleasurable interaction rather than a quick walk to urinate/defecate, or even an opportunity to escape the confinement of the kennel.

Despite non-significant results for both urine and fecal cortisol metabolite levels over time and between the two treatment groups, the urine GCM/creatinine analysis appears to be more telling than the fecal GCM analysis. This may be a consequence of dogs preferentially excreting cortisol metabolites via urine [42] or perhaps a reflection of differences in the biological averaging by the two sample types. Because feces are present in the gut for a longer period of time than urine is in the bladder, fecal GCM values are inherently more stable and resilient to temporal variation than are urine GCM values [42]. This seemed to hold true for the shelter dogs, as a majority of the dogs maintained a constant level of fecal GCM whereas all of the dogs experienced changes in urine GCM/creatinine levels over time (Fig. 4 and 5).

In accord with my prediction, three out of the five three-day dogs exhibited an overall decrease in urine GCM/creatinine levels (Fig. 5A and 5B). For the two three-day dogs that displayed an increase in urine GCM/creatinine, these dogs were fixed (i.e. spayed or neutered) and moved to kennels accessible to the public between interaction day 2 and day 3. This was also evident for the two-day dogs, all of which exhibited an increase in urine GCM/creatinine (Fig. 5A and 5B). These results intriguingly parallel the back-half behavioral data that illustrated an increase in time spent in the back half of the kennel between interaction day 2 and day 3 (Fig. 8B).
Sound levels in an animal shelter regularly exceed 100 dB [50]. A noise level greater than 70 dB is considered ‘loud’ [51], and because sound is measured in decibels, 100 dB is 8 times the intensity of 70 dB and is comparable to a passing subway train [52]. It has long been demonstrated that high auditory levels are disturbing to animals and can lead to physiological, behavioral, and even anatomical responses [52]. Dogs have much better hearing abilities than humans and can hear sounds four times quieter than can the human ear [52]. Coppola et al. (2006) found that adoptable areas in animal shelters are loudest, hold the greatest number of animals in close proximity, and induce a surge in cortisol levels in the dogs housed in these areas [52]. In connection with the current study, the physical change in environment in conjunction with more noise and human presence when moved to publicly accessible kennels could have been unnerving to the dogs, perhaps causing them to respond in ways that counterbalanced any positive response to working with a human volunteer. The influences of an environmental change, increased noise levels, and greater human exposure on stress levels in shelter dogs have been inadequately studied, but the impacts of these variables on shelter dog welfare certainly warrant further consideration.

With regards to behavior, even though there was no overall significant difference between the two treatments, behaviors associated with poor welfare, such as inertness and self-grooming, generally decreased whereas behaviors associated with good welfare, such as exploration, generally increased over time (Fig. 8A, 8C, and 8D). What was particularly striking and unexpected is that dogs in the consistent treatment group spent remarkably less time resting than dogs in the rotating treatment group prior to any interaction with the human volunteers (Fig. 8A). Because shelter acquisition was relatively similar for the dogs in both treatment groups, the cause of this difference being the dogs’ history is unlikely (Table 1). A more plausible
justification for these results is that the consistent dogs spent a majority of time displaying other behaviors associated with poor welfare (e.g. vocalizing and alertness) on interaction day 1 (Fig. 7). These types of behaviors were not measured because other studies failed to find significant results; additionally, dichotomizing different vocalizations from a video, especially when multiple dogs are vocalizing, is a difficult task and can be unreliably coded. One must also bear in mind that individual animals manifest stress in different ways and that canine behavior varies with age, sex, breed, history, and differences in personality and temperament [12]. Hence, each dog will exhibit individualized changes in behavior despite being exposed to and experiencing similar environmental stimuli.

The American Society for the Prevention of Cruelty to Animals (ASPCA) estimates that 60% of kennel dogs are euthanized in shelters [53]. Even if a dog is housed in a non-kill shelter, remaining in a shelter for a prolonged period of time exerts profound effects on the animal’s welfare [54]. In-kennel behavior has been consistently demonstrated to be a principal predictor of adoption [55]. Hormonal measures may or may not be manifested in outward behavior, but shelter visitors unwaveringly report in-kennel presentation as the main reason for adopting or not adopting a dog [55]. Potential adopters on average spend only 20-70 seconds evaluating a dog in its kennel [56], and a vast majority of potential adopters only take out a single dog to interact with based on the dog’s in-kennel behavior [55]. Playfulness and friendliness are the top two characteristics associated with a high likelihood of adoption, whereas inertness and aloofness are the top two characteristics associated with a low likelihood of adoption [55]. The significant decrease in resting behavior in response to human interaction, regardless of the experimental treatment group, is thus an incredibly promising outcome of this study. Nonetheless, the increase in time spent in the back half of the kennel is concerning, as reclusiveness and unsociability are
viewed as highly unfavorable traits in a dog and may adversely affect a dog’s likelihood of adoption. Normando et al. (2009) mitigated these behaviors by subjecting shelter dogs to an enhanced human interaction program in which the dogs interacted with humans for fifteen minutes once a week: after six interaction weeks, the dogs spent more time in the front of the kennel and displayed increased tail wagging towards people [57]. On that account, enhanced human interaction for an extended period of time may reduce undesirable behaviors whilst encourage desirable behaviors, thereby leading to an increased likelihood of adoption.

*Experimental Limitations*

As with all studies, there are certain limitations of this study that must be noted and considered. In spite of working with twenty-six dogs over the course of several weeks, only eight dogs met the three-day data collection criterion, therefore making the sample size appreciably smaller than the originally intended twenty-dog sample size. Accompanied by the fact that hormonal data are extremely variable, both within a single individual and results obtained from different studies, a small sample size has the potential to introduce sampling bias that undermines the reliability of the results. Moreover, the urine and fecal samples were not true first morning samples, which yield the highest cortisol metabolite concentration and represent the cortisol production rate over twenty-four hours [12]. Nonetheless, this would exert a larger impact on urine GCM levels than on fecal GCM levels, as feces have a longer intestinal passage time so fecal GCM levels are more robust to temporal variation [42]. Perhaps greater differences in urine GCM/creatinine levels between the first and last interaction day could have been observed if the sample size was larger and if true first morning urine samples were collected.
In addition to the small sample size, the amount of data that could have been collected on an individual dog was limited. It was initially intended to collect data on an individual subject three times a week for three weeks to give nine data points per dog. However, the average length of stay for dogs at the HLLC is nine days, hence the data collection time period was reduced to three times a week for one week. Consequently, the amount of data that could have been collected per subject was reduced to three data points, and the amount of time for a meaningful dog-human bond to form was curtailed. Of the eight dogs that did meet the data collection criteria, three of the dogs were fixed in between interaction days and all of the dogs were moved to publicly accessible kennels at some point during the study. These two factors would certainly provoke stress-related responses in the dogs and could explain the increased GCM levels observed in some of the dogs as well as the increased percentage of time spent in the back half of the kennel for both treatment groups.

**Future Directions**

The objective of this study was to determine if and what type of interaction more effectively reduces stress in shelter dogs in hopes to create a welfare model that rescue facilities can employ to minimize stress in their animals whilst promoting positive dog-human relations. Even though the experimental results did not show a significant difference between the two treatment groups, having the dogs fixed and/or moved to publicly accessible kennels are undeniably stressful events. The HLLC is arranged in such a way that there are kennels accessible and inaccessible to the public. When the dogs are first admitted to the shelter, they are housed in the inaccessible kennels but as soon as they are deemed ‘adoptable’, they are moved to the accessible kennels where potential adopters can view the dogs (Fig. 9A). Rather than housing
the dogs in one kennel and then moving them to another kennel, it may be less stressful for the dogs if they are kept in a single kennel throughout their stay at the shelter. By designing a facility in which certain rows of kennels can be made accessible and inaccessible to the public at different times, this would avoid having to move the dogs to the select publicly accessible kennels (Fig. 9B).

**Fig. 9.** A schematic representation of (A) the facility layout of the Humane League of Lancaster County and (B) a proposed facility layout in which the kennels can be made accessible or inaccessible to the public at different times.

Social housing, kennel size, and kennel location within a shelter have all been shown to impact shelter dog welfare, but surprisingly few studies have evaluated the sensory aspects of the kennel environment [58]. Some studies have looked at the effects of the visual environment on kenneled dogs, but given that olfactory communication and investigation is a fundamental
feature of canine behavior [59], the lack of research on the olfactory environment of the kennel is staggering. Only two studies have investigated olfaction in animal shelters, yet these studies examined the influences of additional aromas as opposed to the diminution of noxious ones on the shelter dogs [58]. For example, Graham et al. (2005) reported that various aromatherapy odors exerted differential effects on shelter dog behavior: dogs spent more time resting when exposed to lavender vapors and more time moving and vocalizing when exposed to peppermint vapors [60]. Tod et al. (2005) found that when exposed to the dog appeasing pheromone (DAP), a synthetic analogue of a pheromone produced by the lactating bitch [58], dogs spent more time resting and sniffing and less time barking [61]. The canine olfactory epithelium expresses 30% more olfactory receptors than and is 20 times as large as that in humans [62-64]. Although studying olfaction in dogs proves to be a complicated and complex task, how canine olfactory sensitivity, which is profoundly greater than that of humans [65], may influence welfare when in a kennel requires more attention.

Because dogs have an extraordinary need for human contact and interaction, this may induce rapid and intense interspecies bond formation [31]. The benefits of using human socialization to optimize kennel dog welfare are two-fold: a human interaction session can reduce stress in shelter dogs and also shed light on a dog’s personality, information that can be exploited to consummate compatible adoptions [66]. This study investigated the type of interaction between the shelter dog and a human volunteer, but no study has examined if the activity during an interaction session (i.e. walking, petting, playing, training) differentially affects physiological and behavioral measures of well-being. Furthermore, minimal research has been done on chronically stressed dogs, such as puppy mill rescue dogs, dog fighting rescue dogs, or even dogs rescued from cruel and neglectful homes. Using positive and nurturing human
relations to reduce stress in these dogs is an important avenue of exploration and can provide insight into how chronic stress impacts well-being and if its effects can be counteracted.

**Conclusion**

There is much discussion revolving around what constitutes the basic needs and proper care of animals. Animal welfare is comprised of multiple statutes that aim to address the objectively-set standards for quality animal care, but more work must be done to ensure that our animals have a high quality of life [67]. The overarching purpose of studying animal welfare is to learn about the demands and preferences of animals with the intention to better our tending to these necessities. Physiological and behavioral indicators of stress are only proxy parameters of welfare status, and because no individual welfare measure can comprehensively describe an animal’s subjective emotional state, multiple welfare indicators must be used concomitantly in welfare studies. And finally, because of our emotional love for and attachment to our companion animals, concern and legislation for their welfare is emphasized, but consideration of animal well-being must also extend to farm animals, laboratory animals, and wildlife [68].

Dogs enrich our lives with affection, loyalty, security, and joy [69]. They help humans in innumerable ways, including but not limited to reducing anxiety and depression in the emotionally distressed, encouraging the physically disabled to perform motor tasks, and providing companionship and love for the terminally ill and the general public [70]. More than 63% of U.S. households currently have at least one pet, with dogs being the most common [1,71], yet even so, a majority of companion dogs will at some point in their lifetime be subjected to the kennel environment, be it for veterinary treatment, boarding, or rehoming. Though animal shelters are extremely limited in funding, staff, and space [69], it is an ethical
obligation to tend to our animals’ needs, maximize their well-being, and, in the case of shelter dogs, find them a loving and forever home.

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References


(*Canis familiaris*): A new application of Ainsworth’s (1969) Strange Situation 

kenneling: Is barking a welfare problem for dogs?. *Applied Animal Behaviour 


Building design and the effects of daily noise exposure. *Journal of Applied 
Animal Welfare Science, 9*(1), 1-7.


Behavioral and contextual predictors of adoption. *Applied Animal Behaviour 

animal rescue shelter. *Anthrozoös: A Multidisciplinary Journal of The Interactions of 
People & Animals, 14*, 12-18.

of an Enhanced Human Interaction Program on shelter dogs’ behavior analysed 


STPM Press; 1978.

the behavior of dogs housed in a rescue shelter. *Applied Animal Behaviour 
Science, 91*, 143-153.

reducing stress and fear-related behaviour in shelter dogs. *Applied Animal Behaviour 
Science, 93*, 295-308.


Appendix: Data collection protocol instructions for human volunteers

A coin will be flipped in order to randomly assign dogs to an experimental condition (consistent or rotating).

Videotaping procedure:

Dogs will be videotaped 35 minutes before the interaction session. To videotape a dog:

1. Set up a tripod and video camera in the staff room. Once set up, place the tripod and camera against the wall facing the front of the dog’s kennel.
2. Press ‘Record’ on the camera and leave the kennel area so that you are completely out of the dog’s sight and sound.
3. Start a stopwatch and time 35 minutes.
4. After 35 minutes, re-enter the kennel area and press ‘Stop’ on the camera.
5. Place the tripod and camera in front of another dog’s kennel and repeat steps 2–4.
6. Take the first dog out of his/her kennel according to shelter/rescue protocol and commence the 30-minute interaction session.

If a dog is being videotaped, try not to walk in front of the dog’s kennel i.e. find an alternative route to get outside.

Interaction session procedure:

Volunteers will be interacting with their respective dog for 30 minutes. After the dog has been videotaped for 35 minutes:
1. **Make sure the leash, urine catch tray, and urine/fecal collection equipment are out of the dog’s sight before you enter the kennel area.** Slowly open dog’s kennel door towards you and at the same time as you open the door, start the stopwatch.
2. Wait for the dog to approach you. When the dog makes contact with you, stop the stopwatch and greet the dog.
3. Write down the approach time somewhere handy and record the time in the Google spreadsheet at your earliest convenience.
4. Take the dog out of his/her kennel according to shelter protocol and take the dog on a 15-minute walk, during which you will collect urine and fecal samples (see the below procedure). Try to remain isolated from other volunteers and their dogs.
5. Following the 15-minute walk, play with your dog for 15 minutes. Play may include tossing a ball or toy, basic positive reinforcement training, and petting. Try to remain isolated from other volunteers and their dogs.
6. After playing with your dog for 15 minutes, return your dog back to his/her kennel.

*Though volunteer behavior will be consistent in that all volunteers will spend 15 minutes walking and 15 minutes play with their dog, how you act during the session will be at your discretion in response to your dog’s behavior.*

**Urinary and fecal collection procedure:**

Please collect urinary and fecal samples when dogs are first let out of their kennel. To collect a sample:

1. Label the urinary collection tube and fecal collection cup and lid with the **animal name, date, and time collected** prior to collecting the samples using the provided waterproof marker.
2. Put on a glove (or gloves).
3. To collect urine:
   a. Place the urine catch tray under the dog to catch the urine midstream. Set the urine catch tray aside until the interaction session is over. If not enough urine (between 1 and 4 mL) is caught the first time, collect again on the same walk.
   b. When the dog is returned to his/her kennel, enter the staff room and, with a sterile syringe, move between 1 and 4 mL of urine into the labeled urinary collection tube.
   c. Twist the lid on securely and place the tube in the cooler. Samples should be frozen as soon as possible, with a maximum acceptable time between urination and freezing of 3 hours.
   d. Rinse the urine catch tray with water, spray with trifectant, and dry completely before using again.
4. To collect feces:
   a. Select the freshest available feces that the dog has produced on the walk and fill the cup about ¾ full. If possible, try to get some feces from several different parts of the pile. Once the feces have been collected, snap the lid on securely and set the collection cup aside until the interaction session is over.
b. When the dog is returned to his/her kennel, enter the staff room, put the cup in a Ziploc bag and place in the cooler. Samples should be frozen as soon as possible, with a maximum acceptable time between defecation and freezing of 3 hours.

*If you are unable to collect urine and/or fecal samples, write “No sample collected” on the urinary collection tube and/or fecal collection cup and place in the cooler.*