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Not all mice are alike: Mixed-strain housing alters social behaviour

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ABSTRACT

The use of millions of mice in scientific studies worldwide emphasises the continuing need for a reduction of sample sizes, however, not at the expense of scientific validity. Split-plot designs have been suggested to enhance statistical power while allowing a reduction of animal numbers in comparison to traditional experimental designs. Recently, a promising approach of a split-plot design has been implemented and proven useful using mixed-strain housing of at least three different mouse strains. However, the impact of co-housing different strains of mice in one cage on animal welfare has still to be defined. This study aimed at comparing the effects of mixed-strain and same-strain housing of female C57BL/6J and DBA/2N mice on welfare and behaviour in two experimental phases. In a first phase, mice were housed in either mixed- or same-strain pairs. Home cage behaviour, activity rhythm, body weight, and faecal corticosterone metabolites were assessed. Furthermore, tests for anxiety-like and exploratory behaviour as well as spatial learning were performed. In a second phase, sociability was investigated in newly formed mixed-strain quartets. Mixed-strain housing did not induce alterations in anxiety, locomotion, learning, stereotypic behaviour, and stress hormone levels. However, changes in social behaviours and activity rhythm were observed. Increased agonistic and decreased socio-positive behaviours might point towards mild impacts on welfare in C57BL/6J mice under co-housing conditions. Altogether, scientific research may greatly benefit from co-housing mice of different strains within the same cages (e.g. for the realisation of a split-plot design), provided that strains are carefully selected for compatibility.

1. Introduction

Each year millions of mice are subject to scientific studies worldwide [1]. The largescale use of animals for research purposes not only triggers ongoing scientific and economic discussions, but also continuously generates concerns about animal ethics [2]. Whilst at the present time, it is not yet possible to replace all animal studies, substantial efforts are made to reduce animal numbers as much as possible (see 3R principle [3]). However, in order to ensure the lowest possible number of experimental subjects while retaining the study's validity [4], a thorough design is required that allows for conducting sufficiently powered experiments. As an achievable solution, the use of split-plot designs has been highlighted [5]. In this concept, experimental subjects that differ in a variable of interest (e.g., genotype) share an experimental unit (e.g., cage or mother). The treatment, for instance a drug, diet, or environmental enrichment, is then applied to these experimental units (see refs. [6,7]).

Walker and colleagues [6] were the first to apply the idea of a split-plot design empirically to mice of different strains housed in cages of varying enrichment. Specifically, mice of the strains C57BL/6, DBA/2, and Balb/c – three of the most common laboratory mouse strains – shared an experimental unit, i.e. were systematically mixed in cages, to which a treatment, i.e. different levels of enrichment, was applied. These mixed-strain housed mice were then screened for a range of behavioural, physiological and haematological variables. The results of this validation study proved that co-housing mice of at least three different strains within the same cage represents a powerful strategy to realize such a split-plot design, which can, in turn, successfully enhance statistical power, and thereby, allow a reduction of animal numbers in comparison to traditional same-strain housing designs. Please note however that only when three or more strains are used, meaningful power benefits can occur, as this effect critically depends on the number of strains, cages,

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and treatments (see [6] for further information).

The initial studies [6,8] did not detect increased levels of stress, as measured by faecal corticosterone metabolites (FCMs), or negative impacts on welfare, reflected by increased aggressive, stereotypic, and depressive-like behaviour, however, the results of other studies have raised concerns [9,10]. Anhedonia and a reduction of body weight were reported in C57BL/6N mice, but not in Balb/c mice, when co-housing mice of these two strains [10]. Similarly, another study found increased anxiety-like behaviour as well as signs of anhedonia and impaired learning in C57BL/6 mice when housed in high-density mixed-strain groups with DBA/2 mice compared to when raised in same-strain groups housed at lower density [9] (but see [11] for methodological concerns). Furthermore, mixed-strain housing of C57BL/6 and 129S mice was associated with increased anxiety-like responses in both strains and an overall higher incidence of agonistic behaviours [12,13]. These findings suggest one-sided or reciprocal influences in mixed-strain housing conditions that might be traced back to diverging physiological and behavioural profiles of different laboratory mouse strains [14].

So far, a detailed assessment of behaviours in the familiar home cage including 24 h activity cycle in mixed-strain housing groups compared to same-strain groups, however, is missing. Since even subtle alterations in socio-positive behaviours (also referred to as prosocial behaviour [15]; e.g., huddling), agonistic behaviours (e.g., chasing), and activity rhythm (e.g., hyperactivity) can be indicative of welfare issues, this assessment might provide further important insights into the impact of mixed-strain housing [16,17].

Thus, for a more comprehensive picture of the animals' welfare state [18], the present study aimed at integrating behavioural, physical, and endocrinological measures for a profound comparison of the effects of mixed-strain and same-strain housing in two experimental phases. In a first phase, female mice of the C57BL/6J and DBA/2N strain were housed in either mixed- or same-strain pairs. Please note that mice of only two strains were involved in this study, as we did not aim at proving the benefits of a split-plot design again, but instead concentrate on the welfare effects of such housing conditions. Specifically, we assessed home cage behaviour, body weight, FCMs, and conducted tests to assess anxiety-like and exploratory behaviour as measures of welfare. In addition, we investigated activity rhythm and spatial learning. Increased levels of agonistic, stereotypic, and anxiety-like behaviour, higher FCMs as well as loss in body weight are commonly regarded as indicators of poor welfare. Exploratory behaviour and spatial learning reflect locomotor activity or cognitive abilities, respectively, and were additionally assessed for gaining a more complete picture of the behavioural phenotype. In order to investigate the effect of mixed-strain and same-strain housing on C57BL/6J and DBA/2N mice, it was hypothesised that mice living in same-strain and mixed-strain housing differ in the above-mentioned measures.

To assess whether there are differences in sociability toward one strain or the other, in a second phase, the aim was to investigate socio-positive behaviour and spatial organization in newly formed quartets. These groups consisted of two individuals from each strain, originating either from same-strain or mixed-strain housing, thus allowing for the detection of an influence of previous experiences on sociability. We hypothesised that mice show higher sociability toward members of their own compared to the other strain, as displayed by increased display of socio-positive behaviour and shared cage space.

2. Animals, materials, and methods

2.1. Animals and housing

48 female mice of the C57BL/6J strain and 48 female mice of the DBA/2N strain were provided by Charles River Laboratories (Research Models and Services, Germany GmbH, Sulzfeld, Germany) at the age of four weeks. Both inbred strains were selected because of their widespread application in behavioural and biomedical research. Upon arrival, mice were pseudo-randomly allocated to cages with an unfamiliar partner of either the same (same-strain condition) or different strain (mixed-strain condition). The allocation was performed in a quasi-random way that accounted for a factor that could have influenced the results (familiarity). All animals were housed in an open top cage system in standard Makrolon cages type III (floor space: 38 cm × 22 cm, height: 15 cm). Each cage contained 1.5 l softwood granules (Tierwohl, Wilhelm Reckhorn GmbH & Co. KG, Warendorf, Germany) as bedding material, a paper tissue as nesting material, a red transparent plastic house (Mouse House™, Tecniplast Deutschland GmbH, Hohenpeißenberg, Germany), a freely hanging red transparent plastic tunnel (Mouse Tunnel Red, Plexx B.V., Elst, Netherlands), which was attached to the cage lid via wire hangers (Stainless Steel Wire Hanger for Mouse Tunnel, Plexx B.V., Elst, Netherlands), and a wooden stick (1.5 cm x 1.5 cm x 10 cm). Food pellets (Altromin 1324, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and tap water were provided ad libitum. Housing rooms were maintained at a reversed 12/12 h light-dark cycle with lights off at 10:00 a.m., a temperature of about 22 °C, and a relative humidity of about 50%. In order to avoid a position bias due to variation in proximity to ventilation, lights, and human traffic, cage position was counterbalanced with respect to strain and housing condition, i.e. each row of the rack contained two cages with mice of the mixed-strain condition and two cages with mice of the same-strain condition. The latter comprised one cage with only C57BL/6J and one cage with only DBA/2N. The experiment was conducted in two independent batches that were counterbalanced with respect to experimental groups.

2.2. Ethics statement

All procedures complied with the regulations covering animal experimentation within Germany (Animal Welfare Act [19]), the ARRIVE guidelines [20,21], and the EU Directive 2010/63/EU for animal experiments [22], and were approved by the local (Amt für Gesundheit, Veterinär- und Lebensmittelangelegenheiten, Münster, Nordrhein-Westfalen) and federal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen "LANUV NRW", reference number: 84–02.04.2015.A245). After the study, the animals remained in the animal facility of the institute.

2.3. Experimental design

The present study aimed at investigating the impact of same-strain versus mixed-strain housing of female mice of two different laboratory inbred strains, C57BL/6J and DBA/2N, on parameters related to welfare (anxiety-like, exploratory, stereotypic, and social behaviours, corticosterone metabolite concentrations, body weights), activity rhythm, and spatial learning abilities in two experimental phases (Phase I and II, Fig. 1).

In Phase I, two mice of the same or different strain lived together in pairs ($n = 24$ animals/ group; Fig. 1). 24 h video recordings and home cage behaviour observations were performed repeatedly over the course of Phase I. Tests for anxiety-like behaviour, exploratory locomotion, and spatial learning were conducted after 7 weeks of co-housing. Body weight and faecal corticosterone metabolites (FCMs) were assessed repeatedly (see Fig. 1).

In Phase II, four mice with either same-strain or mixed-strain prior experience were housed together in two cages that were connected via a short transparent plastic tube ($n = 24$/ group; Fig. 1). The quartets either consisted of two established pairs or of four unfamiliar animals. Again, home cage behaviour was assessed repeatedly (see Fig. 1), focussing on social behaviours and spatial organisation. There were no significant main or interaction effects of familiarity or housing condition on these parameters, meaning that behaviours did not differ based on whether quartets consisted of two established pairs or four strangers (ANOVA; huddling: main effect of familiarity: $F_{(1,88)} = 2.329$, $p = 0.131$;

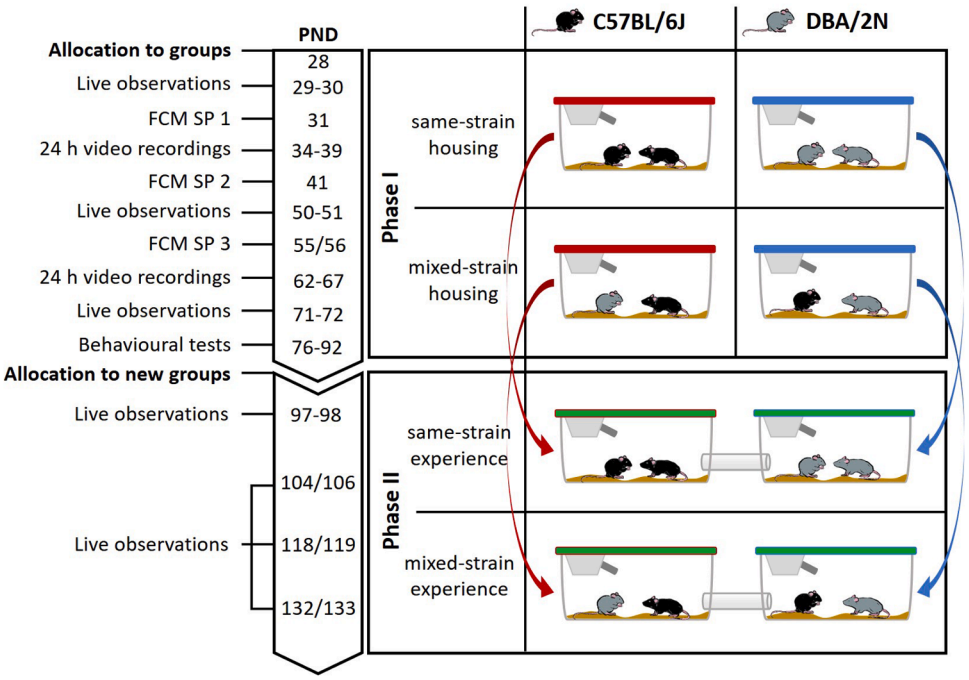

Fig. 1. Combinations of strains and housing conditions in experimental phases I and II. In Phase I, two mice of the same or different strain lived together in pairs, whereas in Phase II, four mice with either same-strain or mixed-strain previous experience lived together in two cages that were connected via a short transparent plastic tube. C57BL/6J mice are shown in black (cages shown in red), DBA/2N mice are depicted in grey (cages shown in blue). Arrows indicate allocation of groups for Phase II (green cages). PND = Postnatal day, FCM SP 1–3 = Faecal corticosterone metabolites sampling points 1–3. Sample size: $n = 24$/ group.

familiarity x strain: $F_{(1,88)} = 0.045$, $p = 0.833$; familiarity x housing: $F_{(1,88)} = 1.856$, $p = 0.177$; familiarity x strain x housing: $F_{(1,88)} = 0.132$, $p = 0.718$; time spent in same cage: main effect of familiarity: $F_{(1,88)} = 0.044$, $p = 0.835$; familiarity x strain: $F_{(1,88)} = 2.034$, $p = 0.157$; familiarity x housing: $F_{(1,88)} = 0.055$, $p = 0.815$; familiarity x strain x housing: $F_{(1,88)} = 2.428$, $p = 0.718$). Therefore, data from familiar and unfamiliar groups were pooled for the statistical analysis.

Due to the experimental design requiring social compatibility of unfamiliar mice of different strains in different stages of life, only female mice were used as agonistic interactions between females are usually less pronounced than in males [23,24].

2.3.1. Activity rhythm

Video recordings took place over the course of the light and dark phase between PND 34 and PND 40 as well as between PND 62 and PND 68 using infrared lamps and surveillance cameras sensitive to infrared wavelengths (EH1000H-4 Nano cameras, AVer Information Inc., Taiwan). Every 10 min, it was recorded whether mice were active or inactive (see Table 1 for definitions) using instantaneous sampling [25]. The order in which mice were observed was pseudo-randomised, meaning that mice of different strains and housing conditions were recorded and observed alternatingly. For technical reasons, 24 h activity patterns were only analysed for mice of the first batch ($n = 12$/ group).

2.3.2. Home cage behaviour

Live observations were performed under red light conditions by an experienced observer (MW). Four observation sessions were performed for each cage per day. The timepoints were spread across the dark phase of the light-dark cycle, covering phases of peak activity and phases of low activity, starting at 10:15 am, 12:15 am, 2:15 pm, and 4:15 pm. The ethogram was adopted from Gross et al. [26] and Bodden et al. ([27]; Table 1).

Phase I: Home cage behaviour was observed live on PND 29–30, 50–51 and 71–72. Each of the 96 observations per cage lasted 20 s during which behaviours of both mice were recorded using one-zero sampling, meaning that the occurrence (one) or non-occurrence (zero) of selected behaviour(s) during sequential sample intervals is recorded [25]. At the end of each 20 s interval, it was documented whether the mice were *active* or *inactive*. This was done using instantaneous sampling, which refers to the recording of an individual's behaviour at sequential, predetermined points in time [25].

Phase II: Observations took place on PND 97–98, 104, 106, 118–119, 132–133. One-zero sampling was performed for each observation of 20 s duration, during which only one focus animal was observed (64 observations per focus animal). At the end of each 20 s

Table 1
Ethogram.

General activity	
Active	The mouse is *active* when it is not *inactive*.
Inactive	The mouse is lying or sitting motionlessly [26], except for head or tail movements. When the mouse is covered by the paper tissue or bedding material, it is inactive when no movement of this material can be seen. If the mouse is covered to an extent that no part of its body is visible, it is considered inactive when no movement of the cover material can be seen and additionally, a time-out (TO) is noted (TOs are not further analysed as it is assumed that the mouse is inactive).
Social behaviour - Affiliative interactions	
Allo-grooming	The mouse touches the body (excluding tail) of a recipient with its snout for at least 2 s while performing licking movements with its head.
Huddling	Two or more mice are *inactive*. At least one entire side of the body of a participating mouse is pressed up against at least one entire side of the body of another participating mouse. Each participating mouse needs to be pressed up against at least one other participating mouse.
Naso-anal contact	The mouse approaches the anogenital region of another mouse with its snout to a distance of less than one snout-length for at least 2 s.
Naso-nasal contact	The mouse approaches the face of another mouse with its snout to a distance of less than one snout-length for at least 2 s.
Fleeing	The mouse runs away from another mouse, with the latter following at a distance of less than one body length.
Chasing	The mouse approaches another mouse which thereupon runs away, with the distance between the mice not exceeding one body length.
Resting alone	The mouse is *inactive* and does not have close body contact to any conspecific (as defined for *huddling*).
Stereotypic behaviour	
Barmouthing	The mouse has a metal bar of the cage lid in its open mouth at least three times in a row.

interval, the position of the focal animal was documented. For data analysis, the percentage of scans or intervals, respectively, in which each behaviour occurred was calculated and corrected for the number of partner animals per strain.

2.3.3. Anxiety-like behaviour, exploration, and spatial learning

In order to assess anxiety-like behaviour, exploratory locomotion, and spatial learning, the elevated plus-maze (EPM), dark-light (DL), open-field (OF), free-exploration (FE), and labyrinth-maze tests (LM) were performed as previously described in Bodden et al. (2019). Parameters assessed in the EPM, DL, and OF are indicative of state anxiety and exploratory locomotion, while FE measures reflect trait anxiety. LM parameters demonstrate spatial learning abilities. Each test was conducted under dim light conditions in a testing room a few meters away from the housing room. During the transport, the cage was protected from light. While the sequence of tests was the same for all subjects, we pseudo-randomised the order in which animals were tested per testing day. Before each trial, the apparatus was thoroughly cleaned with 70% ethanol. The animals' movements were recorded by a webcam (Webcam Pro 9000, Logitech, Europe S.A., Lausanne, Switzerland) in the absence of the experimenter and automatically analysed by the video tracking system ANY-maze (Version 4.99, Stoelting Co., Wood Dale, USA). Due to different fur colours of mice (C57BL/6 mice: black, DBA/2N mice: brown) blinding was not possible. However, the use of automated tracking and data collection for behavioural tests diminished the influence of a potential experimenter effect.

Elevated plus-maze test (EPM): The EPM was performed on PND 76/77. The plus-shaped apparatus consisted of two opposing open arms (30 cm × 5 cm), two opposing closed arms (30 cm × 5 cm), and a central square (5 cm × 5 cm). While the closed arms were equipped with 20 cm high walls, the open arms were surrounded by only a small lip (4 mm) that prevented mice from falling off. The EPM was elevated 50 cm above the ground. Illumination intensity in the centre square was 25 lx. After spending 1 min in an empty box protected from light, each mouse was individually placed on the central platform facing a closed arm and could freely explore the apparatus for 5 min. The parameters measured were the percentage of time spent on the open arms (*(time on open arms/(time on open + time on closed arms)) × 100*), the percentage of entries into the open arms (*(entries into open arms/(entries into open + entries into closed arms)) × 100)* to assess anxiety-like behaviour. The sum of entries into the open and closed arms, the total distance as well as the number of protected head dips (mouse lowers its head over the side of an open arm with its ears protruding over the edge, while at least the hind limbs remain in the closed segment or central platform) were assessed as indicators of exploratory locomotion.

Dark-light test (DL): The DL was performed at the age of 78/79 using a modified Makrolon cage type III, which was separated into two compartments by a dark plastic panel comprising a sliding door. The dark compartment (17 cm × 27 cm × 15 cm) made up approximately one third of the cage, had opaque walls and an opaque lid and was unlit, while the light compartment (26 cm × 27 cm × 15 cm) had transparent walls and no lid. Illumination (40 lx) was provided by an LED lamp. Each mouse was placed inside the dark compartment with the lid and sliding door closed and remained there for 1 min before the sliding door was opened and the mouse could freely explore the apparatus for 5 min. The parameters analysed were the latency to enter and the time spent in the light compartment as indicators of anxiety-like behaviour, and the number of entries into the light compartment as well as the distance travelled in the light compartment to assess exploratory locomotion.

Open-field test (OF): The OF was conducted at the age of 80/83 days. The apparatus consisted of a square arena (80 cm × 80 cm) surrounded by walls (42 cm) and made of white coated plywood. The illumination level was set to 35 lx. After spending 1 min in an empty box protected from light, each mouse was individually placed in one corner of the OF facing the wall and could freely explore the arena for 5 min. The parameters analysed were the time spent in the centre of the arena (defined as the area of the OF being located at least 20 cm distant from the walls), the number of entries into the centre, and the distance travelled in the centre to measure anxiety-like behaviour. The total distance travelled in the OF was assessed as a measure for exploratory locomotion.

Free-exploration test (FE): At the age of 84±2 days, the mice were tested in the FE. The apparatus consisted of a square arena (60 cm × 60 cm) surrounded by walls (36 cm) and made of white coated plywood. An LED lamp set to 35 lx was mounted above the testing apparatus to provide illumination. The arena was connected to the home cage of the mouse via a sliding door and a transparent plastic tunnel (24 cm × 15 cm × 10 cm). Prior to testing, the cage mate of the test subject was removed from the home cage and placed in another cage containing a small amount of bedding material from the home cage, a red house, a paper tissue, and food and water ad libitum. After spending 1 min in an empty box protected from light, the mouse was placed in its home cage, with the sliding door open, allowing the animal to freely explore the FE for 15 min. The parameters measured were the latency to enter the arena, the number of excursions into the arena, and the time spent in the arena. After the test, the cage mates of the test subject were placed back into the home cage and the cage was left undisturbed for at least one day before the next animal of the cage was tested.

Labyrinth-maze test (LM): The LM was performed on PND 91±2. The apparatus consisted of a white platform (40 cm x 24 cm) with several transparent acrylic glass walls (15 cm), partly with passageways to form a labyrinth. Altogether, there were 7 passageways, with only a restricted number leading to the home cage, which was connected via a short tunnel (8 cm). Before testing, the cage mate of the test subject was placed in another cage for the time of testing. The empty home cage was connected to the end of the LM while the test subject was placed in an empty box protected from light for 1 min prior to testing. Thereafter, it was placed in the start position of the LM, allowing it to freely explore the apparatus and find its way to the home cage within 5 min. After having solved the task by reaching the home cage, the mouse had a 5 min pause in its home cage, while the LM was thoroughly cleaned with 70% ethanol. Subsequently, the mouse was again placed in the start position to perform a second trial for 5 min maximum. After the second test, the cage mate of the test subject was placed back into the home cage and the cage was left undisturbed for at least 1 day before the next animal of the cage was tested. The parameters measured were the time needed to exit the LM and the number of errors, meaning all transits through passageways that did not lead to the exit.

2.3.4. Body weight

Each individual's weight was recorded on a weekly basis (PND 31, 38, 45, 52, 59, 66, 73, 80, 87, 94, 101, 108, 115, 122, 129, 136, and 143) in gram, rounded to one decimal place, using a digital scale (CM 150–1 N, Kern, Balingen, Germany).

2.3.5. Faecal corticosterone metabolites (FCMs)

The stress hormone level of mice in different housing conditions was monitored non-invasively by measuring FCM concentrations as an indicator of baseline hypothalamic-pituitary adrenal (HPA) axis activity [28]. Numerous studies have shown that FCMs reliably reflect environmental manipulations [29–31]. Faeces were collected at three time points during Phase I, i.e. on PND 31, 41, and 55/56 (see Fig. 1). Touma and colleagues [32] determined that it takes around 4 h from corticosterone secretion to excretion of its metabolites. To avoid an influence of the collection procedure itself, the duration of the faeces sampling procedure was limited to 3 h – from 12 am to 3 pm. Subsequently, all faeces defecated were collected and frozen at −20 °C. Samples were dried and homogenized, and aliquots of 0.05 g were extracted with 1 ml of 80% methanol. For the analysis of the samples, a 5α- pregnane-3β, 11β,21-triol-20-one enzyme immunoassay was used, which was established and successfully validated to measure FCMs in mice (for details see refs. [32,33]). Intra- and inter-assay coefficients of variation were

below 10% and 12%, respectively.

2.4. Statistical analysis

Linear mixed models were used to analyse parameters assessed in behavioural tests as well as corticosterone metabolites and body weights. To meet the assumptions of parametric analysis, residuals were graphically examined for normal distribution, homoscedasticity and outliers, and the Shapiro-Wilk test was applied. When necessary, raw data were transformed using square root or logarithmic transformations (see Table 2). Specifically, linear mixed models were used to analyse dependant variables (EPM, DL, OF, and FE parameters) with fixed between-subject factors 'strain' and 'housing' and random factor 'cage'. Likewise, linear models for repeated measures were performed for the analysis of spatial learning, body weight development, and FCM. The fixed within-subjects factor was 'trial' or 'time', respectively, with the fixed between-subject factors 'strain' and 'housing', and the interaction of 'trial' or 'time', respectively, and 'strain' and 'housing', with the random factor 'cage'. Main effects and interaction terms were tested on local significance level alpha (α) = 0.05. If there were significant main or interaction effects, *post hoc* pairwise comparisons were computed from the contrasts between factors using the lsmeans package with Tukey adjustments [34].

Since not all home cage behaviour data were normally distributed and could not be transformed adequately, non-parametric statistics were applied. Specifically, the Kruskal-Wallis test was performed to reveal differences between all four experimental groups (Phase I: general activity, social behaviour – affiliative behaviour, stereotypic behaviour; Phase II: sociability). Whenever significant differences were detected, the Mann-Whitney U test for between-group comparisons with Bonferroni-Holm correction was applied. To test for significant deviations from chance level (50%), home cage behaviour data were submitted to one-sample t-tests (Phase II: huddling and sharing the same cage).

A statistical power analysis for sample size estimation was performed using G*Power [35]. Taking into account all parameters that yielded large effect sizes, we could ensure that a total sample size of 96 mice (n = 24/ group) could detect biologically relevant differences with a power of 80% [36]. Statistical analyses were conducted using the statistical software IBM SPSS Statistics (IBM Version 26, Release 26.0.0.0) or R [34]. Graphs were created using the software GraphPad Prism (Version 8.3.0 (538) for Windows, GraphPad Software, San Diego, California USA).

3. Results

Data were assessed in two separate experimental phases (Phase I and Phase II, see Fig. 1). Phase I served to reveal possible differences in welfare-related parameters between female C57BL/6J and DBA/2N mice housed in either mixed-strain or same-strain conditions. Phase II served to assess whether mice prefer members of the own or the other strain in a new setting of mixed-strain groups.

3.1. Phase I

3.1.1. Mixed-strain housing influenced activity rhythm and social behaviour

24 h activity profiles as well as live home cage observations revealed distinct effects of mixed- compared to same-strain housing. Differences in the 24 h activity rhythm between strains and same- or mixed-strain housing were analysed qualitatively. ImageJ analysis of areas (ImageJ 1.52a, National Institutes of Health, USA) revealed generally more activity in the mixed-strain housing conditions compared to same-strain housing (Fig. 2). Same-strain housed C57BL/6J mice were found to be active 50.7% of the time (Fig. 2**a**) and DBA/2N mice in same-strain housing exhibited lowest overall activity (45.9%; Fig. 2**b**). In mixed-strain conditions, however, C57BL/6J mice showed the highest overall activity (57.2%; Fig. 2**c**), with enhanced phases of increased activity and the absence of a distinct break in activity between two phases of peak activity during the dark phase. Similarly, mixed-housed DBA/2N mice (51.6%) did not display breaks in activity between their three phases of peak activity (Fig. 2**d**) compared to same-strain housing. Moreover,

Table. 2
Statistical details on the linear mixed model for behavioural tests. Females of the strains C57BL/6J and DBA/2N mice living either in same-strain or mixed-strain housing conditions were subjected to the elevated plus-maze, dark-light, open-field, free-exploration, and labyrinth-maze test. Shown are df values (numerator and denominator), F-ratios, and p-values. Linear mixed models demonstrate a significant effect of strain on most parameters (indicated in bold numbers), while the effect of housing condition was not statistically significant. A significant interaction between strain and housing condition was detected regarding one parameter (indicated in bold). Transf. = Transformation, Log = logarithmic transformation, SqRt = square root transformation.

Test - Parameter	Transf.	Strain				Housing				Strain x housing			
		NumDF	DenDF	F	p	NumDF	DenDF	F	p	NumDF	DenDF	F	p
Elevated plus-maze													
Time on open arms (%)	–	1	91.679	54.699	**<0.001**	1	45.000	0.100	0.753	1	91.679	0.009	0.925
Entries into open arms (%)	–	1	90.536	14.154	**0.020**	1	45.000	0.020	0.889	1	90.536	0.038	0.847
Sum of entries into open and closed arms (#)	Log	1	87.385	15.720	**<0.001**	1	45.000	0.062	0.804	1	87.385	0.199	0.656
Total distance (m)	–	1	92.000	78.057	**<0.001**	1	92.000	1.272	0.262	1	92.000	0.143	0.706
Dark-light													
Time in light compartment (s)	–	1	86.862	12.092	**<0.001**	1	45.000	0.001	0.980	1	86.862	0.219	0.641
Entries into light compartment (#)	–	1	91.783	0.663	0.418	1	45.000	3.123	0.084	1	91.783	0.201	0.655
Latency to enter light compartment (s)	Log	1	92.000	16.153	**<0.001**	1	92.000	0.963	0.329	1	92.000	0.267	0.607
Open-field													
Time in centre (s)	SqRt	1	89.486	8.284	**0.005**	1	45.000	0.039	0.844	1	89.486	1.897	0.172
Entries into centre (#)	SqRt	1	77.842	28.800	**<0.001**	1	45.000	0.335	0.566	1	77.842	1.473	0.228
Latency to enter centre (s)	SqRt	1	87.445	145.080	**<0.001**	1	45.000	0.014	0.908	1	89.486	6.123	**0.015**
Total distance (m)	SqRt	1	82.001	20.378	**<0.001**	1	45.000	0.054	0.817	1	82.001	0.123	0.727
Free-exploration													
Time in open arena (s)	SqRt	1	92.000	20.506	**<0.001**	1	92.000	1.057	0.307	1	92.000	1.049	0.309
Distance in open arena (m)	SqRt	1	92.000	23.954	**<0.001**	1	92.000	1.062	0.305	1	92.000	0.753	0.388
Labyrinth-maze													
1st trial: Time to reach exit (s)	Log	1	75.893	17.059	**<0.001**	1	45.000	0.834	0.366	1	75.893	0.038	0.847
2nd trial: Time to reach exit (s)	Log	1	92.000	15.178	**<0.001**	1	92.000	0.424	0.517	1	92.000	0.369	0.545
1st trial: Errors (#)	Log	1	81.817	12.648	**<0.001**	1	45.000	1.217	0.276	1	81.817	0.020	0.887
2nd trial: Errors (#)	Log	1	91.487	5.032	**0.027**	1	45.000	0.154	0.697	1	91.487	1.544	0.217

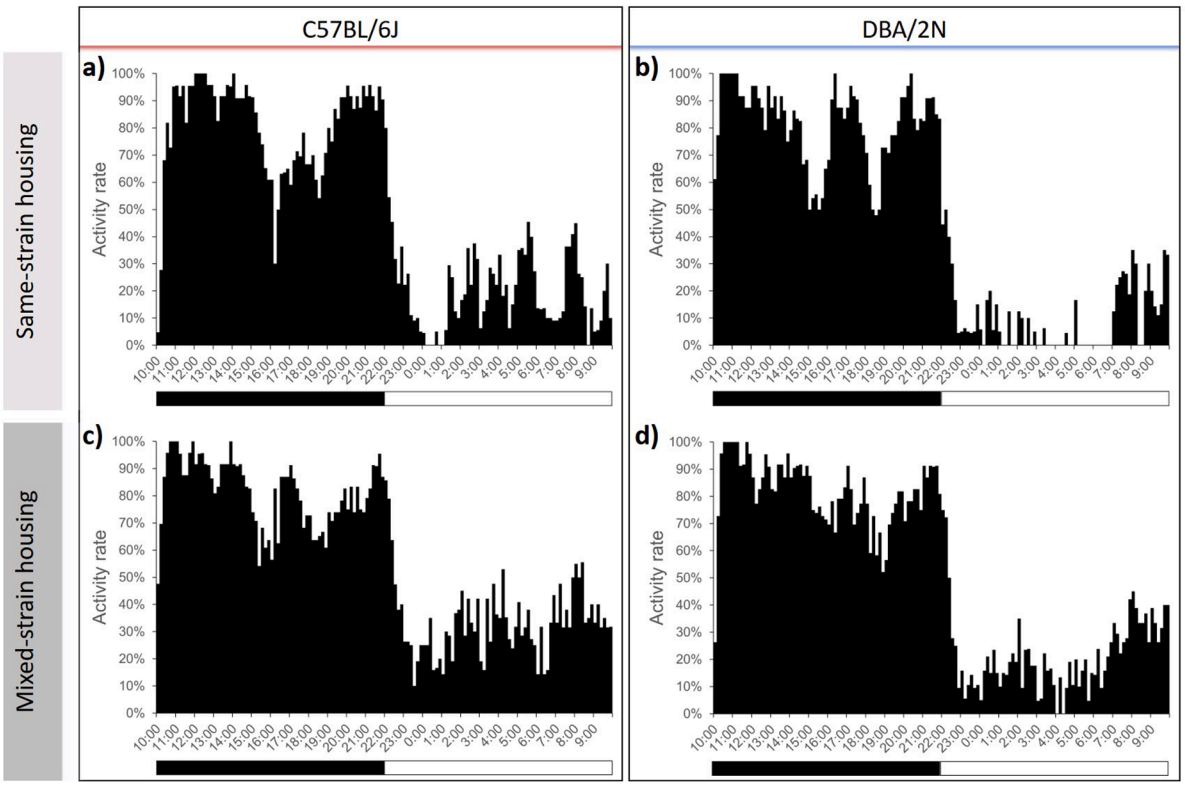

Fig. 2. Activity rhythm of female C57BL/6J and DBA/2N mice in same-strain and mixed-strain conditions. Individual home cage activity was analysed using video-recordings over a period of 24 h during Phase I (PND 34 and 40). Activity was assessed at an interval of 10 min. The focal animal was considered 'inactive' if it was lying or sitting motionlessly (except for tiny whisker, ear, or tail movements). Whenever the level of activity exceeded aforementioned tiny movements, the mouse was considered to be active (black). The order in which mice were observed was pseudo-randomised, meaning that mice of different strains and housing conditions were recorded and observed alternatingly. Illustrated is the relative frequency of being active (in percent) at each sampling point for 24 h for same-strain housed mice of the strain (a) C57BL/6J (total activity: 50.7%) and (b) DBA/2N (45.9%) as well as for mixed-strain housed (c) C57BL/6J (57.2%) and (d) DBA/2N (51.6%) mice. The horizontal black/white bar represents the reversed 12/12 h dark-light cycle, with lights off at 10 a.m. Sample size: 12 mice per strain and housing condition.

activity during the light phase was increased in mixed-strain housing conditions.

Home cage behaviours (Fig. 3) were initially analysed for differences between the four experimental groups. Significant effects can therefore reflect either strain or housing effects and were observed with regard to socio-positive behaviours (allo-grooming: $H = 10.611$, $p = 0.014$; huddling: $H = 41.025$, $p < 0.001$) and agonistic behaviours (chasing: $H = 13.393$, $p = 0.004$; fleeing: $H = 14.908$, $p = 0.002$), exploratory behaviours (nasonasal & nasonasal sniffing: $H = 23.656$, $p < 0.001$; climbing, rearing & digging: $H = 14.635$, $p = 0.002$), maintenance & avoidance behaviours (self-grooming: $H = 13.596$, $p = 0.004$; resting alone: $H = 33.819$, $p < 0.001$; being in tube: $H = 22.092$, $p < 0.001$), stereotypic behaviours (barmouthing: $H = 60.036$, $p < 0.001$), and being active ($H = 40.489$, $p < 0.001$; Fig. 3).

Post hoc testing showed that, within C57BL/6J mice, mixed-strain housed mice displayed less socio-positive (huddling: $U = 54.0$, $p < 0.001$) and more agonistic behaviours (chasing: $U = 150.0$, $p = 0.002$) compared to same-strain housed animals. Furthermore, mixed-strain housed C57BL/6J mice displayed more social exploration behaviour (naso-nasal & naso-anal sniffing: $U = 89.0$, $p < 0.001$) and more maintenance or avoidance behaviour (resting alone: $U = 172.0$, $p = 0.016$). Mixed-housed C57BL/6J mice were also more frequently observed to be active ($U = 130.0$, $p = 0.001$) than same-strain housed animals.

Regarding DBA/2N mice, mixed-strain housed mice exhibited higher amounts of maintenance (self-grooming: $U = 156.0$, $p = 0.006$) and avoidance behaviour (being in tube: $U = 97.0$, $p < 0.001$; fleeing: $U = 141.0$, $p = 0.001$) compared to same-strain housed mice.

Although not in the focus of this investigation, there were several significant differences between strains. Specifically, within same-strain housed animals, C57BL/6J mice displayed higher amounts of socio-positive behaviours (huddling: $U = 28.0$, $p < 0.001$), but also more avoidance (resting alone: $U = 124.0$, $p = 0.001$), less exploratory behaviour (climbing, rearing & digging: $U = 125.5.0$, $p = 0.001$; naso-nasal- and nasoanal sniffing: $H = 164$, $p = 0.009$), but also less stereotypic behaviours (barmouthing: $U = 40.0$, $p < 0.001$) compared to same-strain housed DBA/2N mice.

The comparison within mixed-strain housed animals revealed higher amounts of maintenance or avoidance behaviour (resting alone: $H = 84.0$, $p < 0.001$), fewer stereotypic behaviour (barmouthing: $U = 26.0$, $p < 0.001$), and less frequent activity ($H = 149$, $p = 0.004$) in C57BL/6J compared to DBA/2N mice.

3.1.2. *Mixed-strain housing did not distinctly affect anxiety, exploration, learning, body weight, and corticosterone metabolites*

Only one out of 15 parameters revealed an effect of housing conditions on behavioural strain differences (latency to enter centre of OF: $F_{(1,92)} = 6.151$, $p = 0.015$; Table 2), with mixed-strain housing being associated with an increased latency to enter the centre of the OF in C57BL/6J mice but a decreased latency in DBA/2N mice when compared to same-strain housed groups (Table 3).

Albeit not being the focus of the present study, several significant main effects of strain were revealed (Table 2), showing higher anxiety-like behaviour and less exploratory locomotion in DBA/2N compared to C57BL/6J mice (Table 3). No significant differences were detected with regards to housing condition (Table 2).

While body weight analysis did not reveal a significant main effect of housing or strain, there were significant effects of time ($F_{(9,828)} =$

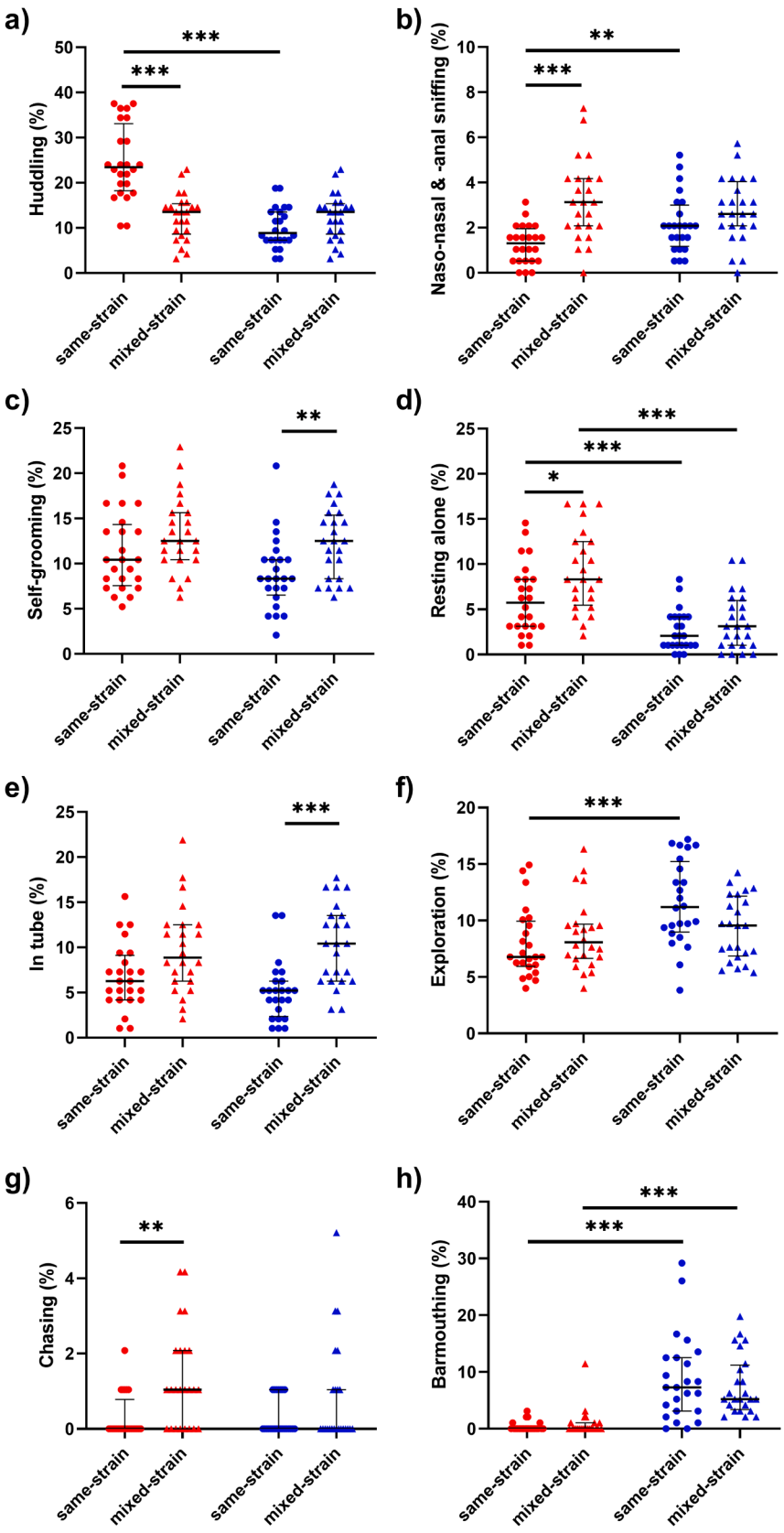

Fig. 3. Home cage behaviour of C57BL/6J (red) and DBA/2N (blue) mice in same-strain (circles) and mixed-strain (triangles) housing conditions in Phase I. Percentage of intervals in which (a) huddling, (b) naso-nasal and naso-anal sniffing, (c) self-grooming, (d) resting alone, (e) in tube, (f) exploration, (g) chasing, and (h) barmouthing occurred. Statistics: Kruskal-Wallis test, Mann-Whitney U test with Bonferroni-Holm correction. Sample size: $n = 24$/ group. The figure shows the dataset presented as scatterplot with medians and interquartile range.

Table. 3

Descriptive statistics for behavioural tests and body weights. Presented are the means and standard deviations (SD) of common parameters assessed in the elevated plus-maze (EPM), dark-light (DL), open-field (OF), free-exploration (FE), and labyrinth-maze test (LM) as well as body weights at different time points over the course of the experiment. Shown are group means for females of the C57BL/6J and DBA/2N strain in mixed-strain and same-strain housing conditions.

Test - Parameter	C57BL/6J				DBA/2N			
	Mixed-strain		Same-strain		Mixed-strain		Same-strain	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Elevated plus-maze								
Time on open arms (%)	29.68	11.71	30.16	10.00	13.83	9.34	14.71	10.25
Entries into open arms (%)	34.40	11.18	35.21	10.50	25.94	12.67	25.83	11.96
Sum of entries into open and closed arms (#)	29.75	5.47	28.67	4.01	25.08	6.97	25.08	5.48
Total distance (m)	11.29	2.07	11.04	1.17	8.44	1.64	7.93	1.60
Dark-light								
Time in light compartment (s)	98.13	51.14	101.67	33.58	73.98	34.47	70.00	35.00
Entries into light compartment (#)	13.62	3.93	15.00	4.08	14.00	4.95	16.29	6.63
Latency to enter light compartment (s)	4.40	6.65	5.26	4.67	8.48	6.66	9.75	11.93
Open-field								
Time in centre (s)	16.91	7.78	19.23	8.11	15.38	11.05	12.72	10.05
Entries into centre (#)	11.13	3.65	11.75	4.02	7.62	4.86	6.42	6.58
Latency to enter centre (s)	45.15	28.09	31.04	16.87	125.47	57.44	153.42	60.42
Total distance (m)	34.18	7.16	34.10	4.47	28.29	6.54	27.75	9.69
Free-exploration								
Time in open arena (s)	101.50	129.42	157.06	157.05	204.11	79.93	213.74	95.40
Distance in open arena (m)	8.31	11.19	12.31	13.04	18.21	8.38	19.76	10.85
Labyrinth-maze								
1st trial: Time to reach exit (s)	122.13	95.98	99.63	74.45	192.96	95.62	163.50	81.36
2nd trial: Time to reach exit (s)	32.67	21.89	51.71	75.56	73.42	67.17	83.92	65.56
1st trial: Errors (#)	17.50	11.68	14.63	10.55	24.88	9.63	23.79	13.61
2nd trial: Errors (#)	6.13	4.24	8.79	13.08	9.96	9.64	12.71	10.90
Body weights (g)								
PND 31	14.55	0.78	14.83	0.88	15.35	1.68	14.67	1.60
PND 38	17.37	0.95	17.19	0.66	17.57	1.27	17.18	1.26
PND 45	18.33	1.07	18.27	0.75	18.97	1.30	18.31	1.10
PND 52	19.71	1.01	19.81	0.77	19.93	1.43	19.15	1.22
PND 59	20.28	0.91	20.39	0.69	20.18	1.57	19.71	1.42
PND 66	20.94	1.01	20.89	0.78	20.89	1.60	20.25	1.15
PND 73	21.70	1.01	21.53	0.77	21.58	1.90	20.88	1.48
PND 80	22.00	0.99	21.78	0.75	21.83	1.69	21.71	1.77
PND 87	22.38	1.03	22.02	0.86	22.15	1.79	21.28	1.54
PND 94	22.63	0.80	22.35	0.95	22.53	1.88	22.05	1.68

1119.784; $p < 0.001$) and strain-by-time interactions ($F_{(9,828)} = 4.300$; $p < 0.001$), indicating that C57BL/6J mice gained more weight over time compared to DBA/2N mice.

The analysis of baseline FCM levels did neither reveal a significant effect of housing condition nor a significant strain-by-housing interaction. However, a significant effect of strain was detected ($F_{(1,87.007)} = 60.676$, $p < 0.001$), with DBA/2N mice having higher values than C57BL/6J mice. Furthermore, there was a significant effect of sampling point ($F_{(2,184.002)} = 9.391$, $p < 0.001$, Fig. 4), with values increasing over time.

Post hoc comparisons within strains revealed increasing FCM concentrations between the first week (sampling point, SP 1) and the third week (SP 2, $p < 0.001$) of the experiment in DBA/2N mice.

Between-strain comparisons showed that DBA/2N mice displayed significantly higher values than C57BL/6J mice at SP 2 ($t = -4.926$, $p < 0.001$) and SP 3 ($t = -9.573$, $p < 0.001$).

3.2. Phase II

3.2.1. C57BL/6J mice showed higher sociability toward mice of their own strain, DBA/2N mice did not

In Phase II, all mice were allocated to new groups of four mice. The new groups consisted of mice that originated from either same-strain or mixed-strain conditions. This part of the experiment was supposed to unveil differences in home cage behaviour depending on whether cohabitation with a different strain preceded or not.

A significant difference between groups was detected with regard to the preferred partner for sharing the same cage in the double cage-

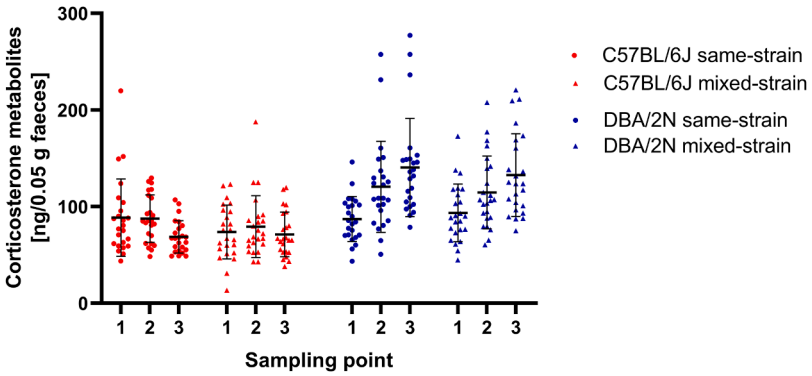

Fig. 4. Baseline corticosterone metabolite concentrations at three sampling points as indicator of HPA axis activity. Faeces from each animal were collected at three sampling points (SP 1–3) over the course of Phase I: at the beginning (PND 31; SP 1), two weeks (PND 41; SP 2), and five weeks into Phase I (PND 55/56; SP 3). There was a significant effect of strain ($F_{(1,87.007)} = 60.676$, $p < 0.001$) and sampling point ($F_{(2,184.002)} = 9.391$, $p < 0.001$. However, there was no significant main effect of housing. DBA/2N mice displayed significantly higher values than C57BL/6J mice at SP 2 ($t = -4.926$ $p < 0.001$) and SP 3 ($t = -9.573$, $p < 0.001$). Furthermore, values changed over time, with increasing values between SP 1 and 2 ($t = -4.240$, $p < 0.001$) in DBA/2N mice. Scatterplot shows means ± SD. Statistics: Linear mixed models. Sample size: $n = 24$/ group.

system ($H = 27.523$, $p < 0.001$, Fig. 5**a**) and for huddling ($H = 17.273$, $p < 0.001$, Fig. 5**b**). C57BL/6J mice showed higher sociability, i.e. deviation from chance level (50%), in terms of sharing a cage (mixed-strain experience: $t = 6.699$, $p < 0.001$; same-strain experience: $t = 6.654$, $p < 0.001$) and huddling (mixed-strain experience: $t = 6.472$, $p < 0.001$; same-strain experience: $t = 11.090$, $p < 0.001$) with members of their own strain. In contrast, DBA/2N mice with mixed-strain experience did not show higher sociability towards mice of their own strain, neither with regards to sharing a cage nor regarding huddling. However, DBA/2N mice with same-strain experience displayed increased sociability toward C57BL/6J mice compared to mice of their own strain (huddling, $t = 3.615$, $p = 0.001$).

Post hoc testing revealed that C57BL/6J mice shared a cage and huddled more often with C57BL/6J than with DBA/2N mice compared with DBA/2N mice, independent of whether they experienced same- or mixed-strain housing before (sharing a cage: same-strain: $U = 108.0$, $p < 0.001$; mixed-strain: 113.5, $p < 0.001$; huddling: same-strain: $U = 161.0$, $p = 0.009$; mixed-strain: 165.0, $p = 0.011$).

4. Discussion

This study investigated the welfare effects of mixed-strain housing as a promising method for the application of split-plot designs (for details please see [6]). For this purpose, female C57BL/6J and DBA/2N mice were kept in either mixed- or same-strain groups and screened for commonly assessed anxiety-like and exploratory behaviours, spatial learning, home cage behaviour, and physiological parameters. Our results, on the one hand, confirm previously published findings regarding mixed-strain housing and strain differences: Mixed-strain and same-strain housed groups did not differ in their anxiety, exploration, learning, stereotypic behaviour, stress hormone levels, and body weights [6,8]. Strain-wise, C57BL/6J mice clearly differed from DBA/2N mice by exhibiting lower levels of anxiety-like behaviour, more exploratory locomotion, increased sociability, less stereotypic behaviour, and lower FCM levels, which is in line with prior research [14,28,37,38]. However, on the other hand, our comprehensive analysis of home cage behaviours unveiled various distinct differences in activity rhythm and sociability between mixed-strain housed C57BL/6J and DBA/2N mice that could potentially point towards subtle welfare issues.

Specifically, mixed-strain housing was associated with altered activity patterns in mice of both strains, with an increased proportion of active bouts and the absence of phases with clearly reduced activity compared with mice in same-strain housing conditions (see ref. [7]). Similarly, changes in activity have previously been reported for mixed-strain housed C57BL/6 and Balb/c mice in fully automated home-cage systems [10]. Such mixed-strain housing-induced alterations in activity patterns should not be disregarded, since more severe shifts in activity rhythms can, at worst, negatively impact brain, behaviour, and physiology [39]. Although these consequences are unlikely in the present study, as the changes are rather subtle, activity should be closely monitored in future studies. A more detailed assessment of activity and inactivity of mixed-house mice could give interesting insights into sleep patterns and their possible disruption and should enable a distinction between 'inactive and asleep' and 'inactive but awake' [40]. The latter is increasingly being discussed in relation to boredom, and thus compromised welfare [40]. The present study recorded activity on the basis of physical movements only and did hence not assess actual sleep.

Mixed-housed C57BL/6J mice displayed less socio-positive behaviour, but more social exploration compared to the same-strain housed controls. Social avoidance and agonistic behaviours were shown more frequently in mixed-housing conditions compared to same-strain conditions by mice of both strains, which may have welfare implications for these mice [23]. A possible explanation for higher amounts of agonistic behaviours (chasing, fleeing) between mice of different strains involves a kin recognition process, allowing individuals to discriminate between their own strain and the other strain using olfactory cues [13]. Relevant odour cues are emitted from urine, faeces, scent glands, and saliva, and likely allow for the recognition of unknown individuals of the same strain as being familiar to some extent [41]. This process could also explain the finding that, when given a chance for spatial separation and partner choice, C57BL/6J mice chose their own kind over others, unlike DBA/2N mice that did not seem to differentiate between strains. Specifically, mice of the C57BL/6J strain displayed more sociability toward peers of their own strain as opposed to members of the DBA/2N strain. However, rather than reflecting increased sociability toward other C57BL/6J mice *per se*, this behaviour could simply indicate higher sociability toward mice of a similarly social strain. Furthermore, huddling involves at least two mice, with one mouse approaching another one. Since it was not assessed which mouse initiated the huddling, it is unknown which mouse initiated the huddling in the mixed-strain cages. Yet again, C57BL/6J mice also spent markedly more time in the same cage with members of their own strain compared to members of the

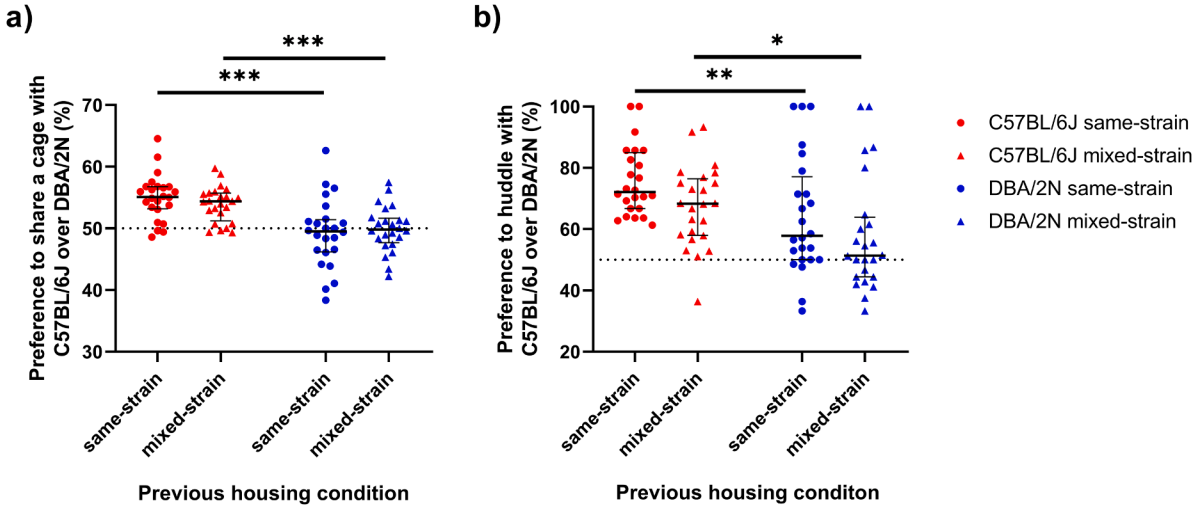

Fig. 5. Level of sociability measured as sharing a cage and huddling with mice of the C57BL/6J compared to mice of the DBA/2N strain in Phase II. **a**) C57BL/6J mice showed higher sociability toward another C57BL/6J mouse compared to mice of the DBA/2N strain in terms of sharing the same cage. Previous experience of mixed-strain experience did not have a significant effect on sociability. **b**) C57BL/6J mice displayed higher sociability toward other C57BL/6J mice in comparison with DBA/2N as measured by huddling. Statistics: Kruskal-Wallis test, Mann-Whitney U test with Bonferroni correction. Sample size: $n = 24$/ group. Scatterplot shows medians with interquartile range. Grey dotted horizontal line indicates chance level. Please note, the vertical (y) axis does not start at zero.

DBA/2N strain, supporting the differences in sociability. Altogether, the observed strain-dependant differences highlight the impact of genotype-specific phenotypes on one another. For future studies, it would be highly valuable to include mice of additional strains, for example Balb/c, since previous investigations found that these mice received the most aggression from their mixed-strain cage mates [42]. At the same time, the involvement of a third strain would enable a more comprehensive investigation of welfare effects in a social setting needed for the implementation of split-plot designs. Furthermore, the fact that mice from different strains differ in their neurochemical profiles [43] leaves the possibility of differential influences on behaviour and sociability in response to mixed-strain housing.

It should be noted though that only female mice were used in this study. In female mice, agonistic behaviours are usually less pronounced compared to their male counterparts [23,24]. Therefore, the present results are not directly transferable to males. A separate experiment is needed to investigate the potential of mixed-strain housing in male mice in order to make conclusions about the general applicability of this paradigm for the purpose of a split-plot design.

In summary, C57BL/6J mice appeared to be more sensitive to their social environment in the mixed-strain housing as they, unlike DBA/2N mice, showed more agonistic and less socio-positive behaviours, resulting in significantly less huddling and more resting alone when housed with the other strain. Consequently, co-housing of C57BL/6J mice with mice of another, less sociable strain [44], could lead to welfare issues due to more agonistic interactions and reduced amounts of socio-positive behaviours [23].

5. Conclusion

Altogether, mixed-strain housing, as a promising approach of a split-plot design, did not induce alterations in anxiety, locomotion, learning, stereotypic behaviour, and stress levels in C57BL/6J and DBA/2N mice. It was, however, associated with differences in activity patterns and altered socio-positive as well as agonistic behaviours in the home cage. Interestingly, mice of the C57BL/6J strain were more affected by the mixed-strain housing than mice of the DBA/2N strain, which is likely a result of the observed sociability of C57BL/6J mice to socialise with members of their own strain. As such, the present findings do not point towards severely compromised welfare, yet neither do they rule out the emergence of behavioural alterations in mixed-strain housed mice that may be associated with mildly impacted welfare. Thus, in order to make full use of the advantages a split-plot design has to offer – including its beneficial effects regarding power, sample size, and reproducibility – the three strains that are the minimum requirement for power benefits have to be very carefully selected to avoid any potential welfare issues [6]. This way, split-plot designs based on mixed-strain housing might considerably advance the quality of scientific research.

CRediT authorship contribution statement

Carina Bodden: Conceptualization, Formal analysis, Project administration, Supervision, Visualization, Writing - original draft, Writing - review & editing. **Maximilian Wewer:** Data curation, Writing - review & editing. **Niklas Kästner:** Project administration, Supervision, Writing - review & editing. **Rupert Palme:** Resources, Writing - review & editing. **Sylvia Kaiser:** Resources, Supervision, Writing - review & editing. **Norbert Sachser:** Resources, Supervision, Writing - review & editing. **S. Helene Richter:** Funding acquisition, Conceptualization, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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