



Assessment of Stress in Laboratory Beagle Dogs Constrained by a Pavlov Sling

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Summary

The 3Rs – Replacement, Reduction and Refinement – have become increasingly important in 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 that had been trained to tolerate restraint by a Pavlov sling are stressed by this procedure and, furthermore, to analyze their behavior during this period. Five male and five female Beagle dogs were used, each three years of age. Animals were restrained in the Pavlov sling for 30 min on six days with an interval of at least two days. The following behaviors 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, including 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 for most behavioral parameters, e.g., body, leg, head, tail, and ear posture, the frequency of changes between different behavior patterns, as well as cortisol concentration, were not influenced by restraint 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 for more ordinary methods of immobilization, e.g., the use of narcotics.

Keywords: Pavlov sling, laboratory dogs, stress assessment, refinement

1 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 experiments include direct measurements in the conscious dog, such as taking blood samples, intravenous dripping, imaging, or measuring heart frequency, body temperature or other physiological parameters. For imaging techniques, especially, it is very important to restrain the animal in its movements, sometimes over a long period of time. Despite many efforts to find alternatives for animal models in science, it is unrealistic to expect a complete end to laboratory animal use in the near future. Therefore, the 3Rs – Replacement, Reduction and Refinement – (Russell and Burch, 1959) are still valid, and the third R becomes increasingly important.

If neither replacement nor reduction is possible, refinement is of utmost importance. Refinement should minimize any pain and/or distress for a laboratory animal in the experiment and during housing. Over recent years a lot of work has been done to

improve experimental conditions and to enrich the environment, not only for the benefit of the animals but also for the validity and reliability of the experiment (European Convention, 1986; TierSchHuV, 2001; Hubrecht, 2002; MUFV, 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 early, during the imprinting period of puppies (Feddersen-Petersen, 1991). To keep laboratory dogs calm and immobile during long-term investigations, e.g., imaging procedures, they usually are anaesthetized or deeply sedated. One method to replace narcotics or sedatives is for an assistant to restrain the dog. 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 restraint and is expected to reduce the stress level caused by the experiment. Dogs should first be carefully trained by classical conditioning so they associate restraint in the sling with a positive experience. Nevertheless, in the Pavlov sling dogs are restrained in their movements and

cannot escape from a situation that impedes their normal resting positions, such as 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, and not only for ethical reasons. The reaction of an individual to stress is complex, integrating 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 that is yet to be determined (Ritskes-Hoitinga et al., 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. The analysis of saliva provides a suitable method to assess changes in cortisol concentration. Traditionally, cortisol concentration is measured in the plasma. However, collecting blood samples is an invasive method that may itself cause stress in the animal (Beerda, et al., 1998), which 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 also can be measured by the occurrence of certain behavioral patterns. Dogs learn submissive behavior as puppies, and signals indicating submission are normally used to avoid a threat or prohibit aggressive behavior (Beaver, 1999). Many of the behavioral 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 behavior shown by habituated Beagle dogs during the Pavlov sling restraining. Secondly, we investigated whether the animals are

stressed by this procedure and looked for stress-related behavioral signs, such as body shaking, sweating of the paws, increased saliva production, piloerection, blinking of eyes, snout licking, yawning, and panting. Additionally, changes in the behavioral pattern have been analyzed at the beginning, middle, and end of restraining, since high levels of change 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 of dogs without causing additional stress during experiments.

2 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, Germany. The dogs were kept in the same room divided into two separate pens (size 25 m² and 26 m², respectively) with visual, acoustic, and olfactory contact possible between the groups. The pens were illuminated by daylight and by an additional artificial light scheme (lights on: 6:00-18:00 h). 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 at about 13:00 h with 200 g of

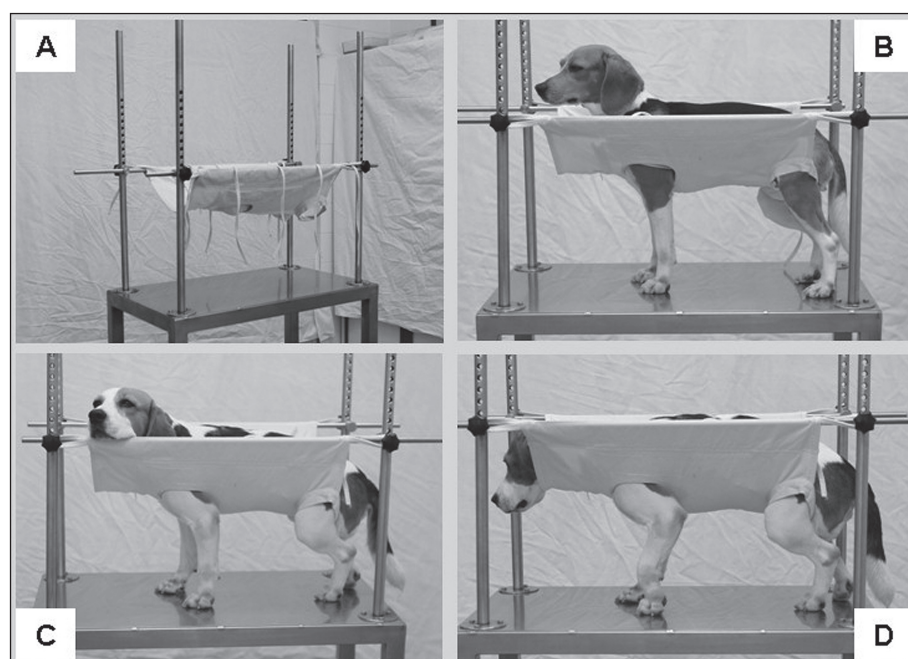


Fig. 1: Picture of the Pavlov sling used in our experiments (A), dog with head in upright position, body leaning against one side, tail hanging (B), dog with head lying on one side, pelvis and tail hanging (C), dog with head, body and tail completely hanging (D)

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 Eurican® 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. 1A).

Basic training

At the age of one year, 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 h without trying to exit the sling. After the dogs had mastered the basic training, the level was maintained at our institute by encouraging the animals to stand or lie in the Pavlov sling for 30 min twice a week.

Experimental procedure

Our experiments, i.e., behavioral observation and sampling of saliva for cortisol concentrations, took place during the regular Pavlov sling training. Two months prior to the experiments, the investigator began to handle the dogs to ensure familiarity. Handling was conducted in the normal surroundings 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 twice 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 the room where the Pavlov sling training was carried out. Normal cotton swabs were used for the saliva extraction. 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 min and was afterwards placed in the Pavlov sling for 30 min. At the end of the experiment the dog was taken out of the sling, was given a reward, and was again allowed to move freely in the room for another 5 min before it was returned to its group. Each dog was tested on 6 days at intervals of at least 2 days. Males and females were tested on different days. The actual acquisition of behavioral data and saliva sampling was carried out on days 2-6. Within each experimental day, the order of the tested dogs was randomized to avoid time dependency.

Acquisition of behavioral data

The dogs were observed by the investigator during the 30 min sessions, and the following main behavioral parameters were

measured and classified according to the code numbers indicated in Table 1: posture of body, head, tail, legs, and ears, and state of eyes and mouth. The occurrence of any of these behavioral 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 behavioral parameter and described as percentage for each session (Fig. 2) and as an average for all sessions (Fig. 3).

Since changes from one behavioral 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 behavioral 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), set on a tripod 1.60 m high, 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. Behavioral parameters indicating stress or submission (Beerda et al., 1998; Haberland, 2002; Rugaas, 2002), such as 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. The program Etholog 2.2 (free-software by © 1995-99 Eduardo B. Ottoni) was used for the analysis of the occurrence of these parameters.

Saliva cortisol

In order to measure changes in cortisol concentration, three saliva samples were taken from each dog on days 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 min after the beginning of the experiment, and a basal sample taken in the pen 30 min 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. The investigator wore new rubber gloves each time 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 the tubes achieved room temperature, they were centrifuged for five minutes at 3,000 g (Eppendorf 5403). Thereafter, they were stored at 4°C. The final ELISA procedure was done at the Institute for Pharmacology and Toxicology of the University of Veterinary Medicine Han-

**Tab. 1: Investigated behaviors including different states and positions encoded from I-V**

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 held 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

nover, Germany, 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 that did not ensure the required amount were excluded from the analysis (1 out of 50 pre-experimental samples, 5 out of 50 experimental samples, and 9 out of 50 basal samples). The ELISA procedure was conducted according to 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 450 nm. 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-ex-

perimental, 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 behavioral parameters listed and encoded in Table 1 and the behavioral parameters indicating stress or submission were analyzed by descriptive statistics (Tab. 2, Fig. 2, 3).

The data for the behavioral changes from one state/position to another for the behavioral 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 behavioral parameter were analyzed by one-way ANOVAs on repeated measures followed by the *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

Tab. 2: Occurrence of behavioral signs indicating stress or submission

Data are presented as means \pm SD for all dogs and sessions.

Signs	Description	Number of occurrences
Body shaking	Shaking of the body muscles	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 \pm 43.6
Snout licking	Tongue licking the mouth	9.8 \pm 5.0
Yawning	Opening the mouth to yawn	0.1 \pm 0.3 (observed in 1 dog only)
Panting	Breathing with mouth opened	3.0 \pm 4.7 (observed in 6 dogs only)

analyzed 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.

3 Results

3.1 Behavior

A characteristic behavioral pattern Beagle dogs display during restraining in the Pavlov sling is shown in Figure 1B-D. The frequency of occurrence of each main behavioral parameter was fairly similar for the five sessions (Fig. 2). Therefore, the percentages for the different states were summarized in Figure 3.

The behavioral 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 most often was 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 was it held “between the legs”

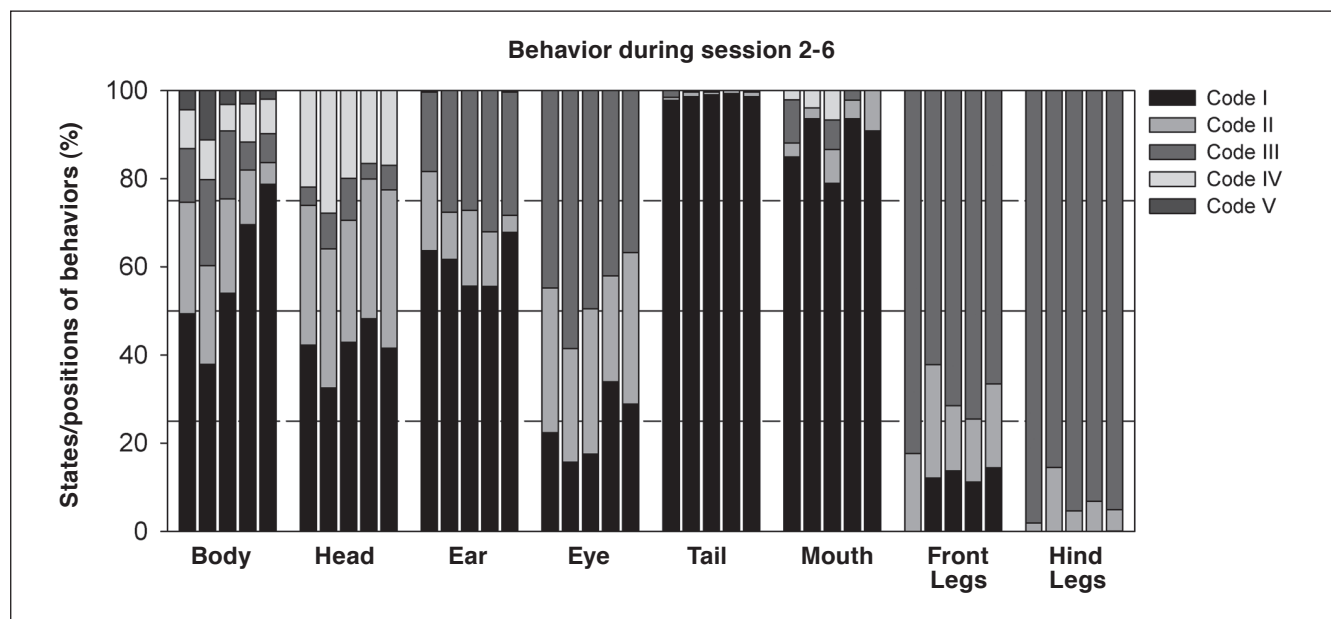


Fig. 2: Frequency of positions or states of the different behaviors (as indicated and coded in Tab. 1) displayed in the five experimental sessions

Data are presented as mean percentages (%) for all dogs.

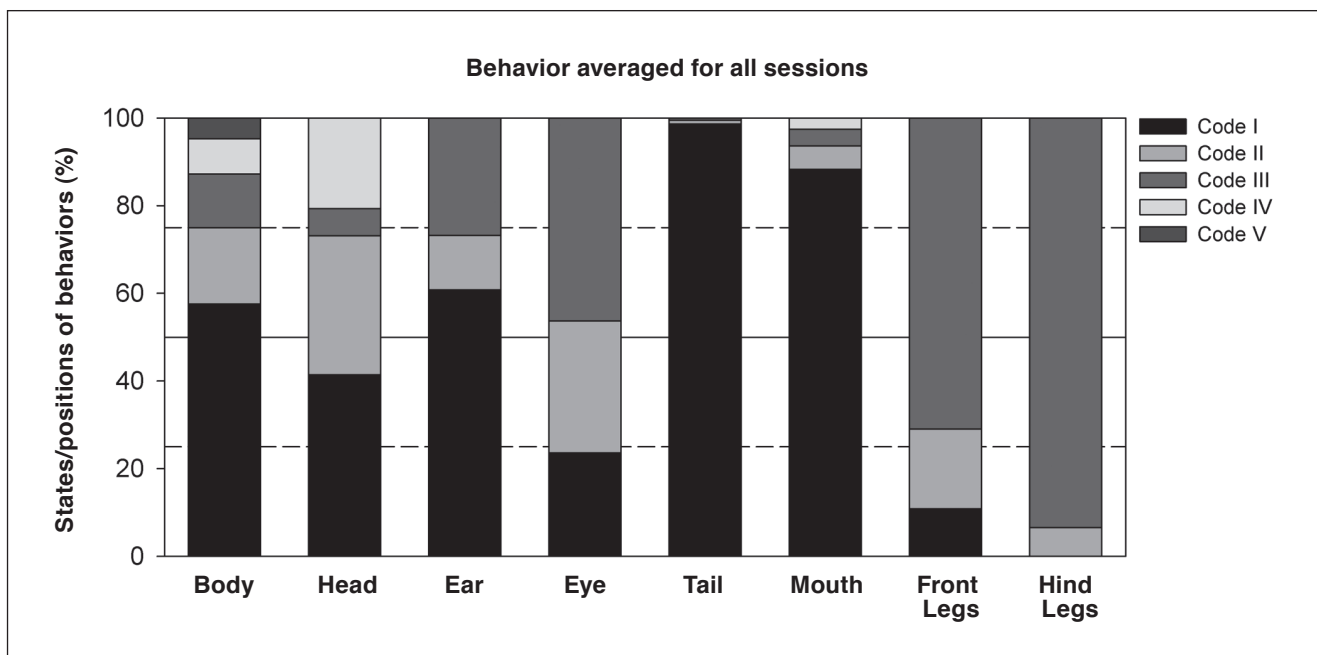


Fig. 3: Frequency of positions or states of the different behaviors (as indicated and coded in Tab. 1)

Data are presented as mean percentages (%) for all sessions and dogs.

or “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 behavioral 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, such as 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 min of the observation period. Six dogs displayed the behavior pattern panting, 3.0 times on average. Yawning was observed in only one dog, and thus with extremely low frequency (0.1 times) (Tab. 2).

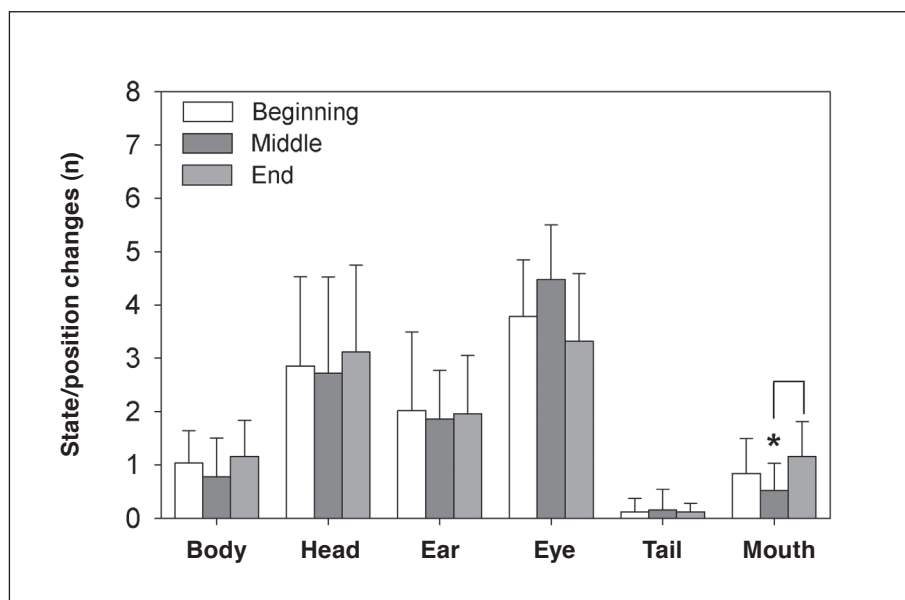


Fig. 4: Number of position or state changes of behaviors at the beginning (1-10 min), in the middle (11-20 min), and at the end (21-30 min) for all five sessions

Data are presented as means \pm SD;

* $p < 0.05$.

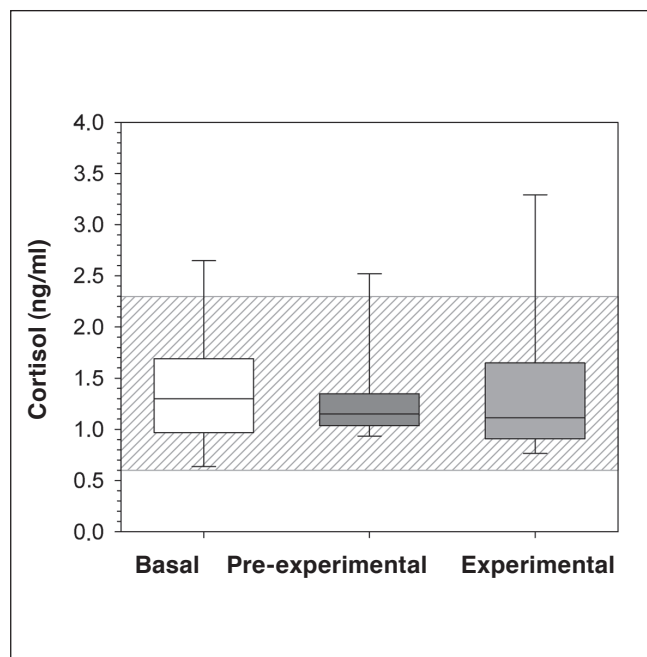


Fig. 5: Salivary cortisol concentrations [ng/ml] of the basal, pre-experimental, and experimental samples for all five sessions and dogs

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 24 h recording according to Koyama et al. (2002).

3.2 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 experimental ($p = 0.647$) sample (data not shown); the data, therefore, was averaged for all five sessions (Fig. 5). Cortisol concentration of the basal 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).

4 Discussion

This is the first study to reveal, review, and discuss the behavior of trained Beagle dogs that are restrained by a Pavlov sling. Overall, the analysis of all behavioral patterns and the physiological parameter cortisol clearly indicate that the dogs did not suffer undue stress in that situation.

The frequency of various behavioral states and positions the animals displayed while restrained in the Pavlov sling revealed no marked differences across the five sessions and confirms that the dogs were already familiarized with this procedure and did not need further habituation. This observation is underscored by the finding that changes of state and positions did not vary significantly between the beginning, middle, and 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 lying mainly on the hammock or on the side metal rods and body either completely hanging or, less often, just pelvis hanging in the hammock (Fig. 1B-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 behavioral 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 increased walking, nosing, and changing from one state of locomotion to another. In our study we regarded the number of changes from one behavioral category to another as a measure of restlessness. We expected that dogs should be more restless, i.e., show an increased number of behavioral 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 the mouth, occurred with only marginal frequency. Most changes were observed in the position of the ears and head and in the state of the 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 were caused by external, mainly acoustic, stimuli from the adjacent rooms and hallway, an assumption which we could confirm by direct observation on several occasions.

Behavioral patterns that indicate some kind of discomfort and that could be displayed by the dogs restrained in the Pavlov sling can be divided into distinct stress signals, such as 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, such as eye blinking, snout licking, yawning, and panting (Beerda et al., 1998; Feddersen-Petersen, 2004; Haberland, 2002; Rugaas, 2002). The quantitative analysis of these behavioral 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 acute stress. Blinking of the eyes occurred



2.7 times a minute on average, 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 that 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 behavior only three times per 30 minutes. With 9.8 instances 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 of stress during the time the dogs were restrained in the sling. In addition, we have surveyed the behavior for any signs of increased aggression and stress when the dogs were reintegrated into their respective groups 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 behavior 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 conclusions drawn from the behavioral data are 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 in 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 Michell, 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 obviously does not cause additional stress in the trained animal. This advantage of the Pavlov sling has implications not only 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), the animals should be accustomed to an experimental situation before the actual experiment is run, a point of view certainly supported by the present study. Our results show that a well-practiced experimental procedure and 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 for more ordinary but often discomfort-producing methods of immobilization – and even for the use of narcotics in some cases.

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. The size of the hammock and height of the Pavlov sling should be fitted 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 of raising or lowering the position of the hammock, as well as using the optimal hammock size, is very important for enabling the animals to stand or lie down comfortably.

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

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