

Assessment of stress in laboratory Beagle dogs constrained by a Pavlov sling

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Abstract

The 3R-principle - Replacement, Reduction and Refinement - gets increasingly important for designing animal experiments. The Pavlov sling is thought to be a non-invasive method to restrain dogs for examinations. The aim of our study was to investigate whether laboratory Beagle dogs, which had been trained to tolerate the fixation by a Pavlov sling, are stressed by this procedure and furthermore to analyse their behaviour during this period. Five male and five female Beagle dogs at an age of three years were used. Animals were restrained in the Pavlov sling for 30 min on six days with an interval of at least two days. Following behaviours were recorded every minute for each session: postures of body, head and ears as well as state of eyes, tail, legs, and mouth. Additionally, the animals were observed for the occurrence of particular stress signs like body shaking, sweating of the paws, increased saliva production, piloerection, blinking of eyes, snout licking, yawning, and panting. As an indicator for stress, salivary cortisol levels were measured before, during and after each session. Our results show that most behavioural parameters like body, leg, head, tail, and ear posture, the frequency of changes between different behaviour patterns, as well as cortisol concentration have not been influenced by restraining in the Pavlov sling. Therefore, the Pavlov sling does not seem to be perceived as a stressful situation by the Beagle dogs. Our study demonstrates that under certain conditions the use of the Pavlov sling in trained dogs can substitute more ordinary methods of immobilisation, e.g. the use of narcotics.

Keywords

Pavlov sling; laboratory dogs; stress assessment; refinement

Introduction

A total of 3,832 dogs were used as laboratory animals in Germany in 2009 (BMELV, 2011), mostly for the development of new products or instruments used in human medicine or dentistry, for toxicological studies, and for fundamental biological research (BMELV, 2011; Ritskes-Hoitinga et al., 2006). Many of the respective experiments include direct measurements in the conscious dog, such as taking blood samples, intravenous dripping, imaging or measuring other physiological parameters like heart frequency or body temperature. Especially for imaging techniques it is very important to restrain the animal in its movements, sometimes over a longer period. Despite many efforts to find alternatives for animal models in science, it is unrealistic to refrain completely from laboratory animals in the near future. Therefore, the 3R-principle - Replacement, Reduction and Refinement - (Russel and Burch, 1959) is still valid and the third R gets more and more important.

If replacement or reduction is not possible, refinement is of utmost importance. Refinement should minimize pain or stress of a laboratory animal in the experiment and during housing. Over the last years a lot of work has been done to improve the experimental conditions as well as to enrich the environment not only for the benefit of the animal but also for the validity and reliability of the experiment (European Convention 1986; Hubrecht, 2002; TierSchHuV, 2001; Vollzugshinweise zur Tierschutz- Hundeverordnung, 2003).

One important issue in refinement is the reduction of stress during the experiment and it can be very useful to develop training programs for habituating dogs (as well as other laboratory animals) to the experimental situation (Adams et al., 2004; Hubrecht, 2002). If possible, a lot of handling and training should take place already in the imprinting period of puppies (Feddersen-Petersen, 1991). In order to keep laboratory dogs calm and immobile during long-term investigations, e.g. imaging procedures, they are usually anaesthetised or deeply sedated. One method to replace narcotics or sedatives is fixing the dog by an assistant. Reduction of discomfort caused by this procedure can be achieved by using the Pavlov sling, in which the dogs are trained to stand nearly motionless. The Pavlov sling is an established alternative to fixation and is expected to reduce the stress level caused by the experiment. Preliminary dogs should be carefully trained by classical conditioning, so dogs associate fixation in the sling with a positive experience.

Nevertheless, in the Pavlov sling dogs are restrained in their movements and can not escape from a situation that impedes their normal resting positions, like sitting or lying down, and it is possible that the Pavlov sling itself causes stress (Mikkelsen et al., 2003). Avoiding stress during the experimental situation is very important not only for ethical reasons. The reaction of an individual to stress is complex and integrates responses of the central and the autonomic system, the hypothalamic-pituitary-adrenal axis, and the target organs. Each reaction can falsify the experimental results in an interactive way, which has not been determined yet (Ritskes-Hoitinga, 2006).

To evaluate the degree of stress of an animal, the assessment of physiological parameters, especially of stress hormones, are diagnostically conclusive (Nagel and von Reinhardt, 2003; Knies, 2005; Taylor, 2000). One important hormone for the identification of a stress reaction is the glucocorticoid cortisol. A suitable method to assess changes in cortisol concentration is the analysis of saliva. Traditionally, cortisol concentration is measured in the plasma. However, collecting blood samples is an invasive method and may already cause stress in the animal (Beerda, et al., 1998) and can be a confounding variable. The concentration of free cortisol in saliva correlates significantly with levels measured in the blood (Beerda et al., 1996; Vincent and Michell, 1992). Therefore, working with salivary cortisol provides a non-invasive and currently very popular method for measuring stress related changes in cortisol concentration (Kobelt et al., 2003).

Stress can also be measured by the occurrence of certain behavioural patterns. Submissive behaviour is learnt by dogs already as puppies, and signals indicating submission are normally used to avoid a threat or prohibit aggressive behaviour (Beaver, 1999). Many of the respective behavioural patterns, e.g. calming signals, are regarded as signs of stress (Rugaas, 2002).

The first aim of our study was to quantify and qualify the behaviour shown by habituated Beagle dogs during the Pavlov sling restraining. Secondly, we investigated if the animals are stressed by this procedure and looked out for stress-related behavioural signs like body shaking, sweating of the paws, increased saliva production, piloerection, blinking of eyes, snout licking, yawning, and panting. Additionally, changes in the behavioural pattern have been analysed at the beginning, middle, and end of restraining since high level of changes can be regarded as signs of restlessness. Moreover, salivary cortisol concentrations were measured before, during, and after the experiment. Our results should help to

evaluate the Pavlov sling as an adequate instrument for long-term restraining dogs without causing additional stress during experiments.

Animals and methods

Animals

The study was conducted on ten trained Beagle dogs (five males, five females) originally obtained by Harlan Winkelmann SARL Gannat, France. At the beginning of our experiments the dogs were three years of age. They were group-housed by sex under standard conditions at the Institute for Pharmacology and Toxicology, Faculty of Veterinary Medicine of the Free University of Berlin. The dogs were kept in the same room divided in two different pens (size 25 m² and 26 m², respectively) with visual, acoustic, and olfactory contact possible between both groups. The pens were illuminated by daylight and by an additional artificial light scheme (lights on: 6:00-18:00h). Room temperature was 18 ±1°C and the relative humidity 55 ±10%. Each group had access to its own outdoor enclosure (approx. 67 m² for the males and 60 m² for the females) for five hours a day. Several times a week the dogs were brought to a large natural outdoor enclosure of approximately 750 m² and were walked on a leash in and outside of the university campus. The animals were fed once a day about 13:00 h with 200 g of ssniff Hd-H 10MM diet (Soest, Germany). They had access to tap water *ad libitum* by an automatic water supply. Dogs were weighed once the week. Once a year, they were vaccinated with Euritan[®]Merial SHP L and regularly de-wormed with Drontal plus[®]. Housing was in accordance with the German Tierschutz-Hundeverordnung, 05/02/2001 and permitted by the Landesamt für Gesundheit und Soziales - LAGeSo - Berlin, 10/22/2011).

Apparatus

The Pavlov sling was made of a cotton-hammock with four holes for the dog's legs (with 12 cm between the holes for the front or hind legs, respectively, and 28 cm between the holes for a front and a hind leg). The sling was fixed between four metal rods (arranged in a rectangle of 32 x 38 cm) on a table 90 cm above the floor (Fig. 1 A).

Basic training

At an age of one, all dogs underwent training for three months by a professional dog training service (DWSS Blumberg, Ahrensfelde, Germany) to stand or lie calmly in the Pavlov sling for 1 hour without trying to exit the sling. After the dogs had mastered the basic training, the level was ensured at our institute by encouraging the animals to stand or lie in the Pavlov sling for 30 minutes twice a week.

Experimental procedure

Our experiments, i.e. behavioural observation and sampling of saliva for cortisol concentrations, took place during the regular Pavlov sling training. Two months prior to the experiments the dogs have been handled by the investigator to ensure familiarity. Handling was conducted in the normal surrounding of the dogs, i.e. in the pens or outside enclosures. The investigator sat or stood between the freely moving dogs, allowing them to make contact while calmly patting and talking to them. This procedure was repeated two days a week for one month. In the second month the dogs were habituated to the procedure of saliva sampling. The sampling took place in the pens and room where the Pavlov sling training was carried out. For the saliva extraction normal cotton swabs were used. The dogs were patted and the cotton swabs were put into the mouth during play. After removing the swab the dog got a reward, i.e. a piece of commercial dog food.

In the actual experiment each dog was tested individually. The investigator took one dog from the pen and carried it to the experimental room. Here it was allowed to move freely for 15 minutes and was afterwards placed in the Pavlov sling for 30 minutes. At the end of the experiment the dog was taken out of the sling, receiving a reward and was again allowed to move freely in the room for another five minutes before it was returned to its group. Each dog was tested on six days at intervals of at least two days. Males and females were tested on different days. The actual acquisition of behavioural data and saliva sampling was carried out on day 2-6. Within each experimental day, the order of the tested dogs was randomised to avoid time dependency.

Acquisition of behavioural data

The dogs were observed by the investigator during the 30 min sessions and the following main behavioural parameters were measured and classified according to

the code numbers indicated in Tab. 1: posture of body, head, tail, legs, and ears, and state of eyes and mouth. The occurrence of any of these behavioural states and positions was recorded once every minute for 30 min (instantaneous sampling). The counts were added up for each dog and 30 min session. Then the frequency of the different states and positions was calculated for each behavioural parameter and described as percentage for each session (Fig. 2) and as an average for all sessions (Fig. 3).

Since changes from one behavioural state to another can be regarded as an indicator for restlessness and stress (Haberland, 2002; Nagel and von Reinhardt, 2003; Rugaas, 2002), we also measured the course of sequential changes between the different states and positions of a behavioural parameter at the beginning (1-10 min), the middle (11-20 min), and at the end (21-30 min) of the experiments. The number of changes was averaged for all dogs and sessions.

Additionally, the dogs were filmed by two cameras: One camera (Canon HG 10; 40 GB HDD) was set up on a tripod 1.60 m high and filmed the animal from the left side. The second camera (Panasonic, NV-GS 1) was placed next to the researcher and recorded the front view about 1 m away. The analysis of the recorded material was performed by the same investigator conducting the experiments and the appearance of specific stress and submission signals were measured for the 30 min by continuous sampling. Behavioural parameters indicating stress or submission (Beerda et al., 1998; Haberland, 2002; Rugaas, 2002) like body shaking, sweating of the paws (visible signs on the metal table), increased saliva production (visible dribbling of saliva), piloerection (erection of the hair), blinking of the eyes (closing of the eyes with duration less than one second), snout licking (visible licking of the mouth), and yawning were measured as instant events for all dogs and sessions. Additionally, panting (heavy breathing with open mouth) was measured by the number of minutes in which it occurred. For the analysis of the occurrence of these parameters the program Etholog 2.2 (free- software by © 1995-99 Eduardo B. Ottoni) was used.

Saliva cortisol

In order to measure changes in cortisol concentration, three saliva samples were taken from each dog on day 2-6: A pre-experimental sample a few minutes before the animal was placed in the experimental room in the Pavlov sling, an experimental

sample 20 minutes after the beginning of the experiment, and a basal sample taken in the pen 30 minutes after the last individual was tested. Saliva was collected by using a “Salivette blau” cotton rope (Sarstedt, Nümbrecht, Germany). The rope was placed into the dog’s cheek pouch for one to two minutes and removed when it was soaked with saliva. Every time the investigator wore new rubber gloves to avoid external contamination. The saliva samples were put on ice until all dogs of the respective experimental day had been tested. In the evening after achieving room temperature, the tubes were centrifuged for five minutes at 3,000 rpm (Eppendorf 5403). Thereafter, they were stored at 4°C. The final ELISA procedure was done in the Institute for Pharmacology and Toxicology of the University of Veterinary Medicine Hannover, to which the samples were transported on dry ice and then frozen at -20°C until the next day. An ELISA kit for salivary cortisol based on the competitive principle (IBL International GmbH, Hamburg, Germany) was used according to Schalke et al. (2009). The kit cross-reacted with cortisone (4.2%), prednisone (2.5%), corticosterone (1.4%), desoxycorticosterone (0.9%) and 17-OH-progesterone (0.4%). The analytic sensitivity was 0.05 ng/ml and the functional sensitivity (20% coefficient of variation) 0.3 ng/ml.

Contaminated (e.g. by blood) saliva samples and samples which did not ensure the required amount were excluded from the analysis (one out of 50 pre-experimental samples, five out of 50 experimental samples, and nine out 50 basal samples). The ELISA procedure was conducted as stated in the protocol of IBL. The optical density was measured by a microplate reader (MRX microplate reader by Dynatech, Denkendorf, Germany) using a wave length of 450nm. The standard curve was generated by plotting the optical density of the standards against their concentrations. Cortisol values were calculated using Probit transformation and the concentrations of basal, pre-experimental and experimental samples were checked for normal distribution. The average concentrations for the samples were calculated for each dog and averaged for all sessions, respectively.

Data analysis

The appearance of the main behavioural parameters listed and encoded in Tab. 1 and the behavioural parameters indicating stress or submission were analysed by descriptive statistics (Tab. 2, Fig. 2 and 3).

The data for the behavioural changes from one state/position to another, for the behavioural stress and submission signs as well as for the salivary cortisol concentrations were tested for normal distribution by Shapiro-Wilk methods. Thereafter, the respective parametric or non-parametric tests were used: The sequential changes for each behavioural parameter were analysed by one-Way ANOVAs on repeated measures followed by post-hoc Holm-Sidak method in order to detect differences between the beginning, middle, and end of the experiment (Fig. 4). The data for the salivary cortisol concentrations were analysed by Friedman tests for one-Way repeated measures analysis of variance by ranks to detect differences between the five sessions and between the averaged basal, pre-experimental and experimental sample (Fig. 5). SigmaPlot 11.0 (Systat Software, Inc. SigmaPlot for Windows) was used for creating the graphs and for statistical analysis. P values < 0.05 were considered to represent statistical significance.

Results

Behaviour

A characteristic behavioural pattern Beagle dogs display during restraining in the Pavlov sling is shown in Fig. 1 B-D. The frequency of occurrence of each main behavioural parameter was quite similar for the five sessions (Fig. 2). Therefore, the percentages for the different states were summarised in Fig. 3. The behavioural states shown most often by the dogs were 'body and tail hanging', 'head low', 'ears low', 'mouth closed', 'eyes opened', and 'leg on the ground' (Fig. 3). Body position was most frequently observed as 'completely hanging' (58%), followed by 'pelvis hanging' (17%), 'shoulder hanging', 'leaning right', 'upright standing', and 'leaning left' (each < 10%). The head was most often held 'low' (41%), followed by 'on side' (32%), 'upright' (21%), and, least frequently, 'in middle position' (< 10%). Pronounced quantitative differences occurred in the status of the ears, which were mostly held 'low' (60%), less upright (27%), and more infrequently in the 'middle position' (13%). Ears were moving only rarely (< 1%). The mouth was predominantly 'closed' (88%), whereas 'licking', 'tongue visible' and 'panting' each occurred less frequently (10%). The eyes were 'open' in 45%, 'half closed' in 30%, and 'completely closed' in 24% of all observations. In 99% the tail was 'hanging', and only rarely it was hold 'between

the legs' or was 'moving' (each < 1%). In the front as well as in the hind legs the position 'down' was dominant (front = 73%, hind = 92%). From time to time the legs were relaxed and just the toes were touching the ground (front < 20%, hind < 10%). The carpal joints were only buckled in the front legs (< 20%).

In general, the number of changes from one behavioural state or position to another did not differ between the beginning, the middle, and the end of the sessions apart from the state of the mouth ($p = 0.047$, Fig. 4).

The dogs did not show any clear sign of stress, like body shaking, sweating of the paws, an increased saliva production, or piloerection. However, some signals indicating submission were observed. Eye blinking was most prominent and occurred, on average, 81.1 times, while snout licking was shown far less often with 9.8 times in the 30 minutes of the observation period. Six dogs displayed the behaviour pattern panting, 3.0 times on average. Yawning was observed in only one dog, with extremely low frequency (0.1 times) (Tab. 2).

Cortisol

The standard curve was generated using six standard concentrations. Linearity of the standard curve was shown for concentrations between 0.6 ng/ml and 40 ng/ml ($r=0.86$). The salivary cortisol values were not normally distributed for all dogs. There were no significant differences between the five sessions for the basal ($p = 0.056$), pre-experimental ($p = 0.092$), and ($p = 0.647$) sample (data not shown), therefore the data was averaged for all five sessions (Fig. 5). Cortisol concentration of the base samples ranged between 0.8 and 2.7 ng/ml, of the pre-experimental samples between 0.7 and 3.4 ng/ml, and of the experimental samples between 0.9 and 2.6 ng/ml. The median for all dogs was 1.3 ng/ml for the basal sample, 1.1 ng/ml for the pre-experimental sample, and 1.2 ng/ml for the experimental sample, with no significant differences between the three groups ($p = 1.000$; Fig. 5).

Discussion

This is the first study to reveal, review, and discuss the behaviour of trained Beagle dogs which are restrained by a Pavlov sling. Overall, the analysis of all behavioural

patterns as well the physiological parameter cortisol clearly indicate that the dogs did not suffer undue stress in that situation.

The frequency of various behavioural states and positions which the animals displayed while restrained in the Pavlov sling revealed no marked difference between the five sessions and confirms that the dogs were already familiarised with this procedure and did not need any further habituation. This observation is underlined by the finding that changes of state and positions did not vary significantly between the beginning, middle and the end of the sessions. Haug (2004) describes a relaxed dog as sitting or lying with muscles not tensed, body not shaking, tail held low, and ears hanging. All of these characteristics were frequently observed in our dogs during the experiment. Additionally, most of the time the animals had their paws touching the ground, the mouth closed, and the eyes totally or half closed. Although, we cannot quantify our observation by EEG, respiratory and heart rate recording, it appeared that most dogs were at least dozing while their eyes were half or fully closed. Further indications that our dogs were relaxed during the experiments are: head mainly lying on the hammock or on the side metal rods and body either completely hanging or, less often, just pelvis hanging in the hammock (Fig. 1 B-D).

However, it is sometimes difficult to decide whether an immobilized dog is truly relaxed or has simply “surrendered” to an unavoidable situation. Therefore, we investigated additional parameters, such as the frequency of changes of behavioural patterns as well as unambiguous stress signals. According to Beerda et al. (1997) stress, i.e. unavoidable electrical shock, results in an increase of restlessness in dogs, such as high levels of walking, nosing and changing from one state of locomotion to another. In our study we regarded the number of changes from one behavioural category to another as a measure for restlessness. We expected that dogs should be more restless, i.e. show an increased number of behavioural changes, at the beginning, and would calm down at the end of the experiment, if they were stressed or agitated during restraining. However, changes in body and tail position as well as in the state of mouth occurred only in marginal frequency. Most changes were observed in the position of ears and head and in the state of eyes. Since the experiment could not be carried out in a soundproof room it is quite likely that these marginal changes in position/state of head, ears, and eyes do not indicate stress or discomfort but caused by external, mainly acoustic, stimuli from the

adjacent rooms and hallway, an assumption which we could confirm by direct observation on several occasions.

Behavioural patterns which indicate some kind of discomfort and which could be displayed by the dogs restrained in the Pavlov sling can be divided in distinct stress signals, like body shaking, sweating of the paws, an increased saliva production and piloerection (Beerda et al., 1997; Beerda et al., 2000; Nagel and von Reinhardt, 2003), and more subtle submission signals, like eye blinking, snout licking, yawning and panting (Beerda et al., 1998; Feddersen-Petersen, 2004; Haberland, 2002; Rugaas, 2002). The quantitative analysis of these behavioural patterns indicates a non-stressed state of the dogs while standing in the sling. Distinct stress signals were not shown at all. Some submission signals occurred, but in frequencies definitely below levels indicating an acute stress. Blinking of the eyes occurred, on average, 2.7 times a minute, which is far below a frequency given by Harmer and Williams (2003) for relaxed dogs (13.7 times a minute).

Beerda et al. (1997) measured stress of freely moving dogs which were exposed to acoustic signals (noise blasts of 3,000 Hz at levels of 70, 78, and 87 dB), and report a snout licking frequency of approximately 26 times per 30 minutes, whereas control dogs showed such behaviour only three times per 30 minutes. With 9.8 times of snout licking in the 30 minute period our dogs in the Pavlov sling displayed a higher frequency than the control dogs used in the study of Beerda et al. (1997), but did not reach the frequency of the stressed dogs. Furthermore, yawning and panting were not observed in all dogs and occurred in less than 10% of the observation time. Therefore, there was no indication for stress during the time the dogs were restrained in the sling. We have additionally surveyed the behaviour for any signs of increased aggression and stress when the dogs were reintegrated in their respective group after the sessions. Separating a dog from and returning it to its group always leads to agitation. Although we did not quantify our observations, this agitation did not differ from the behaviour when a dog was taken out of the group for examinations (e.g. control of body weight and health status) or returning from a walk on a leash outside. There were also no differences in body weight before, during, and after the experiments indicating no additional stress for the animals.

Our conclusion drawn from the behavioural data is confirmed by the measurement of the stress hormone cortisol. Firstly, salivary cortisol concentrations of the experimental samples ranged on average from 0.9 to 2.6 ng/ml. This is line

with salivary cortisol concentrations of unstressed Beagle dogs at a similar age (one to four years of age, between 0.6 and 2.3 ng/ml) (Koyama et al., 2002; Vincent and Mitchell, 1992). Secondly, there was no difference in average cortisol levels between the basal, pre-experimental, and experimental samples, clearly indicating that the dogs were neither stressed by the experimental preparation nor by the experiment itself, i.e. spending 30 minutes standing or lying in the Pavlov sling.

Hubrecht (2002) and Adams et al. (2004) point out that an experimental situation should be as comfortable as possible for the tested animal. Therefore, the results of our study could be helpful in improving future experiments with laboratory dogs, because the Pavlov sling does obviously not cause additional stress in the trained animal. This advantage of the Pavlov sling does not only have implications for the ethics concerning the use of laboratory animals but also for the overall validity of the desired data: The less stress an animal suffers the more reliable the experimental data are (Shuy et al., 1987; Vogel, 1993). According to Hubrecht (2002) and Adams et al. (2004) an experimental situation should be trained with the animals before the actual experiment is run, a point of view certainly supported by the present study. Our results show that a well practised experimental procedure and a habituation to the special test situation can even lead to relaxation of the animal, as indicated by a completely hanging body, eyes closed for longer periods of time. The Pavlov sling, therefore, can substitute more ordinary – and often discomfort producing – methods of immobilisation, in some cases even the use of narcotics.

The setup of the Pavlov sling should always be optimized in detail, to make it as comfortable as possible for the dogs (Mikkelsen et al., 2003). In some cases it may be best, if compatible with the aim of the experiment, to have the dogs sitting instead of standing or lying flat in the sling, so the hammock should be constructed accordingly. Especially the size of the hammock and height of the Pavlov sling should fit to the individual dog as our experience shows that even dogs of the same breed can differ considerably in body height and length. The possibility to raise or lower the position of the hammock as well as using the optimal hammock size is very important to have the animals standing or laying down comfortably.

In summary, our study demonstrates that under certain conditions the usage of a Pavlov sling in trained animals can substitute other methods of immobilisation with more discomfort, e.g. the use of narcotics or sedating drugs.

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Acknowledgement

We are grateful to Prof. Dr. Kietzmann, Institute of Pharmacology, University of Veterinary Medicine Hannover, for the supply of the equipment, the instruction, and assistance for the performance of the ELISA. Additionally we want to thank PD Dr. Hauff, Bayer HealthCare Pharmaceuticals Berlin, for his contribution to the discussion. We also want to thank Dave O’Gorman and Sascha Helfrich for proof-reading the manuscript.

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Fig. 1

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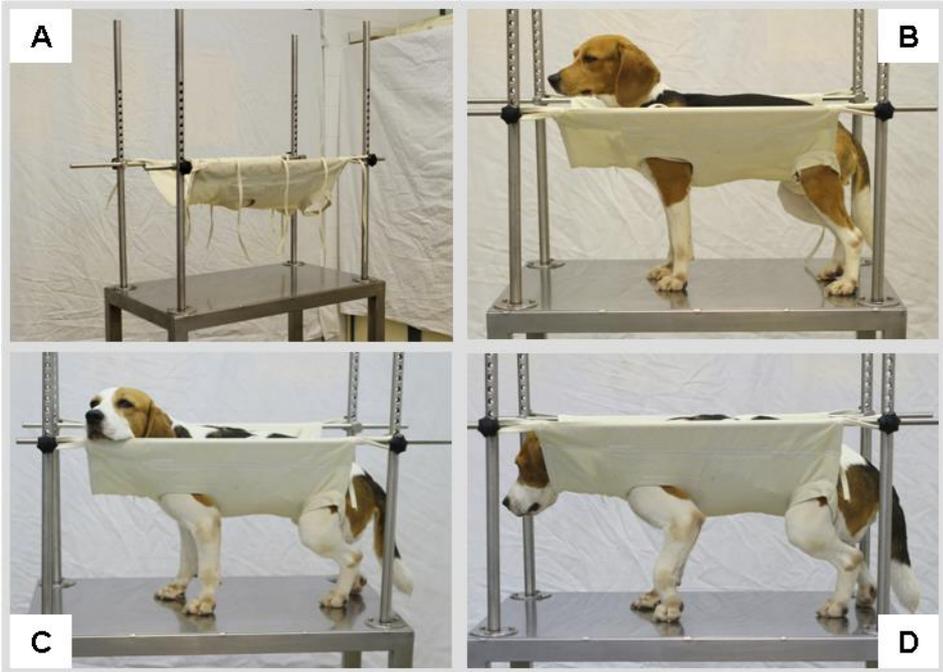


Fig. 2

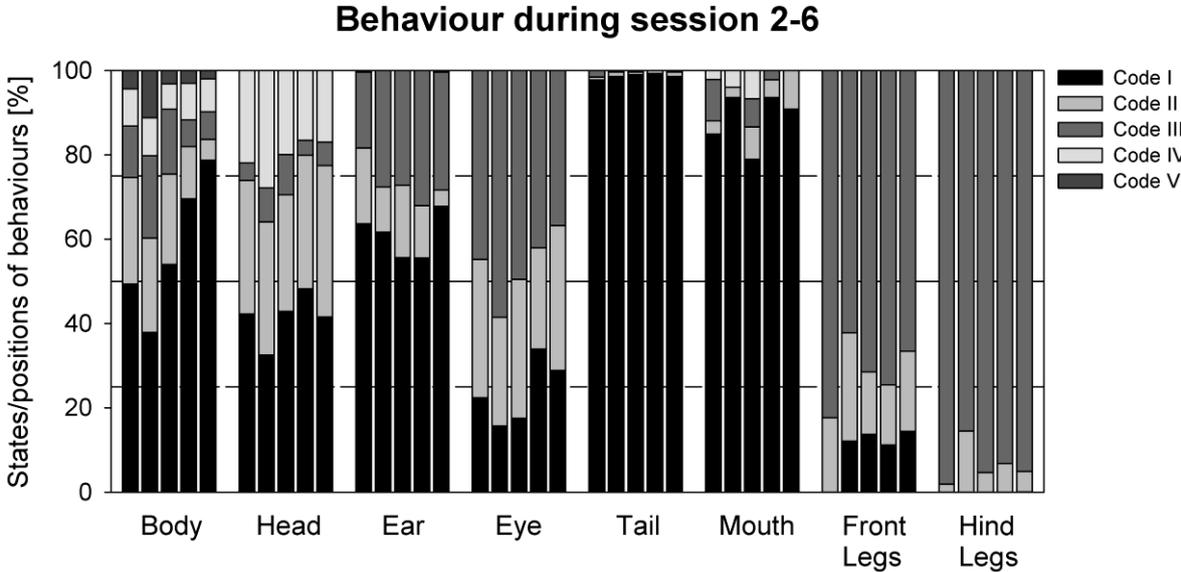


Fig. 3

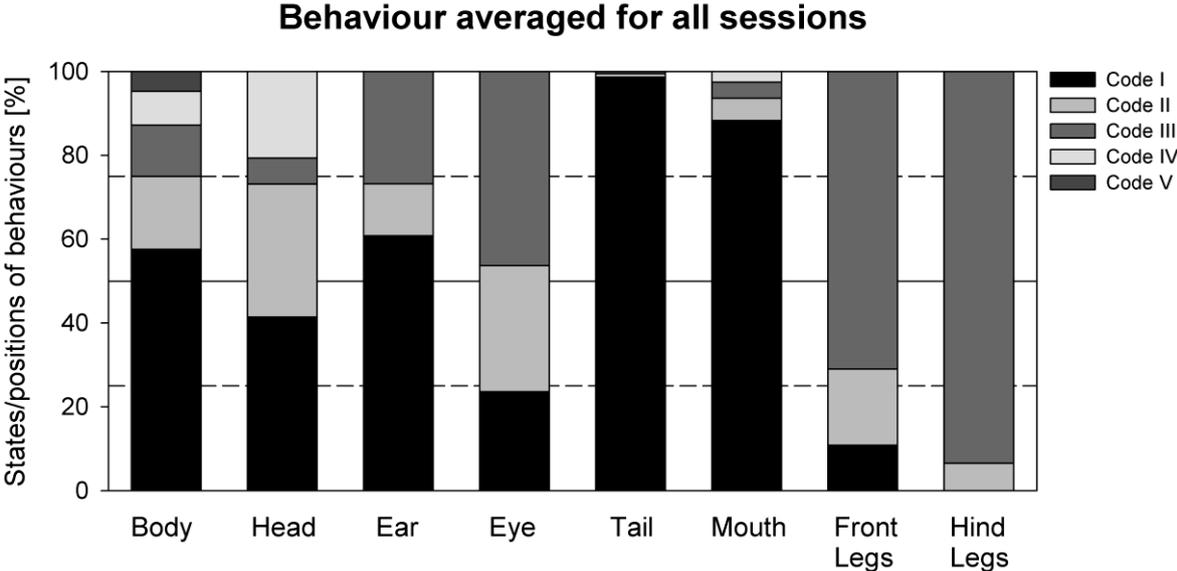


Fig. 4

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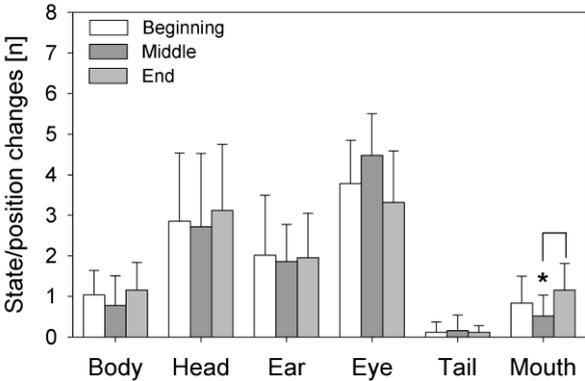
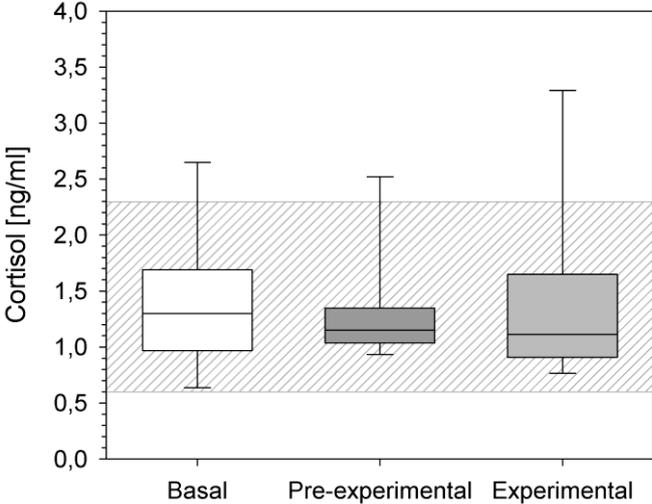


Fig. 5

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Tab. 1

| Parameter | State/Position | Definition | Code |
|------------------|-----------------------|--|-------------|
| Body | Completely hanging | Whole body is hanging in the hammock | I |
| | Pelvis hanging | Only pelvis is hanging in the hammock | II |
| | Leaning against side | Dog leans against one side of the hammock | III |
| | Shoulder hanging | Only breast is hanging in the hammock, so shoulder is down | IV |
| | Upright/ standing | Dog stands with stretched legs and does not put any weight on the hammock | V |
| Head | Low | Head lying down on the hammock | I |
| | Side | Head lying down on one of the side metal rods | II |
| | Middle | Head put in forward position, but not placed on the hammock | III |
| | Upright | Head held up | IV |
| Ear | Low | Ears completely hanging (typical for the Beagle breed) | I |
| | Middle | Ears between hanging and erected | II |
| | Up | Ears erected as far as possible, attentive | III |
| | Moving | Ears moving | IV |
| | Laid back | Laid back tight to the head, so that the ear is behind the vertical line of straight hanging | V |
| Eye | Closed | Eyes completely closed | I |
| | Half closed | Eyes half closed | II |
| | Open | Eyes completely opened | III |
| Tail | Hanging | Tail relaxed in a nearly vertical position | I |
| | Movement | Tail wagging | II |
| | Hold between the legs | Tail hold between the legs and pressed against the belly | III |
| Mouth | Closed | Mouth is completely closed | I |
| | Licking | Licking the mouth | II |
| | Tongue visible | The tongue can be seen without panting | III |

| | Panting | Panting | IV |
|--------------------|---------|---------------------------------------|-----|
| Front and hind leg | Joint | Joint is buckled (yes/ no) | I |
| | Toe | Only the toes are touching the ground | II |
| | Down | Whole foot on the ground | III |

Tab. 2

| Signs | Description | Number of occurrence |
|-----------------------------|---|-------------------------------------|
| Body shaking | Shaking of the muscles of the body | 0 |
| Sweating of paws | Visible signs of sweat on the metal table | 0 |
| Increased saliva production | Visible dribbling of saliva | 0 |
| Piloerection | Erection of the hair | 0 |
| Blinking of eyes | Eyes are closed very briefly (< 1 s) | 81.1 ± 43.6 |
| Snout licking | Tongue licking the mouth | 9.8 ± 5.0 |
| Yawning | Opening the mouth to yawn | 0.1 ± 0.3 (observed in 1 dog only) |
| Panting | Breathing with mouth opened | 3.0 ± 4.7 (observed in 6 dogs only) |

Figure legends / Table headings

Fig. 1. Picture of the Pavlov sling used in our experiments (A), dog with head in upright position, body leaning against one side, tail hanging (B), dog with head lying on one side, pelvis and tail hanging (C), dog with head, body and tail completely hanging (D).

Fig. 2. Frequency of positions or states of the different behaviours (as indicated and coded in Tab. 1) displayed in the five experimental sessions. Data are presented as mean percentages [%] for all dogs.

Fig. 3. Frequency of positions or states of the different behaviours (as indicated and coded in Tab. 1). Data are presented as mean percentages [%] for all sessions and dogs.

Fig. 4. Number of position or state changes of behaviours at the beginning (1-10 min), in the middle (11-20 min), and at the end (21-30) for all five sessions. Data are presented as means + SD; * $p < 0.05$.

Fig. 5. Salivary cortisol concentrations [ng/ml] of the basal, pre-experimental, and experimental samples for all five sessions and dogs. Data are presented as medians and 25./75. and 5./95. percentiles. The shaded area indicates the salivary cortisol range of unstressed Beagle dogs during a 24h recording according to Koyama et al. (2002).

Tab. 1. Investigated behaviours including different states and positions encoded from I-IV.

Tab. 2. Occurrence of behavioural signs indicating stress or submission. Data are presented as means \pm SD for all dogs and sessions.