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Effect of a partial cage dividing enrichment on aggression-associated parameters in group-housed male C57BL/6NCrl mice

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ABSTRACT

Group-housing is highly important for social animals. Group-housing of male mice in captivity though often leads to aggression with partially disastrous consequences for the animals as well as for the quality of experimental data. In this study we investigated the effect of a novel "cross-enrichment", i.e. a colored partial cage divider, which is provided in transparent or black and which is partly separating the cage in four small areas. Group-housed male C57BL/6NCrl mice (three per cage) were maintained under either standard conditions (nestlet group) or enriched conditions (nestlet + cage divider in black [EB-group] or in transparent [ET-group]) for eight weeks. Several physiological parameters (body weight, blood glucose, stress induced hyperthermia, fecal corticosterone metabolites and organ weights) and behavioral tests (Nest test, Openfield/social Novel-Object, Dark-Light-Box, Hotplate and Resident-Intruder test) were measured/performed to determine enrichment-induced effects. In comparison to nestlet- and ET-group animals, EB-mice showed significant increased stress-associated parameters, i.e. in the blood glucose concentration. Furthermore, EB animals seemed to have enhanced emotional stress with a poorer outcome in the nest test and a higher amount of fecal boli at the end of the social Novel-Object test. Additionally, EB-mice behaved more aggressively towards conspecifics after cleaning cages. We conclude that the opacity of the tested partial cage dividers has a huge impact on aggressive behavior and therefore may lead to significant changes in behavioral and physical measures potentially altering research outcomes.

1. Introduction

Aggression in group-housed laboratory male mice is a problem concerning not just animal welfare but also quality of experimental data. If conventional strategies for aggression limitation fail, the only possibility to prevent critical traumatization between the animals can be animal separation and individual housing, raising housing costs and human resources. However, for a highly social species, such as mice, this housing form is not sustainable because also male mice prefer the proximity of another conspecific to individual housing (Van Loo et al., 2001). As mice are the most commonly used species for biomedical research, researchers and facility managers are looking desperately for a solution to solve the problem of aggression and its resulting consequences, so far in vain (Weber et al., 2017).

In the wild, aggressive behavior of the species *Mus musculus* is part of their social organization, which varies depending on local resource availability (Latham and Mason, 2004; Gray et al., 2002). Male mice social organization has been studied in semi-natural bawns and differs between i) individual males defending established territories, to ii) groups of males living in the same area and exhibit dominant-subordinate relationships and to iii) males defending territories while other males -not owning a territory- co-exist peacefully between defended territories (Wolff, 1985).

Under laboratory conditions federal guidelines regulate the cage size and density. Up to four mice < 30 g body weight and 3 mice > 30 g body weight are allowed in a standard type II cage of 370 cm^2 and 12 cm height. This standard cage, even if it satisfies the legal norms, does not offer sufficient opportunities to show innate natural behavior such as exploration, burrowing or hiding and might especially be disadvantageous for male mice, where social dominance is inherent and escape behavior cannot be performed (Tallent et al., 2018). Furthermore, the suppression of the innate behavior may increase aggression

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from the dominant mouse. This does not only affect physiological, but also psychological welfare of all cage mates, especially pertaining to the subordinate mouse, which might experience pain and distress caused by injuries (Council, 1992). An aggressive interaction is typically started by a thrust behavior (i.e. tail rattling, thrust) of the dominant companion and is followed by an aggressive behavior (i.e. attack, bite) if the subordinate mouse does not react appropriately (i.e. submissive upright, fleeing) (Joseph Garner et al., 2018). Such an adequate reaction though is not always possible under laboratory housing conditions as a non-enriched cage does provide only limited means of escape, leaving the subordinate male almost unprotected. Consequently, the dominant male gets separated to prevent further damage and the victim suffers from either isolation stress or its injuries and might even end up dead.

One approach to solve the problem of aggression is by providing environmental enrichment, which has been defined as “an improvement in the biological function of captive animals resulting from modifications to their environment” (Newberry, 1995). As a result of increased public and regulatory pressure, the improvement of animal welfare is no longer only on the basis of standardizing housing to minimize variability of experimental outcomes. Previous research verified enrichment to enable mice to interact with and partially control their surrounding by manipulating the device, which has a positive impact on their stress level (Wiepkema and Koolhaas, 1993). Subsequently structural enrichment is provided by most facilities (Hutchinson et al., 2005) and several different forms of them exist on the market (Howerton et al., 2008). However, most refinement efforts for laboratory rodent husbandry are evaluated from an anthropomorphic point of view non-regarding physiological parameters and the biological relevance for the animals. Environmental enrichment may not only influence animal’s behavior, but also might affect physiological parameters (i.e. body temperature, glucose, immune system) as outlined before (Meijer et al., 2006; Haemisch et al., 1994; Kingston and Hoffman-Goetz, 1996). Additionally, strain- and sex-specific effects associated to the added cage enrichment can occur (Nevison et al., 1999; Van de Weerd et al., 1994; Martínez-Cué et al., 2002; Tsai et al., 2003; Bayne, 2005; Tsai et al., 2006). Therefore an evaluation of each form of enrichment is necessary to prove their beneficial effect on mice (Benefiel et al., 2005). Weber et al. published a review paper summarizing all methods to cope with aggression in group-housed male mice to date (Weber et al., 2017). Aggression is a complex field and affected by many factors. For example an enrichment which is valued by and advantageous for individually-housed mice could have the opposite effect in group-housed animals as it may become a defensible resource, increasing aggressive behavior (Howerton et al., 2008). A review of the literature reveals few behavioral investigations of the effects of environmental enrichment on aggression in mice and the results have generally been inconsistent. Some demonstrating an increase (Barnard et al., 1996; Haemisch and Gartner, 1994, 1997; Henderson, 1976; Marashi et al., 2003; Tsai et al., 2002; Van de Weerd et al., 2004; Bergmann et al., 1995) others a reduction (Ambrose and Morton, 2000; Van Loo et al., 2002; Vestal and Schnell, 1986; Belz et al., 2003) and still others no effect regarding aggressive behavior (Haemisch and Gartner, 1997; Marashi et al., 2003; Van Loo et al., 2002, 2004; Van Loo et al., 2003; Van der Meer et al., 2004).

So far the only well-established enrichment known to decrease aggression is nesting material: Mice spend over 60 % of their wake phase (Van de Weerd et al., 1997a) with the provided material, building nests which allows them to thermoregulate in a surrounding where ambient temperatures are set below the mice’s thermoneutral zone (Van Loo et al., 2003; Van de Weerd et al., 1997b). Also transferring old nesting material at cage changing is recommended to minimize aggression. With the Appendix A of the European Convention of the Council of Europe (ETS 123) coming into force, nesting material therefore became nearly indispensable.

Structural enrichments, such as shelters, had a mixed outcome regarding aggression with studies reporting both increases and decreases (Olsson and Dahlborn, 2002). However, studies explicitly focusing on cage dividers are rare and only one so far evaluated such a type of enrichment with respect to aggressive behavior: Talent et al. (Tallent et al., 2018) tested 18 male Balb/c mice, which were assigned at an age of eight weeks in groups of three and put either to a standard or a divided cage. The cage divider created a three-burrow partition and mice behavior was recorded on day one, two and seven. Findings indicated a significant decrease in events of aggressive behaviors, both in the light and dark cycles.

The present study was conducted to evaluate the suitability of a novel partial cage dividing enrichment to reduce stress- and aggressive-related parameters. Studies in group-housed males, in which an enrichment allowed one mouse to gain control over it led to an increase in the variance of the collected experimental data (Gärtner, 1999). Therefore, the invented cage divider enables no exclusive control of the device. Up to four mice < 30 g can be held in a Macrolon type II cage and the created cross-enrichment, which consisted of equal chambers with an area of 33 cm^2 each, allowed all animals to use it contemporaneously. Besides that, it is known that mice do prefer specific cage opacities over others (Sherwin and Glen, 2003). With no study investigating the effect of different colors of cage dividers on aggression-associated parameters, we custom-designed the enrichment in two different colors, one translucently for mice (Enrichment transparent, ET), the other one opaque for mice (Enrichment black, EB). In a previous experiment, in which the effect of different handling forms on the aggression behavior of C57BL/6NCrl mice was investigated, it was found that only the handling of the test animals with forceps had a negative effect on the aggression behavior (compared to tube and hand handling, (Mertens et al., 2019)). Therefore, all handling procedures were performed by hand.

We tested the hypothesis if both enrichment items, independent of their opacities, would significantly decrease aggressive behavior in group housed male mice compared to mice housed in a standard, non-divided cage.

2. Materials and methods

2.1. Ethics statement

The study was conducted according to the guidelines of the German Animal Welfare Act and was approved by the Karlsruhe State Authority (project licence number: G-154/17).

2.2. Animals

Male mice (n = 54) of the inbred strain C57BL/6NCrl were obtained from Charles River Laboratories at the age of three weeks. The animals were maintained under standard laboratory conditions (reversed 12 h light/ 12 h dark cycle, 22 ± 2 °C, 55 ± 10 % humidity, aspen wood bedding (ABEDD LTE-001, Lab & Vet Service, Vienna, Austria)), provided with pelleted food (Rod 16-A LasVendi, Soest, Germany)) and access to water *ad libitum* throughout the whole experimental. A specific pathogen-free (SPF) hygienical status according to Federation of European Laboratory Animal Science Associations (FELASA) recommendations was given (rodents, F.w.g.o.r.o.g.f.h.m.o. et al., 2014). Mice of the inbred strain C3H/HeJ were obtained from Janvier Laboratories (Laval, France), to serve as interaction partners for 2 behavioral tests. They were selected due to its brown color (easier to distinguish from the black C57BL/6NCrl mice in the RIT) and because of their even tempers (personal communication with commercial breeders). They were housed under the same abiotic conditions as the experimental mice, but in a separate room (no reversed light/dark cycle) to prevent an impact on the experiment by the influence of odor particles.

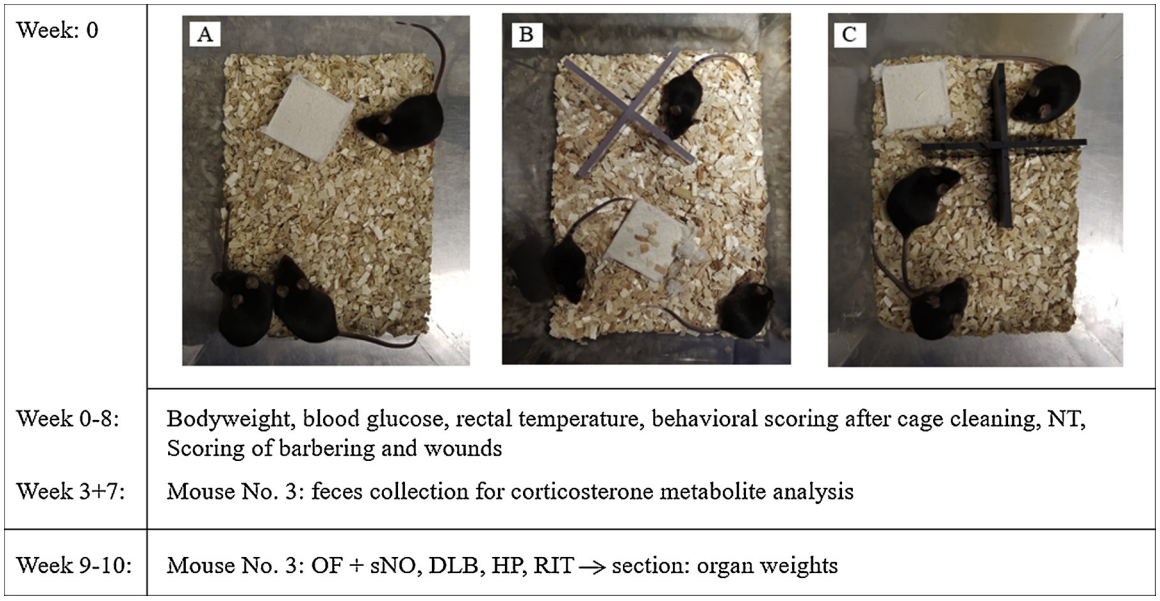

Fig. 1. Study design.
Mice were housed on arrival (week 0) in groups (n = 3) in a standard cage (A), with a transparent enrichment (B) or with a black enrichment (C). Measurements of clinical parameters were conducted from week 0–8, followed by behavioral testing (week 9–10) and sections for organ weight assessment. NT = Nest test, OF = Openfield, sNO = social Novel-Object test, DLB = Dark-Light-Box test, HP = Hotplate test, RIT = Resident-Intruder test

2.3. Study design

The Experimental setup was performed as illustrated in Fig. 1. All experiments and measurements were performed during the dark phase and the animals were handled by hand.

Upon arrival the 54 animals were randomly allocated into three different housing conditions of groups of n = 3 per cage. A total of 18 mice were maintained in standard housing conditions serving as controls (C), only provided with a cotton nesting pad and 36 mice in enriched housing conditions (18 mice housed with a transparent enrichment (ET) and 18 mice housed with a black enrichment (EB)). Mice were marked by ear punches (1–3) and additionally for easy differentiation on their tails (mark on the tail refreshed weekly). The average body weight of the animals, measured the first time on week 1 was 10.62 g (Control =10.40 g; ET =11.14 g; EB =10.33 g; SD = 1.27, p = 0.1). Only one initially randomly assigned mouse per cage (number 3) was used for the behavioral testing battery in weeks 9–10 and the feces collection. The aspen wood bedding and additional enrichment of all cages (Makrolon type II cages, 370 cm^2, Tecniplast, Milan, Italy) were changed on a seven day cycle with a small portion (± 0.5 g) of old nesting material transferred with the mice and a fresh nestlet added to the new cage.

Mice were housed in respective conditions for ten weeks and cage cleaning, behavioral scoring after cage cleaning plus the nest test (NT) were performed once a week between week one to eight. On the following day the clinical parameters blood glucose, body weight ant the rectal temperature were measured. For feces collection the mouse was placed on an empty Macrolon Typ II cage in the third and 7^{th} week, fecal boli for fecal corticosterone metabolite (FCM) measurements were collected and the animal relocated in its home cage. Furthermore, all procedures were performed by a female researcher.

2.3.1. Physiological parameters

The animals were weighed weekly for the duration of the experiment. Each mouse was picked up individually by hand and transferred to an empty Macrolon Type II cage, which was wiped with 70 % ethanol. The animals were then placed on the grid of a bedded cage and their coat status checked for signs of barbering or bite wounds.

Thereafter the blood glucose was measured: Blood was sampled from the tail vein by puncture and the blood glucose measured using an automatic glucose meter (Medisana ® MediTouch 2, Promed GmbH, Germany). FAD-binding glucose-dehydrogenase converts the glucose in the blood to glucoconolactone, which is measured by the device and which is in proportion to the blood glucose volume. Levels of glucose were compared within the groups to estimate an effect of stress.

After blood drop collection, a rectal thermometer was inserted to test for stress-induced hyperthermia. For this purpose, a thermistor probe was inserted 1 cm deep into the rectum of the mice (Testo 108, Testo SE & Co. KGaA, Lenzkirch, Germany + MLT1404 Rectal Probe, ADInstruments Ltd, Oxford, United Kingdom), after dipping it into a lubricant. The first rectal temperature measurement (T_1) was followed by a second temperature measurement (T_2) 30 min later. The difference ΔT ($=T_2-T_1$) is the stress-induced hyperthermia (Van der Heyden et al., 1997).

In order to analyze fecal corticosterone metabolites (FCM) fecal samples of mouse number three were collected in the third and seventh week of housing. Samples were taken by placing the mouse in a separate empty cage for approximately 45 min (14.00–14.45 h), fecal boli were collected and the mouse replaced in its home cage. FCM were extracted according to the method described by (Palme et al. (2013)). In brief, each sample was homogenized and an aliquot of 0.05 *g* was shaken for 5 min by hand with 1 ml of 80 % methanol (if less feces was available proportionally less methanol was used). After centrifugation the supernatant was frozen at −20 °C until analysis. The samples were analyzed using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay as described and validated for mice by (Touma et al. (2004), 2003).

As an index of the degree of stress, which may arise from sub-optimal housing conditions, we final weighed the thymus, spleen, both adrenal glands, both vesicular seminales and both testes of the mice. Under deep anesthesia induced with ketamine (195 mg/kg i.p., Bremer Pharma GmbH, Warburg) and xylazine (30 mg/kg i.p., Ecuphar GmbH, Greifswald) the organs were dissected, weighted and the weight set in relation to the body weight.

2.3.2. Behavioral parameters

The acclimatization time before the behavioral testing was at least 25 min. Between two tests a pause of 24 h was abided. Additionally,

animals were tested in the experiments ranked as less stressful, following earlier recommendations for repetitive behavioral testing (Maier, 2001; Chourbaji et al., 2008a; McIlwain et al., 2001).

2.3.2.1. Behavioral parameters tested week 0–8. Each week a nest test was performed. Nest building behavior is an indicator of well-being, as the behavior is reduced by pain and stress (Jirkof, 2014). After changing the bedding, the mice received approximately 0.5 g of the old nesting material as well as a new cotton nest pad (Plexx B.V, AB Elst, The Netherlands). During the test the cage divider remained in the cage all the time. The nests were then scored after 5 h and 24 h by using a modified protocol developed by Deacon on a 6-point scale (1 = nestlet untouched, 2 = more than 90 % of the nestlet intact; 3 = < 50 % intact; 4 = identifiable, but flat nest, 5 = nearly perfect nest, more than 90 % shredded, less than 50 % of its circumference is higher than mouse body height when curled up; 6 = perfect nest, more than 50 % of its circumference is higher than mouse body height when curled up).

Between the change of bedding and the 5 h scoring, the animals were filmed to analyze the behavior of the animals after the transfer in regard to aggression. Cage cleaning disrupts odor cues, which are emanated from the body, deposited on the bedding largely by mice's urine, and mediate aggression between mice (Gray and Hurst, 1995). Therefore, the mice were recorded for 20 min under light illuminated conditions (35 lux) and the behavioral interactions i) latency to the first attack, ii) duration of the first attack and iii) total amount of attacks were analyzed.

2.3.2.2. Behavioral parameters tested week 9 - 10. A series of behavioral tests were carried out after two months of keeping the animals in the different conditions in the following order: Openfield test (OF) test combined with a social novel-Object test (sNOT), Dark-Light-Box test (DLB), hotplate test (HP) and a Resident intruder Test (RIT).

Mice were subjected to an Openfield (OF) test between 10.30 and 11.50 a.m. on two consecutive days to monitor their exploration, activity and anxiety (Chourbaji et al., 2008b). They were placed in the center of the OF on an infrared light surface. Light intensity during testing was 25 lux floor level (Domanskyi et al., 2011). The test was combined with a sNOT to investigate for exploratory-, neophobic- and social behavior towards an unfamiliar male mouse (C3H/HeJ strain). The C3H/HeJ mouse, sitting in a metal cage (7 cm x 7 cm x 8 cm), was placed in the middle of the OF wherefore the test is named social Novel-Object (sNO) test. Each test was recorded with a camera-videosystem (Ikegami Digital) for 10 min and mice's movement tracked using a tracking software (Ethovision, Noldus Information Technologies, Wageningen, The Netherlands). In both parts of the OF test several parameters were measured, i.e. distance moved, time in center or the velocity. Additionally, while testing for social interaction with the new object parameters like 'latency to the sNO', 'frequency of visiting the sNO' and 'time spend at the sNO' were evaluated. In the end of the testing period, the number of fecal boli was counted as an indicator of emotionality. In between animal changes the apparatuses were cleaned with 70 % ethanol.

A Dark-Light-Box (DLB) test was conducted to measure anxiety-like behavior (Chourbaji et al., 2008b), as aggression provokes the development of anxiety in male mice (Kudryavtseva et al., 2002). The DLB consisted of an arena partitioned into two compartments, a dark (approximately 1 lux) and a lit compartment (600 lux), connected by a small entry. Animals were placed in in the dark compartment and recorded for 5 min by a video camera (Ikegami Digital) positioned overhead. The latency until entering, the number of entries into- and the time spent in the lit compartment was scored. Furthermore, the number of fecal boli was counted at the end of each trial and the arena cleaned with 70 % ethanol.

Enrichment may alter pain sensitivity (Pham et al., 2010), wherefore a Hotplate (HP) test was performed using an electronically controlled hot plate (Ugo Basile Hot/Cold Plate 35100, Ugo Basile, Gemonio, Italy) heated to 53 °C (± 0.1 °C). Latency until hind paw flinching or licking movements occurred was measured and the animal immediately removed from the hot plate if the behavior was presented. Cut off time was set at 45 s to avoid tissue damage (Chourbaji et al., 2005).

The last behavior test performed, the Resident-Intruder test (RIT), is based on the territorial behavior against unfamiliar intruding conspecifics. Prior to testing, the mice bedding was left unchanged for 12 days. Two mice were set out of the cage and the remaining resident was confronted in its home cage by an unfamiliar, lighter and smaller intruder male of the C3H/HeJ strain for 10 min. Behavioral interactions during each confrontation were recorded by a camera from above (Ikegami Digital) and subsequently scored. Thrust (tail rattling, thrust, mounting) and aggressive behavior (boxing, attack latency, aggressive bite, attack, fighting, chase) were analyzed following a previously published mouse ethogram (Joseph Garner et al., 2018).

2.4. Statistical analysis

All statistical analyses were performed using SPSS 26.0 for Mac (IBM). Due to the exploratory character of the experiment, P-values are to be interpreted only descriptively, thus no formal adjustment for multiple testing was performed. P-values smaller than 0.05 were considered to be statistically significant. If our data was normally distributed, we used a one- way ANOVA followed by Tukey post hoc testing. If not, we used nonparametric statistical tests, i.e. the Kruskal-Wallis test or the Mann-Whitney-U-test. Body weight and blood glucose were analyzed by using a repeated measurement ANOVA. Additionally, we calculated the standard deviation of all values, serving as a measure of variation observed in the data

2.5. Cross-Enrichment

The enrichment was hand-fabricated from either Polycarbonate (transparent) or, due to availability, recycled Polycabonate (black) and designed to fit in a Macrolon Typ II cage. Composed of two parts, each piece had the dimension of 12 × 5 × 0.5 cm with a centered pit (0.5 × 2.5 cm) and could be easily combined in the middle, creating four equal compartments **(**Fig. 1**).** Total floor space was unaffected by the addition of the partial cage divider and daily observation and health checks easily feasible.

3. Results

Table 1 illustrates all conducted tests with significant and non-significant results.

3.1. Clinical parameters

Presence of the enrichment did not alter body weight of male mice compared to mice housed in standard cages when analyzed with a repeated measures ANOVA. Furthermore, the enrichment opacity had no effect on the course of body weight. No barbering or biting wounds were noted during the experiment.

Defined as a temperature difference $\Delta T > 0.5$ °C, the stress induced hyperthermia was evaluated for each animal from week 3–8. No significant difference measured by one way ANOVA occurred between enriched and non-enriched groups or opacity-differing enrichments. Additionally, chi-square statistic in the last week of housing did not reveal any difference.

The blood glucose level was analyzed by performing a repeated measures ANOVA between week 3 and 8. The additional enrichment had no effect on the course of blood glucose level compared to the standard cage, whereas the opacity of the enrichment did altered the blood glucose level with higher glucose level in EB compared to ET mice ($F_{(1,10)} = 5.881$, $p = 0.036$, Fig. 2**)**. Furthermore, in the third and

Table 1
Statistical results concerning the factors 'enrichment' and 'opacity'.

Test	Parameter	Mean values			Effects		
		Control	ET	EB	Control vs. ET vs. EB	Enrichment (Control vs. both enriched cages)	Opacity (ET vs. EB)
Clinical parameters	Blood glucose						
	glucose, week 3 (mmol/L)	131.5	111.5	132	n.s., p = 0.08	n.s., p = 0.3	**a**
	glucose, week 3–8 (mmol/L)	114.06	106.64	113.58	n.s., p = 0.06	n.s., p = 0.4	**a**
Behavioral parameters	Behavioral scoring of aggression-associated parameters						
	Number of attacks, week 4–8 (n)	0	0	6	**c**	**a**	**b**
	Nest test						
	quality, week 5, 24 h scores	3.83	5	3.33	**a**	n.s., p = 0.62	**a**
	quality, week 6, 24 h scores	4.33	5.33	4	n.s., p = 0.1	n.s., p = 0.78	**a**
	quality, week 3–8, 5 h scores	3.17	2.86	2.61	**c**	no enrichment: **a** enriched cages: **b**	ET: **b** EB: n.s., p = 0.3
	quality, week 3–8, 24 h scores	4.72	4.89	4.19	**b**	no enrichment: n.s., p = 0.06 enriched cages: n.s., p = 0.072	ET: n.s, p = 0.18 EB: n.s., p = 0.1

Identification of the effects of the factors 'enrichment' and 'opacity'. Different letters show significant differences (a: $p \leq 0.05$; b: $p \leq 0.01$; c: $p \leq 0.001$). n.s. = no significance. Data is split in 'clinical parameters' and 'behavioral parameters' for better overview. Non-parametric testing is highlighted in grey. The order of the data presented in the categories reflects the order of the tests as they were conducted. Parameter not appearing in this table revealed non-significant results.

regarding a tendency in the 6th week, analyzed with a one-way ANOVA, the devices opacity influenced the blood glucose level ($F_{(1,10)} = 7.3$, $p = 0.022$; $F_{(1,10)} = 4.54$, $p = 0.059$), which also shows in the 6th week in a tendency comparing enriched and non-enriched cages.

The FCM did not significantly differ neither between enriched and non-enriched nor between ET nor EB-enriched mice when analyzed with a one-way ANOVA in respect to the baseline value (Fig. 2). A repeated measurement ANOVA revealed a tendency for the factor 'opacity' ($F_{(1,10)} = 4.35$, $p = 0.064$) with higher values for EB mice.

All organs were removed by the same animal technician to reduce an unwanted effect and the organ weights analyzed corrected for final body weight. Again, just a trend regarding the factor opacity was detected (spleen, $F_{(1,10)} = 4.665$: $p = 0.056$; testis right, $F_{(1,10)} = 3.748$, $p = 0.082$), with higher data outcomes for EB mice (Fig. 2).

3.2. Behavioral parameters

3.2.1. Behavioral parameters, week 0–8

All mice improve their nest building performance between week three and eight after 5 h and 24 h, analyzed by the Friedman-test. However, only non-enriched and ET mice do so significantly after 5 h. Furthermore after 24 h only C-housed mice show a tendency in preforming better over the time (Chi^2 (2) = 10.6, $p = 0.06$). Kruskal-Wallis-Analysis did reveal a significant overall effect in the 24 h nest scores in week 5 when comparing all 3 housing conditions (Chi^2 (2) = 6.533, $p = 0.038$). Specified with the Mann-Whitney-U-test an effect in the 24 h scores in week 5 (U = 4, $p = 0.026$) and in week 6 (U = 4, $p = 0.026$) for the parameter opacity occurred (Fig. 3).

Analyzing the behavior after cage cleaning from week four on (starting point was set here as the first aggressive interaction was observed in week four) a significant overall effect for the occurrence of attacks in the different housing conditions was seen (Chi^2 (2) = 18.202, $p < 0.001$, Fig. 3). From the 90 total cases, (18 animals observed over 5 weeks), nine times aggressive behavior was seen, all in EB mice. In detail, the factor 'enrichment' (Chi^2 (1) = 4.551, $p = 0.033$) as well as the factor 'opacity' (Chi^2 (1) = 9.153, $p = 0.002$) had a significant impact on the behavioral outcome.

3.2.2. Behavioral parameters, week 9–10

The OF measures anxiety-like behavior as mice naturally prefer to be near a protective wall rather than being exposed to danger out in the open (Christakis et al., 2012). All mice did not show any difference in the measured parameter. After the following sNO test, differences in the mean values of the counted 'fecal boli' were visible, which, however, turned out not be statistically significant comparing the enrichments opacities ($F_{(1,10)} = 4.187$, $p = 0.068$, Fig. 3).

DLB testing did not uncover any anxiety-related differences. Regarding variation differences an effect of enrichment was found (no enrichment, 0.5 ± 1.23; enrichment, 1.75 ± 2.56; C, 0.5 ± 1.23; ET, 2.17 ± 3.06; EB, 1.33 ± 2.16; mean ± SD). Also, the housing condition did not affect the response in the hotplate.

Among all 18 mice enrolled in the RIT, only two mice acted aggressive towards the intruder within 10 min testing time. Both were housed under EB conditions. Chi-square statistical analysis revealed no significant difference within the groups.

4. Discussion

The results show that the male C57BL/6NCrl mice were more aggressive when housed under EB conditions, compared to when housed under C or ET conditions. Partial cage division per se did therefore not decrease aggressive behavior within male mice, contrary to the findings by Tallent et al. (Tallent et al., 2018) and to our expectations. We previously hypothesized that the animals housed under enriched conditions would be less aggressive. The enrichment structures the cage and offers an escaping possibility; hence we assumed the mice to cope

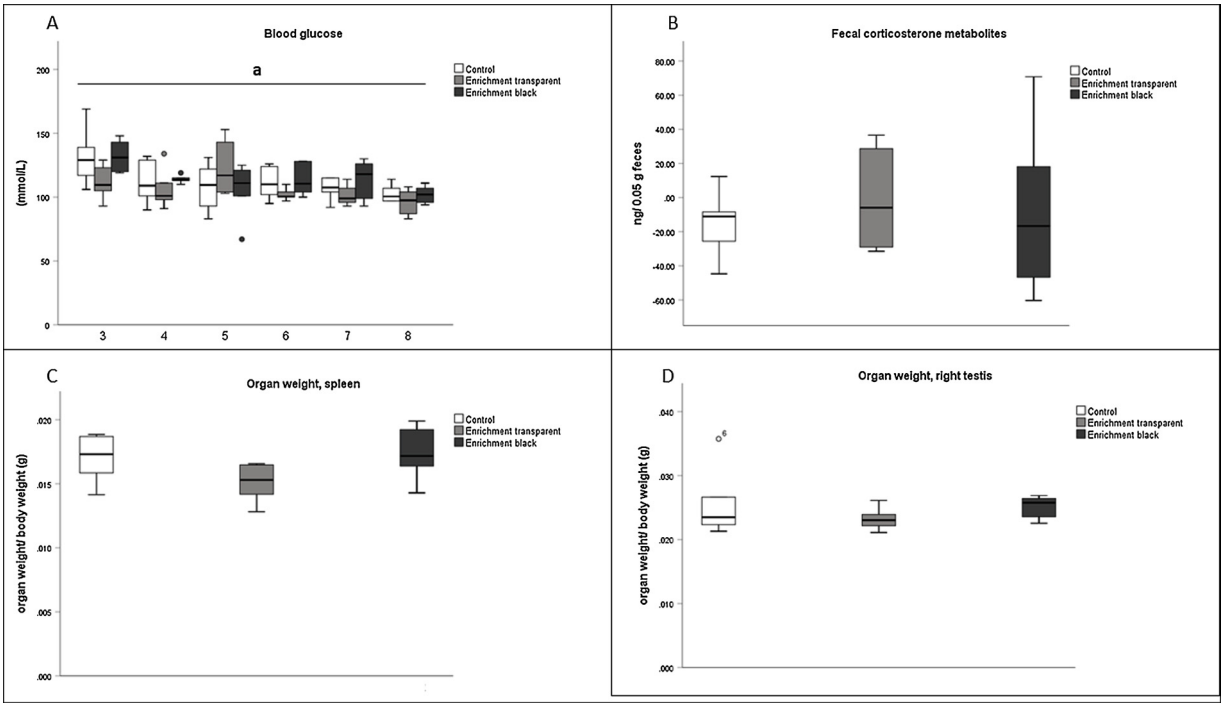

Fig. 2. Physiological analysis.
A) Blood glucose: Evaluation of stress-induced hyperglycemia revealed significant opacity differences between EB- and ET mice. B) Fecal corticosterone metabolites: No opacity effect comparing data values in respect to the baseline value were detected. C–D) Organ weights: Right testis and spleen of EB-mice were heavier than in ET-housed animals. Different letters show significant differences (a: $p \leq 0.05$; b: $p \leq 0.01$; c: $p \leq 0.001$).

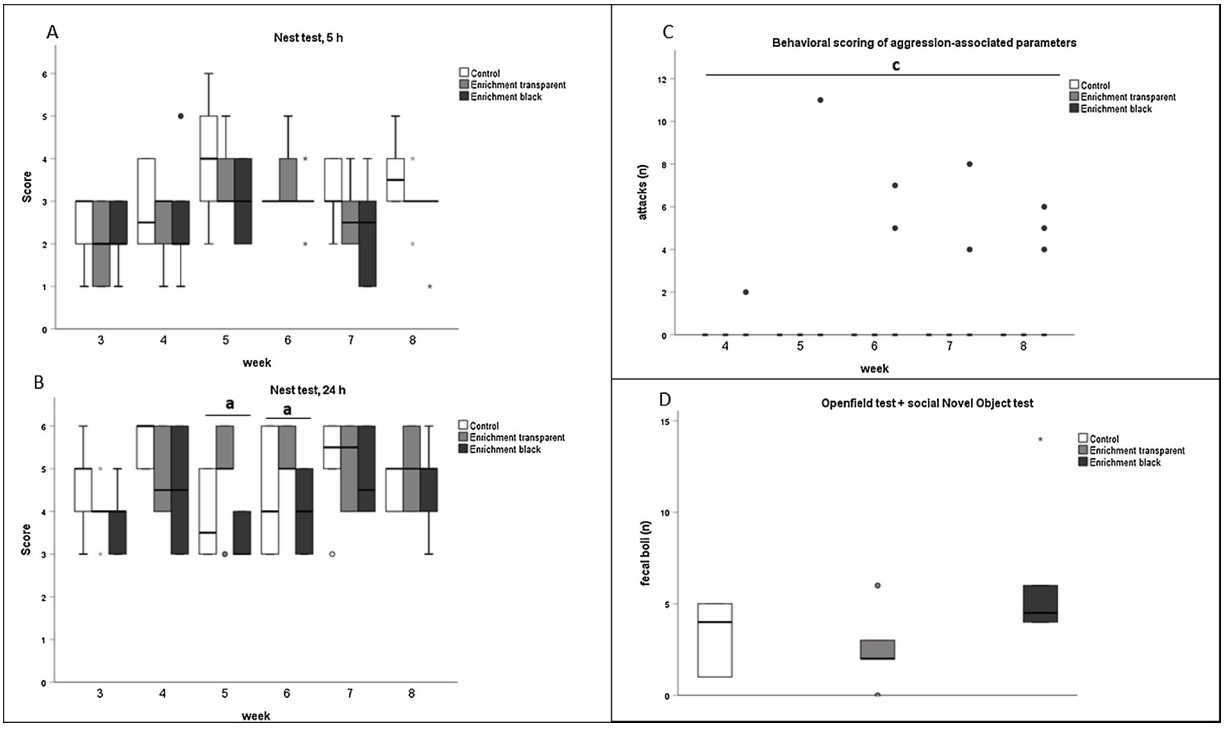

Fig. 3. Behavioral parameters.
A–B) Nest test 5 h and 24 h: The median of both enrichment opacities after 5 h is equally whereas 24 h scores revealed a significant difference in the 5th and 6th week for the factor 'opacity' with lower scores for EB mice. B) Behavioral scoring of aggression-associated parameters: EB-housed mice compared to C- or ET-housed mice attacked conspecifics highly significant more often. C) Openfield + social Novel-Object: Tendentially, the number of the counted fecal boli was higher in EB-housed compared to ET-housed mice. Different letters show significant differences (a: $p \leq 0.05$; b: $p \leq 0.01$; c: $p \leq 0.001$).

more easily with stressful situations. This effect was estimated to be enhanced by black enrichment, because the shelter effect was postulated to be increased by lack of visual contact.

Especially transferring the EB mice into new cages increased fighting and dominance behaviors. This may have been due to the non-transparency as it did not occur in the ET mice. In the wild, male mice defend their established territories and attack male intruders until the rival flees (Crowcroft, 1966). In our study, breaking line of sight is just achievable in EB mice, therefore the statement could also apply for the fighting over the dominance status in laboratory conditions - as soon as the subordinate mouse flees behind the wall of the black enrichment, the dominant one is not able to see it anymore, consequently being the winner of conflict. The minute the subordinate returns in the field of vision of the mouse which has previously defend its territory successfully, it may trigger once again aggressive behavior of the dominant resident. Contrary, the transparent enrichment allows the mice to see through the portioned areas, so the aggression is not provoked again as soon as the mouse emerges from the divided zone. This result raises the presumption of an enhanced territorial behavior of the EB animals. Additionally, our findings of the first aversive interaction being observed in the 5th week of housing, hence the mice being 8 weeks old, correspond to previous findings of territorial aggression, demonstrating that territorial aggression behavior is not displayed prior to sexual maturity, which is approximately around the age of 6–8 weeks (Benus et al., 1992).

However, the pain threshold measured by the HP test was not altered by housing conditions. So far it is unclear whether a break out of aggression is a result of pain, frustration, a failure of dominance relationships to mediate aggression or if it is more closely related to territorial aggression (Weber et al., 2017; Gaskill et al., 2017). As no diversity in pain perception was obvious for the different housing conditions, aggressive behavior in our study does not seem to be a result of pain. Furthermore, we tested the mice for territorial aggression towards an unfamiliar intruder in the RIT. Out of all tested male mice, only 2 attacked the intruder, both originating from EB. Two possible explanations may be assumed: On the one hand, the impossibility of vision after aggression between both subjects might be the reason of a higher aggression values in the EB group. On the other hand, it is also conceivable that this underlines the assumption of black enrichment strengthen aversion behavior due to a greater development of territorial aggression and a breakout of aggression, as seen in the behavioral testing after cage cleaning, being more closely related to territorial aggression. Therefore, this result is consistent with previous findings, showing that an environmental enrichment can lead to an increase of aggressive behavior in male mice (Haemisch et al., 1994; Haemisch and Gartner, 1994; Marashi et al., 2003; Bergmann et al., 1995; McGregor and Ayling, 1990). Interestingly the transparency of the enrichment seemed to have a profound impact, since no significant differences existed between C- and ET-housed mice. This outcome was unexpected as cage dividers so far were not investigated regarding their opacity.

Furthermore, the EB had a clear effect on the stress-induced hyperglycaemia. Thus, individuals housed under EB conditions, differed from C or ET animals. The release of the glucocorticoid corticosterone in mice is a key component of a response to stress and regulates many metabolic processes and glucose homeostasis (Ghalami et al., 2013). Corticosterone stimulates the gluconeogenesis leading to an increase in blood glucose, hence stressed animals show higher blood glucose levels. As the blood glucose level also depends on mice food uptake, we assumed all animals to be replete as food was provided *ad libitum*. Therefore, changes in blood glucose would be explainable with different stress states. However, these findings were not consistent with the measured FMC levels. The FCM values did not reflect the outcomes of the blood glucose measurements, since no significant difference for EB mice was obvious. Thus, EB mice do not seem to be more stressed. Nevertheless a greater variation for enriched animals was observed, consistent with previous research (Gärtner, 1999).

Besides those findings a tendency in the number of fecal boli counted after the sNO test indicate EB mice to exhibit higher emotionality (Lister, 1990; Lerch et al., 2015). This may result from previous stress-related behavior, justifying the assumption of EB mice being more stressed and therefore more anxious. However, the 'distances to walls' in the OF as well as the 'exit latency' in the DLB, 2 parameters testing for anxiety-like behavior, were unaffected by the different housing condition. Thus, anxiety may only manifest in a very slight expression in feces and just towards an unknown conspecific, assuming the alien mouse to stress EB- mice more than ET or standard-housed mice.

Also stress can lead to an increase in aggressive behavior in rodents (Marquez et al., 2013) and a decrease in the nest building performance (Jirkof, 2014). In our cages, the nest-scorings of EB animals were particularly lower after 24 h than in the two other husbandry conditions. It must be kept in mind that in two out of three groups a cage-dividing enrichment was introduced into the housing condition. An influence on the nest-building performance would be conceivable in so far as no nest could be built at exactly this point. However, a reduction in the performance in both enriched housing forms would then be to be expected. In addition, the compartments of the enrichment were also used to build the nests on two protected walls. The NT is an indicator for wellbeing (Jirkof, 2014) and therefore when scorings are decreased, mice seem to be more stressed. Several explanations are conceivable for the considerable differences in the outcome of aggressive behavior compared to the study by Tallent et al. (10), in which the cage dividing enrichment had led to a significant decrease in aggression. First, both studies used different mouse strains and ages. Furthermore, different devices and lengths of enrichment exposure were examined. We used C57BL/6NCrl mice, which were tested over a period of 10 weeks, whereas they observed Balb/c mice for one week. It is known that a variable effect of enrichment exists on different strains (Tsai et al., 2002; Chapillon et al., 1999) and that the duration of enrichment exposure can affect behavioral outcome (Leger et al., 2014). A study conducted by (Leger et al., 2014) aimed at assessing the time in which beneficial effects of an enriched environment appear, by using behavioral tests and neurobiological parameters. After testing the mice following exposition to different durations of enriched environments (24 h, 1, 3, or 5 weeks) they did see alterations for the different time-points, subsequently recommending, based on the results, 3 weeks of enrichment-exposure. So far, no investigation regarding enrichment duration and its effects on aggression was performed. Nevertheless, different outcomes are very likely to exist for different times of enrichment-exposure, too. Therefore, the statement of partial cage dividing decreasing aggression in male mice should be clarified independently for each device, exposure-period and strain. Additionally, the current study was performed involving animal manipulations to gather data relevant to the day-to-day use of mice in experimental research, whereas in Tallent et al. no handling of the animals was performed and therefore could have affected data outcomes. Handling is known to be stressful for animals and can result in impaired test performances (Deacon, 2006). All mice in our experiment experienced the same handling technique and therefore stress, but accumulation of two stressors, one being the black enrichment, the other one being the handling, could have led to the bad results for EB mice.

To prove our suggestion of opacity being important regarding dividing enrichment, it would have been interesting to clarify our results by using a colored Cross-Enrichment, e.g. green, as a fourth group. A study investing cage color preferences and the effect of home cage color on anxiety in 72 female CBA mice revealed after a five week period of housing differences in behavioral outcomes and mice's color preferences (Sherwin and Glen, 2003). Held in groups of three, one mouse was selected arbitrarily at the age of 8 weeks from each home cage and used in the cage color preference test (n = 24). The preference apparatus consisted of a central transparent cage connected by yellow plastic tubing (15 cm length) to four preference cages, each painted one of the

colors of the home cage. The test was filmed by a camera from above and started by placing the mouse in the central cage, in which it could habituate for 24 h to the entire apparatus. Then each 24 h the position of the preference cages was changed until each cage color (red, black, green or white) was tested in each position. Overall the white cages were most and the red least preferred. In addition, anxiety behavior in a raised plus maze was investigated in which mice from red home cages spent most time in the closed arms, hence indicating greater anxiety. Even if we could not detect any anxiety-related differences in our study (OF, DLB), the higher amount of fecal boli after the sNO test points to a higher emotionality in EB mice. Hence, in accordance to Sherwin et al., we assume the reduced occupancy of the black cross-enrichment to induce a negative mental state (Sherwin and Glen, 2003). Likewise, findings in rats demonstrated the importance of taking opacity into account. An experiment performed by Wren-Dail et al. aimed to examine the impact of different colored tunnels (amber, red, clear, or opaque) on the metabolism of pair-housed male Crl:SD rats (Wren-Dail et al., 2016). The colored devices, which were placed for 25 days into the animals' cages, altered the circadian rhythms of plasma measures of metabolism and physiology. Thus, all mentioned opacity-investigating studies, including ours, assumed opacity to affect experimental outcomes (Wren-Dail et al., 2016). Nevertheless, future studies are necessary in mice to investigate opacity related differences to validate the presumption.

5. Conclusion

The opacity of cage dividing enrichment, and likely other types of enrichment, appears not to be trivial, but to have a great impact on the behavioral outcome and therefore may be an important aspect when considering animal welfare, especially in the group-housing of male mice. The potentiation of aggression by a non-transparent enrichment, which already can be purchased commercially, should consequently be closely observed and well evaluated before using in large dimensions. With regard to the results of the study on hand we thus recommend that mice get a transparent enrichment when aggression within caged groups of males is a concern, but further research on this topic and the biological relevance of respective cage equipment is needed.

CRediT authorship contribution statement

Sinja Mertens: Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Peter Gass:** Resources. **Rupert Palme:** Resources. **Bernhard Hiebl:** Supervision. **Sabine Chourbaji:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

None.

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