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The 'Cage Climber' - A new enrichment for use in large-dimensioned mouse facilities

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ABSTRACT

Environmental Enrichment has been shown in experimental contexts to have clear and often beneficial effects on animal physiology and behavior. Housing prior to experiments can represent a large proportion of an animal's living conditions, and improving housing conditions can be seen as "refinement" in a 3R-Context. However, large-dimensional implementation in rodent facilities often lack systematic analysis of respective refinement measures. Although enrichment is a legally binding element for housing animals according to EU law, potential effects on data variability are often neglected or not taken in consideration for experimental designs.

Here, we aimed at implementing a new and innovative tool to improve wellbeing without side effects applicable for male and female mice. This poses a great challenge since the social structure in this species allows group housing in females without the risk of aggressive interaction, while in males despotic dominance hierarchy is often associated with agonistic interactions. Thus, we focused on enrichment-induced changes in behavior and stress physiology emphasizing effects on data variability in both sexes.

Accordingly, recycled cage lids (resembling sex-neutral early environmental experiences in mice) were formed and three shapes of different structures were examined ('Cage Climber'): 'Triangle Climber', 'Bridge Climber' and 'Round Arch Climber'.

The results demonstrate significant preferences of C57BL/6 N mice for any of the three structures in comparison with a neutral object when presented in a Novel Object Test. Despite intense use of enrichment, there were neither behavioral alterations detectable in a test battery assessing locomotion, anxiety and sociability nor in assessment of stress physiological parameters such as stress hormone metabolites, analyzed non-invasively from feces.

Additionally, the structural supplement did not affect general variability of data in both male and female mice.

To promote well-being of mice in a 3R-matched context, our study recommends the use of properly assessed structural enrichment, such as 'Cage Climbers' combined with nesting material to satisfy physical and thermal needs in the cage environment.

1. Introduction

Nowadays, the use of environmental enrichment in the housing of laboratory animals represents 'State of the art' and is mandatory for any vivarium according to the Directive 2010/63/EU. However, there are no defined standards, neither for the type of enrichment nor its precise application. There is definitely a lack of appropriate quality assessment regarding animal welfare and potential effect on data variability and reproducibility.

Complex structural and social environments, especially referring to natural settings can affect the emotional state of rodents (Bardi et al., 2016; Lambert et al., 2016). However, these studies analyze effects only in male animals and do not consider possible sex specific effects, which limits the possibility of generalization and holistic interpretation.

Besides effects on the behavioral phenotype, housing conditions may modulate physiological stress parameters such as corticosterone, e.g. in the context of aggressive interactions (Mesa-Gresa et al., 2016). It furthermore may shape the predisposition to develop particular features of stress-associated conditions such as depression (Chourbaji et al., 2005, 2008). Especially, impoverished housing conditions and thereby induced stress are thought to be the main factors for stereotypic behaviors and other abnormal repetitive behaviors (ARB) (Mason et al.,

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2007). Hence, the application of environmental enrichment has the potential to avoid the development of ARB and other stress-related phenotypes, thus improving wellbeing. (André et al., 2018).

Changes in behavior may not simply be 'beneficial', but can be accompanied by differences in biochemical markers, thus evoking mixed results (Girbovan and Plamondon, 2013). This is especially critical when housing practices are not addressing sex differences (Girbovan and Plamondon, 2013). Notably, environmental manipulations may even exert opposing effects in males and females (Toth et al., 2011), which underlines the necessity to assess any side effect, that could occur due to alterations of cage structures.

Institutional enrichment programs need to fulfill the criteria to avoid any kind of distress, to provide wellbeing of the mice and at the same time not affecting the variability of data (Andre et al., 2018). However, one needs to differentiate between experimental enrichment, which is expected to exert effects and can be referred to as a kind of treatment (e.g. 'rescue' of a phenotype) and housing enrichment, that intends to increase the animals' wellbeing. Importantly, a large-dimensional use of a certain housing protocol always needs to be part of the organizational infrastructure plus experimental design and requires thorough interaction between the animal facility and the scientists. When using enrichments, it is therefore important to consider i.) biological relevance for the animal ii.) hygienic aspects, iii.) handling by caretaker staff, iv.) storage/ sustainability, v.) visibility of the animal and vi.) potential health danger for animals and personnel (Fig. 1d). Being aware of such essential and pragmatic requirements, we developed a new type of enrichment for the group housing of mice of either sex, called the 'Cage Climber'. Since it is an important part of the mouse' behavioral repertoire to be able to climb (Baron et al., 1962; Ishiwaka and Mori, 1999) and build nests (Gaskill et al., 2013a, b; Jirkof, 2014) we recycled cage lids and shaped them in three types of structures. Beside the possibility to use it for physical activity, mice are familiar with the topography of cage lids from a very early developmental stage, which should prevent neophobia. Using standardized material eases the hygienic management and increase compliance of caretaker staff, who were – in our study - involved in the evaluation of handling the equipment. While offering the animals a new structure, the grid structure of the 'Cage Climber' does not hinder daily visual inspections by the staff.

A thorough evaluation of housing measures is a key factor for quality insurance of best practice science and research reproducibility (Toth, 2015) and even seemingly trivial issues like handling (Mertens et al., 2019) or the position of the cage in the rack can influence the outcome of an experiment (Izidio et al., 2005). Despite results like those of a study of Nagy et al. (2002), according to which phenotypic variance in group housed mice is greater than in individually housed animals (Nagy et al., 2002), housing measures are a rather neglected, rather pragmatically handled aspect in experimental animal facilities. In our study we therefore chose group housing of mice of both sexes (separately) to gain insights to further aspects which can impact behavioral and physiological measures (e.g. hierarchy dynamics) (Varholick et al., 2019). In aspects of the laboratory mouse, it is known, that different strains, sexes and e.g. genetically modified mice cannot be considered behaviorally similar, so environmental enrichment may not be used in a one-size-fits-all manner. Often used materials are e.g. nesting material which advantageously allow to create different thermal zones and provide the possibility to manipulate the material. On the other hand, certain nesting materials can be dangerous for pups due to fibers looping and warping around body parts or sticking to the skin of newborns. In contrast, shelters are less manipulative but present a higher risk of inducing or supporting aggressive behavioral patterns, especially in male mice (Bayne, 2018).

Keeping such considerations in mind, validation of the new, environmental enrichment included a large number of behavioral and physiological measurements. In this study, our aim was a) to rule out 'negative' effects regarding potential influences on behavior and b) to decipher positive effects of all or distinct types of 'Cage Climbers'. Therefore, it is important for us, to underline non-significant data as

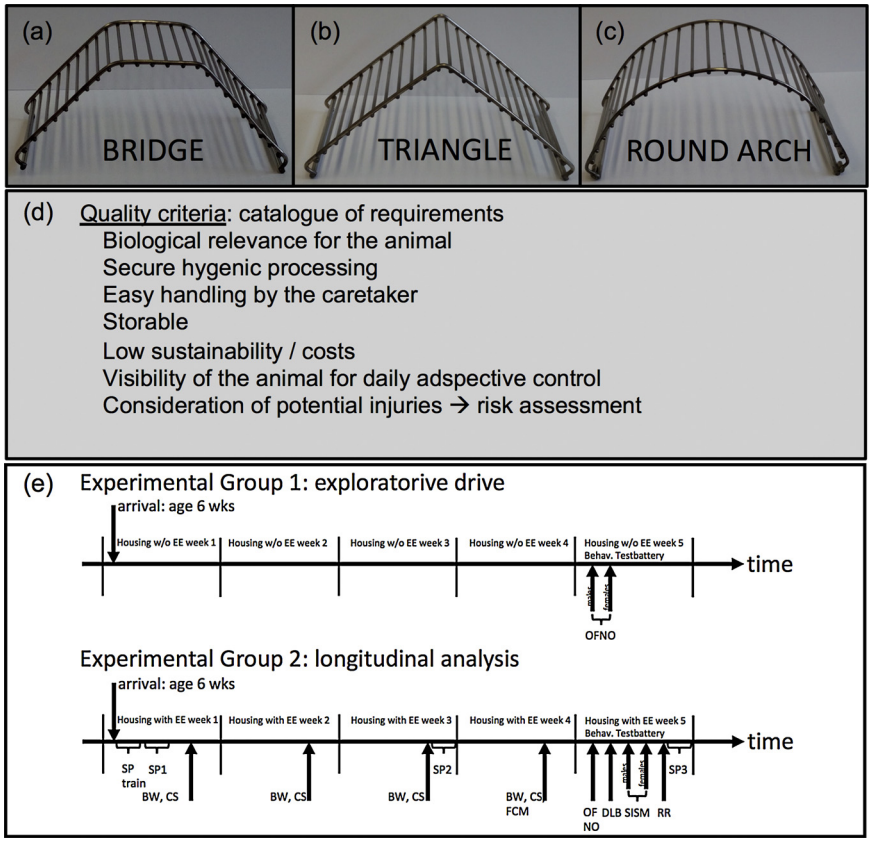

Fig. 1. Types of 'Cage Climbers': (a) 'Bridge' type, (b) 'Triangle' type, (c) 'Round Arch' type. Size is 7 cm wide, 16 cm long and 7 cm high (approximal measures for all types). (d) catalogue of criteria for structural environmental enrichment to be considered. (e) timeline of the experiments: upper time-line: group 1 for assessment of explorative drive: openfield – novel object exploration (OFNO) with one type of 'cage climbers', lower time-line: group 2: housing with one type of 'cage climbers' (or control with nesting material only), assessing behavioral alterations in a behavioral test battery. SP (train or 1,2,3): Sucrose preference training and test 1,2,3; CS: Coat State; BW: Bodyweight; FCM: fecal corticosterone metabolites; OFNO: openfield – novel object exploration (with control object); DLB: Dark-Light Box; SISM Social Interest and Social Memory Test; RR: Rotarod.

they will depict the safe use of the novel environment with regard to artefacts. To emphasize, we examined in a behavioral test battery, i) preference for the structure, ii) locomotoric features (two- and three dimensional), iii) sociability, iv) anxiety and v) analysis of feces collected from the cages for corticosterone metabolites. In particular, bodyweight development, climbing and balance behavior was chosen due to enhanced possibilities of movement within the cage when the enrichment is present; aggression, stress signs or barbering concerning possible higher territorial aggression and amount of sucrose preference at beginning, midterm and end of testing concerning a development of higher hedonic state. From our point of view this is essential to make good laboratory practice part of compliance with regard to animal welfare and raises higher consciousness for the biology of the model organism itself.

2. Materials and methods

2.1. Animals

Male and female C57BL/6 NRj (6 weeks old at arrival) mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). All mice were housed in groups in Macrolon cage type III (425 mm x 276 mm x 153 mm, floor area 820 cm^2, Tecniplast, Buguggiate, Italy) containing wooden aspen chips (ABEDD LTE-001, Lab & Vet Service, Vienna, Austria), tissue nesting material and food (Rod16-A, LASvendi, Soest, Germany) and filtered, irradiated tap water ad libitum. Housing was standardized by 12:12 h dark–light cycle with lights on at 8 pm at a room temperature of 22 ± 2 °C and humidity at 50 % on SPF hygienic conditions. Adspective controls of animals (health condition, wounds) and environmental conditions (room climate, light cycle, food and water supply) were conducted daily. Our study complied with the actual regulations on animal experiments in Germany and was approved by local authorities (Regierungspräsidium Karlsruhe, permit license number: 35–9185.81/G-5/17) following the actual regulations of the European Directive 2010/63/EU.

2.2. Environmental enrichment

The environmental enrichment consisted of differently shaped metal ladders derived from type II cage lids curved in the university's precision mechanics workshop to three different shapes: 'Bridge' with a raising ladder on both sides and a plateau in the middle, a 'Triangle' with a peak in the middle and a 'Round Arch' (Fig. 1a-c). All types were placed in the rear area of the cages to provide a free access to food and water in the front rack. The 'Cage Climbers' in the animals' cages were exchanged, washed and autoclaved once a week, when the cages were changed.

2.3. Experimental groups

Two groups of mice were used (Fig. 1e): (i) the 1st group was naïve to environmental enrichment and (ii) the 2nd cohort was confronted directly after the arrival and kept for the whole period of 5 weeks with one single type of the environmental enrichment (and nesting material) or served as control animals with nesting material only.

Mice of group I were kept in sex-separated groups of 3 and tested at 10 weeks of age. They were tested for their naïve interest in exploring the three different types of enrichment in contrast to a well-established novel object in an unknown arena, the Open Field (N = 6 per type of enrichment (control or one of three types of enrichment) and per sex summarizing to 24 males and 24 females, time-line experiments: Fig. 1e).

The 2nd group was kept in sex-separated groups of 4 mice with one type of the new developed enrichment (or with nesting material only as controls) and tested longitudinal for their change of well-being (anhedonia, stress, body weight development) and, after 5 weeks, for several behavioral and physiological characteristics (Fig. 1e; males: controls N = 32, 'Bridge' N = 36, 'Triangle' N = 28, 'Round Arch' N = 32; females: N = 24/enrichment type). Mice of one control and three enrichment cages per sex arrived weekly and were tested consecutively also weekly in groups of always four cages of males and females. Week of testing was added as statistical co-variance, however, it never had never an effect and will therefore not be further mentioned.

2.4. Behavioral assessments in the longitudinal analysis

Mice of group II were used to assess the following parameters: bodyweight, aggression/stress signs or barbering and amount of sucrose preference at beginning, midterm and end of testing. In week 5, the mice were analyzed first for physiological variations (fecal corticosterone metabolites as group stress biomarker) and followed by a test battery assessing behavioral alterations (Fig. 1e). The behavioral analyses began by testing locomotion in an unknown Open Field, directly followed by novel object exploration to assess locomotion, explorative drive and spatial pattern possible varied by more environmental stimulation. The animals were analyzed 24 h later for anxiety-like behavior concerning emotional changes in the Dark-Light Box. The following 2 days, first males and then females were analyzed for their social interest and their ability to recognize familiar versus unfamiliar mice. At the last day, after the weekly control of bodyweight and coat state, the mice were tested for their ability to run on an accelerating rotating rod (Rotarod) and keep balance reasoned on more climbing possibilities in the cage.

Prior to each behavioral test, mice were cage by cage acclimatized to the experimental room for at least 30 min.

2.4.1. Coat state

Weekly, 8 areas of the body were checked: head, neck, dorsal coat, ventral coat, front paws, hind paws, genital region and tail (Fig. 3c). A score of '0' was assigned when the area was in well-groomed status, a score of '1' was set when the part of the body showed any signs of alteration including: fresh bites, wounds, scurf, fur changes (unkempt, fatty, removed) or removed vibrissae. The score was summarized with a minimum of 0 accounting as normal and well-groomed, and a maximum of 8 accounting for a mouse with distinct alterations at all 8 body areas.

2.4.2. Sucrose consumption

Directly after arrival, animals received two bottles of 1% sucrose solution as training. 24 h later, the bottles were exchanged to (preweighed) bottles of tap water and 1% sucrose solution. After 12 h, the position of the bottles was reversed to avoid a preference due to the position. After 24 h, the bottles were removed and the total fluid intake (TFI) as well as the sucrose preference (% of the TFI) was calculated. The procedure was repeated (without the previous training session) after 2 and 4 weeks to assess hedonic and anhedonic behavior within the experimental groups. Sucrose preference testing was started at light cycle change at 8am, Sucrose bottle start position was balance via cages and treatments and added as co-variate.

2.4.3. Open field and novel object exploration

The Open Field Test examines the locomotoric and explorative characteristics of an animal placed into an unknown arena. Activity monitoring was conducted in a square shaped, black Open Field, measuring 50 cm × 50 cm, illuminated from above by 25 lx and placed on an infrared-light surface. Mice were always tested cage after cage, but placed individually into the arena and monitored for 20 min by a video camera (IkegamiDigital).

The resulting data were analyzed using the image processing system EthoVision XT8 (Noldus Information Technology, Wageningen, the Netherlands). For each sample, the system recorded position and the status of defined events. Parameters assessed for the present study were

total distance moved, velocity, time in center, which was defined as the area 10 cm distant from the walls, and mean distance to the nearest wall.

The subsequent Novel Object Test, representing an extended version of the Open Field experiment, assesses potential neophobic features by analyzing free exploration towards an unknown object. After 10 min Open Field Test, the novel object was introduced into the middle of the Open Field arena. The same test conditions were employed as for the Open Field Test. Object exploration was assessed for 10 min, recording the latency of 1st approach, as well as the total number of approaches. Novel object typed were different between the two groups of mice: Group I was assessed for their explorative drive towards the unknown 'Cage Climbers' in contrast to the control object, a water-filled 50 mL Falcon tube placed upside down. Group II was monitored for the behavior towards the control object only, the number of fecal boli was counted as indicator for emotional stress after the complete session of open field and novel object testing. Between the different animals the apparatus was cleaned with 70 % ethanol.

2.4.4. Dark–Light Box

In the Dark–Light Box, animals are investigated regarding anxiety-like behavior in terms of exploring an aversive bright compartment. The Dark–Light Box consisted of 2 plastic chambers, connected by a small opening. The dark chamber measured 22.5cm × 22.5cm and was covered by a lid. The adjacent chamber, measuring 31.5 cm × 22.5 cm, was white and illuminated from above by 600 lx. Mice were placed into the dark compartment and latency to 1st exit, number of exits and total time in the light compartment were recorded for 5 min. The behavior in the lit area was monitored by video camera and the resulting data were analyzed using the image processing system EthoVision XT8, analyzing distance moved and velocity. Between the different animals the apparatus was cleaned with 70 % ethanol.

2.4.5. Social interest and social memory

The apparatus (grey PVC, 50 cm × 50 cm) to assess socially induced motivation consisted of three compartments (each 16.5cm × 50cm) separated by plexiglas walls which are connected through squared openings at middle position. The testing procedure comprised three phases: acclimatization (5 min), social interest ('sociablity', 10 min) and social memory (10 min). Phase 1: acclimatization to explore the whole arena without further data collection. Phase 2: an unfamiliar mouse of the same sex ('stranger 1') was placed into a wireframe cage in the corner of one outer compartment. Into the opposite diametral corner at the other external compartment an empty wire frame cage was placed. Both wire cages were weight with a water filled mouse cage bottle to avoid climbing/sitting on the top and movements of the wire cage by the caged mouse. The behavioral analysis comprised the time spent in the compartment with either the empty or the caged mouse. Additionally, the time spent in close vicinity (cage – nose distance max. 1 cm) of the wire frame cages and sniffing towards or exploring it was measured. As control measure for general activity, the number of entries to each compartment were counted. Phase 3: the empty wire cage was exchanged with a wire cage containing a novel stranger to observe the ability to recognize the familiar versus the new, unfamiliar mouse ('stranger 2'). The entire test procedure was conducted at a light intensity of 25 lx.

2.4.6. Rotarod

The Rotarod apparatus (TSE, Bad Homburg, Germany) consisted of a rotating accelerating rod (5 − 60 rpm) separated by walls in several compartments. Mice were individually placed on the rod rotating with 5 rpm. After 30 s the rod accelerated continuously for the next 4 min 30 s to reach an end speed of 60 rpm. Mice were placed additional two times after falling down the first time. Complete time on the rod for all three trials were analyzed.

2.4.7. Fecal corticosterone metabolites (FCMs)

Before the behavioral assessment started in the 5th week, the fecal corticosterone metabolite levels were assessed to measure adrenocortical activity via a non-invasive technique (Palme, 2019). The cages were changed 1 − 2 h after beginning of dark phase and all fecal boli inside the group cage were collected 4 h later and frozen directly by − 20 °C. After all experiments were finished, fecal boli were dried at 75 °C for 4 h and homogenized thoroughly by hand. A methanol extraction (80 %) was conducted and the extracts stored at − 20 °C. Later they were analyzed using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay, which proved well suited to assess adrenocortical activity (FCMs) in fecal samples of mice (Touma et al., 2003, 2004; Resch et al., 2018).

2.5. Integration of the environmental enrichment in the cage infrastructure

Although the active usage of the environmental enrichment within the cage was not monitored, the application of the enrichment was assessed by observing the nest position within the cage. Nest position was either monitored as 'under the food rack', in the 'open corners' (not under the food rack), in the 'open area' or 'under the enrichment'. The scoring of nest position was carried out 3 days after the last cage change after the behavioral assessments.

2.6. Removal of cages due to aggression

In distinct cages of group II, aggressive behavior occurred in male mice, reflected by 1–3 conspecifics in the cage with wounds of tail/anogenital region and dorsal part of the body and therefore increased scores of the coat state. Because cages with aggressive behavior would possibly alter behavioral and physiological read-outs, the complete cages were removed from further analysis. This was the case for 4 of the control cages, 4 with the type of 'Bridge' enrichment, 3 with the type of 'Triangle' enrichment and 2 of the type 'Round Arch' (resulting number of male mice: control N = 16, 'Bridge' N = 20, 'Triangle' N = 16, 'Round Arch' N = 24).

2.7. Statistics

Statistical analyses were performed using the statistical program SPSS 25 for Mac (IBM). Inter-group comparisons were calculated by one-way ANOVAs, data comprising a time dependent development by ANOVAs for repeated measurements. Post-hoc analyses were performed with Dunnett's post-hoc tests comparing control mice with the three different types of intervention. Sex effects were not assessed, males and females were analyzed separately. Individual animals were considered as experimental units due to the aim of the study to confirm changes of data variance of the level of single data and not cage means in animals with versus without enrichment.

To assess potential effects of cage order within the rack or the testing order, week of testing, order within the cage (mouse number) or day time, these effects were assessed as co-variates to the statistical models applied but never had significant effects and were therefore not further discussed or analyzed.

To assess variation due to the different types of cage climbers and the control condition, the co-efficient of variation (CV) for each of the parameters based on individual (not cage-wise) based parameters was calculated by dividing the standard deviation by the mean.

3. Results

3.1. Naïve mice were highly interested in the new 'Cage Climbers'

Naïve mice were observed concerning their explorative drive towards the different enrichment types in an open field. During the 10 min acclimatization phase in the empty arena, male and female mice

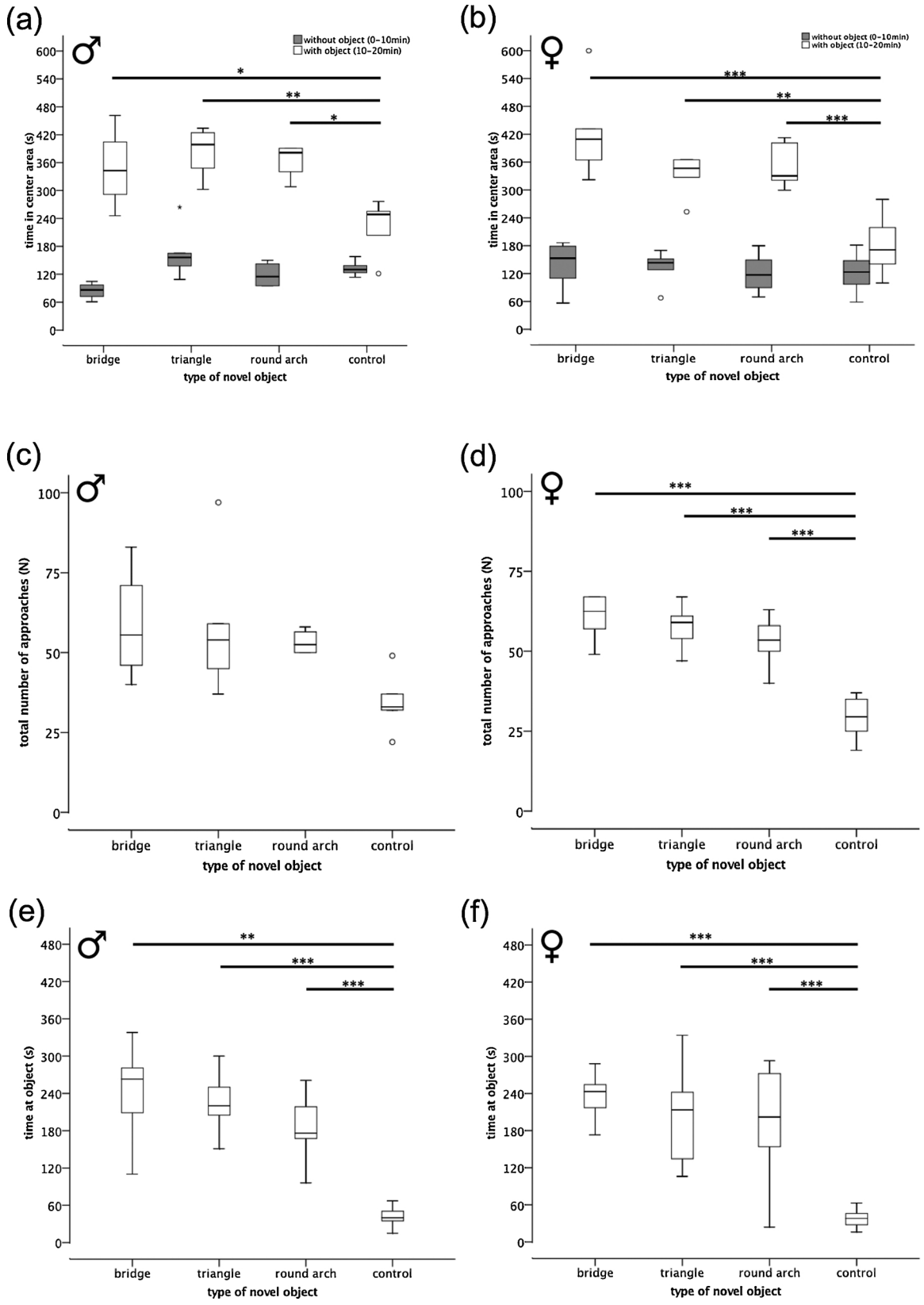

Fig. 2. Interest in the novel enrichment (or falcon tube as control object) in naïve mice in the Open Field: time in center area in males (a) and females (b), number of approaches to the novel object in males (c) and females (d) and time exploring the object in males (e) and females (f). Graphs are Box-plots depicting median (line in box), first and third quartile (box) and minimum and maximum of values (whisker) as well as outlier (dots or stars). Asterisk (*) depict the p-value of Dunnett's post-hoc tests in comparison to the control object: *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$, N = 6/sex/enrichment type.

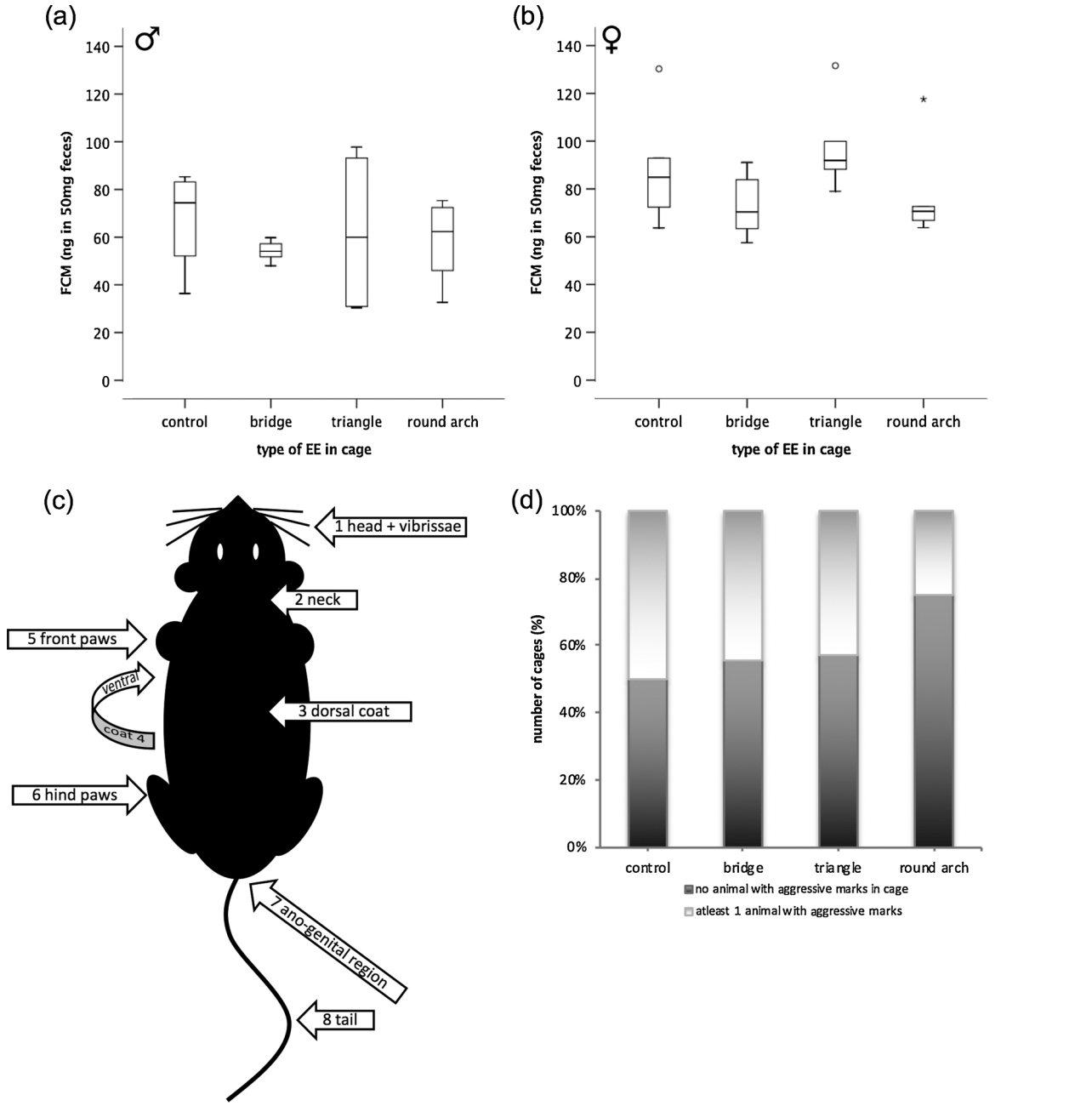

Fig. 3. Stress and aggression in the longitudinal study: Fecal corticosterone metabolites (FCMs) as a non-invasive parameter of adrenocortical activity in male (a) and female (b) mice (males: control N = 16, 'Bridge' N = 20, 'Triangle' N = 16, 'Round Arch' N = 24; females N = 24/enrichment type). (c) the graph depicts the scoring of the coat state with the 8 spots: 1 head incl. vibrissae, 2 neck, 3 dorsal coat, 4 ventral coat, 5 front paws, 6 hind paws, 7 ano-genital region, 8 tail. (d) Number of cages (%) with male mice with at least 1 positive score due to wound, scurf of bite in the regions 2,3,7,8 (indirect measure of aggression).

of the different groups (however naïve at this time-point) behaved comparably in their locomotor activity concerning moved distance, velocity and usage of spatial pattern. After introduction of either one type of the environmental enrichment or the control object (falcon tube), mice showed particular altered behavior depending on the type of object: Although the direct interest in exploring the object (latency) was comparable with all types of objects, the following behavior towards the objects was altered: Mice stayed significantly more in the center of the arena when confronted with any of the 3 enrichment types than mice confronted with the control object (Fig. 2a-b ANOVA: males: $F(3,14) = 6.623$ $p = 0.005$, post-hoc: control vs. 'Bridge' $p = 0.05$, vs. 'Triangle' $p = 0.007$, vs. 'Round Arch' $p = 0.025$; females: $F(3,20) = 14.490$ $p < 0.001$, post-hoc: control vs. 'Bridge' $p < 0.001$, vs. 'Triangle' $p = 0.004$, vs. 'Round Arch' $p = 0.001$) and the mean distance to the walls was consequently enhanced in the mice with environmental enrichment (males: ANOVA: $F(3,14) = 5.818$ $p = 0.008$; post-hoc: control vs. 'Triangle' $p = 0.011$, vs. 'Round Arch' $p = 0.040$; females ANOVA: $F(3,20) = 12.927$ $p < 0.001$; post-hoc: control vs. 'Bridge' $p < 0.001$, vs. 'Triangle' $p = 0.008$, vs. 'Round Arch' $p = 0.002$). Because center time and mean distance to the walls do not reflect directly the exploration of the novel object but more the time spent nearby, the time exploring the object and the number of object-approaches were analyzed: although the number of object-approaches only reached statistical significance in female mice, however the time exploring the object in both sexes depicted the statistically significant enhanced interest of the naïve mice in the enrichment objects in contrast to a control object (Fig. 2c–f, number of approaches: males: one-way ANOVA: ns, females: ANOVA $F(3,20) = 23.357$ $p < 0.001$, post-

Table 1a
Results of the behavioral analyses of the male mice kept 5 weeks with the environmental enrichment 'Bridge', 'Triangle' and 'Round Arch' in comparison to the control group (mean ± SEM). Preference at the Social Interest is calculated as (time at S1)/(time at S1 plus time at empty cage) and preference of the Social Memory Test as (time at S2)/(time at S2 plus time at S1). Control N = 16, 'Bridge' N = 20, 'Triangle' N = 16, 'Round Arch' N = 24.

Test	Parameter	control	bridge	triangle	round arch
Dark-Light Box	Latency (s)	83.63 ± 20.12	76.30 ± 14.09	59.50 ± 10.33	72.00 ± 15.97
	Exits (N)	7.00 ± 0.98	7.00 ± 0.68	7.25 ± 0.64	7.83 ± 0.71
	Time in lit (s)	107.37 ± 11.91	106.45 ± 10.63	102.50 ± 12.28	94.42 ± 9.08
Open Field	Total distance moved (cm)	4932.77 ± 203.50	4904.99 ± 239.25	4543.15 ± 116.39	4859.12 ± 130.61
	Velocity (cm/s)	8.29 ± 0.34	8.25 ± 0.40	7.66 ± 0.20	8.16 ± 0.22
	Time in center area (s)	138.04 ± 13.20	126.56 ± 9.89	139.71 ± 13.28	127.58 ± 6.77
Novel Object	Latency (s)	8.75 ± 1.62	12.80 ± 3.56	9.69 ± 1.66	10.79 ± 2.28
	Number of approaches (N)	26.88 ± 4.09	23.15 ± 2.45	31.31 ± 3.17	29.83 ± 3.22
Social Interest	time at cage of stranger 1 (pref)	0.52 ± 0.02	0.59 ± 0.02	0.56 ± 0.02	0.56 ± 0.02
Social Memory	time at cage of stranger 2 (pref)	0.61 ± 0.03	0.53 ± 0.03	0.64 ± 0.02	0.57 ± 0.02
Rotarod	Time on rod (s)	55.68 ± 5.24	56.83 ± 5.04	49.55 ± 5.21	45.53 ± 4.48

Table 1b
Results of the behavioral analyses of the female mice kept 5 weeks with the environmental enrichment 'Bridge', 'Triangle' and 'Round Arch' in comparison to the control group (mean ± SEM). Preference at the Social Interest is calculated as (time at S1)/(time at S1 plus time at empty cage) and preference of the Social Memory Test as (time at S2)/(time at S2 plus time at S1). N = 24/enrichment type.

Test	Parameter	control	bridge	triangle	round arch
Dark-Light Box	Latency (s)	39.30 ± 4.58	42.83 ± 7.15	28.46 ± 3.53	28.46 ± 3.58
	Exits (N)	9.57 ± 0.79	9.25 ± 0.68	10.00 ± 0.66	10.83 ± 0.80
	Time in lit (s)	101.48 ± 5.94	100.08 ± 7.05	106.29 ± 8.32	119.29 ± 8.78
Open Field	Total distance moved	5703.01 ± 141.69	5503.08 ± 105.58	5767.95 ± 172.10	5511.32 ± 180.90
	Velocity (cm/s)	9.59 ± 0.24	9.29 ± 0.19	9.72 ± 0.28	9.30 ± 0.30
	Time in center area (s)	120.60 ± 6.66	125.76 ± 9.42	134.94 ± 8.85	120.36 ± 8.12
Novel Object	Latency (s)	9.35 ± 2.17	8.79 ± 2.30	8.62 ± 1.81	11.17 ± 2.56
	Number of approaches (N)	35.91 ± 3.27	29.67 ± 2.54	36.04 ± 2.34	33.83 ± 3.66
Social Interest	Preference: time at cage of stranger 1	0.52 ± 0.02	0.57 ± 0.02	0.57 ± 0.02	0.55 ± 0.02
Social Memory	Preference: time at cage of stranger 2	0.59 ± 0.02	0.56 ± 0.03	0.60 ± 0.02	0.59 ± 0.02
Rotarod	Time on rod (s)	63.27 ± 4.49	50.26 ± 4.07	55.30 ± 4.73	54.08 ± 4.33

hoc: control vs. 'Bridge' $p < 0.001$, vs. 'Triangle' $p < 0.001$, vs. 'Round Arch' $p < 0.001$; time exploring object: males: ANOVA: $F(3,14) = 14.537$ $p < 0.001$, post-hoc: control vs. 'Bridge' $p = 0.002$, vs. 'Triangle' $p < 0.001$, vs. 'Round Arch' $p = 0.001$; females: ANOVA: $F(3,20) = 43.410$ $p < 0.001$, post-hoc control vs. 'Bridge' $p < 0.001$, vs. 'Triangle' $p < 0.001$, vs. 'Round Arch' $p < 0.001$).

3.2. The new 'Cage Climbers' did not affect bodyweight development or (an-) hedonia

All mice gained weight the 5th week of measurement after arrival, a normal effect during the age of 6–11 week (ANOVA factor time: male: $F(5,355) = 409.188$ $p < 0.001$, female: $F(5,455 = 735.522$ $p < 0.001$). Presence of 'Cage Climbers' did not reach statistical significance on bodyweight alteration in neither sex (factor EE: males: ns, females: ns). Sucrose preference was measured as hedonic state at arrival, after 2 and 4 weeks. Sucrose preference increased slightly over time in female but not in male mice (factor time: males: ns, females: $F(2,40) = 3.779$ $p = 0.031$), but presence or type of enrichment did not reach statistical significance (ANOVA factor EE: males: ns, females: ns).

The nest position gives an idea of spatial pattern usage in the cage and usage of the enrichment as (additional) resources or shelter. In control mice, both sexes placed nests under the food rack due to the absence of a second shelter-like area. In the experimental groups, in male mice still approximately 80 % of the nests of all 3 enrichment types were located under the food rack. In the female mice, 50 % of the cages of both the 'Triangle' and the 'Bridge' type build nests under the enrichment.

3.3. The new 'Cage Climbers' did not affect locomotion, neophobia, anxiety, social behavior or climbing

Several behavioral tests were conducted to evaluate changes of motor abilities like locomotion, balance and climbing and emotional features like exploration, anxiety and social interests as well as memory abilities (Tab. 1a males, 1b females, Tab 1c for statistical testing).

In the Open Field and the following Novel Object Test, environmental enrichment did not reach statistical significance regarding locomotion concerning total distance moved or velocity (Tab 1c). Time in center area increased significantly in all groups after introduction of the novel object (Table 1c). Consequently was the distance to the walls significantly enhanced during the Novel Object Test in mice of all groups (ANOVA factor time: males: $F(1,72) = 12.672$ $p = 0.001$; females $F(1,92) = 69.584$ $p < 0.001$) although environmental enrichment as factor possibly affecting distance to walls did not reach statistical significance (factor EE: males: ns; females ns). The number of fecal boli, representing an indicator for stressful events, was very low in both males and females, environmental enrichment as factor did not reach statistical significance (ANOVA males: ns, females: ns). Concerning the novel object, mice of all groups demonstrated comparable exploration of the object regarding latency to 1st approach (Table 1a–c, ,), as well as number of approaches (Table 1a–c).

The Dark-Light Box Test was used to assess anxious behavioral outcomes. Neither the latency to enter the lit area, nor the total number of exits, nor the time spent in the lit area exhibited statistically significant with any different environmental enrichment (Tab. 1a-c).

In the Sociability Test, mice were analyzed for interest in investigating a novel (caged) conspecific in comparison to a novel but empty wire cage. Independent of housing conditions, all mice preferred the conspecific ('stranger 1') in comparison to an empty cage, different environmental enrichments did not show statistical significance as an

Table 1c
Statistical outcome for male and female mice in the longitudinal study (5 weeks housing with enrichment): regarding the 'repeated measurement ANOVAs', the two factors *time* and *enrichment* are depicted, for the 'one-way ANOVA', the single factor *enrichment* is depicted. Males and females were analyzed separately. Males: Control N = 16, 'Bridge' N = 20, 'Triangle' N = 16, 'Round Arch' N = 24, females: N = 24/enrichment type.

Test	Parameter	rep. measurement ANOVA		one-way ANOVA	
		males	females	males	females
Dark-Light Box	Latency (s)			F(3,75) = 0.352 p = 0.788	F(3,94) = 2.266 p = 0.086
	Exits (N)			F(3,75) = 0.100 p = 0.960	F(3,94) = 0.772 p = 0.513
	Time in lit (s)			F(3,75) = 0.310 p = 0.818	F(3,94) = 0.884 p = 0.453
Open Field - Novel Object	Total distance moved (cm)	Time: F(1,72) = 0.014 p = 0.907	Time: F(1,91) = 57.473 p < 0.001 ***		
		Enrichment: F(3,72) = 0.555 p = 0.646	Enrichment: F(3,91) = 1.184 p = 0.320		
	Velocity (cm/s)	Time: F(1,72) = 0.107 p = 0.745	Time: F(1,91) = 64.396 p < 0.001 ***		
		Enrichment: F(3,72) = 0.550 p = 0.650	Enrichment: F(3,91) = 1.092 p = 0.357		
	Time in center area (s)	Time: F(1,72) = 14.305 p < 0.001 ***	Time: F(1,91) = 62.122 p < 0.001 ***		
		Enrichment: F(3,72) = 0.746 p = 0.528	Enrichment: F(3,91) = 0.868 p = 0.461		
Novel Object	Latency (s)			F(3,75) = 0.442 p = 0.723	F(3,94) = 0.276 p = 0.843
	Number of approaches (N)			F(3,75) = 1.235 p = 0.303	F(3,94) = 0.989 p = 0.401
Social Interest	Time at cage of stranger 1 (preference)			F(3,75) = 1.377 p = 0.257	F(3,94) = 1.389 p = 0.251
Social Memory	Time at cage of stranger 2 (preference)			F(3,75) = 2.652 p = 0.055	F(3,94) = 0.384 p = 0.765
Rotarod	Time on rod (s)			F(3,74) = 1.262 p = 0.294	F(3,94) = 1.511 p = 0.217

influencing factor (Table 1a–c).

In the subsequent Social Memory Test, the mice could choose to interact with either the familiarized conspecific ('stranger 1') or a new, unfamiliar conspecific ('stranger 2'). Independent of 'Cage Climbers' type, the mice spent more time with the unfamiliar mouse, although preference between the different groups did not reach statistical significance (Table 1a–c).

Rotarod performance was analyzed to gain insights into the (forced) balance and climbing abilities. The type of the environmental enrichment in the cage did not reach statistical significance as an influencing factor (Table 1a–c).

3.4. The new 'cage climbers' did not increase stress, but rather reduced aggression

Measuring adrenocortical activity non-invasively via fecal metabolite samples reached no statistical effects concerning corticosterone metabolites levels due to the different enrichment types in male or female mice (Fig. 3a-b; ANOVA males: ns, females: ns).

The coat state (Fig. 3c-d) was used as direct score of wellbeing and indirect measurement of aggressive behavior (scurf and bite wounds) of one or more animals per cage. Because we could not identify individual mice attacking each other, we counted cages with at least one animal with aggression scores (due to scurf, wounds) in the coat state as 'with aggressive marks'. This was exclusively the case in male mice, female mice never reached scores higher than 1, which was sometimes reached due to a reduced (but never a complete abundance) of the number of vibrissae. The number of cages with wounds due to aggressive attacks was related on the housing condition: while in control mice, 50 % of the cages were found with coat state scores due to aggression, all 'Cage Climber' types had the tendency to reduce this behavior. Although the effect was relatively low in the 'Bridge' (44.44 %) and the 'Triangle' type (42.86 %), the 'Round Arch' reduced this behavior to 25 % (Fig. 3d). This effect is unfortunately not statistically significant; an analysis using the Wilson Score Confidence Limits revealed no significant effects due to the low number of cases/cages.

3.5. The new 'cage climbers' did not increase data variability

To illustrate potential effects of the newly developed cage climbers on data variability, we calculated the coefficient of variation (CV) for parameters based on individual data (Fig. 4a,b). CVs differ to a large extent depending on the parameter with lowest range at the bodyweight measurements and highest range at the number of approaches to the novel object in both males and females. Independent of the type of enrichment, adding enrichment did never increase the CV of any of the measured outcomes (Fig. 4a-b).

4. Discussion

Here, we assessed the short- and long-term effects of a new type of mouse enrichment, i.e. 'Cage Climbers', which was presented to the animals in three different shapes: 'Triangle', 'Bridge' and 'Round Arch'.

While there were significant preferences for the unfamiliar structure when the animals were primarily exposed to it in an Open Field arena, none of the three 'Cage Climbers' evoked changes in weight development, motor features, anxiety, social behavior or fecal corticosterone metabolites in a longitudinal analysis of 5 weeks. This is somewhat surprising since we expected at least differences in locomotion and balancing due to the improved supply within the cage, which did neither occur in males nor in females. Also, the controversially debated increase of data variability (Wurbel, 2001, 2002; Wolfer et al., 2004; Bailoo et al., 2018) did not occur in our hands as statistically assessed and graphically illustrated with the Co-efficient of variation. Therefore large-dimensional use in animal facilities may be suggested as refinement without huge concern about potential side effects (e.g. aggression,

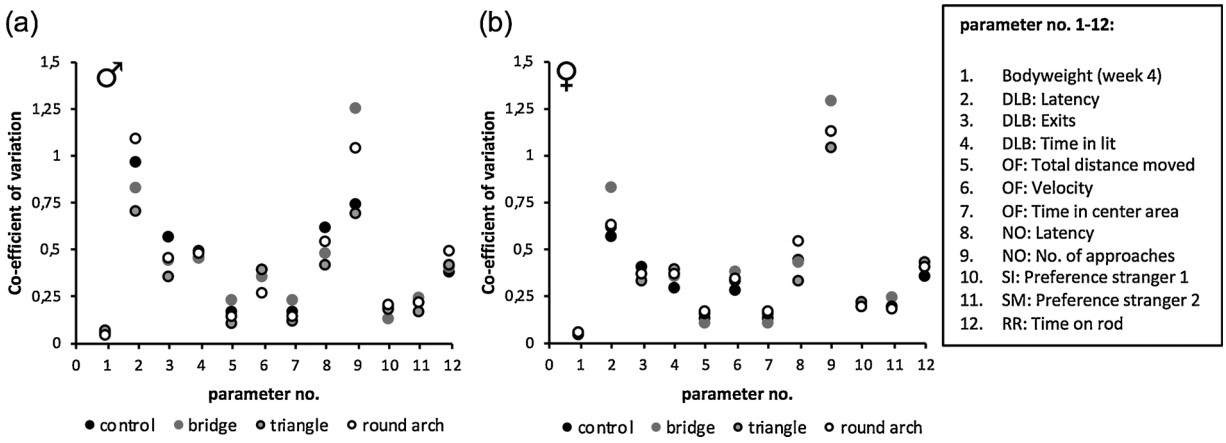

Fig. 4. Co-efficient s of variation estimates by measurements of different behavioral parameter based on individual data of (a) male and (b) female mice depending on enrichment type (males: control N = 16, 'Bridge' N = 20, 'Triangle' N = 16, 'Round Arch' N = 24; females N = 24/enrichment type).

induction of stereotypies, influence on data variability). Contrary, for experimental use in which an enriching factor is used as an instrument to induce behavioral changes (e.g. higher motor abilities), the 'Cage Climbers' may be questionable.

While the major focus of our study assessed the effects of the 'Cage Climbers' on behavior, stress physiology and variability of data, we included, although measuring indirectly, an additional aspect of housing, i.e. aggression, which occurred in some groups of males in all conditions. Male mice housed in groups demonstrate a non-linear despotic dominance hierarchy, with one dominant animal and a group of subordinates (Haemisch et al., 1994; Lee et al., 2017; Kunkel and Wang, 2018), representing a challenge for animal facilities, which want to address animal welfare issues (Holman et al., 2016) but also reduce holding cage numbers. Behavioral interactions in group-housed mice may furthermore lead to an increase in variance of certain parameters such as bodyweight (Nagy et al., 2002).

Considering the effects of our 'Cage Climbers', we realized a decrease of aggressive behavior up to 25 % ('Round Arch'). Including or excluding data from aggressive cages is an interesting aspect with regard to current discussions about the biometrical validity of *in vivo* studies (Holman et al., 2016). We assumed that aggression in the cage will affect data quality somehow (e.g. via increased variability due to stress) and excluded all cages in which at least one male mouse had one wound due to a bite. This strict approach decreases animal numbers to a large extent, but considers the potential psychological threat of not only being attacked but also be a witness of attacks, which can affect mice (Sial et al., 2016). Surprisingly, statistical effects were comparable when all mice were included, probably due to the effects of higher n-numbers (data not shown).

Our newly developed structures, independent of the detailed type of enrichment, offer an interesting and day-to-day-business appropriate approach to deal with our catalogue of requirements (Fig. 1d): By giving the opportunity to climb and use structures to enhance the possibility to use different compartments for e.g. locomotion, nest building or defecation, the biological needs are taken by animals of both sexes. The use of the 'Cage Climbers' with regard to handling, risk assessment and use by the mice, was highly advocated by the staff, who was in charge to score the new enrichment approach according to our catalogue of requirements. Addressing the need of animal facilities to organizationally emphasize a 'one-fits-all' way to enrich the cage environment of mice, one may assure a standardized housing protocol, that is preferred by the animals, may reduce aggression and represents a sustainable tool to create an enriched structure in mouse cages.

The evaluation of behaviour therefore refers only to the results of the behavioural test battery including group-pooled stress hormone levels. We also did not use different cage systems, which could create another context, e.g. IVC cages with an immense reduced cage lid area used for climbing or smaller cages than Macrolon type III. Despite of such limitations, we consider our findings important to sensitize researchers, caretakers and directors of animal facilities for sufficient evaluation of enrichment programs. Neither the direct cost of handling additional equipment nor anthropocentric viewpoints should count for the success (or failure) of any environmental enrichment for rodents. Sometimes, rather trivial-seeming structures may fulfil the needs of animals and caretaker staff without endangering reproducibility. Further studies are needed to evaluate the impact of 'Cage Climbers' in IVC cages with reduced size of the grid top, which could be compensated by additional structures to climb.

4.1. Conclusion

'Cage Climbers' are easy-to-handle, cost effective and from the animal welfare point effective environmental enrichment when using C57BL/6 N mice of both sexes. With the data on hand, we recommend an application of any type of 'Cage Climbers' in holding cages of both experimental and, additionally, breeding cages due to the fact that the animals' visibility does not interfere daily adspective controls. Data quality assessed by variance and behavioural measures of the analysed behavioural outcomes *per se* are not altered, which would bear a risk of using such kind of enrichment. Although the potential to shift stereotypic behaviour back to normal behavioural patterns cannot be judged, housing male mice from an age around puberty on with 'Cage Climbers' of the 'Round Arch' type had in our experimental settings the potential to, at a small degree, reduce aggressive behaviour. Application, beyond our evaluation of 'Cage Climbers' at the level of a larger scale in mouse husbandry, also including effects on IVC housing focusing on different mouse strains of both sexes, definitely needs to be addressed to confirm the potential of our approach. Every mouse counts.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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