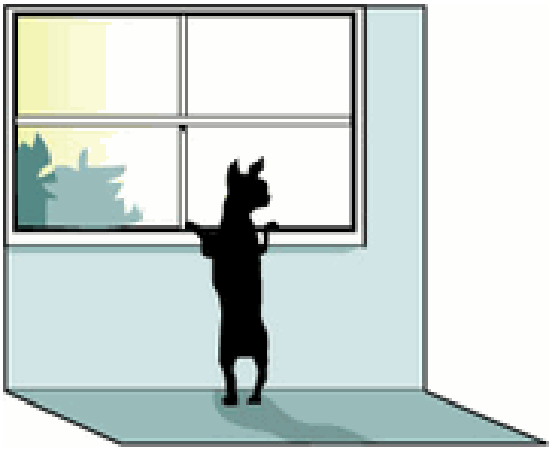
Universiteit Utrecht

Follow-up study: 'Behavioral and physiological responses in dogs with separation-related behavioral problems'.



Internship report; Master of Veterinary Medicine
Laura Reifler, 3404986
Supervisor: Dr. A. Ortolani
Dept Animals in Science and Society, Utrecht University
Animal Welfare and Laboratory Animal Science
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Prefactory note

The aim of the research project within the Master's program of Veterinary Sciences is to enable the students to get a first impression of the world of science and research. This project lasts three months in which the students for fill scientific work. This paper contains the results of a follow-up study carried out by Laura Reifler at the department Animals in Science and Society of the University of Utrecht. The research project was set up for the Honours Program 2011-2012, which was performed by Inge Hoogendam.
Research was performed to get an understanding about the behavioral and physiological responses in dogs with separation-related behavioral problems.
However the project is also part of a broader research program, namely 'Dog Welfare': from a subjective impression to objective measures'. This program is being supported by the Ministry of Economic Affairs, Agriculture and Innovation and the aim is to develop a method to evaluate welfare of individually kept dogs which is scientifically valid and socially accepted. This method will also have practical applications, so it can be useful for a veterinary practice.

Abstract

This paper describes a follow-up study based on the original study performed by Inge Hoogendam on separation-related behavioral (SRB) problems. These problems are thought to be common among domestic dogs and are a problem for both the dog and the owner. The most common problems seen by dog owners are destructive behavior, excessive vocalizations and inappropriate elimination. Characteristic of SRB problems is that the problems only occur in the (virtual) absence of the owner and that these problems are most significant within 30 minutes after departure of the owner.
As SRB problems can negatively affect the dogs' welfare, a better understanding of these problems is important. The ultimate aim of this research project is to contribute to the diagnose of separation anxiety. Moreover, this can contribute to the understanding of this problem and ultimately to the improvement and evaluation of welfare in dogs.

Physiological and behavioral parameters were objectively measured under standardized testing conditions. Physiological parameters include salivary cortisol concentrations and heart rate variability parameters.
This study measured 8 SRB and 2 non-SRB dogs. Data from this study was then combined with the previous study (study 1) for analyses and the total sample is 22 SRB and 11 non-SRB dogs. The dogs were grouped in either the SRB or the non-SRB group based on owner reports.
The observation was divided in three phases: table, separation and reunion phase. All phases lasted five minutes. During the separation phase the dog was left alone in the testing room for five minutes. During both the table as the reunion phase the owner was in the same room as the dog.
Heart rate parameters were measured continuously throughout the observation by a non-invasive Polar® heart rate monitor. Saliva samples for cortisol determination were taken in the morning prior to the test (home sample), five minutes after the end of the table phase (table sample) and five minutes after the end of the reunion phase (separation sample). Three cameras recorded the dog behavior during the test. Dog behavior was scored a posteriori by trained researchers. In this study only the behaviors displayed in the table phase were analyzed.

The results from these combined studies suggest that the behaviors seen during the table phase do not appear to be useful indicators of SRB problems in dogs.
Mean heart rate values in SRB dogs differed across phases. The mean heart rate was significantly higher in the separation phase compared to the table phase.
In SRB dogs cortisol values were significantly higher in the separation phase sample, compared to the table phase sample. However, the cortisol values between SRB and non-SRB dogs did not differ significantly.
The behavior, especially the door-directed behavior, displayed during the separation phase appears to be an useful indicator of the intensity of SRB problems. In SRB dogs the mean heart rate during the separation phase was also significantly correlated with all door-directed behaviors.

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1. Introduction

Separation-related behavioral problems in dogs form a problem for as well as the owner as for the dog.[2] We believe that these problems are not uncommon in the Dutch dog population as a lot of dog owners contacted us to sign up their dogs with separation-related behavioral problems to participate in the observation. Therefore a better understanding of these separation-related behavioral problems is relevant.

Dog owners often name the problematic behavior the dog shows when left alone 'separation anxiety'. Horwitz (2000) defined separation anxiety as: 'the distress response by the dog to separation from the person(s) to whom the dog is most attached, usually the owner'.[2] The term separation anxiety indicates that the motivation of the dog to express behavioral problems when (virtually) separated from their owners is due to anxiety. This, however, is not always the case as other motivations can also lead to problem behavior.[3] Therefore, as suggested by Appleby (2004), in this report behavioral problems of dogs when separated from their owners are preferably referred to as separation-related behavioral (SRB) problems, rather than separation anxiety.

Clinical signs related to separation reactions in dogs can be complex and diverse, between as well as within individuals. Often dogs do not only show a distinct behavioral response to being left alone, but emotional and physiological responses can vary in intensity and clinical appearance. During a period of separation from the owner, the dogs' reaction can vary between agitated states and depressed states. [4]

The most common problems seen by dog owners are destructive behavior, excessive vocalization and inappropriate elimination of the dog.[5,3,2,6,4] Increased repetitive motor activity, excessive salivation, vomiting [7], lack of appetite, repetitive behaviors and self-inflicted trauma are more rarely seen.[2, 3] All problems only emerge while the owner is (virtually) absent and are most significant in the first 30 minutes after the owner's departure. [2, 3] Nervous or even aggressive behavior may also appear any time during the peri-departure period. The SRB problems can persist until the owner returns, but some dogs recover sooner.[4]

There are known risk factors which can trigger a dog's overdependence on humans and could lead to SRB problems. These risk factors mostly include a prolonged period of intense dog-owner contact. Whether hyper-attachment [5-7] of the dog to the owner is correlated to SRB problems is subject of debate. A study by Parthasarathy (2006) suggests that dogs with SRB problems are not necessarily hyper-attached to their owners, however, it is the attachment pattern that is potentially inappropriate.[11] Other reported risk factors could be a traumatic experience while left alone, excessive greetings by the departing owner, changes in the routine of the owner, moving to a new home or a prolonged period of absence of the owner. [3] A combination of these factors can also be the cause of the development of SRB problems in a dog. It is also possible that the SRB problems are caused by factors which are not known yet.

As stated before SRB problems can be problematic for the dog. As welfare is a positive state of mind, SRB problems could affect the dogs' welfare. Dogs suffering from severe SRB problems display a decreased ability to cope with changes in their environment, such as the departure of their owner, which can negatively influence their welfare state.[12] Therefore diagnosing SRB problems in dogs is important.

The diagnosis of SRB problems in dogs is generally based on the owners perception. According to Horwitz (2000) it is important to rule out other (medical) reasons for the problematic behavior (like

house soiling, destruction and/or vocalization) to occur and to determine the timing of the appearance of this behavior.[2,10,23] As already stated before, SRB problems should only occur in the (perceived) absence of the owner. In addition, SRB dogs often show signs of increased attachment towards their owners and are more likely to follow their owners around than non-SRB dogs.[2] An important feature of SRB dogs is their over-excited greeting behavior when the owner returns. This behavior is often prolonged and could take up to 5-10 minutes.[2]

The ultimate goal of this study is to produce results which can contribute to the evaluation of welfare in dogs. Therefore distinguishing separation anxiety in dogs from other behavioral problems and thus finding an objective way to diagnose separation anxiety will contribute to the understanding of this problem and ultimately to improve the evaluation of welfare in dogs.

In this study parameters, like physiological and behavioral indicators, will be measured in SRB and non-SRB dogs under standardized testing conditions. As reported by Beerda et al. (1998) measuring behavioral parameters which identify an acute stress response in dogs is potentially of great importance. However these parameters are easily misinterpret. To improve the interpretation of the behavioral data, Beerda et al. (1998) recommends to simultaneously measure physiological indicators of an acute stress response as well. Therefore in this study saliva cortisol and heart rate (variability) parameters are measured in addition to the behavioral parameters. Beerda et al. (1998) states that saliva cortisol and heart rate measure the activity of the hypothalamic pituitary adrenal axis and the sympathetic nervous system respectively, which respond to an acute stress response in dogs.

Behavioral parameters which could indicate an acute stress response in dogs are a lower body posture, increased vocalization, panting, oral behaviors (like smacking and licking lips) and yawning.[19]

This follow-up study is based on the original study of Inge Hoogendam (study 1). The parameters mentioned above were therefore already measured and analyzed in study 1 for 25 dogs. The aim of this study was to measure the same parameters, as in study 1, in a larger sample size of SRB dogs and 1) compare SRB dogs from this study to study 1; 2) combine dogs from both studies and compare responses in SRB dogs versus non-SRB dogs.

1.1 Hypotheses

The goal of the study was to compare our findings with those of the previous study by Hoogendam et al. (2012). In addition, we expected that when combining dogs from both studies, the results for SRB versus non-SRB dogs would be comparable. The previous study (Hoogendam, 2012) found the following results:

1) Dog behavior (the combined data of study 1 and this study)
    a) During the Table phase
        i) Stress indicators ('Panting', 'Licking lips' and 'Yawning') are higher in SRB dogs compared to non-SRB dogs.
        ii) Exploratory indicators ('Sniffing table') are higher in non-SRB dogs than in SRB dogs.
        iii) SRB dogs direct their attention more often to the owner ('Head to body owner' and 'Head to face owner') than non-SRB dogs.

iv) Non-SRB dogs direct their attention more often to the environment ('Head to environment') than SRB dogs.

v) 'Panting' and 'Sniffing' should be negatively correlated in SRB dogs. As reported by Beerda et al. (1998) panting is an indicator of an acute stress response. Ortolani et al. (2012) reported that sniffing is an explorative behavior. Therefore the behaviors 'Sniffing' and 'Panting' represent two different motivational states, which are incompatible with each other. A negative correlation between these behaviors is therefore expected.

b) Comparison Table versus Separation phase (with explorative analysis)

i) Panting during the table phase should be positively correlated with all door-directed movements during the separation phase.

ii) The heart rate (HR) during the table phase should be positively correlated with all door-directed movements during the separation phase.

iii) The opposite is also expected with respect to the HR, thus: The heart rate during the table phase should be negatively correlated with 'lying' during the separation phase.

2) Heart rate (mean HR) and Heart rate variability (HRV)

a) Across the table, separation and reunion phases

i) The mean heart rate is higher in SRB dogs versus non-SRB dogs, especially during the separation phase.

b) During the Table phase

i) The HRV (RMSSD) is lower in SRB dogs compared to non-SRB dogs.

3) Salivary Cortisol

a) Across the table, separation and reunion phases

i) The salivary cortisol in SRB dogs is higher across the 3 phases (Home sample < Table sample < Separation sample).

b) After the Separation phase

ii) The salivary cortisol is higher in SRB dogs versus non-SRB dogs.

2. Materials and Methods

The study described in this report is based on 10 dogs in total. The subjects were divided in two groups (SRB or non-SRB) based on the owners' answers to our screening list during a previously telephonic interview.

The SRB group contained 8 dogs and the non-SRB group included 2 dogs.

All dogs that participated in this study were older than one year old, were healthy and were not on medication for separation anxiety problems or were not on medicines that could influence the test results. Dogs with behavioral problems other than SRB problems were also excluded from this study.

As already mentioned this study is based on the original study of Inge Hoogendam (study 1).

Besides the results obtained from the 10 dogs observed in the current study, results of study 1 were

used as well. In total 23 (14 SRB and 9 non-SRB) dogs were used from the previous study. As study 1 included 25 dogs in total, we excluded two (non-SRB) dogs as these dogs did not appear to be suitable non-SRB dogs.
From the total of 33 dogs (study 1 combined with the current study), 14 were female and 19 were male. 19 dogs were castrated (10 male dogs) or sterilized (9 female dogs) in both the SRB group as the non-SRB group.

2.2 Screening

As already mentioned a screening for suitable subjects was done by a screening list. Based on the answers the owner gave to questions of the screening list the dog and owner were invited to participate in the test or not. The screening list was filled in during a telephone conversation between the owner and a researcher. The screening list is added in this paper in appendix 7.1. A positive answer at the points 'barking, howling or whining' , 'destructiveness', 'elimination' and 'over-excitement greeting' of question 4 of the screening list made that the dog was more likely to be invited to participate in the study in the SRB group.[12] Especially the combination of excessive greeting behavior of the dog and vocalization, destructiveness and/or inappropriate elimination in the owners absence formed an important indication that the dog suffers from SRB problems rather than from other behavioral problems.[12] The screening procedure was the same as the screening in the previous study done by Inge Hoogendam (study 1).
Prior to the telephone conversation the owners contacted the research team by e-mail or phone to show their interest in participating in this study.

In the case the dog seemed suitable to participate in this study an appointment was made to observe the dog. In addition a saliva kit and a letter, containing information about the procedure and the saliva kit, was send to the owners address. The owner was asked to take a saliva sample from the dog on the morning the dog was observed within 10 minutes of waking up to determine basal salivary cortisol concentrations.
In appendix 7.3 the instructions for the saliva sample, which were send to the owner, are added. The saliva sample was taken by using a braided 'Cotton Rope' (Part no. 5016.00, Salimetrics®). This rope had to be held in the mouth of the dog for at least 60 seconds. After the sample was taken, the rope was stored in a 'Swab Storage Tube' (Part no. 5001.05, Salimetrics®). The procedure of saliva sampling in this study was exactly the same as in the previous study of Inge Hoogendam (study 1).[12] The owner was asked to hand us the saliva sample at the testing location. Additionally the owner was asked to note the time the sample was taken and provide us this information.

A day before the observation would take place our research team contacted the owner again. This was necessary to check whether the owner had received the saliva kit and to remind the owner to take the sample. This contact also served as a reminder for the observation appointment.

2.3 Testing procedure

All observations took place in polyclinic consultation room 13 and 15 at the Utrecht University Animal Behavior Clinic. The observations were performed in the afternoon at approximately the same time, as the polyclinic consultation rooms (poly) were not in use for other purposes. The dogs

were mostly observed between 14:30 and 15:00 pm. All the dogs in this study were observed on different days and usually two dogs were observed in one week.

The testing procedure was the same for each dog. At first the owner and the dog were guided from the general waiting room to poly 13 by a researcher. In this poly the researcher gave the owner a short briefing of the procedure. After the procedure was clear to the owner, the researcher left the owner and the dog alone in poly 13 for five minutes. The dog was able to acclimatize to the situation and the owner was asked to fill in an additional questionnaire, named the owner-questionnaire (see appendix 7.2). The dog and the owner were able to interact freely during this period.

Table phase

After the five minutes were over, the researcher led the owner and the dog to poly 15. In poly 15 another researcher was waiting for the dog to enter the room and to start recording the dog. The dog was placed on the examination table, where a non-invasive polar heart rate monitor was strapped around the dogs' chest. It was necessary to wet the dogs' chest with water and place ultrasound gel on the electrodes before the monitor was fitted to the chest as this is crucial for the electrodes to make contact.

A Polar RS800CX training computer, a polar Wearlink® W.I.N.D. transmitter and straps were used to record inter-beat intervals in the dog.[12] The materials and the method of recording these intervals were based on the previous study of Inge Hoogendam (study 1).[12]

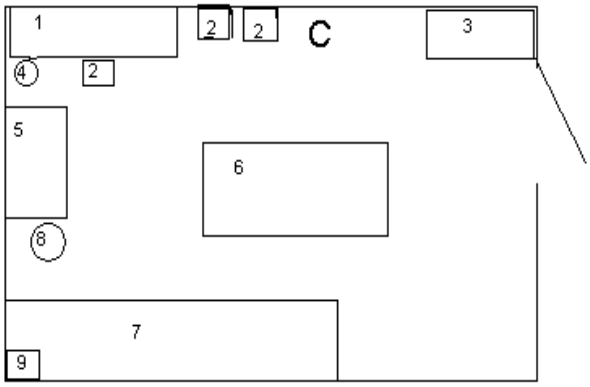
After the polar showed a heart rate, the dog was ready for the observation to begin.

One researcher left the polyclinic consultation room so the owner, the dog and the other researcher were alone in poly 15. The door was locked to ensure nobody would disturb the observation by entering the room.

The dog had to stand on the table for five minutes while two cameras (an overview camera and camera 1) recorded the dogs' behavior. The owner was asked to stand by the dog to ensure that the dog would stay on the table. It was possible for the owner to interact with the dog. This five-minute phase was termed the table phase.

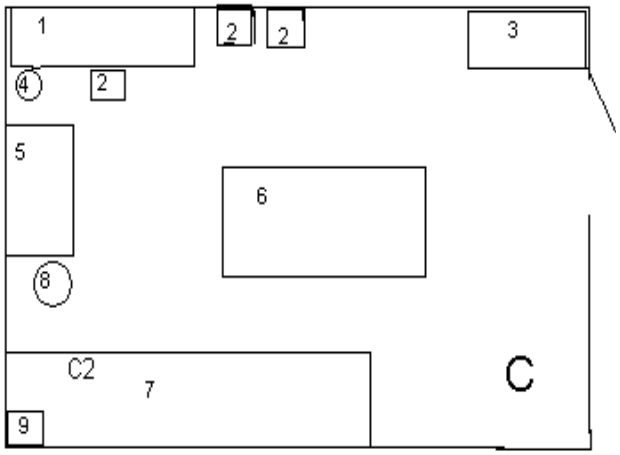
Fig.1. Floor plan of poly 15[12]

Legend
1. table
2. chairs
3. mat
4. stool
5. sink
6. examination table
7. dresser
8. water bowl
9. overview camera
C. Camera 1



This phase started as soon as the heart rate recording began, just after the door was locked.
In these five minutes the adjusting abilities of the dog were observed.
See figure 1 for the floor plan of the observation room (poly 15).[12]

Fig.2. Floor plan of poly 15 after displacing camera 1



Legend
1. table
2. chairs
3. mat
4. stool
5. sink
6. examination table
7. dresser
8. water bowl
9. overview camera
C. Camera 1
C2. Camera 2

When the five minutes passed, the researcher who was absent during the table phase re-entered the room. The dog was allowed to move of the table and to walk freely around the polyclinic consultation room for another five minutes. After these five minutes were over, the owner was asked to take a saliva sample from the dog. The procedure of sampling was the same as by the saliva sample the owner took at home. Camera 1 (a portable Sony CX550VE digital HD video camera), which recorded the dog standing on the examination table, was now displaced to another position to record the dog for the remaining phases of the observation. Camera 2 (a portable Sony CX550VE digital HD video camera) was switched on by the researchers during these five minutes of waiting. See figure 2 for the floor plan of the test room after displacing camera 1.

Separation phase
After the saliva sample was taken, the second phase began. This phase was termed the separation phase, where the dog was left alone in poly 15 for five minutes. Prior to leaving the dog alone, the owner said goodbye to the dog in a- for the dog- usual manner. The researchers left poly 15 before the owner. As soon as the owner left poly 15, the separation phase began. Three cameras recorded the dogs' behavior while left alone. One camera, the overview camera, recorded the whole room and was positioned high in a corner of poly 15. The two other cameras, camera 1 and 2, respectively recorded the door and the mat (camera 1) and the chairs and the water bowl (camera 2).
Camera 1 and 2 were necessary for recording the dog in more detail in comparison to the overview camera.
Prior to the observation both the real time displayed on the small as the big camera were synchronized to the real time displayed on the heart rate polar. This adjustment was important for us to be able to link the polar time to the time on the cameras and so to the movements or to other behaviors the dog made.
The overview camera in poly 15 was linked to a monitor in poly 13. During the separation phase it was possible for the owner and the researchers to watch the dogs' behavior while the dog was left alone in poly 15.

Reunion phase
The third phase, the reunion phase, began as soon as the five minutes of the separation phase were

over. The reunion phase started at the moment the owner re-entered poly 15. The researchers stayed behind in poly 13 and watched the greeting behavior of both the owner and the dog when the owner entered poly 15. The owner was asked to greet the dog in the same manner the owner was used to greet the dog at home. The owner had to stay alone with the dog for five minutes where the dog and owner could interact freely. It was possible for the owner to sit down on a chair and read magazines. As already mentioned before, camera 2 was focused on the chairs where most owners took place for the remaining five minutes of the reunion phase.

As soon as these five minutes were over, the researchers entered poly 15. This marked the end of the reunion phase. The dog still was able to move around freely while the researchers and owner waited for another five minutes. After this period, the owner was asked to take a second saliva sample. This sample was taken in the same way as the previous saliva samples.
In the previous study of Inge Hoogendam (study 1) the second saliva sample was taken right after the reunion phase, 10 minutes after the separation phase begun. In this study the second sample was taken 15 minutes after the beginning of the separation phase, as the cortisol concentrations in the saliva of the dog are believed to take 10-15 minutes to reach the maximum values (Beerda et al. 1998).
After the second saliva sample was taken, the observation ended. The non-invasive polar heart rate monitor around the dogs' chest was removed and the dog and owner were allowed to go home. Camera 1 was switched off after the dog and the owner left poly 15.
The room temperature was measured by a thermometer during the whole observation. The temperature was noted twice, once at the beginning of the observation and once at the end of the observation. The mean of these two values was calculated.

2.4 Data analysis

2.4.1 Behavioral analysis
The behavioral analysis was done by scoring the dogs' behavior from videos using an ethogram. There were three different ethograms; one for the table phase (appendix 7.5.1), one for the separation phase (appendix 7.5.2) and one for the reunion phase.
The table phase ethogram was split up in three groups, namely head orientation, mouth behaviors and tail directions. The durations and frequencies of behaviors in these three groups were scored by the same trained researcher.
For the 23 dogs, which were used from study 1, the table phase was scored by the previous research team by Inge Hoogendam (HP student). The previous research team scored the durations and frequencies of the behaviors in the table phase from videos using the JWatcher 1.0 software program. The 10 dogs which were observed by the current research team were scored by a trained researcher of the current research team. The durations and frequencies of the behaviors of the table phase of these 10 dogs were scored manually from videos, without using the JWatcher program. The videos, which were used to score the durations and frequencies of the behaviors in the table phase, were recorded by camera 1.
The ethogram for the table phase was developed by the previous research team. In the current study some small adjustments have been made in this ethogram, these adjustments are marked in appendix 7.5.1.

The scored behaviors – both the frequencies as the durations- have been corrected for 'Out of Sight' before any statistical analysis has been done. This was done by calculating the proportions of both the frequencies as the durations. The proportion is the duration and/or frequency of a behavior divided by the Inside. The Inside is calculated by diminishing 300 seconds (which is equal to five minutes) with the duration (in seconds) of 'Out of Sight'. This correction was done in study 1 as well. As already mentioned before, the researchers who scored the behavior were trained researchers. Training was necessary as the current research team was inexperienced in how to score behaviors using an ethogram. Therefore the current research team first trained their scoring qualities by scoring videos taken from the previous research team and comparing the results of the previous research team with the scoring results of the current researcher. After a period of practicing how to score behavior, the inter-observer and intra-observer reliability were calculated. These reliability scores needed to be over 85% before the current researcher could be a trained researcher. In appendix 7.4 the inter- and intra-observer reliability scores are shown.

The separation phase ethogram was split up in groups as well, namely movement and mouth behaviors (including the vocalization). (Appendix 7.5.2)
The scoring of durations and frequencies of the behaviors in the separation phase was done by a blind, trained researcher. This researcher was (in both the previous as in the current study) a different person than the researcher who scored the behavior in the table phase. The training of the researcher who scored the separation phase was comparable to the training of the researcher who scored the behavior in the table phase.
The 23 dogs (from study 1) were scored by the previous research team as well. The remaining 10 dogs were scored by a blind researcher from the current research team. Both the researcher from the previous research team as the researcher from the current research team manually scored the behavior from videos of three different cameras. The overview camera, camera 1 and camera 2 were used to score the durations and frequencies of the behaviors in the separation phase.

In the analysis of the behavior displayed in the separation phase, some behaviors were combined. The term 'all vocalizations' includes 'barking', 'howling' and 'whining' scored in the separation phase. 'All door-directed behaviors' includes the behaviors: 'standing door', 'approach door', 'walking towards door', 'scratching door', 'climbing door', 'jumping door', 'digging door', 'small jump door' , 'circling door' and 'moving door'.
The following behaviors are listed in the term 'all movement behaviors':

- 'approach door', 'approach mat'
- 'climbing', 'climbing door', 'climbing chair' and 'climbing table'
- 'digging', 'digging bag', 'digging door' and 'digging mat'
- 'circling', 'circling door' and 'circling mat'
- 'leave door' and 'leave mat'
- 'chasing tail'
- 'jump' and 'jump door'
- 'moving', 'moving door', 'moving table' and 'moving mat'
- 'scratching door'
- 'walking', 'walking door', 'walking mat', 'walking on table', 'walking table' and 'walking

water'

The reunion phase was not scored by the current research team, as we had not enough time to do so. Therefore the behaviors during the reunion phase are not analyzed in this paper.

2.4.2 Heart rate analysis

The heart rate analysis was done by using parameters obtained by Polar® inter-beat intervals (RR or NN).

The mean heart rate (BPM) was calculated by dividing the mean of the mean HR (provided by the polar heart rate monitor) by 60.000.

Time domain parameters and frequency domain parameters were calculated in the same manner as in the previous study of Inge Hoogendam (study 1).

Time domain parameters of the heart rate variability are the mean IBI (ms), the SDNN, the RMSSD and the NN50.

The mean IBI (ms) is the sum of the difference between two consecutive mean HR values. The RMSSD is the square root of the mean squared differences between adjacent N-N intervals (square of mean IBI (ms)). The RMSSD gives an indication of the short-term HRV [8]. The lower the value of the RMSSD is, the more variation there is between the heartbeats. A high variation between heartbeats indicates a higher stress level in the dog. [9]

The SDNN is the standard deviation of RR intervals. [8] The NN50 is the number of pairs of successive RRs (which is equal to NNs) that differ by more than 50 ms.

The frequency domain parameters of the heart rate variability are the LF, HF and the LF/HF ratio. As physical activity has a strong influence on changes in cardiac activity, frequency domain analysis is only possible in stationary conditions. So only during the table phase the frequency domain analysis is possible. Both the frequency as the time domain parameters were calculated by using the Kubios® software program. This is the same method which is used in the previous study by Inge Hoogendam (study 1). However due to the time it was not possible to include the frequency domain parameters for the dogs which were observed by this research team in this paper. For the time domain parameters, only the RMSSD could for this reason by analyzed in this paper.

Due to loss of contact between the electrodes and the body, the heart rate data of both the separation as the reunion phase contained errors. Only the so-called 'red errors' were adjusted.

All the mean HR which had an IBISdif (the difference between two consecutive mean HR values) greater than 450 ms were marked as a red error. These mean HR were replaced by a value which was the mean of mean HR values surrounding the red errors.

The table phase did not contain 'red errors' as the heart rate data in this phase was measured under stationary conditions where there was no loss of contact between the electrodes and the dog.

2.4.3 Cortisol analysis

The cortisol analysis was done from saliva collected from the saliva samples. At the end of the observational procedure there were three saliva samples taken from the dog. The first sample was a home sample, which the owner took the morning of the observation. The second and third samples are respectively the sample taken five minutes after the table phase (termed table sample) and the sample taken five minutes after the reunion phase (termed separation sample). Note that in the 23

dogs of study 1, the third sample was taken right after the reunion phase without a five minute waiting period.
The samples were centrifuged at 3000 rpm for at least 15 minutes. This procedure is adapted from the previous research team (study 1). After centrifuging the samples, the saliva had been squeezed from the cotton rope and could therefore be transported to another test tube. The volume of the sample was estimated by weighing the saliva. After estimating the volume, the saliva was stored at -20 degrees Celsius until further cortisol analysis (ELISA). [12]
The ELISA was done at the end of the observational period for all of the 10 saliva samples, from the dogs which were observed by the current research team, at once. The saliva samples of the 23 dogs of study 1 were determined by the previous research team by Inge Hoogendam following the same procedure as the current research team.
To make a double ELISA for a sample, the sample had to contain at least 50 micro liters saliva. For a single determination 25 micro liter saliva was necessary. If possible a double determination of a sample was preferable. Some samples, however, did not contain enough saliva for such a determination and therefore had to be diluted to reach a volume of 50 micro liters.
Some samples contained less than 25 micro liters and could not be determined twice. Therefore these samples were diluted so a single determination was possible. Some dogs were excluded from cortisol value analysis as their saliva production was to minimal for the ELISA test.
After diluting the samples the ELISA was done following the procedures of the ELISA kit. All 10 saliva samples of the dogs observed by the current research team were transferred to the same ELISA plate.
The optical densities of the samples on this plate were read out using a computer program.
The average was taken from the outcomes of the samples which had a double determination.
The analysis of the ELISA was done following the instructions which were included in the ELISA kit.
With help of a computer program named 'graph pad program', the optical densities (OD) of the samples obtained from the ELISA analysis were transformed into cortisol concentrations.
The cortisol concentrations in the ELISA sample were –if necessary- corrected for the factor of dilution to get the real cortisol concentration in the saliva sample.
The cortisol concentrations of all tested samples were higher than the detection limit.

2.4.4 Statistical analysis

The data obtained in this study is analyzed with the SPSS version 2.0 program.
First descriptive statistics have been used to look at the data of the behavioral, heart rate and cortisol analysis.
With descriptive statistics it was possible to see whether the results obtained from this current study followed the same trend as seen in study 1. Whenever this was the case, the dogs of both studies were combined and the results of the total SRB group were compared to the non-SRB group.
In addition, the results of study 1 were compared to the results obtained from all dogs combined.
For these comparisons, non-parametric tests were used as the data was not normally distributed.

The Mann-Whitney U test has been used to compare two numeric, independent samples. This test is used to compare the parameters of SRB dogs with the parameters of non-SRB dogs. The test has also been used for the comparison between the data of study 1 and the data of all dogs combined.

A different test has been used to compare the cortisol levels across the three phases in either theSRB or the non-SRB group. This test was a Friedman ANOVA, as the comparison was between dependent samples. In case of a significant Friedman ANOVA test, a Wilcoxon Signed Rank test was used to follow up this finding. A Bonferroni correction (for the three phases) was applied to the results of the Wilcoxon Signed Rank test and the effects are reported at α=0.0167.
The mean heart rate and the RMSSD across phases in either SRB or non-SRB dogs were analyzed in the same way. Besides comparing SRB dogs with non-SRB dogs and dogs of study 1 with all dogs, statistical analysis has been done to find correlations between parameters as well.
After making a scatterplot, a Spearman's Rho test has been used to analyze whether the correlation was significant.

3. Results

3.1 Behavior

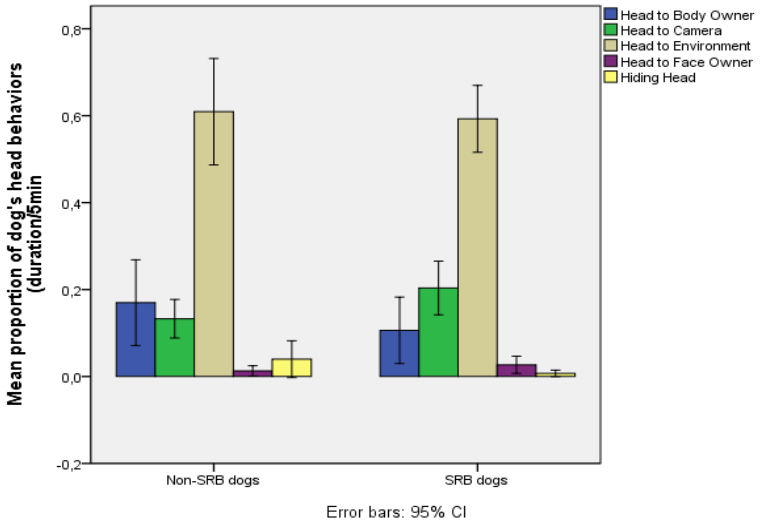
Especially the behavioral parameters obtained during the table phase are mentioned in this paper. The behavioral parameters of the separation phase were scored by a different (blind) researcher and are described in another paper.[14] However correlations between the behavior displayed in the table phase and in the separation phase are noted in this paper.
As already mentioned the behavior displayed by the dogs observed by the current research team during the reunion phase was not scored. The behavioral analysis of the reunion phase was therefore not included in this paper.

3.1.1 Table phase

The duration and frequency of dog behavior obtained from study 1 was compared with the results obtained from the current study (1). Afterwards all dogs from study 1 and the current study were combined. Comparisons were made between the combined SRB and non-SRB dogs (2).
Figure 3 shows an overview of all head orientation behaviors displayed in the combined SRB and non-SRB dogs. Figure 4 and 5 show an overview of the mouth behaviors displayed in SRB and non-SRB dogs during the table phase.

Analysis of the head orientation behavior 'Head to Camera' showed;
1) In the previous study (study 1) SRB dogs directed their head to the camera significantly longer in comparison to non-SRB dogs. It is interesting to note there is a significant difference between the SRB dogs (Mdn=0.24) described in the previous study (study 1) and the SRB dogs (Mdn=0.07) observed in the current study with respect to this behavior (Mann-Whitney U test: U=8.00, z=-3.278, n=22, p=0.00, r=-0.69).

Fig. 3: All head orientation behaviors (duration) of SRB and non-SRB dogs displayed during the table phase (Head to body owner: SRB Mdn=0.05 and non-SRB Mdn=0.15 (U=67.500, z= -2.05, n=33, p(one-tail)=0.02, r=-0.36).



2) When all dogs were combined there was no significant difference between the duration 'head to camera' between SRB (SRB Mdn=0.17) and non-SRB dogs (non-SRB Mdn=0.13) (Mann-Whitney U test: U=84.000, z=-1.41, n=33, *ns*, r=-0.25).

Analysis of the head orientation behaviors 'Head to Body Owner' and ' Head to Face Owner' showed;
1) In the previous study (study 1) non-SRB dogs directed their head significantly longer to the owner's body in comparison with SRB dogs. There was no significant difference between the non-SRB dogs in study 1 (Mdn=0.151) and the non-SRB dogs in the current study in respect to this behavior (Mdn=0.195) (Mann-Whitney U test: U=6.000, z=-0.707, n=11, *ns*, r=-0.21).
2) When all dogs were combined, non-SRB dogs (Mdn=0.15) also directed their head significantly longer to the owners body in comparison to SRB dogs (Mdn=0.05) (Mann-Whitney U test: U=67.500, z= -2.05, n=33, p(one-tail)=0.02, r=-0.36).
When all dogs were combined, no significant difference in the duration of the behavior 'Head to Face Owner' was found between SRB (Mdn=0.0115) and non-SRB (Mdn=0.0077) dogs (Mann-Whitney U test: U=103.50, z=-0.681, n=33, *ns*, r=-0.12). In addition, there was no significant difference in the frequency of this behavior between SRB (Mdn=0.0067) and non-SRB dogs (Mdn=0.0033) (Mann-Whitney U test: U=84.0, z=-1.451, n=33, *ns*, r=-0.25).
No further differences in head orientation behavior between SRB and non-SRB dogs were significant.

Analysis of the mouth behaviors (sniffing, panting, licking lips and yawning) showed:
1) In study 1 none of these mouth behaviors displayed during the table phase were significantly different between SRB and non-SRB dogs.
The durations and frequencies of these mouth behaviors analyzed in study 1 were not significantly different compared to the results of the current study.

2) After combining all dogs it appeared that only the frequency of yawning was significantly lower in SRB (Mdn=0.0033) compared to non-SRB dogs (Mdn=0.0067) (Mann-Whitney U test: U=63.00, z=-2.27, n=33, p=0.026, r=-0.40) (appendix 7.6.).

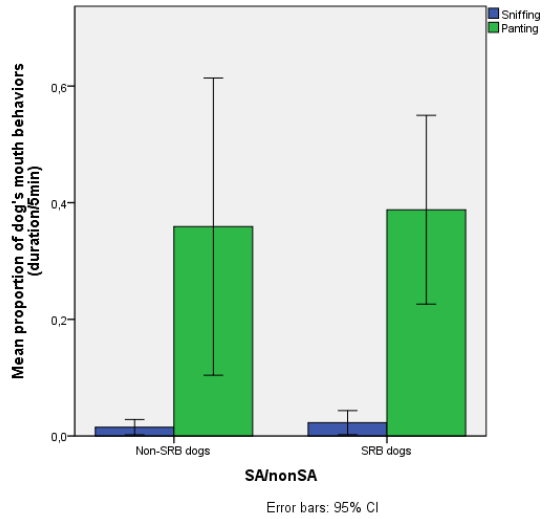
It was not possible to compare most of the tail behaviors displayed during the table phase in study 1 with the current study as the ethogram had been adjusted for these behaviors. The 'Tucked Tail' was a new behavior which influenced the scoring of the 'Tail Low' in the current study.

A tail behavior which was scored in the same way in both studies is 'Tail Between Legs'.

1) In study 1 there was no significant difference between SRB and non-SRB dogs in respect to this behavior.

2) When all dogs were combined there was no significant difference between SRB (Mdn=0.01) and non-SRB (Mdn=0.01) dogs in respect to the duration of the behavior 'Tail Between Legs' (Mann-Whitney U test: U=119.500, z=-0.06, n=33, p=0.955, r=-0.01).

Fig. 4: Mouth behaviors (duration) of SRB and non-SRB dogs displayed during the table phase.



The analysis of a correlation between the behaviors 'Sniffing' and 'Head to Camera' showed:

1) In the previous study a significant, negative correlation was found between 'Sniffing' and 'Head to Camera' in SRB dogs.

2) However this correlation is not significant when all SRB dogs are combined (Spearman's Rho: r_s= -0.114, n=22, p=0.614).

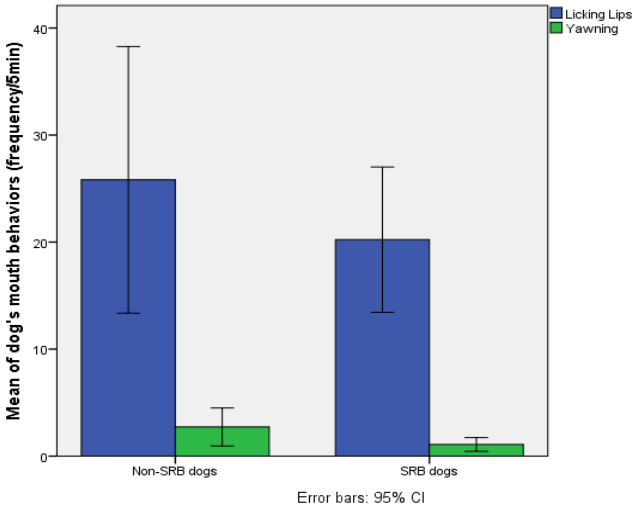
A correlation between 'Sniffing' and 'Panting' was analyzed as well, this showed:

1) These behaviors were significantly negatively correlated in SRB dogs in the previous study.

2) This correlation was also significant when all SRB dogs were combined (Spearman's Rho: r_s=-0.517, n=22, p=0.014).

Moreover this correlation was also significant across all dogs combined (both SRB and non-SRB dogs) (Spearman's Rho: r_s= -0.407, n=33, p=0.019).

Fig. 5: Mouth behaviors (frequency) of SRB and non-SRB dogs displayed during the table phase (Yawning: SRB Mdn=0.0033 and non-SRB Mdn=0.0067 (U=63.00, z=-2.27, n=33, p=0.026, r=-0.40).



As in the previous study panting was not significantly correlated with the mean room temperature during the table phase (Spearman's Rho: r_s= 0.312, n=33, p=0.087).

3.1.2 Separation phase

Dog behavior during the separation test was scored by a separate researcher (Anne Gijsbertsen [14]) who scored behavior blindly as to whether the dogs were classified as SRB or non SRB. The behavioral analysis of the separation phase is therefore not mentioned in this paper.

However, in this study the owner-questionnaire (Appendix 7.2) was analyzed and compared to the results of the separation phase. There was a significant positive correlation between the frequency of vocalizations displayed by the dogs during the separation phase and the rating of vocalizations displayed by the dog at home while left alone (Scale 1-5; never to always) reported by the owners in the owner-questionnaire (Spearman's Rho: r_s=0.612, n=33, p=0.000).

3.1.3 Comparisons between the table phase and the separation phase

All comparisons between the behavioral data from the table and the separation phase were done using an exploratory analysis.

When all dogs were combined there was a significant negative correlation between all door-directed behaviors (page 12) in the separation phase and 'Head to Body Owner' in the table phase in SRB dogs (Spearman's Rho: r_s=-0.556, n=22, p=0.007).

A significant positive correlation in SRB dogs was found between all door-directed behavior (page 12) in the separation phase and 'Head to Camera' in the table phase (Spearman's Rho: r_s=0.458, n=22, p=0.032).

In addition, there was a marginally significant negative correlation in SRB dogs between 'Lying Door' in the separation phase and the mean heart rate during the table phase (Spearman's Rho: r_s=-0.292, n=33, p=0.099) and with panting during the table phase (Spearman's Rho: r_s=-0.30, n=33, p=0.09).

In addition, there was also a marginally significant negative correlation between 'Lying Mat' in the separation phase and 'Panting' during the table phase (Spearman's Rho: r_s=0.293, n=33, p=0.098).

3.2 Heart rate

3.2.1 Mean heart rate

The mean heart rate (BPM) in SRB dogs was analyzed across the 3 phases:

1) In the previous study (study 1) the mean heart rate of SRB dogs differed significantly across the 3 phases, in particular between the table and separation phase.

2) After combining all dogs, there also was a significant difference in the mean heart rate (BPM) in SRB dogs between the table, the separation and the reunion phases (Friedman ANOVA: χ^2=11.000, df=2, n=22, p=0.004). Post-hoc Wilcoxon tests were used to follow up this finding. A Bonferroni correction was applied and all effects are reported at α=0.0167. After corrections it appears that the mean heart rate is significantly different only between the table (Mdn=115.73) and the separation phase (Mdn=133.69) (Wilcoxon Signed Ranks test: Z= -2.841, n=22, p=0.005, r=-0.61). These results fit the results seen in the previous study.

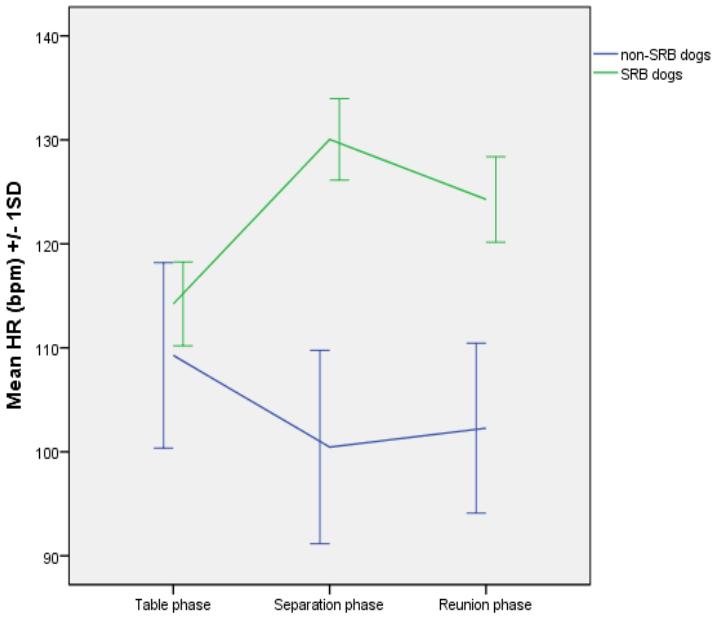
Analysis of the mean heart rate in non-SRB dogs across the 3 phases showed:

1) In the previous study there was no significant difference in the mean heart rate of non-SRB dogs during the table, the separation and the reunion phase.
2) The mean heart rate of all non-SRB dogs combined did not differ significantly between the three different phases as well (Friedman ANOVA: χ^2=3.818, df=2, n=11, p=0.148).

Comparisons of the mean heart rate in the table phase between SRB and non-SRB dogs showed:
1) In the previous study there was no significant difference in the mean heart rate during the table phase between SRB and non-SRB dogs.
2) After combining all dogs, this was also not significant (SRB Mdn= 115.73 and non-SRB Mdn=115.04) (Mann-Whitney U test: U=103.000, z=-0.687, n=33, *ns*, r=-0.12).
Comparisons of the mean heart rate in both the separation and reunion phase between SRB and non-SRB dogs showed:
1) In study 1 the mean heart rate in the separation phase was significantly different between SRB and non-SRB dogs. In the reunion phase this difference was only marginally significant.
2) When all dogs were combined, the mean heart rate of SRB dogs was significantly higher in both the separation phase (SRB Mdn=133.69 and non-SRB Mdn=105.21) (Mann-Whitney U test: U=47.000, z=-2.83, n=33, p=0.004, r=-0.49) and reunion phase (SRB Mdn=121.81 and non-SRB Mdn=106.09) (Mann-Whitney U test: U=60.000, z=-2.33, n=33, p=0.019 r=-0.41) compared to non-SRB dogs.

Analysis of a correlation between the mean heart rate during the separation phase and (all) door-directed behaviors displayed in the separation phase showed:
1) In the previous study (study 1) a significant positive correlation was found in SRB dogs between the mean heart rate during the separation phase and door-directed behaviors (only: 'Standing Door', 'Approach Door', 'Walking towards Door' and 'Scratching Door') displayed in the separation phase. This was not found in non-SRB dogs.
2) After combining all dogs, this correlation was not significant in both SRB (Spearman's Rho: r_s=0.139, n=22, p=0.536) and non-SRB dogs (Spearman's Rho: r_s=0.027, n=11, p=0.937).
However, not all door-directed behaviors were taken into account in the previous analysis. When all door-directed behaviors (see page 12) were taken into account there was a significant correlation between these behaviors and the mean heart rate during the separation phase in SRB dogs (Spearman's Rho: r_s=0.713, n=22, p=0.000). This correlation was not significant in non-SRB dogs (Spearman's Rho: r_s=0.478, n=11, p=0.137).

Fig. 6: Mean heart rate (bpm) during the different phases; SRB and non-SRB dogs compared



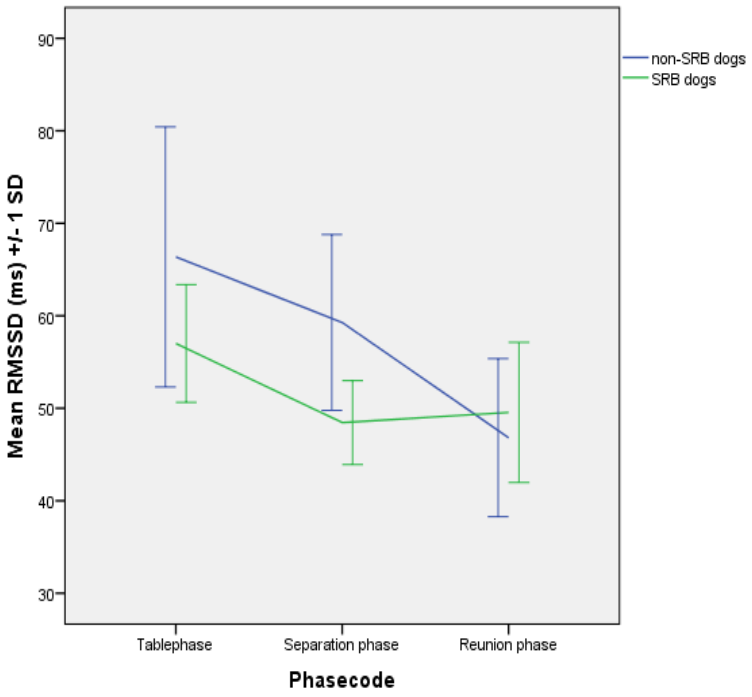
A correlation between the mean heart rate in the separation phase and all movement behaviors displayed in the separation phase in SRB dogs was also analyzed:
1)In study 1 there was a significant positive correlation between the mean heart rate in the separation phase and all movement behaviors displayed in the separation phase in SRB dogs. In the non-SRB dogs in study 1 this correlation was not significant.
2) When all dogs were combined, this correlation was significant in SRB dogs (Spearman's Rho: r_s=0.640, n=22, p=0.001), but not significant in the non-SRB dogs (Spearman's Rho: r_s=0.282, n=11, p=0.401).

3.2.2. Time domain parameter; RMSSD

As already mentioned the RMSSD was the only time domain parameter which was calculated in the current study. Therefore this was the only time domain parameter that could be compared with study 1.

1) In the previous study the RMSSD declined significantly in the SRB dogs across all three phases (table, separation and the reunion phase). The RMSSD in non SRB dogs from study 1 also declined across the phases, but this was not significant. The RMSSD of the SRB dogs (n=8) of the current study did not decline significantly across the phases (Friedman ANOVA: χ^2=0.250, df=2, n=8, p=0.882).
2) When all dogs were combined, the RMSSD of the SRB dogs differed marginally significant (Friedman ANOVA: χ^2=5.182, df=2, n=22, p=0.075) and the RMSSD of the non-SRB dogs (Friendman ANOVA: χ^2=1.273, df=2, n=11, p=0.529) did not differ significantly between the three phases.

Fig. 7: Mean RMSSD (ms) during the different phases; SRB and non-SRB dogs compared



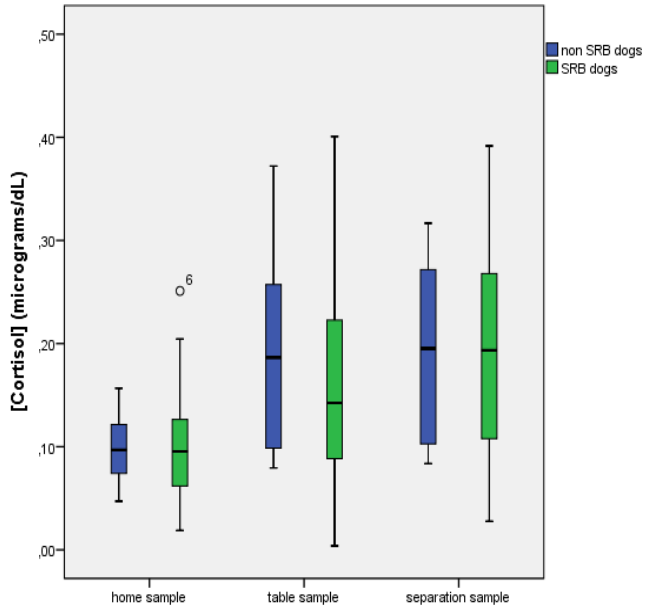
Comparisons between RMSSD of SRB and non-SRB dogs showed:

1)In study 1, there were no significant differences between the RMSSD of SRB and non-SRB dogs.
2)When all dogs were combined, there also were no significant differences between SRB and non-SRB dogs during the table (SRB Mdn= 52.31 and non-SRB Mdn=46.71) (Mann-Whitney U test: U=116.00, z=-0.19, n=33, *ns*, r=-0.03), the separation (SRB Mdn= 45.51 and non-SRB Mdn=66.33) (Mann-Whitney U test: U=99.00, z=-0.84, n=33, p=0.418, r=-0.15) and the reunion phase (SRB Mdn= 39.88 and non-SRB Mdn=39.43) (Mann-Whitney U test: U=121.00, z=0.00, n=33, p=1.000, r=0.00).

3.3 *Salivary cortisol*

The salivary cortisol values of the 3 samples (home, table and separation sample) in both SRB and non-SRB dogs were analyzed:

Fig. 8: Salivary cortisol values (microgram/dL) during the different phases; SRB and non-SRB dogs compared



1) The salivary cortisol values of SRB dogs in study 1 were significantly different between the home (basal), table and the separation samples (Figure 8). In study 1 these differences were found especially between the home and separation samples and between the home and table samples. The salivary cortisol values of the non-SRB dogs in study 1 did not significantly differ across the three samples.
2) When all dogs were combined, the salivary cortisol values of SRB dogs were also significantly different across the three samples (home, table and separation) (Friedman ANOVA: χ^2=7.125, df=2, n=16, p=0.028). Wilcoxon tests were used to follow up this finding. A Bonferroni correction was applied and all effects are reported at α=0.0167. After corrections it appears that the salivary cortisol values are significantly different only between the home and the separation sample (home SRB Mdn=0.10 and separation SRB Mdn=0.19) (Wilcoxon Signed Rank test: Z= -2.689, n=16, p=0.007, r= -0.67). The salivary cortisol values in non-SRB dogs did not differ significantly between the home, table and separation samples (Friedman ANOVA: χ^2= 4.222, df=2, n=9, p=0.121).

Comparisons between the salivary cortisol values of SRB and non-SRB dogs were analyzed:
1) In study 1 the mean salivary cortisol values were not significantly different between SRB and non-SRB dogs in any sample (home, table and separation).
2) After combining all dogs, these cortisol values also did not differ significantly between SRB and non-SRB dogs in the home (SRB Mdn=0.10 and non-SRB Mdn=0.10) (Mann-Whitney U test:

U=78.000, z=-0.105, n=26, p=0.938, r=-0.02), the table (SRB Mdn=0.16 and non-SRB Mdn=0.19) (Mann-Whitney U test: U=88.000, z=-0.90, n=31, p=0.381, r=-0.16) and the separation sample (SRB Mdn=0.19 and non-SRB Mdn=0.17) (Mann-Whitney U test: U=90.000, z=0.00, n=28, p=1.000, r=0.00).

When all dogs were combined no significant correlation was found between the table cortisol levels and panting during the table phase in both SRB (Spearman's Rho: r_s=0.117, n=20, p=0.622) and non-SRB dogs (Spearman's Rho: r_s= 0.385, n=11, p=0.242). In addition, no significant correlation was found between the separation cortisol levels and panting during the separation phase in both SRB (Spearman's Rho: r_s=-0.24, n=18, p=0.925) and non-SRB dogs (Spearman's Rho: r_s=0.175, n=10, p=0.629).
However all door-directed behaviors were marginally significantly negatively correlated with the cortisol levels in sample 3 (separation sample) in SRB dogs (Spearman's Rho: r_s= -0.461, n=18, p=0.054). This correlation was not significant in the non-SRB dogs (Spearman's Rho: r_s= -0.006, n=10, p=0.987).

4. **Discussion:**

4.1 Behavior

4.1.1 Table phase

In both the previous study (study 1) and in this study there was a significant difference in the head orientation behavior 'Head to Body Owner' between SRB and non-SRB dogs during the table phase. Non-SRB dogs directed their head significantly longer to the body of the owner than SRB dogs. This is a puzzling finding as we expected SRB dogs to seek more support by their owners in a novel, potentially stressful situation than non-SRB dogs.
A possible explanation for this finding was reported by Hoogendam (2012). Hoogendam (2012) reported that the owner may be an important factor during the five minute table phase. It is possible that SRB dogs benefit more from the attachment bond to their owners during the table phase, as the owners are in close proximity of the dog, than non-SRB dogs and SRB dogs may therefore be more calm and display less stress-induced behaviors during the table phase than non-SRB dogs. This would be the case only if the attachment between the dog and owner plays a role in developing SRB problems. However in the literature the hyper-attachment theory is still subject of debate. It is therefore not clear whether the degree of dog-owner attachment is correlated with the level of stress the dog experiences when left alone. [3,4,6,15]
In addition Hoogendam (2012) reported that the owner may be able to influence the dogs' behavior by displaying more control and comfort talk during the table phase. Hoogendam (2012) reported that it is possible non-SRB dogs looked longer to the owners' body in an attempt to actively seek comfort by their owners in this stressful situation, as these owners may have displayed less comfort and control talk.
However during this study we noticed that there may have been an effect of our scoring rules on 'Head to Body Owner' during the table phase as the position of the owner towards the dog influences the scoring of the duration of this behavior. We have noticed differences in the position of the owner towards the dog, as some owners stand behind the dog (from the researchers' point of

view) whereas some owners stand next to the dog, nearby the dogs head. When the owner stands next to the dog, it could be difficult to distinguish whether the dog actively directs its head to the owners' body or not. Therefore the behavioral category 'Head to Body Owner' may not be useful in distinguishing SRB versus non-SRB dogs. Although there were no significant differences in duration or frequency of 'Head to Face Owner' between SRB and non-SRB dogs, it seems that this behavior may be more interesting to look at than 'Head to Body Owner'. It is possible that these differences were not significant due to the small sample size we used in this study.
So in the future it may be better to look at the duration of the behavior 'Head to Face Owner' or to score the frequency in which the dog actively directs its head to the face of the owner.

It is interesting to note there was a significant difference in the duration of 'Head to Camera' between study 1 and our study. SRB dogs in study 1 directed their heads longer to the camera (and therefore to the researcher, who is standing behind the camera) than non-SRB dogs in study 1. This difference was not significant in both the dogs from this study as in all the dogs combined. This is interesting because there might be a 'researcher effect' that may explain this difference between studies. It is possible that one researcher may be more attracted for dogs to look at or more frightening to look at than another researcher. This may explain the significant difference in this behavior between the SRB dogs of study 1 and the SRB dogs of this study.
If there is a researcher effect, the behavior 'Head to Camera' is not an interesting behavior to analyze in future studies.

In both the previous study and in this study there were no significant differences in mouth behaviors ('Sniffing', 'Panting' and 'Licking lips') between SRB and non-SRB dogs.
According to Ortolani (2012) panting, yawning and licking lips are some indicators of an acute stress response in dogs. We expected that SRB dogs would be more sensitive to 'stress' in novel situations, such as the table phase, and therefore would show more 'stress indicators' as panting and licking lips. Hoogendam (2012) reported that it is possible that the close proximity of the owner during the table phase may have moderated an acute stress response in some SRB dogs. Gácsi (2013) supports this theory as Gácsi reported that the owner may provide security for the dog in potentially stressful situations.
It is also reported by Hoogendam (2012) that 'stress responses' may be shown for other reasons than SRB problems. This might explain why no significant differences in 'Panting' and 'Licking lips' were found between SRB and non-SRB dogs during this phase.
There was a significant difference in 'Yawning' between the combined SRB and non-SRB dogs in this study. Non-SRB dogs displayed more yawning than SRB dogs during the table phase. As stated above yawning is described as an indicator of an acute stress response in dogs (Ortolani 2012). However, if these dogs experienced an acute stress response we would expect to see more parameters (such as mean HR, HRV and/or cortisol) to differ as well. As this is not the case, it is possible that yawning reflects an uncertain state of mind rather than an acute stress response. In addition the overall frequency of yawning in dogs during the table phase was low.

In both study 1 and this study 'Sniffing' and 'Panting' were found to be significantly negatively correlated. This significant correlation supports the theory that panting is an indicator of an acute stress response (Beerda at all (1998)) whereas sniffing is an exploratory behavior (Beerda et al

(1998), Ortolani et all. (2012)) that is not displayed in stressful situations.
As reported by Hoogendam (2012) panting is also a behavior necessary for thermoregulation. It is unlikely that the displayed panting behavior of the dogs during the table phase was due to thermoregulation, as there was no significant correlation between panting during the table phase and the mean room temperature.

4.1.2 Separation phase
As the separation phase is scored and analyzed by a different researcher (Anne Gijsbertsen), the behavioral results are not presented in this study.

However in this study we did find a significant positive correlation between the frequency of vocalizations displayed by the dogs during the separation phase and the owners' ratings of whether the dog produced vocalizations at home when left alone (scale 1-5: never-always) as reported in the owner-questionnaire. As most SRB owners report that their dogs vocalize frequently when left home alone, we expected that the frequency of vocalizations during the separation phase would be higher as well in SRB dogs versus non-SRB dogs.
The vocalizations displayed during the separation phase which were analyzed in this study include 'Howling', 'Barking' and 'Whining'. Palestrini (2010) reported that howling is used in wolves to communicate when separated from the pack, in dogs this could be a natural response when feeling the discomfort of being left alone. In addition Palestrini (2010) reported that whining and barking may respectively be an attention-seeking signal or may reflect arousal. Whining could reflect an emotional state of fear when the dog is experiencing emotional isolation (as during the separation phase). Hence, according to Palestrini (2010), dogs when left alone display vocalizations as a consequence of discomfort, fear and/or anxiety. Displaying vocalizations by the dog when left alone may therefore be an indication of an acute stress response (Parthasarathy (2006), Palestrini (2010)). Gijsbertsen (2013) reported that the SRB dogs in this study showed a significantly higher frequency of vocalizations ('Howling', 'Barking' and 'Whining') during the separation phase than the non-SRB dogs. In addition Palestrini (2010) reported that SRB dogs spent most of their time vocalizing while left home alone. Therefore a positive correlation between the vocalizations displayed during the separation phase and the owners' ratings of whether the dog produced vocalizations at home when left alone (scale 1-5: never-always) was expected.

4.1.3 Comparison table and separation phase
Both Hoogendam (2012) and Gijsbertsen (2013) reported that SRB dogs displayed significantly more door-directed behavior during the separation phase than non-SRB dogs. Therefore significant positive correlations between this behavior and 'Panting'/'mean HR' during the table phase were expected. However, in this study these correlations were not significant as the differences between SRB versus non-SRB dogs in 'Panting' and mean heart rate during the table phase were not significant as well. It is possible that the sample size in this study was too small. In the future it would be interesting to test whether these correlations exist in a bigger sample size.
The significant negative correlation between all door-directed behaviors in SRB dogs and 'Head to Body Owner' may be due to the significant differences between SRB and non-SRB dogs in these behaviors. SRB dogs displayed significantly more door-directed behaviors during the separation

phase than non-SRB dogs and non-SRB dogs displayed more 'Head to Body Owner' during the table phase. A negative correlation is therefore found.

We expected a negative correlation between the mean heart rate during the table phase and the behavior 'Lying (Door)' during the separation phase. This correlation was only marginally significant. This could be due to a too small sample size. However both Hoogendam (2012) as Gijsbertsen (2013) reported that non-SRB dogs significantly lied down more (especially by the door) than SRB dogs during the separation phase. Lying behavior is seen as a resting behavior.[12] It is expected to see this lying behavior more in non-SRB dogs than in SRB dogs as Palestrini (2010) reported that SRB dogs were more active when separated from their owner compared to non-SRB dogs. Both Hoogendam (2012) as Gijsbertsen (2013) reported this finding as well. Palestrini (2010) also reported that SRB dogs display more stress-related behavior (such as yawning and licking lips) when locked in a closed area (like a bench) while being alone compared to SRB dogs that can move around freely. This data suggests that movement in SRB dogs may be on way of coping with being left alone by their owner. Restricting movement in SRB dogs could possibly therefore affect their welfare.
In this study, however, we have seen 2 SRB dogs which differed from the others with respect to movement during the separation phase. These SRB dogs –which were used to being locked in a bench while left home alone- lied on a mat for the whole five minutes of the separation phase. As these dogs showed behavior which was in discordant with the behavior seen in most of the SRB dogs, this could be due to a 'bench-effect'. These SRB dogs displayed excessive vocalizations, which indicate an acute stress response.[15] This finding suggests that lying behavior during the separation phase does not necessarily indicate a relaxed state of mind.
The 'bench effect' may be a reason for the correlation between the mean heart rate during the table phase and the behavior 'Lying (Door)' to be only marginally significant.

4.2 Heart rate

4.2.1 Mean heart rate (BPM)

The mean heart rate was expected to be higher in SRB dogs versus non-SRB dogs during the table, separation and reunion phases due to diminished coping abilities in SRB dogs in novel situations. However the mean heart rate in SRB dogs was not significantly different compared to the mean HR in non-SRB dogs during the table phase. It is possible that the statistical power necessary to perform the test was too low.

The mean heart rate during the separation phase in SRB dogs was significantly higher than in non-SRB dogs. In addition the mean heart rate in SRB dogs increased significantly between the table and separation phases. Physical exercise causes the mean heart rate to increase (Newton et al. (1982)). As SRB dogs were significantly more active during the separation phase than non-SRB dogs (Gijsbertsen (2013)), an increase in mean HR in SRB dogs versus non-SRB dogs during the separation phase is not surprising. This could also explain the increased mean HR in SRB dogs in the separation phase versus the table phase.
The significant positive correlations in SRB dogs between the mean HR during the separation phase and both all door-directed behavior and all movement behavior displayed in the separation phase also indicate that physical activity may influence the mean HR.

However, the correlation between the mean HR during the separation phase and all movement behavior was not significant in non-SRB dogs. Hoogendam (2012) reported this finding as well and therefore suggested that the increase in physical activity may not be the only explanation for these results.
In this study no further data analyses were done to compare the heart rate changes between movement and non-movement periods during the separation phase in SRB dogs, as suggested by Hoogendam (2012). These data analyses may further our understanding on this issue in the future.

4.2.2 Heart rate variability (RMSSD parameter)
The RMSSD was the only time domain parameter which was analyzed in this study. According to Von Borrell et al. (2007), the RMSSD is an informative HRV time domain parameter. The RMSSD gives an indication of the variation between heartbeats that represent vagal (parasympathetic) regulatory activity (Von Borrel et al. (2007), Reed (2005)).
We expected the RMSSD to be lower in SRB dogs during the table phase compared to non-SRB dogs as Von Borrell et al (2007) reported that a lower HRV implicates physiological 'stress' and therefore may implicate a compromised welfare state. However the RMSSD during the table phase was not significantly different between SRB versus non-SRB dogs in this study. It is possible that the statistical power necessary to perform the test was too low. In addition, although not expected, it is also possible that there was no reduction in vagal tone in SRB dogs and therefore there was no difference between SRB and non-SRB dogs in RMSSD levels during the table phase.
Moreover we found the RMSSD in SRB dogs to differ in a marginally significant way across the three phases (table phase > separation phase > reunion phase). However, according to Von Borrel et al. (2007) stationary conditions are most desirable to measure the heart rate data. Therefore only the heart rate data measured during the table phase in SRB versus non-SRB dogs could be compared in a reliable manner. The heart rate data of the separation and reunion phase contained errors due to physical activity. Therefore this data should be interpreted with caution.
Hoogendam (2012) reports the occurrence of errors in the separation and reunion phases as well.

4.3 Cortisol

SRB dogs show an increase in salivary cortisol levels between the home and separation sample. This finding was the same as in study 1 and indicates that SRB dogs experience an acute stress response during the five minute separation phase. Non-SRB dogs show no significant increase across the three phases, which we expected as the acute stress response is not evoked in non-SRB dogs as much as in SRB dogs when left alone.

In the dogs observed in this study, the separation sample (third sample) was taken 15 minutes after the beginning of the separation phase. In the previous study, the separation sample was taken only 10 minutes after the beginning of the separation phase. Since cortisol levels are known to peak in saliva 10 to 15 minutes after presenting a stressor (Beerda et al., 1999), the 10 minute period in the previous study may not have been sufficient to detect a peak, if present. Therefore, results for the two studies cannot be combined for the separation sample and our sample size may have been too small to detect any significant effects. In the future, a greater sample size of dogs sampled 15 min after the separation phase would need to be observed and compared to the dogs in this study.

Hoogendam (2012) states that it is possible that owners misinterpret their dogs with respect to SRB problems and therefore it can happen that some of the dogs belonging to the non-SRB group may actually show signs of separation-related behaviors when left alone in a novel environment, such as our test setting. The dogs in our study show variability in the intensity of their behavioral responses during the separation phase, confirming Hoogendam's (2012) previous suggestion that the prevalence of separation-related behavioral problems in dogs should be measured using a gradual scale.[12]

4.4 Further considerations

In future studies it would be interesting to consider the following possibilities as these factors may have influenced our testing results;

1) It is possible that the owners provide support for their SRB dogs during the table phase. By looking at the reunion phase, classifying the dogs based on the behavior seen in this phase and then looking at the table phase again, it may be possible to test this theory.
2) As SRB problems are believed to follow a gradual scale, it may be interesting to classify the dogs as low, medium and high based on the behavior seen in the separation phase. After classifying these dogs and relooking at the table phase, significant differences between SRB and non-SRB dogs may show.

4.5 Further recommendations

This study is being continued by a third research team suing the exact same protocol. At the end of the study, it would be interesting to combine the cortisol, heart rate and behavioral data of all dogs at the end to confirm these results and get a better understanding of SRB problems in dogs. Furthermore an analysis of the dog-owner relation during the reunion phase is currently under way. This study might provide further information about the dog-owner relationship in SRB dogs. Moreover, the behavior displayed by the owner towards the dogs during the table phase is interesting as well as we hypothesized that the owner may have a pronounced supporting role in SRB dogs during potentially 'stressful' situations. Such analyses have already been performed by Inge Hoogendam en Ellis ten Hove in previous studies and are currently underway also for the dogs in this study.

4.5.1 Heart Rate
As stated before the HRV parameter (RMSSD) was not significantly different during the table phase between SRB and non-SRB dogs. This could be due to low statistical power as reported by Hoogendam (2012). Hoogendam (2012) reported that to achieve 80% power, sample size should be n=29 in each group. Future studies should be conducted using this sample size in order to determine whether the RMSSD during the table phase is a useful parameter for indicating SRB problems in dogs.

4.5.2 Salivary Cortisol
Scientific evidence (Beerda et al. (1999)) suggests that within 80 minutes from a stressor the salivary cortisol values reach a baseline in dogs. Therefore it is possible that we measure a salivary cortisol value which does not only represent the stress response the dog experiences during the observation,

but also the potential stress response which may have been evoked in the dog in the hour prior to the observation.
The research team has no control on what happens to the dog in the hour prior to the observation.
Travel distance may influence salivary cortisol values. We should have corrected for travel distance in all dogs to check whether such effect exists. However if travel distance has an effect on salivary cortisol values we would expect to see no differences between salivary cortisol values from the table and separation samples in these dogs. We should therefore also have checked whether the salivary cortisol values in those dogs did not differ between the table and separation sample.

4.5.3 Developing a scale of SRB

As recommended by Inge Hoogendam it would be important to create a scale of SRB, based on the behaviors seen during the separation phase.[12]
The interpretation of the owner could be misleading in grouping the dogs in the non-SRB group.

5. Conclusion

During the table phase non-SRB dogs directed their head to the owners' body significantly longer compared to SRB dogs. This behaviour was negatively correlated with all door-directed behaviors displayed during the separation phase, which was found to be a clear indicator of SRB symptoms. During this phase, the frequency of yawning was also significantly higher in non-SRB dogs than in SRB dogs. No other behaviours displayed during the 'table phase' were significantly different, suggesting that SRB symptoms are not evident during this phase, however a greater sample size is needed to confirm this finding.

Second, the mean heart rate (mean HR) differed significantly in SRB dogs across the three phases and in particular between the table and the separation phase. In addition, the mean HR in the separation and the reunion phases in SRB dogs is significantly higher than in non-SRB dogs.
In SRB dogs, there was a positive correlation between both all door-directed behavior and all movement behavior and the mean HR during the separation phase. However in non-SRB dogs no significant correlation was found between all movement behavior and the mean HR during the separation phase. This suggests that the increase in mean HR in SRB dogs in the separation phase could be explained not only by an increase in physical activity, but maybe by the separation of the owner as well.
The HRV (RMSSD) in SRB and non-SRB dogs was not significantly different during the table phase, probably due to low statistical power.

Third, salivary cortisol values in SRB dogs differed across samples, in particular between the home and the separation sample. This was not found in non-SRB dogs. There were no significant differences between SRB and non-SRB dogs in salivary cortisol levels.

In summary, the behavior displayed during the table phase is probably not an indicator of SRB problems. On the contrary, dog behavior displayed during the separation phase seems to be a clear indicator of the severity of SRB problems further suggesting that SRB should be assessed on a gradual scale. The heart rate and cortisol data during the three phases suggest that SRB dogs experienced an acute stress response during the 5 minute separation from their owners. However, no differences between SRB and non-SRB dogs were found in these parameters. A greater sample size needs to be analyzed to confirm these results since statistical power was too low to detect significant differences in these parameters.

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7. APPENDIX

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7.1 APPENDIX 1: The Screening list (Dutch)

Checklist eigenaren met zowel SRB als non-SRB honden

Naam …… Tel ……

Naam hond …… Ras ……

Geslacht ☐ teef ☐ reu E-mailadres: ……

Vertellen aan eigenaren dat ze brief met speekselmonstername krijgen

Adres

Straat: ……huisnr: ……..

Postcode…….. Woonplaats ……

1. **Ouder dan een jaar?** ☐ Ja ☐ Nee
2. **Gezond?** ☐ Ja ☐ Nee

Zo nee, wat zijn de klachten? ……

3. **Medicijnen**? ☐ Ja ☐ Nee

Indien ja, wat voor medicijnen?……..

4. Waarom denkt u dat uw hond verlatingsangst heeft? Wat doet hij/zij dan precies?

- Blaffen/huilen/piepen ☐ Ja ☐ Nee
- Buren klagen ☐ Ja ☐ Nee
- Vernielzucht ☐ Ja ☐ Nee
- Poepen en plassen in huis ☐ Ja ☐ Nee
- Hyperactief ☐ Ja ☐ Nee
- Eten snel opeten als eigenaar thuiskomt ☐ Ja ☐ Nee
- Volgen door het huis ☐ Ja ☐ Nee
- Aandacht vragen ☐ Ja ☐ Nee
- Uitbundig begroeten (ook bij 5min weg?) ☐ Ja ☐ Nee

5. Wordt uw hond of is uw hond pas geleden behandeld door een gedragstherapeut voor 'verlatingsangst'?

☐ Ja ☐ Nee

Hoe lang? …… medicijnen? ……

6. **Heeft u hond andere gedragsproblemen, bijvoorbeeld 'agressie' of andere 'angstproblemen'?**

☐ Ja ☐ Nee

7. **Is uw hond ooit agressief geweest tegen een dierenarts?**

☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

8. **Welke dagen en tijden bent u beschikbaar om naar de faculteit Diergeneeskunde in Utrecht te komen voor ons onderzoek**?

7.2 APPENDIX 2: The Owner-questionnaire (Dutch)

AANVULLENDE VRAGENLIJST over uw hond.

Uw naam …… **Naam hond** ……

Tel …… **E-mail** ……..

Ras …….. **Geslacht** ☐ teef ☐ reu

Gecastreerd/gesteriliseerd ☐ Ja ☐ Nee **Geboortedatum hond** ……..☐ Onbekend

9. Laat u uw hond wel eens alleen thuis? ☐ Ja ☐ Nee

10. Hoe lang laat u gemiddeld uw hond alleen thuis per keer?

……

11. Waar is uw hond als u hem/haar alleen thuis laat?
☐ Binnen ☐ Buiten ☐ Anders, ……

Indien 'binnen', kunt u aangeven waar uw hond zich bevindt?
☐ Los rondlopend ☐ In een afgesloten ruimte, ……..☐ In een afgesloten bench
☐ In een kennel ☐ Anders, ……..

12. Volgt uw hond u door het huis wanneer u *op het punt staat weg te gaan*?
☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

13. Valt u nog andere zaken op aan het gedrag van uw hond als u weggaat?

……

14. Blaft, piept en/of huilt uw hond als hij/zij alleen thuis is?
☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

Hoe weet u dat? ……..

15. Klagen uw buren over het geluid dat uw hond maakt als u hem/haar alleen thuis laat?
☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

16. Vernielt uw hond in uw huis als hij/zij alleen thuis is?
☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

17. Is uw hond hyperactief als hij/zij alleen thuis is? (krabben aan deuren en ramen, rusteloos rondlopen, hijgen)
☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

Hoe weet u dat? ……..

18. Poept of plast uw hond in het huis als hij/zij alleen thuis wordt gelaten?
☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

19. Is uw hond moe/uitgeput wanneer u thuiskomt (na de begroeting)?
☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

20. Hoe begroet *u* de hond wanneer u thuiskomt?
Helemaal niet uitbundig ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 *Heel erg uitbundig*
☐ Onbekend

21. Hoe begroet *de hond* u wanneer u thuiskomt?

Helemaal niet uitbundig ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 *Heel erg uitbundig*

☐ Onbekend

22. Begroet *de hond* u ook zo als u maar heel even weg bent gegaan? (bijv. 5 min)

☐ Onbekend ☐ Ja ☐ Nee ☐ Anders, ……..

.

23. Eet uw hond wanneer hij/zij alleen thuis is? (bijv. voer, botten, beloningskoekjes etc.)

☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

24. Drinkt uw hond wanneer hij/zij alleen thuis is?

☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

25. Heeft uw hond een sterke band met 1 bepaald lid van uw huishouden?

☐ Ja (met wie?)…… ☐ Nee ☐ Anders, ……..

26. Heeft uw hond de neiging om u (of anderen uit uw huishouden) te volgen door het huis van kamer naar kamer?

☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

27. Heeft uw hond de neiging om dicht naast u (of anderen uit uw huishouden) te gaan zitten? ☐ Onbekend

☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

28. Heeft uw hond de neiging om aandacht te vragen als u zit, door een poot te geven of met haar/zijn neus tegen u aan te duwen?

☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

29. Wordt uw hond onrustig (blaft/jankt, springt op of probeert tussenbeide te komen) wanneer u (of anderen uit uw huishouden) aandacht geeft aan *andere personen*?

☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

30. Wordt uw hond onrustig (blaft/jankt, springt op of probeert tussenbeide te komen) wanneer u (of anderen uit uw huishouden) aandacht geeft aan *andere honden of dieren*?

☐ Onbekend ☐ Nooit ☐ Zelden ☐ Soms ☐ Vaak ☐ Altijd

31. Als u hond problemen heeft met niet alleen thuis kunnen zijn, wanneer is dit dan begonnen?

…… ☐ n.v.t.

32. Hebben er bepaalde gebeurtenissen (bijv. een verhuizing, een geboorte,uw hond naar een pension, het langdurig thuisblijven van de eigenaar) plaatsgevonden waarna uw hond problemen kreeg met alleen thuis blijven?

☐ Ja, specificeer …… ☐ Nee ☐ n.v.t.

Dit is het einde van deze vragenlijst. Hartelijk dank voor het invullen.

Indien we nog vragen hebben aan de hand van dit onderzoek, mogen wij dan contact met u opnemen?

☐ Ja ☐ Nee

7.3 APPENDIX 3 : Saliva sampling instructions (Dutch)

Hoe verzamelt u speeksel bij uw hond?

Lees voor te beginnen de instructies helemaal door. Zie ook de achterzijde voor de instructies met illustraties.

Benodigdheden:
- Schaar
- Pen of stift
- Bijgeleverde envelop met inhoud

In de bijgeleverde envelop zitten:
- 2 handschoenen
- 2 touwen (waarvan 1 reserve)
- 1 testbuis (testbuis bestaat uit 2 delen; een binnenste en buitenste buis. Deze buizen graag in elkaar laten zitten)
- 1 etiket

Voordat u gaat beginnen, laat uw hond wat lekkers ruiken, bv. hondenvoer of hondenkoekje (hij/zij mag dat niet opeten of eraan likken), zodat de speekselproductie op gang komt.

De touwen graag te allen tijde met de bijgeleverde handschoenen vastpakken
1. Trek de bijgeleverde handschoenen aan
2. Open het zakje en pak 1 van de 2 touwen aan een van de uiteindes vast.
3. Stop circa 5 cm van het andere uiteinde van het touw in de bek van uw hond
Let op: *Als uw hond op het touw gaat sabbelen/kauwen is dit prima. Als uw hond het touw wil uitspugen, houdt de snuit dan voorzichtig, maar wel stevig dicht.*
4. Houdt het touw 60 seconden in de bek van de hond. Tel hardop mee. Als de hond het touw uitspuugt, stop met tellen en stop het touw terug in de bek. Tel dan verder waar u bent gebleven. Om het speeksel op gang te krijgen, kunt u voer of een snoepje laten ruiken, niet eten.
5. Stop na de 60 seconden het deel van het touw dat in de bek van de hond heeft gezeten in het bovenste deel van de testbuis en knip het andere uiteinde van het touw af met een schaar.
6. Zorg ervoor dat u een schone schaar gebruikt. Maak de schaar eventueel schoon met water en zeep.

Let op: *Het buisje bestaat uit twee delen; een binnenste en een buitenste buis. Wanneer het touw in de buis wordt gestopt, moeten de twee buisjes in elkaar blijven zitten. Om de buisjes in elkaar te laten zitten, houdt u het buisje net onder het dopje vast, zodat de twee buizen in elkaar geklemd blijven. Haal het dopje hierna met een draaibeweging van het buisje af.*

7. Sluit de testbuis met het dopje
8. De handschoenen mogen nu uit
9. Schrijf de datum, naam van de hond en tijdstip afname met pen of stift op het etiket en plak op de testbuis (bv: naam hond, dag/maand/jaar, tijd h:mm)
10. Neem de testbuis mee naar de Faculteit Diergeneeskunde
Nadat de test is uitgevoerd mogen de handschoenen weggegooid worden. Het reserve touw gelieve mee terugnemen naar de Faculteit Diergeneeskunde.

Stap 1
Trek handschoenen aan

Stap 2
Pak het touw aan een van de uiteindes vast

Stap 3 + 4
Stop circa 5 cm van een van de uiteindes van het touw in de bek van de hond en houd het touw 60 sec in de bek

Stap 5
Stop na de 60 sec het deel van het touw dat in de bek van de hond heeft gezeten in de testbuis

Stap 6
Knip het uiteinde van het touw af

Stap 7
Sluit de testbuis met het dopje

Stap 8
Schrijf de datum en de naam van de hond op het bijgeleverde etiket en plak het op de testbuis

Stap 9
Neem de testbuis mee naar de Faculteit Diergeneeskunde

7.4 APPENDIX 4: Intra and inter observer reliability scores

Legend

L : Laura Reifler (observer current study) scored for the first time
I : Inge Hoogendam (observer study 1)
T : Laura Reifler (observer current study) scored for the second time
S : Sjoukje (observer Vet study)
B : Brigit (observer Vet study)
InterO1: the first inter observer reliability between two observers
InterO2: the second inter observer reliability between two observers

Head:

E : head directed to environment
C : head directed to camera
B : head directed to body owner
F : head directed to head owner
L : head low
H : head high
I : hiding head
S : head shake
U : head out of sight

Mouth:

P : panting
L : licking lips
Y : yawning
U : out of sight
X : nothing
Io : licking owner
Is : licking self
Fo: sniffing owner
Fs : sniffing self
Ft : sniffing table

Tail:

L : Tail low
L/W: Tail low and wagging
L (tot): Sum of L and L/W
W (tot): the total amount of wagging scored
T : Tail on table
B : Tail between legs
M : Tail middle
H : Tail high
U : Tail is out of sight

7.4.1 Head:

Date	Code	Dog	Inter observer reliability (head)				
		Nano1	**InterO1:0,97**		**Nano2**	**InterO2: 0,96**	**IntraO:0,99**
04/12	**E**	L29, I31	0,94	06/12	**E**	T28, I31	E: 0,9 A:0,97
	C	L24, I23	0,96		**C**	T24, I23	E:0,96 A:1
	I	L1, I1	1		**I**	T0, I1	E:0 A:0
	B	L10, I11	0,91		**B**	T11, I11	E:1 A:0,91
	S	L1, I1	1		**S**	T1, I1	E:1 A:1
	L	L4, I4	1		**L**	T4,I4	E:1 A:1
	U	L1, I1	1		**U**	T1, I1	E:1 A:1
		Loena1	**InterO1:0,78**		**Loena2**	**InterO2: 0,75**	**IntraO:0,96**
04/12	**E**	L 13,I16	0,81	06/12	**E**	T12, I16	E: 0,75 A:0,92
	C	L3,I8	0,38		**C**	T3, I8	E: 0,38 A:1
	B	L7,I7	1,0		**B**	T7, I7	E: 1 A:1
	L	L2,I1	0,5		**L**	T2, I1	E: 0,5 A:1
		Muftis1	**InterO1:0,98**		**Muftis2**	**InterO2:0,98**	**IntraO:1**
03/12	**E**	L14, I16	0,88	06/12	**E**	T13,I16	E:0,81 A:0,93
	C	L12, I13	0,92		**C**	T12, I13	E:0,92 A:1
	B	L8, I6	0,75		**B**	T9, I6	E:0,67 A:0,89
	F	L9, I8	0,89		**F**	T8, I8	E:1 A:0,89
	L	L0, I1	0,5		**L**	L1, I1	E:1 A:0
		Boomer1	**InterO1:0,85**		**Boomer2**	**InterO2:0,81**	**IntraO:0,96**
03/12	**E**	L19, I16	0,84	06/12	**E**	T19, I16	E: 0,84 A:1
	C	L13, I11	0,85		**C**	T14, I11	E:0,79 A:0,93
	B	L8, I9	0,89		**B**	T8, I9	E: 0,89 A:1
	F	L2, I0	0		**F**	T3, I0	E:0 A:0,67
	I	L3, I2	0,67		**I**	T3, I2	E:0,67 A:1
	H	L0, I1	0		**H**	T0, I1	E:0 A:1
	L	L1, I0	0		**L**	T1, I0	E:0 A:1
		Dexter1	**InterO1:0,91**		**Dexter2**	**InterO2:0,89**	**IntraO:0,97**
29/11	**E**	L16, I17	0,94	06/12	**E**	T15, I17	E:0,88 A:0,94
	C	L14, I16	0,88		**C**	T14, I16	E:0,88 A:1
	L	L2, I2	1		**L**	T2, I2	E:1 A:1

Tommie1		**InterO1:0,96**		**Tommie2**		**InterO2:0,89**	**IntraO:0,85**	
29/11	**E**	L12, I11	0,92	06/12	**E**	T11, I11	E:1	A:0,92
	C	L8, I8	1		**C**	T8, I8	E:1	A:1
	B	L5, I4	0,8		**B**	T3, I4	E:0,75	A:0,6
	L	L1, I2	0,5		**L**	T0, I2	E:0	A:0
	F	L1, I1	1		**F**	T1, I1	E:1	A:1

Total inter and intra observer reliabilities of the head behaviors:

InterO1 : total I (Inge)/ total L (Laura1) : 243/248 = **0,98**

InterO2 : total I (Inge)/ total T (Laura2) : 238/248 = **0,96**

IntraO : total T (Laura2)/ total L (Laura 1) : 238/243 = **0,98**

7.4.2 Mouth:

Date	Code	Dog	Inter observer reliability (mouth)				
		Baika1	**Inter01:0,93**		**Baika2**	**InterO2:0,91**	**IntraO: 0,98**
26/11	**P**	L17, I17	1	05/12	**P**	T17, I17	E:1 A:1
	L	L37, I34	0,92		**L**	T38, I34	E:0,89 A:0,97
	U	L1, I0	0		**U**	T1, I0	E:0 A:1
		Laila1	**InterO1:,86**		**Laila2**	**InterO2:0,98**	**IntraO: 0,87**
29/11	**P**	L18, I17	0,94	05/12	**P**	T17, I17	E:1 A:0,94
	L	L37, I33	0,89		**L**	T30, I33	E: 0,91 A: 0,81
	Y	L2, I2	1		**Y**	T2, I2	E: 1 A: 1
	IO	L2, I1	0,5		**IO**	T2, I1	E: 0,5 A: 1
	FO	L4, I2	0,5		**FO**	T4, I2	E: 0,5 A: 1
	U	L6, I5	0,83		**U**	T5, I5	E:1 A: 0,83
	X	L1, I0	0		**X**	T1, I0	E:0 A: 1
		Arrow1	**InterO1:0,93**		**Arrow2**	**InterO2: 1**	**IntraO: 0,93**
29/11	**P**	L12, I11	0,92	05/12	**P**	T11, I11	E:1 A:0,92
	L	L13, I13	1		**L**	T12, I13	E:0,92 A: 0,92
	Y	L2, I2	1		**Y**	T2, I2	E:1 A:1
	U	L1, I1	1		**U**	T1, I1	E:1 A:1
	X	L1, I0	0		**X**	T1, I0	E:0 A:1
		Sjef1	**InterO1:0,94**		**Sjef2**	**InterO2:0,94**	**IntraO:1**
29/11	**P**	L17, I18	0,94	05/12	**P**	T17, I18	E:0,94 A:1
	L	L43, I45	0,96		**L**	T43, I45	E:0,96 A:1
	U	L3, I3	1		**U**	T3, I3	E:1 A:1
	X	L0, I1	0		**X**	T0, I1	E:0 A:1
		Harrie 1	**InterO1:0,98**		**Harrie2**	**InterO2:0,98**	**IntraO:1**
28/11	**L**	L38, I39	0,97 `	05/12	**L**	T38, I39	E:0,97 A:1
	IS	L3, I3	1		**IS**	T3, I3	E:1 A:1
	IO	L1, I1	1		**IO**	T1, I1	E:1 A:1
	FT	L1, I1	1		**FT**	T1, I1	E:1 A:1

Total inter and intra observer reliabilities of the mouth behaviors:

InterO1 : total I(Inge)/ total L (Laura1) : 249/260 = **0,96**

InterO2 : total I (Inge) / total T (Laura2) : 249/250 = **0,99**

IntraO : total T (laura2) / total L (Laura1) : 250/260 = **0,96**

7.4.3 Tail:

Date	Code	Dog	Inter observer reliability (tail)			
		Zappa	**InterO1: 0,94**	**Zappa**		**InterO2: 0,93**
	L	L6, S9	0,67	**L**	L6, B9	0,67
	L/W	L10, S9	0,9	**L/W**	L10, B10	1
	L (tot)	L16, S18	0,89	**L (tot)**	L16, B19	0,84
	T	L3, S3	1	**T**	L3, B3	1
	U	L6, S6	1	**U**	L6, B4	0,67
	W (tot)	L10, S9	0,9	**W (tot)**	L10, B10	1
		Ricky	**InterO1: 0,75**	**Ricky**		**InterO2: 0,75**
	L	L2, S1	0,5	**L**	L2, B1	0,5
	U	L2, S2	1	**U**	L2, B2	1
		Tigo	**InterO1: 0,86**	**Tigo**		**InterO2: 0,86**
	L	L3, S4	0,75	**L**	L3, B4	0,75
	L/W	L1, S1	1	**L/W**	L1, B1	1
	B	L1, S1	1	**B**	L1, B1	1
	W (tot)	L1, S1	1	**W (tot)**	L1, B1	1
		Catootje	**InterO1: 1**	**Catootje**		**InterO2: 1**
	L	L1, S1	1	**L**	L1, B1	1
		Spot	**InterO1: 1**	**Spot**		**InterO2: 1**
	L	L2, S2	1	**L**	L2, B2	1
	M	L1, S1	1	**M**	L1, B1	1
	H	L2, S2	1	**H**	L2, B2	1

Total inter and intra observer reliabilities of the tail behaviors:

Total InterO1: total L (Laura)/ total S (Sjoukje) : 51/52 = **0,98**

Total InterO2: total L (Laura)/ total B (Brigit) : 51/52 = **0,98**

7.5 APPENDIX 5: Ethograms

The ethograms of both the table and the separation phases are similar to those used in study 1. However, in this study certain behaviors were added, changed or deleted in some categories to optimize the table phase ethogram. Adjustments in the separation phase ethogram are reported by Gijsbertsen (2013).
In the category 'Head orientation' in the table phase ethogram the behavior 'Glance camera' was added. In the category 'Mouth' the behavior 'Vocalization' and the 'Vocalization modifiers' were added. In the 'Tail position' category 'Low', 'Middle', 'High' and 'Tucked tail' were added.
Every behavior and modifier of each category was abbreviated to a unique letter and in the 'scored as' columns 'frequency' was abbreviated as 'F' and 'duration' as 'D'. [12]

General scoring rules[12]
In this study the same scoring rules were used as in study 1, although some rules were optimized for the differences in the table phase ethogram. General scoring rules were used when scoring each behavioral category.
'Out of sight' was scored if the dog or the mouth or tail of the dog was out of sight for at least 2 seconds. Only in the 'head orientation' category it is not possible to score 'Out of sight'.
'Nothing' was defined by Hoogendam (2012) as 'the dog exhibits none of the behaviors listed'.[12] This behavior was especially used to mark the stop of a duration behavior when it was not followed by another listed behavior.[12]
The way behaviors were scored could be divided in events (frequency) or states (duration). State behaviors were not scored if they lasted for only one second. Behaviors that were scored as states (duration) were scored as events (frequency) as well.

Head orientation[12]
The head orientation towards the camera, owner (body and/or face) and environment were based on the gaze direction and the nose direction of the dogs. As is most dogs the eyes were difficult to see, the nose direction was mostly used. However when the eyes were visible, the gaze direction generally took precedence over the nose direction.
'Head high' and 'head low' were based on the direction of the dogs' head, neck and part of the upper body. For example 'head low' was scored when a dog had its nose on the table or attended to do so (for example seen in long leg breeds).
'Hiding head' was scored only if the dog puts its head voluntarily in the arms or coat of the owner. This behavior was not scored if the owner forced its arm(s) around the dogs' head.
All the head orientation behaviors were scored as durations, except 'Glance camera' and 'Head jerk' which were scored as a frequency. As reported by Hoogendam (2012) the behaviors were scored only if they were undoubtedly visible and not part of a continuous movement. For example, if the dog moves its head from left to right and thereby passes the camera, this was not scored as 'Head to camera' as the movement did not stop at the moment the dog looked in the camera.

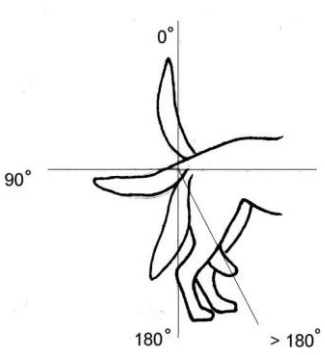
Mouth behaviors[12]
All mouth behaviors were scored as frequencies, except for 'Panting', 'Licking' and 'Sniffing' which were scored as durations. Panting was scored by looking at the tongue and the mouth. If the dogs' mouth was out of sight but clear inhalations and exhalations were still seen on the thorax of the dog, panting was scored as well.
As smacking was frequently seen in a sequence of licking lips, 'Smacking' was only scored if there were no licking lips (or any other mouth behaviors) within two seconds before and after the smacking occurred. These rules were made because smacking quickly before or after licking lips has no interesting value, whereas smacking on its own has.

'Licking' and 'Sniffing' were scored in combination with a modifier to distinguish what the dog was licking or sniffing. 'Vocalizations' were also scored in combination with a modifier to distinguish which vocalization the dog made.

Tail position[12]

All tail position behaviors were scored as durations. 'Between legs' was scored if the tip of the dogs' tail was visible cranial from the hind legs (>180°). 'Tucked tail' was scored if most of the tail (from the basis) was tucked to the dogs' body. The tip of the tail is in a horizontal line (180°) or is in a position a little smaller than 180°. 'Tucked tail' was added as the distinction between 'Tail low' and 'Between legs' was sometimes difficult to make. In addition some dogs may physically not be able to tuck the tail completely between the legs. Therefore some tail positions could be falsely scored as 'Tail low', which has an effect on the interpretation of the tail position.

Fig.9: Illustration of the scoring rules used for the tail position[13]



In figure 9 the scoring rules for the tail position are illustrated. 'Tail low' is scored if the dogs' tail is into a position of approximately 90 till 180°, except if most of the dogs' tail is tucked to the body. 'Tail middle' is scored if the dogs' tail is into a position of approximately 90°. If the dogs' tail is into a position of approximately 0 till 90°, 'Tail high' is scored.

'Wagging' was only scored when the tail moved clearly repetitively from side to side and was scored as a modifier. If there was no special behavior to score, but the dog was wagging its tail, then 'Nothing' was scored with the modifier 'Wagging'.

7.5.1 Table phase ethogram

General scoring rules	**Code**	**Score as:**	**Definition**
Out of sight	U	D	Out of sight for at least 2 seconds
Nothing	X	D	The dog exhibits none of the behaviors listed
Head orientation	**Code**	**Score as:**	**Definition**
Head directed to owner body	B	D	The dog's head is orientated to any part of the owner's body (except the face)
Head directed to camera/observer	C	D	The dog's head is directed to the camera for at least 2 seconds
*Glance to camera**	Gc	F	The dog glances in the camera for less than 1 second
Head directed to environment	E	D	The dog's head is directed to the environment, the dog's attention is directed to something in the consulting room, without the owner or camera/observer
Head directed to face owner	F	D	The dog's head is directed to the owner's face; based on the direction of the nose (kop-as)
Head high	H	D	The dog's head is high and directed to the ceiling; direction of nose 45° and less
Hiding head	I	D	The dog is voluntarily hiding its head in the owners coat or

			arms and the head is (partly) out of sight
Head jerk	J	F	The dog jerks its head back in a swift, powerful motion
Head low	L	D	The dog's head and part of the body is low and directed to the table or sniffing its legs; head and neck are 135° and more
Head shake	S	D	The dog shakes his head from side to side; also as part of a body shake
Mouth orientation	**Code**	**Score as:**	**Definition**
Licking lips	L	F	Every time the dog extrudes its tongue from its mouth and runs it over its lips, with or without smacking; when the tongue is visible
Panting	P	D	An increased inhalation and exhalation in combination with the opening of the mouth, also scored if out of sight but the thorax movements are seen
Smacking	M	F	The dog presses its lips together and then opens its mouth quickly and noisily, without licking lips; 2 seconds before and after no other mouth behaviors are seen
Yawning	Y	F	The dog opens its mouth wide, gaping
Licking	I	D	The dog licks the table, itself or the owner with its tongue
Sniffing	F	D	The dog moves its nose along objects, such as the table, its own body or the owner; clear sniffing movement are exhibited
*Vocalizations**	V	F	The dog produces sounds such as barking, whining, yelping, growling or grunting
Bare teeth	B	F	The dog pulls its upper lip upwards and lower lips downwards, revealing its teeth
Sneezing	Z	F	The dog expels air forcibly from the mouth and nose
Vocalization modifiers			
*Barking**	b		
*Whining**	w		
*yelping**	y		
*growling**	g		
*grunting**	r		
Mouth modifiers			
Table	t		
Self	s		
Air	a		
Owner	o		
Tail position	**Code**	**Score as:**	**Definition**
Between legs	B	D	The dog's tail is between the legs completely, where the tip of the tail is >180°
*Tail low**	L	D	The dog's tail is into a position of approximately 90° till <180°
*Tail middle**	M	D	The dog's tail is into a position of approximately 90°

*Tail high**	H	D	The dog's tail is into a position of approximately 0° till 90°
*Tucked tail**	D	D	Most of the dog's tail (from the basis) is tucked to the body. The tip of the tail may be in a horizontal line (180°) or be in a position of approximately 175° till 180°.
Tail on table	T	D	The dog's tail is lying on the table, when the dog is sitting or lying on the table
Tail modifiers			
Wagging	w		Repetitive side to side movements of the tail; if present specified as modifier

** = Behaviors which were added or changed in the table phase ethogram of this study compared to the ethogram of study 1.*

7.5.2 Separation phase ethogram

General scoring rules	Code	Score as:	Definition
Out of sight	U	D	Out of sight for at least 2 seconds
Nothing	X	D	The dog exhibits none of the behaviors listed
Movement			
Behaviors of 'still'	**Code**	**Score as:**	**Definition**
Standing	S	D	The dog is standing on 4 paws
Sitting	I	D	The dog is sitting with front legs extended and hind legs flexed
Lying	L	D	The dog is lying; its head may or may not be in contact with the ground/carpet
Modifiers of 'still'			
door	d		< 1 body length
on mat	m		< 1 body length
table	t		< 1 body length
on table	ot		< 1 body length
water	w		< 1 body length
Behaviors of 'moving'	**Code**	**Score as:**	**Definition**
Walking	W	D	The dog moves around in the room with a direction towards something
Circling	C	D	The dog turns on its axis, the loop should be closed and within 3 seconds the dog should be completely on its axis
Scratching	R	D	The dog raises both front paws and moves them in a quick, powerful motion with the nails on the surface
Climbing	B	D	The dog raises both front paws and puts them on the door. Its hind legs are kept on the ground
Digging	D	D	The dog moves its front legs quickly in a powerful motion without the nails on the surface. The dog does not raise its front legs
Chasing tail	H	D	The dog's head is orientated to its tail and the dog tries to catch the tail with its mouth while moving in circles
Jumping	J	F	The dog lifts its legs from the ground in a fast powerful motion
Small jump	SJ	F	The dog lifts its front legs from the ground in the air. The hind legs are kept on the ground
Moving	M	D	The dog shows short, non directional, movements, more or less on the same spot, within 5 seconds
Approach	A	D	All small movements within 2 body lengths towards the door or mat. Approach takes precedence over leave
Leave	E	D	All small movements within 2 body lengths away from the door or mat. Approach takes precedence over leave
Body shake	BS	F	The dog moves its whole body from side to side with short, quick movements
Modifiers of 'moving'			
(towards) door	d		
mat	m		

table	t
water	w
chair	c
owner stuff (i.e belt, bag, jacket etc)	o

Mouth

Behavior of 'mouth'	**Code**	**Score as:**	**Definition**
Licking lips	LL	F	Every time the dog extrudes its tongue from its mouth and runs it over its lips, with or without smacking; when the tongue is visible
Panting	P	D	An increased inhalation and exhalation in combination with the opening of the mouth, also scored if out of sight but the thorax movements are seen
Smacking	MK	F	The dog presses its lips together and then opens its mouth quickly and noisily, without licking lips; 2 seconds before and after no other mouth behaviors are seen
Yawning	Y	F	The dog opens its mouth wide, gaping
Licking	LK	D	The dog licks the table, itself or the owner with its tongue
Sniffing	F	D	The dog moves its nose along objects, such as the table, its own body or the owner; clear sniffing movement are exhibited
Sneezing	Z	F	The Dog expels air forcibly from the mouth and nose
Biting	T	F	The dog put his teeth in an object

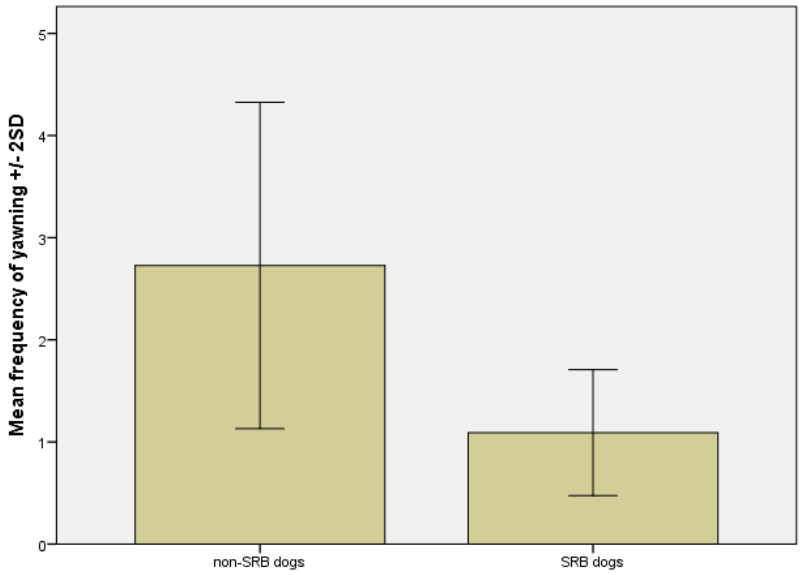
Modifiers of 'mouth'

Door	d	< 1 body length
Mat	m	< 1 body length
Table	t	< 1 body length
Self	s	< 1 body length
Air	a	< 1 body length
Chair	c	< 1 body length
Owner stuff (belt, bag, jacket etc)	o	< 1 body length

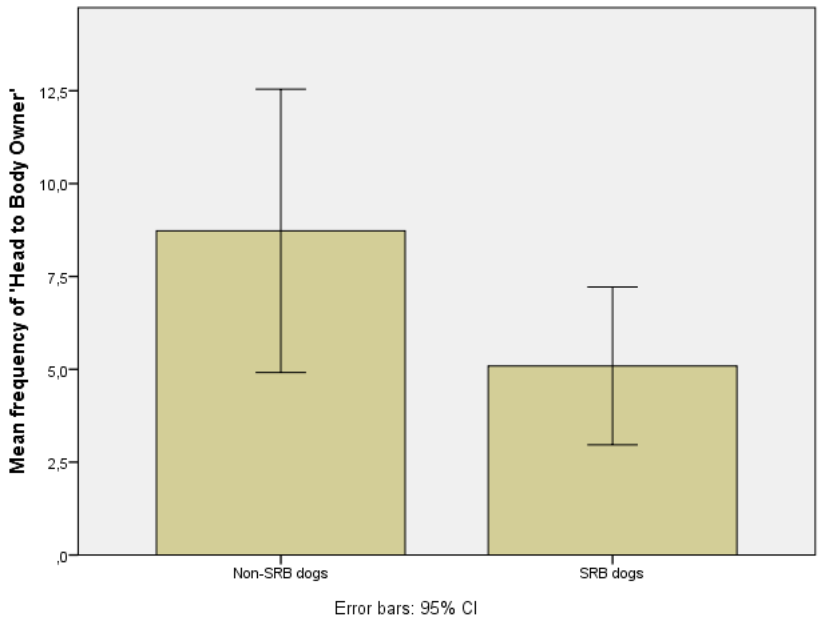
Vocalizations	**Code**	**Score as:**	**Definition**
Barking	BK	F	Low frequency vocalizations
Whining	JW	F	Soft, high pitched vocalizations with raised frequency
Howling	HW	F	Loud, high pitched vocalizations

7.6 APPENDIX 6: Table phase

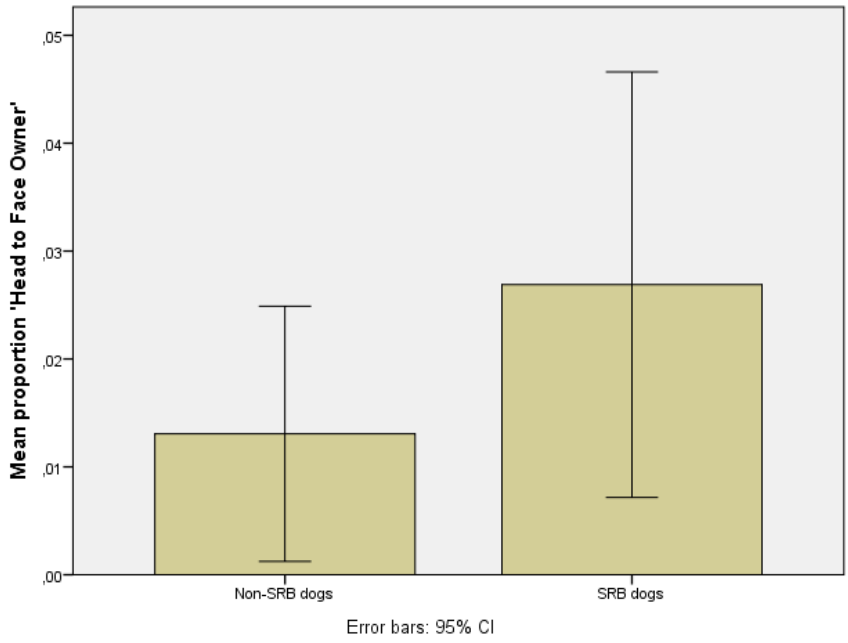
7.6.1 Fig.10: Yawning (frequency) displayed by SRB (Mdn=0.0033) and non-SRB (Mdn=0.0067) dogs during the table phase (U=63.00, z=-2.27, n=33, p=0.026, r=-0.40)



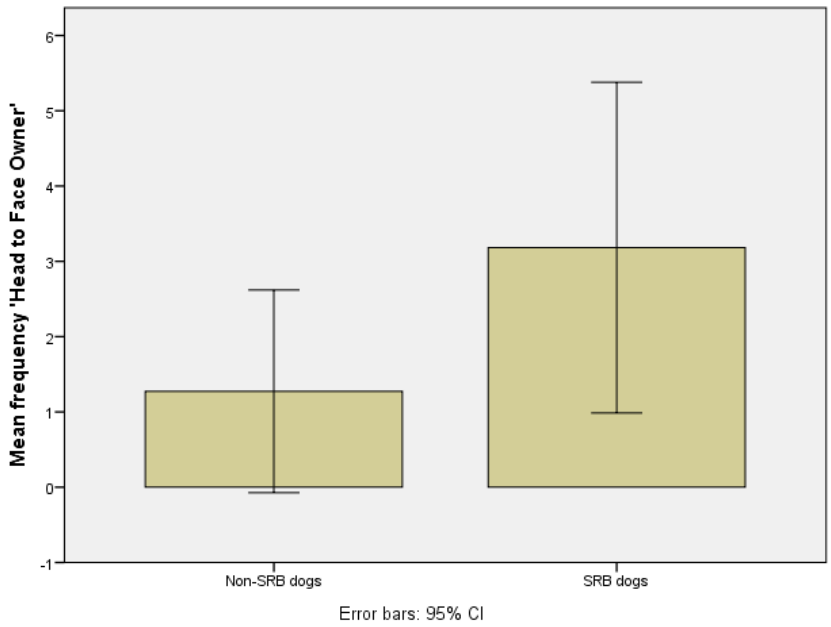
7.6.2 Fig.11: Head to Body Owner (frequency) displayed by SRB (Mdn=0.013) and non-SRB (Mdn=0.023) dogs during the table phase (U= 75.50, z=-1.746, n=33, p=0.08, r=-0.30)



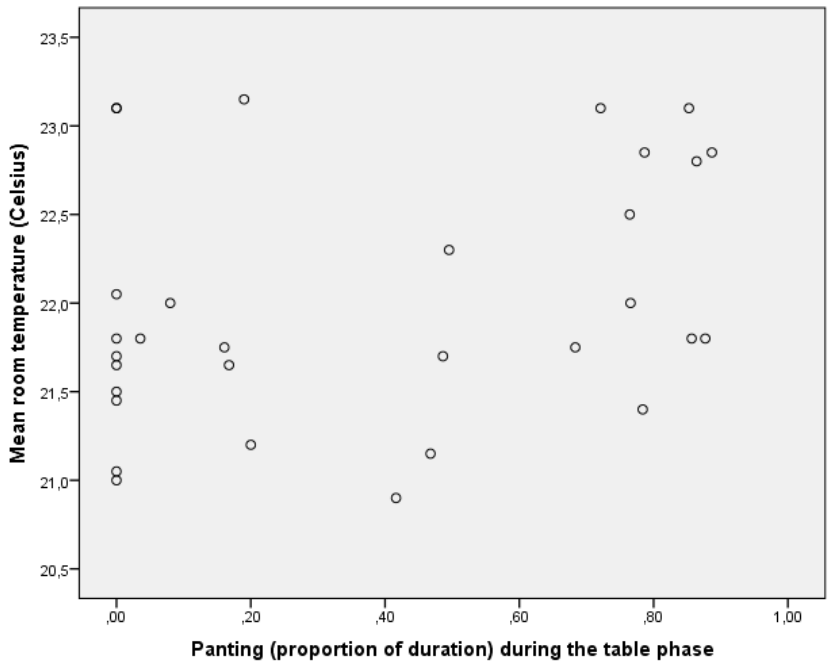
7.6.3 Fig 12: Head to Face Owner (duration) displayed by SRB (Mdn=0.0115) and non-SRB (Mdn=0.0077) dogs during the table phase (U=103.50, z=-0.681, n=33, *ns*, r=-0.12)



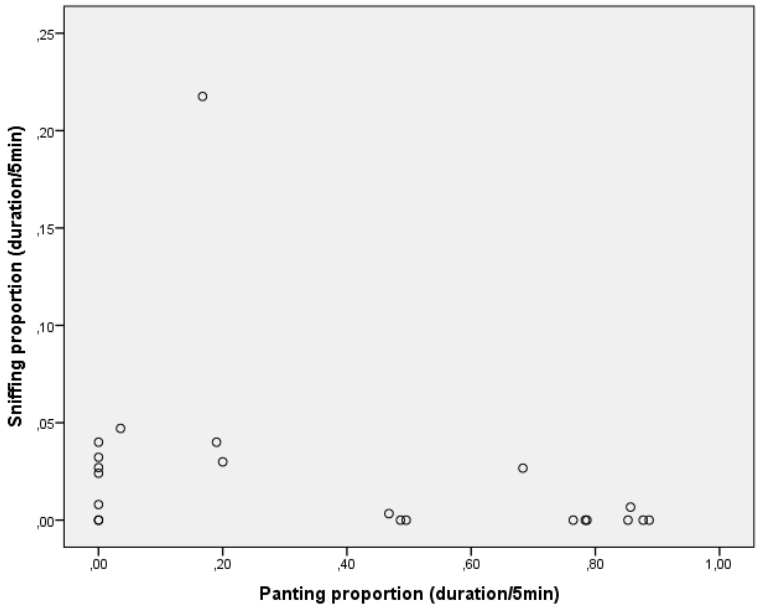
7.6.4 Fig 13: Head to Face Owner (frequency) displayed by SRB (Mdn=0.0067) and non-SRB (Mdn=0.0033) dogs during the table phase (U=84.0, z=-1.451, n=33, *ns*, r=-0.25)



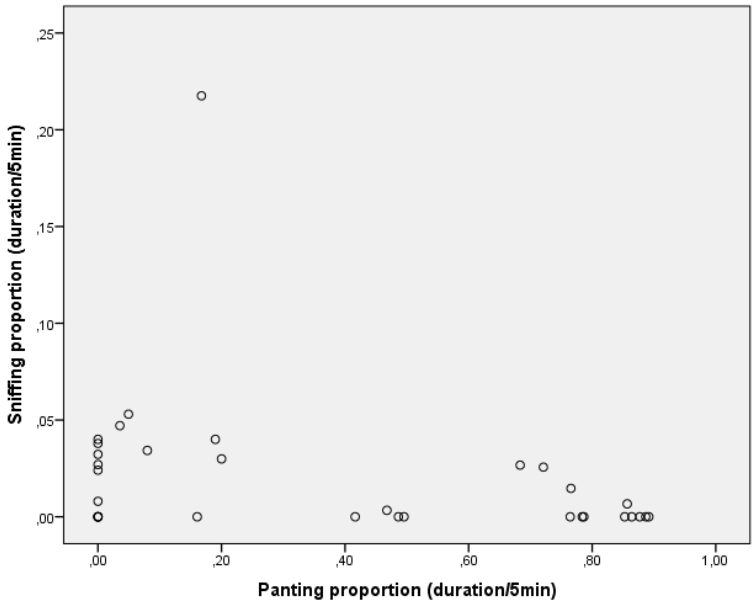
7.6.5 Fig.14: Mean room temperature (in Celsius) versus Panting (proportion of duration) during the table phase (r_s= 0.312, n=33, p=0.087)



7.6.6 Fig.15: Panting (proportion of duration) versus Sniffing (proportion of duration) during the table phase in SRB dogs (r_s=-0.517, n=22, p=0.014)

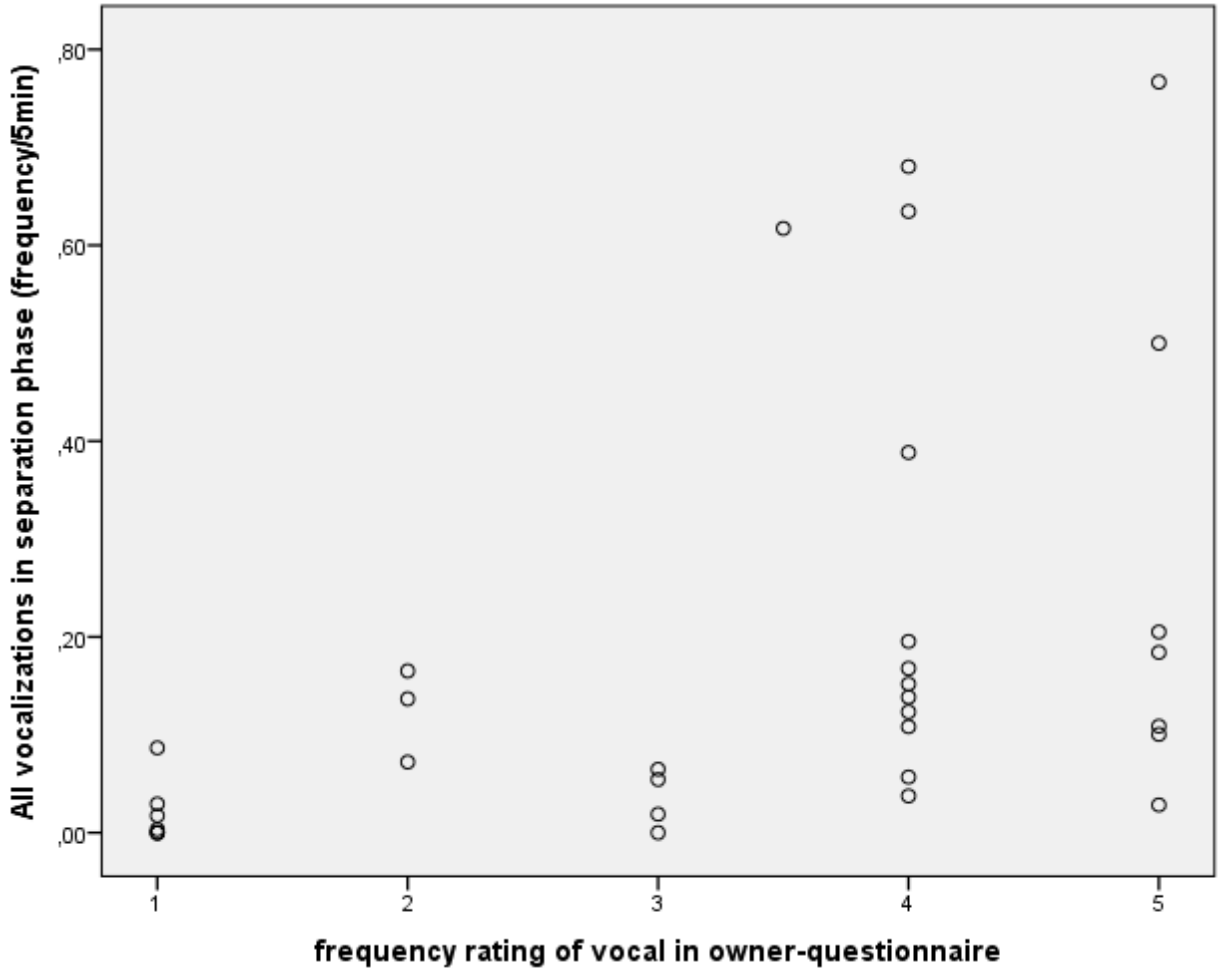


7.6.7 Fig.16: Panting (proportion of duration) versus Sniffing (proportion of duration) during the table phase in all dogs (r_s= -0.407, n=33, p=0.019)



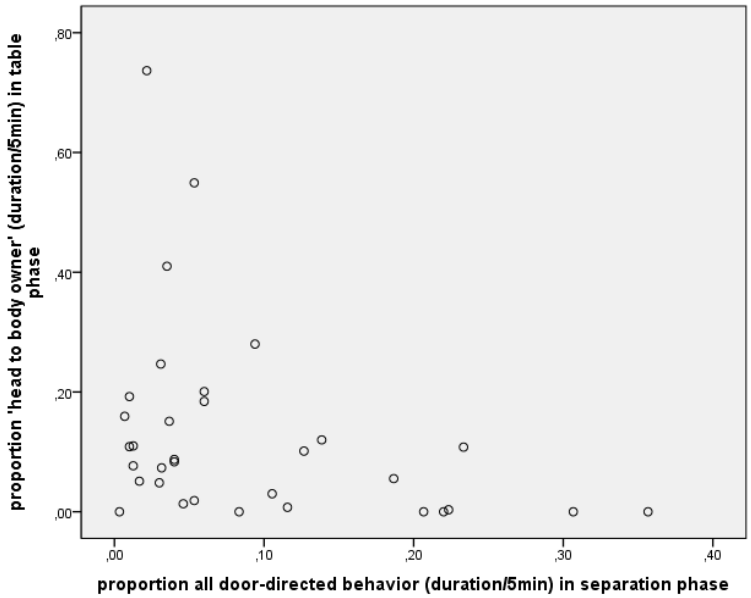
7.7 APPENDIX 7: Separation phase

7.7.1 Fig.17: All vocalizations in separation phase (frequency) versus frequency rating (1-5; never-always) of vocal in owner-questionnaire (r_s=0.612, n=33, p=0.000)

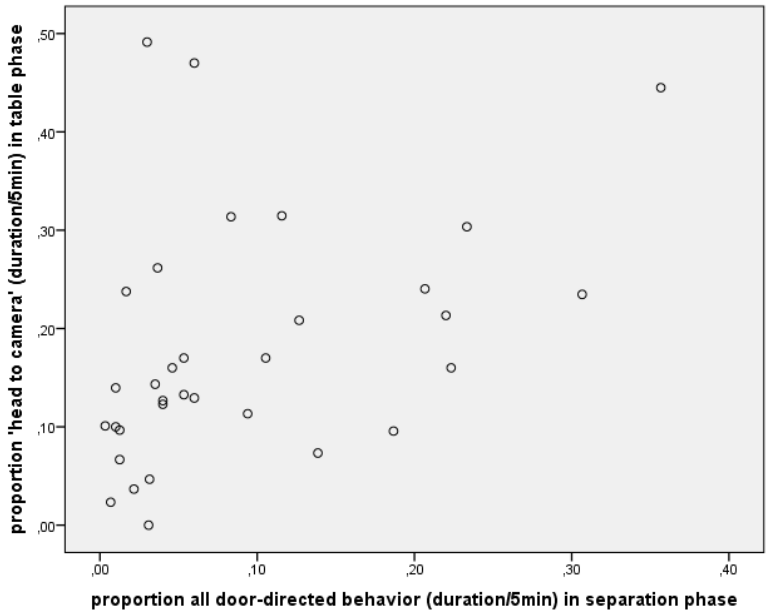


7.8 APPENDIX 8: Comparison table and separation phase

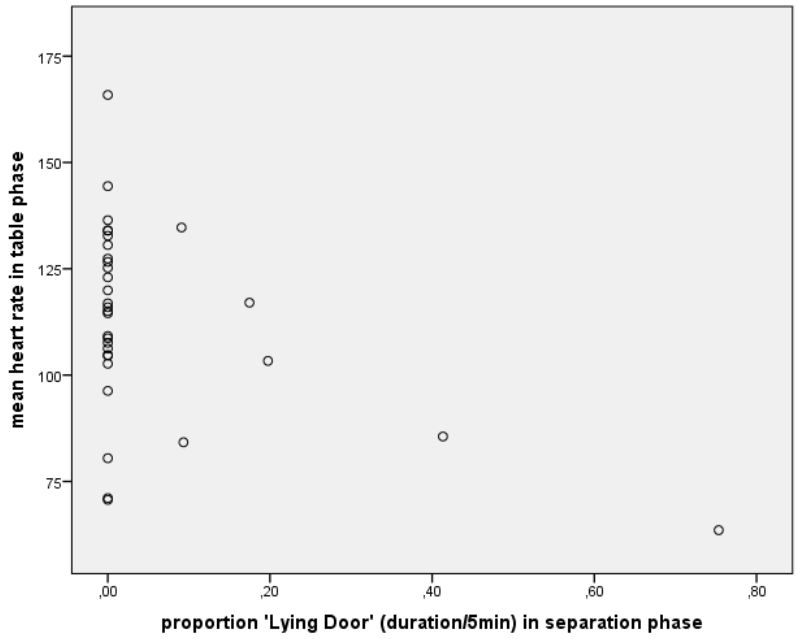
7.8.1 Fig.18: All door-directed behavior (duration) in the separation phase versus 'head to body owner' in the table phase (r_s=-0.556, n=22, p=0.007)



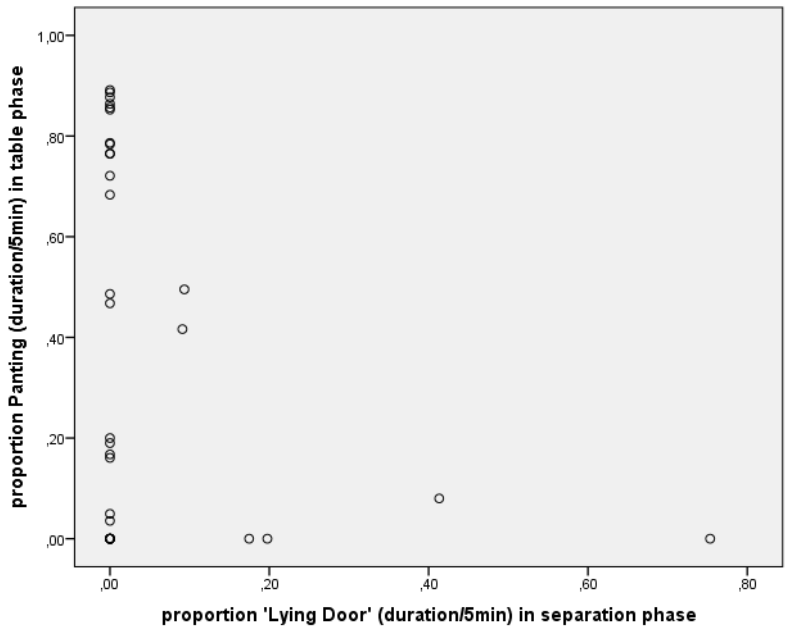
7.8.2 Fig.19: All door-directed behavior (duration) in the separation phase versus 'head to camera' in the table phase (r_s=0.458, n=22, p=0.032)



7.8.3 Fig.20: 'Lying Door' (duration) in the separation phase versus the mean heart rate in the table phase (r_s=-0.292, n=33, p=0.099)

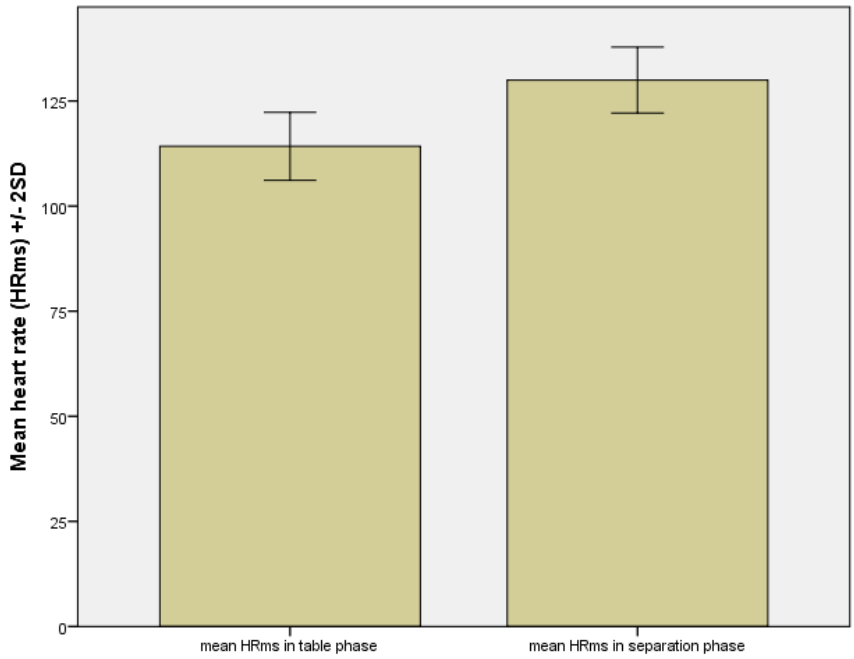


7.8.4 Fig.21: 'Lying Door' (duration) in the separation phase versus Panting (duration) in the table phase (r_s=-0.292, n=33, p=0.099)

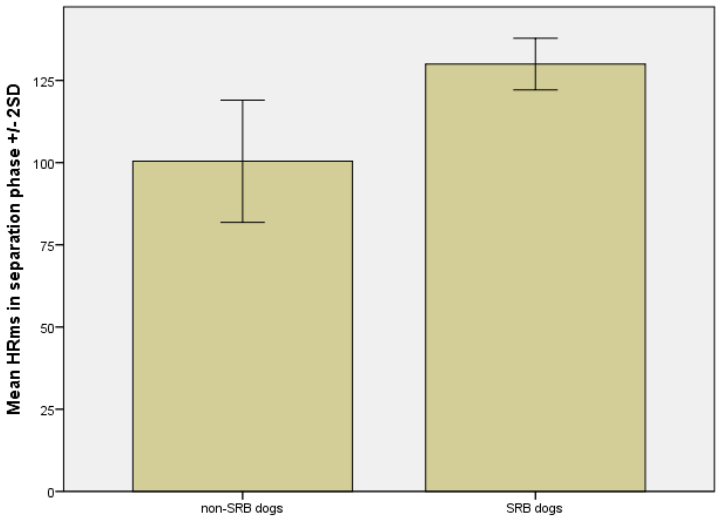


7.9 APPENDIX 9: Heart Rate

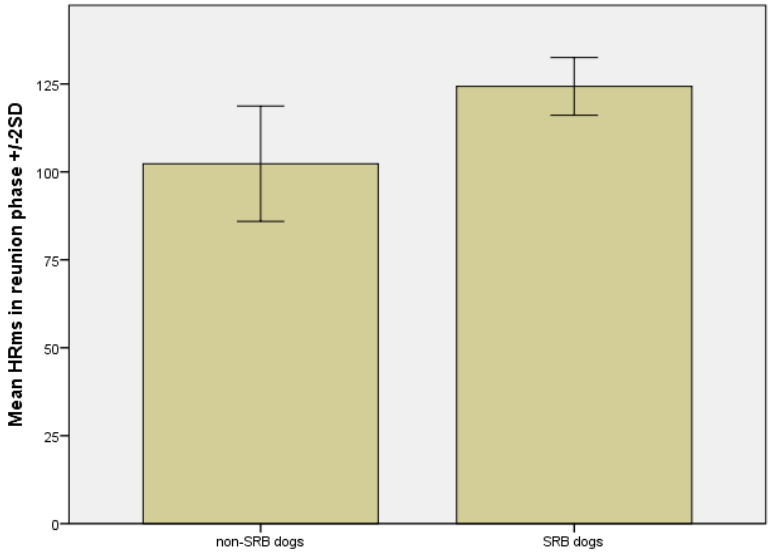
7.9.1 Fig.22: The mean heart rate in the table phase (Mdn=115.73) versus the separation phase (Mdn=133.69) in SRB dogs (Z= -2.841, n=22, p=0.005, r=-0.61)



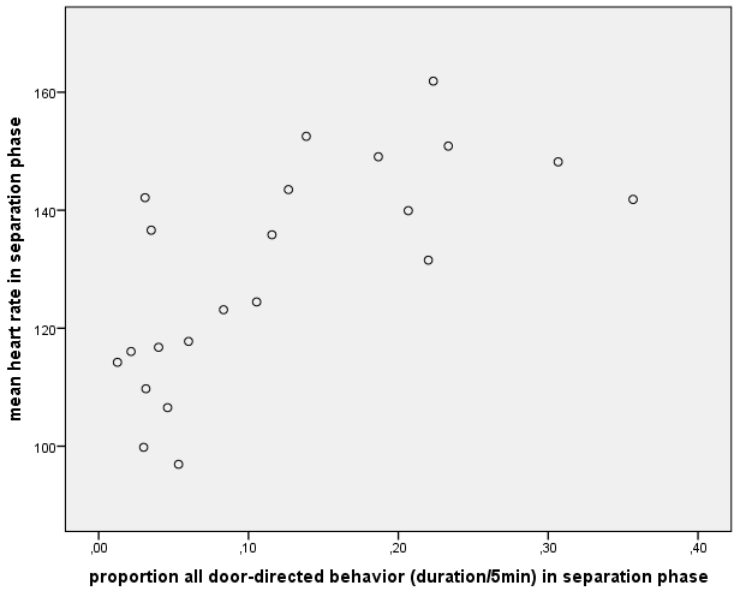
7.9.2 Fig.23: The mean heart rate in the separation phase in SRB (Mdn=133.69) and non-SRB (Mdn=105.21) dogs (U=47.000, z=-2.83, n=33, p=0.004, r=-0.49)



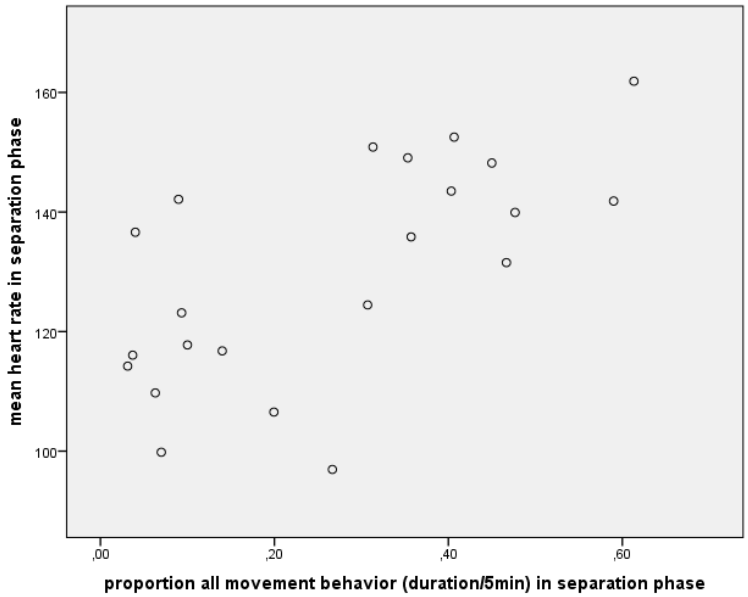
7.9.3 Fig.24: The mean heart rate in the reunion phase in SRB (Mdn=121.81) and non-SRB (Mdn=106.09) dogs (U=60.000, z=-2.33, n=33, p=0.019 r=-0.41)



7.9.4 Fig.25: All door-directed behavior (duration) in the separation phase versus the mean heart rate in the separation phase in SRB dogs (r_s=0.713, n=22, p=0.000)

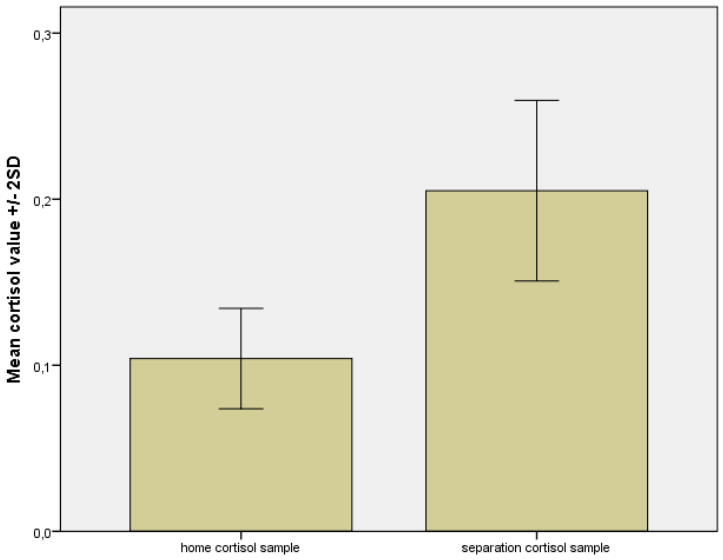


7.9.5 Fig.26: All movement behavior (duration) in the separation phase versus the mean heart rate in the separation phase in SRB dogs (r_s=0.640, n=22, p=0.001)



7.10 APPENDIX 10: Cortisol

7.10.1 Fig.27: The home cortisol values (Mdn=0.10) versus the separation cortisol values (Mdn=0.19) in SRB dogs (Z= -2.689, n=16, p=0.007, r= -0.67)



7.10.2 Fig.28: All door-directed behavior (duration) in the separation phase versus the separation cortisol value in SRB dogs (r_s= -0.461, n=18, p=0.054)