Assessing physiological and behavioral responses over time in a group of laboratory Marshall beagles

Lisa Dietz

3258858

Supervisor: Dr. A. Ortolani

Faculty of Veterinary Medicine

Department of Animals in Science and Society

Utrecht University

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Universiteit Utrecht

Summary

The aim of this study was to assess adaptive capacity in a group of purpose-bred Marshall beagles by means of a standard procedure that measures heart rate variability, salivary cortisol and behavior. Heart rate variability was measured with a Polar® heart-rate monitor and behavior was recorded with a video camera during two successive 5 minute observation periods; a 'restrained' observation period in which the dogs stood on a table while being restrained by a technician, and an 'unrestrained' observation in which the dogs were allowed to roam freely on the table. At the end of the standard procedure a saliva sample was taken to measure cortisol levels and compare with morning cortisol levels.

A large variation was present in heart rate variability and behaviors observed in this sampled group of Marshall beagles. For the most part this variation could not be attributed to gender, age or previous experience in experimental studies, although more experienced dogs appeared to exhibit more *tail between legs*, which is considered fearful behavior.

Physiological and behavioral responses over time were assessed for each individual dog using mean heart rate and the behavior *tail between legs*. Some dogs showed clear adaptive responses over time while others did not. Dogs that do not show an adaptive response over time may be at risk of poor welfare when exposed to a stressor, such as the environmental stimuli they may encounter when being used for laboratory research.

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1. Introduction

1.1. The concept of animal welfare

Animal welfare first became an issue when certain people started questioning the way livestock was managed to achieve ever higher production rates. In response to the book 'Animal machines', published by Harrison in 1964, the British government put the 'Brambell Committee' in charge of a research on animal welfare (Harrison, 1988). This resulted in the publication of 'the five freedoms' in 1965. These five freedoms stated that animal welfare was assured if animals were:

- Free from hunger, thirst and improper nutrition
- Free from thermal and physical discomfort
- Free from injuries or diseases
- Free from fear and chronic stress
- Free to display normal, species-specific behavioral patterns (Brambell, 1965)

This concept of animal welfare was commonly accepted for decades and has been implemented in European legislation, which has led to some serious and positive changes for the welfare of farm animals (Yeates & Main, 2008). However, in the last two decades a need has developed to define animal welfare not only as the absence of negative stimuli, but to also implement positive aspects of welfare in the concept. Several concepts of animal welfare have been proposed since then.

According to Ohl and van der Staay (2012) welfare is not just about the absence of aversive stimuli, but about an animal's capacity to adapt to changes in its environment and about positive feelings of the animal. Recently, they proposed an enhanced version of the five freedoms as a new concept of animal welfare (Ohl and van der Staay, 2012). They state that '*An individual is in a positive welfare state when it has the freedom adequately to react to hunger, thirst or incorrect food; thermal and physical discomfort; injuries or diseases; fear and chronic stress, and thus, the freedom to display normal behavioral patterns that allow the animal to adapt to the demands of the prevailing environmental circumstances and enable it to reach a state that it perceives as positive.*'

This means that presence of negative stimuli is not an indicator of poor welfare per se (and absence of these stimuli is not an indicator of good welfare), provided that the animal is capable of adequately adapting to the changes.

1.2. Welfare assessment

When assessing welfare, measurements can be made in three different domains: resource-based measures (e.g. the dog has sufficient food and water), management-based measures (e.g. the dog is regularly taken for a walk) and animal-based measures (e.g. the dog is healthy, in good condition and displays natural behavior). Resource-based and management-based measurements are relatively easy to assess but say little about the internal state of an animal. Animal-based measurements are therefore more valuable because they are a direct reflection of an animal's internal state. However, animal-based measurements are also more difficult to measure since we cannot see inside the animal and the animal cannot tell us how it is doing.

Welfare Quality® is a research project that has developed standardized checklists with largely animal-based measurements for assessing welfare in farm animals (Blokhuis, 2010). Checklists are made specifically for each farm animal species and are therefore not applicable to companion animals. Similar checklists specifically for dogs have yet to be made. However, it should be evident that animal-based measures are most valuable for companion animals as well and should therefore be used for welfare assessment.

1.3. *Stress and adaptive capacity as a welfare indicator*

The concept of stress has been defined by Selye as follows: "Stress is the non-specific response of the body to any demand upon it" (Selye, 1971). Physiological processes in an animal's body are maintained around a certain set point, which is called homeostasis. When environmental challenges are encountered a stress response occurs in the form of elevated heart rate and respiratory rate, and increased cortisol levels through activation of the hypothalamic-pituitary-adrenal axis (HPA axis) (Yeates and Main, 2008; Koolhaas et al., 2011). It is thought that these physiological changes mobilize more energy for the animal to respond and adapt to the environmental changes. However, because the stress response is not-specific (i.e. it occurs in situations that animals perceive as positive as well as situations they perceive as negative), it has been proposed that these physiological parameters should only be interpreted in relation to other physiological parameters, the animal's behavior and the environmental challenges (Yeates and Main, 2008).

By measuring the physiological parameters that are involved in the stress response (e.g. heart rate, respiratory rate and cortisol levels) the magnitude of an acute stress response can be measured. However the magnitude of an acute stress response does not have to be an indication of poor welfare per se, if the animal shows adaptation over time. According to Ohl and van der Staay (2012), an animal is in a state of positive welfare when it is able to adequately adapt to environmental changes. It is therefore not so much the magnitude of the response as it is the temporal dynamics of the response that are indicative of an individual's ability to cope with the situation (Koolhaas et al., 1997). For the assessment of adaptive capacity it is important to observe how an individual's behavioral and physiological response to a challenging stimulus changes over time, i.e. the 'shape' of the response. Only when observing whether an animal adapts to a stimulus over time it is possible to make a statement on the welfare state of this animal. A prolonged (acute) stress response with no adaptation over time indicates poor adaptive capacity (Ohl and van der Staay, 2012) and, consequently, indicates that the individual may be at risk of poor welfare.

1.4. *Dog welfare*

To ensure good welfare in dogs some species-specific needs must be fulfilled. Dogs need a proper diet suitable for its breed which may not lead to malnutrition or obesity. They need good housing conditions with enough space to allow exercise and expressing of normal dog behavior and with sufficient enrichment to avoid boredom. Also, dogs are social animals and should therefore be housed with conspecifics or allowed intensive social contact with humans (Broom and Fraser, 2007; Yeates, 2012). When any of these requirements is not met the welfare of the dog is compromised.

Dogs serve a variety of purposes in our modern society. They are used as companion animals, working dogs, but also as laboratory animals. The most commonly used dog breed for laboratory research is the beagle, due to their small size, their co-operative nature and the ease of housing them in kennels (MacArthur Clark and Pomeroy, 2010). Because laboratory dogs may be subjected to welfare compromising conditions it is especially important for these dogs that good welfare is ensured where possible.

The concept of the three Rs - reduction, replacement and refinement - has been developed to improve welfare in laboratory animals. This concept implies that wherever possible reduction and replacement of laboratory animals and refining of experimental design (i.e. minimizing discomfort for test subjects) should be applied (Russell and Burch, 1959). However, even when applying this concept, in some cases laboratory animals are still needed. For these animals impairment of welfare is inevitable to a certain extent, but efforts should be made to optimize their welfare. European legislation concerning husbandry and management of laboratory animals provides a good foundation on this matter.

Optimizing welfare in laboratory animals is important not only for the animals themselves and the increasing concern of society on the topic, but also for the quality of scientific research.

(Chronic) stress may lead to altered enzyme activities, hematologic parameters and glucocorticoid metabolism, cardiovascular changes and reduced gastro-intestinal activity (Koolhaas et al., 1997; Kuhn et al., 1991; Ritskes-Hoitinga et al., 2006). In addition, animals with poor adaptive capacity may develop stereotypic behavior when environmentally challenged (Hubrecht, 2002). Consequently this may lead to altered energy and muscle metabolism (Ritskes-Hoitinga et al., 2006). It should be clear that stereotypic behavior could interfere with the interpretation of behavioral studies and that altered metabolic processes could interfere with many scientific studies measuring physiological parameters. The influence exerted by the physiological alterations caused by (chronic) stress could lead to wrong interpretations by researchers, thereby reducing the quality of scientific research.

1.5. Aim of this study

The aim of this study was to assess adaptive capacity in two groups of purpose-bred Marshall beagles used for laboratory research.

Ortolani et al. (2013) have developed a standard procedure for measuring adaptive capacity in dogs, which measures behavior, heart rate variability and cortisol levels to assess an individual's adaptive capacity in a potentially challenging situation, such as a visit to the vet. The standard procedure has been used to assess adaptive capacity in a study of companion dogs visiting a vet practice and in a study of dogs with separation related behavioral problems.

A similar standard procedure, with some modifications, was used in this study to measure behavioral and physiological responses and assess adaptive capacity in two groups of purpose-bred Marshall beagles. We compared responses in beagles of different ages (0,95-5,85 years) and different experience with participation in research studies (1-9 studies). The goals of the study were: 1) to characterize the variation in behavioral and physiological responses to the standardized procedure in this group of purpose-bred Marshall beagles; 2) to assess adaptive capacity by looking at the pattern of the heart rate and behavioral responses over a 5 minute period; and 3) to investigate if there is a significant effect of age, gender and/or experience on the responses exhibited by the dogs.

2. Materials en methods

2.1. Subjects

This research took place at WIL Research Laboratory in Den Bosch, the Netherlands, over a two-month period. Twenty six purpose-bred Marshall beagles (13 males, 13 females, all intact) were used as test subjects. These dogs had not been part of a study for at least one week prior to beginning of observations. The dogs were divided in two groups, described further below. Lights went on at 0700h in the morning and off at 1900h. All dogs were fed once a day around 0800h. After the study described here, all dogs were re-used in other studies at WIL Research.

2.1.1. Group 1

The first group consisted of 12 relatively young and inexperienced beagles (6 male, 6 female) of 1,16±0,04 years (mean±SE, range 0,95-1,30). All dogs in this group had been previously used in one other study at WIL Research Laboratory, except for one female who had been used in two previous studies. These studies were mainly pharmacokinetic studies. All 12 dogs were purpose-bred Marshall beagles imported from Upstate New York.

Three male dogs were socially housed with other dogs in cages measuring 1.9m x 1.85m, with raised platforms of different heights and concrete floors. They were able to see and hear but not have physical contact with dogs in other cages. The other dogs were housed in pairs in cages measuring 2.69m x 1.46m and were able to have physical contact with dogs in adjacent cages through the bars of the cage. None of the test subjects were litter mates or shared a cage with each other.

2.1.2. Group 2

The second group consisted of 14 relatively older and more experienced beagles (7 male, 7 female) of 4,27±0,24 years (mean±SE, range 3,02-5,85) used in 5,79±0,42 previous studies at WIL Research (mean±SE, range 4-9). All males were bred in Italy except for one who was bred in the USA. Four females were bred in Italy, one in the USA and two at WIL Research.

All dogs were housed in pairs in cages measuring 2.69m x 1.46m and were able to have physical contact with dogs in adjacent cages through the bars of the cage. None of the test subjects were litter mates. Two dogs in this group shared a cage with each other.

2.2. Standard Procedure

The purpose-bred Marshall beagles were tested by means of a standardized procedure, developed by Ortolani et al. (2013) to assess the welfare of individually kept dogs. With this standard procedure three parameters are measured: heart rate variability, salivary cortisol levels and behavior.

During the standard procedure the dog stands on a table and a non-invasive Polar® heart-rate monitor (RS8000cx) is strapped to the dog's chest. The accuracy of the Polar® heart-rate monitor (RS8000cx) compared to ECG has been previously validated (Jonckheer-Sheehy et al., 2011) in stationary dogs. Dogs were not previously habituated to wearing the heart rate monitor.

The dogs were observed in two 5-minute observation periods: 1) restrained on table and 2) unrestrained on table. During these observation periods the heart rate was measured continuously and the dog's behavior was recorded with two video cameras, a Sony HDR-CX550 placed in front of the table and a Sony HDR-CX160 placed on the side of the table (Photo 1 and 2).

Once the heart rate monitor was in place, which took approximately 90 seconds, the first observation period began in which the dogs were restrained on a table. In order to keep the dog stationary a technician remained next to the table and was instructed to hold the dog to keep it from moving, but they were asked not to pet the dog. After this 'restrained' period the dog was released and was allowed to roam freely on the table for an additional 5 minutes. During this

second ‘unrestrained’ observation period the technician stepped back from the table but stayed nearby to keep the dogs from falling or jumping of the table.
During the entire 10 minutes the researcher stood behind and controlled the camera in front of the table. The temperature in the observation room was measured by means of a room thermometer during the procedures and varied overall from 19,6 to 20,1°C, but there was never more than a 0,2°C difference during one standard procedure.

Two salivary samples were obtained for each dog, one in the morning before feeding (between 0700 and 0830h) on the day of testing to assess morning (baseline) values for each individual, and an additional one 10 minutes after the start of the standard procedure (right after the Polar® heart-rate monitor was strapped on). Saliva samples were taken using Salimetrics® cotton ropes held in the dog’s mouth for at least 90 seconds. The total duration of the whole standard procedure was 15 minutes. Standard procedures were performed between 1100 and 1300h on all dogs.

Photo 1. Overview of the room seen from the camera in front of the table. Notice the second camera on the right side.

Photo 2. View from the camera on the side of the table.

2.3. Behavioral analysis

Dog behavior displayed during the standard procedure was analyzed afterwards from videos using an ethogram initially developed in a previous study which measured adaptive capacity in dogs during a veterinary consult (Ortolani et al., 2013), but modified and extended for use in this study. The ethogram for the 'restrained' observation period consists of four behavioral categories: mouth behaviors, tail behaviors, body movements and head orientations. A description list of all behaviors scored and their definitions can be found in Appendix I. Although many behaviors were scored, only 11 were selected for further analysis. Some behaviors occurred infrequently or not at all and were therefore excluded. Other behaviors reflected more or less the same situation (e.g. *head to own body* and *sniffing self*) in which case only one of the behaviors was selected. The selected behaviors are listed in Table 1.

The ethogram for the 'unrestrained' observation period consists only of mouth behaviors, tail behaviors and body movements and only a selection of behaviors was scored. Only those behaviors that occurred frequently and showed some significant correlations during the 'restrained' period were selected. The ethogram for the 'unrestrained' observation period can be found in Appendix II.

Behaviors were scored either as frequency or as both duration and frequency. Durations were adjusted for 'out of sight' occurrences and converted into the 'proportion of time' that the behavior occurred in 5 minutes for statistical analysis.

One observer scored all dogs' behaviors. The observer was trained with previous footage of other dogs under similar circumstances and an inter-observer reliability of at least 0,9 for all behaviors was reached before scoring of these dogs began. Intra-observer reliability was measured by scoring three or four dogs of the first group again and comparing both results. Sniffing behaviors, *tucked tail* and *low tail* did not reach an intra-observer reliability of 0,85 or higher and therefore mouth and tail behaviors were scored again.

2.4. Heart rate variability analysis

The date collected from the Polar® heart rate monitor consisted of RR intervals, aka inter beat intervals (IBIs). Using these IBIs mean heart rate (bpm) was calculated and heart rate variability (HRV) was measured for different time domain and frequency domain parameters using the software program Kubios HRV, version 2.1 (Tarvainen et al., 2008). Time domain parameters consisted of mean inter-beat interval (meanRR), standard deviation of all IBIs (SDNN), root mean square of successive differences (RMSSD), number of pairs of successive IBIs differing by more than 50 ms (NN50) and proportion of beats differing by 50ms (pNN50). Frequency domain parameters consisted of very low frequency (VLF, 0-0,04 Hz), low frequency (LF, 0,04-0,15 Hz), high frequency (HF, 0,15-0,6 Hz) and LF/HF ratio (Von Borell et al., 2007).

Since HRV parameters are largely correlated with each other, for statistical analyses RMSSD was chosen as being representative for time domain parameters and LF/HF ratio as representative of the frequency domain parameters. In addition, mean heart rate (bpm) was used for further analysis.

During both observation periods there were three dogs with more than 15% errors in their heart rate data (IBIs). This data was therefore excluded from further statistical analysis (see Jonckheer-Sheehy et al., 2011).

2.5. Salivary cortisol analysis

Saliva samples were centrifuged at 3000 rpm during 15 minutes at 20°C, usually within 8 hours after sample collection. When the time span between collection and processing of the samples was expected to exceed 8 hours the saliva samples were stored on ice. Centrifuged samples were weighed with a Sartorius SAR/1801 scale to establish if enough saliva was collected - the minimal amount required for a single reading by the Salimetrics kit is 25 µL - and then stored at -20°C until further processing (Dreschel and Granger, 2009). Salivary cortisol was measured using highly sensitive enzyme immunoassay (ELISA) kits, following the protocol of the manufacturer (Salimetrics, State College, PA). All samples were analyzed in duplicate to validate results, unless sample volume only allowed a single reading. The mean of the two values was used for statistical analysis.

Of the 52 saliva samples in total, seven samples had a volume between 10 and 50 µL and were diluted to gain enough volume for a duplicate reading. Nine samples had a volume smaller than 10 µL: three of these were diluted to gain enough volume for a single reading, the other six were noted as a missing value. As a result, two dogs had both saliva samples noted as missing values and two other dogs had only the post-observation saliva sample noted as a missing value.

Inter- and intra-assay variation was calculated following the guidelines published by the kit manufacturer (Salimetrics, State College, PA). The inter-assay coefficient of variation (%CV) was 2,9%, with 15% being the acceptable maximum limit. Intra-assay %CV was on average 1,5% and has a maximum acceptable limit of 10%.

One of the dogs had a test cortisol value below the lower bound of the confidence interval and could therefore not be measured reliably. Statistical analysis with cortisol values was performed both including and excluding this value and it did not have a large effect on the results.

Of three dogs the morning saliva sample did not contain enough saliva and sampling was repeated on other days at the same time in the morning.

2.6. Adaptive capacity

Adaptive capacity was assessed by means of plotting both heart rate and behavioral responses over time. The heart rate data (RR intervals) from the 5 minute 'restrained' observation period was divided in ten bouts of 30 seconds for each individual dog and the mean heart rate was calculated for each bout. Next, these ten data points were plotted in a graph to view the shape of an individual dogs' heart rate response.

In order to assess adaptive capacity using behavioral indicators, we chose to look at *total tail between legs.* Total tail between legs is the sum of the behaviors *tail between legs*, *wagging tail between legs* and *tail on table between legs*. This behavior is a clear indicator of fear/anxiety in dogs (Kiley-Worthington, 1976; Bradshaw and Nott, 1995; Overall, 1997) and in this sampled group of purpose-bred Marshall beagles it showed a large within group variation.

2.7. Statistical analysis

Behavioral, heart rate and salivary cortisol data were statistically analyzed using the software program SPSS (version 20 for Windows). To assess the relationship between behavioral and physiological parameters and to assess the relationship between age and/or experience and behavioral and/or physiological parameters, Pearson's (normally distributed parameters) or Spearman's Rho (non-normally distributed parameters) correlation coefficients were used.

Differences in behavior and heart rate parameters between the 'restrained' observation period and the 'unrestrained' observation period were assessed with a Friedman ANOVA.

To assess differences in sex and age group paired Student's t tests were used for normally distributed and homoscedastic data and Mann Whitney U tests were used for data that were not normally distributed. Differences between experience groups were assessed with a one-way ANOVA for normally distributed and homoscedastic data, otherwise a Kruskal-Wallis test was tested.

Results are reported after Field (2009).

3. Results

To control for age effects, dogs' responses in two age groups were compared: the 'young' group (1,16±0,04 years, mean±SE) was also the first observed group, and the 'old group' (4,27±0,24 years, mean±SE) was the last observed group. To control for the effect of experience dogs' responses were compared across three groups: the first group consisted of dogs that had only participated in one research study previously (n=11), the second group consisted of dogs that had participated in 2 to 5 previous studies (n=7) and the third group consisted of beagles that had been in 6 studies or more (n=8). These studies were mainly pharmacokinetic studies.

3.1. 'Restrained' observation period

3.1.1. Behavior

Although many behaviors were scored during the 'restrained' observation period (see Appendix I), only 11 were selected for further analysis. The selected behaviors are listed in Table 1.

Gender

No significant differences in gender were found in any of the eleven selected behaviors.

Age

A significant difference between both age groups was found only for the behavior *tail between legs* (Mann Whitney U: U=44,0, z=-2,20, p=0,028, r=0,43), with older dogs exhibiting more of this behavior. Significant negative correlations of age were found with *sniffing table* (Spearman's Rho: r_s=-0,46, p=0,018), *tail wagging* (r_s=-0,40, p=0,044) and *head to technician* (r_s=-0,51, p=0,009).

Experience

No differences between experience groups (no. of studies) were found in any of the eleven selected behaviors. Experience was positively correlated with *tail between legs* (Spearman's Rho: r_s=0,48, p=0,014).

Many behaviors were significantly correlated with each other. *Head movement* was positively correlated with *sniffing self* (r_s=0,62, p=0,001), *sniffing table* (r_s=0,51, p=0,008), *head to technician* (r_s=0,62, p=0,001), *hiding head* (r_s=0,50, p=0,009), *paw lifting* (r_s=0,61, p=0,001), and *moving* (r_s=0,74, p=0,000).

Also, the behavior *moving* was significantly positively correlated with *sniffing self* (r_s=0,46, p=0,019), *sniffing table* (r_s=0,40, p=0,020), *head to technician* (r_s=0,63, p=0,001) and *paw lifting* (r_s=0,48, p=0,014).

Furthermore *sniffing self* was positively correlated with *sniffing table* (r_s=0,62, p=0,001), *head to technician* (r_s=0,43, p=0,027), and *hiding head* (r_s=0,53, p=0,006). *Sniffing table* was positively correlated with *licking lips* (r_s=0,46, p=0,019).

In addition *tail between legs* was negatively correlated with *tail wagging* (r_s=-0,51, p=0,008) and positively correlated with *crouching* (r_s=0,51, p=0,008).

Individual variability

Mouth behaviors

Figure 1 shows that sniffing behaviors were exhibited more frequently by younger dogs, in particular *sniffing table* and *sniffing technician*. However, this difference was not statistically significant (Mann Whitney U: U=54,0, z=-1,54, ns, r=0,30). Mouth behaviors scored as frequency were not exhibited frequently with the exception of *licking lips,* see Table 2. Figure 2 presents a histogram showing within group variation of the behavior *licking lips*.

Tail behaviors

Figure 3 shows all tail behaviors and positions that were scored. Although over 10 distinct tail positions and movements have been scored, the most common were *tail between legs* and *tail wagging*. *Tail between legs* was exhibited more by the older dogs (Mann Whitney U: U=44,0, z=-2,20, p=0,028, r=0,43) and *tail wagging* was seen more in the 'young' group, although this was not statistically significant (U=55,0, z=-1,52, ns, r=0,30). Moreover, the whole group of 26 beagles showed a wide variation in both *tail wagging* and *tail between legs,* as is showed in Figure 4*.* Some beagles did not exhibit these behaviors at all, while others showed them almost continuously during the observation time.

Body movement

The body behaviors are presented in Figure 5. 'Young' dogs sat down on the table about twice as much as the older dogs (Mann Whitney U: U=52,0, z=-1,79, ns, r=0,35) and exhibited more *moving* than older dogs (Mann Whitney U: U=74,5, z=-0,49, ns, r=0,10), but this was not a significant difference. The other behaviors don't appear to differ much between the groups. However, there was clear variation in body behaviors in the whole group of 26 dogs. For example, the behavior *moving* was exhibited ranging from 1,0 to 56,3% of the time.
Data on the occurrence of *paw lifting* is presented in Table 2.

Head orientation

The head orientations that were scored are shown in Figure 6. *Head to environment* is not included in this figure because all dogs showed a high rate of this behavior. As a consequence, differences between both age groups in other head behaviors were not clearly visible in the bar chart. *Head to technician* and *head to camera* show opposite trends in the 'old' group compared to the 'young' group. Younger dogs appear to look more to the technician than older dogs whereas older dogs appear to look more to the camera and/or the observer behind the camera, although this result was only marginally statistically significant (Mann Whitney U: U=46,0, z=-1,96, p=0,051, r=0,38). The behavior *head low* was exhibited more in the younger dogs (U=37,0, z=-2,42, p=0,011, r=0,47), which could be explained by the fact that younger dogs also exhibited more *sniffing table.*

3.1.2. Heart rate variability

A large individual variation in heart rate variability parameters was present in the sampled group of purpose-bred Marshall beagles, as shown in Table 3.

Gender

No differences in gender was found in mean heart rate (Mann Whitney U: U=44,0, z=-1,35, ns, r=0,26), RMSSD (U=62,0, z=-0,25, ns, r=0,05) or LF/HF ratio (U=41,0, z=-1,54, ns, r=0,30).

Age

No differences in age group were found in mean heart rate (Mann Whitney U: U=61,0, z=-0,25, ns, r=0,05), RMSSD (U=58,0, z=-0,43, ns, r=0,08) or LF/HF ratio (U=59,0, z=-0,37, ns, r=0,07). Also, no significant correlations between age were found with mean heart rate (Spearman's Rho: r_s=-0,11, ns), RMSSD (r_s=0,24, ns) or LF/HF ratio (r_s=-0,01, ns).

Experience

No significant differences between groups of experience were found with mean heart rate (Kruskal-Wallis: H(2)=1,83, ns), RMSSD (H(2)=0,02, ns) or LF/HF ratio (H(2)=1,17, ns).
No significant correlations were found between experience and mean heart rate (Spearman's Rho: r_s=0,07, ns), RMSSD (r_s=0,06, ns) or LF/HF ratio (r_s=-0,07, ns).

3.1.3. Physiological and behavioral parameters

No significant correlations were found between cortisol parameters (morning cortisol, post-observation cortisol and cortisol difference) and heart rate variability parameters (mean heart rate (bpm), RMSSD and LF/HF ratio). Also, no significant correlations were found between any of the eleven selected behaviors listed in Table 1 and the cortisol parameters (morning cortisol, post-observation cortisol and cortisol difference). In addition, no significant correlations were found between any of the eleven selected behaviors and HRV parameters (mean heart rate (bpm), RMSSD and LF/HF ratio).

3.2. 'Unrestrained' observation period

3.2.1. Behavior

Of the total of seven behaviors that were scored during the 'unrestrained' observation period (see Appendix I) all but one were used for further statistical analysis. The body movement *still* was left out because it is the inverted equivalent of *moving*.

Gender

No significant differences in gender were found in any of the six behaviors. There was only a marginally significant difference in *sniffing table* (Mann Whitney U: U=47,0, z=-1,92, p=0,054, r=0,38) with female dogs exhibiting this behavior more often during the 'unrestrained' observation period.

Age

Similar to the 'restrained' observation period, 'old' dogs showed significantly more *tail between legs* than 'young' dogs (Mann Whitney U: U=53,0, z=-2,04, p=0,041, r=0,40). A significant negative correlation of age with *panting* (Spearman's Rho: r_s=-0,44, p=0,023) and with *tail wagging* (r_s=-0,44, p=0,024) was found and a significant positive correlation with *tail between legs* (r_s=0,39, p=0,047).

Experience

There was a significant difference between the first and third experience group (1 previous study and 6 or more previous studies respectively) in the behavior *tail between legs* (U=18,0, z=-2,35, p=0,019, r=0,46). Also, experience and *tail between legs* correlated positively (Spearman's Rho: r_s=0,55, p=0,004).

Tail between legs and *tail wagging* were significantly negatively correlated (Spearman's Rho: r_s=-0,53, p=0,006), similar to the results in the 'restrained' observation period. *Moving* was positively correlated with *sniffing table* (r_s=0,40, p=0,042), *panting* (r_s=0,42, p=0,032) and *tail wagging* (r_s=0,44, p=0,027).

3.2.2. Heart rate variability

For the parameter mean heart rate there were no significant gender differences (Mann Whitney U: U=58,0, z=-1,36, ns, r=0,27), age differences (U=68,0, z=-0,82, ns, r=0,16) or experience differences (Kruskal-Wallis: *H*(2)=1,83, ns). No significant correlations of age with mean heart rate were found (Spearman's Rho: r_s=-0,35, ns), nor of experience with mean heart rate (r_s=-0,28, ns).

3.2.3. Physiological and behavioral parameters during the 'unrestrained' period

A positive correlation was found between mean heart rate and *moving* (Spearman's Rho: r_s=0,42; p=0,045) and a negative correlation was found between mean heart rate and *change of locomotion* (r_s=-0,44; p=0,037).

3.3. Comparing behaviors in both observation periods

There was a significant positive correlation between the 'restrained' and 'unrestrained' observation period for the following behaviors: *sniffing table*, *licking lips*, *tail between legs* and *tail wagging*, see Table 4. In addition, *sniffing table* during restraint was significantly positively correlated with *moving* in the 'unrestrained' observation period (Spearman's Rho: r_s=0,54, p=0,004).

Mouth behaviors

All dogs but one showed significantly more *sniffing table* during the 'unrestrained' period (a mean of 20,0% of the time) compared to the 'restrained' period (a mean of 3,9% of the time) (Friedman ANOVA: $F(1)$=21,2, p=0,000).

The behavior *licking lips* was not significantly different in both observation periods ($F(1)$=0,15, ns); 30,2 times on average during the 'restrained' observation period and 31,3 times on average during the 'unrestrained' observation period.

Tail behaviors

Tail between legs was only exhibited during the 'unrestrained' observation period by dogs that also showed this behavior during the 'restrained' observation period (n=7). For some dogs the proportion of time exhibiting this behavior increased and for some it decreased. Six dogs showed *tail between legs* during the 'restrained' observation period but not during the 'unrestrained' observation period. Overall, tail between legs was seen more in the 'restrained' observation period (21,3% of the time by 13 dogs) compared to the 'unrestrained' observation period (13,9% by 7 dogs), but this was not a significant difference (Friedman ANOVA: $F(1)$=3,77, ns).

Tail wagging was exhibited by 17 dogs during the 'restrained' observation period for a mean of 30,7% of the time. During the 'unrestrained' observation period it was exhibited by 19 dogs for 40,7% of the time. There was no significant difference between both observation periods ($F(1)$=1,80, ns).

3.4. Salivary cortisol

Results on cortisol are presented in Table 5. The data shows variation in morning and post-observation cortisol values in the sampled group of purpose-bred Marshall beagles. In addition, the difference in cortisol values was calculated by subtracting morning cortisol values from post-observation values for each dog. Differences in cortisol values are presented as a bar chart in Figure 7. Ten (3M, 7F) beagles showed a decrease in post-observation cortisol compared to morning cortisol, whereas 12 beagles (8M, 4F) showed an increase in cortisol values. This apparent gender difference was not significant (Mann Whitney U: U=39,0, z=-1,41, ns, r=0,28).

Gender

No significant gender difference was found for morning cortisol values (Mann Whitney U: U=44,0, z=-1,62, ns, r=0,32). However, one male dog had a much higher than average morning cortisol value and there would be a significant gender difference without this outlying value, with female dogs showing higher morning cortisol values than male dogs (Mann Whitney U: U=32,0, z=-2,09, p=0,036, r=0,41, Figure 8). Post-observation cortisol values did not differ between male and female dogs (Mann Whitney U: U=46,0, z=-0,95, ns, r=0,19). Although there appears to be a gender difference in Figure 7, this was not statistically significant (Mann Whitney U: U=39,0, z=-1,41, ns, r=0,28).

Age
There was no difference between the 'young' and 'old' group in morning cortisol values (Mann Whitney U: U=50,0, z=-1,17, ns, r=0,23), post-observation cortisol values (U=53,0, z=-0,37, ns, r=0,07), or in the difference between values (U=38,0, z=-1,37, ns, r=0,27). Also, no significant correlations were found between age and morning cortisol (Spearman's Rho: r_s=-0,24, ns), post-observation cortisol (r_s=0,09, ns) or cortisol difference (r_s=0,37, ns).

Experience
No difference between experience groups were found in morning cortisol (Kruskal-Wallis: $H(2)$=0,59, ns), post-observation cortisol ($H(2)$=1,57, ns), or cortisol difference ($H(2)$=3,14, ns). In addition, no significant correlations were found between experience (no. of studies) and morning cortisol (Spearman's Rho: r_s=-0,18, ns), post-observation cortisol (r_s=-0,05, ns) or cortisol difference (r_s=0,27, ns).

3.5. Assessing adaptive capacities

Mean heart rate
When looking at the adaptive responses of the heart rate during the 5 minute 'restrained' period three main distinct patterns emerged:

1. A fairly constant low or average heart rate response (n=7)
2. A fairly constant high heart rate response (n=4)
3. A fluctuating heart rate response with peaks and valleys (n=4)
4. A decreasing heart rate response over time (n=11)

The graphs of heart rate response over time are presented for each individual dog in Appendix III. Heart rate responses were also defined as low, average or high based on the mean heart rate of all dogs, which was 127,66 bpm as can be seen in Table 3. Figure 9 shows the mean heart rate response of all 26 dogs: the overall trend in heart rate response over the 'restrained' period is a decrease over time.

Behavior: tail between legs
When looking at the *tail between legs* response during the 5 minute observation period a number of specific patterns emerged:

1. An (almost) constantly high *tail between legs* (n=3)
2. An increase in *tail between legs* over time (n=4)
3. A decrease in *tail between legs* over time (n=1)
4. A fluctuating high-low pattern (n=3)
5. Mostly no *tail between legs* with the exception of one peak (n=4)

Eleven dogs did not show *tail between legs*. The graphs of *tail between legs* over time are presented for each individual dog in Appendix III.

4. Conclusion

- No significant gender, age or experience differences in behaviors were found with the exception of the behavior *tail between legs*.
    - *Tail between legs* was exhibited more by younger dogs than by older dogs. It was also significantly positively correlated with experience. Older, more experienced dogs appear to exhibit more *tail between legs*.
    - *Tail between legs* was negatively correlated with *tail wagging* and positively correlated with *crouching*.

- Negative correlations of age were found for the behaviors *sniffing table*, *tail wagging* and *head to technician*.

- In a previous similar study by Ortolani et al. (2013) in a heterogeneous group of 105 Dutch pet dogs, the behaviors *panting*, *sniffing table* and *licking lips* were seen most common.
    - In the sampled group of purpose-bred Marshall beagles studied here *panting* was not seen at all during the 'restrained' observation period.
    - *Sniffing table* and *licking lips* was frequently seen in this study but a large within group variation was present.

- No correlations of behaviors with heart rate variability parameters were found; for the time being no conclusions can be drawn from the heart rate variability data. However, a large within group variation in heart rate variability parameters was present.

- Based on preliminary analyses four different patterns of mean heart rate response over time could be distinguished. Dogs showing a constant high heart rate (n=4) appear to be unable to adequately adapt to the environmental challenge they were subjected to or these dogs may need a longer period of time to adapt.

- Based on preliminary analyses five different patterns of the behavior *tail between legs* over time could be distinguished. Dogs showing an (almost) constantly high tail between legs or dogs that showed an increase in tail between legs over time appear to be unable to adequately adapt to the environmental challenge they were subjected to.

- Some dogs show adaptive heart rate and behavioral responses over time while others do not. Dogs that appear to have diminished adaptive capacities may be at risk of poor welfare. A close monitoring of the behavior and heart rate of these dogs is recommended when using them in experimental studies.

Table 1. Behaviors used for further analysis of the 'restrained' observation period

	Behaviors	Description
Mouth	Sniffing self	Dog runs nose along own body
	Sniffing table	Dog runs nose along table
	Licking lips	Dog extrudes tongue from mouth and runs it along its lips
Head	Directed to technician	The dog's head is directed to the technician
	Hiding head	The dog is hiding its head against the technicians body or arm
	Head movement	Number of times the dog changes the direction of its head
Tail	Between legs	The tail is tucked between the legs
	Tail wagging	The tail is wagging in any position
Body	Paw lifting	Dog lifts front paw and keeps it up for more than a second
	Crouching	Hind part of the body is low, tail is tucked and knees are bend
	Moving	The dog is moving its feet and body with high intensity

Table 2. Mean, standard error of the mean, range and *N* of the behaviors that were scored as frequency.

	Mean	±SE	Range	*N*
Licking technician	0,35	0,28	0-7	2
Licking self	0,15	0,15	0-4	1
Licking table	0,31	0,31	0-8	1
Licking lips	30,2	3,24	4-71	26
Smacking	0,50	0,19	0-4	7
Head movement	81,5	5,34	18-120	26
Jumping	0,96	0,60	0-15	5
Paw lifting	5,31	1,18	0-20	20

Table 3. Mean, standard error of the mean, median and range of time and frequency domain HRV parameters of the 'restrained' observation period. N=23 for all parameters.

	Mean	±SE	Median	Range
Mean heart rate (1/min)	127,66	4,30	124,4	70,30 - 170,08
Mean RR (ms)	493,26	21,30	492,2	356,9 – 874,9
RMSSD (ms)	69,80	5,66	70,0	29,8 – 159,7
SDNN (ms)	69,06	4,28	69,2	40,0 – 139,4
NN50	153,57	8,56	152	70 - 235
pNN50 (%)	26,35	2,59	25,1	8,3 - 68,7
LF (ms^2)	887,65	140,2	770	209 - 3448
HF (ms^2)	1231,57	214,06	972	357 - 5493
LF/HF ratio	0,773	0,08	0,63	0,29 - 1,99

Table 4. Spearman's Rho correlation coefficients and *p* values of correlations between the same behaviors performed during the 'restrained' observation period and the 'unrestrained' observation period.

Restrained → Unrestrained ↓	Sniffing table	Licking lips	Tail between legs	Tail wagging
Sniffing table	r_s 0,62 p 0,001			
Licking lips		r_s 0,43 p 0,027		
Tail between legs			r_s 0,82 p 0,000	
Tail wagging				r_s 0,87 p 0,000
Moving	r_s 0,54 p 0,004			

Table 5. Mean, standard error of the mean and range of cortisol parameters.

	Mean	±SE	Range	n
Morning cortisol (µg/dL)	0,10	0,008	0,06-0,22	24
Post-observation cortisol (µg/dL)	0,10	0,006	0,04-0,14	22

Figure 1. Bar chart of the means of all mouth behaviors that were scored as duration. The y-axis represents the proportion of time that the behaviors were exhibited. The error bars represent ± 2 SE.

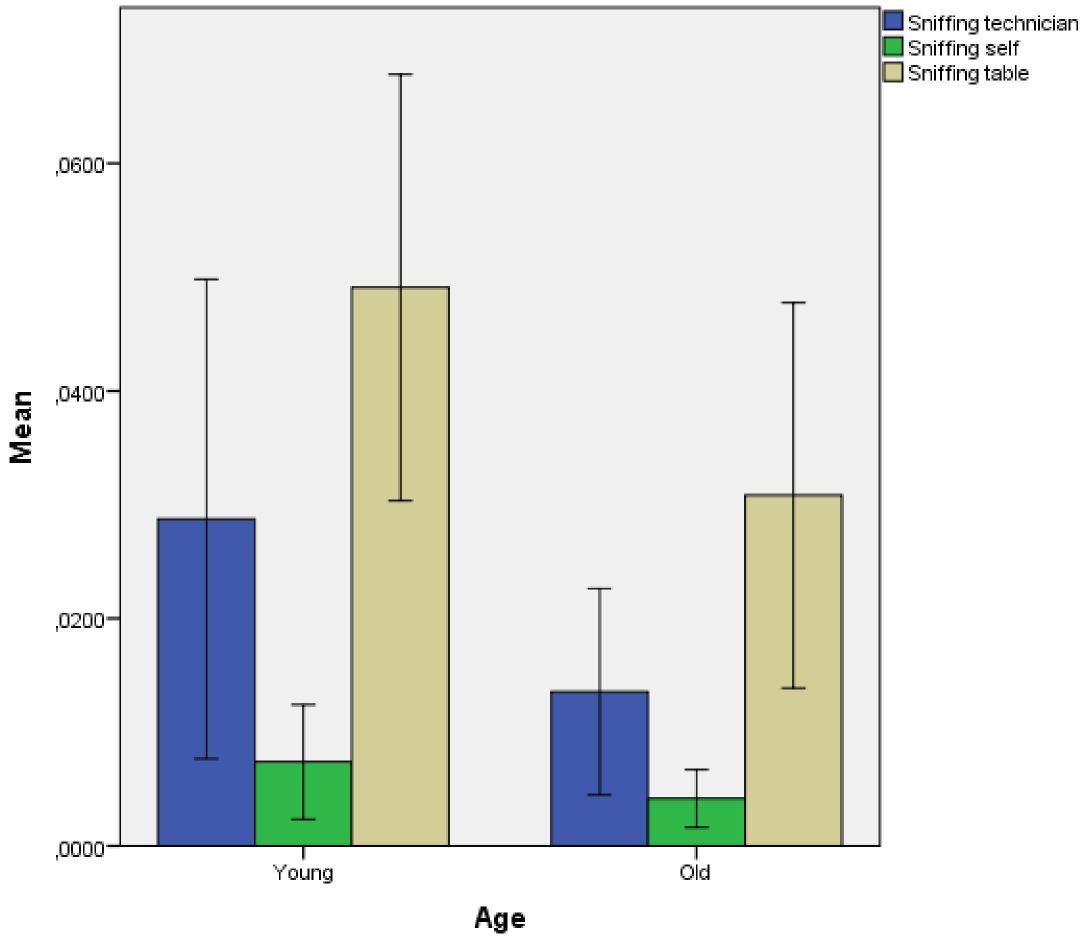


Figure 2. Variation within the group of dogs in the behavior *licking lips*. The x-axis represents the individual dogs and the y-axis represents the frequency of the exhibited behavior.

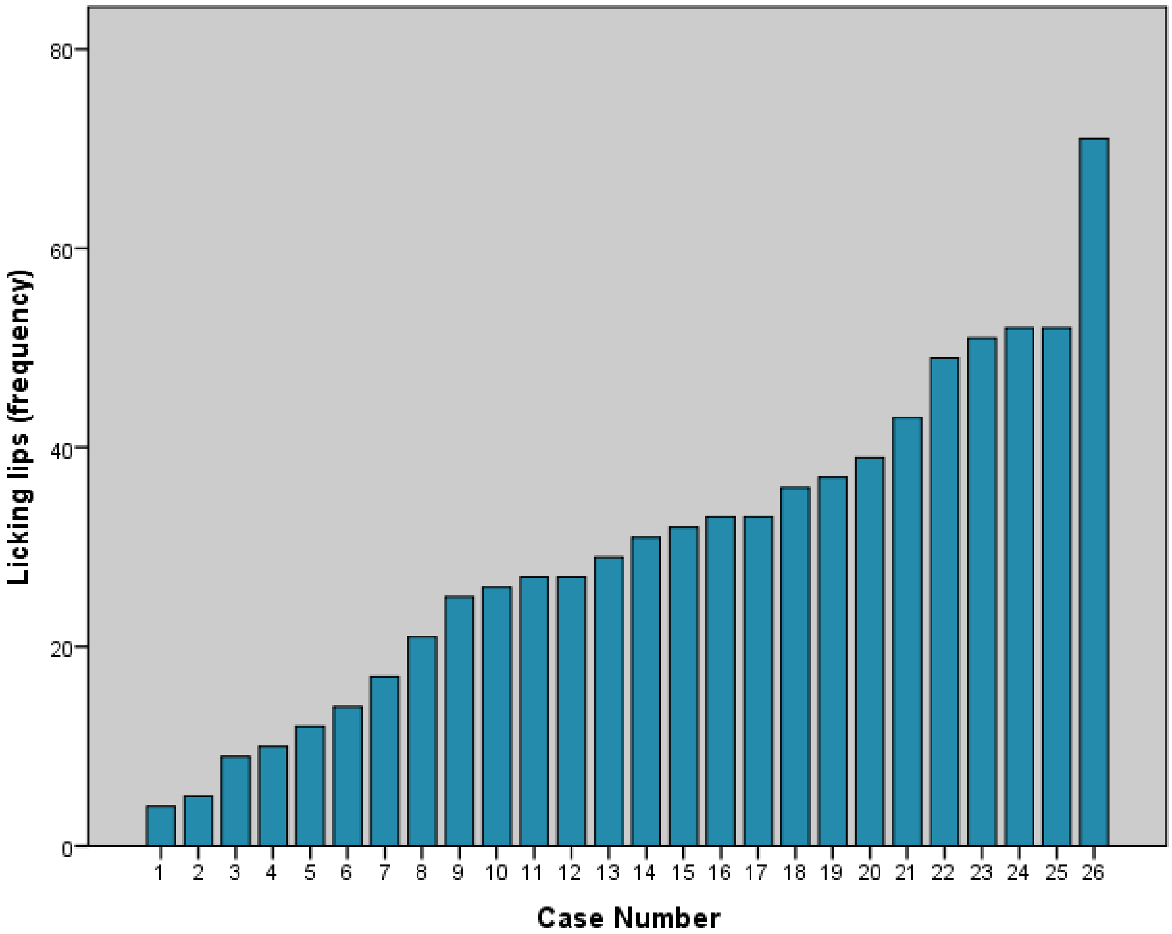


Figure 3. Bar chart of all tail behaviors scored as duration. The y-axis represents the mean proportion of time that the behaviors were exhibited during the 5 min observation.

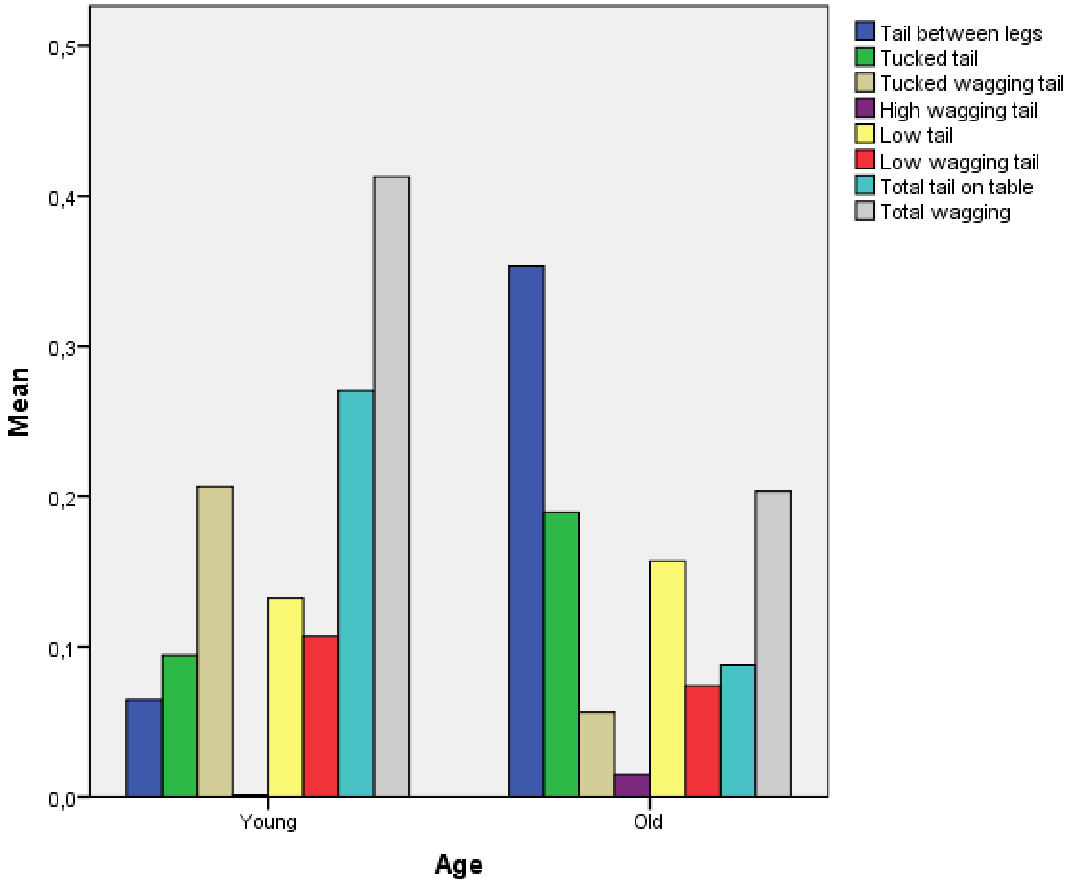


Figure 4. Bar chart showing within group variation and age differences in the behavior *tail between legs* and *tail wagging*. The y-axis represents the mean proportion of time that the dogs exhibited this behavior.

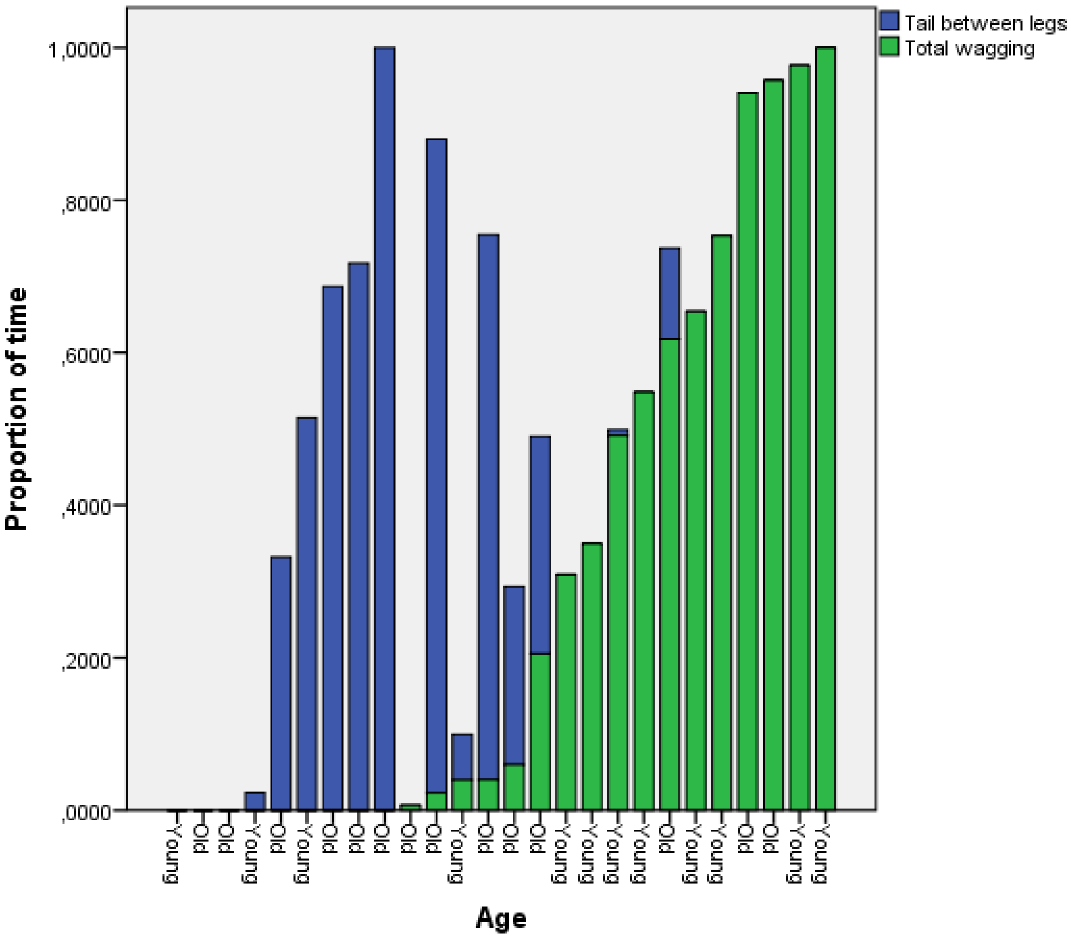


Figure 5. Bar chart of all body movement, scored as duration. The y-axis represents the mean proportion of time that the behaviors were exhibited.

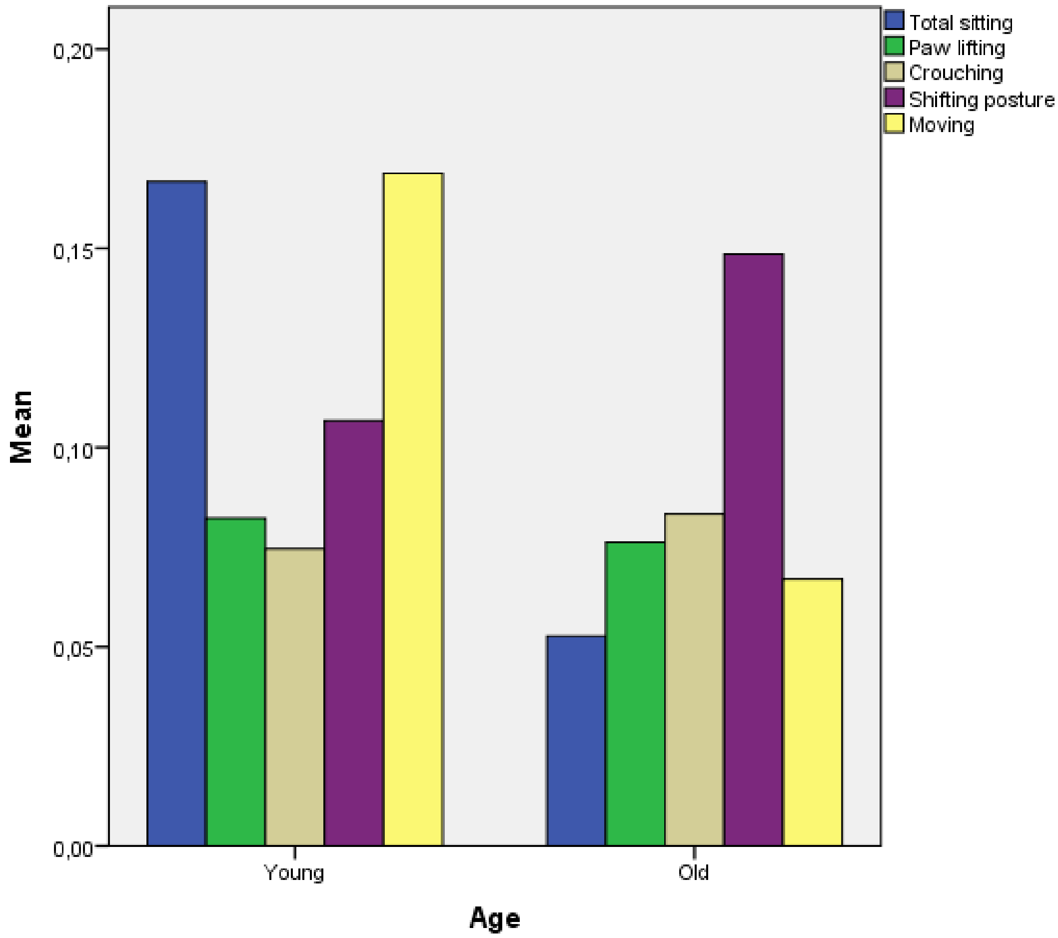


Figure 6. Bar chart all head behaviors that were scored as duration, except for *head to environment*. The y-axis represents the mean proportion of time that the behaviors were exhibited.

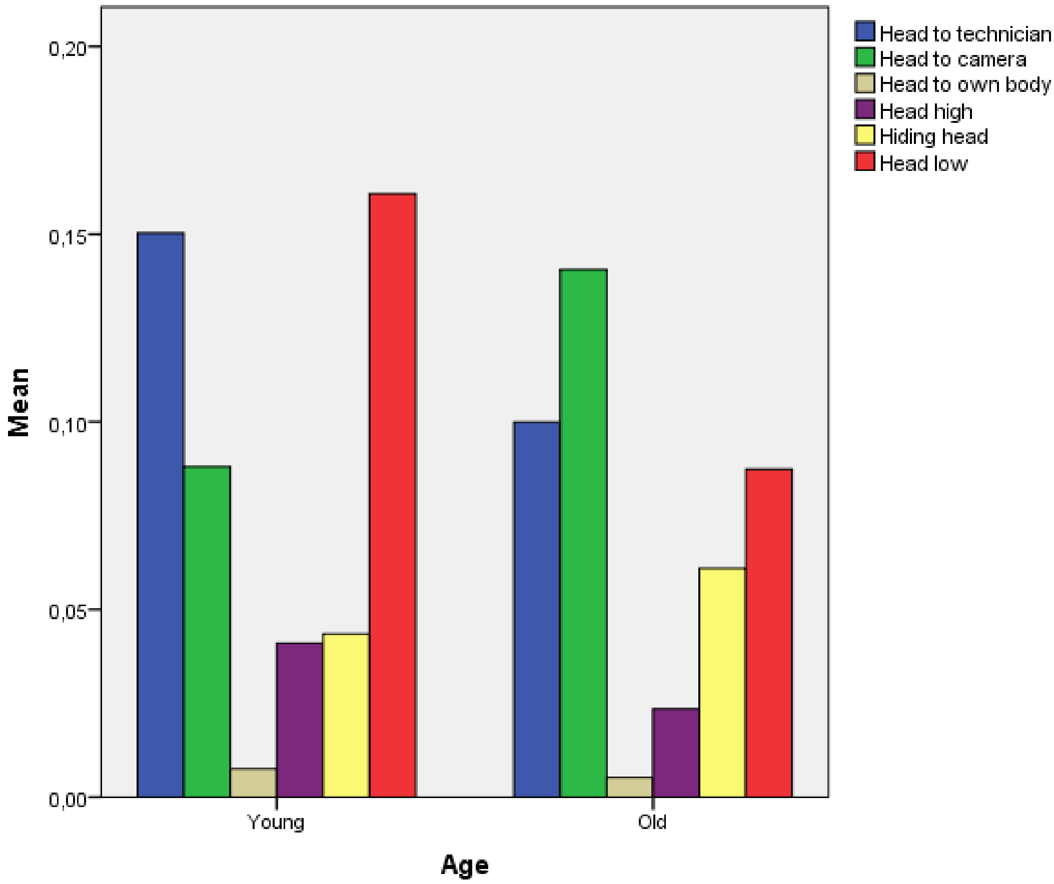


Figure 7. Bar chart presenting the value of cortisol difference in μg/dL for all beagles (n=22).

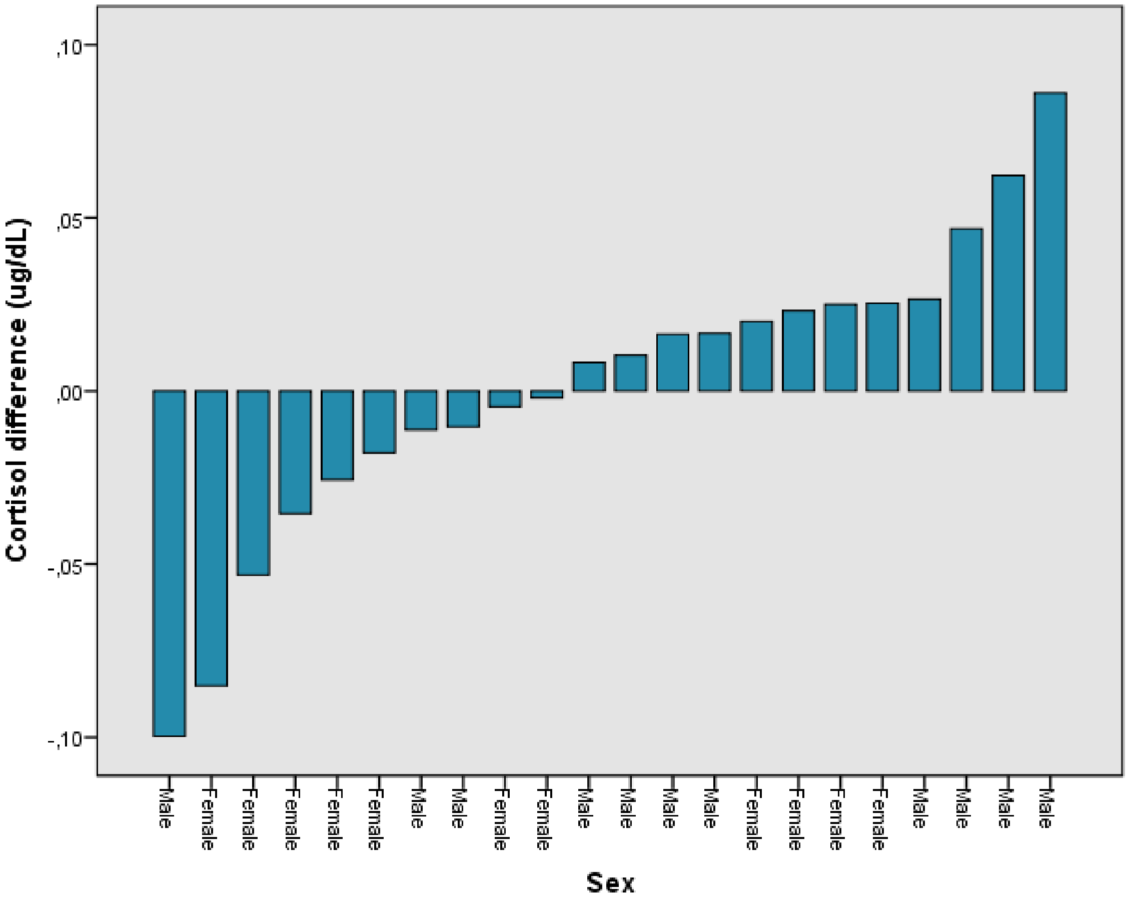


Figure 8. Difference between male and female beagles in morning cortisol values in the sampled group of purpose-bred Marshall beagles studied here.

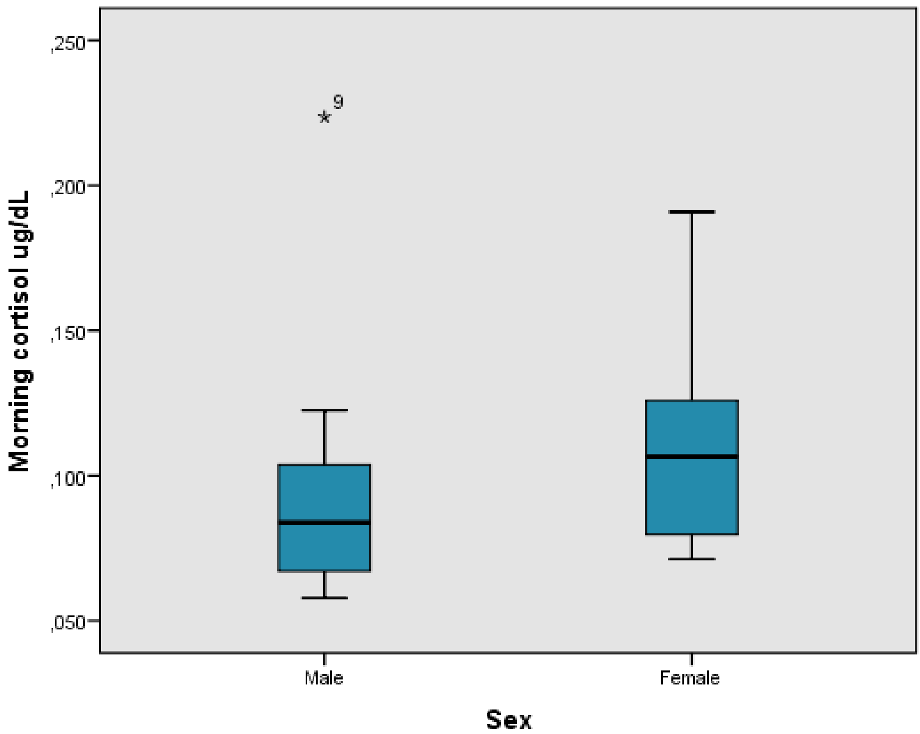
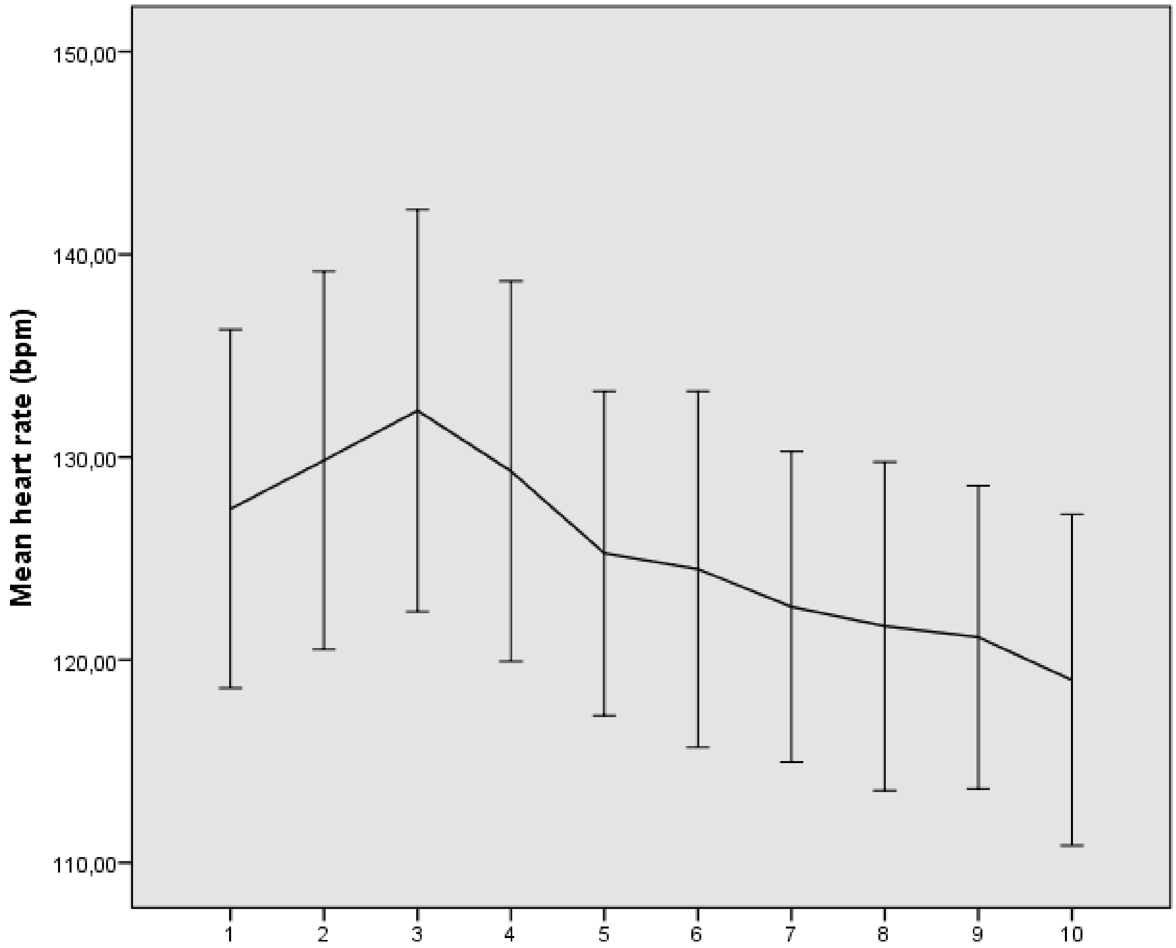


Figure 9. Shape of the mean heart rate response of all 26 dogs during the 'restrained' observation period. The 5 minute observation period is divided in 10 bouts of 30 seconds, represented on the x-axis. The line represents the mean heart rate and the error bars represent ± 2 SE.



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Appendix I. Ethogram for the restrained period

This appendix further explains the behaviors that were observed during the 5 minute observation period. Some behaviors occurred as an event and the frequency of occurring was scored (i.e. how often did the behavior occur in 5 minutes). The other behaviors were scored as both duration (i.e. for how long did the behavior occur in 5 minutes) and frequency.
Out of sight was scored when the targeted body part(s) could not be seen on camera for 2 seconds or longer. The seconds that a dog or part of the dog was out of sight were subtracted from the total 300 seconds that the observation period lasted to obtain the parameter 'time in sight'. Durations were then divided by 'time in sight' to obtain the proportion of time that a behavior was displayed. The parameter proportion of time was used for further statistical analysis.
All behaviors that were scored are listed in Table 1 to 4. Type F stands for behaviors scored as frequency and type D stands for behaviors scored as duration. Behaviors within each category are mutually exclusive.

Mouth behaviors

Mouth behaviors that were scored are listed in Table 1. *Sniffing* and *licking* were scored separately for technician, itself and table.

Table 1. Mouth behaviors.

Behavior	Type	Description
Coughing	F	The dog stretches its neck while opening the mouth and producing a dry throat sound
Sniffing	D	The dog runs its nose along the technician, itself or the table. Clear sniffing movements are made
Licking	F	The dog licks the technician, itself or the table with its tongue
Licking lips	F	The dog extrudes its tongue from its mouth and runs it over its lips, with or without smacking
Smacking	F	The dog presses its lips together and then opens the mouth quickly and noisily, without licking lips
Panting	D	An increased frequency of inhalation and exhalation in combination with the opening of the mouth and/or the movements of the dogs chest
Vocalization	F	The dog produces a soft high pitched sound (whining) or loud high pitched sound (yelping)

Table 2. Tail behaviors

Behavior	Type	Description
Low	D	Dog's tail is held in a position between 90 and 180 degrees, tail base is relaxed
Middle high	D	Dog's tail is held in a position of approximately 90 degrees
High	D	Dog's tail is held in a position between 0 and 90 degrees
Between legs	D	Dog's tail is held in a position between 180 and 270 degrees
Tucked tail	D	Tail base is tucked close to the body but the tip of the tail may be in a position of 180 degrees or less
Tense tail	D	Tail is held away from body, so not tucked, but tail base is not relaxed
Tail on table	D	The tail is laying on the table while the dog is sitting and may or may not be between legs
Wagging	D	Modifier that can be used with all other tail behaviors. Repetitive side to side movements of the tail for at least one second.

Tail behaviors

Tail behaviors that were scored are listed in Table 2. *Low, middle high, high* and *between legs* are used to indicate the position of the tail, using a circle of degrees with 0 degrees on north. In all positions the dog may or may not be wagging its tail, therefore *wagging* was scored as a modifier of these behaviors. All durations of *wagging* were added up to obtain *tail wagging*.

Head orientations

Behaviors of the head that were scored are listed below in Table 3. This category was designed to represent what the dogs attention was focused on, reflected by the direction in which the dogs gaze pointed. However, since the dogs gaze was often not visible on camera, the direction of the nose was scored unless it was clearly visible that the gaze was directed somewhere else.
Head high and *head low* were based on a circle of degrees with 0 degrees on north.

Table 3. Head behaviors.

Behavior	Type	Description
Directed to technician	D	The dog's nose (gaze) is directed to any part of the technician's body
Directed to camera	D	The dog's nose (gaze) is directed to the camera
Directed to environment	D	The dog's nose (gaze) is directed to the environment, attention is directed to something in the room
Directed to self	D	The dog's nose (gaze) is directed to its own body
Directed to researcher	D	The dog’s nose (gaze) is directed towards the researcher
Hiding head	D	The dog is hiding his/her head in the technician’s or arms and the head is (partly) out of sight
Head high	D	The dog's nose (gaze) is directed to the ceiling, in a position of 45° and less
Head low	D	The dog's head and part of the body is low and directed to the table. The position of head and neck is 135° or more
Head shake	F	The dog's shakes his head from side to side

Body movements

Body movements that were scored are listed in Table 4.

Table 4. Body behaviors.

Behavior	Type	Description
Standing	D	Dog is standing still on the table.
Sitting	D	Dog is sitting still on the table.
Lying	D	Dog is lying on the table, head may or may not be in contact with the table
Shifting posture	D	Dog changes its body posture or position by lifting its feet and displacing its body. Scored as one continuous event, unless the dog stands still for at least 2 seconds
Moving	D	Dog moves with a higher intensity than the small movements seen in *shifting posture.* Scored as one continuous event, unless the dog stands still for at least 2 seconds
Crouching	D	Used as a modifier of *standing.* Posture is low on the back, tail is held close to the body, knees are bend.
Paw lifting	D	Used as a modifier of *standing* and *sitting*. The dog raises one or both front paws and holds it above the ground or one the technician. Changing lifted paw is scored in the same event, unless there is at least two seconds in between.
Jumping	F	Dog raises both front paws in the air or puts them on the technician

Appendix II. Ethogram for the unrestrained period

This appendix further explains the behaviors that were observed during the unrestrained 5 minute observation period. Some behaviors occurred as an event and the frequency of occurring was scored (i.e. how often did the behavior occur in 5 minutes). The other behaviors were scored as both duration (i.e. for how long did the behavior occur in 5 minutes) and frequency. Durations were then divided by the total seconds that the observation period lasted to obtain the proportion of time that a behavior was displayed. The parameter proportion of time was used for further statistical analysis.
All behaviors that were scored are listed below in tables per category. Type F stands for behaviors scored as frequency and type D stands for behaviors scored as duration. Behaviors within each category are mutually exclusive.

Mouth behaviors

Behavior	Type	Description
Sniffing table	D	The dog runs its nose along the table and clear sniffing movements are made
Licking lips	F	The dog extrudes its tongue from its mouth and runs it over its lips, with or without smacking
Panting	D	An increased frequency of inhalation and exhalation in combination with the opening of the mouth and/or the movements of the dogs chest

Tail behaviors

Tail between legs is used to indicate the position of the tail, using a circle of degrees with 0 degrees on north. The position of the tail during *wagging* was not scored.

Behavior	Type	Description
Tail between legs	D	Dog's tail is held in a position between 180 and 270 degrees
Wagging	D	Repetitive side to side movements of the tail for at least one second.

Body movements

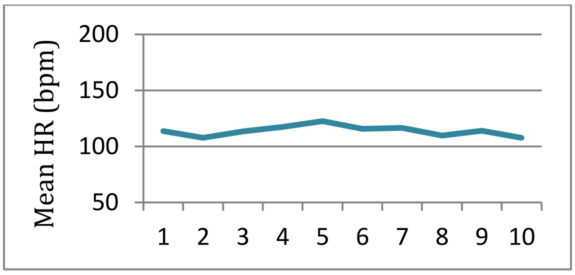
Behavior	Type	Description
Still	D	Dog is standing still on the table.
Moving	D	Dog is walking or moving its feet in place, scored as one event unless dog stands still for at least 2 seconds in between
Change of locomotion	F	Dog changes its body movement from still to moving or the other way around

Appendix III. Individual cardiac and behavioral responses over time during the 'restrained' period

Dog 1, male

Heart rate response

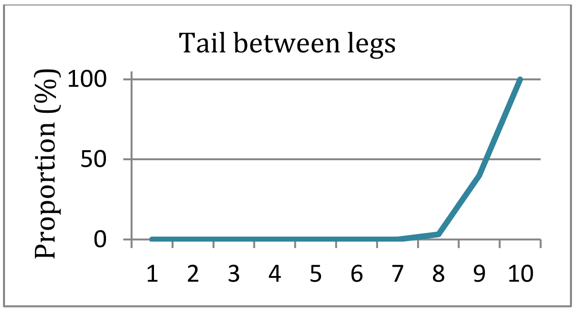
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant low heart rate response. The mean heart rate was 116 bpm.

Behavioral response

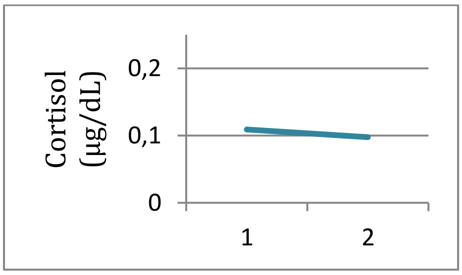
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed an increase in *tail between legs* only at the end of the observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

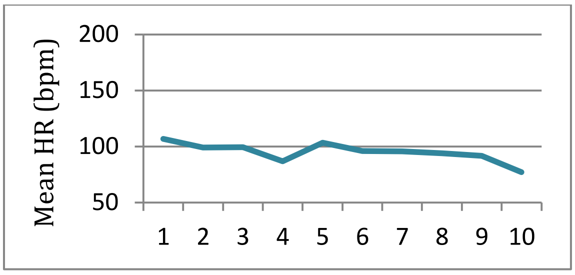


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 2, male

Heart rate response

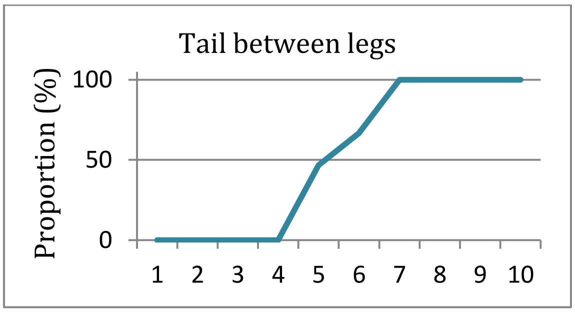
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a decrease in heart rate. The mean heart rate was 108 bpm during the first bout and 78 bpm during the last bout.

Behavioral response

Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed an increase in *tail between legs* over time.

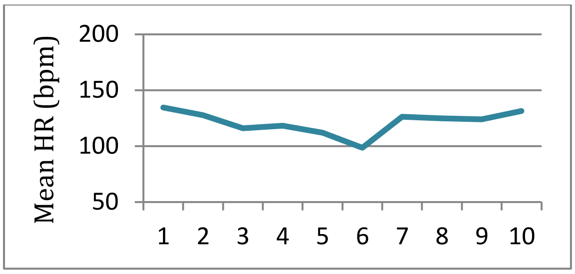
Cortisol response

Unfortunately, both saliva samples of this dog did not contain enough volume to measure cortisol values.

Dog 3, male

Heart rate response

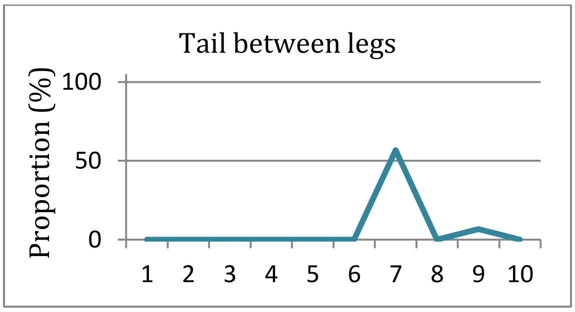
Figure 1. The x-axis represents the 30-second bouts of the 5 minute ‘restrained’ observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fluctuating heart rate response with no netto change at the end of the 5 minute observation period. The mean heart rate was 124 bpm.

Behavioral response

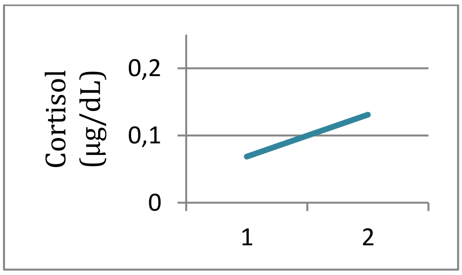
Figure 2. The x-axis represents the 30-second bouts of the 5 minute ‘restrained’ observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed a peak in *tail between legs* during the second half of the 5 minute observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

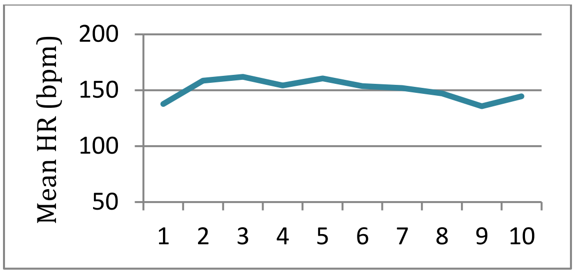


The post-observational cortisol value was increased compared to the morning cortisol value.

Dog 4, female

Mean heart response

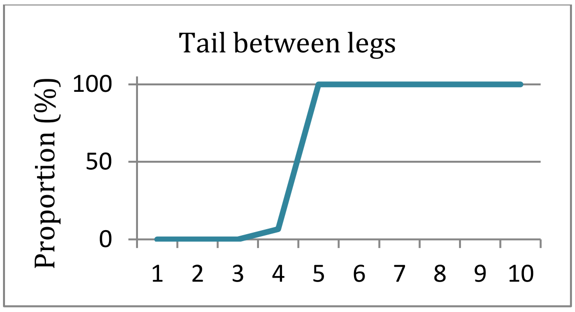
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant high heart rate response with a mean heart rate of 154 bpm.

Behavioral response

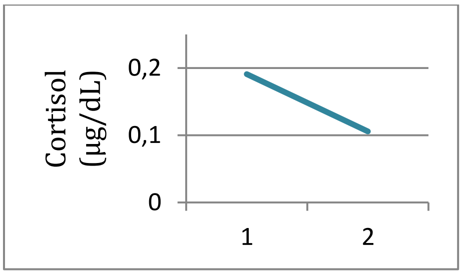
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed an increase in *tail between legs* over time.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

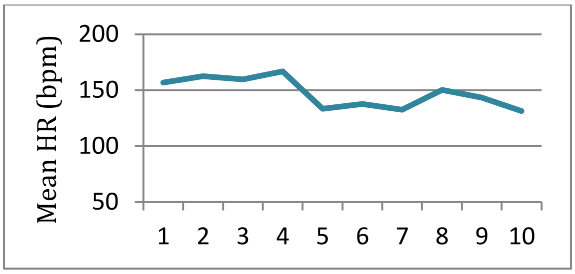


The post-observational cortisol value was decreased compared to the morning cortisol value.

Dog 5, female

Heart rate response

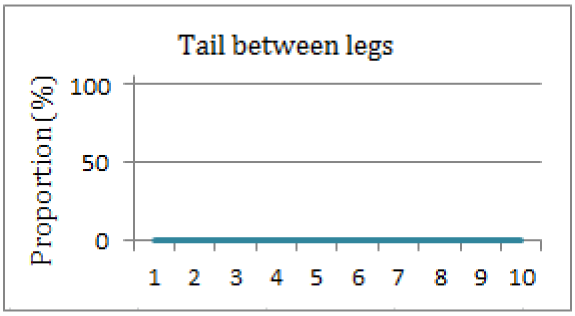
Figure 1. The x-axis represents the 30-second bouts of the 5 minute ‘restrained’ observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a decrease in heart rate over time, with some fluctuation. The mean heart rate was 158 bpm during the first bout and decreased to 132 bpm during the last bout.

Behavioral response

Figure 2. The x-axis represents the 30-second bouts of the 5 minute ‘restrained’ observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

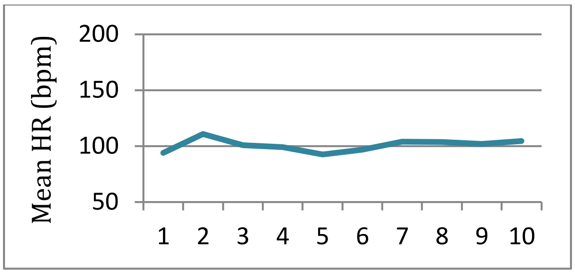
Cortisol response

Unfortunately, both saliva samples of this dog did not contain enough volume to measure cortisol values.

Dog 6, female

Heart rate response

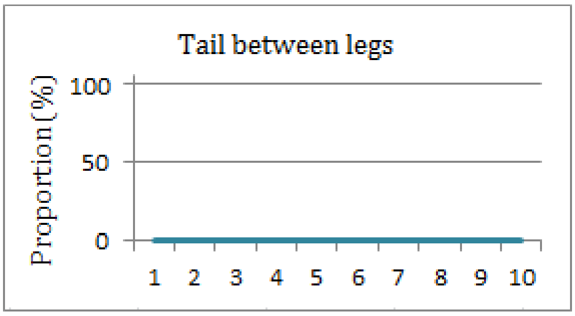
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant low heart rate response with a mean heart rate of 102 bpm.

Behavioral response

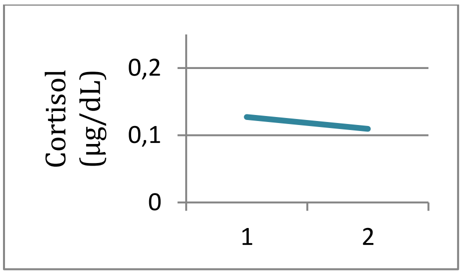
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

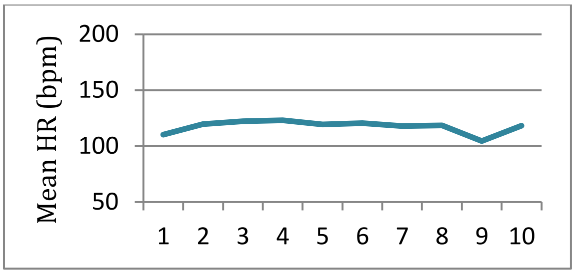


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 7, male

Heart rate response

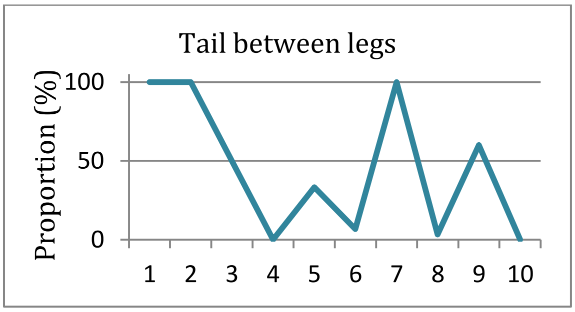
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant average heart rate response with a mean heart rate of 119 bpm.

Behavioral response

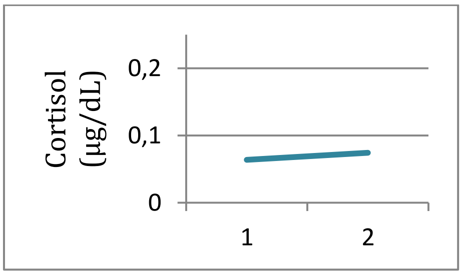
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed a decreasing but fluctuating rate of *tail between legs*.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

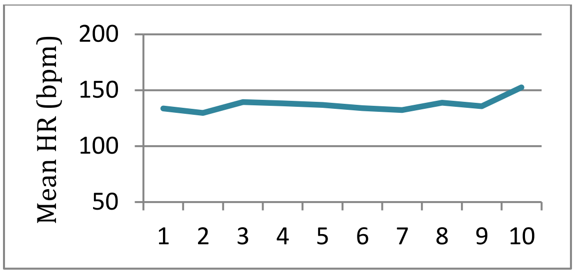


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 8, male

Heart rate response

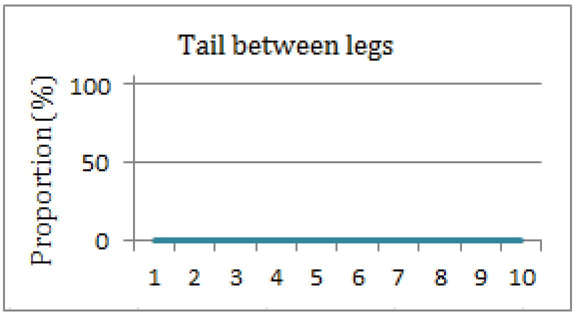
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant high heart rate response, slightly increasing at the end of the 5 minute observation period. Mean heart rate during the first bout was 136 bpm and during the last bout 154 bpm.

Behavioral response

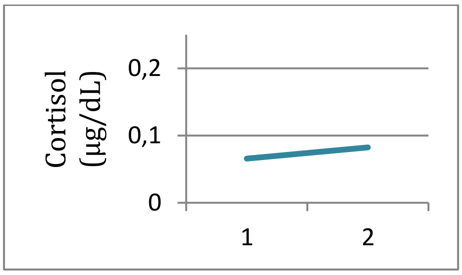
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

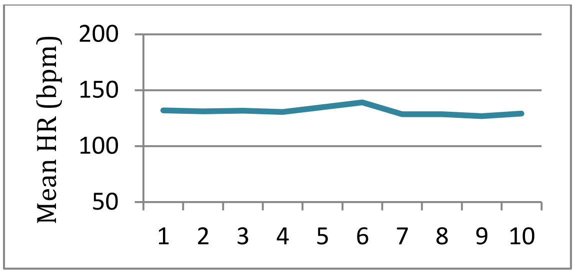


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 9, male

Heart rate response

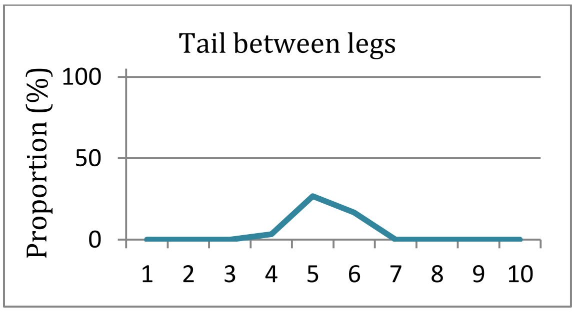
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant average heart rate response with a mean heart rate of 132 bpm.

Behavioral response

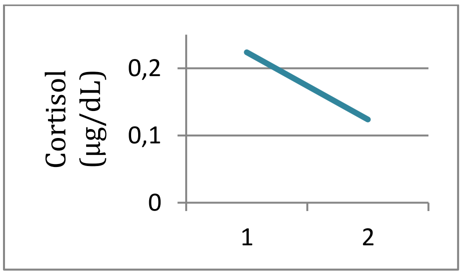
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed a small peak halfway the observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

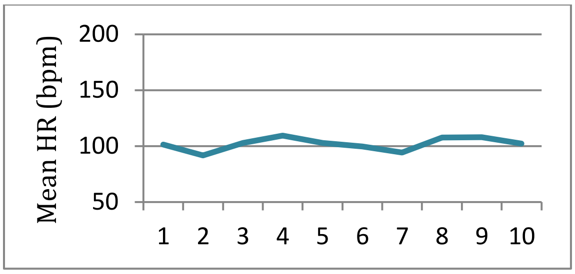


The post-observational cortisol value was decreased compared to the morning cortisol value.

Dog 10, female

Heart rate response

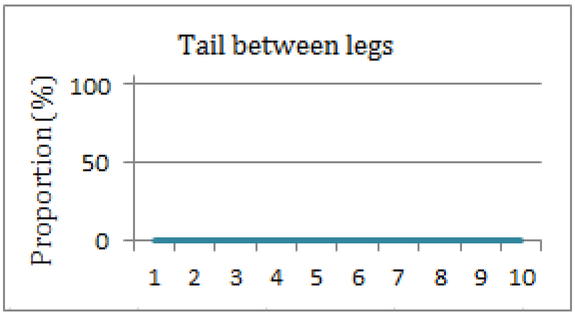
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a low, mildly fluctuating heart rate response with no netto change at the end of the 5 minute observation period. Mean heart rate was 104 bpm.

Behavioral response

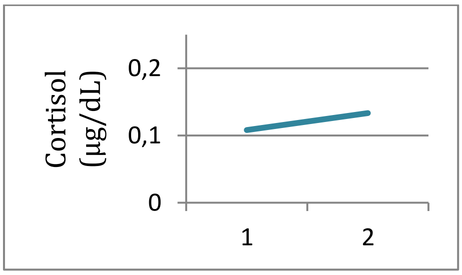
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

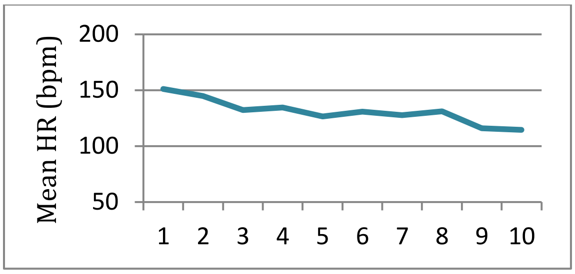


The post-observational cortisol value was slightly increased compared to the morning cortisol value.

Dog 11, female

Heart rate response

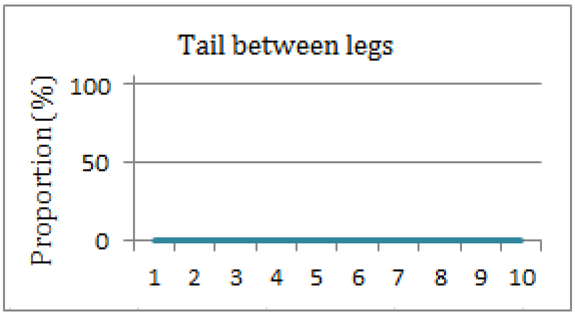
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a decrease in heart rate over time. Mean heart rate was 152 bpm during the first bout and decreased to 116 bpm during the last bout.

Behavioral response

Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

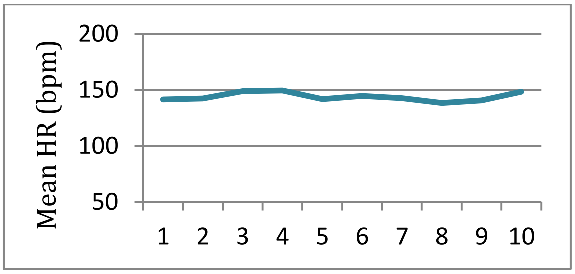
Cortisol response

The post-observation saliva sample of this dog did not contain enough volume to measure cortisol; therefore a graph with change in cortisol values could not be presented.

Dog 12, female

Heart rate response

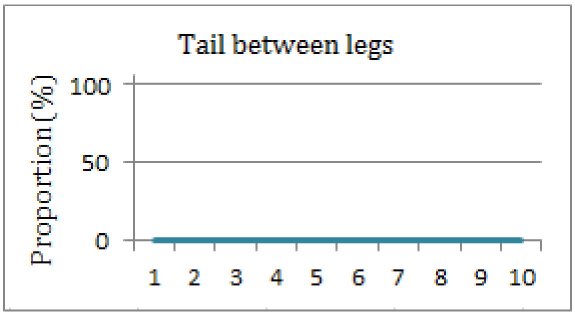
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant high heart rate response with a mean heart rate of 146 bpm.

Behavioral response

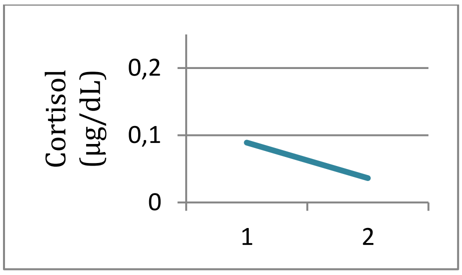
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

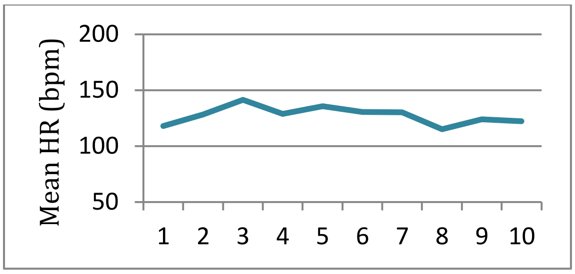


The post-observational cortisol value was decreased compared to the morning cortisol value.

Dog 13, male

Heart rate response

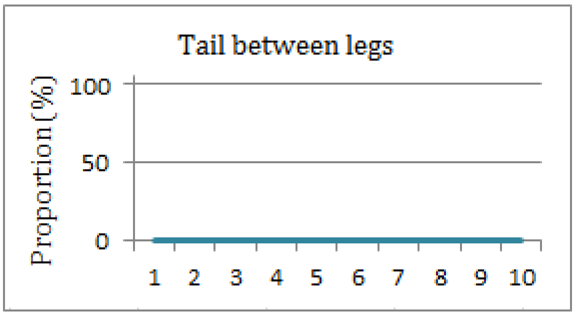
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant average heart rate response with some fluctuation. Mean heart rate was 130 bpm.

Behavioral response

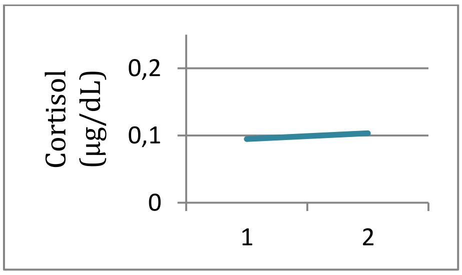
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

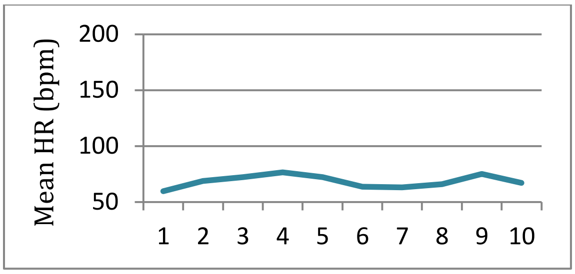


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 14, male

Heart rate response

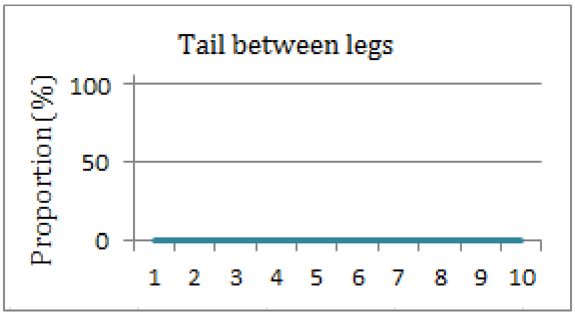
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant low heart rate response with a mean heart rate of 70 bpm.

Behavioral response

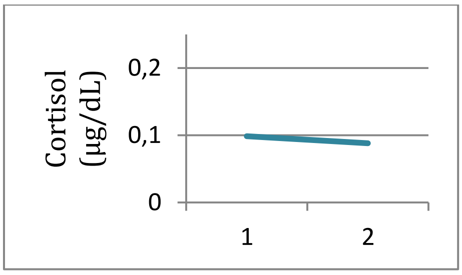
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

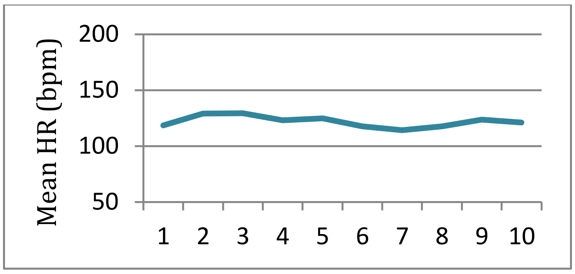


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 15, male

Heart rate response

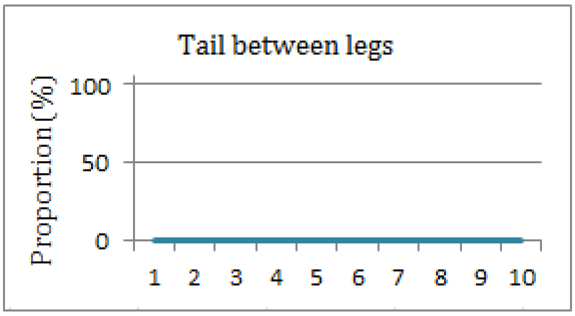
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant average heart rate response with a mean heart rate of 123 bpm.

Behavioral response

Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

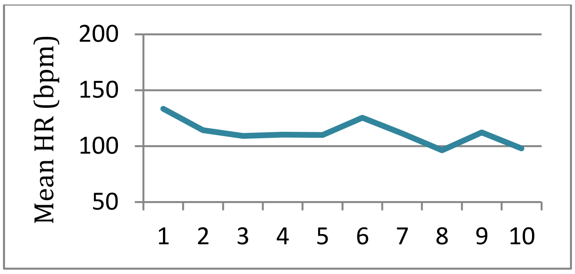
Cortisol response

The post-observation saliva sample of this dog did not contain enough volume to measure cortisol; therefore a graph with change in cortisol values could not be presented.

Dog 16, male

Heart rate response

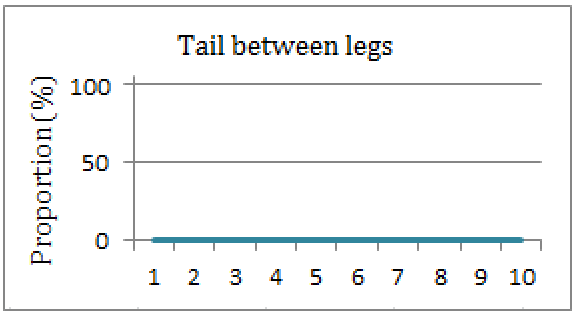
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a decrease in heart rate, with some fluctuation. The mean heart rate was 136 bpm during the first bout and 99 bpm during the last bout.

Behavioral response

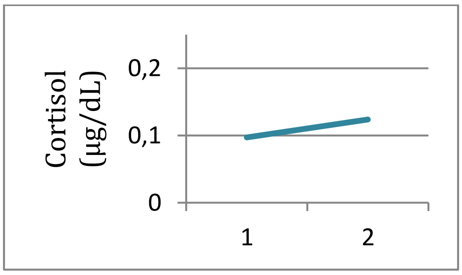
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in μg/dL.

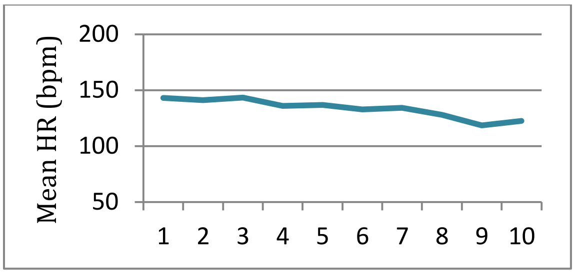


The post-observational cortisol value was slightly increased compared to the morning cortisol value.

Dog 17, female

Heart rate response

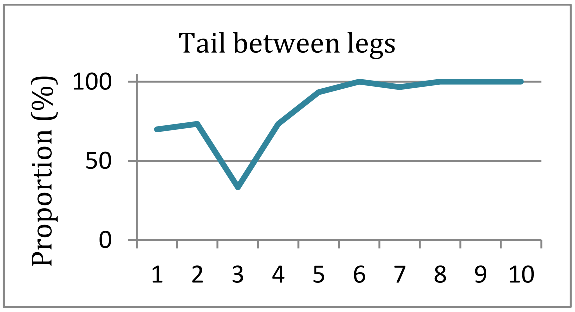
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a decrease in heart rate over time. Mean heart rate was 144 bpm during the first bout and 125 bpm during the last bout.

Behavioral response

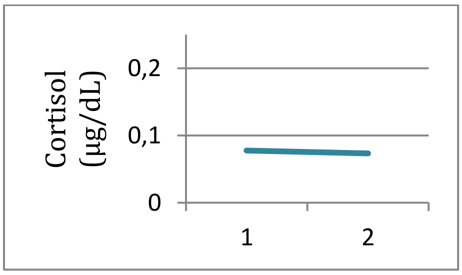
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed an initial decrease in *tail between legs*, followed by an increase.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

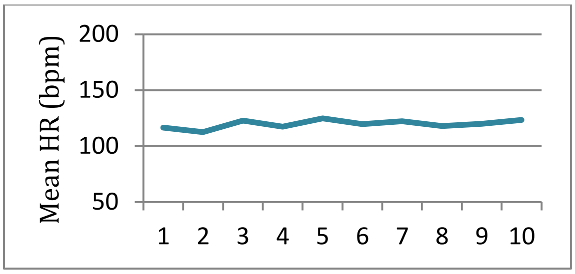


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 18, female

Heart rate response

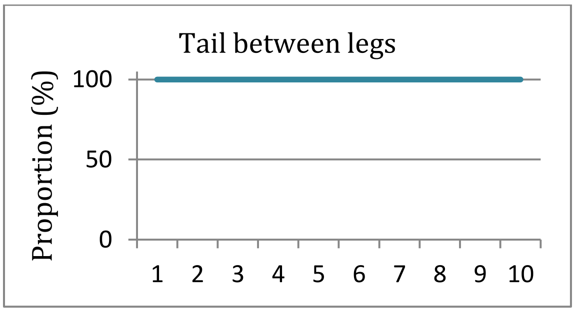
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant average heart rate response with a mean heart rate of 122 bpm.

Behavioral response

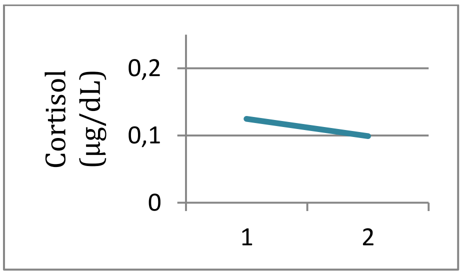
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed a continuous *tail between legs* response.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

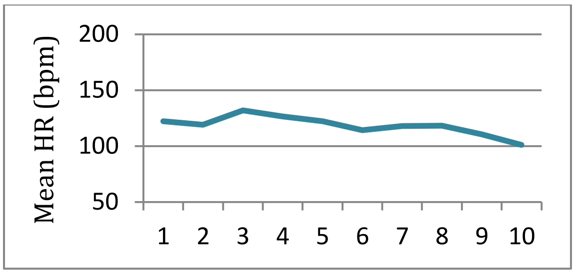


The post-observational cortisol value was slightly decreased compared to the morning cortisol value.

Dog 19, female

Heart rate response

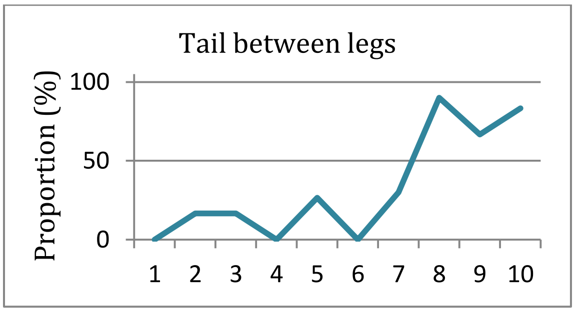
Figure 1. The x-axis represents the 30-second bouts of the 5 minute ‘restrained’ observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a decrease in heart rate over time. Mean heart rate was 124 bpm during the first bout and 103 bpm during the last bout.

Behavioral response

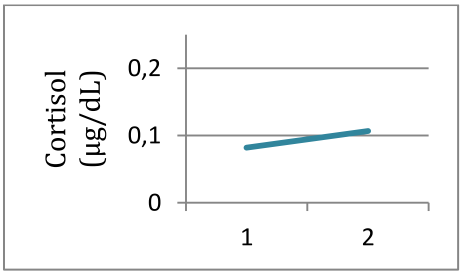
Figure 2. The x-axis represents the 30-second bouts of the 5 minute ‘restrained’ observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed an increase in *tail between legs* over time.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

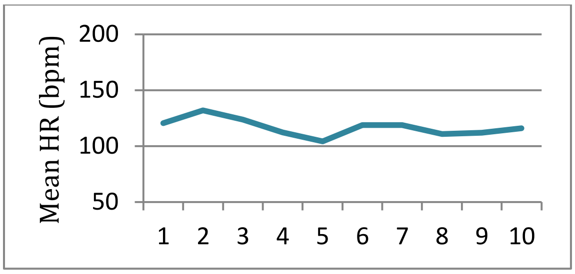


The post-observational cortisol value was slightly increased compared to the morning cortisol value.

Dog 20, male

Heart rate response

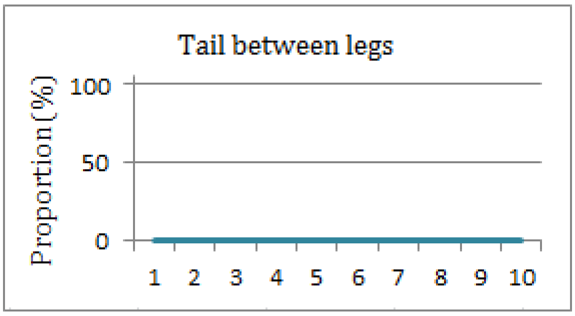
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fluctuating average heart rate response with no netto change at the end of the 5 minute observation period. Mean heart rate was 119 bpm.

Behavioral response

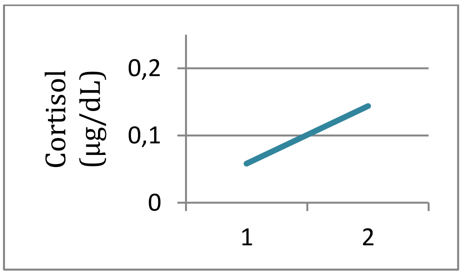
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed no *tail between legs* during the 5 minute observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

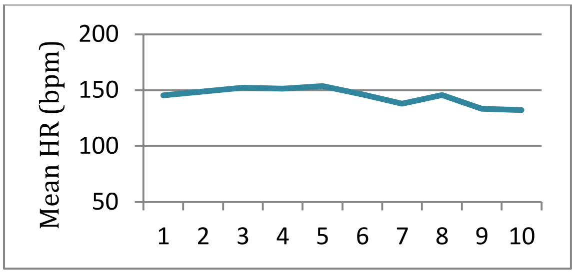


The post-observational cortisol value was increased compared to the morning cortisol value.

Dog 21, male

Heart rate response

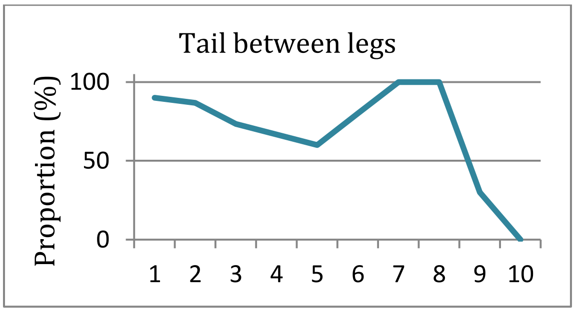
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant high heart rate with a slight decrease at the end. Mean heart rate was 147 bpm during the first bout and 134 bpm during the last bout.

Behavioral response

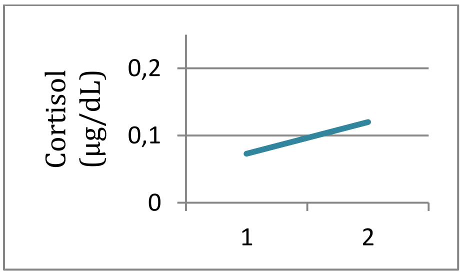
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed a decrease in *tail between legs* over time.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

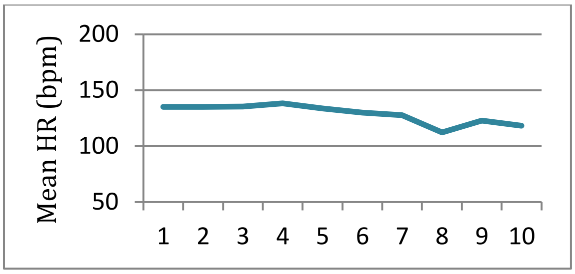


The post-observational cortisol value was increased compared to the morning cortisol value.

Dog 22, male

Heart rate response

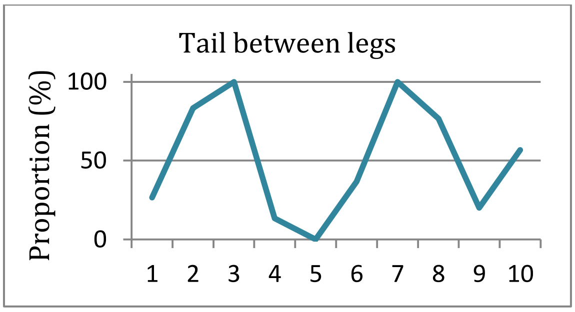
Figure 1. The x-axis represents the 30-second bouts of the 5 minute ‘restrained’ observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a decrease in heart rate over time. It should be noted that the first three bouts of heart rate data contained a high error rate and therefore only the shape of bouts 4 to 10 should be interpreted. Mean heart rate was 139 bpm during the fourth bout and 121 bpm during the last bout.

Behavioral response

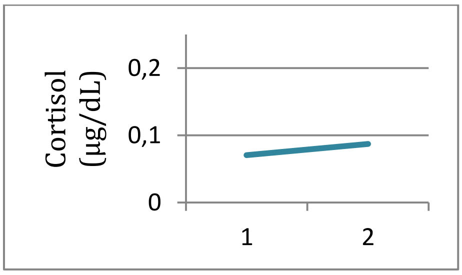
Figure 2. The x-axis represents the 30-second bouts of the 5 minute ‘restrained’ observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed a fluctuating response of the tail.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

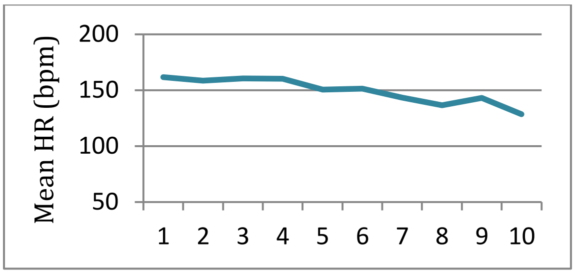


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 23, female

Heart rate response

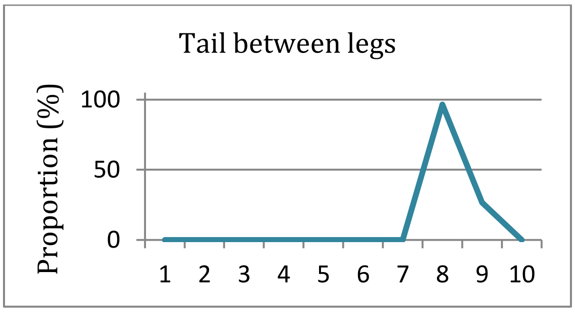
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a decrease in heart rate over time. Mean heart rate was 162 bpm during the first bout and 131 bpm during the last bout.

Behavioral response

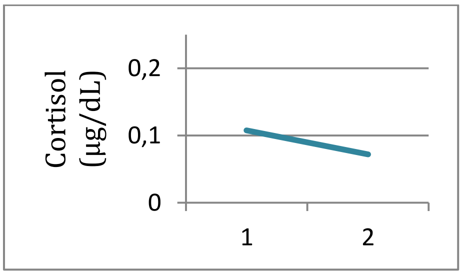
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed a peak in *tail between legs* at the end of the observation period.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

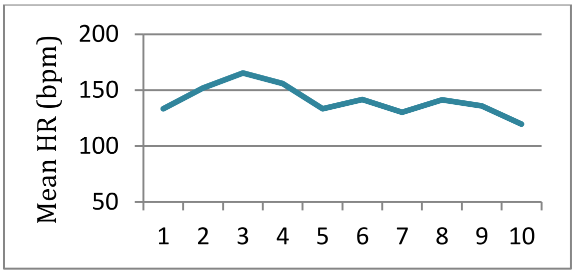


The post-observational cortisol value was decreased compared to the morning cortisol value.

Dog 24, female

Heart rate response

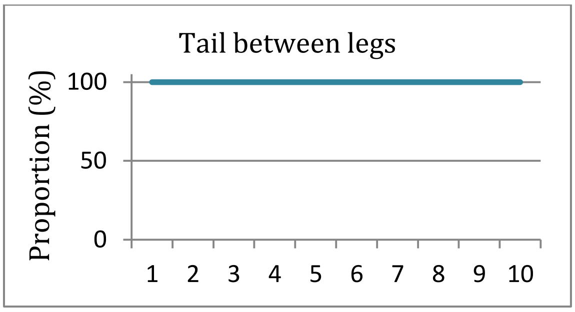
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fluctuating high heart rate response with a decrease at the end of the 5 minute observation period. Mean heart rate was 135 during the first bout and 121 bpm during the last bout. Overall mean heart rate was 144 bpm.

Behavioral response

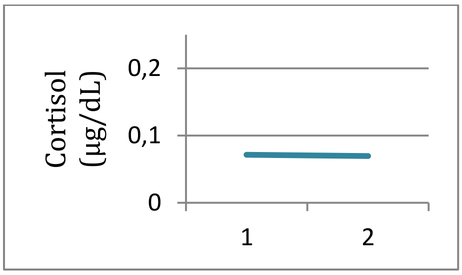
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed a continuous *tail between legs* response.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.

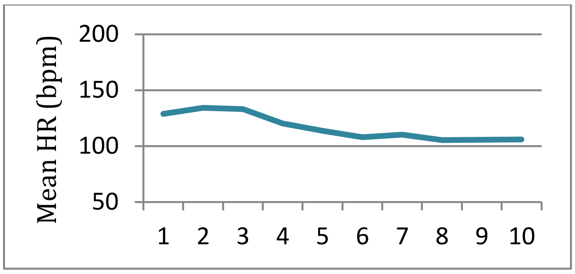


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 25, female

Heart rate response

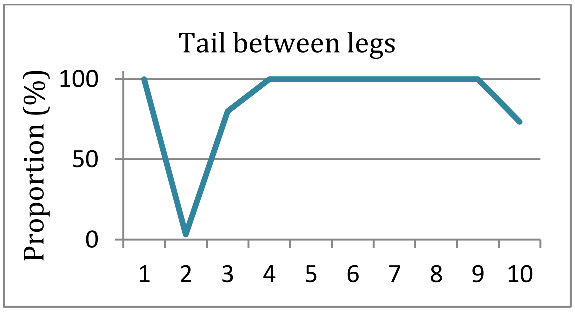
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a decrease in heart rate over time. It should be noted that the first bout of heart rate data contained a high error rate and therefore only the shape of bouts 2 to 10 should be interpreted. Mean heart rate was 136 bpm during the second bout and 110 bpm during the last bout.

Behavioral response

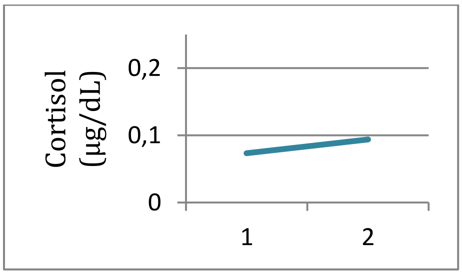
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed an initial decrease in *tail between legs*, followed by an increase.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in μg/dL.

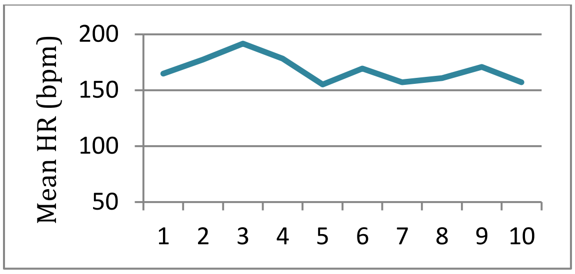


The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.

Dog 26, female

Heart rate response

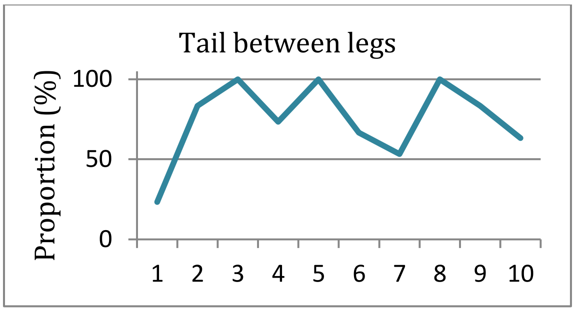
Figure 1. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis presents the mean heart rate in bpm.



This dog showed a fairly constant high heart rate response with a mean heart rate of 170 bpm.

Behavioral response

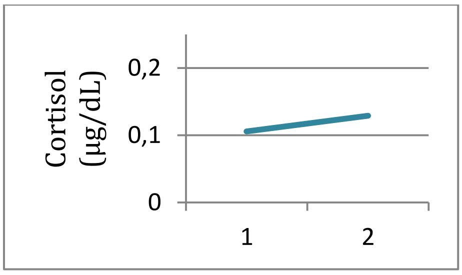
Figure 2. The x-axis represents the 30-second bouts of the 5 minute 'restrained' observation period. The y-axis represents the proportion of time (%) that *tail between legs* was exhibited in each bout.



This dog showed an increase in *tail between legs* over time, with fluctuation.

Cortisol response

Figure 3. The x-axis represents saliva sampling points; 1=morning cortisol and 2=post-observation cortisol. The y-axis represents cortisol values in µg/dL.



The post-observational cortisol value showed no prominent change compared to the morning cortisol value in this dog.