



Utrecht University

**Relationship between behaviour and tail temperature of tickled and gentled rats before, during and after manual restraint stress**

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## Abstract

Many routine procedures in experiments, such as handling, cause stress in rats. Rat tickling is a technique which aims to reduce handling stress. In a previous study, the effect of tickling or gentling on tail temperatures of rats was investigated, using infrared thermography. The present study analyses the behaviour of the rats from the mentioned study, to investigate possible effects of stress-related and other behaviours on tail temperature. Moreover, the effect of two different handling techniques, tickling and gentling, on behaviour was analysed. Sixteen male Wistar rats (WU:CrI) received one of the following treatments for 14 weeks: tickling (n = 8), gentling (n = 4) or no handling (control, n = 4). Mid-tail temperature was measured before, during and after manual restraint, and videos for behavioural analysis were recorded. Results showed that higher groom, scratch and rear frequencies are associated with higher tail temperatures, while no effect of freezing or huddling on tail temperature was found. Moreover, gentled rats froze less than control rats and tickled rats during the stressor, and less than control rats post-stressor. Gentled rats also huddled the most pre-stressor. Control rats spent the most time grooming pre-stressor, while they had the highest groom frequency post-stressor. Post-stressor, the average groom bout duration was longer for Tickled rats than for Gentled rats. The results of freeze durations suggest that tickling did not reduce fear in response to manual restraint, at least not in all rats. Tickling might not always be a positive experience for rats. This study is limited by its sample size, with possible cage effects influencing the results. Additional research is suggested.

**Keywords:** Rats – Behaviour – Tickling – Gentling – Manual restraint stress – Tail temperature – Infrared thermography

**Abbreviations:** ACTH: adenocorticotrophic hormone; AUC: area under the curve; DOI: 2,5-dimethoxy-4-iodoamphetamine; HPA: hypothalamic-pituitary-adrenal; IRT: infrared thermography; SAM: sympathetic-adrenal-medullary; SIH: stress induced hyperthermia

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# 1 Introduction

## 1.1 3Rs in laboratory research

Rats are frequently used in research and teaching. In 2019, roughly 81.600 experiments with rats were performed in the Netherlands.<sup>1</sup> Many procedures in these experiments, such as handling, insertion of a needle or orogastric gavage, are stressful for the animals.<sup>2,3</sup> Prolonged or severe stress can cause distress, a state in which an animal is unable to adapt to stressors, leading to compromised welfare.<sup>4</sup> Moreover, stress influences cardiovascular, hormonal, immunological and behavioural parameters, and may in this way increase the variability of research results.<sup>3,5</sup> In order to minimise distress in laboratory animals, guiding principles were formulated by Russel & Burch in 1959 in the form of the 3Rs: Replacement, Reduction and Refinement.<sup>6</sup> Reducing stress can, in addition to improving welfare for the involved animals (Refinement), decrease data variability, thereby increasing the robustness and reproducibility of experiments and reducing the number of animals needed (Reduction).<sup>7</sup> This is especially true for research in which the stress response itself is investigated.

## 1.2 Rat tickling

Rat tickling, a handling technique which mimics parts of the rough-and-tumble play of rats,<sup>8</sup> is seen as a refinement of rat experiments. Social play is an essential behaviour: rats experience play as rewarding, and it is important for emotional development.<sup>9</sup> Rough-and-tumble play consists of two main components: pounce and pin. A rat initiates play by touching the nape of another rat (pounce). The other rat can respond by rolling over to a supine position and being pinned down (pin) (Fig. 1A).<sup>10</sup> Rats take turns pouncing and pinning each other. Humans can mimic the pounces and pins of rough-and-tumble play with rapid finger movements, alternating contact with a rat's dorsal and ventral surface (Fig. 1B). Although tickling is not exactly similar to rough-and-tumble play, the idea is that tickling can make handling a positive experience.<sup>8</sup>

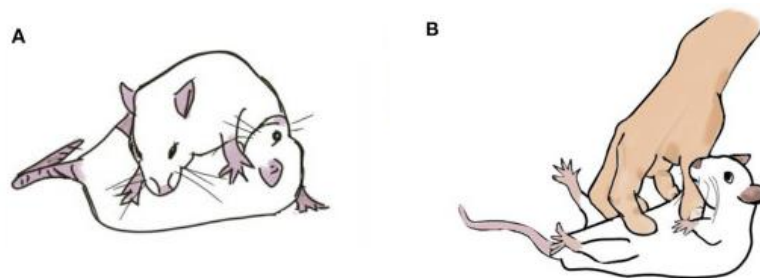


Fig. 1. (A) Pin and pounce, the main components of rough-and-tumble play in rats.  
(B) Rat tickling. From Bombail et al. (2021).<sup>11</sup>

To verify the supposed positive effects of tickling, research is necessary. LaFollette et al.<sup>12</sup> conducted a systematic review of articles on rat tickling. The reviewed articles used multiple behavioural parameters: 50-kHz vocalisations, which is used as a measure of positive affect,<sup>13</sup> approach or fear behaviours, and stress associated with handling. Results were largely positive: of the sixteen experiments that investigated vocalisation, fifteen (94%) found that tickling increased 50-kHz vocalisations; nine out of ten experiments (90%) found increased approach behaviour in tickled rats; five out of seven experiments (71%) found decreased fear behaviours; and lastly, five

out of six experiments (83%) found decreased stress associated with handling in tickled rats.<sup>12</sup> These findings suggest tickling can be used as a refinement of experiments with rats. It should be noted, though, that most research has been done with individually housed rats. Tickling might have less effect on socially housed rats, since they have the opportunity to play with each other. Habituation to handling could also be the cause of reduced fear of humans, instead of tickling specifically. This should be further investigated.

### **1.3 Measuring stress**

To investigate whether a procedure such as tickling or handling can reduce stress from experimental procedures, a reliable method to measure stress is necessary. Stress parameters that can be measured include biochemical and physiological parameters. Taking Refinement into account, the least invasive method that is also both reliable and available, should be used.

#### **1.3.1 Plasma glucocorticoid or catecholamine levels**

Plasma corticosterone or catecholamine levels are often used to measure stress.<sup>14-16</sup> This method is based on the activation of the sympathetic-adrenal-medullary (SAM) and hypothalamic-pituitary-adrenal (HPA) axes that stress induces. Activation of the SAM axis leads to the release of catecholamines (adrenaline and noradrenaline) from the adrenal medulla,<sup>17</sup> whereas the HPA axis is responsible for the release of adenocorticotrophic hormone (ACTH) from the pituitary. ACTH in turn stimulates the release of glucocorticoids (corticosterone in rodents) from the adrenal cortex into the bloodstream.

To obtain blood plasma, a blood sampling procedure is necessary. All methods of blood sampling induce a certain amount of stress. In the case of blood vessel puncture without anaesthesia, stress arises from restraint, handling and needle insertion.<sup>3</sup> This causes a rise in plasma corticosterone and catecholamine levels, leading to confounding results.<sup>18</sup> Placement of a permanent intravenous cannula avoids the stress from repeated sampling; blood samples can then be taken in freely moving rats.<sup>14,16,19</sup> However, the necessary surgical procedure makes this an invasive method.

#### **1.3.2 Physiological parameters**

Besides biochemical alterations, stress also induces physiological alterations. Heart rate, blood pressure and respiratory rate are sometimes used to measure stress in rats.<sup>5,20,21</sup> These parameters can be measured with the use of telemetric implants.

In addition, body temperature can be used to measure stress. The increase in core temperature following a stressful event is called stress induced hyperthermia (SIH),<sup>22</sup> or “psychogenic fever”.<sup>23</sup> However, this term is misleading: unlike infection-induced fever, which is mediated by pro-inflammatory cytokines and prostaglandin, SIH is sympathetically mediated.<sup>23,24</sup> In response to psychological stress, signals are transmitted from the dorsomedial hypothalamus to sympathetic premotor neurons in the medullary raphe region, activating brown adipose tissue thermogenesis, tachycardia and cutaneous vasoconstriction.<sup>22</sup> SIH is thought to be beneficial to the “fight-or-flight” response, since the increase in core temperature warms up muscles and the central nervous systems, and leads to an increase in physical and neurocognitive performance.<sup>25</sup> The reduced blood flow to the skin and extremities resulting from vasoconstriction has multiple

presumed functions: in addition to contributing to the increase in core temperature,<sup>25</sup> it also minimises the risk of blood loss after injury.<sup>26</sup>

Core temperature changes following stressors have been observed in rats: handling or exposure to novel environments can raise core temperature up to 2 °C.<sup>23</sup> Core temperature can be measured with a rectal probe or permanent thoracic,<sup>27</sup> peritoneal<sup>25</sup> or retroperitoneal<sup>28</sup> implant. Rectal temperature measurement is easy to perform, but this method causes a quick increase in core temperature which can last for more than an hour<sup>27</sup> and does not diminish with repeated measurements.<sup>28</sup> This problem can be avoided by using surgical implants. As with blood sampling, however, the invasive surgical procedure itself is a source of distress.

### **1.3.3 Infrared thermography**

Instead of core temperature, peripheral temperatures can possibly also be used to measure SIH. Decreases in peripheral temperatures following stress have, for example, been observed in the fingers and nose of humans,<sup>29</sup> the comb and wattle of hens,<sup>30</sup> the ears of rabbits<sup>26</sup> and the tail and paws of rats.<sup>31,32</sup> In hens and rats, this temperature decrease was found to be proportional to stressor intensity. Additionally, a rebound increase in temperature following the initial decrease was observed in the comb and wattle of hens<sup>30</sup> and in the tail (not the paws) of rats.<sup>31</sup> One of the methods used to measure peripheral body temperature is infrared thermography (IRT).<sup>24,30-32</sup> An IRT camera detects the infrared radiation emitted by an object or animal and converts this into a thermal image or thermogram.<sup>24</sup> With analysis software, temperatures of specific body areas can be selected and measured. The greatest advantage of IRT is its non-invasiveness: no contact with the animal is necessary.<sup>33</sup> This makes it a possible refinement if it can be applied instead of invasive methods for stress research. Further research on the relationship between stress and tail temperatures in rats is necessary to validate this method.

## **1.4 Relationship between behaviour and stress response**

The stress response is not only observable in biochemical and physiological parameters, but also in behaviour. Like the physiological alterations, behavioural alterations are meant to increase the rat's chances of survival.<sup>34</sup> The relationship between behaviour and stress response works two ways. On the one hand, certain behaviours are induced or repressed by stress; on the other hand, behaviour can influence the stress response. The magnitude of the stress response is not only dependent on stressor severity, but also on individual coping style.

### **1.4.1 Stress-related behaviour**

Certain behaviours occur more frequently in stressful situations and might be used as indicators of stress. Examples of these behaviours are freezing, shaking and scratching. Freezing, a commonly used behaviour to measure fear or anxiety in rats, is a rigid body posture with the absence of movement.<sup>35</sup> It is part of the response to both conditioned fear (e.g. foot shocks<sup>36</sup>) and unconditioned fear (e.g. predator odour<sup>37</sup>). The freezing response can be weakened by anxiety-reducing drugs and enhanced by anxiety-inducing drugs.<sup>36</sup> The second example, shaking, includes both head shakes and wet-dog shakes. The evidence for shaking as a response to stress is less clear than for freezing. One study found an increase in wet-dog shakes after exposure to chronic stress<sup>38</sup>; however, other studies did not find a difference in the frequency of wet-dog shakes between stressed and non-stressed rats.<sup>39,40</sup> The third behaviour, scratching, is considered

a displacement activity. Displacement activities are behaviours which occur when two motivations (for example, fight or flight) conflict, leading to the display of behaviour that seems out of context. Displacement activities can be seen in stressful conditions.<sup>41</sup> In rats, fighting, chewing, drinking and possibly grooming are additional examples of displacement activities, when performed out of context.<sup>41,42</sup>

Grooming is a behaviour which can be either induced or repressed by stress. In rats, grooming is a complex ritual consisting of several stages, including licking the paws, washing the head, cleaning the fur and licking the genital region.<sup>43</sup> Rats stop grooming when faced with a highly aversive stressor, whereas in moderately aversive situations, grooming increases.<sup>44</sup> This increased duration of total grooming is the result of more frequent grooming bouts, while the average bout duration remains unchanged.<sup>43,45</sup> The function of stress-induced grooming is not completely understood. It is unclear whether grooming is a displacement activity, helps with de-arousal after a stressor, or is simply an expression of the transition into a state of low arousal.<sup>41,45,46</sup>

Like grooming, yawning is seen in periods of low arousal,<sup>47</sup> but also following stress. One study found that conditional fear conditioning induced yawning.<sup>48</sup> The function of yawning is still unclear. In primates, it is seen as a displacement activity.<sup>49</sup> In rats, yawning possibly increases at the moment the rat enters a state of low arousal.<sup>50</sup> Yawning also seems to play a role in thermoregulation of the brain in rats.<sup>51</sup>

#### **1.4.2 Social behaviour**

In highly social animals such as rats, the presence of conspecifics can help the recovery from an aversive experience. This phenomenon is termed social buffering.<sup>52</sup> Social buffering in rats is well documented; for example, rats that are exposed to a stressor on their own show a greater fear response than grouped rats.<sup>53,54</sup> Conversely, stressed rats are more drawn to conspecifics than non-stressed rats are.<sup>53</sup> Both tactile<sup>52,54,55</sup> and olfactory<sup>56,57</sup> stimuli are important for social buffering. Examples of social behaviour are allogrooming, huddling and playing.

#### **1.4.3 Coping styles**

Not all rats react the same towards a stressful situation. Rats with different coping styles exhibit different stress responses. Two main coping styles are recognised: pro-active and reactive.<sup>58</sup> Pro-active animals are more aggressive, often dominant, and demonstrate higher exploratory activity, while reactive animals are slower explorers and tend to freeze more in response to a stressor. While pro-active animals show a more sympathetic reaction to stress, reactive animals show more HPA-axis activation.<sup>32</sup> Following social defeat, for example, rats displaying low fight and low guard behaviour have higher peak cortisol values than more aggressive rats.<sup>59</sup> Additionally, rats that react less aggressively towards intruders have higher plasma ACTH values.<sup>60</sup>

Since physiological responses of pro-active and reactive copers differ, it can be expected that core and peripheral temperature changes also differ between rats with different coping styles. The relationship between coping styles and tail temperatures has been investigated in rodents. Ågren et al<sup>61</sup> analysed the predictive value of tail skin temperatures for the classification of coping styles. Rats were exposed to different stressors and subsequently divided in A-rats and B-rats, based on tail temperatures. These groups were consistent with pro-active coping (A-rats) and reactive



coping (B-rats), although this determination of coping style was made based on organ weight instead of behavioural tests. Duparcq et al.<sup>32</sup> performed behavioural tests with mound-building mice. They divided the animals in groups based on exploration tendency, a component of the coping styles used by Koolhaas et al.<sup>62</sup> In their study, ‘fast explorers’ had lower tail temperatures than ‘slow explorers’ after handling. These studies indicate that individual differences in behavioural response towards stress correlate with differences in tail temperatures.

## **1.5 Aim of this study**

In a previous study in our lab,<sup>63</sup> the effect of repeated handling on tail skin temperatures of rats and possible differences between gentled, tickled and control rats were investigated. Results showed that gentled rats had lower baseline (pre-stressor) tail temperatures than control rats, while differences between tickled and control rats were not significant. The baseline tail temperatures also showed high variation between individuals. This can be partly explained by fluctuations in tail temperatures throughout the day,<sup>72</sup> in addition to inter-individual variation in core temperature. Moreover, the rats could differ in their reaction to the same stressor. Observing stress-related behaviour could give an indication of the stress levels of individual rats.

In another study,<sup>64</sup> the same rats as in the previous study were exposed to the same stressor, and their behaviour before and after the stressor was studied. No significant differences in freeze, rest and rear durations between treatment groups were found. The behaviour during the stressor period itself was not analysed, while behaviours such as freezing are expected to occur the most during this period. Also, other behaviours which were not scored could be interesting, such as scratching, shaking and yawning. If differences in behaviour between the treatment groups would be found, this might explain the differences in tail temperatures between these groups.

In the present study, video footage from the previous two studies was used to analyse the behaviour of rats before, during and after manual restraint. The aims of the study were:

- 1) To investigate the effect of stress-related and other behaviours on tail temperature;
- 2) To analyse possible differences in behaviour between gentled, tickled and control rats.

## **2 Materials & Methods**

### **2.1 General outline**

In the present study, behavioural analysis was performed on video footage obtained in two earlier experiments: Experiment 1 was performed by Weitkamp,<sup>63</sup> while Experiment 2 was performed by Edwards.<sup>64</sup> Mid-tail temperatures from Experiment 1 were also used to analyse the relationship between behaviour and temperatures. The procedures of Experiment 1 and 2 will be described in the next paragraphs, followed by the methods of the present study. More details on the tail temperature measurements in Experiment 1 can be found in Appendix A.

### **2.2 Ethical note**

Both experiments were performed within an educational project approved by the Central Authority for Scientific Procedures on Animals (CCD).

## 2.3 Animals and housing

16 male Wistar rats (WU:CrI; Charles River, Germany), three weeks old at arrival, were used in this experiment. They received a pen marking on their tail for identification. The rats were housed in Eurostandard type IV S cages (480 x 375 x 210 mm; floor area: 1500 cm<sup>2</sup>) in groups of four. The cages contained wood shavings as bedding and a transparent orange shelter (151 x 90 x 90 mm) and paper tissues as enrichment. The rats were kept in a room with a controlled temperature of 22.0 °C ± 0.6 °C and humidity of 48.9% ± 5.4%, in a 12-hour day-night cycle with lights on between 7 a.m. and 7 p.m. The radio was always playing. Food (Rat/Mouse maintenance, 10 mm, Ssniff Spezialdiäten GmbH) was available *ad libitum*, as was tap water. The cages were changed weekly.

The rats were used in a practical three times in the first week after arrival. In this experiment, Methylphenidate was administered and behaviour was observed. The animals were also used in practicals in which handling, restraint and oral gavage was taught.

## 2.4 Treatment groups

Directly following their arrival, the rats were habituated for two weeks by removing the cage lid and placing a hand in the cage for five minutes every day. Next, the rats were divided in treatment groups. Cage 1 was the control group (n = 4), cage 2 and 3 received the tickling treatment (n = 8), and cage 4 received the gentling treatment (n = 4). Treatment sessions took place between 9:00 and 17:00, first five times weekly for 3 weeks, then 4 times weekly for one week, and ultimately 3 times weekly for 10 weeks. The last week of treatment was the week in which Experiment 1 took place.

The procedure of a treatment session was as follows: each of the four cages was randomly assigned to one experimenter. The rats remained in their home cage, which was located at either the housing location or test room. The experimenters wore no gloves. A session started with habituation, during which a hand was placed in the cage for one minute. This was followed by seven minutes of treatment, divided between the four rats. For the tickling treatment, the method of Cloutier et al.<sup>8</sup> was used. First, a pounce was performed by touching the dorsal surface of the rat's nape using quick finger movements for two to four seconds. Next, the rat was turned on its back and a pin was performed using quick finger movements on the rat's ventral surface. Pinning stopped when the rat turned back on its feet. For the gentling treatment, the rat was briefly lifted by scooping or gripping its body from underneath. The rat was then free to stay on the hand or walk away. The control group received no treatment and their cage remained in the wall rack.

## 2.5 Experiment 1

### 2.5.1 Cameras

For recording of thermal videos, a FLIR T430sc thermal camera was used. More details on camera placement and settings are given in Appendix A. For recording of real-time video, a Bascom camera was placed next to the thermal camera. These video recordings were used for identification of the rats and behavioural analysis.

### **2.5.2 Experimental procedure**

When the rats were 15 weeks old, Experiment 1 was performed. This experiment was divided between two consecutive days, between 12:00 and 16:00, with cage 3 (Tickled) and 4 (Gentled) on the first day and cage 1 (Control) and 2 (Tickled) on the second. An overview of the experimental timeline is presented in Fig. 2A. The rats remained in their home cage, which was placed in the test room. First, all objects were removed from the cage and the lid was replaced by a 40 cm high plexiglass wall. Then, the habituation period of 30 minutes started, followed by a pre-stressor period of 10 minutes. The rats were picked up at 0, 5 and 10 minutes pre-stressor, just before measurement of tail-temperatures, to ensure all rat tails were visible. The experimenter left the room after each wake-up moment.

The wake-up at 10 minutes pre-stressor indicated the start of the stressor period. During the stressor period, the experimenters stood around the cage. The rats were picked up by one experimenter and given to the other three experimenters, following a pre-determined rotation system. Then, the rats were manually restrained six times. One restraint consisted of twice performing all the steps in the restraint technique (as taught in the Laboratory Animal Science Course of Utrecht University). The rats were placed back in the cage for 45 seconds after each stressor and the stressor period ended 45 seconds after the last restraint. A stressor period lasted 9 minutes and 45 seconds in total.

The stressor period was followed by a post-stressor period of 30 minutes. Rats were again picked up at 0, 5, 10, 20 and 30 minutes post-stressor, coinciding with the measurement moments of tail temperatures. Except for these wake-up moments, no experimenter was present in the room during the post-stressor period.

In Appendix A, the method of determination of mid-tail temperatures is described.

## **2.6 Experiment 2**

### **2.6.1 Animals**

Experiment 2 was performed with 30 rats in total, but only the rats used in Experiment 1 are included in the present study. Two rats, one from cage 1 (Control) and one from cage 3 (Tickled) became ill after Experiment 1 and were euthanised because the humane endpoint was reached. Consequently, 14 rats were included in Experiment 2.

### **2.6.2 Experimental procedure**

Experiment 2 took place on one day, two days after Experiment 1. The experimental procedure, depicted in Fig. 2B, differed from Experiment 1. The first difference was that the stressor period was preceded by treatment. This treatment was either tickling, gentling or no treatment, corresponding to the treatment the rats normally received. Treatment started with holding a hand in the cage for one minute, as normal, and ended with one minute of rest. The second difference was the omission of wake-up moments, and the third was the length of the periods: 5 minutes habituation, 10 minutes pre-stressor and 15 minutes post-stressor. The stressor period was

similar to Experiment 1. Mid-tail temperature measurements were made, but not used in the present study.

## 2.7 Welfare assessment

Once a week, welfare parameters were measured, preferably on Monday and after treatment. Body weight, body condition, body posture, fur condition and porphyrin levels were scored.

## 2.8 Behavioural analysis

For the behavioural analysis, an ethogram was created (Table 1). Video scoring was performed by one observer, using Solomon Coder beta 19.08.02.<sup>65</sup> All behaviours except huddling were scored by continuous focal sampling, and both state and event behaviours were scored simultaneously. Huddling was scored separately, because it could co-occur with other behaviours. Huddling was scored by instantaneous scan sampling at ten second intervals. All moments (Fig. 2) were scored, except treatment in Experiment 2, since this period was not comparable between treatment groups.

Before scoring, the observer practiced scoring on the video footage of the rats in Experiment 1 and 2. The goal of the training was to learn to code in Solomon, recognize all behaviours and refine the definitions in the ethogram. After training, within-observer reliability was determined. For this, 16-minute clips of eight rats were scored twice, and a Pearson correlation between the two scores of durations (for states) and frequencies (for events) was performed in Excel. Pearson correlation coefficients are presented in Appendix B.

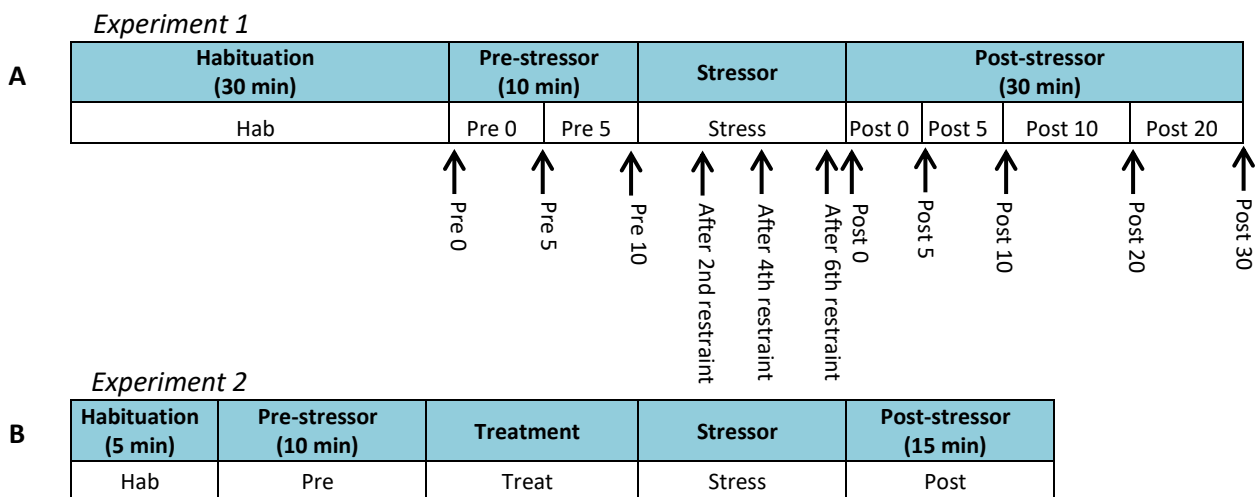


Fig. 2. Timeline of Experiment 1 (A) and 2 (B). Each cell indicates a moment (time period) in the experiment as used in the behavioural analysis. Arrows indicate the moments at which the measurements of mid-tail temperatures took place.

Behaviour	State/ event	Description
<b>Active non-social behaviour</b>		
Rear	State	De rat is standing on its hind paws, with or without leaning against the plexiglass wall. Rearing is usually accompanied by sniffing. Huddling with other rats against the wall is not counted as rearing.
Walk	State	The rat is moving forward with four legs.
Dig	State	The rat uses its front paws to move the bedding.
Eat	State	The rat eats pellets lying in the cage or nibbles on a piece of bedding.
Explore (hand)	State	The rat touches the hand of the researcher with its snout.
Climb (hand)	State	De rat climbs on the hand of the researcher with its front paws.
Flee (hand)	Event	The rat runs away from the hand of the researcher when they are trying to pick it up.
Groom	State	The rat washes its face, usually by first licking the front paws and then rubbing the face, or the rat rubs its fur. Scratching is also counted as grooming when a scratch is followed by licking the paw.
Scratch	Event	The rat scratches itself. Scratching can occur during self-grooming or as a quick, isolated behaviour. When scratching occurs during grooming, one scratch is counted.
Shake	Event	The rat quickly shakes its whole body or its head.
Yawn	Event	The rat briefly opens its mouth wide.
<b>Inactive non-social behaviour</b>		
Immobile	State	The rat stays in one place, either standing or sitting, and is not performing any of the other state behaviours. The rat may be looking around, sniffing or turning its body. It may also be motionless without being rigid, for example between periods of resting.
Rest	State	De rat is motionless, lying on the ground or on top of another rat. The eyes, if in view, are (partially) closed. When a rat is huddling with its head tucked in, this is also counted as resting. When another rat lays itself on top of the resting rat, the rat is assumed to still be resting if it is not seen moving, even though it is out of view.
Freeze	State	The rat is completely motionless and rigid, and is either standing, sitting or leaning against the plexiglass wall, with its eyes open.
<b>Social behaviour</b>		
Allogroom	State	The rat is licking or nibbling the fur of another rat.
Be groomed	State	The rat's fur is licked or nibbled by another rat.
Huddle	State*	The rat is lying closely together with one or more conspecifics.
<b>Play behaviour</b>		
Pounce	State	The rat nuzzles the neck of another rat with its snout or front paws.
Receive pounce	State	The rat's neck is nuzzled by another rat's snout or front paws.
Pin	State	After the recipient of a pounce has turned on its back, the rat tickles the recipient on its belly. The pinning stops when the rat removes its front paws from the recipient.
Receive pin	State	After being pounced, the rat fully rotates around the longitudinal axis of its body, ending in supine position with the conspecific standing over it. The rat stops being pinned when it has rotated back to its original position.
Evasion of pin	Event	After being pounced, instead of rotating, the rat runs or turns away from the initiating rat.
Box	State	The rat is standing on its hind paws, facing another rat. Both rats are pushing, pawing or grabbing at each other, or one rat holds the other rat with it front paws.
Chase	State	The rat is following another rat that is walking away from it.
Bouncy gait	Event	The rat makes a quick, jumping movement.
<b>Experimental procedures</b>		
Handled	State	Outside of the stressor period, the rat is picked up by the researcher.
Manual restraint	State	During the stressor, the rat is picked up by the researcher and brought out of view. The rat is restrained out of view. Manual restraint stops when the rat is no longer handled.
<b>Out of view</b>		
Out of view	State	The rat is in its cage, but behaviour cannot be scored. This can happen when the researcher or other rats block the view of the rat.

Table 1. *Ethogram.* \*Huddling was scored separately by scan sampling.

## 2.9 Statistical analysis

Statistical analyses were performed using R 4.0.0.<sup>66</sup> The significance level was set at  $p = 0.05$ .

### 2.9.1 Behaviour durations and frequencies

For each state behaviour scored by focal sampling, the percentage of time spent performing this behaviour was calculated for each time period, as follows:

$$\text{Duration (\%)} = \frac{\text{total duration (s)}}{\text{total time (s)} - \text{manual restraint (s)} - \text{out of view (s)}} \cdot 100\%$$

The durations of Allogroom and Be Groomed were summed, creating the composite variable Social Groom. Since huddling was scored by scan sampling, the percentage of time spent huddling was calculated as follows:

$$\text{Huddle duration (\%)} = \frac{\text{Total moments spent huddling}}{\text{Total number of sampling moments}} \cdot 100\%$$

A sampling moment was only included when all rats were in view and none were being handled.

Event behaviours, as well as Rear and Groom, were analysed as frequencies (the number of times a rat performed this behaviour). Since the time periods differed in length, these frequencies were converted to frequencies per 5 minutes, as follows:

$$\text{Frequency/5 min} = \frac{\text{frequency}}{\text{total time (s)} - \text{manual restraint (s)} - \text{out of view (s)}} \cdot 300$$

Additionally, for Experiment 2, the average duration of one grooming bout (in seconds) was calculated by dividing the total groom duration by groom frequency.

### 2.9.2 Experiment 1, correlations

To get a first idea of the relationship between both freezing and tail temperature, and huddling and tail temperature, the correlation between the average behaviour duration (freezing or huddling, in %) and the overall mid-tail temperature of each rat was analysed. Overall mid-tail temperature was approximated as the area under the curve (AUC) of a temperature-time plot. The AUC was calculated by first multiplying the average of two consecutive temperature measurements by the time difference in minutes, giving the area under the curve between two measurements, and then taking the sum of all the areas. Normality of data was assessed by visual inspection of histograms, qq-plots and the Shapiro-Wilk test. Since the distribution of Freeze duration, Huddle durations and AUC's were non-Normal, the nonparametric Spearman Rank correlation coefficient was calculated.

### 2.9.3 Experiment 1, mixed models

The behaviours Walk, Eat, Dig, Climb hand, Explore hand, Flee hand, Yawn, and all play behaviours were not analysed due to very low occurrence. The effects of Freeze, Rest, Huddle,

Social Groom, Groom duration, Groom frequency, Rear, Shake and Scratch on tail temperature of Experiment 1 were analysed with linear mixed models, using the *lme* package in R.<sup>67</sup> Mixed models are suitable for unbalanced data with repeated measures. Also, both categorical and continuous variables can be included as fixed effects. Separate models for each behaviour were made with Temperature as dependent variable, Rat nested in Cage as random effect and Behaviour (duration or frequency), Treatment and Moment as fixed effects. The interaction Behaviour\*Moment was initially included, but was removed if not significant. Significance was calculated using the *lmerTest* package,<sup>67</sup> which applies Satterthwaite's method to estimate degrees of freedom. Post-hoc pairwise comparisons were performed with Tukey adjusted p-values for performing multiple comparisons. In case of a significant interaction effect, post-hoc pairwise comparisons were performed on each level of Moment separately, and a Bonferroni correction was applied to the significance level.

From one rat, only its huddle duration was included for the moment 'post 5', because it was only in view for 0.3% of the time. The view was blocked by other huddling rats. Huddle duration was included, because that behaviour could be scored.

Mixed models do not require the variables to be Normally distributed; however, Normal distribution of residuals is assumed. To test this, histograms and qq-plots of residuals were visually inspected, in addition to a Shapiro-Wilk test. In the case of non-Normal residuals, data was transformed to better fit a Normal distribution. Freeze durations were cube root transformed and Rear frequencies were log-transformed. While this improved the distribution of the histogram and qq-plot, the Shapiro-Wilk test was still significant. The models with Groom, Scratch and Shake frequencies had residuals which deviated from Normality according to the Shapiro-Wilk test, which did not improve with transformation. However, the visual inspections did not show great deviations from Normality: histograms were roughly bell-shaped and the qq-plots resembled a straight line (see Appendix C for distributions of the Freeze model). These models were still analysed, since there is no suitable nonparametric alternative that can analyse with a similar model.

An additional model with Freeze durations was made, with exclusion of very low values, as this might improve the distribution of residuals. This meant that the moments 'pre 5' and 'post 20', and four zero-values of moment 'post 5' were excluded. However, since this made the distribution of residuals less Normal (see Appendix C), this model was not analysed further. The moment 'post 20' was also automatically excluded by R in the complete model, since all values were 0 at that moment. This was also true for the moment 'stress' in the model with Rest.

#### **2.9.4 Experiment 2, mixed models**

The same behaviours as in Experiment 1 were analysed in Experiment 2. This time, Behaviour was the dependent variable. Rat nested in Cage was again used as random effect, and Treatment and Moment as fixed effects. The interaction Treatment\*Moment was initially included, but was removed if not significant.

Freeze, Rest and Groom Bout durations were cube root transformed to make distribution of residuals Normal (in case of Rest and Groom Bout) or more Normal (Freeze). Besides the model

for Freeze, models without Normal distribution of residuals (according to Shapiro-Wilk test) were Social Groom, Scratch (log-transformed) and Shake (square root transformed).

### 3 Results

All behaviour durations and frequencies for each moment are presented in Appendix D.

#### 3.1 Experiment 1, correlations

There was no significant correlation between Freeze duration and AUC of tail temperatures ( $r_s = 0.24$ ,  $p = 0.36$ , Fig. 3A) or between Huddle duration and AUC of tail temperatures ( $r_s = -0.29$ ,  $p = 0.28$ , Fig. 3B).

#### 3.2 Experiment 1, mixed models

The fixed effects Moment and Treatment had a significant effect on tail temperatures in all models, while the interaction Behaviour\**Moment* was not included in any of the models. Groom Frequency ( $F_{1,71.0} = 5.0$ ,  $p = 0.028$ , Fig. 3A), Scratch ( $F_{1,68.7} = 5.5$ ,  $p = 0.022$ , Fig. 4B) and Rear ( $F_{1,55.3} = 5.6$ ,  $p = 0.022$ , Fig. 4C) had an effect on tail temperature, in which higher Groom, Scratch or Rear frequencies were associated with higher tail temperatures. The following behaviours had no significant effect on tail temperature: Freeze ( $F_{1,71.0} = 3.5$ ,  $p = 0.07$ , Fig. 4D), Rest ( $F_{1,68.9} = 1.09$ ,  $p = 0.30$ ), Huddle ( $F_{1,70} = 0.03$ ,  $p = 0.87$ ), Groom Duration ( $F_{1,69.5} = 3.3$ ,  $p = 0.07$ ), Social Groom ( $F_{1,69.6} = 1.0$ ,  $p = 0.32$ ) and Shake ( $F_{1,67.0} = 1.0$ ,  $p = 0.31$ ).

#### 3.3 Experiment 2, mixed models

The models of Experiment 2 investigated the effect of Treatment and Moment on Behaviour. Freeze duration (cube root transformed) was affected by Treatment ( $F_{2,11} = 7.0$ ,  $p = 0.011$ ), Moment ( $F_{3,33} = 32.4$ ,  $p < 0.001$ ), and interaction Treatment\**Moment* ( $F_{6,33} = 4.8$ ,  $p = 0.0013$ ).

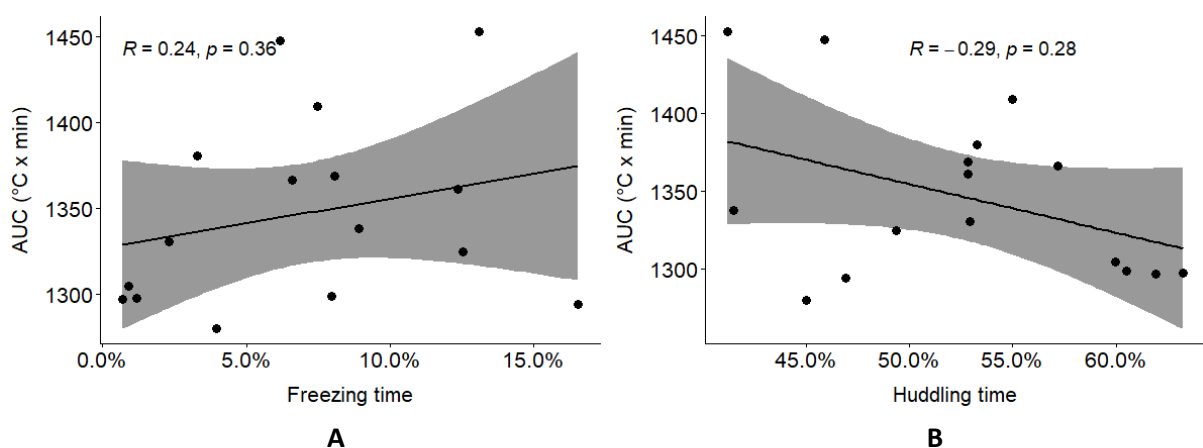


Fig. 3. Correlation between A) Freeze duration (%) and B) Huddle duration (%) and tail temperature AUC (°C x min) of each rat. Freeze and Huddle durations are given as percentages of the total time in view. The shaded area depicts the 95% confidence interval of the regression line.



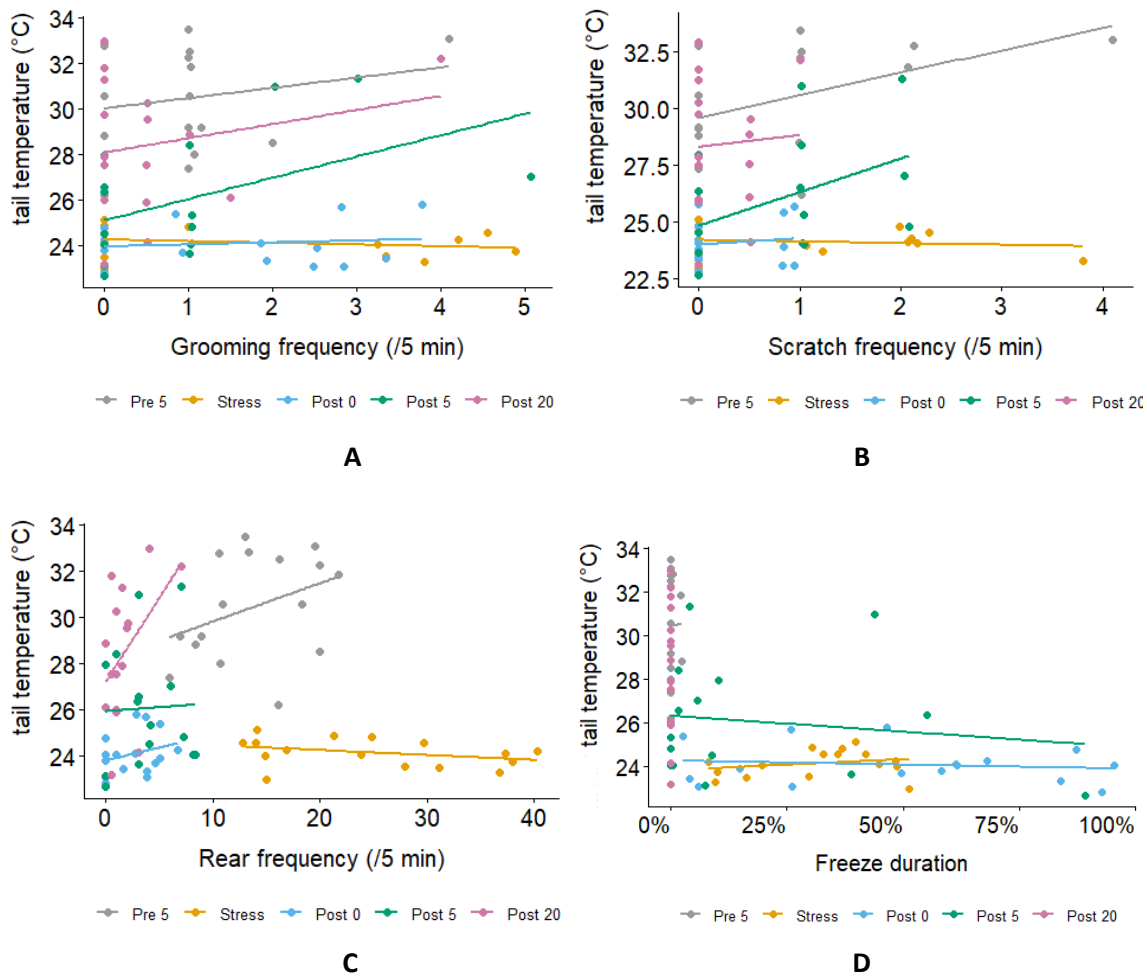


Fig. 4. Scatterplots of mid-tail temperature ( $^{\circ}\text{C}$ ) vs. A) Groom frequency/ 5min, B) Scratch frequency/ 5min, C) Rear frequency/ 5min and D) Freeze duration (%). Regression lines are given per moment, as Moment affected tail temperatures. The untransformed data is used. Groom, Scratch and Rear frequency had a significant effect ( $p < 0.05$ ) on tail temperature.

Gentled rats froze less than Control rats ( $t_{36.3} = 5.0$ ,  $p < 0.0001$ ) and Tickled rats ( $t_{36.3} = 4.6$ ,  $p = 0.0001$ ) during the stressor, and less than Control rats post-stressor ( $t_{36.3} = 3.8$ ,  $p = 0.0016$ ) (Fig. 5A). The complete pairwise comparisons of treatment groups per moment, for all models with a significant interaction effect, are presented in Appendix E.

Treatment ( $F_{2,44} = 3.7$ ,  $p = 0.034$ ) and Moment ( $F_{3,44} = 7.2$ ,  $p = 0.0005$ ) affected Groom duration, with a significant interaction Treatment\**Moment* ( $F_{6,44} = 4.5$ ,  $p = 0.0013$ ). During the pre-stressor moment, Control rats spent on average 11.3% more time grooming than Tickled rats ( $t_{44} = 4.0$ ,  $p = 0.0008$ ) and 9.9% more than Gentled rats ( $t_{44} = 3.1$ ,  $p = 0.0081$ ) (see Fig. 5B and Table E2).

Groom frequency was affected by Treatment ( $F_{2,44} = 7.6$ ,  $p = 0.0015$ ) with a significant interaction Treatment\**Moment* ( $F_{6,44} = 3.1$ ,  $p = 0.012$ ). During the post-stressor moment, Control rats groomed 2.8 times per 5 minutes more than Tickled rats ( $t_{44} = 3.4$ ,  $p = 0.0046$ ) and 3.6 times per 5 minutes more than Gentled rats ( $t_{44} = 3.7$ ,  $p = 0.0015$ ) (see Fig. 5C and Table E3).

The median duration of a groom bout was 13.0 seconds (IQR 12.4 s). There was no significant effect of Treatment ( $F_{2,39} = 2.3$ ,  $p = 0.11$ ) or Moment ( $F_{3,39} = 2.5$ ,  $p = 0.075$ ) on Groom bout duration (cube root transformed), but there was a significant interaction Treatment\*Moment ( $F_{6,39} = 2.6$ ,  $p = 0.030$ ) (Fig. 5D). During the post-stressor moment, the average groom bout duration was longer for Tickled rats than for Gentled rats ( $t_{39} = 3.5$ ,  $p = 0.0032$ ) (see Fig. 5D and Table E4).

Huddle duration was affected by Moment ( $F_{3,33} = 88.7$ ,  $p < 0.0001$ ), with a significant interaction Treatment\*Moment ( $F_{6,33} = 4.4$ ,  $p = 0.0023$ ). During the pre-stressor moment, Gentled rats huddled on average 39.3% more than Control rats ( $t_{35.5} = 4.1$ ,  $p = 0.0006$ ) and 26.8% more than Tickled rats ( $t_{35.5} = 3.4$ ,  $p = 0.0045$ ) (see Fig. 5E and Appendix E).

The models with Social Groom, Rest, Scratch, Shake and Rear did not include the interaction Treatment\*Moment. Social Groom was affected by Moment ( $F_{3,50} = 52.9$ ,  $p < 0.0001$ ), not by Treatment ( $F_{2,50} = 0.43$ ,  $p = 0.66$ ). Pairwise comparisons of Moment are presented in Table 2.

Comparison	Estimate (SE)	df	t-ratio	p
hab-pre	-0.11167 (0.0104)	50	-10.773	<.0001***
hab-stress	0.00129 (0.0104)	50	0.124	0.9993
hab-post	-0.02423 (0.0104)	50	-2.338	0.1031
pre-stress	0.11296 (0.0104)	50	10.897	<.0001***
pre-post	0.08744 (0.0104)	50	8.435	<.0001***
stress-post	-0.02552 (0.0104)	50	-2.462	0.0786

Table 2. *Post-hoc test of Social Groom duration model, with Tukey adjusted p-values.*

Rest duration (cube root transformed) was also affected by Moment ( $F_{3,50} = 31.5$ ,  $p < 0.0001$ ), not by Treatment ( $F_{2,50} = 2.1$ ,  $p = 0.13$ ). Pairwise comparisons of Moment are presented in Table 3.

Comparison	Estimate (SE)	df	t-ratio	p
hab-pre	-0.1842 (0.0685)	50	-2.687	0.0466
hab-stress	0.0426 (0.0685)	50	0.622	0.9245
hab-post	-0.5541 (0.0685)	50	-8.086	<.0001***
pre-stress	0.2268 (0.0685)	50	3.309	0.0091**
pre-post	-0.3700 (0.0685)	50	-5.399	<.0001***
stress-post	-0.5967 (0.0685)	50	-8.708	<.0001***

Table 3. *Post-hoc test of Rest duration model (cube root transformed), with Tukey adjusted p-values.*

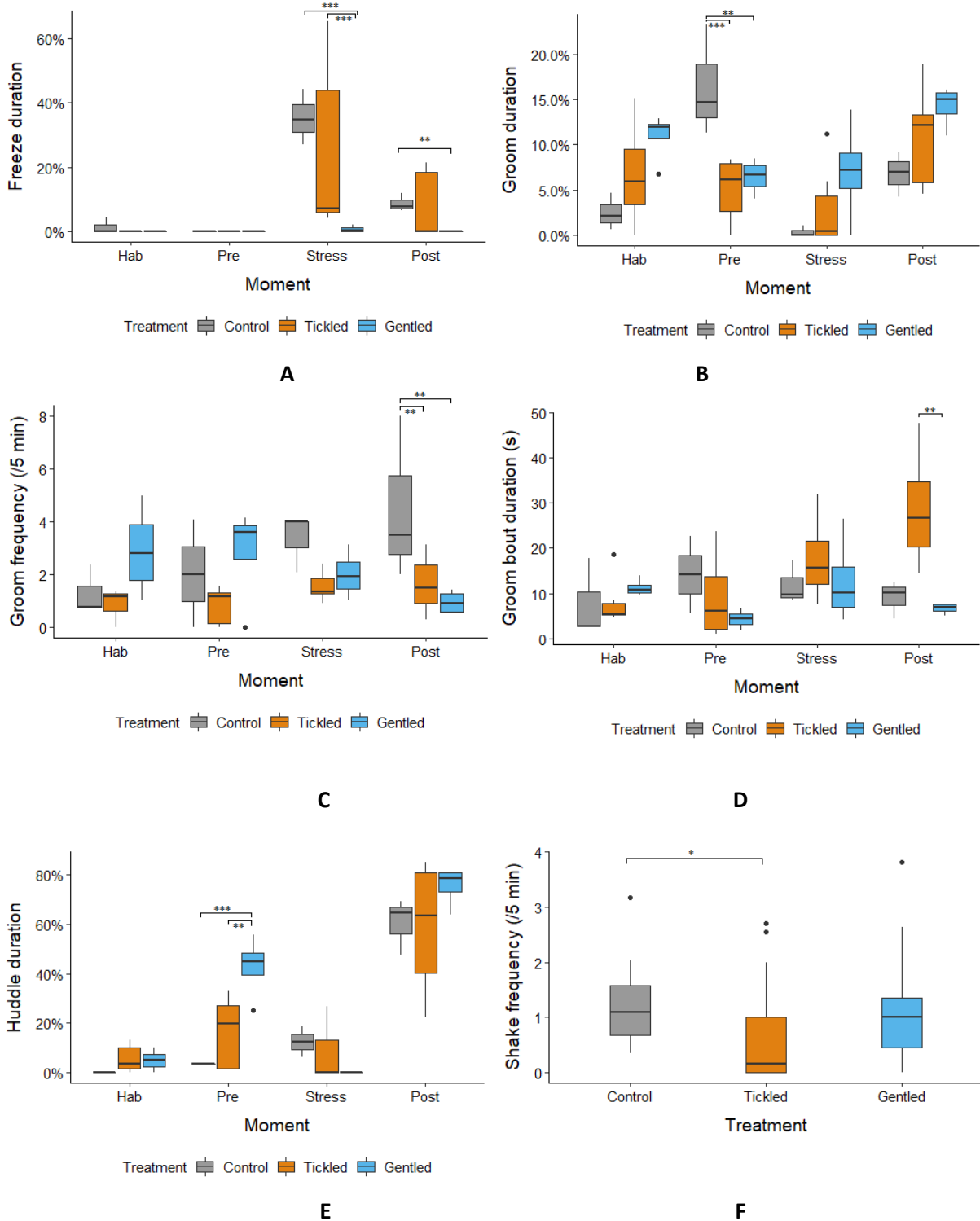


Fig. 5 .Boxplots of A) Freeze duration (%); B) Groom duration (%); C) Groom frequency/5 min; D) Groom Bout duration (s); E) Huddle duration (%), grouped by Moment and Treatment, and F) Shake frequency/5 min, grouped by Treatment. Durations and frequencies re presented asmedian  $\pm$  IQR. Data are untransformed. Significant differences between treatment groups are indicated by asterisks: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

Scratch frequency (log-transformed) was affected by Moment ( $F_{3,50} = 3.1$ ,  $p = 0.036$ ) and Treatment ( $F_{2,50} = 3.5$ ,  $p = 0.039$ ). However, in the pairwise comparisons, no comparisons of Treatment groups were significant (Table 4).

Comparison	Estimate (SE)	df	t-ratio	p
Control-Tickled	0.2796 (0.151)	50	1.846	0.1655
Control-Gentled	-0.0487 (0.168)	50	-0.290	0.9546
Tickled-Gentled	-0.3283 (0.138)	50	-2.386	0.0535
hab-pre	-0.0783 (0.166)	50	-0.472	0.9650
hab-stress	0.1664 (0.166)	50	1.002	0.7487
hab-post	0.3867 (0.166)	50	2.330	0.1049
pre-stress	0.2446 (0.166)	50	1.474	0.4605
pre-post	0.4649 (0.166)	50	2.801	<b>0.0352*</b>
stress-post	0.2203 (0.166)	50	1.327	0.5502

Table 4. *Post-hoc test of Scratch frequency model (log-transformed), with Tukey adjusted p-values.*

Shake frequency (square root transformed) was affected by Moment ( $F_{3,50} = 6.3$ ,  $p = 0.0011$ ) and Treatment ( $F_{2,50} = 4.9$ ,  $p = 0.011$ ). Control rats shook slightly more frequently than Tickled rats ( $t_{59} = 2.8$ ,  $p = 0.018$ , Fig. 4F). Pairwise comparisons of Treatment and Moment are presented in Table 5.

Comparison	Estimate (SE)	df	t-ratio	p
Control-Tickled	0.2535 (0.0893)	50	2.840	<b>0.0176*</b>
Control-Gentled	0.0768 (0.0988)	50	0.777	0.7187
Tickled-Gentled	-0.1768 (0.0811)	50	-2.180	0.0846
hab-pre	0.0332 (0.0978)	50	0.339	0.9864
hab-stress	-0.2413 (0.0978)	50	-2.467	0.0776
hab-post	0.1751 (0.0978)	50	1.790	0.2902
pre-stress	-0.2745 (0.0978)	50	-2.807	<b>0.0347*</b>
pre-post	0.1419 (0.0978)	50	1.451	0.4746
stress-post	0.4164 (0.0978)	50	4.257	<b>0.0005***</b>

Table 5. *Post-hoc test of Shake frequency model (square root transformed), with Tukey adjusted p-values.*

Rear frequency was affected by Moment ( $F_{3,39} = 24.0$ ,  $p < 0.0001$ ), not by Treatment ( $F_{2,11} = 0.2$ ,  $p = 0.82$ ). Pairwise comparisons of Moment are presented in Table 6.

Comparison	Estimate (SE)	df	t-ratio	p
hab-pre	9.70 3.01	39	3.228	0.0129*
hab-stress	3.42 3.01	39	1.140	0.6676
hab-post	23.59 3.01	39	7.850	<b>&lt;.0001***</b>
pre-stress	-6.28 3.01	39	-2.088	0.1748
pre-post	13.89 3.01	39	4.622	<b>0.0002***</b>
stress-post	20.16 3.01	39	6.710	<b>&lt;.0001***</b>

Table 6. *Post-hoc test of Rear frequency model, with Tukey adjusted p-values.*

## 4 Discussion

### 4.1 Relationship between behaviour and tail temperature

In this study, the effect of behaviour on tail temperature was investigated. The hypothesis was that rats that showed more stress-related behaviour, such as freezing, scratching and shaking, would have lower tail temperatures, since stress induces vasoconstriction in the rat tail and lowers tail temperature.<sup>31</sup> Huddling was expected to increase temperatures, because of the close physical contact between rats. The results did not support the hypotheses. No effect of freezing, shaking or huddling on tail temperature was found.

During Experiment 1, scratching significantly influenced tail temperatures, but opposite to what was expected: rats that scratched more frequently had higher tail temperatures. The same was seen for rats that groomed more frequently. Grooming and scratching are interrelated behaviours; rats can perform a scratch as an isolated behaviour, but also during grooming. The relationship between grooming and stress is difficult to interpret. When rats are faced with a mild stressor (e.g. habituation to a novel environment), groom frequency increases. However, when the stressor is highly aversive (e.g. exposure to light and noise, or foot shocks), rats stop grooming.<sup>44</sup> Groom durations, groom frequencies and groom bout durations varied highly between treatment groups at different moments (Fig. 5). From these results, it is not clear if the manual restraint was aversive enough to repress grooming. If so, rats that groomed more might have been less stressed. Some researchers state that grooming helps with de-arousal.<sup>46</sup> Then, grooming could help to increase tail temperatures to pre-stressor levels. It is remarkable, however, that only groom frequency had an effect on temperature, not total groom duration. Another curious finding is that in Experiment 2, scratch frequencies almost did not differ between different moments. Scratching was expected to increase during and after the stressor, if it is a stress reaction. To the author's knowledge, the effect of a stressor on scratch frequency has not been studied before. Therefore, it is uncertain whether scratch frequency is a reliable indicator of stress levels.

The finding that more frequent rearing was related to higher tail temperatures was as expected. Rearing is an exploratory behaviour and a sign of low fear or anxiety.<sup>68</sup> Interestingly, Duparcq et al.<sup>32</sup> found that in mound-building mice, 'fast explorers' had lower tail temperatures after handling than 'slow explorers'. Their findings can be explained by considering coping styles. Pro-active animals, of which higher exploratory tendency is a trait, show a more sympathetic reaction to stress, which induces a stronger vasoconstriction in the tail.<sup>32</sup> This effect was not found in the present study. However, not enough behavioural analyses were performed to be able to assign coping styles to the rats in this study. As a result, higher rear frequencies due to either pro-active coping or lower stress levels cannot be distinguished.

### 4.2 Effect of tickling and gentling on behaviour

This study also investigated possible differences in behaviour between gentled, tickled and control rats. The objective of both tickling and gentling is to habituate the rat to handling. Tickling mimics play behaviour, which is a rewarding behaviour for rats.<sup>8</sup> Hence, the tickled and gentled rats were expected to experience the manual restraint as less stressful, and show less

stress-related behaviour, than the control rats. Interestingly, gentled rats froze less than tickled or control rats during the stressor, and less than control rats after the stressor. In fact, the gentled group almost did not freeze. In Experiment 1, differences in behaviour between treatment groups were not analysed, but it was observed that the gentled rats froze less during Experiment 1 as well. This seems to suggest that the gentled rats were less fearful and experienced the manual restraint as less stressful. All rats started freezing during and after the stressor period, which supports the assumption that freezing is induced by fear.<sup>37</sup> It is important to note that freezing is also a characteristic of a reactive coping style.<sup>62</sup> It is possible that the gentled rats had a more proactive coping style on average. However, as mentioned before, coping styles could not be distinguished in the present study.

Another interesting finding is the great individual variation in the freeze response between rats, visible as a wide spread in freeze durations. The variation is most pronounced in the tickled group. In this study, rats froze in response to the manual restraint. High levels of freezing in the tickled group suggests that the tickled rats have not developed a more positive association with handling than control rats, which was the goal of tickling. Moreover, it was observed that some rats froze during the tickling treatment in Experiment 2 as well. Not all rats enjoy being tickled to the same degree.<sup>11</sup> While many rats seem to derive pleasure from tickling,<sup>12</sup> some rats might even experience it as aversive. Bombail et al.<sup>11</sup> argued that the extensive use of pinning in current tickling protocols does not allow rats to express their enjoyment of tickling. In future research, when the effect of tickling is investigated, inclusion of a gentling group is recommended, to investigate whether gentling is just as, if not more stress-reducing for rats. It would also be interesting to focus more on individual variation between rats.<sup>69</sup>

Bowen et al.<sup>70</sup> showed that rats huddled more after exposure to cat odour, an unconditioned fear-inducing stimulus, compared to non-exposed rats. In the present study, rats huddled more after manual restraint, but because no comparison with non-stressed rats is made, it is unclear whether the increased huddling is a reaction to the stressor, or simply a result of the rats being less active at the end of the experiment. The increased rest durations post-stressor seem to suggest the latter. The finding that gentled rats huddle more, but only pre-stressor, cannot be well explained. At any rate, huddle durations are difficult to interpret, because of the strong dependence on the behaviour of cage-mates. As will be discussed later, more cages should be used to investigate huddling behaviour.

Rats performed more shakes during the stressor than pre- or post-stressor in Experiment 2. Shaking therefore seems to be a reaction to the stressor. The control group shook more frequently than the tickled group. This could suggest that the control group was more stressed than the tickled group. However, it is remarkable that the gentled group did not differ from the other two groups, since they froze the least. The question is whether shake frequency is a reliable measurement of stress. Chaouloff et al.<sup>71</sup> found an increase in 2,5-dimethoxy-4-iodoamphetamine (DOI) induced wet-dog shakes after rats were restrained for three hours. DOI, a serotonin receptor agonist, is administered to rats in other studies examining wet-dog shakes and head shakes as well.<sup>38-40</sup> Moreover, most studies investigate the effect of chronic instead of acute stress on head shakes, because the increase in shakes is thought to arise from an increase in the number

of serotonin receptors in the brain, which is not an acute response.<sup>38-40</sup> It has not been proven that shake frequency increases after acute stress without pharmacological influence.

### **4.3 Variation in tail temperatures**

Analysis of tail temperatures by Weitkamp<sup>63</sup> showed that the gentled rats had the lowest baseline tail temperatures. Behavioural analysis in the present study did not provide an explanation for this finding. Before the stressor in Experiment 2, gentled rats only differed from tickled and control rats in huddle duration. However, in Experiment 1, there was no visible difference in huddle duration between control and gentled rats. For additional research, it would be interesting to perform both analyses, the relationship between behaviour and treatment group, and the differences in behaviour between groups, on data of the same experiment.

Besides behaviour, other factors could explain the difference in baseline temperatures between treatment groups and the high variation between individual rats. Vianna and Carrive<sup>31</sup> remarked that tail blood flow is sensitive to small disturbances, such as noise. In the present study, the circumstances in the test room were not equal for all cages: background noise and movement of people in the room were not constant. In addition, the circadian rhythmicity of body temperature<sup>72</sup> and individual differences in temperature set point, demonstrated in humans,<sup>73</sup> could give rise to variation in baseline temperatures.

### **4.4 Limitations and recommendations for further studies**

A limitation of this study is the small sample size. Specifically, the control and gentling group only consisted of one cage. Therefore, correcting for cage effects in the mixed model was almost impossible. A cage effect which could influence the results is 'behavioural contagion': the presence of a stressed rat can increase stress levels of cage-mates.<sup>74</sup> For example, untrained rats freeze in response to a cue when their cage-mate, that has been trained to associate that cue with foot shocks, freezes.<sup>75,76</sup> In other words, the presence of a freezing rat could influence the freezing behaviour of the whole cage. The same could be true for other behaviours. Therefore, in a next experiment, each treatment group should consist of multiple cages.

In Experiment 1, the rats were picked up by the researcher before each temperature measurement to ensure that all tails were visible. These wake-up moments influence behaviour; for example, rats might have rested and huddled more if they were not roused. Moreover, rats that found handling more stressful might have had a more pronounced stress reaction to the wake-up moments, which could have influenced tail temperatures.

In Experiment 1, tail temperatures reached a similar lowest level during the stressor in all rats.<sup>63</sup> Vianna and Carrive found the same when they placed fear-conditioned rats in the chamber in which they had received electric shocks.<sup>31</sup> This maximal drop in tail temperatures can be a limitation of the use of infrared thermography. If the minimal tail temperature is reached, differences between stress levels of individual rats could go undetected. Perhaps these differences are more pronounced when a less aversive stressor is used. In future research, it could also be interesting to focus on the rate in which tail temperatures return to baseline levels after a stressor.

## 5 Conclusion

In this study, an effect of groom, scratch and rear frequency on tail temperatures was found. Gentled rats also froze less in reaction to manual restraint than tickled or control rats. Additional research is necessary to support the findings, since the power of the present study was small. The relationship between freezing and tail temperatures remains unclear and is an interesting subject for further research. In addition, the results seem to suggest that tickling does not always increase positive associations with human interaction. This should also be investigated in the future.

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## Appendix A: Excerpt from Materials & Methods of Weitkamp et al. (2020)

### 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).

### 3.4 THERMAL VIDEO ANALYSIS

#### 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.



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

## Appendix B: Within-observer reliability

	Pearson
Allogroom	0.993
Be groomed	0.998
Be pounced	0.921
Dig	0.987
Eat	1.000
Freeze	0.999
Groom	0.998
Handled	1.000
Immobile	0.978
Out of view	1.000
Pounce	1.000
Rear	0.997
Rest	0.999
Scratch	1.000
Shake	0.982
Walk	0.984

Pin, Be pinned, Evade pin, Chase and Yawn were only observed once; therefore, no correlation coefficient could be calculated. Explore hand, Flee hand, Climb hand and Box were not observed.

## Appendix C: Distribution of residuals

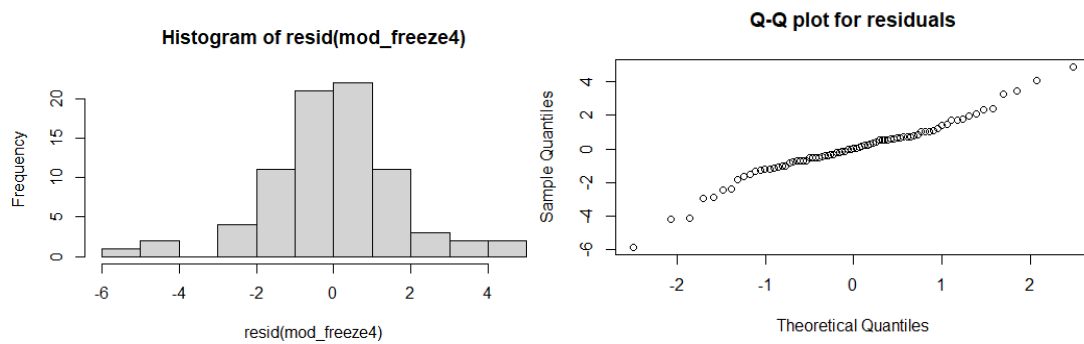


Figure C1. Histogram and *QQ*-plot for residuals of the model with Freeze duration, after cube root transformation.

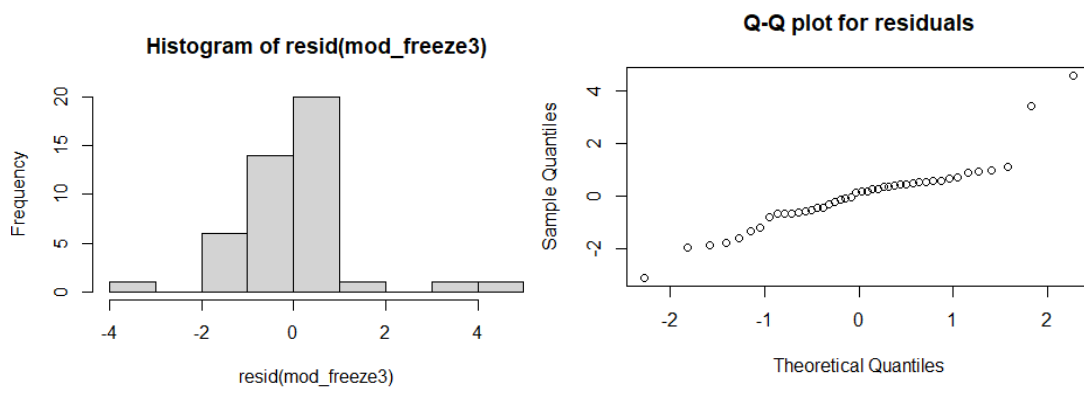


Figure C2. Histogram and *QQ*-plot for residuals of the model with Freeze duration, after cube root transformation, when all Freeze durations of 0% are excluded.

## Appendix D: Behaviour durations and frequencies

	hab	pre 0	pre 5	stress	post 0	post 5	post 10	post 20	Total exp
<b>Rest</b>	29.5% (10.6%)	28.8% (36.4%)	33.3% (30.9%)	0.0% (0.0%)	0.0% (21.4%)	15.4% (52.4%)	44.0% (41.2%)	74.4% (18.7%)	<b>34.1%</b> <b>(15.7%)</b>
<b>Immobile</b>	32.4% (8.1%)	40.7% (15.6%)	36.6% (14.6%)	36.2% (16.9%)	22.2% (21.3%)	35.6% (21.1%)	35.0% (23.8%)	20.4% (9.9%)	<b>32.2%</b> <b>(6.9%)</b>
<b>Rear</b>	25.4% (6.7%)	22.3% (22.3%)	22.0% (9.4%)	25.1% (10.3%)	3.1% (5.0%)	5.5% (8.0%)	7.6% (6.7%)	1.5% (3.2%)	<b>16.6%</b> <b>(6.3%)</b>
<b>Freeze</b>	0.1% (0.2%)	0.0% (0.1%)	0.0% (0.0%)	34.5% (23.8%)	54.0% (49.0%)	6.7% (38.4%)	0.5% (3.6%)	0.0% (0.0%)	<b>7.0%</b> <b>(6.7%)</b>
<b>Groom</b>	6.6% (4.1%)	0.0% (4.1%)	1.1% (3.2%)	0.1% (2.7%)	5.0% (14.8%)	0.4% (3.7%)	0.0% (9.4%)	0.2% (3.3%)	<b>5.7%</b> <b>(1.8%)</b>
<b>Allogroom</b>	3.0% (1.4%)	0.0% (1.0%)	0.0% (0.8%)	0.0% (0.8%)	0.0% (0.0%)	0.0% (0.0%)	0.2% (0.7%)	0.0% (0.0%)	<b>1.5%</b> <b>(0.9%)</b>
<b>Walk</b>	1.8% (0.3%)	1.7% (0.8%)	1.7% (0.9%)	4.5% (1.0%)	0.5% (0.5%)	0.7% (1.0%)	1.1% (0.7%)	0.4% (0.3%)	<b>1.5%</b> <b>(0.3%)</b>
<b>Be groomed</b>	3.1% (2.6%)	0.0% (1.4%)	0.0% (2.0%)	0.0% (1.1%)	0.0% (0.0%)	0.0% (0.0%)	0.3% (0.8%)	0.0% (0.0%)	<b>1.4%</b> <b>(1.4%)</b>
<b>Eat</b>	0.1% (0.4%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.1%)	0.0% (0.0%)	<b>0.2%</b> <b>(0.7%)</b>
<b>Dig</b>	0.1% (0.2%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.1%</b> <b>(0.1%)</b>
<b>Pin</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0%</b> <b>(0.0%)</b>
<b>Pounce</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0%</b> <b>(0.0%)</b>
<b>Be pinned</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0%</b> <b>(0.0%)</b>
<b>Be pounced</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0%</b> <b>(0.0%)</b>
<b>Box</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0%</b> <b>(0.0%)</b>
<b>Chase</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0%</b> <b>(0.0%)</b>
<b>Climb hand</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0%</b> <b>(0.0%)</b>
<b>Explore hand</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0%</b> <b>(0.0%)</b>

Table D1. Behaviour durations of Experiment 1 (median (IQR)).

	hab	pre 0	pre 5	stress	post 0	post 5	post 10	post 20	Total exp
<b>Rear</b>	13.4 (3.9)	12.0 (9.7)	13.2 (8.4)	24.3 (17.6)	2.8 (4.1)	3.0 (5.6)	3.0 (2.0)	1.0 (1.5)	<b>9.7 (3.5)</b>
<b>Shake</b>	0.7 (0.6)	0 (1.0)	0 (1.0)	3.1 (2.7)	0 (0)	0 (1.0)	0 (0.1)	0 (0)	<b>0.7 (0.2)</b>
<b>Scratch</b>	0.6 (0.8)	0 (1.0)	0.5 (1.0)	0.5 (2.1)	0 (0.8)	1.0 (1.0)	0 (0.5)	0 (0.5)	<b>0.5 (0.4)</b>
<b>Groom</b>	1.7 (0.8)	0 (1.0)	1.0 (1.0)	0.5 (3.5)	1.4 (2.6)	0.5 (1.0)	0 (1.5)	0.3 (0.5)	<b>1.2 (0.7)</b>
<b>Yawn</b>	0 (0.04)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.1)	<b>0.0 (0.1)</b>
<b>Evade pin</b>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	<b>0.0 (0.0)</b>
<b>Flee hand</b>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	<b>0.0 (0.0)</b>
<b>Bouncy gait</b>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	<b>0.0 (0.0)</b>

Table D2. Behaviour frequencies (in count per 5 minutes) of Experiment 1 (median (IQR)).

	hab	pre	treat	stress	post	Total exp
<b>Immobile</b>	31.7% (7.7%)	33.4% (16.0%)	27.5% (11.1%)	47.5% (10.1%)	41.1% (15.3%)	<b>35.8% (5.6%)</b>
<b>Rear</b>	55.9% (13.1%)	37.9% (15.9%)	35.3% (15.4%)	25.6% (22.6%)	9.1% (7.5%)	<b>27.0% (3.0%)</b>
<b>Rest</b>	0.0% (0.0%)	0.9% (5.3%)	0.0% (0.0%)	0.0% (0.0%)	31.0% (29.9%)	<b>16.1% (10.7%)</b>
<b>Groom</b>	6.4% (8.5%)	7.5% (4.0%)	2.0% (1.9%)	0.7% (6.6%)	11.6% (7.2%)	<b>7.5% (3.9%)</b>
<b>Allogroom</b>	0.0% (0.0%)	5.2% (7.0%)	0.0% (0.0%)	0.0% (0.0%)	1.0% (1.1%)	<b>2.2% (1.5%)</b>
<b>Walk</b>	4.1% (0.7%)	1.8% (5.8%)	4.6% (5.6%)	5.8% (2.7%)	0.5% (6.4%)	<b>2.2% (1.2%)</b>
<b>Be groomed</b>	0.0% (0.0%)	6.1% (6.1%)	0.0% (0.0%)	0.0% (0.0%)	1.2% (1.0%)	<b>2.1% (2.1%)</b>
<b>Explore hand</b>	0.0% (0.0%)	0.0% (0.0%)	5.0% (6.8%)	0.4% (0.9%)	0.0% (0.0%)	<b>0.9% (1.4%)</b>
<b>Freeze</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.5%)	6.6% (39.0%)	0.0% (10.9%)	<b>0.6% (6.4%)</b>
<b>Dig</b>	0.0% (0.0%)	0.1% (0.2%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0% (0.2%)</b>
<b>Pounce</b>	0.0% (0.0%)	0.0% (0.5%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0% (0.1%)</b>
<b>Box</b>	0.0% (0.0%)	0.0% (0.2%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0% (0.1%)</b>
<b>Eat</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0% (0.0%)</b>
<b>Pin</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0% (0.0%)</b>
<b>Be pinned</b>	0.0% (0.0%)	0.0% (0.2%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0% (0.0%)</b>
<b>Be pounced</b>	0.0% (0.0%)	0.0% (0.2%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0% (0.0%)</b>
<b>Chase</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0% (0.0%)</b>
<b>Climb hand</b>	0.0% (0.0%)	0.0% (0.0%)	0.0% (2.3%)	0.0% (0.0%)	0.0% (0.0%)	<b>0.0% (0.0%)</b>

Table D3. Behaviour durations of Experiment 2 (median (IQR)).

	hab	pre	treat	stress	post	Total exp
<b>Rear</b>	30.8 (6.8)	21.2 (11.6)	34.0 (25.9)	25.0 (19.2)	5.7 (5.4)	<b>17.4 (6.3)</b>
<b>Groom</b>	2.2 (2.7)	3.5 (2.1)	1.2 (0.7)	0.7 (1.5)	1.4 (0.9)	<b>1.8 (0.7)</b>
<b>Scratch</b>	1.0 (1.7)	1.0 (1.2)	0.6 (1.0)	0.6 (1.3)	0.3 (0.3)	<b>0.6 (0.6)</b>
<b>Shake</b>	1 (0.9)	0.7 (1.3)	0 (1.0)	1.3 (1.7)	0.3 (0.6)	<b>0.7 (0.5)</b>
<b>Yawn</b>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.3)	<b>0.1 (0.1)</b>
<b>Flee hand</b>	0 (0)	0 (0)	0 (0)	0 (1.5)	0 (0)	<b>0.1 (0.1)</b>
<b>Evade pin</b>	0 (0)	0 (0.4)	0 (0)	0 (0)	0 (0)	<b>0.0 (0.1)</b>
<b>Jump</b>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	<b>0.0 (0.0)</b>

Table D4. Behaviour frequencies (in count per 5 minutes) of Experiment 2 (median (IQR)).



## Appendix E: Post-hoc tests per moment, Experiment 2

Comparison	Estimate (SE)	df	t-ratio	p
<b>Moment 'hab'</b>				
Control - Tickled	0.118 (0.1054)	36.3	1.115	0.5110
Control - Gentled	0.118 (0.1167)	36.3	1.008	0.5768
Tickled - Gentled	0.000 (0.0957)	36.3	0.000	1.0000
<b>Moment 'stress'</b>				
Control - Tickled	0.141 (0.1054)	36.3	1.339	0.3832
Control - Gentled	0.582 (0.1167)	36.3	4.988	<.0001***
Tickled - Gentled	0.441 (0.0957)	36.3	4.604	0.0001***
<b>Moment 'post'</b>				
Control - Tickled	0.193 (0.1054)	36.3	1.834	0.1730
Control - Gentled	0.441 (0.1167)	36.3	3.779	0.0016**
Tickled - Gentled	0.247 (0.0957)	36.3	2.585	0.0362

Table E1. *Post-hoc test of Freeze duration model (cube root transformed). After bonferroni correction, the new significance level is <0.0167.*

Comparison	Estimate (SE)	df	t-ratio	p
<b>Moment 'hab'</b>				
Control - Tickled	-0.0420 (0.0285)	44	-1.473	0.3137
Control - Gentled	-0.0843 (0.0316)	44	-2.671	0.0279
Tickled - Gentled	-0.0423 (0.0259)	44	-1.634	0.2424
<b>Moment 'pre'</b>				
Control - Tickled	0.1130 (0.0285)	44	3.963	0.0008***
Control - Gentled	0.0994 (0.0316)	44	3.149	0.0081**
Tickled - Gentled	-0.0136 (0.0259)	44	-0.526	0.8589
<b>Moment 'stress'</b>				
Control - Tickled	-0.0254 (0.0285)	44	-0.890	0.6493
Control - Gentled	-0.0669 (0.0316)	44	-2.120	0.0974
Tickled - Gentled	-0.0415 (0.0259)	44	-1.603	0.2551
<b>Moment 'post'</b>				
Control - Tickled	-0.0370 (0.0285)	44	-1.296	0.4047
Control - Gentled	-0.0741 (0.0316)	44	-2.349	0.0595
Tickled - Gentled	-0.0372 (0.0259)	44	-1.435	0.3322

Table E2. *Post-hoc test of Groom Duration model. After bonferroni correction, the new significance level is <0.0125.*

Comparison	Estimate (SE)	df	t-ratio	p
<b>Moment 'hab'</b>				
Control - Tickled	0.406 (0.857)	44	0.473	0.8841
Control - Gentled	-1.596 (0.948)	44	-1.683	0.2229
Tickled - Gentled	-2.002 (0.778)	44	-2.572	0.0355
<b>Moment 'pre'</b>				
Control - Tickled	1.218 (0.857)	44	1.421	0.3389
Control - Gentled	-0.812 (0.948)	44	-0.857	0.6700
Tickled - Gentled	-2.030 (0.778)	44	-2.609	0.0325
<b>Moment 'stress'</b>				
Control - Tickled	1.801 (0.857)	44	2.102	0.1010
Control - Gentled	1.357 (0.948)	44	1.431	0.3340
Tickled - Gentled	-0.444 (0.778)	44	-0.571	0.8363
<b>Moment 'post'</b>				
Control - Tickled	2.871 (0.857)	44	3.351	<b>0.0046 **</b>
Control - Gentled	3.551 (0.948)	44	3.745	<b>0.0015 **</b>
Tickled - Gentled	0.680 (0.778)	44	0.874	0.6595

Table E3. *Post-hoc test of Groom Frequency model. After bonferroni correction, the new significance level is <0.0125.*

Comparison	Estimate (SE)	df	t-ratio	p
<b>Moment 'hab'</b>				
Control - Tickled	-0.248 (0.414)	39	-0.599	0.8214
Control - Gentled	-0.659 (0.447)	39	-1.472	0.3154
Tickled - Gentled	-0.410 (0.378)	39	-1.085	0.5289
<b>Moment 'pre'</b>				
Control - Tickled	0.594 (0.490)	39	1.211	0.4539
Control - Gentled	0.931 (0.535)	39	1.741	0.2030
Tickled - Gentled	0.338 (0.428)	39	0.789	0.7118
<b>Moment 'stress'</b>				
Control - Tickled	-0.314 (0.404)	39	-0.776	0.7199
Control - Gentled	0.080 (0.447)	39	0.179	0.9826
Tickled - Gentled	0.394 (0.367)	39	1.072	0.5369
<b>Moment 'post'</b>				
Control - Tickled	-1.075 (0.404)	39	-2.660	0.0297
Control - Gentled	0.213 (0.447)	39	0.477	0.8825
Tickled - Gentled	1.289 (0.367)	39	3.509	<b>0.0032 **</b>

Table E4. *Post-hoc test of Groom Bout duration model (cube root transformed). After bonferroni correction, the new significance level is <0.0125.*

Comparison	Estimate (SE)	df	t-ratio	p
<b>Moment 'hab'</b>				
Control - Tickled	-0.05747 (0.0863)	35.5	-0.666	0.7847
Control - Gentled	-0.05000 (0.0956)	35.5	-0.523	0.8605
Tickled - Gentled	0.00747 (0.0784)	35.5	0.095	0.9950
<b>Moment 'pre'</b>				
Control - Tickled	-0.12538 (0.0863)	35.5	-1.452	0.3257
Control - Gentled	-0.39314 (0.0956)	35.5	-4.114	<b>0.0006 ***</b>
Tickled - Gentled	-0.26775 (0.0784)	35.5	-3.414	<b>0.0045 **</b>
<b>Moment 'stress'</b>				
Control - Tickled	0.04881 (0.0863)	35.5	0.565	0.8393
Control - Gentled	0.12500 (0.0956)	35.5	1.308	0.4001
Tickled - Gentled	0.07619 (0.0784)	35.5	0.972	0.5993
<b>Moment 'post'</b>				
Control - Tickled	0.01588 (0.0863)	35.5	0.184	0.9815
Control - Gentled	-0.14870 (0.0956)	35.5	-1.556	0.2777
Tickled - Gentled	-0.16458 (0.0784)	35.5	-2.099	0.1045

Table E5. *Post-hoc test of Huddle duration model. After bonferroni correction, the new significance level is <0.0125.*