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# Response, use and habituation to a mouse house in C57BL/6J and BALB/c mice

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**Abstract:** Animal welfare depends on the possibility to express species-specific behaviours and can be strongly compromised in socially and environmentally deprived conditions. Nesting materials and refuges are very important resources to express these behaviours and should be considered as housing supplementation items. We evaluated the effects of one item of housing supplementation in standard settings in laboratory mice. C57BL/6J<sup>01a</sup>Hsd (B6) and BALB/c<sup>01a</sup>Hsd (BALB) young male and female mice, upon arrival, were housed in groups of four in standard laboratory cages and after 10 days of acclimatization, a red transparent plastic triangular-shaped Mouse House™ was introduced into half of the home cages. Animals with or without a mouse house were observed in various contexts for more than one month. Body weight gain and food intake, home cage behaviours, emotionality and response to standard cage changing procedures were evaluated. The presence of a mouse house in the home cage did not interfere with main developmental and behavioural parameters or emotionality of BALB and B6 male and female mice compared with controls. Both strains habituated to the mouse house in about a week, but made use of it differently, with BALB mice using the house more than the B6 strain. Our results suggest that mice habituated to the mouse house rather quickly without disrupting their home cage activities. Scientists can thus be encouraged to use mouse houses, also in view of the implementation of the EU Directive (2010/63/EU).

**Key words:** animal facility, environmental enrichment, housing supplementation, inbred strains, mouse house

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## Introduction

A growing interest in animal welfare, both in the scientific community and by the public, has recently drawn attention to animal needs. For the sake of the animals and to improve the quality and reproducibility of results, laboratory animals' well-being (psychological and physical health) needs to be particularly cared for in both husbandry and experimental setting (i.e. breeding, housing, manipulation and experimental procedures) [31].

Although somewhat subjective and not standardized, evaluation of animal welfare is usually based on several parameters including: growth and reproductive parameters (e.g. body weight, food intake, fertility rate), physiological measures (e.g. hormones), behavioural profile (e.g. day-light activity cycles, emotionality, cognition, stereotypical behaviours) and experimenter's subjective evaluation based on his/her experience and knowledge of the biology of the species. Reports in the literature on the effects of novel techniques to improve

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welfare and assessment of good practices are limited to few journals and poorly acknowledged in the methods section [33].

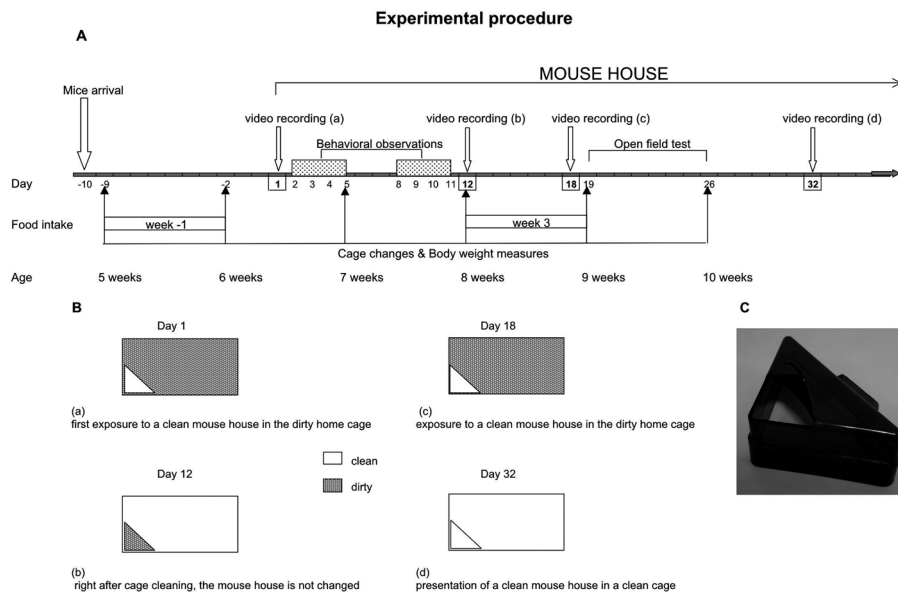
It is now recognized that animal welfare depends on the possibility to express species-specific behaviours [1] and can be strongly compromised in socially and environmentally deprived conditions. To provide proper conditions, personnel training and husbandry facilities for laboratory animals have been improved focusing on sanitation, nutritional needs and enrichment items. All international and national regulations for the accommodation and care of animals used for experimental and other scientific purposes are along this line. The European Directive (2010/63/EU) on the protection of animals used for scientific purposes states that “all animals shall be provided with space of sufficient complexity to allow expression of a wide range of normal behaviour” (article 33, annex III, 3.3. Housing and enrichment) and, in particular, “bedding materials or sleeping structures adapted to the species shall always be provided”(article 33, annex III, 3.6. Resting and sleeping areas). Differently shaped objects can be introduced into the animals’ cages to provide secure locations (i.e. refuges). A protected area can help the individual to temporarily conceal from stress-full stimulations (e.g. aggressive cage-mates, experimenter handling, sudden external noises).

The introduction of objects in the animals’ home cage has already been realized for experimental purposes. Differently shaped objects, together with larger cages and social groups, represent a well known experimental condition firstly recognized by Hebb [9] and then developed by Rosenzweig [20] in laboratory rodents and named “environmental enrichment”. Environmental enrichment (EE) was in fact classically defined [9, 20] as a complex sensory-motor stimulation that provides the animal with an increased opportunity of physical exercise, learning experience and social interactions. Till now many studies have demonstrated that EE induces neurobehavioral changes through changes in gene expression [17, 18, 21, 22, 26]. This experimental condition, highly variable across studies, once applied to animals previously reared in standard husbandry, is able to revert and/or prevent pathological conditions resulting from genetic, environment and pharmacological insults [13]. The results of these studies emphasized thus the importance and the potential therapeutic effects of complex environments and, at the same time, the understimulating/deprived conditions that have been applied

till now to laboratory animals. The opposite experimental condition, being reared from birth in EE and moved to standard (deprived) housing thereafter, resulted in behavioural abnormalities [12].

The relative roles of the increased physical and/or social complexity have been scarcely investigated and it is not possible to extrapolate from studies in the literature the importance of refuges only, to EE induced neurobiological changes. The great variability and complexity that characterize EE studies in terms of additional space, objects and partners provided, are hardly replicable and difficult to be reproduced in laboratories’ and breeders’ animal facilities [5, 28, 32]. In order to harmonize animal housing conditions, several mouse/rat houses, serving as possible nests/refuges, have been produced by different suppliers to easily improve animal welfare and several studies have been conducted to evaluate the animals’ preference [14, 23, 29]. The red transparent mouse house was not always the preferred item by all the strains tested but it is one of the most used in large animal facilities.

The aim of this study was to evaluate whether the presence of the mouse house in the home cage, induced modifications on basic growth and behavioural parameters from adolescence to adulthood. We decided to conduct this study on young animals to give them time to recover from transfer and habituate to the new housing environment, before starting experiments. The mouse houses we used were autoclavable and re-usable, had more than one entry/exit, were large enough to allow more animals to fit inside. These red houses provided the animal a place to hide, as the mice perceive them as being dark and give them a better control of the physical and social environment. Moreover the red transparent material allows the experimenter continuous monitoring of the animals even inside the house. Specifically, two main questions were addressed: 1) Were these objects used by the animals as refuges? 2) Did the introduction of secure refuges modify the behavioural and physiological profiles of the animals? This study was carried out on young males and females of two inbred mouse strains, the BALB and the B6, never exposed to such houses before in their lives. BALB and B6 mice were selected because they are commonly used as reference strains in biomedical research. We found that the use of the houses differed between the two strains, was sex dependent and no major effects were observed on growth and emotionality.



**Fig. 1.** Experimental procedure. (A) Time line of experimental procedure. Salient events along over a month of study: body weight and food intake measures, behavioural observation periods, cage changes and videorecording times at different time points. Age (weeks) of the animals is also indicated. (B) Depiction of different time points/conditions at cage changing times when mice were observed and behaviours scored for 10 min. (C) Mouse house.

## Materials and Methods

### Subjects and housing

C57BL/6J<sup>01a</sup>Hsd and BALB/c<sup>01a</sup>Hsd male and female mice (Harlan, San Pietro al Natisone, UD, Italy) were used in this study. After their arrival, at five weeks of age, animals were weighed, individually ear tagged and housed in groups of four same-sex mice in 1264C Eurostandard Type II cages (26.7 × 20.7 × 14.0 cm - floor area 370 cm<sup>2</sup>, Tecniplast, Buguggiate, VA, Italy) with bedding (Scobis 2, Mucedola, Italy), food (Diet 2018, Harlan, Italy) and water available *ad libitum*. A total of 96 subjects to form 6 cages × strain × sex were used. Cages were cleaned once a week. According to the animal breeder, animals had never been exposed to a mouse house. The animal room had a controlled 12-h light cycle (lights on at 0700 h), lux level (on average 100 lux), temperature (21 ± 1°C) and relative humidity (50 ± 5%). Animals were subjected to experimental protocols approved by the Veterinary Department of the Italian Ministry of Health, and experiments were conducted according to the ethical and safety rules and guidelines for the use of animals in biomedical research provided by the relevant Italian law and European Union Directive (Italian Legislative Decree 26/2014 and 2010/63/EU) and

the International Guiding Principles for Biomedical Research involving animals (Council for the International Organizations of Medical Sciences, Geneva, CH). All adequate measures were taken to minimize animal pain or discomfort.

### Mouse house

A red transparent plastic triangular-shaped Mouse House™ (150 × 110 × 77 h mm, Tecniplast, Buguggiate, VA, Italy, Fig 1C) was introduced in half of the home cages (always in the same position in one of the corners) after a period of 10 days of acclimatization in our animal house facility. The mouse house has two entrances, one on a side with a small tunnel (50 × 25 × 25 h mm) and one on the flat top (60 × 45 × 45 mm). No additional nest material was provided in the cages.

### Experimental procedure

From their arrival the animals were monitored for food intake and body weight. After ten days from arrival, animals in the different experimental conditions, with and without mouse house, were observed on different occasions during the study period: 1) at specific time points during cage cleaning/mouse house cleaning; 2) continuously for eight days (4 + 4 days) during the

first 2 weeks starting the day after the mouse house introduction; 3) in a 5 min open field test to evaluate emotionality after habituation to the mouse house. See Fig. 1A for time line of experimental procedure.

#### *Body weight and food intake*

Once a week, at the time of cage cleaning, each animal was weighed. The total amount of food, pellet consumed before mouse house presentation (week -1) and at the end of behavioural observations (week 3), were measured for each cage.

#### *Behavioural observations at cage or mouse house cleaning*

Each cage was video-recorded (DCR-TRV320E PAL; SONY) for 10 min right after cage or mouse house cleaning procedures (Fig. 1). Four conditions/time points were considered: (a) first presentation of a clean mouse house in the home cage (Day 1), (b) right after cage cleaning (Day 12), the mouse house is not changed, (c) presentation of a clean mouse house in the dirty home cage (Day 18), (d) presentation of a clean mouse house in a clean cage (Day 32). Due to a technical problem the video recording of a cage on day 12 was missing. Video files were analysed by an expert observer. The number of mice (max 4) displaying specific behaviours directed either towards or outside the mouse house were simultaneously scored for each cage/condition by observational sampling every 15 seconds. Behaviours directed to the house: approach, contact, inside (in), through top or side entrances (doors), on top of the mouse house (on), digging toward the mouse house (dig), i.e. "burying behaviour". Behaviours not directed to the mouse house (outside): general activity outside the mouse house such as grooming, climbing to the roof of the cage, fighting, feeding or drinking. No fighting events however occurred during the observation sessions.

#### *Behavioural observations during habituation to the mouse house*

Behavioural observations were also conducted on four continuous days/week during the first two weeks of habituation to the mouse house (Days 2–5 and 8–11, Fig. 1A). Two daily 30-min sessions of observations were conducted in the animal facility room (from 0830 h to 0900 h and from 1230 h to 1300 h) without moving the cages from their racks standing at a distance of 60–80 cm from the cages. The experimenters started

collecting data 10 min after entering the room to habituate the animals to their presence. Behavioural sampling consisted of recording the behaviour of the four animals of each cage simultaneously once every 5 min for a total of six observations  $\times$  session, 12 observations a day, for a total of  $12 \times 8$  observations per cage. The following behaviours for each subject were recorded on check sheets as resting (IN or OUT) or active in contact with the house. No fighting events occurred during the observation sessions.

#### *Emotionality*

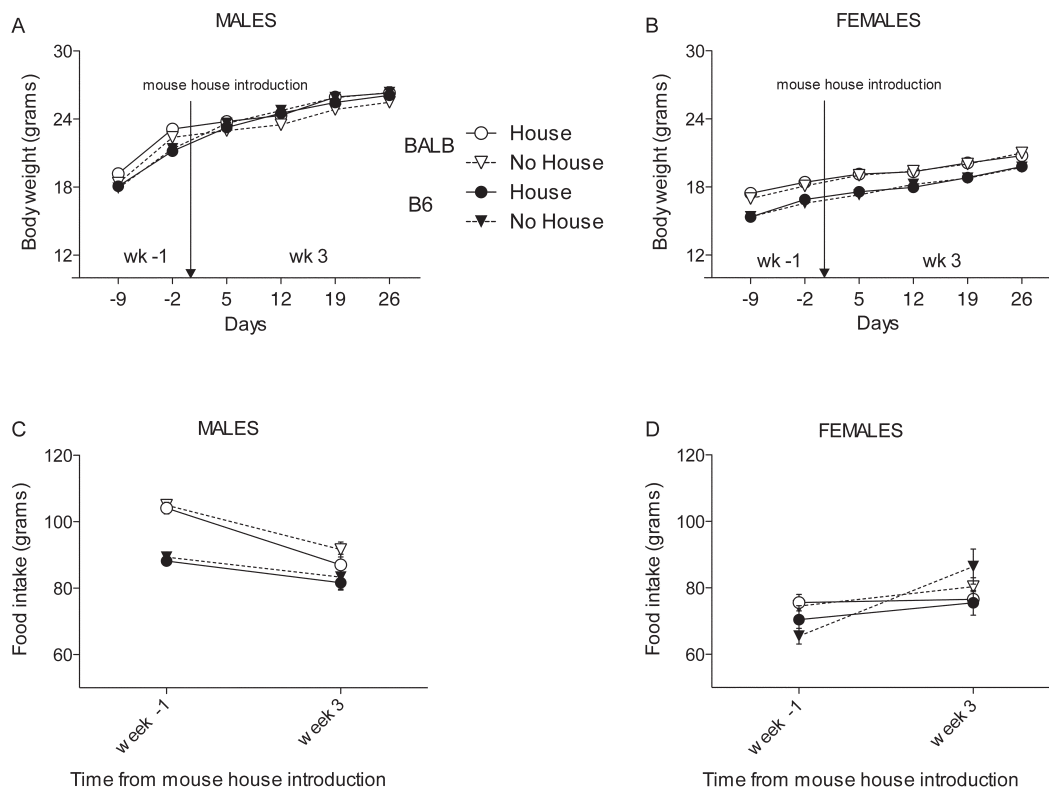
Emotionality was measured in an open field test (5 min) between days 19 and 26 after mouse house introduction (Fig. 1A). Each mouse was introduced in a clean squared arena ( $60 \times 60 \times 25$  h cm) and left free to explore for a 5 min period. Each session was video recorded and the percentage of time and number of entries in the centre of the arena were used as dependent variables using an automated video-tracking system (Smart, PANLAB, Cornell, Spain).

#### *Statistical analysis*

The sum of frequencies of single behaviours per mouse was calculated for each cage across four days/conditions (Fig. 1B) and during the 8 days of observation pooling AM and PM data (Days 2–5 and 8–11, Fig. 1A). Cluster analysis of variables (oblique principal components) [7], principal component analysis (PCA) [19], and inferential analyses (Chi-square and ANOVA) were performed using the SAS System (Cary, NC). Statistical significance was set at  $P < 0.05$ .

Multidimensional statistics (variable clustering, PCA) had as variables the cages and as statistical units the behavioural measures, allowing for a well-conditioned data structure (statistical units outnumbering variables) and, analogously to the approach used in gene expression microarray [19] pattern recognition, instead of separately considering the different behavioural aspects had as main focus the discrimination between an invariant behavioural pattern (or size component) and specific differences between profiles (shape components), classically registered by minor components [10].

The cages are considered as specific profiles in terms of behavioural variables and their mutual correlations gave rise to both cage clustering and PCA structure. The interpretation of the components was based upon the measures with the highest scores on the different com-



**Fig. 2.** Body weight and food intake. (A-B) Body weight and (C-D) food intake, in respectively male and female mice of BALB and B6 strains with and without a mouse house measured before and after mouse house introduction. Data are expressed as mean  $\pm$  SEM.

ponents [19] while the inferential statistics were based upon chi-square (relation between cage cluster and strain and time respectively) and ANOVA having as source of variation strain and time and as dependent variable component loadings. Repeated measures (RM) ANOVA, performed on data from the eight observations (this in the usual mode having cages as statistical units and behavioural descriptors as variables) had time (RM) and strain, sex and house presence as sources of variation.

## Results

### *Body weight and food intake*

The presence of the mouse house did not affect body weight and food intake in either males or females of both strains (Fig. 2). No effect of strain, mouse house presence or their interaction was detected in male's body weight increase. A significant time effect is evident during development ( $F_{5,222}=872.64$ ,  $P<0.001$ ) with a significant strain  $\times$  time effect ( $F_{5,220}=18.67$ ,  $P<0.01$ ) with B6 males showing, in comparison with BALB males, lower body

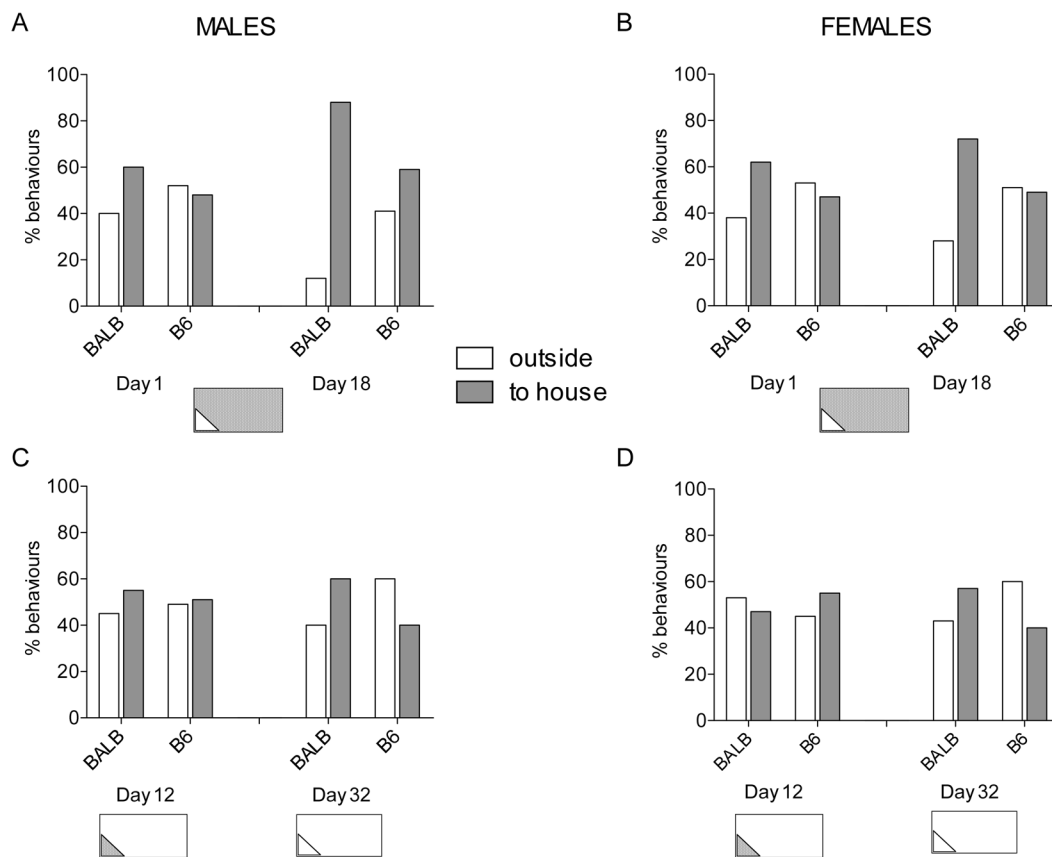
weight upon arrival and similar weight at the end of the experiment. No other significant effects emerged.

Females' body weight increase is shown in Fig. 2B. The three-way ANOVA indicates significant effect of strain ( $F_{1,220}=55.00$ ,  $P<0.001$ ), time ( $F_{5,220}=444.72$ ,  $P<0.001$ ) and time  $\times$  strain ( $F_{5,220}=4.10$ ,  $P<0.01$ ). The presence of the mouse house did not significantly affect female body weight gain, neither as main nor in interaction with other independent variables.

Food intake measured per cage during the week before mouse house introduction (week -1) and on week 3 (Figs. 2C and 2D) did not seem to be affected by the mouse house.

### *Behavioural observations at cage or mouse house cleaning*

Mouse behaviour during the routine cage cleaning was observed to evaluate if the animals habituate to the mouse house. Salient behaviours directed outside or towards the mouse house were scored (Fig. 3). In general no differences in behavioural profile was observed



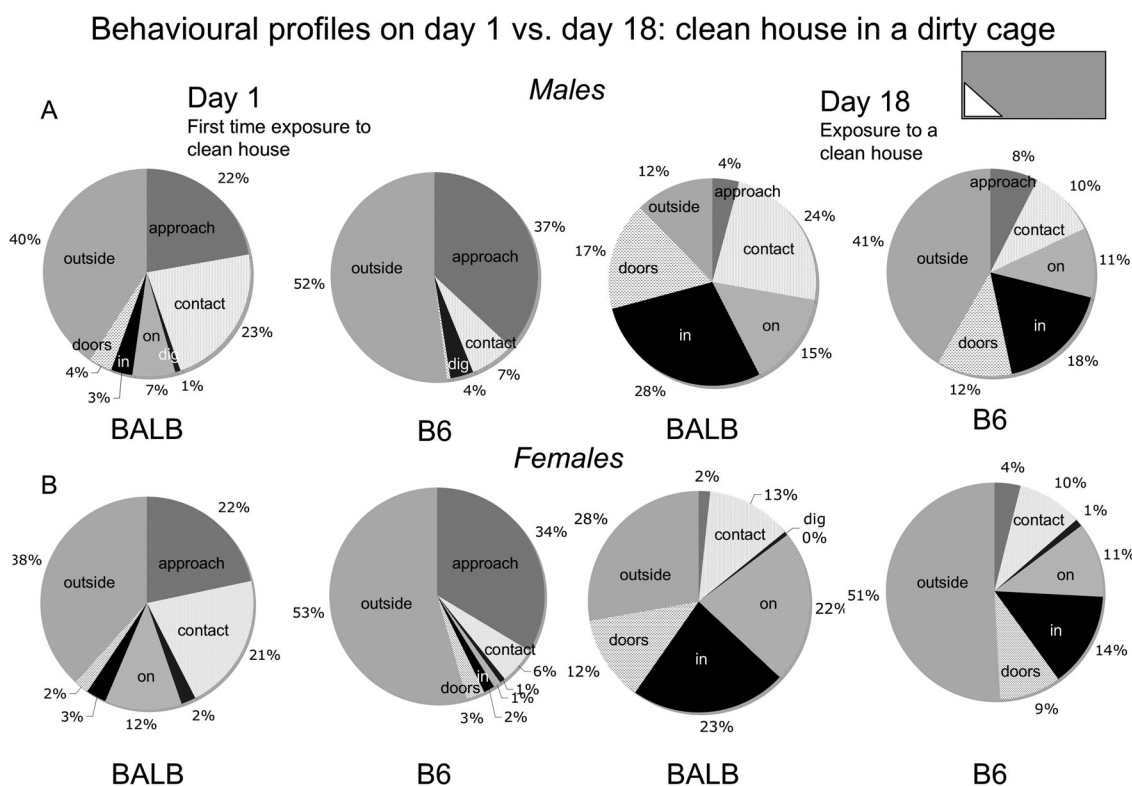
**Fig. 3.** Behaviours observed right after cage changing. Percentage of behaviours performed outside or directed to the house in respectively (A-C) male and (B-D) female BALB and B6 mice observed at cage or mouse house changing times. (A-B) Day 1 and Day 18 clean mouse house in a dirty home cage. Day 12, dirty (C) or Day 32, clean (D) mouse house in a clean cage.

between males and females regardless of strain in the four experimental conditions/time points (Figs. 4 and 5). However, different behavioural profiles depending on the strain or the experimental condition, emerged from the variable cluster analysis (Oblique Principal Components). The clustering is driven by the construction of cluster made of variables (behavioural profiles relative to different cages) maximally correlated among them and independent from the cages pertaining to other classes. Cages clustered in four groups (Supplementary Table 1) with a 94.8% of total variation explained. The obtained clustering was demonstrated to have a statistically significant relation with both strain and day/condition (Supplementary Table 2; Strain:  $\chi^2=8.716$ ,  $P<0.05$ ; Day:  $\chi^2=55.38$ ,  $P<0.0001$ ).

Having demonstrated the general relation between 'behavioural profile' and both strain and time in an unsupervised way (we only based on general resemblance

between profiles as emerging from cage clustering) and the inferential analysis was applied *a posteriori* on the obtained 'natural' clusters, we go more in depth into the interpretation of the nature of the differences.

We compared mouse behaviour on DAY 1 (first exposure) with DAY 18, when clean mouse houses were introduced in dirty home cages (Figs. 3A–3B, males and females respectively). All mice directed between 40 and 60% of their behaviours to the mouse house on DAY 1 with BALB mice spending slightly more time with the house. A detailed behavioural profile (Fig. 4) showed that B6 mice approached the house from a distance whereas BALB mice touched, climbed on top or even entered the house within the first 10 min of exposure. On DAY 18 (Figs. 3A and 3B), contrary to B6 mice, BALB males and females increased the frequency of interaction with the mouse house (about 80%) making contact, climbing, entering, or staying inside the mouse house,



**Fig. 4.** Behavioural profiles on day 1 vs day 18: clean house in a dirty cage. Percentage of behaviours scored in respectively (A) male and (B) female BALB and B6 mice observed upon first exposure to a clean mouse house (Day1) and to same cage/house condition on Day 18 of exposure. The percentage of behaviours performed outside the house (grooming, climbing to the roof of the cage, fighting, feeding or drinking) and the percentage of behaviours directed to the house (approach, contact, digging, on top, inside, through the doors) are shown.

therefore reducing the approach behaviour (Fig. 4).

When a mouse house was introduced in a clean cage (Figs. 3C and 3D), the behaviour of both mouse strains was very similar when the house was dirty (DAY 12). However, on DAY 32, when both cages and houses were clean, the two strains seemed to behave differently towards the house with BALB showing about 60% of behaviours directed to the house, compared to about 40% in B6 mice. The presence of the clean mouse house in a clean cage (DAY 32) did not affect BALB mice behaviour compared to DAY 12, whereas B6 mice spent more time outside. However in B6, after 32 days of exposure to the house, direct physical contact with the clean house was observed with almost no approach from a distance (Fig. 5).

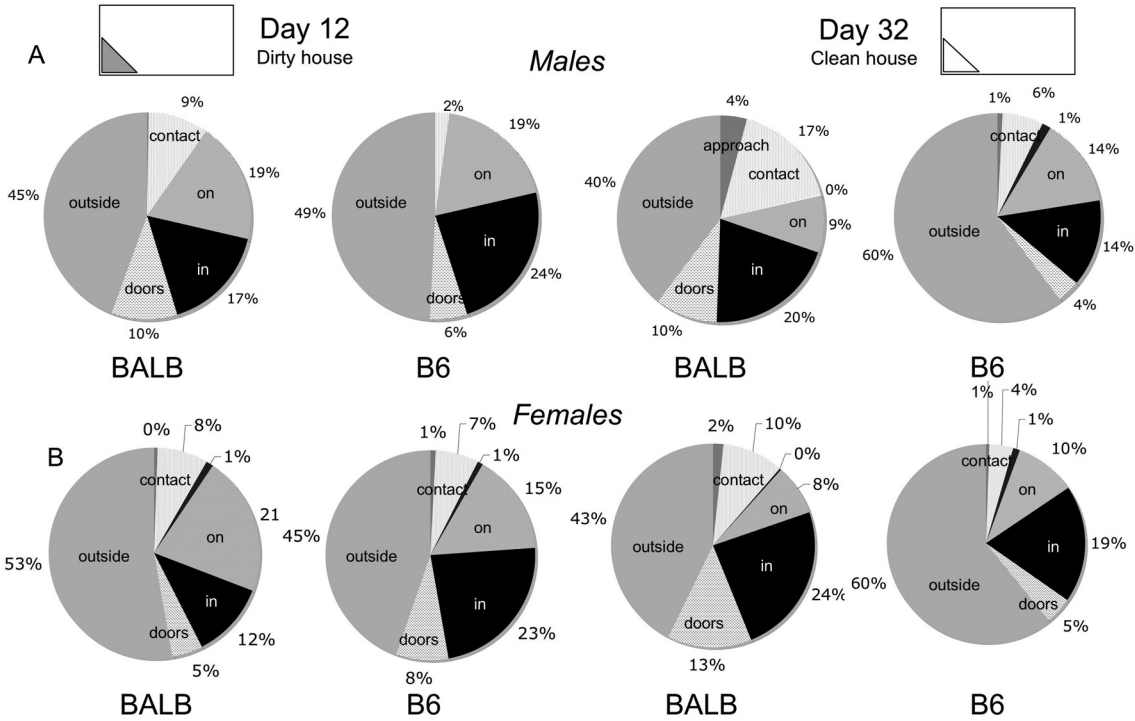
In conclusion, B6 mice appeared to be more ‘cautious’ than BALB every time they were exposed to a clean mouse house, even after 32 days of familiarization with this object.

In order to have a more comprehensive view of the

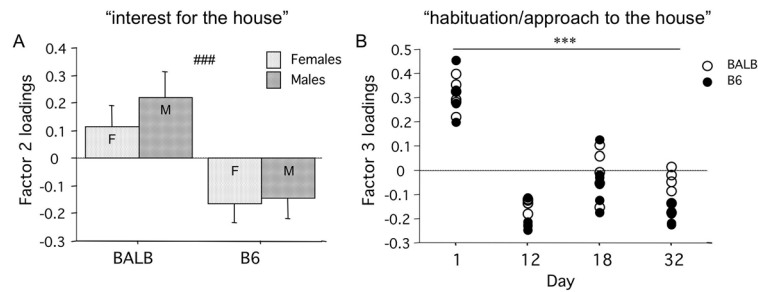
above description, a PCA was computed over the data set, again having cages as variables and behavioural measures as statistical units. This data organization will lead the loadings to play the role of dependent variables for Analysis of Variance and component scores as the descriptors of the relative weight, of each specific behaviour, in the component meaning [4, 19].

PCA gave rise to a three-component solution accounting for 97.9% of the total variance (Supplementary Table 3). Factor 1 accounted for 84.07% of the variance which is clearly a size component [10] having all positive (and near to unity) loadings and thus representing the commonality among all the cages. The subsequent analysis of the scores (Supplementary Table 4) showed for Factor 1, variables related to “general activity” both towards and outside the house. It is worth noting that variables like “out” and “house” pointing to an opposite direction of motion have the same score sign as for Factor 1 giving a proof of concept of the interpretation of Factor 1 as “general activity”. This interpretation is re-

Behavioural profiles on day 12 vs. day 32: dirty vs. clean house in a clean cage



**Fig. 5.** Behavioural profiles on day 12 vs. day 32: dirty vs. clean house in a clean cage. Percentage of behaviours scored in respectively (A) male and (B) female BALB and B6 mice observed upon exposure in a clean cage, to a dirty mouse house (Day 12) and to a clean house on Day 32 of experiment. The percentage of behaviours performed outside the house (grooming, climbing to the roof of the cage, fighting, feeding or drinking) and the percentage of behaviours directed to the house (approach, contact, digging, on top, inside, through the doors) are shown.



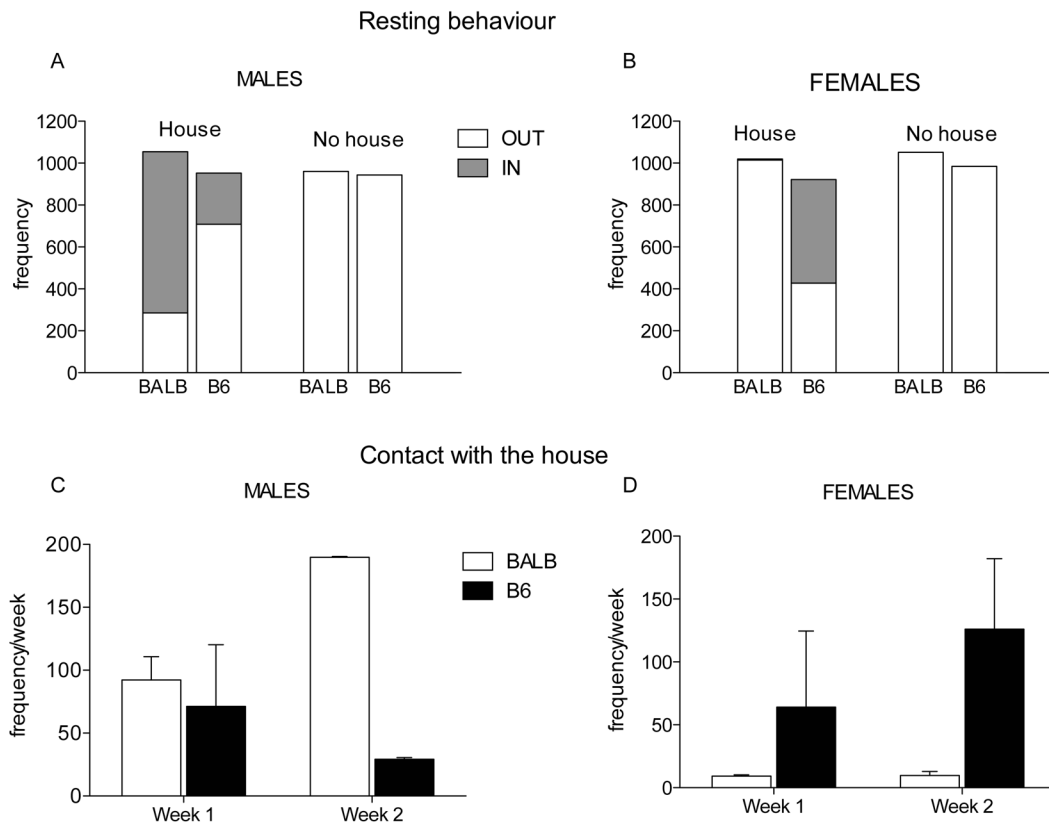
**Fig. 6.** Principal Components Analysis: Factor 2 and Factor 3 loadings. (A) Loadings for Factor 2 (9.45% of variance) that was interpreted as an “interest in the house” factor. ###  $P < 0.0005$  BALB vs B6, ANOVA. Data, from Supplementary Table 3, are expressed as mean  $\pm$  SEM (n=3 cages per sex/strain). (B) Loadings for Factor 3 (4.38% of variance) that was interpreted as ‘habituation/approach to the house’ and varied over time, \*\*\* $P < 0.0001$ , ANOVA. Each point represents one cage (data from Supplementary Table 3).

inforced by a relatively high score of the “active” variable. The presence of a common “size component” [4, 10] accounting for more than 80% total information is the image in light of the presence of a “strong invari-

ant core” of behavioural profile across different cages and thus giving a proof-of-concept of the reliability of the proposed strategy.

Factor 2 (9.45% of variance) is a shape component





**Fig. 7.** Resting and contact with house behaviours. Frequency of resting behaviours (A-B) and contact with the mouse house (C) observed in male and female mice of BALB and B6 strains during the two weeks of habituation to the mouse house. Resting behaviour frequency was scored when occurring either in or out of the house (A-B). Data are expressed as mean ( $\pm$  SEM) frequency of contacts per week (C).

(both positive and negative loadings with cages), this implying we are no more measuring the “commonality” (components are each other independent by construction) but the profile differences between cages. Factor 2 showed a positive and high score mainly for the behaviours directed toward the house while outside had a high but negative score with Factor 2 (Supplementary Table 4). This allows us to interpret this Factor has a preference toward “near house” activity with respect to outside and could be interpreted as an “interest for the house” factor (Fig. 6A).

Factor 3 (4.38% of variance) is again a shape component (both positive and negative loadings), and showed higher scores for “approach” behaviours and high but negative score for behaviours “in” the house (Supplementary Table 4). We interpreted Factor 3 as a sort of ‘habituation/approach to the house’ that varied over time (Fig. 6B).

An ANOVA performed on the Factors showed a sig-

nificant difference between strains for Factor 2 (“interest to the house”,  $F_{1,43}=16.18$ ,  $P<0.0005$ ; Fig. 6A) and among days for Factor 3 (“habituation/approach to the house”,  $F_{1,46}=41.15$ ,  $P<0.0001$ , Fig. 6B). This suggests that BALB and B6 have a different “interest” in the house and that in general the ‘approach’ or interaction with the house changes over time. Interestingly, a statistically significant difference due to sex was never observed.

#### *Behavioural observations during habituation to the mouse house*

In general, it was observed that the presence of the house did not affect the total amount of home cage resting behaviour (Figs. 7A and 7B). However, strains and sex showed different preference in the use of the house for resting. In particular, BALB male mice, but not females, preferred to sleep inside the house (ca 70% vs. 0%). On the other hand, B6 male mice rested inside the

house about 25% of the total frequency of resting behaviours compared to about 50% in females. The RM ANOVA showed that BALB rested more than B6 mice ( $F_{1,18}=6.78$ ,  $P<0.05$ ). It is of note that resting behaviour showed a very high frequency in general since animals were observed during the light phase of the light/dark cycle. As for the contact with the house (Figs. 7C and 7D), the RM ANOVA showed a significant interaction (sex  $\times$  strain  $\times$  week:  $F_{1,8}=6.86$ ,  $P<0.05$ ). Only two groups of mice (BALB males and B6 females) increased their contact with the house between the two weeks of habituation. On the other hand, BALB female mice inside or in contact with the house showed no change in frequency between the two weeks of habituation and as for B6 males, their contact with the house was even reduced from the first to the second week.

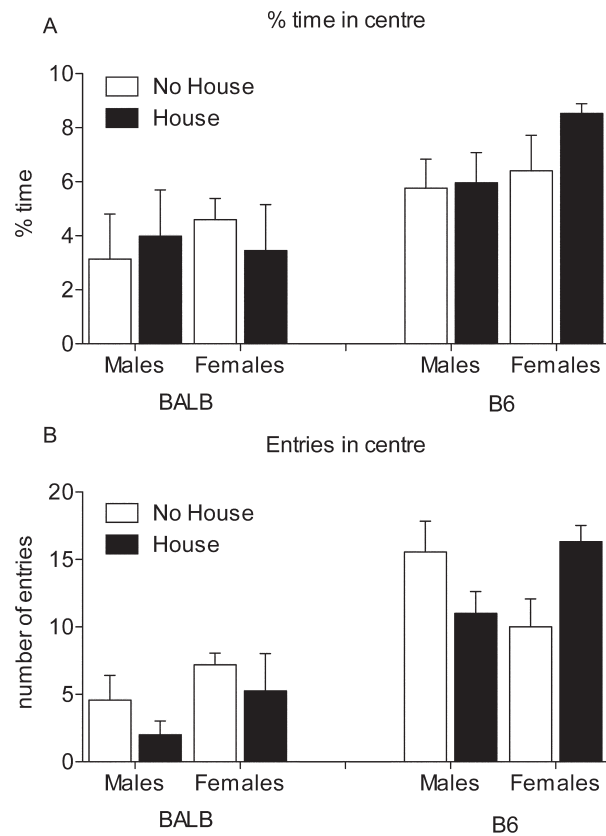
### Emotionality

Exposure to the mouse house did not affect emotionality as measured by number of entries and percentage of time spent in the centre of the open field (Fig. 8). A 3-way ANOVA indicated a statistically significant effect for the factor strain only for the number of entries in the centre of the arena ( $F_{1,59}=35.67$ ,  $P<0.0001$ ). Neither sex, nor house factors, significantly affected mouse behaviour in the open field and no interaction reached statistical significance.

## Discussion

This study demonstrates that the presence of a mouse house in the home cage did not interfere with main developmental and behavioural parameters of BALB and B6 mice. Additionally, both strains habituated to the house in about a week. Even if a general invariance of the behavioural profiles was observed pointing to a high level of commonality (accounting for around 80% of total variance) across cages, we were able to single out subtle but statistically significant differences between strains as for the use of house.

The presence of the mouse house did not affect body weight gain or food intake, neither in males nor in females of both mouse strains (Fig. 2). Some differences observed between strains or sexes in food intake were within the expected range at that age [15]. This result is particularly relevant considering that animals in this study were observed during development, from young age (five weeks) to adulthood (10 weeks), and moving



**Fig. 8.** Emotionality (open field test). Percentage of time spent (A) or number of entries (B) in the centre of the open field in male and female mice of BALB and B6 strains exposed (House) or not exposed (No House) to a mouse house for about three weeks. Data are expressed as mean  $\pm$  SEM.

developing animals from the supplier breeding colony to the new facility, may have a strong impact on animal physiology and behaviour.

Behavioural observations conducted during cage/house changing, indicated that BALB mice made contact with the house much more than B6, especially from the very first day of exposure (Fig. 4). Contrary to what is expected on the basis of literature, that considers B6 less anxious than BALB mice in several behavioural tests [2, 6, 11], the B6 seemed more “cautious” toward the house from the beginning (high approach behaviour on Day 1) and almost ‘indifferent’ at the end of the experiment. These mice showed no approach and a low percentage of behaviours directed to the house in comparison with the BALB mice (Figs. 3–5). Other strain differences were also observed in the use of the house (Fig. 7): BALB male mice preferred to sleep inside the house more than B6 males, suggesting that they con-

sider it as a protected area. We had confirmation from the supplier that animals of this study had never been exposed to the house before. Therefore we can state that they habituated to it in about one week and from the second week onward they showed a stable “interaction” with the mouse house.

Sex differences were observed in the use of the house during habituation. In particular, BALB males’ contact with the house increased overtime while BALB females did not use it, even for sleeping, possibly reflecting lower anxiety levels compared to males. High variability observed in B6 behaviour did not allow a clear interpretation, but our data suggest a time  $\times$  sex opposite trend in this strain (Fig. 7). Interestingly, not so many sex differences in behaviours directed to the house were observed during cage/house changing (Figs. 4–6). It should be noted however that active behaviours (contact with the house) occurred in a very low frequency compared to resting behaviours (Fig. 7), due to the fact that behavioural observations were conducted during the light phase of the light/dark cycle, as already mentioned.

Mice emotionality, after three weeks from mouse house supplementation was also evaluated using the open field test (Fig. 8). A lower number of entries in the central part of the apparatus was considered as an index of higher emotionality during exploration of a new environment [2]. Our findings confirmed that BALB mice entered less frequently in the centre of the arena as reported extensively in the literature to support strain differences in emotionality [8, 27]. Similar results were reported elsewhere [24] indicating that BALB mice housed with a shelter did not change their strain-dependent behavioural phenotype.

Data discussed above seem to be discordant with the idea of “anxious” BALB as reported here (open field test) and in the literature. However, this finding stresses the importance of considering the context while measuring emotionality. In effect, BALB mice general activity was reported higher than the B6 ones, when measured in the home cage [3, 25], but lower in an unfamiliar environment [6]. Limits and inconsistency of association between standard anxiety tests (open-field, elevated plus-maze and emergence tests) in mice has been discussed [11] and an attempt to correlate behaviour with physiological parameters (heart rate, body temperature) failed to reach successful results in BALB mice [6]. B6 seemed more “cautious” and dug more on day 1. Digging in this case could be considered as an avoidance behaviour [16]

or alternatively as a strain specific behaviour by which B6 have been considered “burrowers” as opposed to BALB considered instead as “surface nestlers” [30].

According to the EU Directive, cage enrichment should be adopted by all animal facilities and thus represents the new standard for all laboratory animals. It is thus important that breeders and laboratory animal facilities agree to use similar cage enrichment, such as a refuge/nest, to reduce stress and time of habituation when animals are transferred among facilities. Transfer of animals between laboratories for scientific purposes is a very common procedure due to the greater interdisciplinary approach of current research that needs multiple competence, not easily available in a single institution. The introduction of a mouse house, such as the one described here could represent an easy and relatively inexpensive way to standardize cage environments among animal facilities. Therefore, the mouse house inserted in the home cage from the arrival of the animals, is part of the physical environment after a few days and does not elicit additional investigation/exploration, also when removed and substituted with a clean one. This would reassure scientists that this implementation does not modify the animal behavioural profile and body weight gain, in comparison with animals tested in the absence of the mouse house.

New regulations on the use of animals for scientific purposes recommend and promote the use of alternative models, without imposing an *a priori* definition of standardized environment, and it stresses the importance of providing adequate housing conditions to improve animal welfare. However, some concern was raised especially within the behavioural neuroscience community, about the effect of arbitrary cage enrichment on variability among laboratories, and reproducibility of experimental findings [5, 28, 32].

Based on our findings, these are a few suggestions for scientists facing the adoption of housing supplementation in their animal facilities to improve animal welfare. Firstly, be informed about the housing conditions animals had before arriving at the facility, i.e. whether any kind of environmental enrichment/supplementation was used. As far as we know, suppliers do not use any type of mouse house, and if they use environmental enrichment in breeding colonies, most often they limit it to nest paper. It would be a good practice for animal suppliers to communicate if and which type of housing supplementation is used in their facilities. Secondly, according

to our results, one week was sufficient in five week-old mice, to habituate to a mouse house. Thirdly, the presence of the mouse house did not modify the home cage resting and feeding profile of our experimental groups. Fourthly, even if a difference in mouse house use is reported in this study between strains and sexes, no objection for the use of the house can be raised, as it did not alter typical behavioural profiles.

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