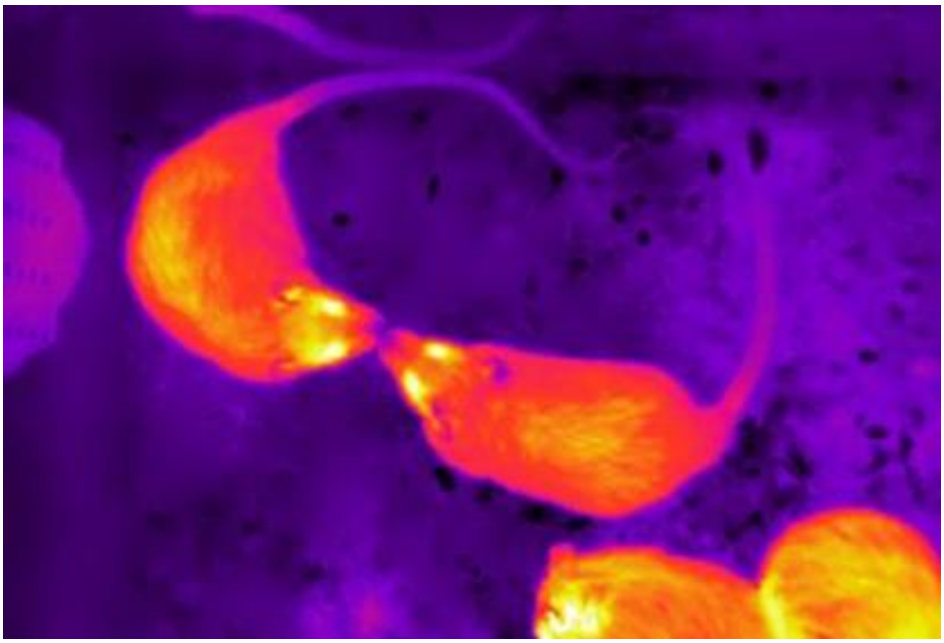
Effect of tickling and gentling on eye and tail temperature of laboratory rats during manual restraint, using infrared thermography



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Contents

1 ABSTRACT

Laboratory rats can experience acute stress during handling procedures, which can negatively affect animal welfare. The purpose of this research was to investigate if rats that had been regularly tickled or gentled from a young age experienced less acute stress during manual restraints due to the social buffer effect these procedures may provide. As read-out parameters for stress levels, maximum eye temperature and mid-tail temperature were used to reflect the stress-induced hyperthermia. Sixteen male Wistar rats (WU:Crl) with an age of three weeks at arrival in the lab were included in this study. Maximum eye temperature and mid-tail temperature were measured before, during and after a stressor period containing several manual restraints, using a FLIR T430sc thermal camera. Due to limitations, no reliable measurements were made for the eye temperature experiment and were, therefore, excluded from the results. For the mid-tail temperature, results showed that the gentled rats (G, *n* = 4) had a lower baseline tail temperature than the tickled rats (T, *n* = 8), where the control group (CONTROL, *n* = 4) showed the highest baseline temperature. During the stressor period, the tail temperatures of all rats dropped to a comparable lowest point temperature, suggesting repeated manual restraint is a stressful experience for all rats. Differences in temperature between the treatment groups during this period could not be distinguished, due to the differences in baseline temperature. Without additional parameters to assess stress levels and a behavioral study, no conclusions for this study could be made yet. However, future research may confirm the suggestion that tickling and especially gentling rats may positively affect animal welfare by improving the human-animal bond, lower the general fear of humans, decrease stress levels during manual restraints, and maybe lower chronic stress levels.

***Keywords**: Rats; Tickling; Gentling; Manual restraint; Stress-induced hyperthermia (SIH); Eye temperature; Tail temperature; Infrared thermography (IRT).*

***Abbreviations**: CONTROL, the group of rats that received no treatment; T, the group of rats that received the tickling treatment; G, the group of rats that receive the gentling treatment; IRT, infrared thermography; ANS, autonomic nervous system; SIH, stress-induced hyperthermia; ROI, region of interest; ANOVA, analysis of variance.*

2 INTRODUCTION

2.1 HUMAN-ANIMAL INTERACTION DURING LABORATORY PROCEDURES

Frequently, laboratory animals are used for experiments that involve certain handling and restraint techniques, for example, to take a blood sample or to place an injection (1). Physiological and emotional stress can arise as a result of such aversive experiences (2–5). These stress reactions are coping mechanisms (6), however, when stress responses are more frequent or long-lasting (chronic stress) it can affect the utility of animals in biomedical research by altering animals' metabolism, by interfering with the learning ability, cognition and behavior of the animals and by, therefore, limiting the external validity of the research data (7–9). Also, stress makes the animals more vulnerable to several diseases, delays the recovery from injuries, and will negatively affect animal welfare (7).

However, animals can also relate to human contact as a positive experience. For instance, studies in rats and rabbits suggest that these animals can quickly adapt to positive interaction with humans and that this can lower their fear of humans (8,10). Rats' social grooming appears to have a physiological effect on stress responses (7) and appears to be rewarding, even when it is performed by humans (3,11). Therefore, the stress associated with negative experiences may be reduced if the stressor is followed by a rewarding experience, such as gentle stroking (7,12).

Additionally, between conspecific animals, rats perform a rough-and-tumble play, where one participant is making contact with their partner's nape so the recipient will roll over to a supine position and the other one can stand on top of the recipient in a pinning position (13,14). The participants are taking turns being on top and beneath, where they especially take pleasure in the brief restraint and release phases in this play (13,15). Playful behavior works as a social buffer for the rats to reduce the intensity of a fear response and to provide a better recovery from stressful events. It is also socially rewarding and actively solicited by rats (16–18). Supposing humans can replace a conspecific, they can mimic this type of social play by using vigorous, rapid finger movements alternating between the rat's nape (dorsal contact) and ventral surface (pinning) (13). According to some authors (12,16,19–21), this 'tickling' performed by humans showed comparisons with the social buffer effect between conspecifics, where, amongst others, dopamine and opioids are released which are important factors to achieve the buffering effect. Therefore, tickling addresses the natural characteristics of social animals and may be used to reduce stress responses caused by standard laboratory procedures. This serves the principle of Refinement of the Three Rs of Russell and Burch (22) since less stress can increase the animals' well-being and can contribute to a better quality of research data. Additionally, this enables researchers to use fewer animals during experiments, addressing to the Principle of Reduction.

2.2 MEASURABLE STRESS PARAMETERS

To investigate the possible effect of positive interaction with humans, like tickling, on stress during laboratory procedures, it is important to apply a proper method to measure stress levels. In most experiments, acute stress in laboratory animals is measured by more or less invasive methods, like determining stress hormone levels from a blood sample. Stress hormones in rats, known as glucocorticoids and primarily corticosterone, are being released via the hypothalamic-pituitary-adrenal (HPA) axis and play an important role in the stress response (23). The same applies to catecholamines because they are released via the sympathetic-adrenomedullary axis when the sympathetic nervous system is activated. Therefore, corticosterone levels in blood and plasma catecholamine concentrations are often used to measure acute stress (24,25), although these methods are invasive and repeatedly taking blood samples from the animals may initiate stress hormone release by itself (26). Also, these hormone concentrations are unstable parameters due to their rapid change, plasma half-life time, and diurnal variation (27,28). This makes it a less reliable parameter to measure stress caused by only the experiment itself, apart from the stress that is induced during the blood sampling and altered by the time the sample was taken.

Core body temperature is also a parameter successfully used to indicate stress levels (29,30). Acute stress responses are linked to transient changes in, among others (31), the autonomic nervous system (ANS). This can lead to 'stress-induced hyperthermia' (SIH), measurable in the core body temperature. Non-shivering thermogenesis in brown adipose tissue (30) and an altered distribution of blood flow due to sympathetically-mediated peripheral vasoconstriction contributes to SIH via reducing heat loss and increasing core temperature (32,33). SIH is a physical reaction to the flight and fight mechanism since it ensures the blood flow to redirect to the muscles and CNS to boost physical and neurocognitive performance (30,33,34). Additionally, the peripheral vasoconstriction that occurs during SIH may be a precaution for possible blood loss at the most exposed parts of the body in case of injury (30). SIH occurs in various species, including rats (35). When used during medical studies, core temperature is mostly measured from the rectum by implanting a thermosensitive device (36). However, this technique is not preferable to use, due to the additional stress and temperature increase during and after the implantation process, and the altered physiology of the animal (37). Even so, determining parameters like heart rate and blood pressure require animal contact and may lead to the induction of a stress response and may, therefore, interfere with the results (38–41).

2.3 INFRARED THERMOGRAPHY

Since the focus on the refinement of experimental studies increases, alternative, non-invasive and objective methods to asses stress in laboratory animals are desired (37). Infrared thermography (IRT) is a tool to measure body temperatures and can be used to investigate stress levels in a non-invasive way. IRT is based on, for humans non-visible, infrared radiation which is, in this case, emitted by the surface of animals due to changes in peripheral blood flow (42–44). A higher subject temperature leads to a greater intensity of the infrared energy emitted. This type of radiation can be processed by infrared measuring devices that turn the intensity of the radiation into a colored image on a monitor, named a thermogram (37,45–47). Therefore, IRT can convert infrared radiation of an object into a temperature value. Due to real-time measurements, IRT can be used on fast-moving targets and can detect minimal temperature changes and fast-changing thermal patterns. Also, the radiation of IRT is harmless for the 'object' and is, therefore, safe and limitless to use, without interfering with the animals' behavior during recording (30,48). For these reasons, IRT has been suggested as an alternative tool that can be used for diagnostic and research aims in veterinary medicine and animal welfare research, such as measuring animals' surface temperature changes in response to stress (25,26,37,49,49–51).

2.4 EYE AND TAIL TEMPERATURE

IRT can be used for measuring rats' eye and tail temperature, as an indication of the core temperature. A stress-related rise in core temperature could be measured by changes in eye temperature since an altered blood flow may also be noticeable in the capillary beds of the conjunctiva (26). Further, the mainly cutaneous and well-vascularized tail can reflect the amount of peripheral vasoconstriction that occurs due to stress (30,37). When open, blood flow in the tail can increase enormously which can lead to a rise of more than 10°C in tail temperature (52). Furthermore, besides identifying the acute stress, measuring tail temperature may also quantify the stress levels and, therefore, measure the intensity of the stressor (53). Further, eyes and tails can be convenient targets to measure temperature via IRT since the eyes and tail lack fur and are easily accessible, where the tail has a large surface which accounts for approximately 7% of the body surface (52). Therefore, differences in the eye and tail temperature can theoretically indicate the ANS activity and could be used in the assessment of stress and animal welfare (26).

The use of IRT for these purposes is investigated in several species, especially in horses. However, the number of studies in this area is still limited, even more in laboratory animal science. In some of the available studies, eye temperature showed significant and positive correlations with stress indicated parameters such as cortisol or corticosterone, core body temperature, heart rate, and stress-related behaviors and is generally confirmed as a reliable indicator of stress in different species (6,38–41,54–57). On the other hand, a study in cattle (58) showed no association between eye temperature and HPA axis activity and there are even some controversial results found in eye temperature response to stress in sheep (59). Peripheral body temperature changes due to stress have also been little investigated. However, a study in rabbits (6) detected a decrease in ear temperature, even as Vianna and Carrive (2005) (30) observed a drop in paw and tail temperature of rats in stressful conditions. Also, the nasal temperature of rhesus monkeys and the temperature of the fingers of humans showed a correlation between a negative emotional state and a decrease in temperature of the extremities (60,61).

2.5 LABORATORY RATS FOR PRACTICAL LESSONS

In this experiment, a group of rats was studied that experienced repeated stress due to their use in practical lessons for inexperienced veterinary students and young researchers practicing manual restraints. To improve animal welfare (principle of Refinement) and to expand the usage time of

these animals (principle of Reduction), this pilot study aimed to investigate the effects of positive interaction with humans, like tickling and gentling, on acute stress levels of the rats. We hypothesized that during stressful circumstances eye temperature will rise, while the tail temperature will decrease. Additionally, rats that have been tickled and gentled every week will be less stressed during a stressor, where tickled rats will show the most reduction in their stress levels. To measure stress responses without interfering with the results, IRT was used as a non-invasive tool to measure SIH via eye temperature (experiment 1) and tail temperature (experiment 2).

3 MATERIALS AND METHODS

3.1 ANIMALS

A group of 16 male Wistar rats (WU:Crl; Charles River, Germany) with an age of 3 weeks at arrival in the lab were used for this study. The animals were housed in groups of four in Eurostandard type IV S cages (480 x 375 x 210 mm; Floor area: 1500 cm^2) with wood shavings as bedding and a transparent orange shelter (151 x 90 x 90 mm) and paper tissues as enrichment and nesting material. The animals were kept under a 12-hour day-night cycle with lights on between 07.00 h and 19.00 h and with controlled temperature (22.0°C ± 0.6°C) and humidity (48.9% ± 5.4%) in the room. The rats had *ad libitum* access to food (Rat/Mouse maintenance, 10 mm, Ssniff Spezialdiäten GmbH) and tap water and the cages were changed once a week. Radio music was on as background noise (channel Q-music, the Netherlands).

The animals were used for educational purposes in the same period this experiment took place. They were used during practical lessons teaching handling and restraint techniques, and restraint and basic techniques (oral gavage). They were also used in two lessons teaching behavioral studies. During one of these lessons, which only took place three times in the first week of arrival, the rats were injected with Methylphenidate to investigate their social behavior while under this drug. The lessons were participated by first-year bachelor and master students of the study veterinary medicine and by students of the International laboratory animal science course for researchers (FELASA A & B).

This experiment was performed within an education project that was approved by the Central Animal Experiments Committee of Utrecht University (CCD approval: AVD1080020171926).

3.2 CAMERAS

A FLIR T430sc thermal camera was used to record thermal videos of the animals. The camera was situated in the test room at a distance of 160 cm above the cage. To obtain accurate results, the camera was turned on at least five minutes before the start of the measurements of experiment 1, as recommended by FLIR. During experiment 2 the camera was turned on exactly 1,5 hours before the first measurement of that day began. Non-uniformity correction (NUC) was automatically performed by the camera during recording. The FLIR ResearchIR program was used to process the recording whereby five frames per second were recorded and, during experiment 2, the focus was manually adjusted to the tail level. Room temperature and humidity of the room were determined before the start of recording and were included in the object parameters of the FLIR ResearchIR program afterwards, during the video analysis. Additionally, the room temperature was used as an estimate for the reflected temperature, since the experiment included moving animals who weren't allowed to be disturbed by measuring tools such as mirrors, which would be needed to determine the reflected temperature more precisely (62). The emissivity was set to 0.98 (30).

A Sony Handycam camera (during experiment 1) and a Bascom camera (during experiment 2) were placed next to the FLIR T430sc thermal camera to record a real-time video of the rats to visually differentiate them by the number on their tail. Additionally, these videos will be used later to perform behavioral observations as an addition to this study. This will include the scoring of stress-related behavior, such as freezing (63,64) and stress-evoked grooming (65–67).

3.3 EXPERIMENTAL PLAN

3.3.1 Animals' habituation and treatment

At arrival, the rats were randomly divided into four groups of four rats per cage. The rats were habituated every day from arrival for two weeks by taking the lid of the cage and holding a hand in the cage for 5 minutes. After the habituation period, the treatment was started. Each cage was assigned to a different treatment: cage 1 received no treatment (CONTROL, *n* = 4), cage 2 and 3 received the tickling treatment (T, *n* = 8) and cage 4 received the gentling treatment (G, *n* = 4). Treatments were given to randomly assigned cages by, in total four, randomly assigned experimenters. A treatment per cage consisted of one minute of habituation to the hand, followed by seven-minute treatment time. Tickling was performed based on the method of LaFollette in Cloutier et al. (2018) (68). A 2-4 second pounce was performed by touching the rat's dorsal surface of the neck with rapid finger movements. Then the rat was turned over and a pin was performed by using rapid, vigorous, but gentle finger movements on the belly. Pinning stopped when the rat turned himself around again. For the gentling treatment, each rat was briefly lifted by the experimenter by scooping or gripping the whole body from underneath, to prevent a possible threatening movement. The rat was allowed to sit on the experimenter's hand till it left by himself. For both treatments, the experimenter continuously alternated between all rats in the cage to equally divide the treatment time. Also, no gloves were worn when in contact with the animals. The control group did not receive any treatment and stayed in the wall rack situated in the same room. Treatments were given 5 times a week in the beginning and were gradually lowered to 3 times a week, continuing on this frequency for the rest of the study period (see Figure 1). The treatments took place between 9.00 h and 17.00 h, on different days, and when possible well spread over the week and before the practical lessons. Treatments were given in the rats' own cages, but the location differed between their housing location and the test room, depending on their use during the practical lessons.

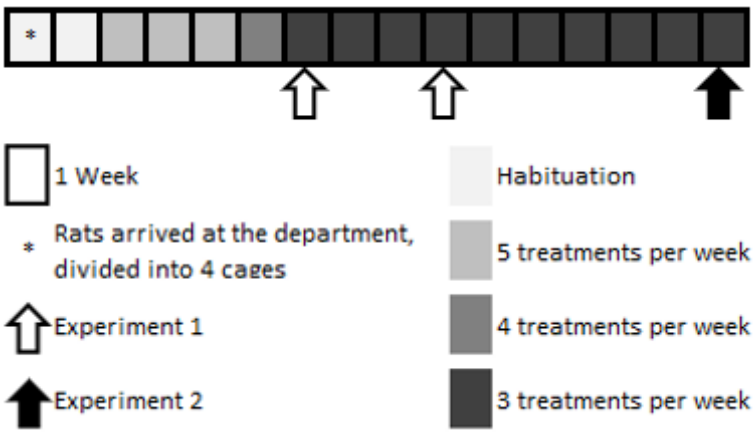

Figure 1 The timeline of the study period from the rats' arrival in the lab, at an age of 3 weeks. A habituation period of two weeks took place before the treatments began. The frequency of the treatment started from 5 times a week and was gradually lowered to 3 times a week. During experiment 1 consisting of two measurements, the rats were treated for 4, respectively 7 weeks. Experiment 2 took place after a period of 13 weeks of treatment.

3.3.2 Experiment 1, eye temperature

Two measurements for eye temperature were performed in experiment 1 during non-standardized practical lessons for students. An overview of the measurements in the timeline of the study is shown in Figure 1. Per measurement, for two days in a row, two cages per day were measured between 15.30 h and 17.00 h. The cages were semi-randomly assigned to a day and time, where each cage was used once as the first cage and once as the last cage in the practical lessons. Recordings started when the cage was placed underneath the cameras, at least five minutes before the treatment started, and continued when possible till 30 minutes after the end of the practical lesson. The manual restraints during the practical lessons were performed by 2-6 unfamiliar students, blindly assigned to the cage. Lid, shelter, and tissues were removed during recording and 40 cm-tall plexiglass walls were put on the home cage to prevent escaping, except during the treatment and the manual restraints. The room had a temperature of 22.0°C and the humidity was 23.0% during the first measurement and 17.0% during the second measurement. During measurement 2, the autofocus was adjusted several times during the experiment.

3.3.3 Experiment 2, tail temperature

Experiment 2 investigating tail temperature took place during a standardized study, 13 weeks after the start of the treatment (see Figure 1). The measurement took place between 12.00 h and 16.00 h, divided over two consecutive days. Cage 3 (T) and 4 (G) were measured on the first day, and cage 1 (CONTROL) and 2 (T) were measured on the second day. The rats were recorded in the test room from their home cages whose lid was replaced by 40 cm-tall plexiglass walls and from which all enrichment was removed. The radio was on the same channel as at their housing location. The room had a temperature of 20.0°C and on the first day, the humidity was 17%, on the second measuring day it was 20%. No treatment took place on the day of the experiment and the cages were changed on the day before the first measurement day. On the first measuring day around 11.30 h, the cleaning of other animals with the same housing location took place.

Before the start of the experiment, after arrival to the test room, the rats were habituated to the place under the camera for 30 minutes. After this period, a ten-minute pre-stressor time took place to obtain the baseline values of the rats' tail temperatures. To prevent the possibility that the rats' tails were not visible at the fixed timepoints for obtaining the measurements, the rats were awakened at fixed moments. This was performed by the same experimenter each time (exp1), by leaning over the plexiglass walls and gently and moving the rats individually for a few inches. This was performed at minute 0, minute 5 and minute 10 of the pre-stressor period (see Figure 2). During the last wake up, three other experimenters with manual restraint experience gathered around the cage, and with that, the stressor period started. After 15 seconds of habituation to the hand of exp1, the four rats were picked up in turn by exp1 and were given to the other experimenters. In total, six manual restraints were performed by the experimenters as a negative stressor, where all rats were handled by the experimenters in a pre-determined rotation system. Per restraint, at the same time, the experimenters run through the same phases of the restraint technique (as taught in the Laboratory Animal Science course of Utrecht University) twice, which lasted for 45 seconds. In between the restraints and after the last restraint, the rats were put back in their cage for a resting period of 45 seconds. After the stressor period, which lasted 9.45 minutes in total, the post-stressor period started in which the rats were filmed for a total of 30 minutes. Exp1 woke the rats in this period at minute 0, 5, 10, 20 and 30 in the same way as during the pre-stressor period. Two minutes after the last wake up the recording stopped. The timeline of this experiment including the phases, measurement moments, and the wake-ups is shown in Figure 2.

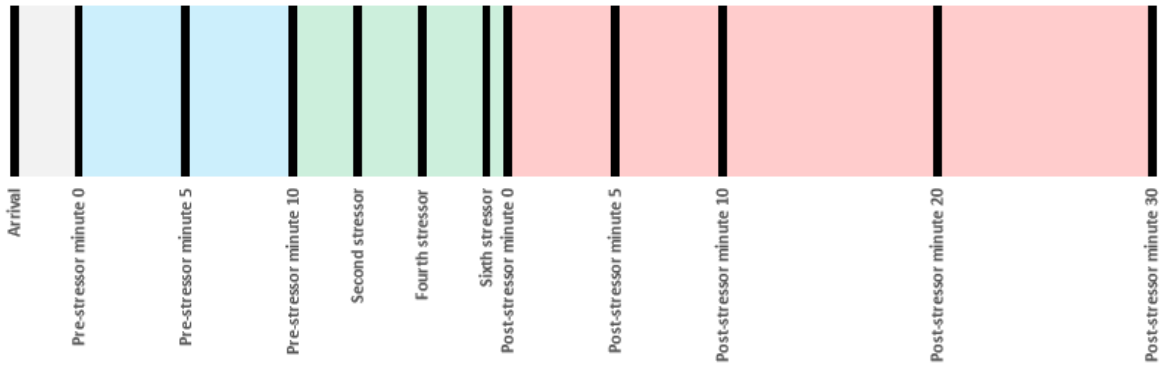

Figure 2 The timeline of experiment 2. Thirty minutes of habituation took place before the start of the experiment (grey). The experiment existed of three phases: the pre-stressor period (blue), the stressor period (green) and the post-stressor period (red). During the pre- and post-stressor period, before each fixed measurement moment (black vertical line, including the relative titles as a description of the measurement moment) the rats were awakened. During the wake-ups, the experimenter moved the rats by gently lifting them for a few inches to place them in another corner of the cage to be sure that the tails were visible during the analysis of the measurement moments in the thermal video. During the stressor period, the rats were simultaneously manually restraint six times by four different experimenters. This period had a total duration of 9.45 minutes, included the resting moments after each restraint.

During the pre- and post-stressor period, the experimenters were not present in the test room. During animal contact, no gloves were worn and the experimenters washed their hands in between the different cages and made sure they did not have cold hands.

3.4 THERMAL VIDEO ANALYSIS

3.4.1 Maximum eye temperature

In each phase of the experiment, different one-minute timepoints (measurement moments) containing 300 frames of thermal video, were selected to collect the maximum eye temperatures per rat (see Table 1). Only usable frames were included in the data, which were selected on specific criteria. Usable frames contained almost equal visible eyes. For that, the rat had to look a bit upwards, holding his head straight but was not allowed to stand on his hind legs. Also, when the rats were lying on top of each other, no eyes could be measured. Further, due to blurriness, rats that were located in the bottom right corner, may not be measured. Examples of usable and unusable frames are shown in Appendix 10.1.

Table 1 Fixed measurement moments to assess the eye temperatures for experiment 1. Each measurement moment of one minute consisted of 300 frames. The frames were evaluated on their usability, wherein the usable frames, the maximum eye temperature was gathered via a box ROI using the FLIR ResearchIR program.

Phase	Measurement moment (minutes)
Habituation	0-1, 4-5, last minute
Treatment	5-6, last minute
Between	12-13, last minute
Practical lesson	11-12, last minute
After	15-16

When a measurement moment did not contain any (or less than 10) usable frames, the measurement moment was extended or a new timeframe was chosen if possible and with a maximum of 600 frames around the original timepoint. To gather the maximum eye temperature in a usable frame with the FLIR ResearchIR program, a box ROI (region of interest) was placed around both eyes of the rat in question to obtain the maximum temperature (see Figure 3).

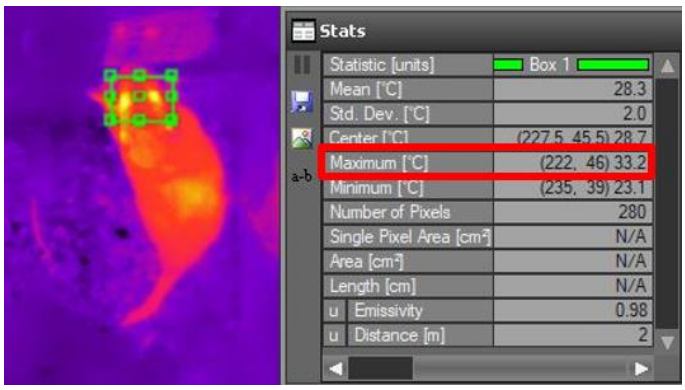

Figure 3 Measuring maximum eye temperature with a box ROI in the FLIR ResearchIR program.

3.4.2 Mid-tail temperature

For each measurement moment, shown in Table 2, a frame was selected in the video close to this timepoint and when possible a frame where all four tails were measurable. Otherwise, 2-4 frames were selected around this point to gather all tail temperatures. In this study, the middle section of the tail was measured, similar to the method of Vianna and Carrive (2005) (30), because the use of the tail base can cause bigger variances, as seen in Gjendal et al. (2018) (37). Before a mid-tail temperature could be gathered, the total length of the tail of the rat in question was determined in the selected frame using a bendable line ROI. After that, the highest tail temperature at half the length of the rat's tail was measured using a 1-pixel cursor ROI, see Figure 4. A more detailed protocol is included in Appendix 10.2.

Table 2 Fixed measurement moments to assess the tail temperatures for experiment 2. Each measurement moment consisted of 1 frame per rat. The mid-tail temperature was gathered using a 1-pixel cursor ROI to measure the highest tail temperature at half of the length of the rat's tail, which was determined using a bendable line ROI.

Phase	Measurement moment (minutes)
Habituation	-
Pre-stressor	0, 5, 10
Stressor	After the second, fourth and sixth restraint
Post-stressor	0, 5, 10, 20, 30

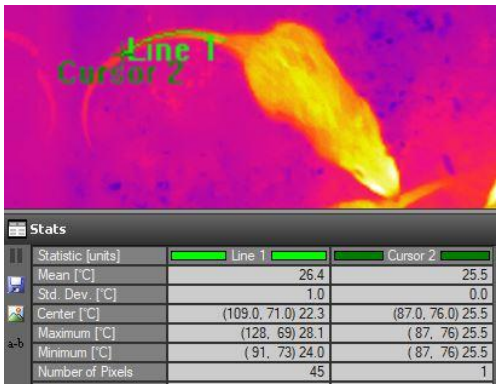

Figure 4 Measuring mid-tail temperature with a bendable line ROI and a 1-pixel cursor ROI in the FLIR ResearchIR program.

3.5 Welfare assessment

Welfare parameters were measured once a week, when possible on the first day of the week and when possible directly after the treatment. All rats were scored on body weight, body condition (69), body posture, fur condition, and the porphyrin level to observe their well-being. To minimize the disturbance of the rats, these evaluations were performed as quickly as possible.

3.6 STATISTICAL ANALYSIS

Experiment 1 was not statistically analyzed due to the large differences in circumstances during the experiment and the large variance in eye temperatures within the measurement moments for the individual rats. For the statistical analysis of experiment 2, the tail temperatures of all measurement moments of all individual rats were imported in the dataset and each rat was labeled by their assigned treatment group (CONTROL, T, G). All statistical analyses on the data were performed using the IBM SPSS Statistics 25 software program. Statistical significance (alfa) was set at p <.05.

Parametric statistical tests were performed on all data. For these statistical tests, assumptions had been met since the dependent variable was continuous, the independent variable consisted of three categorical independent groups, and there were no potential outliers, assessed with box plots. The normality assumption was assessed using a Sharpio-Wilk test, because of the small sample size. All data proved to be normally distributed, except for measurement moment "Post-stressor minute 0" (p = .006) and "Post-stressor minute 5" (p = .015) of the control group. The visual interpretation of quantile-quantile (Q-Q) plots did support the normality assumption. And since the Analysis of Variance (ANOVA) as a parametric test is quite robust to violations of normality, parametric statistical tests were still used. For the performed One-Way Repeated-measures ANOVA, the assumption of sphericity was assessed with Mauchly's test of sphericity. This test indicated that this assumption had been violated ($\chi^2(54) = 191.34$, $p < .001$). Therefore, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε = .26). Furthermore, for the One-Way Independent ANOVA, the assumption of the independence of the observations was met since the individual rats were used only in one treatment group. The assumption of homogeneity was not met since the homogeneity of variances was violated according to Levene's test for the measurement moment "After second stressor" (p = .012), "After fourth stressor"(p = .024), and "Post-stressor minute 30 - Pre-stressor minute 10" (p = .048). Therefore, the Brown-Forsythe test, because of the small sample size, was conducted for these two measurement moments.

The general effect of the treatment group on the tail temperature during the experiment was analyzed using a One-Way Repeated-measures ANOVA, whereby the within-subjects variables were the different measurement moments and the between-subjects factor was the treatment. The effect of treatment on tail temperature during stress for each measurement moment separately was analyzed using a One-Way Independent ANOVA, whereby the dependent factors were the different measurement moments and the independent factor was treatment. Three additional delta measurement moments were tested by the One-Way Independent ANOVA, where the decrease of tail temperature after the stressor period was compared between the treatment groups ("Pre-stressor minute 10" - "After sixth stressor"). Also, the difference between the tail temperature after the stressor period and at the end of the experiment ("Post-stressor minute 30" - "After sixth stressor") and the difference between the tail temperature before the stressor and at the end of the experiment ("Post-stressor minute 30" - "Pre-stressor minute 10") were tested as delta measurement moments.

Because of a specific hypothesis, planned contrasts between CONTROL and G, CONTROL and T, and G and T were performed in case of significant effects. For the measurement moments "After second stressor" and "After fourth stressor" the p-value of the "Does not assume equal variances" was used. For the other (homogeneity) measurement moments, the p-values of the "Assume equal variances" were used.

Plots were executed for the One-Way Repeated-Measures ANOVA for the Mean tail temperature by Treatment and the Mean tail temperature by Moment by Treatment.

4 RESULTS

4.1 MAXIMUM EYE TEMPERATURE, EXPERIMENT 1

The mean maximum eye temperature per measurement moment for the treatment groups and the individual rats is shown in Figure 5. One rat from CONTROL was excluded from the database due to non-measurable eyes since he often buried his head in the wood shaving bedding. No statistical analysis was performed on the data of experiment 1, due to the great individual variance noted (to illustrate this, see individual data in Figure 5B) in in the large standard deviation and the large difference in the time of the different phases of experiment 1. Additionally, there was a large number of missing data. Of almost every measurement moment, at least one rat, and sometimes an entire cage, was not measurable, and in many cases, rats had to be measured at other timeframes than determined in advance.

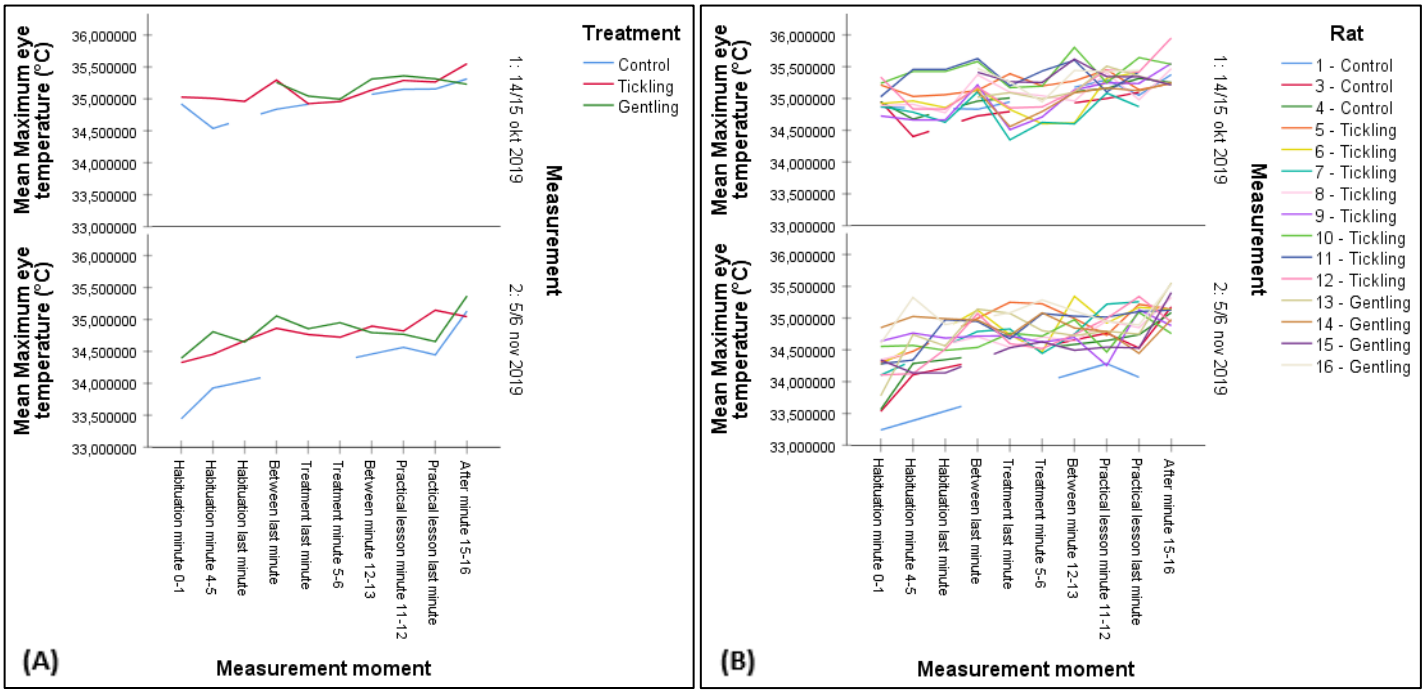

Figure 5 Maximum eye temperature during different timepoints of experiment 1 (see Table 1) vs. the treatment groups (A), and the individual rats (B). Top: results from measurement 1, performed 4 weeks after the start of the treatment. Bottom: results from measurement 2, performed 7 weeks after the start of the treatment.

4.2 MID-TAIL TEMPERATURE, EXPERIMENT 2

One-Way Repeated-measures ANOVA

There was no significant interaction effect between the measurement moments and treatments on the tail temperatures, $F(5.19, 33.76) = 1.08$, $p = .39$. Therefore, the main effects of the measurement moments and treatments were included. The analysis revealed a main effect of the measurement moments on tail temperature, $F(2.60, 33.76) = 32.97$, $p < .001$. No main effect of the treatment on tail temperatures was found, $F(2, 13) = 3.46$, $p = .063$, see Figure 6.

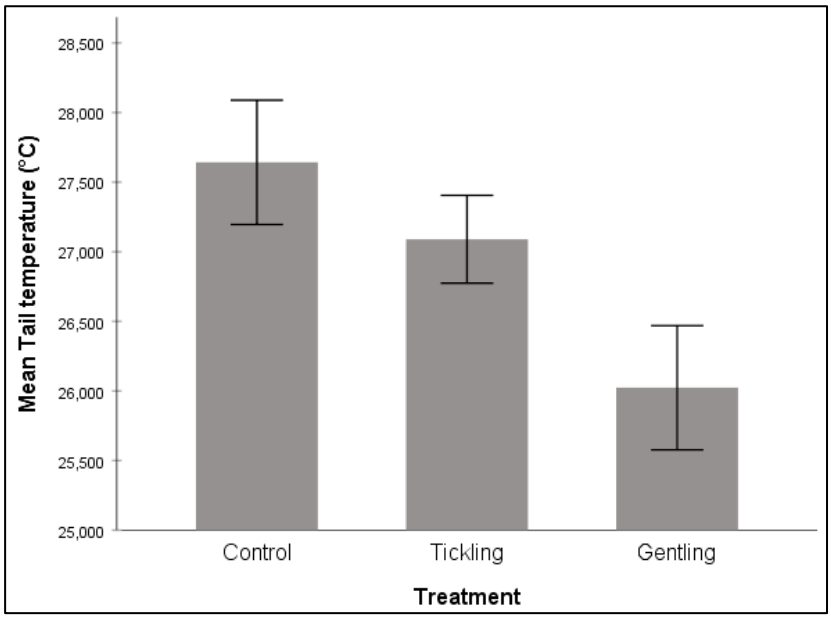

Figure 6 The effect of tickling and gentling on mean +/- SEM. Mid-tail temperature during experiment 2.

One-Way Independent ANOVAs

A significant difference in tail temperature between treatment groups was found for the measurement moment "Pre-stressor minute 10", $F(2, 13) = 4.67$, $p = .030$, and for the delta measurement moment "Pre-stressor minute 10 - After sixth stressor", $F(2, 13) = 4.75$, $p = .028$. Significance was found for the measurement moment "After second stressor", $F(2, 11.70) = 10.62$, $p = .002$, and for the measurement moment "After fourth stressor", $F(2, 10.19) = 10.06$, $p = .004$, with the Brown-Forsythe test. Other measurement moments revealed no significance. All results are shown in Table 3.

Table 3 Descriptives and results of the One-Way Independent ANOVAs of the individual measurement moments for each treatment group. Significance ($p < .05$) was found for the measurement moments "Pre-stressor minute 10", "After second stressor", "After fourth stressor", and for "Pre-stressor minute 10 - After sixth stressor". Since the assumption of homogeneity was not met for the measurement moments "After second stressor", "After fourth stressor", and "Post-stressor minute 30 - Pre-stressor minute 10", the Brown-Forsythe test results were used for these measurement moments, indicated with "◊".

Measurement moment	Group	N	Mean (°C)	SD	df1,df2	F	Sig.
Pre-stressor minute 0	CONTROL	4	31.593	0.885	2, 13	3.32	.16
	T	8	30.585	1.530			
	G	4	29.777	0.796			
Pre-stressor minute 5	CONTROL	4	31.000	1.794	2, 13	2.53	.12
	T	8	29.284	2.332			
	G	4	27.842	1.118			
Pre-stressor minute 10	CONTROL	4	32.404	1.286	2, 13	4.67	**.030**
	T	8	30.511	2.384			
	G	4	28.314	0.809			
After second stressor	CONTROL	4	28.138	0.655	2, 11.70 ◊	10.62 ◊	**.002** ◊
	T	8	27.040	1.446			
	G	4	25.324	0.461			
After fourth stressor	CONTROL	4	25.597	0.352	2, 10.19 ◊	10.06 ◊	**.004** ◊
	T	8	25.300	0.987			
	G	4	23.990	0.217			

After sixth stressor	CONTROL	4	24.501	0.341	2, 13	2.36	.13
	T	8	24.152	0.679			
	G	4	23.652	0.392			
Post-stressor minute 0	CONTROL	4	24.558	0.174	2, 13	1.47	.27
	T	8	24.285	0.699			
	G	4	23.922	0.180			
Post-stressor minute 5	CONTROL	4	24.493	0.791	2, 13	.57	.58
	T	8	23.896	0.973			
	G	4	23.936	1.028			
Post-stressor minute 10	CONTROL	4	25.801	1.850	2, 13	1.01	.39
	T	8	26.793	3.317			
	G	4	24.539	0.621			
Post-stressor minute 20	CONTROL	4	26.707	2.942	2, 13	.28	.76
	T	8	27.320	3.048			
	G	4	28.203	2.289			
Post-stressor minute 30	CONTROL	4	29.273	4.298	2, 13	.89	.44
	T	8	28.823	2.768			
	G	4	26.760	0.895			
Pre-stressor minute 10 - After sixth stressor	CONTROL	4	7.903	1.002	2, 13	4.75	**.028**
	T	8	6.359	1.838			
	G	4	4.662	0.839			
Post-stressor minute 30 - After sixth stressor	CONTROL	4	4.772	4.245	2, 13	.43	.66
	T	8	4.671	2.908			
	G	4	3.108	1.013			
Post-stressor minute 30 - Pre-stressor minute 10	CONTROL	4	-3.131	4.835	2, 6.08 ◊	.26 *	.78 ◊
	T	8	-1.688	4.157			
	G	4	-1.554	0.267			

The planned contrasts between CONTROL and G in combination with Figure 7, showed that G had a significantly lower tail temperature compared to CONTROL for the measurement moment “Pre-stressor minute 10”, *t*(-3.05), *p* = .009 (2-tailed), for the measurement moment “After second stressor”, *t* (-7.02), *p* = .001 (2-tailed), for the measurement moment “After fourth stressor”, *t*(-7.76), *p* = .001 (2-tailed), and for the delta measurement moment “Pre-stressor minute 10 - After sixth stressor”, *t*(-3.08), *p* = .009 (2-tailed). The contrasts between CONTROL and T showed there was no significant different tail temperature between these groups. The last contrast between T and G revealed that G significantly decreased their tail temperature compared to T for the measurement moment “After second stressor”, *t*(-3.06), *p* = .013 (2-tailed), and for the measurement moment “After fourth stressor”, *t*(-3.59), *p* = .007 (2-tailed).

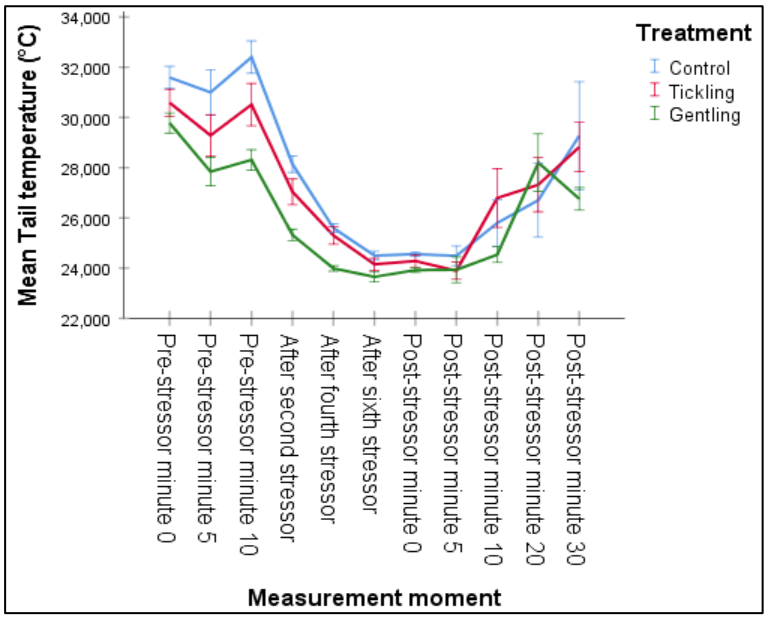

Figure 7 Mean +/- SEM. Mid-tail temperature during different timepoint of experiment 2 (see Table 2) vs. the treatment groups.

Overall, the tail temperatures of all individual rats during the experiment varied between 22.66 and 33.48°C.

4.3 Welfare assessment

All rats had a normal body posture, a well-groomed fur and showed no piloerection during this study. The rats' body weight increased every week, confirm expectations considering their age. Body condition scores stayed in conformity with the standard. The porphyrin levels altered between no pigment visible to a slight presence of red pigment on the eyes and/or nose.

5 Discussion

The purpose of the experiments was to establish whether tickled or gentled rats from a young age experienced less stress from manual restraints than rats without additional human contact. We expected that gentled and/or tickled rats react less severe to the restraints due to the social buffer effect and the improved positive correlation with humans. For this aim, eye temperatures of the rats were assessed using an infrared camera in response to manual restraints practiced by inexperienced students during practical lessons. Although eye temperatures were expected to increase in response to this stressor, the data subtracted from these measurements turned out to be unusable for statistical analyses due to the incomparability of the treatment groups during this experiment. Gjendal et al. (2018) (37) and Vianna and Carrive (2005) (30) also used rats' tail temperatures to assess stress levels, therefore, a second more standardized experiment was designed using tail temperatures to measure the rats' stress responses. In this experiment, the main differences in tail temperature were found between the baseline temperatures of the treatment groups, where the group of the gentled rats (G) had the lowest baseline tail temperature and the rats from the control group (CONTROL) had the highest temperature. The baseline temperature of the group of the tickled rats (T) lay in between G and CONTROL. However, the tail temperatures of all groups decreased to approximately the same temperature near the end of the stressor period.

5.1 MID-TAIL TEMPERATURE

5.1.1 The effect of the stressor

During the stressor period of experiment 2, all rats' tail temperatures decreased in response to the repeated restraint, regardless of the treatment group they had been assigned to. This strongly suggested that all rats did experience stress because of the manual restraints and, consequently, dropped their tail temperature which was well measurable with the infrared camera. This was supported by the subjective behavioral observation of the experimenters during the experiment. The experimenters noted that all cages showed severe signs of stress like freezing and emitting audible calls. Further analysis based on objective behavioral scoring in the future will reveal if this is indeed the case. In the experiment of Vianna and Carrive (2005) (30) a large drop in tail temperature was also found as a reaction on a stressor, supported with a behavioral study and other additional stress parameters.

It is visually noticeable that also in the pre-stressor period a drop in tail temperatures occurred (see Figure 7, measurement moment "Pre-stressor minute 5"). Possibly, the rats experienced some stress at the time the experimenter woke them, which led to a drop in tail temperature. It can be due to habituation that the second and third wake-ups seemed less stressful, as the rise at "Pre-stressor minute 10" suggested. However, it must be taken into account that the temperature that was used as a baseline may, therefore, be lower than it would have been when no interference took place in the pre-stressor period. This may cause an altered outcome of the trend in the decrease of the tail temperatures, especially in the case of G, because these rats' tail temperatures rose less after the second wake up compared to the rats of the other groups. This group likely experienced a high state of arousal during the wake-ups, since this can induce SIH and consequently lower tail temperature. However, SIH happens independently of the state of valence, which can be positive or negative (70–72). Therefore, a behavioral study might confirm the kind of valence the rats experienced. It may also enlighten possible other explanations for the different trends in temperature, like a recent playful interaction between the rats or a fearful response to something that happened in or around the cage.

Furthermore, there was a large variance in the tail temperatures within the treatment groups during the pre-stressor period (see Table 3 for the SD). This may show that in general, the basal tail temperature can differ a lot within and between individuals. Within individuals, core temperature, and, therefore, possibly tail temperature, fluctuate throughout the day on a circadian rhythm (73,74) and spontaneous minor fluctuations in rats' tail temperature can also occur (52). However, basal body temperature can differ between individuals too. This difference could be explained by inter-individual variation in core temperature, since different individuals regulate core temperature to different setpoints, as already demonstrated in humans (75). Also, a congenital or acquired ability to cope with stress may explain interindividual differences. The rats differed from each other on a genetic level since they were outbred rats. Also, epigenetic effects could provide for differences in the vulnerability to stress (76,77). Further, inter-individual differences may also be explained by the fact that all rats were used for the practical lessons during this study too. It may be possible that certain rats received more, or more rough, manual restraints than others. Furthermore, when comparing the individual rats, it must be taken into account that the tail temperature may also differ due to external circumstances. Since the rats were housed together, the temperature of the rats' tails may be affected by the contact they had with each other. Sometimes, all rats were lying on top of each other and sometimes they were lying alone in a corner of the cage. This could interfere with the temperatures measured from the videos. For this reason, behavioral analysis of the video material may give more information to correctly interpret individual differences. To exclude the effect of social housing on the tail temperature in a future experiment it is suggested to house the rats solitary. Besides, Cloutier et al. (2018) (68) described a higher positive effect of tickling when rats

were housed individually, compared to group-housing, which may be due to play deprivation. Since the rats in this experiment were housed with mates, there may be only a little benefit of the treatments. However, housing the rats solitary conflicts with the research aim to improve animal welfare. Therefore, including a habituation period for each rat individually to test room may enable the isolation of the rats during the experiments itself, without causing additional stress.

The (inter-)individual variance in tail temperature disappeared during the stressor period, in which all tail temperatures dropped to a certain range of temperatures. This suggests that there is an absolute minimum value for the tail temperature. This 'lowest point temperature' is in agreement with Vianna and Carrive (2005), who phrased that skin (and tail) temperature could only variate between room temperature and skin blood temperature (± 34°C) (30,78). However, the rats from this experiment did not drop their tail temperature down to the actual room temperature but only came nearby that point. It is not clear yet if the tail temperatures would have decreased even more when the stressor period lasted longer. Further, when visually inspecting Figure 7, it can be noted that the tails of the treatment groups did not decrease to the exact same temperature, although significant differences were not found. When analyzing the different cages instead of the different treatment groups (see Appendix 10.3), it could be noticed that the lowest point temperature of cage 1 and 2, and of cage 3 and 4, were nearly similar. The difference between these sets of groups was the day they were measured. During the second measurement day (cage 1 and 2), the room temperature was mainly 20°C but did fluctuate between 20 and 21°C throughout the experiment, suggesting the room temperature may have been higher than during the first measurement day (cage 3 and 4). Also, a difference of 3% in humidity was found between the days. This may explain the slightly higher lowest point temperature of cage 1 and 2 compared to cage 3 and 4. To prevent this non-conformity, future research needs to make sure the room temperature and humidity are controlled in the test room.

5.1.2 The effect of tickling and gentling

Despite the not significant main effect of the treatments on tail temperature, there was a good possibility that when sample sizes increased, a significant effect may be found since the result was marginally significant (p = .063). However, these almost significant differences were probably solely found because of the large differences in tail temperature before the stressor started. The main differences in the baseline temperatures were found between CONTROL and G. As discussed above, a higher baseline may exist due to interindividual differences. On the other hand, it is investigated that a higher general core temperature is seen in animals with chronic stress (34,53,73,74,79). However, the impact of the chronic elevated basal core temperature on the skin and tail temperate is hardly investigated where it may cause core heat to either preserve or dissipate (32,53,74,79). Both effects were seen in earlier experimental outcomes of humans. For example, a lower peripheral temperature was seen in a lower finger temperature of depressed humans (80), while a relatively higher skin temperature was found in humans experiencing chronic stress due to the chronic fatigue syndrome (81). In rats, it is very likely to adopt that the general tail temperature is higher when the rats are chronically stressed since the tail is the main part of the body used to dissipate heat from elevated core temperatures (30). This may suggest that CONTROL might have experienced a higher level of chronic stress in general compared to T and G, although additional parameters for chronic stress, like core body temperature (29,30), basal hormone level (82), or expression of corticosteroid receptors in the brain (83), are necessary to confirm this.

The trend of the drop in tail temperature during the stressor period may also indicate the level of stress the rats experienced during the manual restraints. However, a comparison in the slope of the drop cannot be made since all groups started from a different baseline and the tail temperatures did not decrease linearly. Therefore, it cannot be stated yet if the tail temperature would decrease in the same pattern irrespective of the baseline temperature. More measurement moments are needed to obtain a more detailed view of this pattern and to be able to compare the slopes of the drops.

Moreover, the recovery during the post-stressor period may distinguish differences between the groups in the rapidity of de-arousal after the manual restraints. However, no significant differences were found between the tail temperatures of the treatment groups. When comparing the trend during the recovery with other experiments that used tail temperature as a parameter for stress, like that of Vianna and Carrive (2005) (30), no rebound effect was seen since the temperatures did not rise beyond the baseline temperature (see Table 3, "Post-stressor minute 30 - Pre-stressor minute 10"). This small recovery in our experiment might be explained by the fact that the rats could still experience a conditioned adversity and stress response to the hand of the experimenter during the wake-ups since the rats were picked up in the same way as when they were picked up for the manual restraint. However, the tail temperature did rise a bit, suggesting the stressor was not equally severe as the restraints and a small recovery did happen. It is necessary that, in a future study, safer recovery circumstances are provided, since tail temperature would only increase after stress when rats are 'returned' to a safe environment (30). Also, tail temperatures must be interpreted in combination with a behavioral study to distinguish between different states of valence, as mentioned before.

The experimenters noticed that CONTROL showed more signs of distress during the practical lessons, which fits with the previously mentioned hypothesis. However, the rats did seek human contact outside of the practical settings (personal observation). Further evidence is needed to confirm this and to investigate how this effect is caused. It can be hypothesized that these rats also associated human appearance with a positive valence since the treatments of the other cages were given in the same room. This effect may have originated from ultrasonic sounds emitted by the other rats (84) or circulated odors (85,86). In another experiment (8), the control group showed also more tameness over time, however, the authors of this study linked it to the habituation of the repeated tests and an age-related effect.

On the other hand, the personal observation of the experimenters revealed also that the tickling treatment sometimes had a negative effect on the rats, in contrast to the literature. Especially near the end of the study period, T seems to show only little interaction with the experimenters and they even showed some freezing behavior during the treatment. This might be explained by the fact that in this experiment, the pinning phase did not stop until the rat turned himself around, wherein the protocol of LaFollette in Cloutier et al. (2018) (68) the pinning was kept brief (2-4 seconds) to minimize negative responses. Also, stress may have affected how the rats responded to their treatment (68) and since the treatment was sometimes given at the practice room, it may, therefore, be experienced as more stressful.

External factors could also have interfered with the outcomes. For instance, the analysis of the video material was solely performed by one experimenter. Fault by human error may have led to less reliable results. Also, the tail temperature was only measured at a few timepoints during the experiment and a measurement moment existed only of one frame. Deviation from the true (mean) tail temperature by fluctuations, as mentioned before, may therefore have altered the outcomes.

As a final point, it is not clear yet whether stress levels of rats showing the same lowest point temperature are comparable, or that tail temperature is a limited parameter when comparing different levels of severe stress. Therefore, it may be true that, despite the nearly similar lowest point temperature of all rats, there were still differences in stress levels between the treatment groups. However, other parameters to measure stress levels are needed to enlighten this.

5.2 EXPERIMENT 1

Experiment 1 in which maximum eye temperature was measured turned out to be unusable for comparing stress levels due to disturbances. Firstly, a main disturbance was found in the time-dependent effects of stress on temperature. Because measurements were taken before, during and after practical lessons it was not possible to standardize the total time of handling and the number of restraints. This depended on the learning curve of the specific student groups. For example, the duration of the practical lesson itself differed between 12 and 23 minutes and the number of manual restraints differed from 2 to 9 restraints per rat. Changes in eye temperature caused by SIH may have a small delay (30) and consequently, the inconsistency in time would make comparing the changes in eye temperatures of the rats in the different groups hard. To prevent this, it would be necessary that all different cages run through the same phases of potential stressors and resting moments at the same time. However, the practical lessons had the main goal of teaching the students, so it was not possible to exclude such confounding factors that could affect the read-out parameter.

Another disturbance could be the treatment the rats from T and G received during experiment 1, just before the practical lessons. During the treatments, it is very likely that the rats experienced a high state of arousal. As mentioned before, this can lead to SIH, which may lead to an increased eye temperature (26,37). Therefore, the baseline eye temperature of the rats before the stressor period started could have been altered due to the treatments, which made it difficult to compare the changes in eye temperature between the groups before and after the practical lesson.

Further, the circumstances during the measurements were not equal within and between measurement days. Background noise and movements in the practical room were both unequally present during the recordings of the different cages. Also, other rats, mice, and rabbits were present in the same room and may have spread stress-related sounds and smells (84–86). This could have interfered with the amount of stress the animals experienced. Besides, during the practical lessons, different students provided for different circumstances. These included, for instance, students' experience with the restraint techniques, the roughness with which they handled the rats, and the students' possible fear for rats.

Lastly, it appeared that even within the same rat the eye temperature data contained a lot of variation. There could be different causes for this variance, but most likely this occurred due to the fixed focus of the infrared camera. This caused a difference in blurriness since the rats moved through the cage, where the level of their eyes differed a lot. Additionally, the focus was poorly identical through the entire cage. Therefore, the used method with this infrared camera was not reliably for measuring eye temperature in this study.

Eye temperature looked like a promising tool to measure non invasively stress in rats (30,37,87), but in our research, it was hard to compare the results from the thermal images between the treatment groups. Besides, the whole procedure of analyzing the videos was very time-intensive, where 300 frames per rat per measurement moment were examined to collect more data to anticipate for the scatter of the analysis. And even with a large number of frames, only a small amount was usable since the frames did not comply with the criteria. For example, there was one rat in the control group that buried his head almost the whole time, which led to no measurable frames and, therefore, a control group of $n = 3$. For future research, the use of IRT measuring mid-tail temperature is preferable to the use of the maximum eye temperature. However, if researchers wanted to use the eye temperature to measure stress levels, it must be taken into account that the eyes must be measured with the same focus to allow quantification, preferably in a non-contact way.

6 CONCLUSION

This study investigated if tickling and gentling could decrease stress levels in laboratory rats during their use in practical lessons for practicing manual restraints. Measuring mid-tail temperature via IRT before, during and after repeated manual restraints revealed only significant differences in tail temperatures between the gentled rats and the control group before the restraints were performed. The highest baseline tail temperature was found in the control group, the tickled rats revealed a lower baseline, and the gentled rats had the lowest baseline tail temperature. Since chronic stress may increase basal core temperature, it might be argued that this provokes a higher baseline tail temperature, since the tail is used to dissipate heat from elevated core temperatures. If that is the case, tickling and especially gentling the rats may lower chronic stress levels. However, this hypothesis must be further investigated and should also be confirmed by additional parameters for chronic stress. Nevertheless, during the manual restraints, it was clear that all rats' tail temperatures decreased to a temperature several degrees beneath the baseline temperature, despite the treatment the rats received for 13 weeks. This strongly suggests that the stressor did provoke acute stress for all rats. Therefore, finding alternatives for the use of living animals to practice restraint techniques or to replace the use of manual restraints in general with another less stressful method may be the aim for future studies. Till then, gentling and tickling may be a good method for rats to ensure a better coping mechanism for stress and provide more positive affective states, all of which can have a positive effect on animal welfare. However, more research is needed to confirm this effect and to measure stress levels beyond the lowest point tail temperature.

7 RECOMMENDATIONS

For future research, the use of mid-tail temperature measured with IRT is recommended as an easy, non-invasive method to obtain an indication of the sympathetically-mediated peripheral vasoconstriction happening during acute stress. However, alterations in study design may expand the power and applicability of the research. This includes the usage of larger sample sizes containing both male and female rats of different ages, expanding the number of measurement moments, increasing the number of frames measured per timepoint, and using a second experimenter for controlling the thermal video analysis. Additionally, better circumstances during the post-stressor period may provide for clearer differences between the recoveries of the rats from the different treatment groups. Since the wake-ups seemed to alter the baseline temperature before the stressor and during the recovery, it is necessary that they are substituted for an alternative, interference-free method to obtain individual tail temperatures. It may also be useful to address the natural behavior of the rats by reversing the day-night cycle, so the animals may be more active during the experiment. Also, behavioral study results are necessary to differentiate between positive and negative arousal and could improve data interpretation, for example, to distinguish outliers due to warmed up tails by conspecifics. Furthermore, it might be interesting to additionally investigate basal tail temperatures without the interference of a stressor to assess chronic stress levels, combined with acknowledged parameters to measure this. Also, investigating the limitations of the tail temperature as a stress parameter during severe stress is needed. Therefore, the time of the stressor period must be expanded and additional standardized and validated methods measuring stress levels have to be measured to investigate if different levels of stress exist beyond the lowest point temperature of the tail.

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9 REFERENCES

1. Balcombe JP, Barnard ND, Sandusky C. Laboratory routines cause animal stress. Contemp Top Lab Anim Sci. 2004;43(6):42–51.

2. Gärtner K, Büttner D, Döhler K, Friedel R, Lindena J, Trautschold I. Stress response of rats to handling and experimental procedures. Lab Anim. 1980;14(3):267–74.

3. Davis H, Pérusse R. Human-based social interaction can reward a rat's behavior. Anim Learn Behav. 1988;16(1):89–92.

4. Brudzynski SM, Ociepa D. Ultrasonic vocalization of laboratory rats in response to handling and touch. Physiol Behav. 1992;52(4):655–60.

5. Hurst JL, West RS. Taming anxiety in laboratory mice. Nat Methods. 2010;7(10):825–6.

6. Ludwig N, Gargano M, Luzi F, Carenzi C, Verga M. Technical note: Applicability of infrared thermography as a non invasive measurements of stress in rabbit. World Rabbit Sci. 2010;15(4):199–206.

7. Cloutier S, Newberry RC. Use of a conditioning technique to reduce stress associated with repeated intra-peritoneal injections in laboratory rats. Appl Anim Behav Sci. 2008;112(1):158–73.

8. Maurer BM, Döring D, Scheipl F, Küchenhoff H, Erhard MH. Effects of a gentling programme on the behaviour of laboratory rats towards humans. Appl Anim Behav Sci. 2008;114(3):554–71.

9. Sherwin CM. The influences of standard laboratory cages on rodents and the validity of research data. Anim Welf. 200;13:S9-15.

10. Podberscek AL, Blackshaw JK, Beattie AW. The effects of repeated handling by familiar and unfamiliar people on rabbits in individual cages and group pens. Appl Anim Behav Sci. 1991;28(4):365–73.

11. Trezza V, Campolongo P, Vanderschuren LJMJ. Evaluating the rewarding nature of social interactions in laboratory animals. Dev Cogn Neurosci. 2011;1(4):444–58.

12. Cloutier S, Wahl K, Baker C, Newberry RC. The social buffering effect of playful handling on responses to repeated intraperitoneal injections in laboratory rats. J Am Assoc Lab Anim Sci JAALAS. 2014;53(2):168–73.

13. Cloutier S, Panksepp J, Newberry RC. Playful handling by caretakers reduces fear of humans in the laboratory rat. Appl Anim Behav Sci. 2012;140(3):161–71.

14. Panksepp J. The ontogeny of play in rats. Dev Psychobiol. 1981;14(4):327–32.

15. Panksepp J. Affective Neuroscience: The Foundations of Human and Animal Emotions. New York: Oxford University Press; 1998.

16. Kikusui T, Winslow JT, Mori Y. Social buffering: relief from stress and anxiety. Philos Trans R Soc Lond B Biol Sci. 2006;361(1476):2215–28.

17. Burgdorf J, Panksepp J. Tickling induces reward in adolescent rats. Physiol Behav. 2001;72(1–2):167–73.

18. Panksepp J, Burgdorf J. 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables. Behav Brain Res. 2000;115(1):25–38.

19. Burgdorf J, Panksepp J, Brudzynski SM, Beinfeld MC, Cromwell HC, Kroes RA, et al. The effects of selective breeding for differential rates of 50-kHz ultrasonic vocalizations on emotional behavior in rats. Dev Psychobiol. 2009;51(1):34–46.

20. Burgdorf J, Knutson B, Panksepp J, Ikemoto S. Nucleus accumbens amphetamine microinjections unconditionally elicit 50-kHz ultrasonic vocalizations in rats. Behav Neurosci. 2001;115(4):940–4.

21. Burgdorf J, Wood PL, Kroes RA, Moskal JR, Panksepp J. Neurobiology of 50-kHz ultrasonic vocalizations in rats: electrode mapping, lesion, and pharmacology studies. Behav Brain Res. 2007;182(2):274–83.

22. Russell WMS, Burch RL. The principles of humane experimental technique. London: Methuen; 1959.

23. Stephens MAC, Wand G. Stress and the HPA axis: Role of glucocorticoids in alcohol dependence. Alcohol Res Curr Rev. 2012;34(4):468–83.

24. Clark G, Magoun HW, Ranson SW. Hypothalamic regulation of body temperature. J Neurophysiol. 1939;2(1):61–80.

25. Rekant SI, Lyons MA, Pacheco JM, Arzt J, Rodriguez LL. Veterinary applications of infrared thermography. Am J Vet Res. 2016;77(1):98–107.

26. Stewart M, Webster JR, Stafford KJ, Schaefer AL, Verkerk GA. Technical note: Effects of an epinephrine infusion on eye temperature and heart rate variability in bull calves. J Dairy Sci. 2010;93(11):5252–7.

27. Hjemdahl P. Plasma catecholamines--analytical challenges and physiological limitations. Baillieres Clin Endocrinol Metab. 1993;7(2):307–53.

28. Zimmermann E, Critchlow V. Effects of Diurnal Variation in Plasma Corticosterone Levels on Adrenocortical Response to Stress. Proc Soc Exp Biol Med. 1967;125(2):658–63.

29. Michel C, Cabanac M. Opposite Effects of Gentle Handling on Body Temperature and Body Weight in Rats. Physiol Behav. 1999;67:617–22.

30. Vianna DML, Carrive P. Changes in cutaneous and body temperature during and after conditioned fear to context in the rat. Eur J Neurosci. 2005;21(9):2505–12.

31. Oka T, Oka K, Hori T. Mechanisms and Mediators of Psychological Stress-Induced Rise in Core Temperature. Psychosom Med. 2001;63(3):476–86.

32. Herborn KA, Graves JL, Jerem P, Evans NP, Nager R, McCafferty DJ, et al. Skin temperature reveals the intensity of acute stress. Physiol Behav. 2015;152:225–30.

33. Lkhagvasuren B, Oka T. The histaminergic system is involved in psychological stress-induced hyperthermia in rats. Physiol Rep. 2017;5(8).

34. Nakamura K. Neural circuit for psychological stress-induced hyperthermia. Temp Multidiscip Biomed J. 2015;2(3):352–61.

35. Adriaan Bouwknecht J, Olivier B, Paylor RE. The stress-induced hyperthermia paradigm as a physiological animal model for anxiety: A review of pharmacological and genetic studies in the mouse. Neurosci Biobehav Rev. 2007;31(1):41–59.

36. Lomax P. Measurement of 'Core' Temperature in the Rat. Nature. 1966;210(5038):854–5.

37. Gjendal K, Franco NH, Ottesen JL, Sørensen DB, Olsson IAS. Eye, body or tail? Thermography as a measure of stress in mice. Physiol Behav. 2018;196:135–43.

38. Redaelli V, Luzi F, Mazzola S, Bariffi GD, Zappaterra M, Nanni Costa L, et al. The Use of Infrared Thermography (IRT) as Stress Indicator in Horses Trained for Endurance: A Pilot Study. Anim Open Access J MDPI. 2019;9(3).

39. Fenner K, Yoon S, White P, Starling M, McGreevy P. The Effect of Noseband Tightening on Horses' Behavior, Eye Temperature, and Cardiac Responses. PloS One. 2016;11(5):e0154179.

40. McGreevy P, Warren-Smith A, Guisard Y. The effect of double bridles and jaw-clamping crank nosebands on temperature of eyes and facial skin of horses. J Vet Behav. 2012;7(3):142–8.

41. Bartolomé E, Sánchez MJ, Molina A, Schaefer AL, Cervantes I, Valera M. Using eye temperature and heart rate for stress assessment in young horses competing in jumping competitions and its possible influence on sport performance. Anim Int J Anim Biosci. 2013;7(12):2044–53.

42. Tattersall GJ. Infrared thermography: A non-invasive window into thermal physiology. Comp Biochem Physiol A Mol Integr Physiol. 2016;202:78–98.

43. Jones BF. A reappraisal of the use of infrared thermal image analysis in medicine. IEEE Trans Med Imaging. 1998;17(6):1019–27.

44. Turner TA. Diagnostic thermography. Vet Clin North Am Equine Pract. 2001;17(1):95–113.

45. Vollmer M, Möllmann K-P. Infrared Thermal Imaging: Fundamentals, Research and Applications. Weinheim: John Wiley & Sons; 2017. 797 p.

46. Modest M. Radiative Heat Transfer [Internet]. 3th edition. Academic Press; 2013 [cited 2019 Jul 15]. 904 p. Available from: https://www.elsevier.com/books/radiative-heat-transfer/modest/978-0-12-386944-9

47. Meola C. Infrared thermography: Recent advances and future trends. Sharjah: Bentham Science Publishers; 2012. 254 p.

48. Usamentiaga R, Venegas P, Guerediaga J, Vega L, Molleda J, Bulnes FG. Infrared Thermography for Temperature Measurement and Non-Destructive Testing. Sensors. 2014;14(7):12305–48.

49. Stewart M, Webster J, Schaefer AL, Cook N, Tracey S. Infrared thermography as a non-invasive tool to study animal welfare. Anim Welf. 2005;14.

50. Yang WJ, Yang PP. Literature survey on biomedical applications of thermography. Biomed Mater Eng. 1992;2(1):7–18.

51. Vogel B, Wagner H, Gmoser J, Wörner A, Löschberger A, Peters L, et al. Touch-free measurement of body temperature using close-up thermography of the ocular surface. MethodsX. 2016;3:407–16.

52. El Bitar N, Pollin B, Karroum E, Pincedé I, Mouraux A, Le Bars D. Thermoregulatory vasomotor tone of the rat tail and paws in thermoneutral conditions and its impact on a behavioral model of acute pain. J Neurophysiol. 2014;112(9):2185–98.

53. Herborn KA, Jerem P, Nager RG, McKeegan DEF, McCafferty DJ. Surface temperature elevated by chronic and intermittent stress. Physiol Behav. 2018;191:47–55.

54. Valera M, Bartolomé E, Sánchez MJ, Molina A, Cook N, Schaefer A. Changes in Eye Temperature and Stress Assessment in Horses During Show Jumping Competitions. J Equine Vet Sci. 2012;32(12):827–30.

55. Warren LK, Cook NJ, Schaefer AL, Burwash L, Anderson M, Baron V, et al. The use of salivary cortisol as an index of stress in horses. In: Proceedings of the 17th symposium of equine nutrition and physiology society. 2001. p. 353–4.

56. Johnson SR, Rao S, Hussey SB, Morley PS, Traub-Dargatz JL. Thermographic Eye Temperature as an Index to Body Temperature in Ponies. J Equine Vet Sci. 2011;31(2):63–6.

57. Ikkatai Y, Watanabe S. Eye surface temperature detects stress response in budgerigars (Melopsittacus undulatus). Neuroreport. 2015;26(11):642–6.

58. Stewart M, Webster JR, Verkerk GA, Schaefer AL, Colyn JJ, Stafford KJ. Non-invasive measurement of stress in dairy cows using infrared thermography. Physiol Behav. 2007;92(3):520–5.

59. Stubsjøen SM, Flø AS, Moe RO, Janczak AM, Skjerve E, Valle PS, et al. Exploring non-invasive methods to assess pain in sheep. Physiol Behav. 2009;98(5):640–8.

60. Nakayama K, Goto S, Kuraoka K, Nakamura K. Decrease in nasal temperature of rhesus monkeys (Macaca mulatta) in negative emotional state. Physiol Behav. 2005;84(5):783–90.

61. Vinkers CH, Penning R, Hellhammer J, Verster JC, Klaessens JHGM, Olivier B, et al. The effect of stress on core and peripheral body temperature in humans. Stress Int J Biol Stress. 2013;16(5):520–30.

62. Harrap MJM, Hempel de Ibarra N, Whitney HM, Rands SA. Reporting of thermography parameters in biology: a systematic review of thermal imaging literature. R Soc Open Sci. 2018 Dec;5(12):181281.

63. Bolles RC, Collier AC. The effect of predictive cues on freezing in rats. Anim Learn Behav. 1976;4(1):6–8.

64. Blanchard RJ, Blanchard DC. Crouching as an index of fear. J Comp Physiol Psychol. 1969;67(3):370–5.

65. Kalueff AV, Stewart AM, Song C, Berridge KC, Graybiel AM, Fentress JC. Neurobiology of rodent self-grooming and its value for translational neuroscience. Nat Rev Neurosci. 2016;17(1):45–59.

66. Kalueff AV, Tuohimaa P. Grooming analysis algorithm for neurobehavioural stress research. Brain Res Protoc. 2004;13(3):151–8.

67. van Erp AMM, Kruk MR, Meelis W, Willekens-Bramer DC. Effect of environmental stressors on time course, variability and form of self-grooming in the rat: Handling, social contact, defeat, novelty, restraint and fur moistening. Behav Brain Res. 1994;65(1):47–55.

68. Cloutier S, LaFollette MR, Gaskill BN, Panksepp J, Newberry RC. Tickling, a Technique for Inducing Positive Affect When Handling Rats. J Vis Exp JoVE. 2018;(135).

69. Hickman DL, Swan M. Use of a Body Condition Score Technique to Assess Health Status in a Rat Model of Polycystic Kidney Disease. J Am Assoc Lab Anim Sci JAALAS. 2010;49(2):155.

70. Moe RO, Stubsjøen SM, Bohlin J, Flø A, Bakken M. Peripheral temperature drop in response to anticipation and consumption of a signaled palatable reward in laying hens (Gallus domesticus). Physiol Behav. 2012;106(4):527–33.

71. Travain T, Colombo ES, Grandi LC, Heinzl E, Pelosi A, Prato Previde E, et al. How good is this food? A study on dogs' emotional responses to a potentially pleasant event using infrared thermography. Physiol Behav. 2016;159:80–7.

72. Chotard H, Ioannou S, Davila-Ross M. Infrared thermal imaging: Positive and negative emotions modify the skin temperatures of monkey and ape faces. Am J Primatol. 2018;80(5):e22863.

73. Kant GJ, Bauman RA, Pastel RH, Myatt CA, Closser-Gomez E, D'Angelo CP. Effects of controllable vs. uncontrollable stress on circadian temperature rhythms. Physiol Behav. 1991;49(3):625–30.

74. Endo Y, Shiraki K. Behavior and body temperature in rats following chronic foot shock or psychological stress exposure. Physiol Behav. 2000;71(3):263–8.

75. van Marken Lichtenbelt WD, Schrauwen P, van de Kerckhove S, Westerterp-Plantenga MS. Individual variation in body temperature and energy expenditure in response to mild cold. Am J Physiol-Endocrinol Metab. 2002;282(5):E1077–83.

76. Kashimoto RK, Toffoli LV, Manfredo MHF, Volpini VL, Martins-Pinge MC, Pelosi GG, et al. Physical exercise affects the epigenetic programming of rat brain and modulates the adaptive response evoked by repeated restraint stress. Behav Brain Res. 2016 Jan 1;296:286–9.

77. Seo MK, Ly NN, Lee CH, Cho HY, Choi CM, Nhu LH, et al. Early life stress increases stress vulnerability through BDNF gene epigenetic changes in the rat hippocampus. Neuropharmacology. 2016;105:388–97.

78. Hertzman AB. Some relations between skin temperature and blood flow. Am J Phys Med. 1953;32(4):233–51.

79. Keeney AJ, Hogg S, Marsden CA. Alterations in core body temperature, locomotor activity, and corticosterone following acute and repeated social defeat of male NMRI mice. Physiol Behav. 2001;74(1):177–84.

80. Lin H-P, Lin H-Y, Lin W-L, Huang AC-W. Effects of stress, depression, and their interaction on heart rate, skin conductance, finger temperature, and respiratory rate: sympathetic-parasympathetic hypothesis of stress and depression. J Clin Psychol. 2011;67(10):1080–91.

81. Pazderka-Robinson H, Morrison JW, Flor-Henry P. Electrodermal dissociation of chronic fatigue and depression: evidence for distinct physiological mechanisms. Int J Psychophysiol. 2004;53(3):171–82.

82. Kim J-G, Jung H-S, Kim K-J, Min S-S, Yoon B-J. Basal blood corticosterone level is correlated with susceptibility to chronic restraint stress in mice. Neurosci Lett. 2013;555:137–42.

83. Orlovsky MA, Dosenko VE, Spiga F, Skibo GG, Lightman SL. Hippocampus remodeling by chronic stress accompanied by GR, proteasome and caspase-3 overexpression. Brain Res. 2014 Dec 17;1593:83–94.

84. Wöhr M, Schwarting RKW. Ultrasonic Communication in Rats: Can Playback of 50-kHz Calls Induce Approach Behavior? PLOS ONE. 2007;2(12):e1365.

85. Valenta JG, Rigby MK. Discrimination of the Odor of Stressed Rats. Science. 1968;161(3841):599–601.

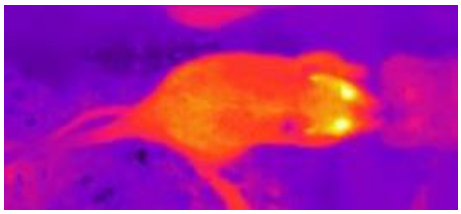
86. Mackay-Sim A, Laing DG. Discrimination of odors from stressed rats by non-stressed rats. Physiol Behav. 1980;24(4):699–704.

87. Lecorps B, Rödel HG, Féron C. Assessment of anxiety in open field and elevated plus maze using infrared thermography. Physiol Behav. 2016;157:209–16.

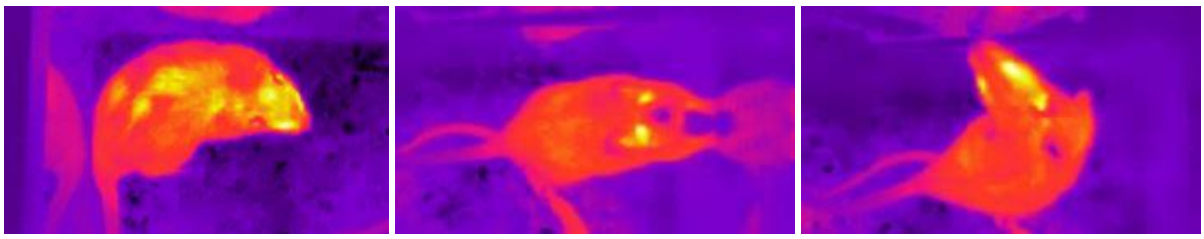
10 Appendix

10.1 Examples of usable and unusable frames in experiment 2

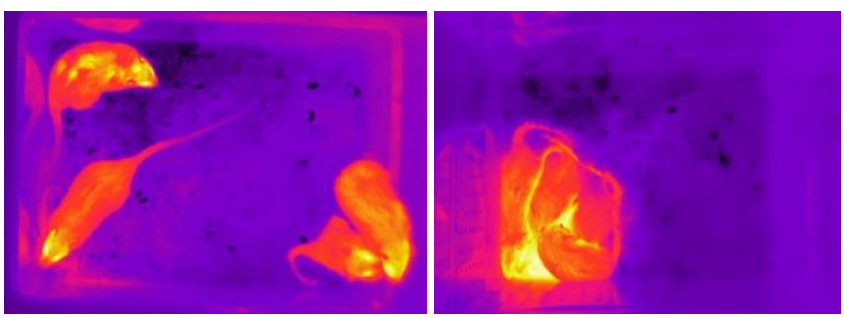
Usable frame: Equally visible eyes, the rat is looking a bit upwards:



Unusable frames: The rat is looking down, too much upwards, or the eyes are not equally visible:

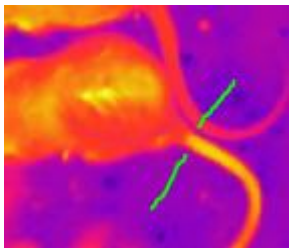


Rat is in the bottom right corner or is lying with the other rats:

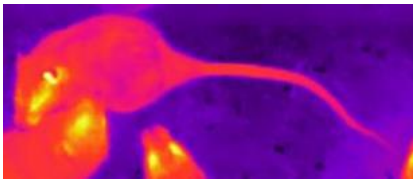
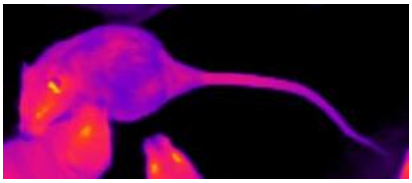
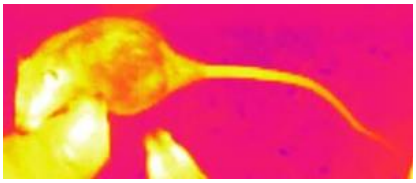


10.2 Measuring mid-tail temperature

Using the FLIR ResearchIR program. To establish the middle of the tail, in each selected frame the length of the tail per individual rat in pixels was measured, via the statistics viewer, by drawing a bendable line ROI following the tail from the base (hairless part, see picture A) to the tip. This length was divided by two and a second bendable line ROI with this amount of pixels was drawn following the tail, starting at the base. If half of the length was not a whole number, the frame and line of the whole tail were judged on which even number was closest to the tail length. At the end of the second line, a cursor ROI (1 pixel) was placed on the tail. The maximum temperature (excepted when the surrounding was warmer) was taken by letting the cursor ROI find the highest temperature on the perpendicular line of this place at the tail. This temperature was detailed saved with the profile plot function. This was repeated for each rat and for each new frame and measurement moment.

A: 

To find the base of the tail when not clearly visible (B), the image enhancement could be manually altered to obtain a better distinction between the rat and the hairless base of the tail (C and D).

B:  **C:**  **D:** 

10.3 Plot experiment 2: tail temperature vs. cages

Mean +/- SEM. Mid-tail temperature during different timepoint of experiment 2 (see Table 2) vs. the different cages and their treatment: