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Body surface temperature of rats reveals both magnitude and sex differences in the acute stress response

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ABSTRACT

Understanding how biological markers of stress relate to stressor magnitude is much needed and can be used in welfare assessment. Changes in body surface temperature can be measured using infrared thermography (IRT) as a marker of a physiological response to acute stress. While an avian study has shown that changes in body surface temperature can reflect the intensity of acute stress, little is known about surface temperature responses to stressors of different magnitudes and its sex-specificity in mammals, and how they correlate with hormonal and behavioural responses. We used IRT to collect continuous surface temperature measurements of tail and eye of adult male and female rats (*Rattus norvegicus*), for 30 minutes after exposure to one of three stressors (small cage, encircling handling or rodent restraint cone) for one minute, and cross-validated the thermal response with plasma corticosterone (CORT) and behavioural assessment. To obtain individual baseline temperatures and thermal responses to stress, rats were imaged in a test arena (to which they were habituated) for 30 seconds before and 30 minutes after being exposed to the stressor. In response to the three stressors, tail temperature initially decreased and then recovered to, or overshot the baseline temperature. Tail temperature dynamics differed between stressors; being restrained in the small cage was associated with the smallest drop in temperature, in male rats, and the fastest thermal recovery, in both sexes. Increases in eye temperature only distinguished between stressors early in the response and only in females. The post stressor increase in eye temperature was greater in the right eye of males and the left eye of females. In both sexes encircling may have been associated with the fastest increase in CORT. These results were in line with observed behavioural changes, with greater movement in rats exposed to the small cage and higher immobility after encircling. The female tail and eye temperature, as well as the CORT concentrations did not return to pre-stressor levels in the observation period, in conjunction with the greater occurrence of escape-related behaviours in female rats. These results suggest that female rats are more vulnerable to acute restraint stress compared to male rats and emphasise the importance of using both sexes in future investigations of stressor magnitude. This study demonstrates that acute stress induced changes in mammalian surface temperature measured with IRT relate to the magnitude of restraint stress, indicate sex differences and correlate with hormonal and behavioural responses. Thus, IRT has the potential to become a non-invasive method of continuous welfare assessment in unrestrained mammals.

1. Introduction

The acute stress response is an adaptive, short-lived emergency reaction to challenges that threaten an organism's homeostasis directly (acute physical stress) or are likely to have a reasonable probability to alter homeostasis (acute psychological stress) [1]. Stress responses can involve multiple physiological, hormonal and behavioural pathways and hence a range of biological markers can be used to characterise them [2]. Stressors can differ in the arousal level induced and/or the magnitude of the response they trigger, which can result in a dose-dependent

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relationship between stressor magnitude and biological markers of the stress response [3] which differ among the different markers [4]. Stressor magnitude may not only influence the peak amplitude of the biological marker response but also the dynamics of the response including the initial rate of increase and the time course of the subsequent recovery to baseline levels [5–10]. Since markers are used to evaluate stress responses (and associated welfare outcomes), it is crucial to understand how they relate to stressor magnitude.

Frequently used measures of acute stress have significant limitations as they are often invasive, interrupt ongoing behaviour, do not reflect real-time changes and/or are time and labour intensive [11–13]. Thus, there is a need to develop more reliable, non-invasive ways to assess acute physiological stress responses. Infrared thermography (IRT) is an emerging non-invasive technique that can be used to measure body surface temperature which is a known marker of stress in vertebrates [14,15]. When under psychological stress such as in fear and anxiety states, increased activity within the sympathetic nervous system (SNS) leads to a prioritisation of blood flow to internal organs, muscles and brain, by rapid peripheral cutaneous vasoconstriction [16–18].This, alongside stress-induced thermogenesis [16], results in a rapid increase in core body temperature; a phenomenon referred to as a stress-induced hyperthermia (SIH) or 'psychogenic fever'. This physiological response is conserved across endothermic species [17,19–27] and has been reported to be proportional to stressor magnitude [17,28,29]. However, the measurement of core body temperature is invasive, and the stress associated with its measurement can confound results [22,28,30]. In addition to increasing core temperature, peripheral vasoconstriction also causes a simultaneous decrease in the shell or body surface temperature, which can be measured reliably, instantaneously and continuously using IRT. IRT can be executed without contact and has the potential to be a real-time continuous automated measure of stress [31]. IRT has been validated as an indicator of stress against SNS parameters and glucocorticoid concentrations [14,32–35]. Several studies have applied IRT showing that the technique is feasible in mammals including humans [36], rodents [37–39], rabbits [40], dairy cows [41,42], sheep [43], dogs [44,45] cats [46], pigs [47,48], elephants [49], otters [50], horses [51–54] and non-human primates [21,25,55].

According to previous work, a distinctive pattern of change in body surface temperature is expected after exposure to an acute stressor. Initially, body surface temperature will rapidly deviate from the baseline, after which it gradually returns to the baseline temperature, but it can sometimes overshoot before finally returning to baseline [56,57]. The body surface temperature response depends on activation of the SNS and therefore may be sensitive over a larger range of changes in stressor magnitude than the glucocorticoid response and be better to be able to distinguish between mild and moderate stressors [4]. The ability to record changes in body surface temperature over time, also allows description of different aspects and metrics of the temporal pattern of change in the body surface temperature in response to acute stressors [58]. Few studies, however, have yet tested whether IRT can also be used to distinguish between stressors of different magnitude. In hens, body surface temperature changes measured with IRT differed between two acute restraint stressors of different magnitude [56], but similar studies are absent in other species. Despite sex differences in behaviour [59], plasma corticosterone [60] and SIH responses to stress [27], the majority of research on the stress response has been done on males [61, 62]. Major research funders now insist that where appropriate studies must consider animals of both sexes [63]. A better understanding of body surface temperature as a marker of acute stress therefore requires consider the responses of both males and females across a range of species.

The aim of the present study was to validate IRT as a non-invasive assessment of stressor magnitude in adult male and female laboratory outbred albino Wistar rats (*Rattus norvegicus*). Rats are the species most often used as models for stress and there is well-established knowledge of their behavioural and hormonal stress responses [18,64–67], against

which to validate IRT measurements. SIH is a well-developed marker of acute stress of rodent in pharmacological research [17,18] and animal welfare assessment [18,27]. Previous rodent studies, that have used IRT, have documented that various types of acute stress temporarily increased dorsal and eye temperature [68–72] and temporarily reduced tail temperature [35,39,71–76]. However, these studies mostly applied a single moderate to severe prolonged stressors, and did not provide information on how IRT signals responses to different mild and brief restraint stressors that rats would encounter more typically during husbandry routines - which are most relevant for welfare assessment. Here we applied three homotypic mild restraint stressors, already known to influence behavioural and hormonal responses of rats [67,77], while standardising the timing and duration of the stressors. Since IRT allows us to measure the dynamics of the response to acute stressors, it is possible to record initial rate of change, peak amplitude and recovery to baseline level, but it is not clear what characteristic (if any) of that dynamic response pattern reliably reflects stressor magnitude. Our study aims to evaluate the validity of markers derived from IRT to assess stressor magnitude in laboratory rats. An understanding of the dynamic physiological response of rats to these three stressors could pave the way to detect and quantify acute stress in real-time, and underpin the refinement of acute stress assessment procedures in accordance with the 3Rs [78].

2. Material and methods

2.1. Animals and husbandry

All experimental procedures and data acquisition were carried out under UK Home Office authorisation (Project licence: PIFD5B3DB). The design and report of the study followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines 2.0 for reporting research [79]. Five-week-old dam-reared outbred albino Wistar rats (101-125 g on arrival) were acquired from Charles River (UK), arriving in batches of 18-24 individuals every 3 weeks. IRT and behavioural data were collected using 18 rats of each sex and plasma corticosterone (CORT) data was collected using a different cohort of 80 rats of each sex. Rats were housed in groups of three individuals of the same sex in a 48 × 37.5 × 21 cm polycarbonate cage (Tecniplast, London, WC1N 3AX, UK). Rats were maintained in a 12:12 h light dark cycle with lights on at 7:00. The mean ± SD temperature and relative humidity of the room were 22.04 ± 1.95°C and 55 ± 10%, respectively. Animals had free access to *ad libitum* water and food (Maintenance and breeder pellets, CRM Special Diet Services, Witham, Essex, UK). In each cage, there was approximately 7 cm deep corn cob and sizzle nest bedding for burrowing, two cardboard tunnels and a 21.5 × 21.5 × 12.5 cm Sputnik rat house enrichment device (®SAVIC nv, Belgium). Rats were handled as part of normal husbandry with non-aversive handling tunnels [80,81]. All rats were inspected daily and found healthy. After the trials, all rats were retained by the research facility and the majority of rats were re-used under another Project Licence after veterinary certification of fitness.

2.2. Experimental protocol

The experimental protocol consisted of three phases; acclimatisation, habituation and testing (Fig. 1). In phase one, all rats were left undisturbed for seven days after arrival to acclimatise to the housing unit. To habituate the rats to transport and the testing arena and overcome some of the stress caused by social isolation in a novel open field-like space, rats were transferred individually from their home cage to the test arena which was located in a separate procedure room on six occasions. Transfers were done using a transport cage (a white opaque polypropylene rat cage sized 56 × 38 × 17 cm; North Kent Plastic Cages, UK) covered with a raised wire lid, supplemented with a handful of the rats' own cage bedding material. The familiar odour of the bedding material
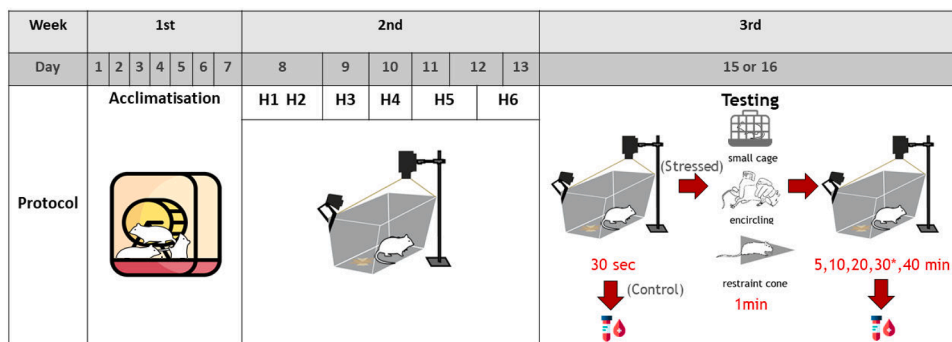
Fig. 1. The experimental protocol consisted of three phases: acclimatisation, habituation and testing. Rats were left undisturbed for the first week after arrival to acclimatise to the home cage. In the second week, six habituation trails of increasing duration of exposure to the test arena (5, 5, 10, 20, 30, 30 -minute habituation trails represented as H1-6, respectively) were completed within 6 consecutive days; for each batch of rats, trials H1 and H2 were completed in one day and trials H5 and H6 were completed over three days. In the third week, each rat was tested by being put into the test arena for 30 seconds to record the baseline temperature, exposed to one of three stressors (small cage or encircling or restraint cone) for one minute and then put back into the test arena for 30 minutes. Throughout, surface temperatures (by IRT) and behaviour were recorded. To collect CORT data, each rat was blood sampled only once in the testing period, either 30 seconds after being put into the test arena without a restraint stressor (control) or after 5, 10, 20, 30 or 40 minutes after having been exposed to one of the restraint stressors (small cage or encircling or restraint cone).

was intended to minimise novelty and maximise habituation [82,83]. The test arena was a grey plastic open topped box (W32.5 cm x L40 cm x H30 cm, Key Industrial Equipment™). Because Plexiglas and stainless-steel mesh blocks infrared radiation, there was no lid on the testing arena. In cases where a rat jumped up to the top edge of the testing arena, it was immediately tunnel handled back into the arena. The habituation trials mimicked the testing period except that the animal was not subjected to a restraint stressor and the duration of exposure to the test arena was gradually increased across the six habituation trials for each rat (Fig. 1). Two transport cages and test arenas were used alternately and were cleaned between rats and trials using tap water and alcohol disinfectant wipes (Medipal®, Pal International Ltd., UK). All habituation was undertaken during the light phase from 08:30 – 17:30, i. e., within 1.5 to 10.5 h after the onset of the light phase. Animals were individually marked with a non-toxic animal marker (Stoelting Co., USA) at the end of the first habituation trial. By the time of testing, rats were 8-week-old and had an average body mass and standard deviation of 258.58±19.68 g for males and 184.97±15.71 g for females.

Testing began one day after the habituation period was completed and was conducted 2.5 – 7.5 h after the onset of the light phase. For testing the response to restraint, and to remove effects of blood sampling (an additional stressor) the rats were divided into two cohorts, one cohort (n = 36) was used to obtain IRT/behavioural response data whereas the other cohort (n = 160) were blood sampled. For both co-horts, one rat at a time, with a handful of its bedding, was placed in the test arena for 30 s in order to obtain individual baseline measure for body surface temperatures or a blood sample in a subset of rats (controls) for baseline CORT assessment. Rats were then exposed to one restraint stressor (see below), for one minute, before being returned to the test arena. Body surface temperature, and behaviour were recorded for 30 mins post stressor exposure (Fig. 1). The trials were monitored by the experimenter standing approximately 2 m from the test arena out of view of the animals. For assessment of the CORT response, blood samples were collected 5, 10, 20, 30 or 40 minutes after rats were exposed to the restraint stressors. Systematic randomisation was used so that each of the three rats within each home cage was exposed to a different re-straint stressor and if blood sampled this occurred at different time-points. The experimenters were aware of the group allocation at the time of testing but were later blinded during the analyses. During all trials air temperature and relative humidity of the test arena were measured at 5 min intervals with an EasyLog USB logger (Lascar Electronics Ltd, Wiltshire, UK). Rats were weighed once only at the end of the experi-ment immediately after the test trial finished.

2.3. Restraint stress

The three acute restraint stressors were (1) confinement in a small novel cage, (2) encircling handling or (3) application of a rodent re-straint cone. These are restraint methods are routinely used in labora-tory animal care [77,84,85] and are recognised as mild stressors, that increase in magnitude from small cage to encircling to restraint cone [66,67,77,86–90].

2.3.1. Small cage

Rats were lifted from the test arena using a handling tunnel into a novel small cage that was an 11.6 W x 20L x 10.5H cm stainless steel confinement box adapted from a rat staircase apparatus (Model 80300, Campden Instruments LTD, LE12 7XT, UK). It had raised welded wire mesh flooring, four sidewalls of opaque stainless steel and a hinged clear polycarbonate lid with two 1.5-cm radius breathing holes. The cage provided the rats enough space to turn around but prevented them from rearing, jumping or running. The cage was cleaned between trials using alcohol disinfectant wipes.

2.3.2. Encircling handling

Rats were picked up from the test arena with the handler's index and middle fingers placed along the sides of the rat's head and with the thumb and ring finger under the forelegs. The rat was then held hori-zontally by supporting the tail and the lower body with the other hand. Thus, the rat was partially physically immobilised and then released back to the test arena directly from the hold.

2.3.3. Rodent restraint cone

Rats were completely immobilised by being placed into a clear cone-shaped disposable plastic Rodent Restraint Bag (model 1802CV, Animal Identification & Marking Systems Inc - AIMS, NY 14843, USA) with a breathing hole at the narrow end. The handler held the rodent restraint cone open and ready for use in one hand. Rats were picked up as with encircling handling and were slid into the cone with their nose towards the breathing hole. Once the rat was in the cone, the handler gently squeezed the wide end closed at the base of the tail and held the rat still in a horizontal position by supporting the chest of the animal with the other hand. Rodent restraint bags were only used once to prevent any carryover effects of odour which could affect the stress response.

2.4. Body surface temperature response

The rats were filmed with both an infrared thermal camera (FLIR A65, f = 25 mm, spatial resolution 0.68 mrad, thermal sensitivity < 0.05 °C @ +30 °C, recording 30 frames per second, FLIR Systems,

Wilsonville, Oregon, USA) mounted on a clamp stand 55 cm above the floor of the test arena and a GoPro HERO 7 Silver 4K Action Camera (GoPro, Inc., San Mateo, USA) attached to the top of the arena with a mount (GorillaPod 500 Action, JOBY, California, USA) (Fig. 1). Both cameras were positioned such that the entire test arena was within their field of views. The experimenter wore insulating gloves (SHOWA 377 Nitrile-Coated Grip Gloves, Manchester, UK) when handling rats including when using the handling tunnel.

From the thermal videos, suitable frames were selected (whole rat visible and in focus) every 10 s for the 30 s pre-stressor and for the first 4 min post-stressor and then every 60 s until the end of the recording (30 min post-stressor). This sampling interval was derived from pilot observations of three rats at 10 s intervals to find the optimum sampling interval using the method of Martin and Bateson [91].For each selected frame, body surface temperatures were calculated from the thermal radiation detected by the camera's sensor and emissivity of bare skin [92], the air temperature and relative humidity at the nearest recording and the distance between the object and the camera (55 cm) using FLIR ThermaCAM Researcher Pro 2.10™ (Fig. 2). Rat tails are well-vascularized with arterio-venous anastomoses and lack fur, thus providing a suitable region of interest (ROI) for IRT [35,71,93–96]. The imaging set-up was therefore designed to prioritise the visibility and accurate measurement of the dorsal side of the whole tail from above, and other non-insulated regions were included that were visible in this top-view. The paws and the nose were rarely seen and the ear temperature was not found to be reliable due to the ear canal opening, i.e., the point of maximum temperature, only being visible in some images, due to head angle. IRT of these body areas was therefore not pursued. The tail, left eye and right eye of the rats were ultimately used as regions of interest (ROI). A line was manually delineated along the tail (only when the whole tail can be seen) and two circles with a radius of 5 pixels centred over the left and right eyes were created (only when the four feet of the rat touch the floor, head angle is down and is parallel to the floor and both eyes are equally visible) using the drawing tool 'bendable line' and 'circle', respectively in the ThermaCAM Researcher software (Fig. 2). The maximum temperature of each ROI was used as the experimental IRT outcome because the minimum and average temperatures could be confounded with background and artefacts such as wet urine, beddings, round-edge effect and movement while there were no other heat sources to confound the maximum temperatures. In addition to body surface temperatures, for each frame that was analysed the location of the rat in the arena, the angle of the head and body, the posture (i.e., grooming, inactive, walking, jumping) were recorded to account for their influence on the variation of body surface temperature measurements [56]. Presence of new faeces or urination ('elimination', see Behavioural analysis below) was also recorded.

The baseline body surface temperature for each rat, was calculated as the average from the three measurements during the pre-stressor period. The post-stressor response in body surface temperature was expressed as the difference from an individual's own pre-stressor baseline temperature (referred to as 'difference from baseline' hereafter). The tail IRT response curve of each individual rat was partitioned into separate components according to Jerem *et al.*, 2019 [58]. Specifically, the amplitudes of the initial decrease and the recovery overshoot (A_{drop}, A_{recov}), their timings (S_{drop}, S_{recov}) and the slope of the initial recovery (M_{recov}) (Fig. 3) were derived for each rat. Eye temperature response curves did not show this pattern (see Results) and were not analysed for curve properties, only the actual eye temperature differences from baseline was use.

2.5. Behavioural response

Based on previous studies in rats exposed to a novel open field arena [97–100] and continuous pilot observations from two rats, an ethogram (Appendix 1) was created that contained 16 mutually exclusive behaviours and a scan sampling interval of 10 s found to be representative. All behaviours were expressed as a proportion of scans per 10 minutes except 'elimination' where counts per 10 minutes was used because it was recorded using thermal images. The proportion of scans per 10 minutes for each behaviour were then grouped into 'Escape', 'Explore', 'Freeze/Groom' and 'Rest' behavioural groups using principal component analysis (Appendix 2) in R package 'FactoMineR' [101] to reduce type I error from separate analysis of a large number of different behaviours.

2.6. Hormonal response

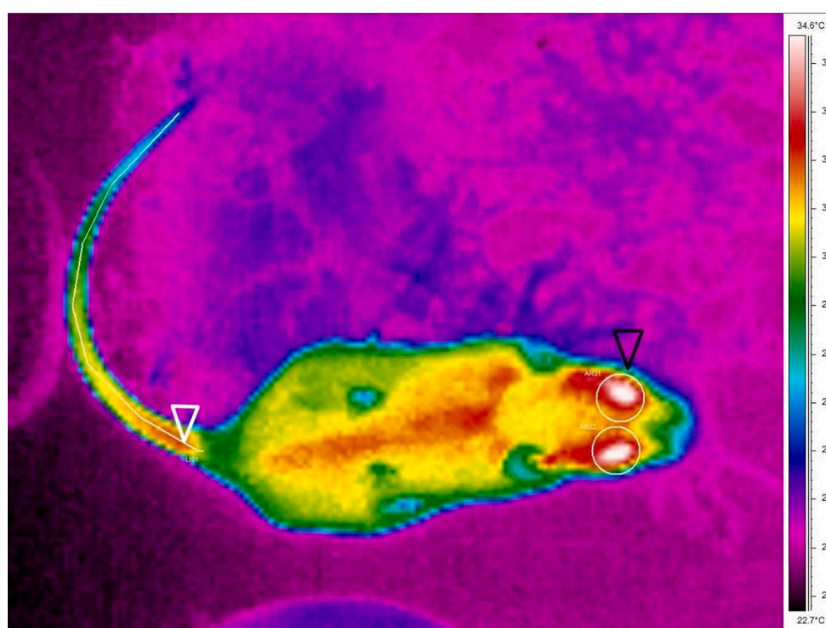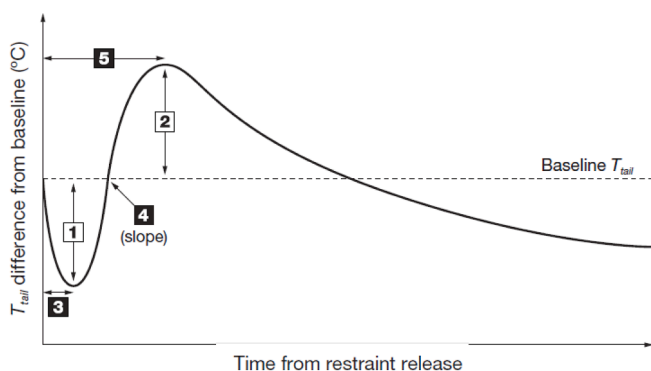To measure the hormonal response to the restraint stressors plasma

Fig. 2. Thermal image of a rat in the testing arena viewed in the 'rain' (rainbow) palette. A bendable line (white arrow) and two circle areas (black arrow) were drawn manually in ThermaCAM Researcher software to extract the maximum temperature of the tail and of the eyes, respectively.

Fig. 3. Schematic standardised body surface temperature response to restraint stressor, identifying five distinct components, adapted from Jerem et al. [58]. The amplitude of the initial decrease in temperature from baseline (A_{drop}, 1.), defined as the minimum value of the temperature difference from baseline (T_{tail} difference) before the first rise of temperature back towards the baseline, and the amplitude of the maximum recovery (A_{recov}, 2.) was defined as the highest T_{tail} difference value recorded after A_{drop}. The time elapsed in seconds to reach A_{drop} was designated as S_{drop} (3.). The rate of change of temperature from A_{drop} to A_{recov} was represented by the slope M_{recov} (4.). The time elapsed in seconds to reach A_{recov} was designated as S_{recov} (5.).

CORT concentrations were assessed in a single blood sample from each rat, collected either at the end of the 30 s pre-stressor period (control rats) or 5, 10, 20, 30 or 40 min after stressor exposure to avoid carry-over effects from previous blood sampling in the same individual. Rats were randomly allocated to one of the sampling points. The 40 min post-stressor assessment period was chosen to encompass the expected onset, peak and recovery of the CORT response [102–105]. To prevent detection of elevated hormone concentrations due to the blood collection procedure itself, the latency from capture to sample collection (blood collection duration - BCD) was recorded and only samples collected within three minutes from capture were used [106–108]. The two rats in which the BCD exceeded three minutes were replaced with two extra rats which were added prior to the experiment to have all rats housed in groups of three of the same sex. For blood collection, rats were encouraged to walk into a rigid red Perspex tube restraint device which provided access to the base of the tail (female rats: AH002AR - 44 × 250 mm 150 – 250 gm, male rats: AH002BR - 59 × 300 mm 250 – 350 gm, Vet Tech Solutions, Congleton, UK). The tail vein was dilated by brief immersion of the tail into warm water and a blood sample (max volume 0.5 ml) collected using a 23-gauge butterfly needle (Venofix, B. Braun, Melsungen, Germany) primed with a sodium heparin solution (1000iu/ml, Wockhardt UK Ltd, Wrexham, UK) into a 1.3 ml screw cap tube containing lithium heparin (International Scientific Supplies Ltd, Bradford, UK). Blood samples were centrifuged at 2,000 rpm at 10 °C for 15 minutes and plasma stored in 0.5 ml Eppendorf tubes at -80 °C until assayed.

Total plasma CORT was extracted from 25 μl plasma/sample using ethyl-acetate, reconstituted and diluted 1:8 with assay buffer and assayed in duplicate using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Michigan, USA) according to the manufacturer's instructions. The duplicated samples from different sexes, treatments and timepoints were randomly distributed over plates. The absorbance at each assay was read at a wavelength of 405 nm with a plate reader (LT-4500, Labtech UK Limited, East Sussex, UK). Plasma CORT concentrations were calculated using AssayZap software v.2.67 (Biosoft, Cambridge, UK) and corrected for dilution. The assay had a range of 8.2-5,000 pg/ml and a sensitivity (80% B/B0) of approximately 30 pg/ml. The intra- and inter-assay co-efficients of variation averaged 7.4 and 7.0%, respectively, and the means between duplicates were used for subsequent statistical analyses.

To determine where the dynamic CORT response differed between stressors and sexes, the peak concentration (Cmax), the time to reach the peak (Tmax, measured in minutes), the rate of increase (slope) from the average baseline to the peak (R_Cmax), the rate of the decrease (slope) from the peak to the lowest level (R_Recov) and the area under the curve (AUC) were analysed. The rate of increase (R_Cmax) and decrease (R_Recov) were assumed to be linear and their values were calculated as the slope and their standard errors by regressing CORT concentrations on time from timepoint 0 to the timepoint when Cmax was reached and from the timepoint when Cmax was reached until the end, respectively,

per stressor and sex. The AUC was calculated from 0 minute (baseline) to 40 minutes post-stressor in MS Excel using the trapezoidal rule.

2.7. Statistical analyses

All analyses were completed in R version 4.1.1 [109]. The sample sizes were determined by power calculations with 80% power and at the 5% significance level using R package 'pwr' [110]. The sample size of n = 6 per sex and treatment group (total n = 36) in the IRT/Behaviour experiment was based on the smallest standardised effect size of 0.9 in a similar study of body surface temperature response to acute restraint stress of different magnitudes in domestic hens [56]. For the CORT analysis, a sample size of 5 per experimental group (total n = 160) was calculated from a power test based on the smallest standardised effect size of 0.998 from a previous study in rats subjected to acute restraint [111]. Analyses of the mean temperature difference from baseline of the maximum tail and eye temperature, the proportion of scans per 10 min showing each behavioural group and the average CORT concentration as response variables were undertaken using general linear mixed models (GLMMs) in R package 'nlme' package [112] with stressor, sex, time-point, ambient temperature, humidity, BCD, time of day (TOD), posture and body mass as explanatory variables where appropriate and animal identity (Rat ID) as a random factor. Outliers were identified using 'boxplot.stats()$out' in R and were kept in the data set after their removal did not affect significant variables in GLMMs (the final model is the same with or without outliers). In all statistical models, non- significant terms were removed with backward-stepwise (from most to least complex) model simplification using the likelihood ratio test (LRT) at a significance level of 0.05. R package 'lsmeans' [113] was used for further post-hoc examination of what groups statistically differed from each other. All the GLMM models were diagnosed with graphical tools and functions in R [114], and when the models did not meet the assumptions, we used log-transformation to meet the normality of residuals, independence of residuals, co-linearity and homogeneity of variance assumptions. The GLMMs models of tail and eye temperatures were examined for temporal autocorrelation using the 'acf' and 'pacf' plotting functions in R. The partial autocorrelations plot of tail and eye temperature models showed a significant autocorrelation at a lag of 1 and much lower spikes for the subsequent lags, and thus the correlation correction 'corAR1()' was added to tail and eye GLMM models. When checking explanatory variables for co-linearity, the rat body mass (g) showed a strong positive co-linearity with sex and high variance inflation factor (VIF) of 6.22, calculated in R package 'car' [115] as male rats had greater body mass than female rats while all other variables had VIF < 3. Therefore, to avoid multicollinearity, 'mass' was excluded from models that included 'sex' and only entered in models analysing male or female rats separately.

3. Results

3.1. Body surface temperature

Female rats had lower baseline tail temperatures (32.11±1.03°C, n = 18) than male rats (33.03±0.82°C, n = 18; LRT, 2ΔLL = 13.87, df = 1, p = 0.0002). Following all three acute stress exposures, tail temperature was initially lower than baseline (Fig. 4a). The tail's thermal response over time, however, was non-linear and differed between the three restraint stressors (stressor-by-time interaction LRT, 2ΔLL = 7.23, df = 2, p = 0.0269, stressor-by-time^2 interaction LRT, 2ΔLL = 8.71, df = 2, p = 0.033) and between the two sexes (sex-by-time interaction LRT, 2ΔLL = 39.01, df = 1, p<0.0001, all other terms: p>0.05 see Supplement Table 1 All rats).

Separate statistical models conducted for each sex, indicated that in males (stressor-by-time interaction LRT, 2ΔLL = 30.15, df = 2, p<0.0001, stressor-by-time^2 interaction LRT, 2ΔLL = 15.21, df = 2, p = 0.0016), but not in females (stressor-by-time interaction LRT, 2ΔLL = 0.23, df = 2, p = 0.8898, stressor-by-time^2 interaction LRT, 2ΔLL = 2.25, df = 2, p = 0.3242), the tail temperature response differed between restraint stressors over time. In the males, the smallest decrease in tail temperature and the highest recovery was seen in rats subjected to the small cage restraint. In the males, encircling resulted in an immediate drop of tail temperature followed by a recovery to the baseline after ca. 20 min. The restraint cone resulted in a similar initial decrease in tail temperature, but then did not recover to baseline within the 30 min post stressor test period. In contrast, the smallest decrease in tail temperature and the highest recovery was seen in rats subjected to the small cage (Fig. 4a). Other factors that were significantly associated with tail temperature in male rats, were jumping posture (LRT, 2ΔLL = 8.36, df = 3, p = 0.0392) after which tail temperature was elevated compared to when they were inactive, walking or grooming (post hoc tests, LSM, p<0.0001) and body mass; tail temperature being higher in heavier rats (LRT, 2ΔLL = 4.04, df = 1, p = 0.0444, all other terms: p>0.05 see Supplement Table 1 Male rats). Tail temperature in females changed non-linearly over time with similar initial tail temperature decreases but then consistently remained below the baseline for the remainder of the 30 min post stressor test period and was not significantly associated with any other explanatory variables (see Supplement Table 1 Female rats).

To further investigate how the tail temperature response differed between the restraint stressors and sexes, each of the curve properties was analysed separately (Fig. 5). When analysing the two sexes together, M_{recov} was higher in rats exposed to small cages than the other two restraint types and was not affected by sex (LRT, stressor: 2ΔLL = 16.45, df = 2, p<0.001). A_{drop} and A_{recov} differed between stressors depending on the sex of the rats (LRT, sex-by-stressor interaction, A_{drop}: 2ΔLL = 7.12, df = 2, p = 0.029; A_{recov}: 2ΔLL = 6.25, df = 2, p = 0.044). When analysing the sexes separately, A_{drop} and A_{recov} differed between stressors only in male rats (LRT, stressor: A_{drop}: 2ΔLL = 6.37, df = 2, p =

0.041; A_{recov}: 2ΔLL = 6.31, df = 2, p = 0.043) with the least drop and highest recovery following restraint in small cages.

Baseline eye temperature did not differ between sexes (females: 36.14±0.48°C, n = 18; males: 35.96±0.56°C, n = 18; LRT, 2ΔLL = 1.41, df = 1, p = 0.235). Eye temperature increased after rats were released from the restraint and either remained high or returned towards the baseline (Fig. 4b). When analysing the sexes together, the post-stressor eye temperature response over time differed between stressors (stressor-by time: LRT, 2ΔLL = 15.97, df = 2, p = 0.0003), between sexes (sex-by-time: LRT, 2ΔLL = 25.77, df = 1, p<0.0001) and between eye sides depending on sex, sex-by-side interaction, LRT, 2ΔLL = 20.84, df = 1, p<0.0001 see Supplement Table 2 All rats). While the eye temperature response in male rats returned towards baseline at the end of the test period, the eye temperatures in female rats remained elevated until the end of the filming period (Fig. 4b). When each sex was analysed separately, the eye temperature response over time varied with restraint stressors only in female rats (LRT, 2ΔLL = 24.69, df = 2, p<0.0001). In females, the eye temperatures remained elevated until the end of the test period with the small cage inducing the highest and encircling the lowest eye temperature during the first half of the filming period (following an initial slight drop at first showing the starting point lower than the baseline due to the one-minute restraint gap). The eye temperature of male rats did not differ between stressors (LRT, 2ΔLL = 0.09, df = 2, p = 0.9555) but varied with location (LRT, 2ΔLL = 4.15, df = 1, p = 0.0417) and posture (LRT, 2ΔLL = 12.06, df = 3, p = 0.0072) where the eye temperature was lower when rats were at the corners of the arena or when they were walking. Male rats also had relatively warmer right eyes than left eyes (LRT, 2ΔLL = 17.22, df = 1, p < 0.0001), whereas in females the left eye was relatively warmer than the right eye (LRT, 2ΔLL = 4.2, df = 1, p = 0.0404). None of the other explanatory variables were related to eye temperature differences from baseline (Supplement Table 2 Male rats and Female rats).

3.2. Behavioural response

The frequency of 'Elimination', 'Escape' and 'Rest' behaviours changed over time and differed between stressors depending on sex (LRT stressor by sex interaction, 'Elimination' 2ΔLL = 9.35, df = 2, p = 0.0093, 'Escape' 2ΔLL = 6.15, df = 2, p = 0.0461, 'Rest' 2ΔLL = 8.02, df = 2, p = 0.0182, see Supplement Table 3). Male rats restrained with the small cage defecated and urinated more than males after the encircling and restraint cone, whereas female rats restrained with the small cage and restraint cone defecated and urinated more compared to those that had been encircled (Fig. 6). Female rats showed more 'Escape' behaviour and less 'Rest' behaviour than male rats (Figs. 7a,c). However, when each sex was analysed separately, male rats restrained with the small cage showed the most 'Escape' behaviour (LRT, 2ΔLL = 9.09, df = 2, p = 0.0106) whereas encircled males rested for a greater proportion of time than males exposed to the other two stressors (LRT, 2ΔLL = 9.57, df = 2,
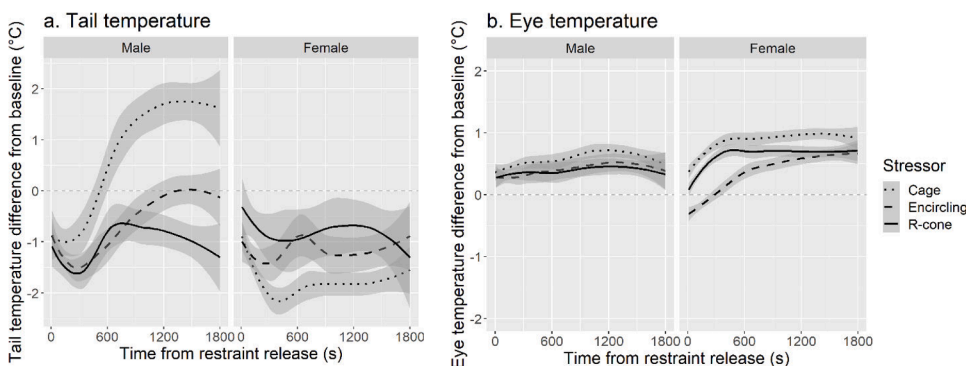

Fig. 4. Sex differences in tail (a) and eye (b) temperature response to three restraint stressors. The figure shows GAM fitted splines and 95% confidence interval (grey bands) of the maximum tail temperature responses of rats after having experienced the small cage (dotted line), encircling handling (dashed line) or the rat restraint cone (continuous line) for 1 minute (n=6 for each response curve). The thermal responses are plotted as the temperature difference from the baseline temperature from being released from the restraint into the test arena (time=0) until 30 minutes post-stressor. The baseline temperature (grey dashed line) is the mean of three measurements taking during the 30-seconds immediately before stress exposure.
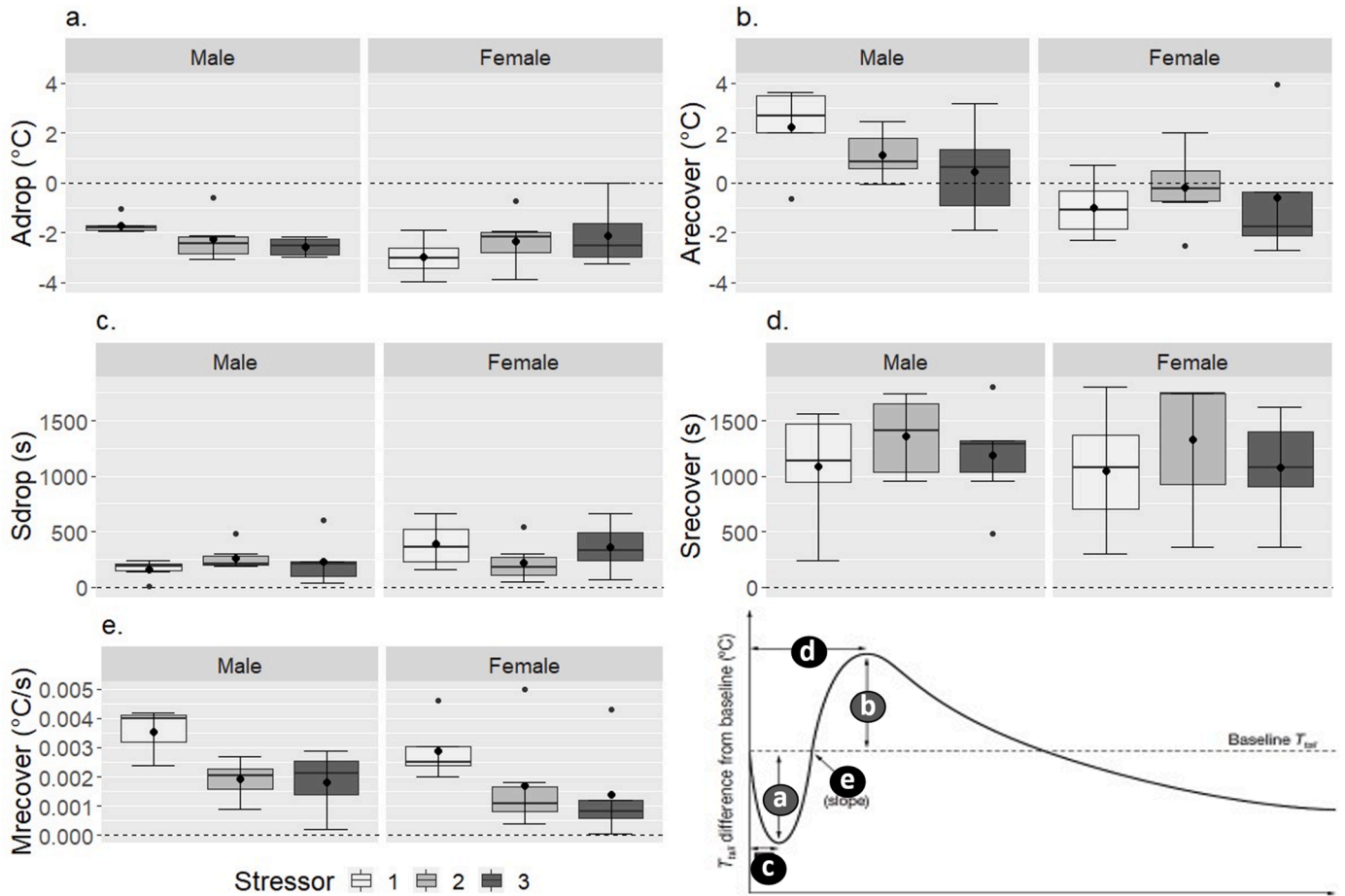
Fig. 5. Boxplots showing the amplitudes and the dynamics of specific components of the tail temperature response of each individual rats to either small cage(1), encircling (2) and restraint cone (3) according to sex (n=6 per box). The median for each dataset is indicated by the black centre line, and the lower and upper hinges of the box are the inter-quartile range (IQR). The extreme values (within 1.5 times the inter-quartile range from the upper or lower quartile) are the ends of the lines extending from the IQR. Outliers are represented as filled circles and whiskers are the standard deviations. The specific thermal response properties plotted are the amplitude of the drop of the temperature (A_{drop}: a) and the rise of the temperature (A_{recov}: b) and the time taken to reach A_{drop} (S_{drop}: c) and the time taken to reach A_{recov} (S_{recov}: d) in seconds. The rate of change of temperature from A_{drop} to A_{recov} was represented by the slope (M_{recov}: e).
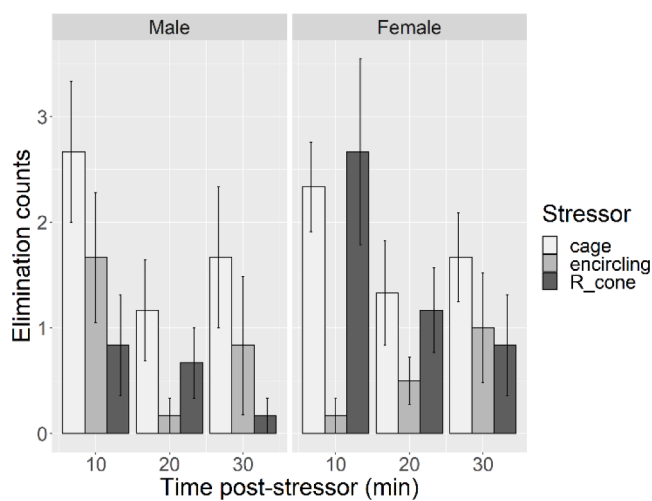

Fig. 6. Bar graphs showing 'Elimination' (event seeing urine or faeces) counts per 10 minutes during the 30-minute filming after exposure to either small cage, encircling or restraint cone (R-cone) stressors for one minute (n=6 per stressor per sex).

$p = 0.0084$, Fig. 7a,7c). Grooming and freezing behaviours were more frequent in females than males regardless of which stressor they were exposed to (LRT, $2\Delta LL = 4.27$, df = 1, $p = 0.0388$), and decreased over time in the testing arena (LRT, $2\Delta LL = 19.79$, df = 1, $p<0.0001$, Fig. 7b). 'Explore' behaviours did not differ between stressors (LRT, $2\Delta LL = 3.67$, df = 2, $p = 0.1593$) and sexes (LRT, $2\Delta LL = 0.69$, df = 1, $p = 0.4066$) nor did they change with time (LRT, $2\Delta LL = 1.69$, df = 1, $p = 0.1942$) in the testing arena (Fig. 7d).

3.3. Plasma corticosterone level

Females had a higher pre-stressor baseline plasma CORT concentration (37.27 ± 9.96 ng/ml, n = 5) than male rats (9.37 ± 3.6 ng/ml, n = 5; t-test, t = 2.63, df = 5.03, $p = 0.04$, Fig. 8). Rats of both sexes exhibited a CORT response to all three stressors (Fig. 8). The CORT response over time differed between stressors (timepoint-by-stressor interaction LRT, $2\Delta LL = 8.46$, df = 2, $p = 0.015$) and CORT concentrations were more elevated in females than in males (LRT, $2\Delta LL = 7.46$, df = 1, $p = 0.006$, Fig. 8, Supplement Table 4 All rats). When each sex was analysed separately (see Supplement Table 4 Male and Female rats), the response profile differed between the restraint stressors depending on timepoint for male (LRT, $2\Delta LL = 10.31$, df = 2, $p = 0.006$) but not female rats (LRT, $2\Delta LL = 1.05$, df = 2, $p = 0.593$). Blood collection duration and time of day were never significant ($p > 0.05$, Supplement Table 4).
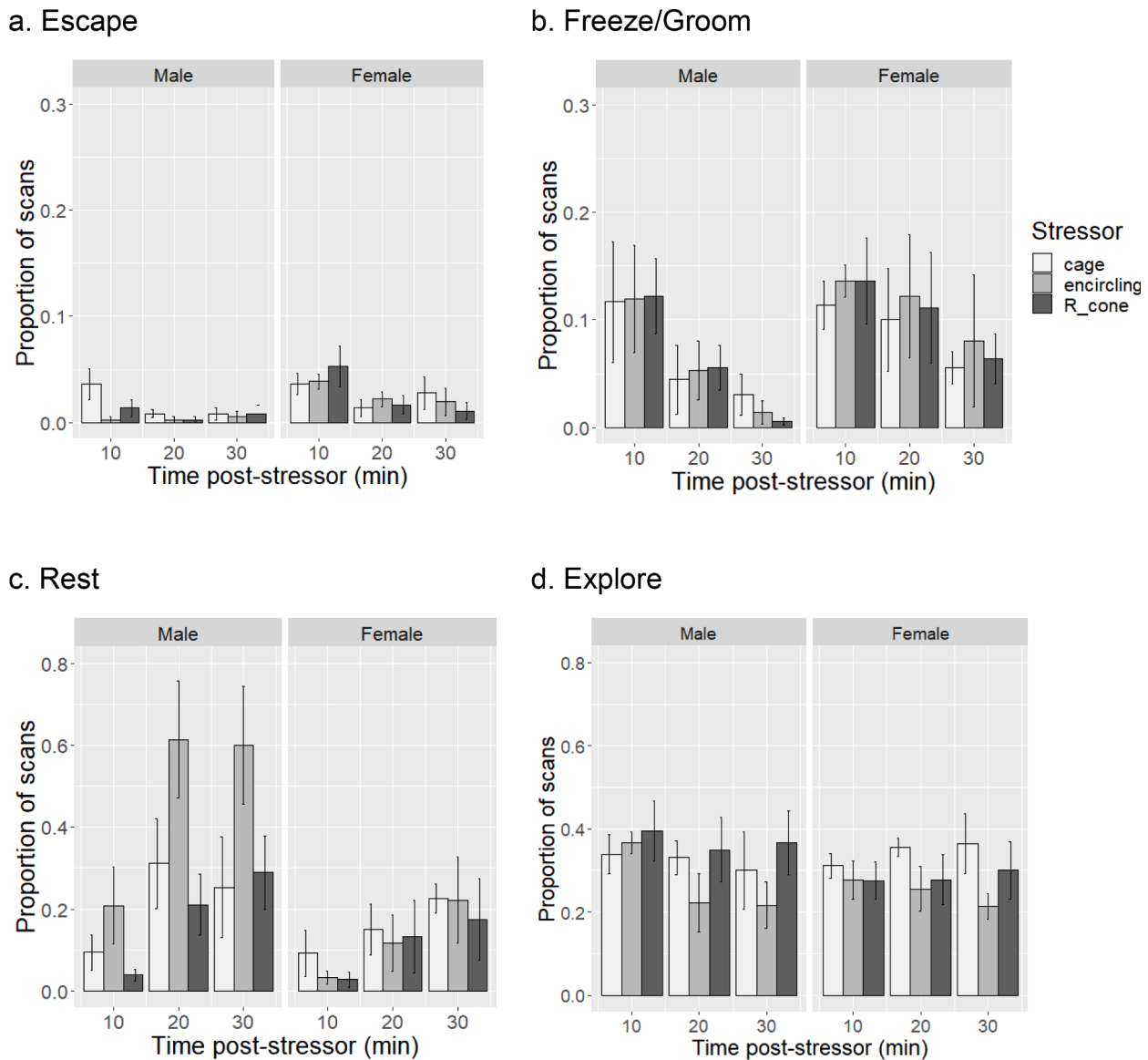
Fig. 7. Bar graphs indicated proportion of scans showing Excape (a.), Freeze/Groom (b.), Rest (c.) and Explore (d.) behaviours during the 30-minute filming after exposure to either small cage, encircling or restraint cone (R-cone) stressors for one minute (n=6 per stressor per sex).

To explore the different dynamics further, the data were subdivided and analysed according to the different phases of the response before (R_Cmax) or after the maximum concentration (R_Recov, Supplement Table 5). The curve component analysis (Fig. 9) showed that the highest Cmax was found in encircled males and in females in restraint cones while the lowest Tmax was found in encircled rats of both sexes. R_Cmax (rate of increase towards the maximum CORT concentration) differed between stressors (LRT, $2\Delta LL = 11.96$, $df = 2$, $p = 0.003$). Post-hoc analysis showed that during the 10 minutes post-stressor, encircling induced significantly higher CORT concentrations than small cage (LSM, $p = 0.003$) and restraint cone (LSM, $p = 0.019$). During the 'Recovery' (R_Recov) male rats had slower declines in CORT concentrations than females (LRT, $2\Delta LL = 5.33$, $df = 1$, $p = 0.021$) but this did not differ between stressors (LRT, $2\Delta LL = 4.61$, $df = 2$, $p = 0.1$). The AUC suggests that the overall CORT responses of the females to all three acute restraint stressors were larger than in the males but that the size of the response in the females was not affected by stressor type (Fig. 9e).

4. Discussion

This study demonstrates, for the first time in unrestrained mammals, that the dynamic response of body surface temperature measured with IRT differs between acute mild restraint stressors of different magnitude and between the sexes. Body surface temperature responses differed between the body regions. Tail surface temperature, measured when the whole tail was visible, was more sensitive to stressor magnitude than eye temperature. The results indicated that the ability to capture the individual dynamics of the tail temperature response and the dynamics of the plasma CORT response (especially M_{recov} and R_C_{max} respectively), were critical to the assessment of the magnitude of acute stress response in female rats. The rate of recovery between the initial surface temperature drop and subsequent surface temperature overshoot (M_{recov}) of the tail was lower for the small cage restraint compared to encircling and the restraint cone, but these measures could not be used to distinguish between the latter two stressors in either sex. Differences in body surface temperature between stressors and sexes were supported by some components of the stressor- and sex-specific behavioural and CORT responses. The thermal, CORT and behavioural responses together
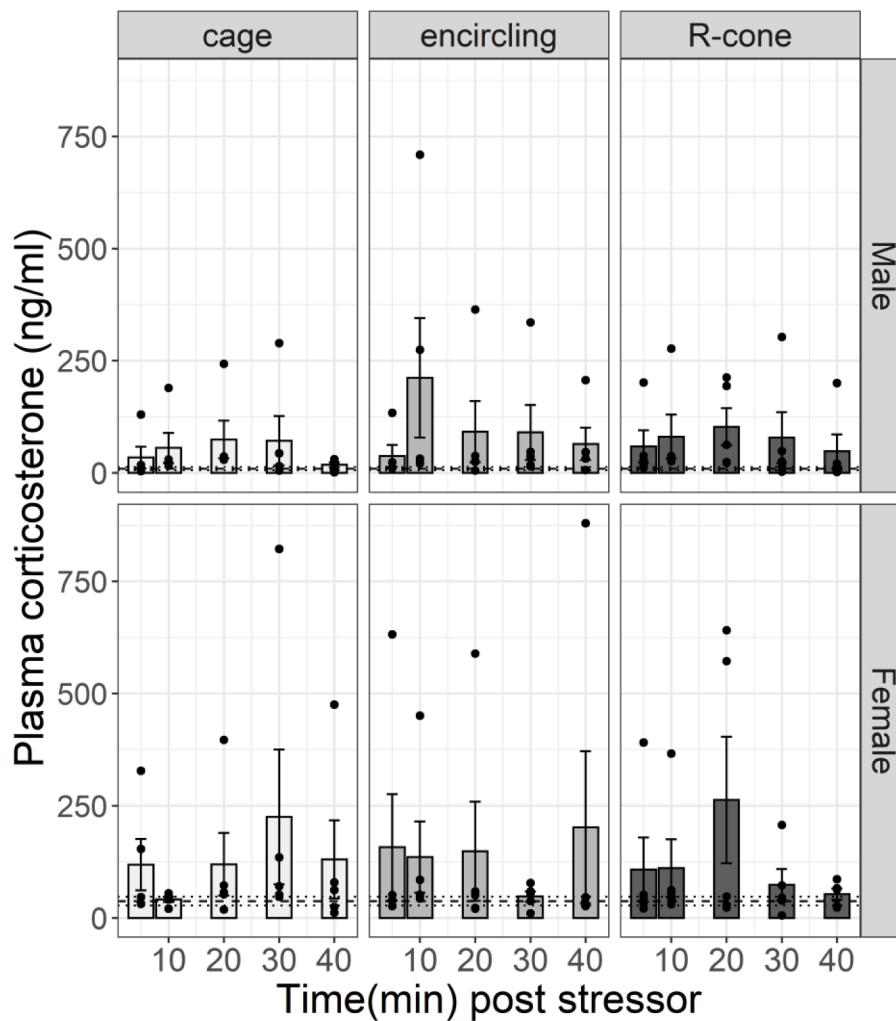
Fig. 8. Mean (± SE) total plasma corticosterone concentrations of the rats in the pre-stressor control group (shown as dashed and dotted horizontal lines, respectively) and at different time points (5, 10, 20, 30 and 40 minutes) after the rats were exposed to one of the three restraint stressors; small cage, encircling, or restraint cone (R-cone) for one minute (n = 5 rats per stressor and sex and timepoint, shown as dots).

suggested the same ranking of stressor magnitude: increasing from small cage, restraint cone to encircling and that female rats were more responsive and/or more vulnerable to mild restraint stressors compared to male rats.

All three short one-minute duration restraint stressors used in our study induced an acute stress response in both male and female rats as demonstrated by the maximum CORT concentration which was approximately 200 ng/ml higher than in the pre-stressor control group. These CORT concentrations were substantially lower than those reported in response to prolonged (30-60 min) and maybe painful immobilisation on wooden boards [9,116,117] and slightly lower than those reported in response to restraint in a transparent plastic cylinder [9,33,118]. The effects of prolonged stress duration and evoking additional severe emotional and physical pain are likely to have contributed to greater CORT responses in other stress studies. We sampled a total of 160 rats with five individuals per stressor, sex and time point. Although variance was high in this data, we log transformed data in our statistical analyses and also verified that data outliers did not affect the results. This analysis showed that there was a CORT response to the stressors but that the response differed between female and male rats. While in males the CORT response differed between stressors with the highest response found in encircled rats, we did not find a difference in the CORT response between the different stressors in female rats without analysing the CORT curve components. It could be that for our mild restraint

stressors the effect size was smaller than predicted by our power-analysis on the basis of previous studies in male rats [111] and larger number of female rats per treatment would be required. Nevertheless, the milder stressors applied in the current study are better candidates to study acute stress responses relevant to laboratory routines, including routine handling, that are of most interest for welfare assessment because it avoided the effects of additional severe emotional and physical pain.

In general, tail temperature initially decreased after the stressor was applied, then recovered and overshot the pre-stress baseline tail temperature before returning toward that baseline. The initial cooling of the tail was as expected and in line with previous restraint stressor studies [33,74,75,118–120] and to foot-shock fear conditioning in rats [35,71]. Unlike previous studies which have examined the SIH responses expressed by rats restrained in a plastic tube for 30 to 90 minutes [74,96, 118,121–125], our results show that IRT can non-invasively detect the surface temperature response to a brief one-minute duration restraint that is relevant to routine laboratory husbandry [88]. The drop in tail temperature observed in the current study in response to mild and brief stressors, however, was smaller and of shorter duration compared to that seen in response to long-lasting stressors [33,74,75,118–120].

In contrast to tail temperature, eye temperature increased following application of the stressors in this study. This was not unexpected as corneal temperature is strongly associated with core temperature in rats
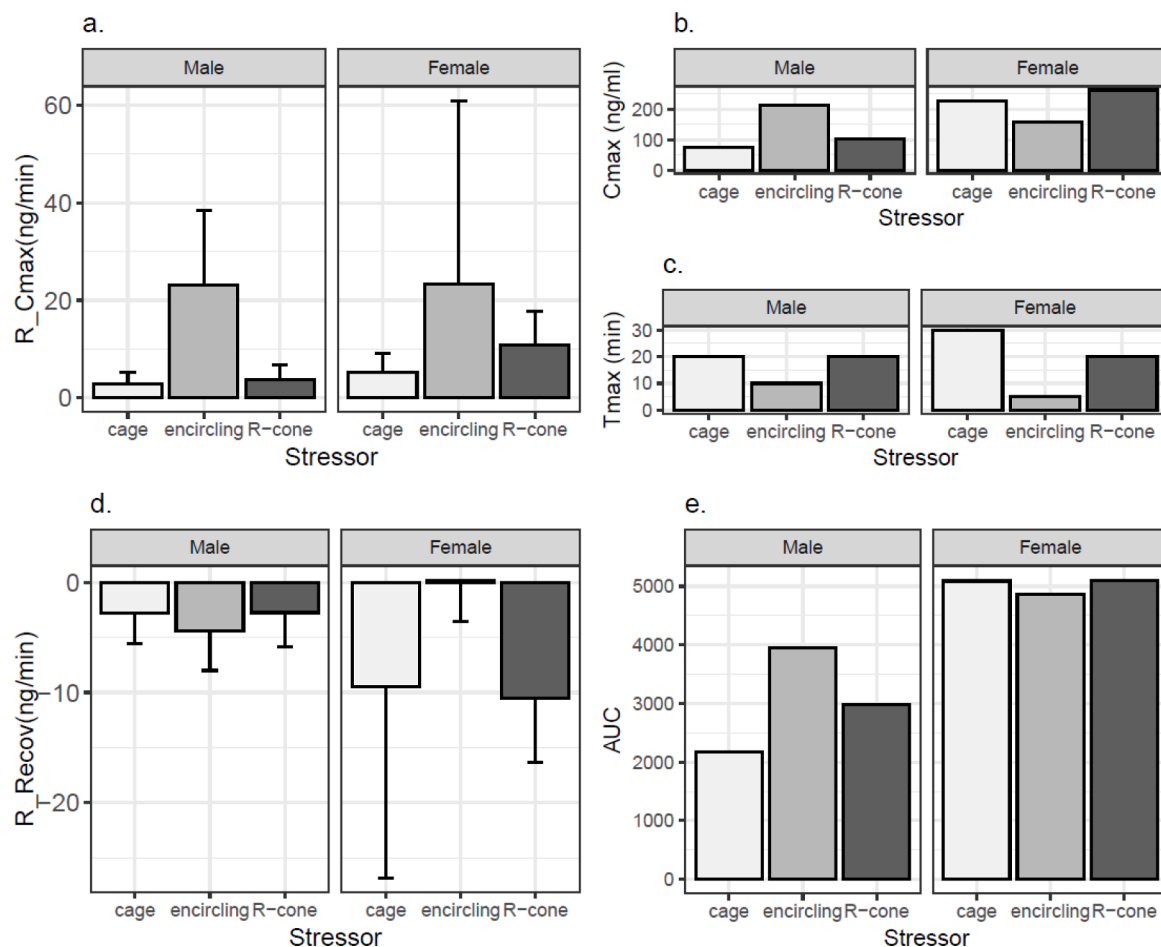
Fig. 9. Mean, and SE where possible, of the curve properties (a.- d.) and area under the curve (e.) extracted from the plasma corticosterone (CORT) plots of each stressor (small cage, encircling, restraint cone) for each sex. Curve properties were: (a.) the rate of the increase of the CORT level from the baseline to the peak (R_C_{max}), (b.) the peak concentration (C_{max}), (c.) time to reach peak concentration (T_{max}), and (d.) the rate of the decrease of the CORT level from the peak to the lowest level (R_Recov).

and humans [126], and an increase in eye IRT temperature, in response to psychogenic stressors has been reported in a range of mammals of varying body size: dogs [127], mice [72], rats [35], horses [51], cattle [128–130], sheep [131], non-human primates [55] as well as humans [132]. One IRT study in mice had reported a decrease in eye temperature, however, this was due to 10 min exposure to isoflurane anaesthesia which lowered the core body temperature overall, as opposed to a psychological stress [39]. In the present study, the eye temperature was greater when the male rats were walking compared to when they were inactive. While this effect could reflect core metabolism and thermogenesis from body activity, it was not found in female rats even though they showed an overall higher level of activity. The variation in eye temperature seen in the current study could be resulting from how the measurements were obtained. It has been suggested that the most accurate way to assesses eye temperature in rats is by imaging the eyes from the front with subjects being restrained with dorsal fixation in front of a close-up lens [133]. In the present study, we avoided restraining the rats for 30-minute filming and prioritised visibility of the whole tail. This overhead filming approach means that the eye temperature measurements were affected by variations in head angle and, although we statistically controlled for different levels of head angle, finer variation in head angle may still have resulted in an underestimate [133] and variation in eye temperature [134,135]. To avoid the confounding effects of probe insertion or surgical implantation of a data logger, the core body temperature of the rats was not measured in this study and therefore the relationship between eye and core temperature was not determined.

None of the other variables that could affect eye temperature were significant except 'location' which was marginally significant in male rats when measured in the corners of the arena, where eyes were cooler compared to when they were in the centre of the test arena. The location effect could be confounded with the angle at which the eyes are exposed to the camera at the different locations. However, since this effect was not found in females, it may also be an artefact.

Eye temperature also differed between the right and left side, but the direction differed between the sexes, strong male right-dominance and weak female left-dominance responses. All vertebrate classes seem to show a similar lateralisation pattern for emotional processing, with a right-hemisphere dominance for processing negative emotions, such as fear and aggression, and a left-hemisphere dominance for processing positive emotions, such as those elicited by a food reward [136]. Lateralised differences in tympanic membrane temperature, possibly due to lateralised cerebral blood flow and neural activities [137], have been associated with stress in humans [138], marmosets [139], macaques [138], chimpanzee [140] and cats [141]. These asymmetries could also be investigated using IRT to assess emotional valence [44]. As with the tympanic membrane temperature, the eye temperature was interpreted in this study to reflect the brain temperature of the same side [142,143]. The mechanism of thermal lateralisation is not clear but it could be due to lateralised cerebral blood flow, sympathetic innervation or activation of corticosteroid receptors [140,141,44,144]. However, sex-related differences in the lateralisation in neurological structures associated with decision making and emotion in humans exert a pattern of

predominantly right-hemisphere lateralisation for men and left-hemisphere lateralisation for women [145,146]. This difference in emotional processing could also exist in rats and be responsible for the sex differences in lateralisation in the present study. Future work on thermal lateralisation and its mechanism is much needed, along with investigation of sex effects and emotional valence on thermal asymmetry.

One of the key aims of this study was to explore the ability of IRT to assess stressor magnitude using the dynamics of surface temperature change in two body parts. It was the relative change in temperature rather than the absolute temperature (Appendix 3) which was of interest in this study in such a controlled laboratory environment. Since the camera angle when filming the tail was relatively stable, the magnitude of stress was, indeed, revealed robustly with the dynamic response of tail temperatures depending on stressor magnitude and sex and was in the same direction with the results of plasma CORT and behavioural changes. Although there was a significant effect of stressor magnitude also on the increase of eye temperature in females, it was a smaller change compared to the pattern in tail temperature and was not supported by the CORT and behavioural results. Based on the analysis of the overall tail response, the male rats responded to the small cage, encircling and restraint cone in ascending order, as expected from previous studies in male rats [67,77]. However, in female rats there was no significant difference in the tail temperature response between the three stressors and the tail temperature never recovered back to baseline level. Therefore, without analysing individual curve components, the stressor magnitude effect emerged only in male rats.

After the initial decrease in tail temperature, only encircled males had returned to baseline while males exposed to small cages remained above baseline for the rest of the 30-minute post stressor period. This rebound of tail temperature to above baseline following exposure to a stressor has been reported in previous studies in rats [35,71]. The tail plays an important role in thermoregulation and the rebound could simply be a result of the tail dissipating the heat accumulated and generated in the core during SIH [147–149]. In addition, mammals overheat easily when they have a low body surface area to volume ratio and a thick subcutaneous fat [150,151]. Therefore, such thermoregulatory responses could explain the positive correlation between body mass and tail temperature in male rats in this study. However, the same effect was not found in females, suggesting the effect of body mass could be either a statistical artefact or the effect of body mass is non-linear only affecting the largest (mainly male) individuals. After the initial cooling, the tail temperature never returned to baseline in female rats or in male rats exposed to the restraint cone. This may indicate an attenuated stress response in males in the small cage compared to other two restraint stressors, and all forms of restraint in the females wherein the larger physiological response would result in the tail temperature remaining below baseline for the entire 30 minutes. This is supported by reports that the stronger the arousal, the stronger and longer the vasoconstriction response [35,147,148]. Another explanation for the non-recovery of tail temperature in female rats could be that females never habituated to the testing arena as the tail temperature of females remained below baseline even after habituation to the testing arena. Nevertheless, the responses to the stressors applied in this experiment were stronger than those observed with just the testing arena at the end of the habituation period (Tail A_{drop}: mean -2.32±1.03°C vs -0.43 ±0.56°C respectively). Furthermore, lack of habituation would have led to baseline temperatures being lower than those observed in our study and the tail temperature could not have decreased further. Therefore, the additive effect of the arena on the stress response of females, if any, was suspected to be minimal.

The thermal curve components provided more detailed information on the dynamic stress response, and identified parts of the response that were sensitive to stressor magnitude in both sexes. The analysis of the recovery rate of tail temperature (M_{recov}) showed that rats recovered faster after being exposed to the small cage than to encircling and the

restraint cone, which was in line with the expectation that the small cage was the mildest stressor for both sexes. The amplitude of the initial dip (A_{drop}) and the overshoot peak (A_{recov}) also differed between stressors but only for males. This emphasises the importance of extracting the individual curve components for M_{recov} when using both sexes or female rats. Since the difference between the temperature response to encircling and restraint cone was not significant, it may be that these two stressors were potentially perceived differently: body restraint as opposed to confinement. However, the interpretation of these tail temperature results and the value of extraction of individual specific components of the tail response to assess stress magnitude was further clarified by the responses of plasma CORT and behaviours.

The CORT response of the males differed between stressor magnitudes with encircling inducing the greatest and the small cage the least amount of CORT secretion. Female rats, on the other hand, secreted similarly elevated levels of CORT in response to each of the three stressors, and in each case, the response in female rats was greater than in male rats. This may suggest either that a ceiling effect was reached in females. The lack of studies in females on the magnitude of these specific restraint stressors and the translation of results from males to females are shown in this study to be problematic. Only when taking the timepoints before C_{max} and the curve components of the CORT response into account, specifically how quickly CORT increased up to the peak (R_C_{max}), the results of the current study showed that encircling was the most severe stressor compared to small cage and restraint cone for both sexes (Fig. 9). Unlike the IRT response, the CORT responses differed between the three stressors during the initial phase of increasing CORT concentrations but not in the recovery phase.

An explanation for the greater change in CORT in response to encircling rather than restraint cone stressor could be that encircling allowed some movements of head and limbs creating the unrealistic perception for the rats of an escape opportunity compared to the restraint cone when their movements were most restricted. This may have caused the rats to struggle more in encircling and might have triggered frustration and emotional stress when the effort to escape from the handling was unsuccessful, leading to further CORT release [152–154]. Alternatively, being fully enclosed in a restraint bag which contoured the whole body might have made the rats feel relatively safe while enduring the stress in line with the tendency of rats to maintain physical contact with surfaces when they perceive environments as threatening [83]. In addition, tactile pressure from being physically enclosed has been reported to produce calming effects in humans [155,156], dogs [157] and pigs [158].

The behavioural observations of 'Elimination' in both sexes, and 'Escape' and 'Rest' behaviours in the male rats differed between stressors. Male rats exposed to the small cage defecated, urinated, and performed escape behaviours more often and rested less than the rats that had been encircled or held in the restraint cone while female rats defecated and urinated the least in encircling group compared to the other stressors. Given that defecation and urination are commonly interpreted as a response to stress or fear [159–161], these results appear to contradict the IRT and CORT results, suggesting that the greatest stress response resulted from the small cage and lowest stress from encircling. However, 'Escape' behaviours included also darting behaviour (Appendix 2), and male rats exposed to the small cage restraint walked more that male rats in other stressors but rarely tried to jump out of the arena which was an escape attempt performed mostly by female rats. The lack of a positive correlation between anxiety level and elimination and negative correlation with locomotor activity [162–165] in this study could be artefact of the method how elimination events were monitored. Fresh faeces or urines were detected using thermal images where they appear as spots that are considerably warmer than the arena surface, but some elimination events may have been missed if subjects blocked the view of their faeces or urine for any length of time. Indeed, it was noted that rats sometimes rested (sternal recumbency) for a long time after release post stressor and once they got up to walk again,

several defecations and urinations which other studies counted as number of faecal boli [160,166–168] would have been counted as one event in our study. This suggests that our scans of 'Elimination' events were possibly confounded with movement behaviour. If indeed the main behavioural difference in the response to the three restraint stressors was in locomotory behaviour and lower locomotor activity indicates anxiety [163], our results would indicate that among the three restraint stressors the small cage was the relatively mildest stressor for male rats and encircling was the relatively most severe stressors for both sexes, which is in line with the IRT and CORT results.

The thermal, behavioural and CORT response differed in what stressors they could distinguish and hence in the range of stressor magnitudes they possibly could detect. For example, although glucocorticoids can be used to differentiate the magnitude of stressors they can do so only for only relatively mild stressors as ACTH quickly reaches a ceiling effect and hence glucocorticoid levels are incapable of rising further at more severe stressors [4]. Our study confirms that vasoconstriction in the rat tail responds rapidly to even brief and mild stressors [169] and thus, that IRT non-invasively measuring changes in body surface temperatures has the greater potential to detect and quantify stress responses over a wider range of stress experiences.

The second aim of this study was to explore sex differences in the response of body surface temperature to acute stress. The results indicated similar sex differences in the thermal, behavioural and hormonal responses to acute stressors. Female rats showed lower baseline tail temperature, a higher proportion of time engaging in escape behaviours, higher baseline CORT levels and greater overall thermal and hormonal responses than male rats. These findings all suggest that female rats were more anxious and/or vulnerable to restraint than males. However, as female baseline tail temperature was already lower and their baseline CORT levels higher than in males, females may have been more anxious to be isolated in the open field-like arena than males, despite the week-long habituation to the testing conditions. Nevertheless, other studies using different protocols also found that female rats showed a greater SIH response to restraint and confinement compared to males in both light and dark periods [27]. Similarly, female, but not male, mice responded to rearing deprivation with increased cutaneous temperature in the head and back, and decreased tail surface temperature [120]. Thus, the greater thermal response to stress in female rats in this study is consistent with their overall greater endocrine, autonomic and behavioural responsiveness [60,170–175]. There is a presumption that the oestrus cycle in females would introduce unwanted variations and, hence, prevents reliable conclusions to be drawn about sex differences [62,176]. In this study, we applied a one-time novel exposure to one of three restraint stressors and it was not possible to obtain vaginal smears to assess the oestrus cycle accurately in the females. Oestrus stage, however, has been recently shown not to contribute to variation in the stress response in female rats [60,177,178].

The environmental temperature and relative humidity of the arena did not influence the body surface temperatures of animals and time of day was not significant in the CORT analysis. This might be because the ambient temperatures and humidity were relatively standardised in the laboratory environment. Similarly, the experimental work was carried out in the light (inactive) phase where the background changes in body temperature [179] and plasma CORT level [180] would be smaller than the dark phase. A limitation of this study was the missing thermal data during the one-minute stressor exposure, because this was performed out of view of the camera recording. This data would also have not been possible to collect as the infrared camera cannot film through the plastic lid of the small cage and for the other stressors the ROIs were covered in encircling handling, or in a plastic restraint cone. Ideally, future acute stress research should develop techniques that allow IRT filming in the home cage (using thermally transparent materials), which would allow acute stressors to be applied inside the home cage environment to which they are habituated and allows the effects of test arena and isolation to be eliminated.

For the first time, this study demonstrates that body surface temperature measured with IRT can reveal the magnitude of acute restraint stress, sex differences and thermal lateralisation in rats consistent with the CORT and behavioural responses to the same stressors. Our results are strictly valid for brief acute stressors and may be stressor- and species-specific, our findings support the notion that IRT has the potential to become a valid, non-invasive method of stress assessment in unrestrained mammals, especially those in routine laboratory husbandry and research. The marked difference in the response of male and female rats also emphasizes the importance of using both sexes in stress-related research which is now been required for many biomedical studies. A validated surface temperature approach to assessing stress magnitude has the possibility to reduce or eliminate invasive procedures (in particular blood sampling and the implantation of loggers to measure core temperature) and hence, refine experimental procedures. It would also increase the quality and quantity of data collection in rat models of stress and anxiety by providing continuous information on the entire stress response and not just single point measures as currently provided by blood samples. Ultimately, IRT could provide a means to develop a non-invasive, continuous method of monitoring welfare throughout the life of laboratory rats.

Data availability

Data are available on DOI:10.17632/8k5z5bx2s8.1.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2023.114138.

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