The influence of different types of behavior on the eye temperature of mice using infrared thermography

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Abstract

Stress is an important factor that affects the welfare of mice used as research animals and therewith the reliability of research results. Currently the most common way of measuring stress levels is testing corticosterone levels from blood samples. However, taking these blood samples is invasive by itself and therefore a less invasive method that is reliable is an improvement.

Using infrared thermography to measure stress-induced hyperthermia could be such a non-invasive method. To use this method properly the different influences on the surface temperature of the body have to be explored. Previous research indicates that other factors may influence the surface temperature of animals. In this research the influence of different types of behavior on the eye and tail temperature of male C3H mice was explored.

16 male C3H mice were put in a testing cage for 30 minutes, for 5 consecutive days. The behaviors of the individual mice were logged continuously and the eye and tail temperature were measured using infrared thermography and were analyzed for every 5 minute interval. The different behaviors were divided into active and inactive behaviors, the duration of behavior of both groups were compared. Spearman's correlation test was run to explore the correlation between the duration of each separate behavior and the two behavior groups and the tail and eye temperature of the mice.

The results show a positive correlation between active behaviors and the eye temperature, and a negative correlation inactive behaviors and the eye temperature. The tail temperature the opposite was found; a positive correlation with the duration of inactive behaviors and a negative correlation with active behaviors.

This shows that behavior and activity level is something to consider during research toward body temperature and eye and tail temperature. However, stress-induced hyperthermia was still measurable in the same data. This means that this method, if explored more thoroughly in the future, might be used to indicate stress in laboratory animals

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Inhoud

Introduction

Protecting the welfare of laboratory animals is a challenge and moral obligation for everyone involved in animal experiments. Ethical considerations have to be made to decide whether the research goals outweigh the distress of the animals. Researchers have to follow the principles of the three R's in experimentation on animals; these are replace, reduce and refine (Centrale Commissie Dierproeven 2015). A very important component in securing the welfare of these animals is predicting and determining the amount of stress that is caused by laboratory procedures and husbandry. Therefore, investigating more accurate and less invasive techniques to measure stress is very relevant in protecting the welfare of these animals. Stress can be described as the state of the body during a potential adverse situation, which can be caused by physical or psychological tension and induces a hormonal and emotional response (Joëls and Baram 2009). Although this response is functional in protection through adaptation to the potential threat, it has negative effects on mental and physical health when the stress is present over a prolonged period of time (Godoy et al. 2018).

Currently blood analysis is commonly used to assess whether or not, and how much an animal is suffering from stress. Important indicators of stress are the glucocorticoid hormones such as cortisol and corticosterone in the blood system. They can be measured invasively from a blood sample and high levels indicate stress (Jerem et al. 2015). However, levels normally fluctuate during the day and high levels can also indicate excitement (McEwen and Wingfield 2003). Taking a blood sample is a stressor by itself, influencing the hormone levels and possibly the research results. These same hormones can also be measured from feces, fur or urine but this is even less time-specific and can only be used for chronic stress (Jerem et al. 2015). Therefore, it is useful to use a technique that has less influence on the animal and in consequence less on the research results.

Physical reaction to stress

After detection of a potential risk the mammalian body responds by activation of the stress systems. The fast responses are mostly caused by the sympatho-adrenal medullary (SAM) axis. The hypothalamus-pituitary-adrenal (HPA) axis is responsible for the more long-term responses (Ulrich-Lai and Herman 2009).

The physiological response to an acute stressor is mediated through activation of the sympathetic nervous system. The SAM system of the autonomic nervous system can increase the blood pressure and heart rate (Ulrich-Lai and Herman 2009). The sympathetic nervous system also causes peripheral vasoconstriction and with that causes blood to go to the core of the animal. Therefore, the temperature in the skin drops and it increases in the core of the animal. The increase of body temperature (BT) due to stress is stress-induced hyperthermia (SIH). Similar to the mentioned stress hormones this hyperthermia is related to the severity of the stressor (Herborn et al. 2015).

When a stressor is recognized by the nucleus paraventricularis (PVN) in the hypothalamus the HPA-axis is activated. This causes the synthesis of oxytocin, vasopressin and corticotropin-releasing hormone (CRH). As a consequence of the increased CRH and vasopressin release the pituitary gland is stimulated to release adrenocorticotropic hormone which subsequently activates the secretion of glucocorticoid by the cortex of the adrenal gland (Goncharova 2013). In rodents the main glucocorticoid is corticosterone (Godoy et al. 2018). Glucocorticoid enters the brain and forms a negative feedback loop by inhibiting the activity of the PVN and pituitary gland within the HPA-axis. This negative feedback loop protects the body from the negative effects of high glucocorticoid levels (Laryea et al. 2014). The HPA-axis can also be activated by inflammatory cytokines. The immune response, including inflammation, is inhibited by an increased glucocorticoids concentration. Furthermore, it decreases growth hormones and reproduction, which is beneficial during periods of

stress, but becomes problematic when glucocorticoid levels remain high over a prolonged period of time (Tsigos and Chrousos 2002).

When a stress response is repeated habituation can occur. The corticosterone response becomes less every consecutive time an animal is exposed to the same stressor. Consequential the physical responses decrease as the hormonal response decreases. This effect can be seen in different animals and for different stressors. However, some types of stressors cause sensitization instead of habituation. This is seen when an animal is exposed to a more severe stimulus or repeated social defeat. In these cases repetitive exposure to these situation increases the stress response with each succession (Barnum, Blandino, and Deak 2007; Bolivar 2009).

C3H mice, which are used in this study, show other habituation results compared to other inbred strains. In an open-field test showed no habituation for this mouse strain during a three-day test, where other inbred strains did show a significant decrease in physical activity (Bolivar et al. 2000). Within sessions, a decrease in activity was seen (Bolivar 2009). However, it is not clear whether the activity level or distance traveled is the same as habituation, as anxiety can also lead to inactivity.

Regulation of body temperature

To protect the homeostasis all homeothermic animals have a strict regulation of their BT to protect the optimal molecular circumstances. Heat is generated by skeletal muscles and through thermogenesis of chemical processes in the body. Heat loss is regulated as well through thermoregulatory cardiac regulation and vasoconstriction and dilation in the skin and the release of adrenocorticotropic hormone (ACTH). These processes are involuntary responses to the signals of the nervous system. This is the afferent sensory part of the thermoregulation. Thermoreceptors are located in the skin, the abdomen and in the brain and spinal cord, which send their information to the brain (Nakamura 2011). There are two types of thermoreceptors, that either sense cold or heat. In the median preoptic area in the anterior hypothalamus of the brain these signals are processed, and the regulatory mechanisms are activated to ensure the BT stays within its equilibrium. The output of the preoptic area is influenced mostly by the heat-sensitive neurons more than the cold sensitive ones. The most common response is therefore decreasing thermogenesis (Morrison and Blessing 2011).

The BT is highly regulated through many different pathways. The regulation of SIH is similarly very complex and is not completely understood. SIH is induced when a stressor is recognized in the medial amygdala. Sympathetic premotor neurons in the medullary raphe region in the brain stem are activated and consequently activate the dorsomedial hypothalamus (Kataoka et al. 2014). This is different from normal thermoregulation which happens in the preoptic and rostral hypothalamus (Vinkers 2009). The dorsomedial hypothalamus has an important function in the regulation of brown adipose tissue. The activation of brown adipose tissue plays a significant part in the thermogenesis during SIH (Kataoka et al. 2014; Mohammed, Ootsuka, and Blessing 2014). The activation of these premotor neurons cause cutaneous vasoconstriction as well (Lkhagvasuren et al. 2011).

When it comes to temporal changes in BT a distinction can be made between fever, SIH and hyperthermia. During fever the set point, the core temperature the body tries to maintain, is increased and the normal regulatory mechanisms take place to increase the temperature and maintain it at the new set point. Hyperthermia is similarly an increase in core BT but the set point is not increased, therefore the body's regulation mechanisms do not protect this new temperature (Olivier et al. 2005). SIH and fever are in contrast to hyperthermia not a passive response to exterior circumstances. In SIH, similar as in a fever, vasoconstriction in the subcutaneous tissue and shivering are observed. The regulatory system causing a fever is connected with SIH because of shared pathways, but is not activated in the same way (Vinkers 2009). An example of this difference is that SIH is not sensitive to

cyclooxygenases (COX) inhibitors. These medications inhibit the production of prostaglandin E2 which initiate fever. Accordingly, COX-inhibitors reduce fevers, but not SIH (Mohammed, Ootsuka, and Blessing 2014). Therefore, fever, hyperthermia, and SIH are three different phenomena.

The normal regulation of BT in mice is heavily influenced by their environment (Fox, Foster, and Small 1981). Because of their high area to mass ratios small animals such as mice have trouble reducing heat loss. These animals retain their BT by adaptations in their behavior such as building of a nest, huddling together and burrowing (Lacy and Lynch 1979).

In addition mice use locomotor activity to increase their core BT (Toth, Trammell, and Ilsley-Woods 2015). The contraction of the fibers in muscles generate heat, which is transferred throughout the body by the circulation of blood. Whereas exercise can cause hyperthermia in different types of mammals, it is unclear whether this is directly caused by the generated heat in the muscles or centrally regulated (Wanner et al. 2014). This is suggested, because the increase of BT caused by exercise is not dependent on the intensity. A moderate or high intensity exercise induces a similar hyperthermia (Wanner et al. 2014).

The conductance of the cells of all animals conform to a 24-hour cycle influencing the distribution of heat and therefore the BT (Fox, Foster, and Small 1981). The fluctuation within this 24-hour period is influenced by multiple genes and differs between different strains of mice. In mice in general the amplitude found was between 1,4℃ and 1,8℃ (Refinetti and Menaker 1992). When this is tested in C3H mice in a twelve hour light/twelve hour dark experiment the temperature follows a unimodal distribution with its peak in the first part of the dark and its low halfway through the light period. The mean low was 35,3℃ and the mean high was 36,9℃ and the mean average during this period was 36℃ (Tankersley et al. 2002).

The use of body temperature to measure stress

Different types of stressful stimuli can induce SIH in a test setting. Methods that have been used to induce stress in laboratory animals include both psychological and physiological discomforts. Widely used stressors are handling including insertion of a rectal thermometer, moving the cage, spreading of predator smell, social isolation, and introducing the animal to an unknown environment or open field (Dallmann et al. 2006; Bouwknecht, Olivier, and Paylor 2007). Dallmann et al. (2006) studied the influence of stressors on rats such as moving the cage and the insertion of a rectal thermometer. The response was an increase in BT measured through telemetry devices implanted in the peritoneum of the rats. This indicates that these types of stressors do induce hyperthermia. Varying degrees of stress caused by social defeat in mice produce a stronger SIH response when the severity of the impulse increases (Keeney, Hogg, and Marsden 2001).

This knowledge can be used if a non-operative, less invasive procedure can be used to measure this hyperthermic response. The use of SIH to indicate stress overcomes some of the flaws of blood tests, but is still invasive when a rectal thermometer or implanted telemetry device is used. Therefore, another method that can measure the temperature from a distance might be more suitable since the sampling method does not influence the measurement. A possibility would be measuring the surface temperature using infrared thermography (Tan et al. 2009).

Infrared thermography

Every surface emits radiation and the wavelength of this light depends on the temperature of the surface. The range of temperatures that the body emits all lye in the infrared region. An infrared camera can detect these radiations and form this information into a thermogram. This thermogram holds the real-time temperature of the surface (Tan et al. 2009; Sniegowski et al. 2015). Thermography

with infrared cameras can be used to measure the temperature of surface areas such as skin and can possibly be used to indicate SIH (McCafferty 2013). So far, this technology has been used on different animals, especially in many different avian species (McCafferty 2013).

Vogel et al. (2016) concluded that the ocular temperature of mice is related to the rectal temperature. Rizzo et al. (2017) made the same conclusion for dogs. As SIH is a temperature shift in the core BT it is necessary that the surface temperature reliably reflects this. The fact that ocular surface temperature is correlated to rectal temperature indicates that it does. In combination with the current knowledge about demonstrating SIH using thermography can improve the use of this technology in mice (Vianna and Carrive 2012). Lecorps, Rödel, and Féron (2016) used this information to measure the SIH in wild house mice induced by an elevated plus maze and open field using infrared thermography. They did not only find an increased eye temperature caused by anxiety, but also a larger increase among the more anxious mice. Rizzo et al. 2017 found an increase in eye temperature using infrared thermography in exercising dogs. They found the mean eye temperature change from 32,39^{o}C before the start of the experiment to 32,70^{o}C after walking, 34,33^{o}C after trotting and 34,90^{o}C after galloping. Indicating that exercise does influence BT as measured through infrared thermography in the dog.

This research

The next step in using infrared thermography to measure stress is to further investigate how the temperature fluctuates during physiological behaviors in both stressful and non-stressful environments. As stated above, the hormones involved in stress responses do fluctuate during distress, but also increase because of excitement. Therefore, in this research the temperature will be analyzed during active and inactive behavior. The goal of this research is to further examine if activity influences the measurements when thermography is used to monitor stress in mice. Secondly, it will be examined if a habituation in active behavior is reflected by a habituation in BT. Hop (2018) focused on the SIH in C3H mice caused by stressors such as new cage and handling. This research uses the data mentioned to answer these research goals.

The execution of the mouse experiment and the collection of the thermography data was already performed. To explore the influence of the behavior and activity of the mice, this previous data is used again and analyzed together with the behavioral data collected in this study. This study will differentiate between active and inactive behavior. Walking, digging and running will be categorized as active behaviors. Sitting, lying down, huddling, grooming and eating will be categorized as inactive behaviors.

The expected results of this study are that both the stress of a novel cage and handling and physical activity of the mice will induce hyperthermia. Furthermore, some behavioral habituation is expected, within and between sessions. The results of different studies on the stress response of mice to different stimuli show habituation between sessions both for novel cage research and with handling. A different result was obtained using C3H/HeJ mice. When observing their level of activity in stressful situation, the activity did not decrease within 3 days of testing (Cabanac and Briese 1992; Barnum, Blandino, and Deak 2007; Bolivar 2009). Therefore, the expectations for the habituation of these mice in this research are uncertain.

The expectations regarding the influence of physical activity on the measured surface temperature of the mice are that there is an activity-induced hyperthermia. However, according to literature the intensity of exercise has little influence on the amount of increase of the BT (Wanner et al. 2014). It is still expected that mice that are more active will have a higher increased surface temperature (Wanner et al. 2014).

Materials and methods

For this research data was used that was previously recorded by Hop (2018). The materials and methods section of Hop (2018) can be found in Appendix 1. Of this research project only the experiment using the test cage was selected for analysis in this research.

Mice

Male C3H/HeOuJ mice that were 12-weeks old at the start of testing were used. In total 16 mice were used, of which 8 mice were born at the Central Animal Facility of Utrecht University (UU mice). They were held under a 12-hour light-dark cycle (lights on at 7AM), room temperature: 18-24℃. These mice have never been transferred and have been in the same room since birth. The other 8 mice were bred at Experimental Animal Center Charles River in Sulzfeld Germany (CR mice) and brought to the University of Utrecht prior to this research. The UU mice and CR mice were housed socially in the same room under the same circumstances in two cages separated by group. Home cages were Makrolon type 3 and 43x27x15cm in size with sawdust bedding. Cage enrichment was a plastic tunnel, cardboard box and tissues. Food pellets (CRM(E) 801730, Special Diet Services) and water was available ad libitum.

Cameras

To measure the temperature the infrared camera FLIR T430sc was used with a resolution of 320 x 240, an emissivity of 0,98 and a frame rate of 5Hz. The camera was placed on a tripod and ± 100 cm from the cages. A regular camcorder (Panasonic HC-V180) was used to collect behavioral data and to identify the mice.

Research setup

Before testing the two groups of mice were divided randomly into four pairs per group, 8 pairs in total. During the experiment the two mice were put in a testing cage (Perspex box, 21x15x15cm) with sawdust bedding. They were not familiar with the cage before the experiment. Each pair stayed in the testing cage for 30 minutes. During the experiment two testing cages were placed next to each other so that two pairs could be recorded simultaneously. After the 30 minutes the next two pairs were put in the same testing cages with clean bedding. This method was repeated on 5 consecutive days with the same pairs. All the pairs were tested twice each day, once in the morning and once in the afternoon. Testing started around 11am for the morning round and at 2pm for the afternoon round. The pairs were tested in the same order every time to ensure that every pair started at the same times each day. After 5 days each pair of mice was recorded in the test cage ten times in total. In this research only the data from day 2 and day 5 are analyzed, which results in 4 test moments per mouse. The infrared and normal video cameras were placed on tripods 1,5 meters above the two testing cages. Both cameras recorded the entire testing period.

Behavioral scoring

To collect the behavioral data of the mice the software Observer XT (Noldus b.v. Wageningen, the Netherlands) was used. The video recordings that were made with the normal video camera were scored using focal-animal sampling. The behavior was scored continuously following a set ethogram (Appendix 1). Each behavior was linked to a key on the keyboard. The behaviors that were continuous, namely eating, grooming, sitting, walking, lying down, huddling and digging, were recorded as state behaviors. Behaviors that start and end in fairly quick succession were recorded as event behaviors. Rearing, jumping, urinating and defecating were logged as event behaviors.

The state behaviors were mutually exclusive, the start of a new behavior ending the previous one. Event behaviors did not stop an active state behavior and only received a timestamp when logged. In the ethogram this division between state and event behaviors is stated for each behavior.

Data processing

Behavioral data

The total observation period was split into 5 minute intervals. For each interval the total duration and number of occurrences of all the different behaviors was collected. Subsequently all active and all inactive behaviors were grouped together. Walking, digging and running were categorized as active behaviors while sitting, lying down, huddling, grooming and eating were categorized as inactive behaviors. This separation was done because of the similarity between some behaviors such as huddling and lying down.

The same behavior data (duration and frequency) was also collected for the complete 30 minute period without the division in intervals. This way the data of the total testing period was collected to compare to the total change in temperature had occurred in each mouse.

Thermography data

The eye and tail temperature were collected using FLIR ResearchIR by Hop (2018) (Appendix 1). The eye temperature at each interval was measured by selecting a region of interest of 3x3 pixels and taking the mean temperature. The temperature in the tail was taken by selecting a 10-pixel-long line and taking the mean temperature as well. The temperature of both tail and eye were taken after each 5 minute interval.

Statistical analysis

Behavioral data and the data from the infrared camera were combined and analyzed using IBM's SPSS Statistics 24. The data was not normally distributed so to explore correlation between different types of behaviors and the eye and tail temperature (T^{eye}, T^{tail}) Spearman's rank-order correlation test was used to calculate the correlation coefficients. All the data of the two days was analyzed together, regardless of group (CR/UU).

The difference between the lowest and highest measured temperature in the total 30 minute testing period was also calculated (ΔT^{eye}, ΔT^{tail}). This was used to explore a potential correlation between the total change within each interval and the behavior of the mouse.

The alpha used in both analyses is $p<.001$.

Results

Behavior

The behavioral data collected is summarized in Table 1 and Table 2. The average duration of each behavior per 5 minute interval can be found in Table 1. In Table 2 the same information can be found of the T^{eye}, T^{tail}, ΔT^{eye} and ΔT^{tail}.

Table 1 The mean duration and standard deviation of every behavior and the two behavior groups in minutes per 5 minute interval. Walking, digging and running were categorized as active behaviors. Sitting, lying down, huddling, grooming and eating were categorized as inactive behaviors.

	Eating	**Grooming**	**Sitting**	**Lying down**	**Digging**	**Walking**	**Huddling**	**Rearing [1]**	**Active**	**Inactive**
Mean duration (minutes)	00:07	00:53	01:56	00:07	00:25	00:35	00:50	19,4	01:00	3:58
Standard deviation (minutes)	00:18	01:16	01:12	00:25	00:28	00:32	1:34	18,5	00:49	00:49

[1] Not the duration but total number of occurrences per interval.

Table 2 The mean temperature and standard deviation of the eye and tail temperatures (^oC) measured. The absolute eye and tail temperature and the average change in temperature within each testing period.

		Eye temperature	**Tail temperature**
Absolute (T^{eye},T^{tail})	Mean (^oC)	33,00	26,79
	Standard deviation (^oC)	0,49	1,17
Change (ΔT^{eye},ΔT^{tail})	Mean (^oC)	1,04	2,51
	Standard deviation (^oC)	0,42	1,10

Habituation

The habituation can be seen in Figure 1 where the mean duration of active behavior is plotted over the different intervals. In this figure the mean duration is compared to the average T^{eye} in those same intervals. Here it can be seen that both are higher in the second interval than in the first. From the second interval onwards both the T^{eye} and average duration of active behavior only decrease over the next intervals. In Figure 2 the median duration of active behavior is compared between day 2 and day 5. Here it is visible that the mice were, on average, less active in every interval on day 5 than on day 2. The median duration of active behavior in the last two intervals was 0. This data is not statistically analyzed.

Figure 1 The mean eye temperature (red) and mean duration of active behavior (blue) per interval in minutes~~l~~. The standard deviation of each value per interval are visualized as well in their respective color.

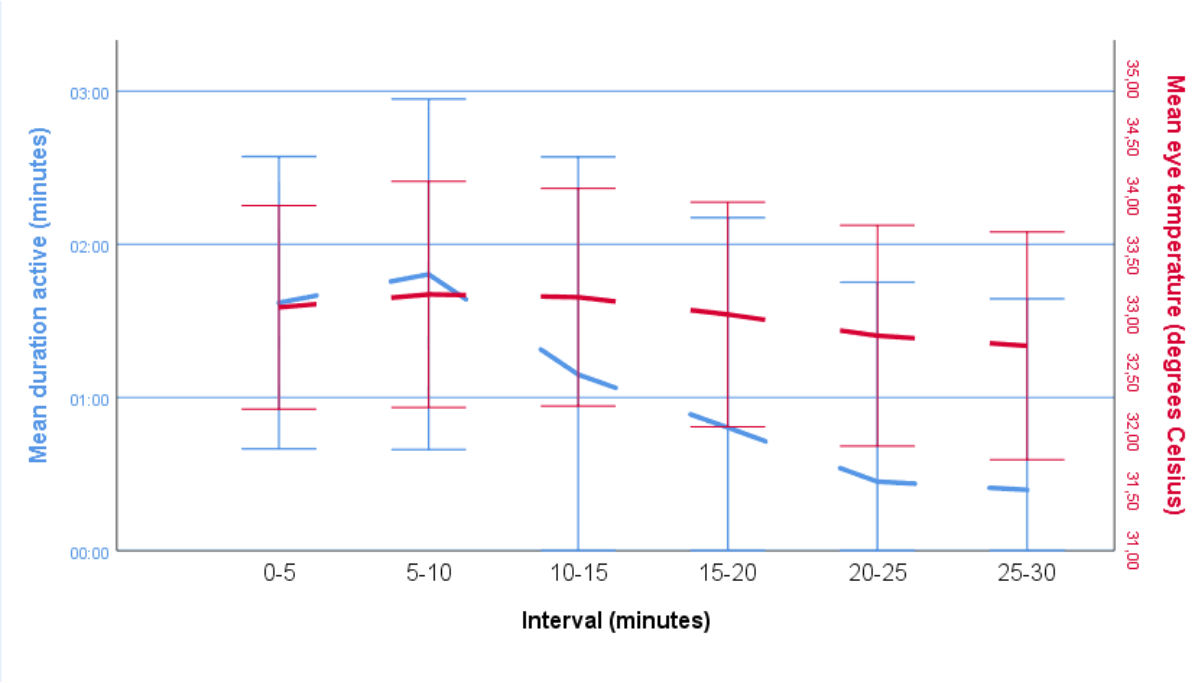
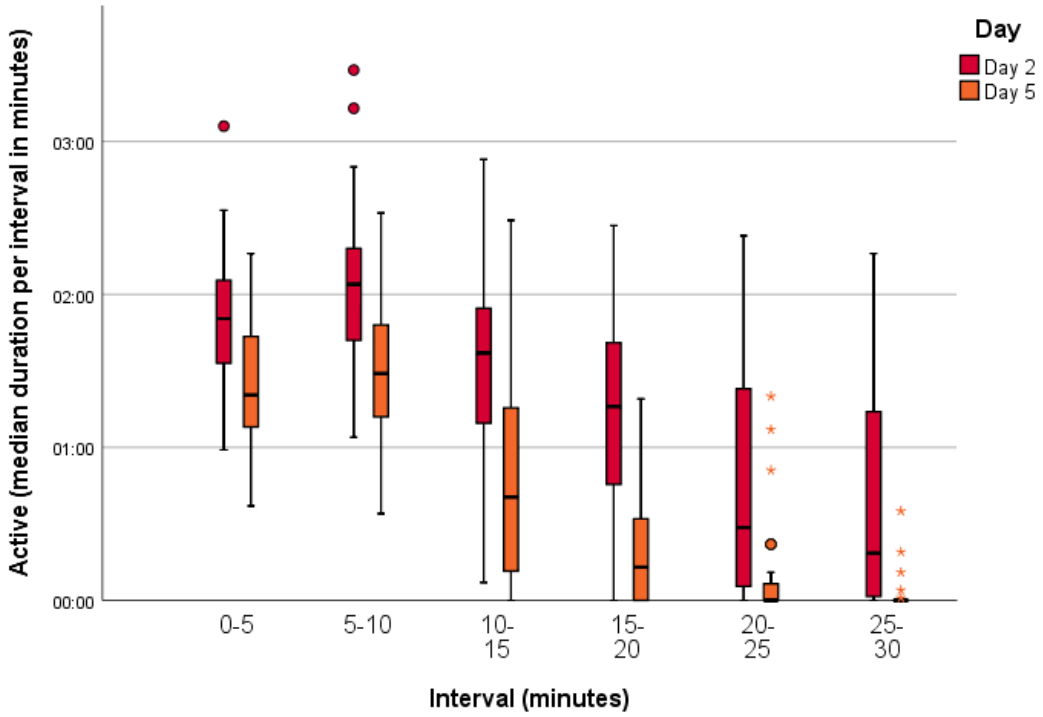


Figure 2 The duration of active behavior per interval in minutes on day 2 compared to day 5 in boxplots.



Eye temperature

The results of the exploration of the data can be seen in the scatterplots in Figures 3 and 4. Each data point represents the eye temperature in relation to the total duration each mouse was active or inactive in the same 5 minute interval. Each interval is one data point and the temperature shown is the temperature measured using infrared thermography at the end of that same interval. As stated above, all mice were tested twice per day and the data is analyzed for two days. Since each mouse was tested for 30 minutes each measurement was divided into 6 of these 5 minute intervals. Therefore, in these scatterplots each mouse provided approximately 24 data points.

Several data points are missing because the temperature data could not be retrieved. In these cases the eye or tail was not visible at the time of measurement. The frequency of this happening increased in the later intervals and later testing days when the mice tended to huddle and sleep more, and after a shorter amount of time.

In Figure 3 the duration of the inactive behaviors was plotted together with the corresponding eye temperature. The linear trend line that is plotted as well shows a negative visual correlation between the inactive behaviors and the eye temperature. A spearman correlation analysis showed that this correlation was significant (ρ(363)=0,336, p<,001). In Figure 2 the same is done for the group of active behaviors. Here, the linear trend line shows the opposite; the mean temperature increases with the increase of the duration of the behavior. This correlation also showed significant (ρ(363)=−0,345, p =,000).

Figure 3 Scatterplot of the duration of inactive behavior per interval (minutes) with the eye temperature (°C) at the end of the corresponding interval. Each dot represents the results of one interval of one mouse. The linear trend line represents a negative correlation.

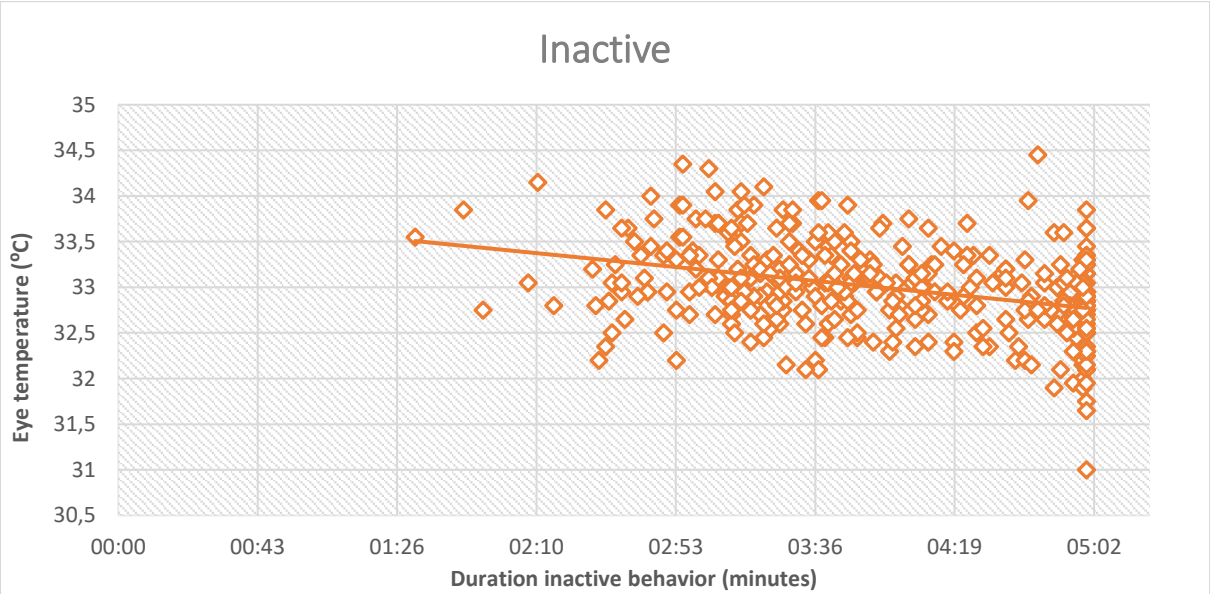
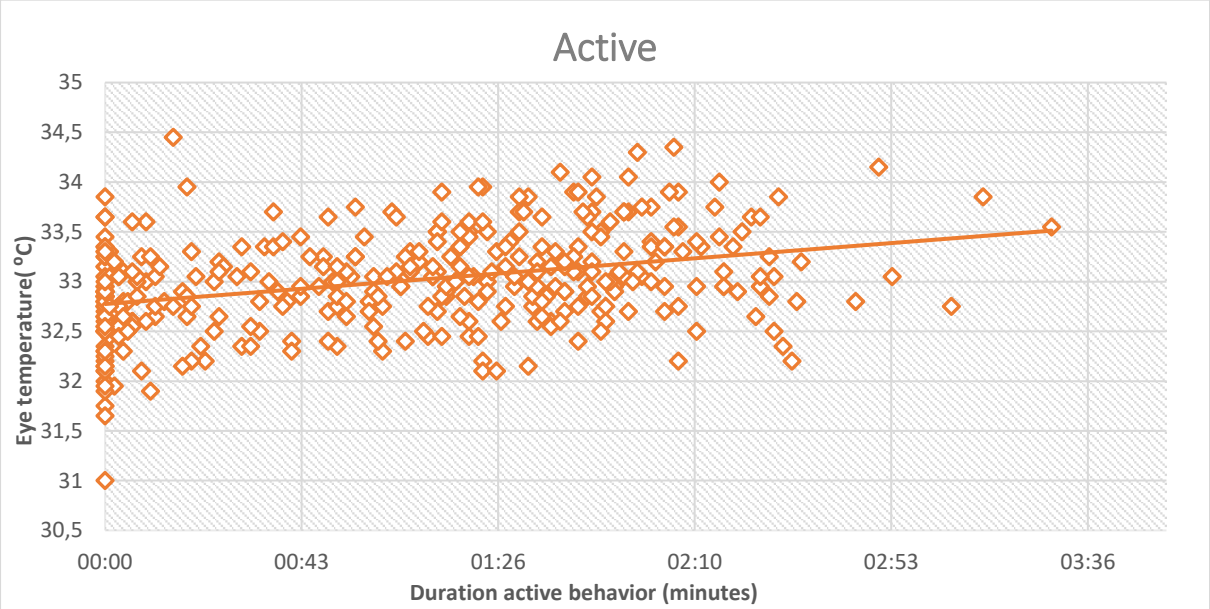


Figure 4 Scatterplot of the duration of active behavior per interval (minutes)l with the eye temperature (°C) at the end of the corresponding interval. Each dot represents the results of one interval of one mouse. The linear trend line represents a positive correlation.



To check these visually increasing and decreasing trendlines, a Spearman's rank order correlation was run to determine the correlation coefficients indicating the relationship between the different behaviors and the eye and tail temperature of the mice. These results can be seen in Table 3. The group of active behaviors showed a weak positive correlation with the eye temperature (ρ(363)=0,345 p<.001) as can be seen in Figure 4.

Table 3 The correlation coefficients between the duration of behaviors and the eye temperature as calculated with Spearman's ranking correlation test.

		Eating	Grooming	Sitting	Lying down	Digging	Walking	Huddling	Rearing	Active	Inactive
T^{eye}	Correlation coefficient (ρ)	-,038	,017	,296[1]	-,191[1]	,251[1]	,385[1]	-,382[1]	,380[1]	,345[1]	-,336[1]
	Coefficient of determination (r^2)	,001	,000	,088	,037	,063	,148	,146	,144	,119	,113
	Sig. (2-tailed)	,464	,752	,000	,000	,000	,000	,000	,000	,000	,000
	df	363	363	363	363	363	363	363	362	363	363

[1] *Significant p < 0.001*

Tail temperature

Data obtained of the T^{tail} of the mice during each interval is plotted against the duration of active and inactive behavior in Figure 5. This information on the tail temperature is made visible in combination with the duration of the two behavior groups per interval. For the active behavior the linear trend line shows a decrease in the average temperature of the tail with an increase of the duration of active behaviors in this time period. The opposite is visible in the plot of the duration of the inactive behaviors and their corresponding T^{tail} in Figure 6. Here there is an increase in temperature when the duration of the behaviors increases.

Figure 5 Scatterplot of the duration in minutes of active behavior per interval with the tail temperature (°C) at the end of the corresponding interval. Each dot represents the results of one interval of one mouse. The linear trend line shows a possible negative correlation.

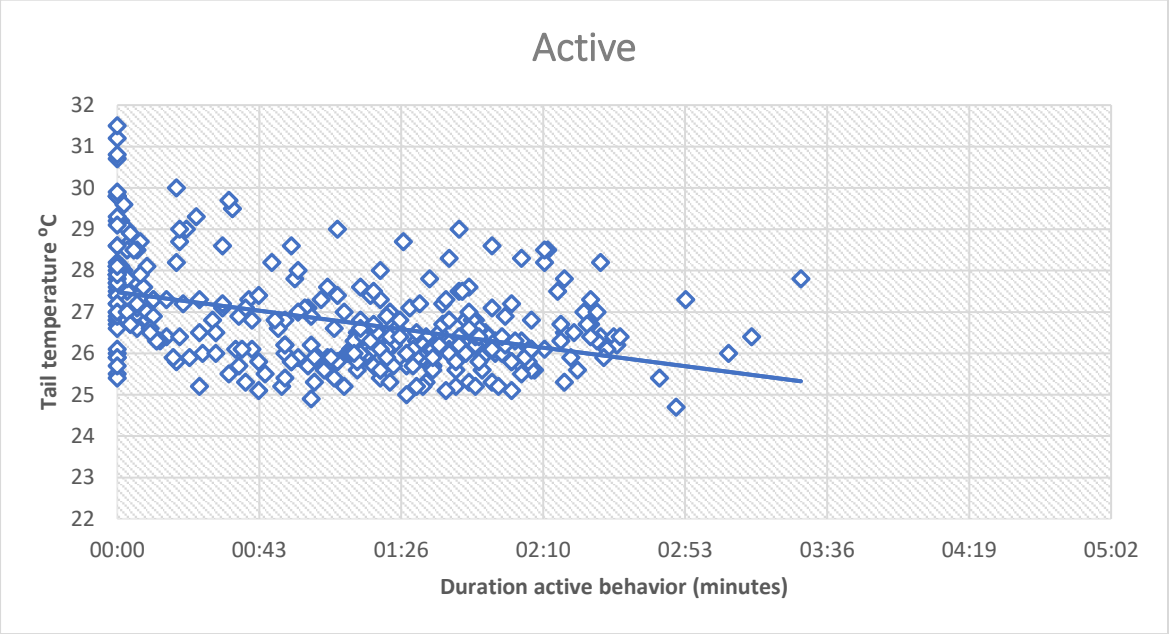
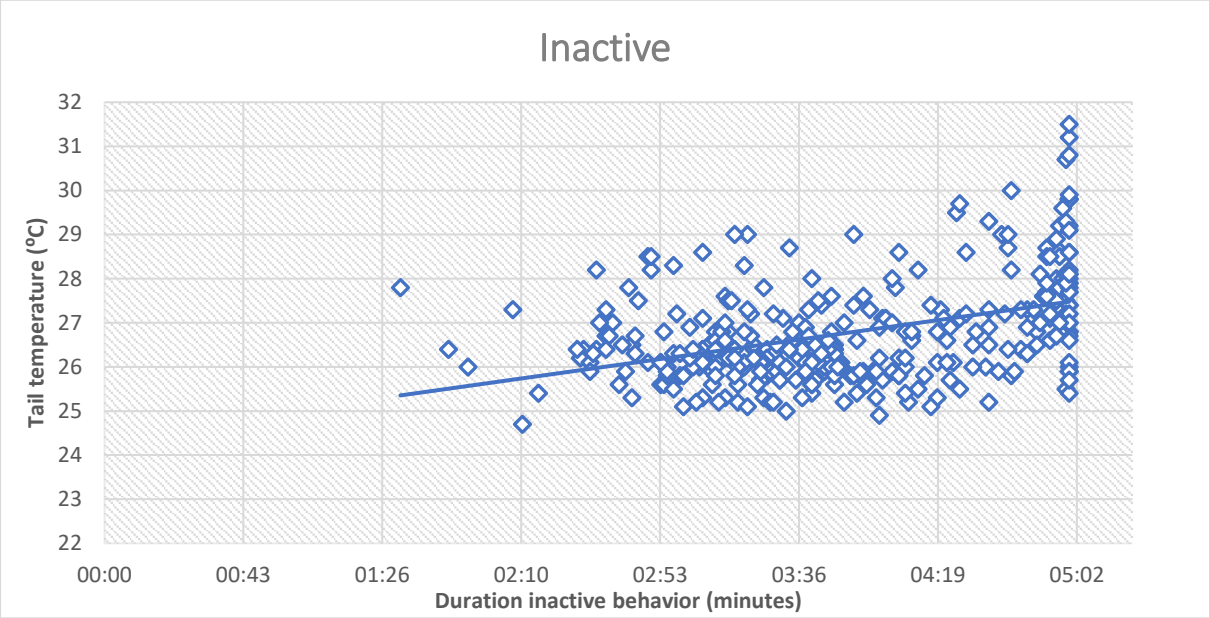


Figure 6 Scatterplot of the duration of active behavior in minutes per interval with the tail temperature (°C) at the end of the corresponding interval. Each dot represents the results of one interval of one mouse. The linear trend line shows a possible negative correlation.



Spearman's rank order correlation was run to determine the correlation coefficients indicating the relationship between the different behaviors and the tail temperature of the mice. These results can be seen in Table 4. This showed a positive correlation between the tail temperature after each interval and the total duration the mice were inactive during it ($\rho(343)=0{,}381$ $p<0.001$). The correlation coefficient for the association between T^{tail} and active behaviors was –0,380 (343) p<.001. Together

these two correlations confirm the visual correlation that can be seen in the trend lines in Figure 5 and 6.

Between the T^{tail} and both walking and sitting a negative correlation was found, the coefficients were respectively –0,443 (343) p<.001 and –0,422 (343) p<.001. Another correlation was found between T^{tail} and huddling; this is a positive correlation (ρ(343)=0,416 p<0.001). The correlation coefficient that was found for the relationship between T^{tail} and both rearing, digging and lying down was significant.

Table 4 The correlation coefficients between the duration of behaviors and the tail temperature as calculated with Spearman's ranking correlation test.

		Eating	Grooming	Sitting	Lying down	Digging	Walking	Huddling	Rearing	Active	Inactive
T^{tail}	Correlation coefficient (ρ)	-,064	,156	-,422[1]	,207[1]	-,299[1]	-,443[1]	,416[1]	-,370[1]	-,380[1]	,381[1]
	Coefficient of determination (r^2)	,004	,024	,178	,043	,089	,196	,173	,137	,144	,145
	Sig. (2-tailed)	,233	,004	,000	,000	,000	,000	,000	,000	,000	,000
	df	343	343	343	343	343	343	343	342	343	343

[1] *Significant p < 0.001*

Change T^{eye} and T^{tail}

Active

The change between the lowest and highest temperature per mouse (ΔT^{eye}, ΔT^{tail}) was calculated to explore possible correlation between the total temperature change in one testing period and the duration of active behaviors that the mice performed. In Figure 7 a scatter plot visualizes the potential relationship between the ΔT^{eye}, ΔT^{tail} and active behaviors. Both linear trend lines show no connection between the behaviors and the measured temperature changes. A spearman correlation test showed that no significant correlation was found.

Inactive

In Figure 8 the same change in temperature within every testing period is visualized in relation to the duration of all the inactive behaviors. There is no parallel visible between either of the two differences in temperature and the sum of the duration of active behaviors, no significant correlations were found.

Figure 7 Scatterplot of the duration of active behavior in minutes per testing period compared to the eye temperature (°C) (orange) and tail temperature (°C) (blue) at the end of the corresponding testing period.

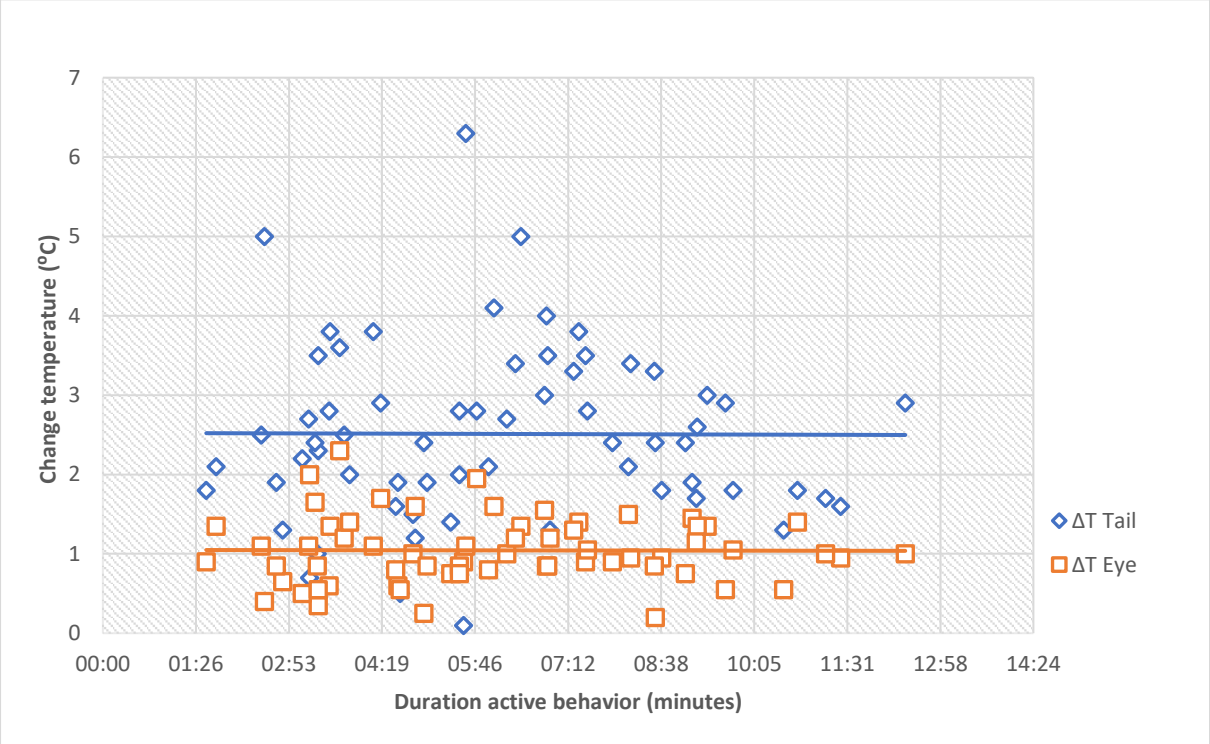
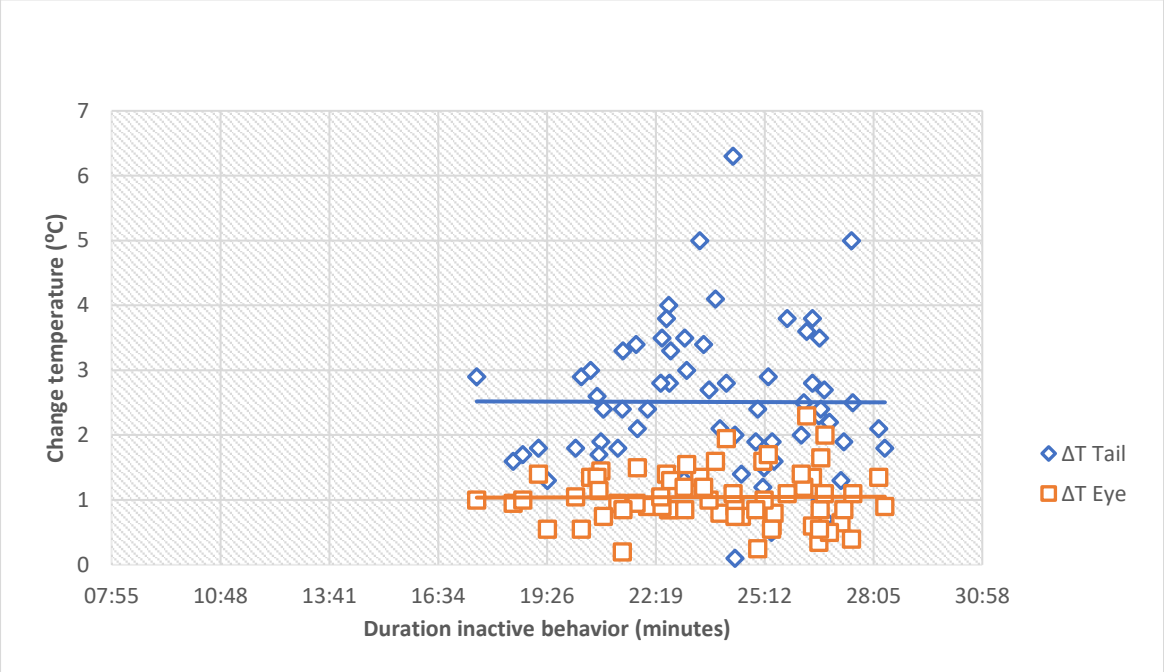


Figure 8 Scatterplot of the duration of inactive behavior per testing period compared to the total change in eye temperature (°C) (orange) and tail temperature(°C) (blue) in the corresponding testing period.



Spearman's correlation ΔT^{eye} and ΔT^{tail}

Running Spearman's correlation test showed non-significant and very weak correlations between all the different behaviors and both the ΔT^{eye} and ΔT^{tail}. From this analysis no correlation can be found between any of the behaviors and these measurements as can be seen in Table 5.

Table 5 The correlation coefficients as calculated with Spearman's ranking correlation test showing the correlation between the duration of behaviors and the change in eye and tail temperature in each testing period.

		Eating	Grooming	Sitting	Lying down	Digging	Walking	Huddling	Rearing	Active	Inactive
ΔT^{eye}	Correlation coefficient (ρ)	,036	-,113	,126	-,098	-,031	,159	,017	,194	,080	-,071
	Coefficient of determination (r^2)	,130	,013	,016	,010	,001	,025	,000	,038	,006	,005
	Sig. (2-tailed)	,780	,376	,321	0,439	0,806	,209	,893	,124	,528	,575
	df	62	62	62	62	62	62	62	62	62	62
ΔT^{tail}	Correlation coefficient (ρ)	-,072	,130	,021	,119	-,018	,045	-,068	,061	,040	-,049
	Coefficient of determination (r^2)	,005	,017	,000	,014	,000	,002	,005	,004	,002	,002
	Sig. (2-tailed)	,571	,304	,867	,350	,887	,722	,593	,633	,755	,701
	df	62	62	62	62	62	62	62	62	62	62

Discussion

The goal of this research was firstly to investigate whether activity influences the measurements when thermography is used to monitor stress in male C3H/HeOuJ mice and secondly if habituation in active behavior was reflected by habituation in BT. The results show a correlation between activity and BT. When looking at the results for the first goal a positive correlation between active behavior and T^{eye} and a negative correlation between T^{eye} and inactive behavior are demonstrated. In line with that, the T^{tail} showed the opposite: a negative correlation with active behavior and a positive correlation with inactive behavior. The decrease in activity during and between sessions implies that it can be concluded that there was habituation during and between these sessions. This was also reflected in BT. That is partly consistent with what was expected before the research took place: that some behavioral habituation was expected, within and between sessions.

Others such as Wanner et al. (2014) have also shown that through physical activity the BT increases and according to Vogel et al. (2016) the eye temperature gives a good representation of the BT in mice. The results of the present research are in agreement with the results in both of these papers, as it shows a correlation between eye temperature and activity. One might argue that the fluctuation of the BT throughout the day as described by Tankersley et al. (2002) could influence these results, but these changes are not so significant compared to other influences such as habituation and exercise.

Further, looking at the habituation of active and inactive behavior then shows that there was habituation during and between sessions. This is however not statistically proven in this research. If true, this does not completely comply with previous research in which C3H/HeJ mice were used (Bolivar 2009). According to this research only a decrease in activity was seen within each session and no significant decrease was seen between subsequent sessions. However, also according to Bolivar, it is not clear whether the activity level or distance travelled is the same as habituation, as anxiety can also lead to activity. Another possible explanation is that the testing methodology used by Bolivar evoked a larger stress response then the methodology used in this research. Furthermore, Bolivar (2009) also looked at different behavioral parameters when looking at animal activity. All these things could explain the lack of habituation in aforementioned study.

The ΔT^{eye} and ΔT^{tail} – the temperature changes over the complete duration of testing – were not correlated to any duration of active or inactive behavior. These results were explored because they might offer an easier way to determine a correlation between BT and activity during a longer period of time. A possible explanation for the lack of correlation in research results regarding the ΔT^{eye} can be found in the duration of each test session. It is possible there is a fluctuation in both temperature and activity, making the peaks indiscernible in the ΔT^{eye} and ΔT^{tail}.

This research then shows that when researching SIH and using measurements such as the temperature of eyes and tail, one cannot ignore the importance of bodily activity. If the goal is to measure stress in the animal in a non-invasive manner through BT, one has to account for the fact that the changes in BT happen not solely because of stress.

Important to note in the results of this research is that the correlation found between T^{eye}, T^{tail} and BT is not proof of causation. It is possible that stress causes both an increase in BT and an increase in activity. This research is not suitable to further illuminate the cause of this correlation. Setting up a methodology that will be able to do this will not be easy, because both stress and activity have influence on BT, whilst it is hard to discern to what effect either activity or stress is the influencing factor. A possible solution to this can be found in the administering of corticosteroids. The iatrogenic-stress hormones could however possibly cause an increase in both BT and activity, therefore, still not necessarily distinguishing which is the primary cause.

Looking at the used methodology, this study had some limitations. First of all only one person performed all the observations. To check the reliability of the observer one video was observed twice and the results were checked for intra-observer reliability. Also, the ethogram was extensively described based on previous studies in our lab. Because there has only been one observer and reliability has been checked it is not likely that the results are severely affected by this. If a certain bias is present it is consistent over all the data.

According to Tan et al. (2009) the angle between the surface of the eye and the camera has to be smaller than 45^{o}. As Tan et al. (2009) focus on human eyes it is, however, possible that this is less of a problem in murine eyes as the visible surface of the human eye is flatter.

In this research the eye temperature was taken from the mean temperature of 4 pixels as measured in FLIR ResearchIR. The highest temperature in this area always seems to be in the eye. When taking the mean of 4 pixels, the area around the eye is unnecessarily taken into account as well. This inconsistency between measurements is avoidable by using the highest temperature in the selected area. The reliability of this method would be higher. However the data provided did not allow for this way of measuring. If further research on this subject would be performed, this methodology would be recommended.

Several data points for temperature in the eyes and tail were missing due to behaviors such as huddling and sleeping. Although these situations were few, they might influence variation and increase the chance of a false negative result.

A difference between the mice that was not examined could be their state at the start of the experiment. If the mice were active before the start this might have had an influence on the results as there could have been less fluctuation in the temperature if the mice stayed active (Lecorps, Rödel, and Féron 2016).

Recommendations for future research

Based on the findings and practical experiences during this study, several recommendations for future research can be made. To know for sure if the ΔT^{eye} and ΔT^{tail} are not correlated to the duration of active or inactive behavior further research is required. Doing a similar experiment with a shorter test session duration, or with greater measurement frequency, might provide evidence for this discrepancy between results and the hypothesis of this research.

The huddling of the mice was recorded as a mutually exclusive state behavior. If the focal-animal was, for example, grooming while huddling with another mouse, only the grooming of the animal was logged. In this situation the huddling was not recorded. As this physical contact between the two mice could influence the BT, logging the huddling separately would have been more suitable. In this research set up the data on huddling is incomplete. For this reason it was only used in the category of restful behaviors and not separately. As influence on the BT is probable (Lacy and Lynch 1979) it is recommended in similar research to document huddling behavior more extensively. Furthermore, in this research the mice were put in the testing cage two at a time. The presence of another mouse might have an influence on both the measured temperatures and the behaviors. The physical contact could influence both the T^{eye} and T^{tail} directly. Behavior can be affected as well.

In this research the mice were housed in a 12-hour light-dark rhythm (lights on 7am), therefore all the testing was done during the day time/lights on. As mice are nocturnal animals this is likely to influence the research results. A problem it caused was that the mouse fell asleep regularly during testing, which resulted in data points missing due to eyes and tail not being visible. Researching the behavior during the night might be more relevant when the goal is to explore natural behavior.

Social behavior can take place and the stress and coping with the unfamiliar environment can differ with another mouse present. Both this change in behavior and stress response can alter the temperature of the eye and tail. This could be a subject for further research. However, mice are social animals and testing on singly housed mice will have certain downsides. If the mouse are placed in an unfamiliar environment alone an increased stress response is expected. This could be advantageous if the response is stronger and can be measured more easily, it is nonetheless an increased discomfort for the animal, and it becomes more difficult to get a measure of the baseline temperature. As mice are group housed in mouse research projects it is uncertain whether the expanded knowledge from this research merits the extra labor and ethical downsides to this method.

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Appendices

Appendix 1. Ethogram

	Name	Description	Key	Properties	Active/inactive
1	Default	Default state	f	State, default	-
2	Eating	Chewing and ingesting food or cage bedding	E	State	Inactive
3	Grooming	Mouse scratches or licks fur, washes face or licks genitalia	G	State	Inactive
4	Rear	Upright posture with forelegs moving into the air (above 45º) or leaning against the wall of the cage	u	Event	-
5	Sitting	Sitting still on one location, all four paws are on the ground. The mouse might move its head or sniff around.	S	State	Inactive
6	Walking	Forward movement with all four paws moving	W	State	Active
7	Lying	Being motionless: animal quietly lies on the floor	L	State	Inactive
8	Huddling	Being motionless: animal quietly lies on the floor while in contact with one or more other mice.	H	State	Inactive
9	Jumping	Animal jumps: none of the four paws touches the floor	j	Event	-
10	Digging	Burrowing in the cage bedding with the front paws	D	State	Active
11	Urinating	Mouse urinates	U	Event	-
12	Defecating	Mouse defecates	P	Event	-

Appendix 2 Materials and Methods section Hop (2018)

Materials and methods

2.1. Animals and housing

All of the described procedures were approved by the Central Animal Experiments Committee of the Faculty of Veterinary Medicine of Utrecht University (CCD license number: AVD1080020171926). The 16 subjects which were used in the experiments were 8- weeks-old male C3H/HeOuJ (C3H) mice. 8 mice were transferred from Experimental Animal Center Charles River in Sulzfeld, Germany (CR mice). The other 8 mice were born and reared in 1 room of the animal facilities of the Department of Animal Sciences at Utrecht University (UU mice). The room had a temperature of 18-24°C, a 12h light-dark cycle (lights on at 07:00h; light intensity at shelf level about 100 lux) and the radio was on during light hours. To identify the mice, different markings with permanent marker were placed on the base of the tail (appendix 1).

2.1.1. Home cage

The mice were housed in same-sex groups (10 CR mice per cage, 8 UU mice per cage) in Makrolon type 3 cages of 43x27x15cm with a bedding of sawdust (figure 1). An orange plastic tunnel, small cardboard box and several tissues were present in the cages. Food pellets (CRM(E) 801730, Special Diet Services) and water were available ad libitum.

Figure 1. A Makrolon type 3 cage of 43x27x15cm in which the mice were housed (Photo: Hop 2017).

2.1.2. Test cage

Test cages were transparent Perspex boxes of 21x15x15cm with no cover, a bedding of sawdust and a transparent demarcation which could be removed (figure 2). The bedding of sawdust was changed every time after a pair of mice has been in the test cage.

Figure 2. A transparent Perspex box of 21x15x15cm in which the mice were placed to measure the eye and tail temperatures (Photo: Hop 2017).

2.2. Infrared thermography

To record the mice, a FLIR T430sc camera on a tripod was used (resolution 640x480, sensitivity <0,03 K). The emissivity is 0,98, this was automatically set. A frame frequency of 5 Hz was used to record

for 30 and 35 minutes. The thermal camera was always at the same distance from the cages (± 100 cm). A digital camcorder (Panasonic HC-V180) was placed next to the thermal camera to record the mice. This way the mice could be kept apart on the recordings, as the mice could not be distinguished on the thermal videos.

2.3. Measuring eye and tail temperatures

To find the best method to measure the basal eye and tail temperatures of C3H mice, 2 different methods were compared. The eye and tail temperatures were measured in the home cage (figure 3) and in a test cage (figure 4) and were assessed every 1 minute for the first 5 minutes, and every 5 minutes for the remaining 25 minutes, which resulted in 20 measurements per mouse per video. When the measuring took 35 minutes, the last 5 minutes were not analysed. A timeline is made to show which test is conducted on which date and when the tunnel and tail handling and marking took place (figure 5).

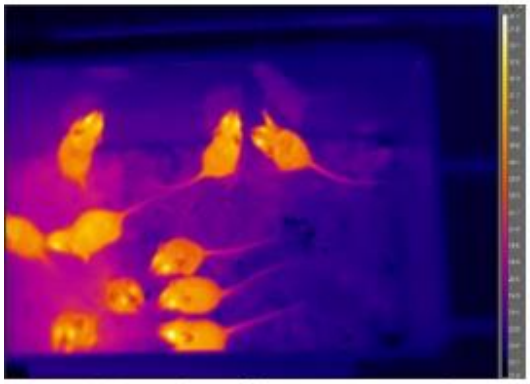

Figure 3. Screenshot of the FLIR ResearchIR program during the assessing of the eye and tail temperatures in the home cage.

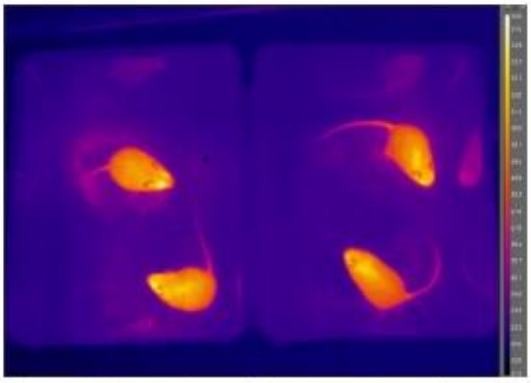

Figure 4. Screenshot of the FLIR ResearchIR program during the assessing of the eye and tail temperatures in the test cages.

Figure 5. Timeline on which is shown when all tests and days of handling and marking took place during this research. The tail handling and marking (orange line) took place from 22-09-2017 to 15-11-2017 and the tunnel handling (blue line) took place from 07-11-2017 to 13-11-2017. The mice were not marked or handled on Saturdays and Sundays.

2.3.1. Home cage test

The lid of the cage was removed and the cage stayed uncovered. The plastic tunnel and cardboard house were removed from the home cage. Between 11:00h and 12:15h thermal videos of the 8 CR mice and 8 UU mice were collected for 30 minutes.

2.3.2. Test cage test

To test if and when the temperatures of the mice habituate to the test cage, the 8 CR mice and 8 UU mice were placed in the test cage in randomized pairs (appendix 2.1). Once there was a mouse at both sides of the test cage, the demarcation was removed, so the mice encountered each other at the same time. There were only pairs of mice from the same home cage, because when mice encounter unfamiliar mice, the corticosterone levels will increase due to social stress (Van Loo et al. 2001). The thermal camera started recording before the mice were placed in the test cage, so the temperatures could be measured from the moment both mice were placed in a test cage. Each pair stayed in the test cage for 35 minutes while recording thermal videos. This was repeated for 5 days, twice every day. The first 4 videos were collected between 10:45h and 13:30h, the second 4 videos were collected between 13:45h and 16:30h. The temperatures of 2 pairs of mice were measured at the same time.

2.4. Tail and tunnel handling

The test cage method was used to determine which method to handle mice will deliver the least amount of temperature changes. 2 handling methods were compared, handling a mouse with an acrylic tunnel (tunnel) and handling a mouse at the base of the tail (tail) (Gouveia & Hurst 2013). 2 equal transparent acrylic tunnels were placed in the UU mice cage and in the CR mice cage 7 days before the actual tests. Every mouse was handled by tunnel without direct contact 4 times spread over these 7 days to let the mice habituate to the tunnel handling.

The mice were handled in a random order spread over 2 consecutive days (appendix 2.2 & 2.3), until every mouse was handled both by tunnel and by tail. The temperatures of 2 pairs of mice were measured at the same time. The thermal camera started recording before the mice were placed in the test cage, so the temperatures could be measured from the moment both mice were placed in a test cage. 4 mice were handled by tunnel or by tail individually for 3 seconds and were placed in the test cages after each other. Once there was a mouse at both sides of the test cage, the demarcation was removed, so the mice encountered each other at the same time. Again there were only pairs of mice from the same home cage. The eye and tail temperatures were assessed every 1 minute for the first 5 minutes, and every 5 minutes for the remaining 25 minutes, which resulted in 20 tail handling and 20 tunnel handling measurements per mouse at the end of the experiment.

2.5. Data-analysis

The eye and tail temperatures were collected using FLIR ResearchIR analysis software. The temperature data was analysed using IBM SPSS Statistics 23 with α=0,05 in all tests. When a two-way Repeated Measures ANOVA was used and the significance obtained from Mauchly's Test of Sphericity was below 0,05, the significance found at Lower-bound was used at the Tests of Within-Subjects Effects. Normality was tested using a Shapiro-Wilk test. Finding the most suitable method to measure the basal eye and tail temperatures of C3H mice Research Report Utrecht University: L. Hop 8

2.5.1. Assessing eye and tail temperatures

The eye-area (the orbital region of the whole eye) of both of the eyes was selected with a cursor of a set surface (9 pixels) from the software program to determine the eye temperature. To assess the

tail temperature, a line of 10 pixels was drawn from the base of the tail (figure 6). The temperature of the tail and the average temperature of both eyes was used for analysis.

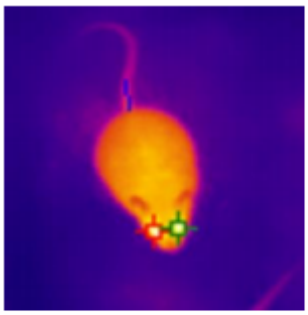

Figure 6. Screenshot of the FLIR ResearchIR program during the assessing of the eye and tail temperatures.

2.5.2. Home cage data-analysis

The eye and tail temperature data measured in the home cage (appendix 3.1 & 3.2) was tested for normality. A two-way Repeated Measures ANOVA was used on the eye and temperature data measured in the home cage to determine whether there were significant differences in eye or tail temperatures between UU mice and CR mice. The timepoints on which the temperatures were measures were used as the within factor and the UU mice and CR mice groups were used as the between factor.

2.5.3. Test cage data-analysis

The eye and tail temperature data measured in the test cage was tested for normality. The average eye and tail temperatures of 8 mice were used for analysis (appendix 4.1 & 4.2). Using two-way Repeated Measures ANOVAs different tests were executed.

It was determined whether there were significant differences between the eye and tail temperatures in the morning and in the afternoon. For this, the timepoints on which the temperatures were measures were used as the within factor and the morning and afternoon groups were used as the between factor.

It was also determined whether there were significant differences between the days of measuring. For this, the timepoints on which the temperatures were measures were used as the within factor and the different days of measuring were used as the between factor. A Post Hoc Bonferroni test was used to see between which days significant differences were present.

To visualize differences in temperatures during all days of measuring in the test cage, the average temperatures per timepoint for the UU and CR mice were calculated and analysed (appendix 4.3 & 4.4). The timepoints on which the temperatures were measures were used as the within factor and the UU mice and CR mice groups were used as the between factor.

2.5.4. Handling methods data-analysis

The average eye and tail temperature data measured during the handling methods tests (appendix 5.1 & 5.2) was tested for normality. Using two-way Repeated Measures ANOVAs different tests were executed.

It was determined whether there were significant differences in eye or tail temperature between the different handling methods tunnel and tail. The timepoints on which the temperatures were measures were used as the within factor and the different handling methods tunnel and tail were used as the between factor.

It was also determined if there were significant differences in eye and tail temperatures between the UU mice and CR mice during the handling tests. For this, the timepoints on which the temperatures were measures were used as the within factor and the UU mice and CR mice groups were used as the between factor.

2.5.5. Comparison home and test cage data-analysis

A comparison between the home cage and the test cage (day 5) is made to determine whether the safe environment (home cage) could be similar to a new environment after getting used to it. The average eye and tail temperatures measured in the home cage and the average eye and tail temperatures measured on day 5 in the test cage were used for this analysis (appendix 6.1; 6.2; 6.3 & 6.4). A two-way Repeated Measures ANOVA was used to determine if there were significant differences in eye or tail temperatures between the home cage test and the test cage test (day 5). The timepoints on which the temperatures were measures were used as the within factor and the different tests were used as the between factor.