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# An examination of nest-building behaviour using five different nesting materials in C57BL/6J and BALB/c mice

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## Abstract

*The aim of our study was to assess the nest-building behaviour of two mouse (*Mus musculus*) strains using different nesting materials and examine possible sex- and housing-specific effects. Adult mice of two strains (C57BL/6J; n = 64 and BALB/cAnNCrl; n = 99) were randomly allocated to the following housing groups: single-housed male, single-housed female, pair-housed male and pair-housed female. One of the following nest-building materials was placed in each home-cage in a random order: nestlets (Plexx BV, The Netherlands), cocoons (Carfil, Belgium), wooden wool, crinklets and compact (all three, Safe, Germany). The following day, nests were rated applying a nest-scoring scale ranging from 0 to 10, the nests were removed, and a different nest-building material provided. In both tested strains, nestlets achieved the highest nest-building scores when compared to the other four nest-building materials. All nest-building materials scored higher in BALB/c mice compared to C57BL/6J animals reaching statistical significance in crinklets only. Sex comparison revealed that female C57BL/6J mice only scored significantly higher using crinklets than males and BALB/c female mice were rated significantly higher using wooden wool, cocoons and compact than their male counterparts. While pair-housed C57BL/6J animals built higher-rated nests than single-housed mice in the C57BL/6J strain in all five materials tested, the scores were not significantly different in the BALB/c strain. Results of the present study reveal significant strain-, sex- and housing-related influences on the complexity of nests using different standardised building materials. Such observations need to be taken into account when planning the optimal enrichment programme for laboratory animals.*

**Keywords**: *animal welfare, behaviour, enrichment, laboratory mice, nest building, nest-building materials*

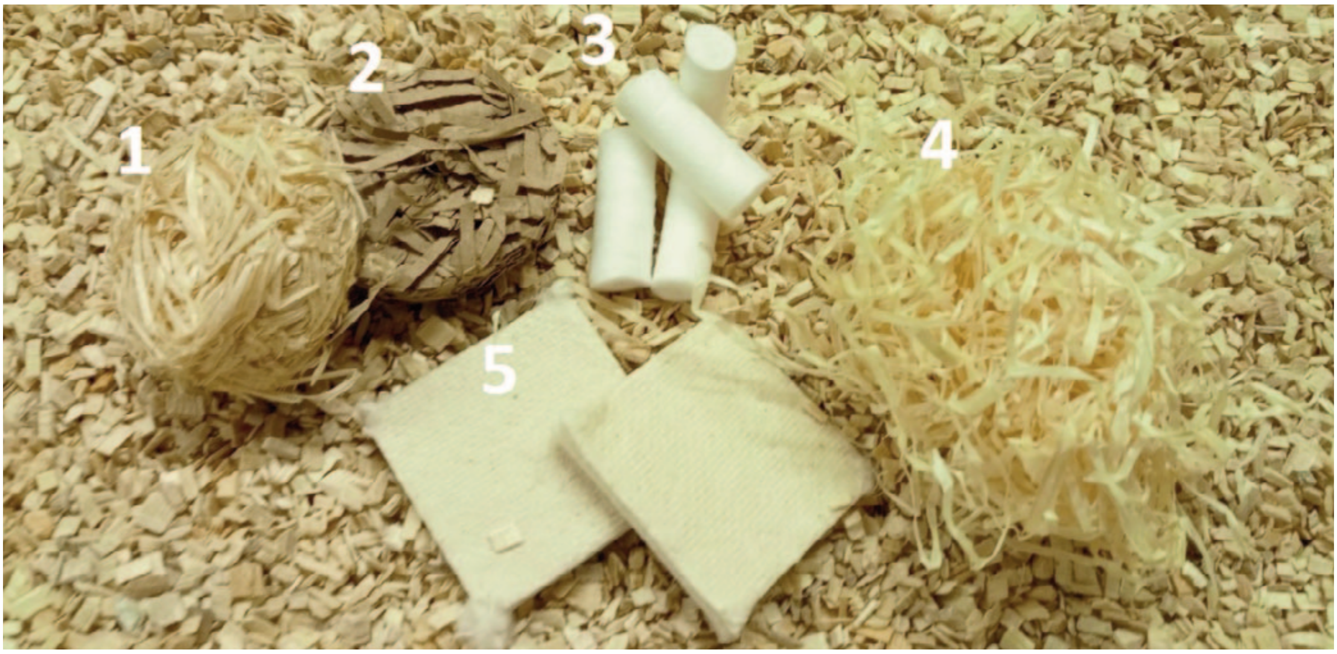
## Introduction

The mouse (*Mus musculus*) represents the most commonly used animal in research. Driven by economic needs (laboratory space, equipment and resulting costs), ergonomics (ease of handling, visibility of animals) and hygiene (easiness to sanitise) these animals have been housed in small cages equipped with bedding material, food and water (Bailoo *et al* 2018). Nevertheless, the principles of 3Rs (replacement, reduction, refinement) governs the provision of environmental stimulation by providing optimal housing conditions (Aske & Waugh 2017). This is supported by European Union (EU) legislation that states "any restrictions on the extent to which an animal can satisfy its physiological and ethological needs are kept to a minimum" (European Union 2010).

To provide optimal housing conditions associated with more comfort than simply regulated temperature and humidity, a growing number of research facilities use nesting materials such as nestlets, wooden wool and fizzle nests. These readily applicable materials facilitate thermoregulation, provide shelter and enable the mice to express their specific nest-building behavioural repertoire (Bult & Lynch 1997; Olsson & Dahlborn 2002; Gaskill *et al* 2012). Concerns that this form of environmental stimulation might negatively influence results in scientific studies have proven unsubstantiated. The scientific literature overwhelmingly supports the notion that housing conditions of laboratory mice can be markedly improved without affecting the standardisation and variability of results (Wolfer *et al* 2004).

In order to choose the optimal enrichment programme, consideration must be given to the possibility that the effects of environmental enrichment on mice behaviour are strain-specific. Goto *et al* (2015) have shown a significantly different nest-building behaviour in three different murine strains (C57BL/6NCrl, DBA/2NCrlCrlj and B6D2F1/Crl) using a 3-D depth camera and a conventional scoring system. Moreover, genetic modification also seems to influence nest-building behaviour. For instance, impaired nesting behaviour was noted by a group using nestlets with mice lacking Dvl1 (a homologue of the *Drosophila dishevelled* gene) (Lijam *et al* 1997) and in Vitamin D receptor mutant mice (Keisala *et al* 2007) in comparison to their unmodified controls. Consequently, it would appear to be a mistake to approach mouse enrichment as a one-size-fits-all husbandry procedure (Bayne 2018).

**Figure 1**




Comparison of the five nest-building materials used in the study: 1) compact, 2) crinklet, 3) cocoons, 4) wooden wool and 5) nestlets.

While the strain-specific effects of different enrichment programmes have already been studied, the influence of provision of different nesting materials on building behaviour remains largely unknown. Additionally, the impact of sex and housing condition remains to be elucidated. Therefore, the aim of the present study was to assess the nest-building behaviour of two mouse strains for different nesting materials. Moreover, possible sex- and housing-specific effects were assessed. We hypothesised that different nest-building materials influence building behaviour examined by nest complexity scoring of laboratory mice. Additional hypotheses were that strain, sex as well as housing conditions significantly influence building behaviour.

## Materials and methods

### Study animals

Adult female (non-pregnant) and male mice of two different strains (C57BL/6J; n = 64 [31 male and 33 female] and BALB/cAnNCrl; n = 99 [70 male and 29 female]) with mean ages of 24 (C57BL/6J) and 23 weeks (BALB/c) were used for the nest-building experiments. Mice were borrowed from other researchers performing unrelated studies and were untreated at the time of housing experiments. C57BL/6J were bred in-house while BALB/c were ordered from the Medical University of Vienna, Austria. Prior to inclusion into the nest-building experiments and during the study, all animals were housed in IVC cages with 60 air changes per hour (Green Line Type II long GM500®, Techniplast, Buguggiate, Italy) measuring 365 × 207 × 140 mm (length × width × height) (Euro Standard) with *ad libitum* access to food (Rat/Mouse maintenance 10 mm V1534-000, Sniff Spezialdiäten GesmbH, Soest, Germany) and water and at a 12 h light: dark cycle (lights went on at 0500 and went out at 1700h) under temperature (22 [± 2]°C) and humidity (55 [± 10]%) control. Standardised irradiated bedding material (Scobis Quattro Bags, Mucedola, Italy) was used for all mice. Prior to inclusion into the nest-building study all mice received standard nesting material (nestlets [n = 1], Plexx BV, The Netherlands and cocoons [n = 2], Carfil, Belgium) as conforming to 3Rs regulations and facility standards.

### Experimental procedure, nesting materials

At the beginning of the nest-building investigation, the standard nesting material was completely removed and mice put into clean cages with new bedding material. Thereafter, mice were randomly allocated to the following housing groups: single-housed male; single-housed female; pair-housed male; and pair-housed female. Housing conditions remained unchanged for the mice throughout the study and mice were not reused for another group.

C57Bl/6J mice were kept in single housing in 22 cages (eleven male and eleven female) and kept in pair housing in 21 cages (ten male and eleven female). BALBc mice were single-housed in 33 cages (24 male and nine female) and pair-housed in 33 cages (23 male and ten female). At 1600h one of the following five different nest-building materials was placed in the front third of the home-cage in a random order: nestlets (made of cotton; two pieces per cage), cocoons (short cotton fibres; four pieces per cage), wooden wool (4 g per cage, Safe, Rosenberg, Germany), crinklets (made of unbleached brown kraft paper; one piece per cage, Safe, Rosenberg, Germany) and compact (made of pressed softwood shavings, one piece per cage, Safe, Rosenberg, Germany) (Figure 1).



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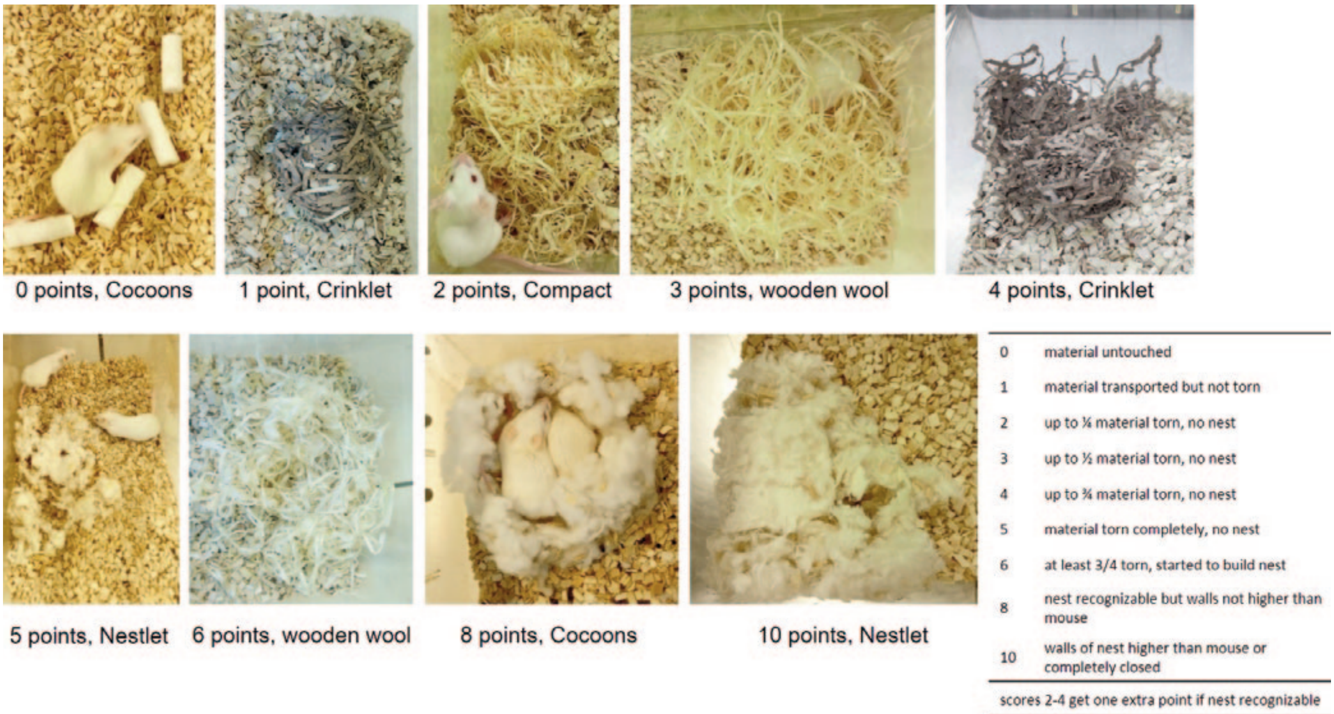
**Figure 2**




Representative examples of the nest-building score in the five different nest-building materials used.

At 1000h on the following day, the nests were photographed and their complexity assessed (always by the same examiner; BO) applying a weighted standardised ordinal scaled nest-scoring system ranging from 0 points (did not interact with nesting material) to 10 points (built complete nest). Exemplary assessments are depicted in Figure 2. A more detailed overview is presented in Supplement 1 (see supplementary material to papers published in *Animal Welfare*: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material). Following removal of the old nest-building material, a different nest-building material in a random order was placed into the cages at 1600h and the following morning the score was assessed again. This procedure was performed twice, always providing the same sequence of the five nest-building materials per cage. The mean of the two scores was calculated for each nesting material.

Ethical review and approval were not required for the animal study since local legislation decreed the animals were receiving no treatment causing any pain, suffering or fear as per Section 1 §2.1a of the Austrian TVG 2012. None of the animals used was ordered for this study and the work was covered by Section 1 §1 (2)3 of the Austrian TVG 2012.

## Repeatability analysis

For repeatability analysis, a randomly chosen subset of 60 photographs was assessed a second time by BO. Moreover, two additional raters (CC, GS) underwent training rating 50 randomly chosen photographs under the supervision of BO. Thereafter, they independently scored the same subset of 60 photographs scored twice by BO. All raters were blinded for the scores of the first measurement and the other raters' scores, respectively.

## Statistical analysis

Data were managed in a Microsoft Excel® spreadsheet. For statistical analysis, IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY, USA) was used. A Friedman test was applied as a dependent test for the comparison of the nest-building scores for the five nest-building materials within each strain examined. In case of global significances this was followed by Wilcoxon signed-rank tests applying a Bonferroni correction for multiple pair-wise comparisons. Pair-wise comparisons of independent variables (sex and housing conditions) were calculated with a Mann-Whitney *U* test. The assessment of the combination of sex and housing in both murine strains was performed with a Kruskal-Wallis test in search of global differences. In case of significant differences, this was followed by a Mann-Whitney *U* test with Bonferroni correction to adjust for multiple testing. Non-parametric tests were chosen because of the ordinal structure of the nest-building score in accordance with the literature (McCrum-Gardner 2008). *P*-values of $< 0.05$ were considered statistically significant.

To assess intra-observer reliability (BO), the intra-class correlation coefficient (ICC) estimates and its 95% confidence interval (CI) were calculated based on a single measure, absolute agreement, two way mixed-effects model (Koo & Li 2016). Inter-observer reliability (three raters) was assessed with ICC (2,1) estimates and their 95% CI based on a single measure, absolute agreement, two way random-effects model (Koo & Li 2016). Interpretation was as follows: < 0.50, poor; between 0.50 and 0.75, moderate; between 0.75 and 0.90, good; above 0.90, excellent (Koo & Li 2016).

## Results

In a first comparison, strain-specific building behaviour was assessed for the five different nest-building materials not considering sex or housing. C57BL/6J mice built significantly more complex nests with nestlets compared to all other materials ($P$ < 0.001 for each pair; Wilcoxon test corrected for multiple testing). In BALB/c mice, nestlets scored significantly higher than wooden wool ($P$ < 0.001; corrected Wilcoxon test), cocoon ($P$ = 0.010; corrected Wilcoxon test) and compact ($P$ = 0.005; corrected Wilcoxon test) (Figure 3[a]). In BALB/c mice, nest-building scores were significantly higher compared to C57BL/6 in crinklets only ($P$ = 0.028; Mann-Whitney $U$ test; Figure 3[b]).

Figure 4 depicts differences between the sexes for the nest-building materials in both strains. While female C57BL/6J mice only scored significantly higher than males ($P$ = 0.028; Mann-Whitney $U$ test; Figure 4[a]) using crinklets, BALB/c female mice were rated significantly higher using wooden wool ($P$ = 0.034; Mann-Whitney $U$ test), cocoons ($P$ = 0.003; Mann-Whitney $U$ test) and compact ($P$ = 0.029; Mann-Whitney $U$ test) than their male counterparts (Figure 4[b]).

In the subsequent comparison, nest-building behaviour was examined according to housing conditions (single or pair). Pair-housed C57BL/6J mice reached significantly higher nest-building scores than their single-housed littermates for nestlets ($P$ = 0.001; Mann-Whitney $U$ test), wooden wool ($P$ = 0.013; Mann-Whitney $U$ test), crinklets ($P$ = 0.047; Mann-Whitney $U$ test) and compact ($P$ = 0.039; Mann-Whitney $U$ test) (Figure 5[a]). The comparisons of the scores between single- and pair-housed BALB/c mice did not reach statistical significance (Figure 5b).

Figure 6 shows the comparison of the combination of sex and housing in both murine strains. In C57BL/6J mice, females in pair-housing uniformly scored the highest in all five nest-building materials tested and the scores of nestlets, cocoons and crinklets reached statistical significance. Furthermore, C57BL/6J pair-housed animals always scored higher than single-housed animals except with cocoons where hardly any differences were found between male single- and pair-housed scoring. BALB/c animals showed significant differences for wooden wool, where single-housed females scored highest and for cocoons where pair-housed females built the most complex nests. More detailed data on all comparisons are provided in Supplement 2 (see supplementary material to papers published in *Animal Welfare*: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material).

In order to assess the reliability of our nest-building score we have performed repeatability analyses. The ICC for the intra-observer reliability of rater 1 (BO) was excellent being 0.99 (95% CI 0.98–0.99). The ICC for the inter-observer reliability of the three raters was excellent being 0.95 (95% CI 0.92–0.97).

## Discussion

The most important finding of our study was that the type of nest-building material provided for mice in laboratory conditions seems to influence the nest-building behaviour. These findings were not only strain-specific but also depended on sex and housing (single or pair).

Standard laboratory cages are designed to fulfil laboratory animals' basic needs. These include the provision of food and water, bedding material, correct temperature and humidity and are regulated by legislation ensuring animal welfare. Nevertheless, laboratory mice undoubtedly have behavioural and social needs that extend beyond the basic requirements that cannot be met in non-enriched standard cages (van de Weerd *et al* 1994). In their *Guide for Care and Use of Laboratory Animals*, the National Research Council (2011) has defined that "the primary aim of environmental enrichment is to enhance animal well-being by providing animals with sensory and motor stimulation, through structures and resources that facilitate the expression of species-typical behaviours and promote psychological well-being through physical exercise, manipulative activities, and cognitive challenges according to species-specific characteristics."

A plethora of publications have already demonstrated the positive effects of environmental enrichment in laboratory mice. These effects include, amongst many others, enhanced neurogenesis and learning, amelioration of cognitive and behavioural deficits and prevention of preterm birth in inflammatory mouse models (Garthe *et al* 2016; Suemaru *et al* 2018; Schander *et al* 2020). The quality of scientific studies using laboratory animals depends on the least possible variability and the highest possible repeatability. Historically, these prerequisites have fuelled concerns that enriched animals may produce results with a high inter-subject variability. Nevertheless, it has been shown that environmental enrichment increases neither individual variability in behavioural tests nor the risk of obtaining conflicting data in replicate studies and the presence of enrichment does not interfere with the main developmental and behavioural parameters of mice (Wolfer *et al* 2004; Wirz *et al* 2015). It can also be argued that animals from enriched environments may be more physiologically and psychologically stable and consequently are better representatives of their species, ensuring more realistic data collection and results (Benn 1995; Van de Weerd *et al* 1997; Baumans 2005). Taken together, the importance of environmental enrichment in laboratory animals' cages cannot be overstressed and by providing enrichment, we are able to increase the animals' welfare without increasing experimental variation (Baumans 2005; Würbel & Garner 2007; Bayne & Wurbel 2014; Andre *et al* 2018). Consequently, we sought to examine the nest-building behaviour of two strains of mice in different housing conditions.

There is a general agreement that environmental enrichment programmes should be tailored to the species of animal of concern (Bayne & Wurbel 2014). Additionally, novelty of enrichment through rotation or replacement of items should be considered (National Research Council 2011). Both



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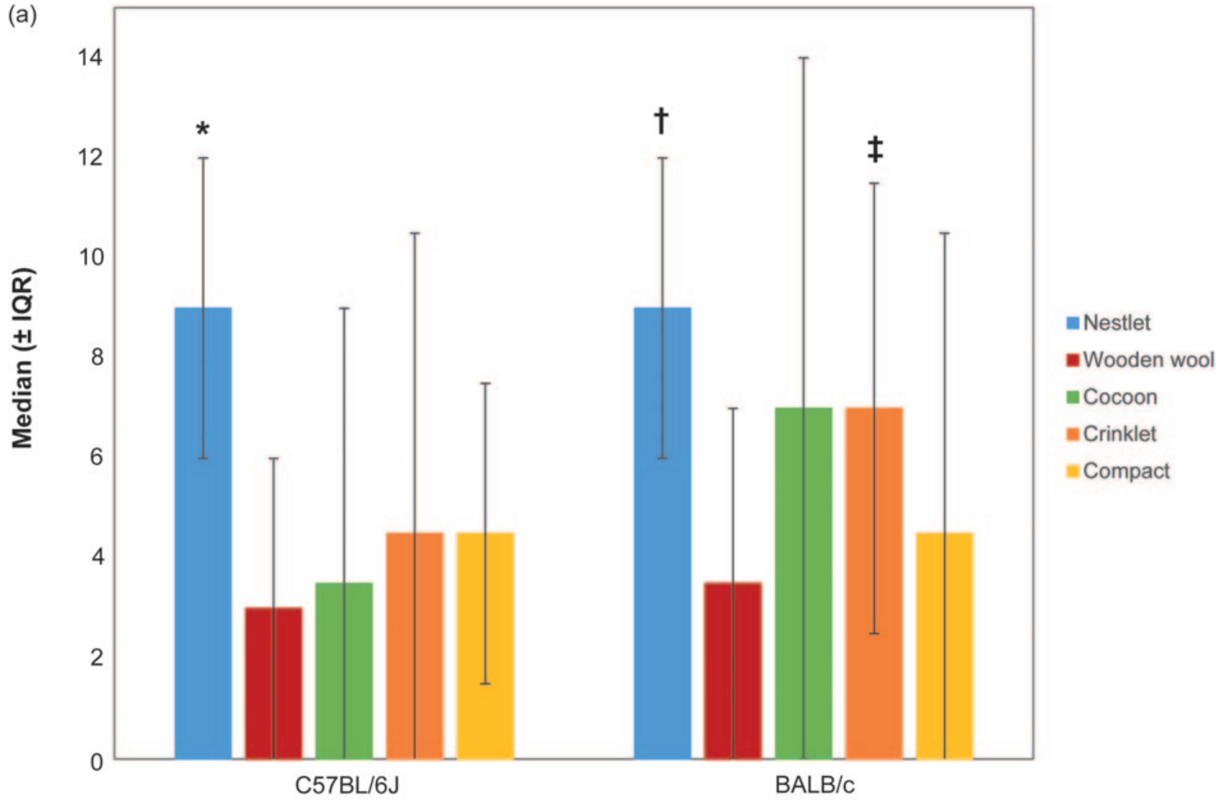
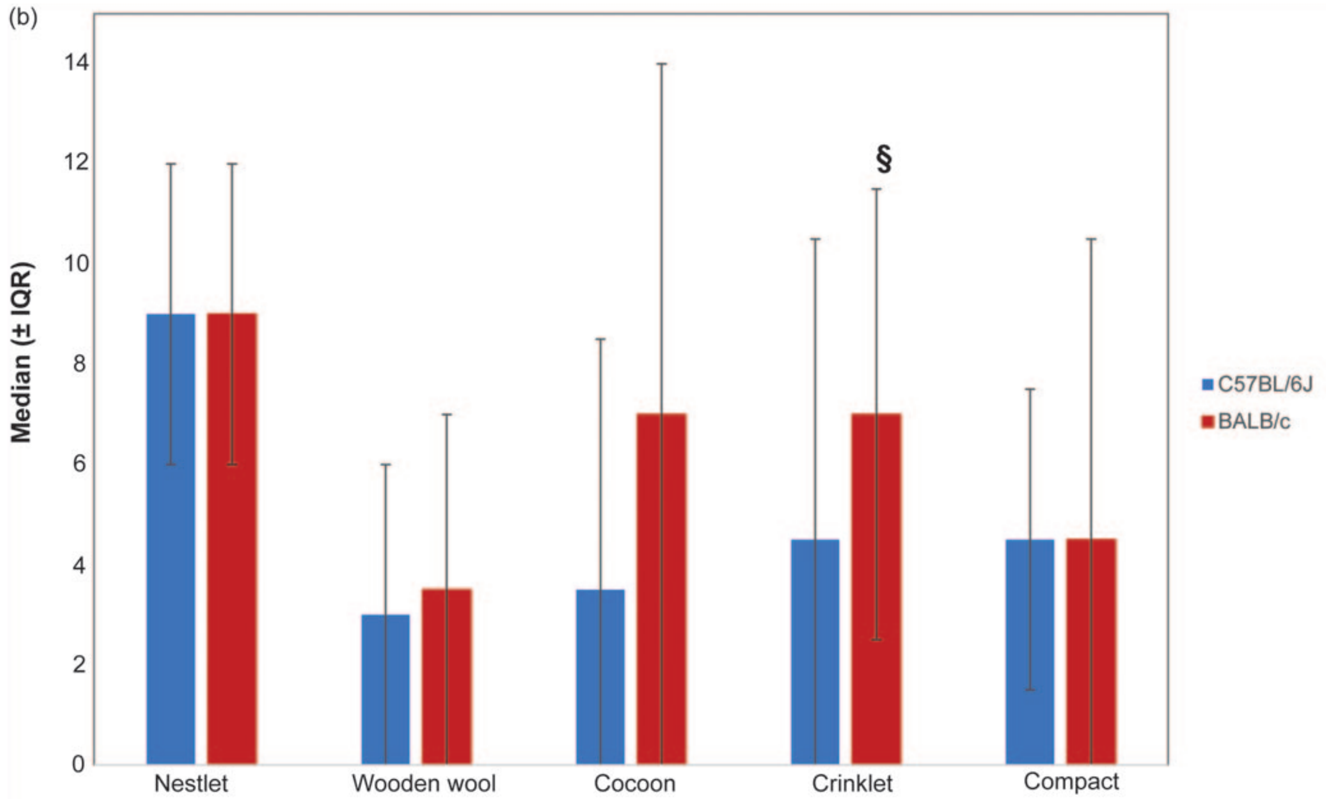
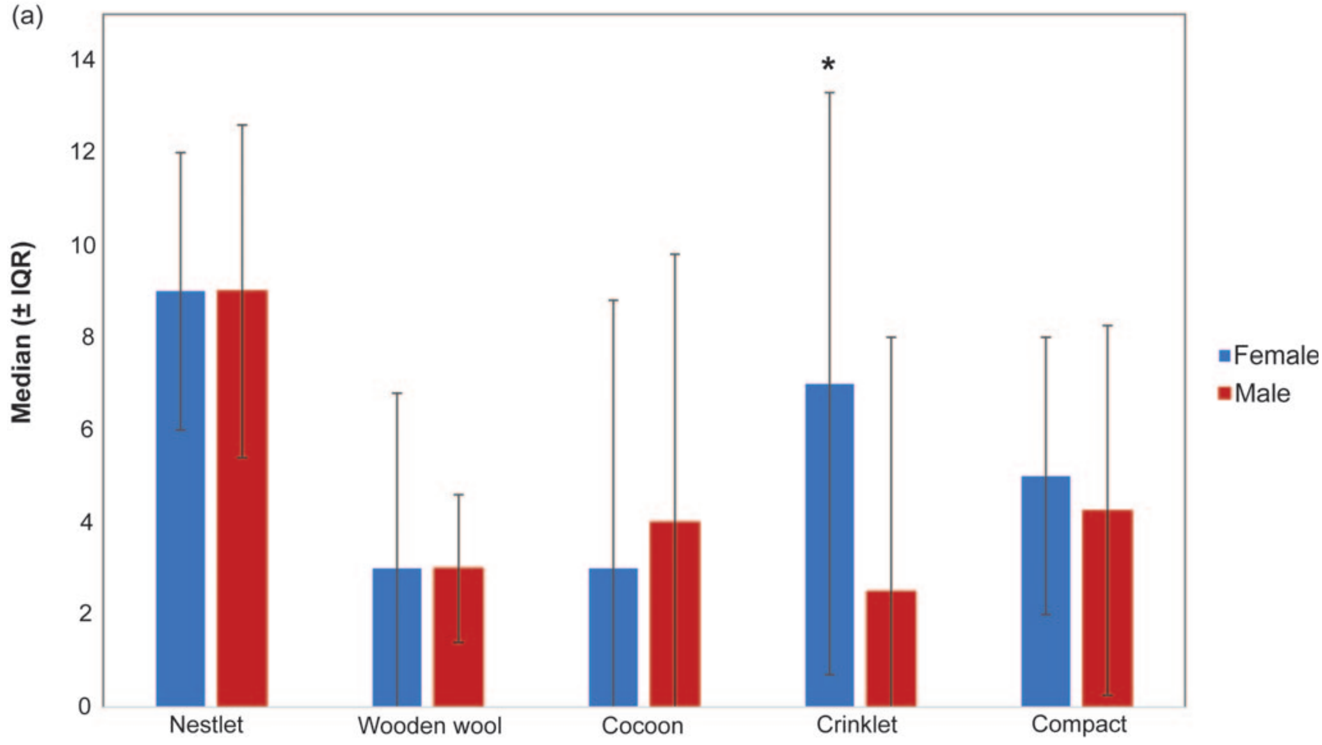
**Figure 3**

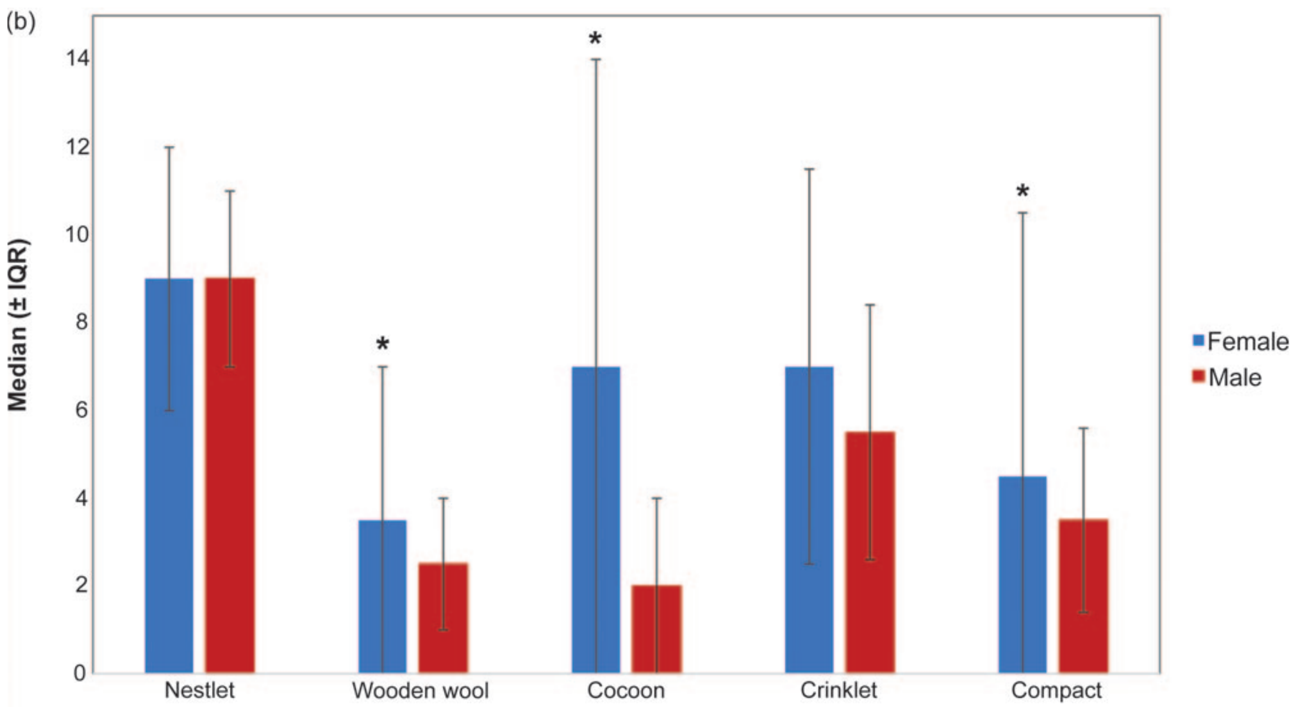



Nest-building scores of five different nest-building materials in two murine strains with respect to (a) mouse strain and (b) type of nesting material. * $P < 0.05$ versus all other four corresponding nest-building materials; † $P < 0.05$ vs wooden wool, cocoon and compact; ‡ $P < 0.05$ vs wooden wool; § $P < 0.05$ versus corresponding strain. Data are presented as median (± IQR).

**Figure 4**




Influence of sex on nest-building scores in (a) C57BL/6J and (b) BALB/c mice for the five nest-building materials used; * $P < 0.05$ vs corresponding sex. Data are presented as median (± IQR).



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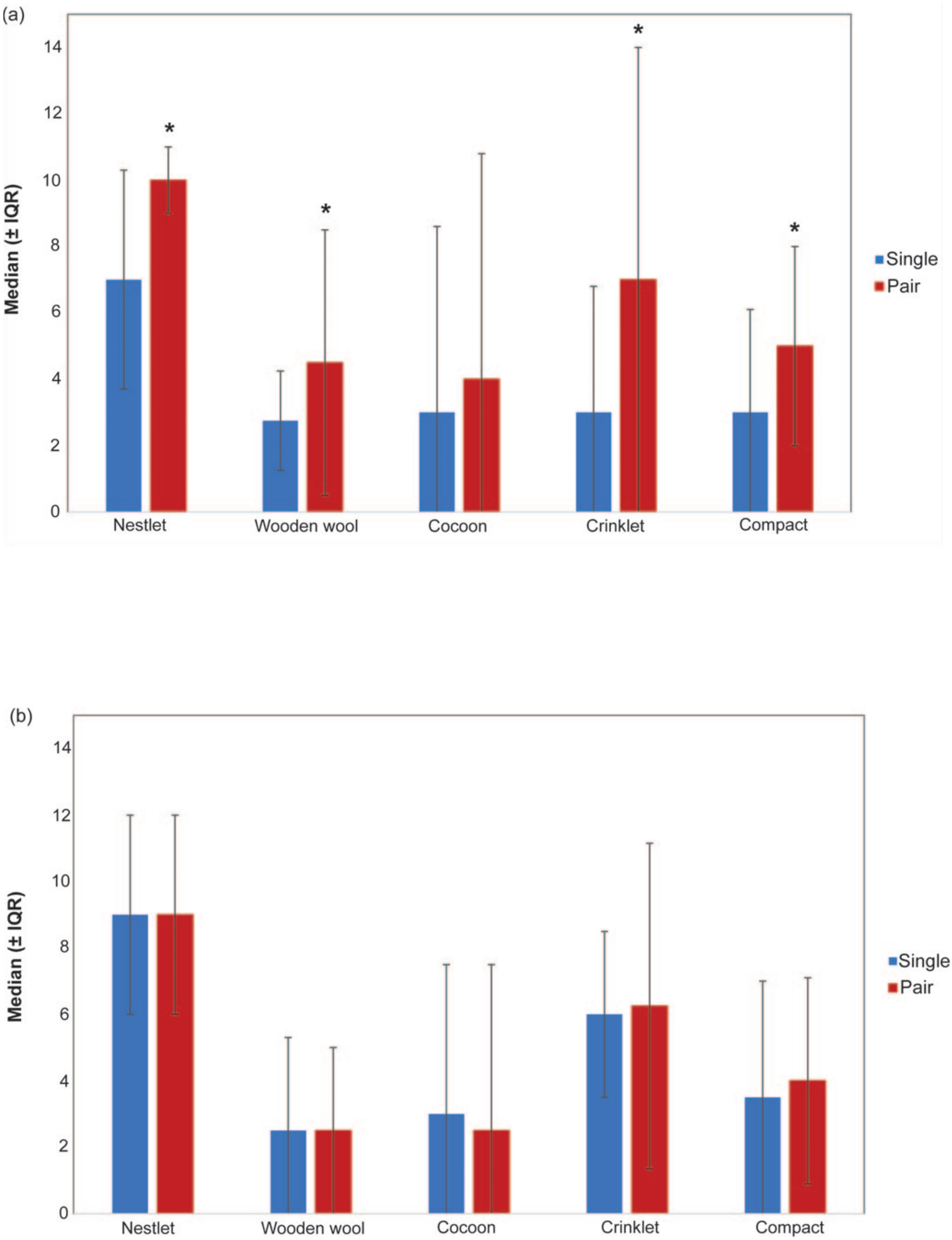
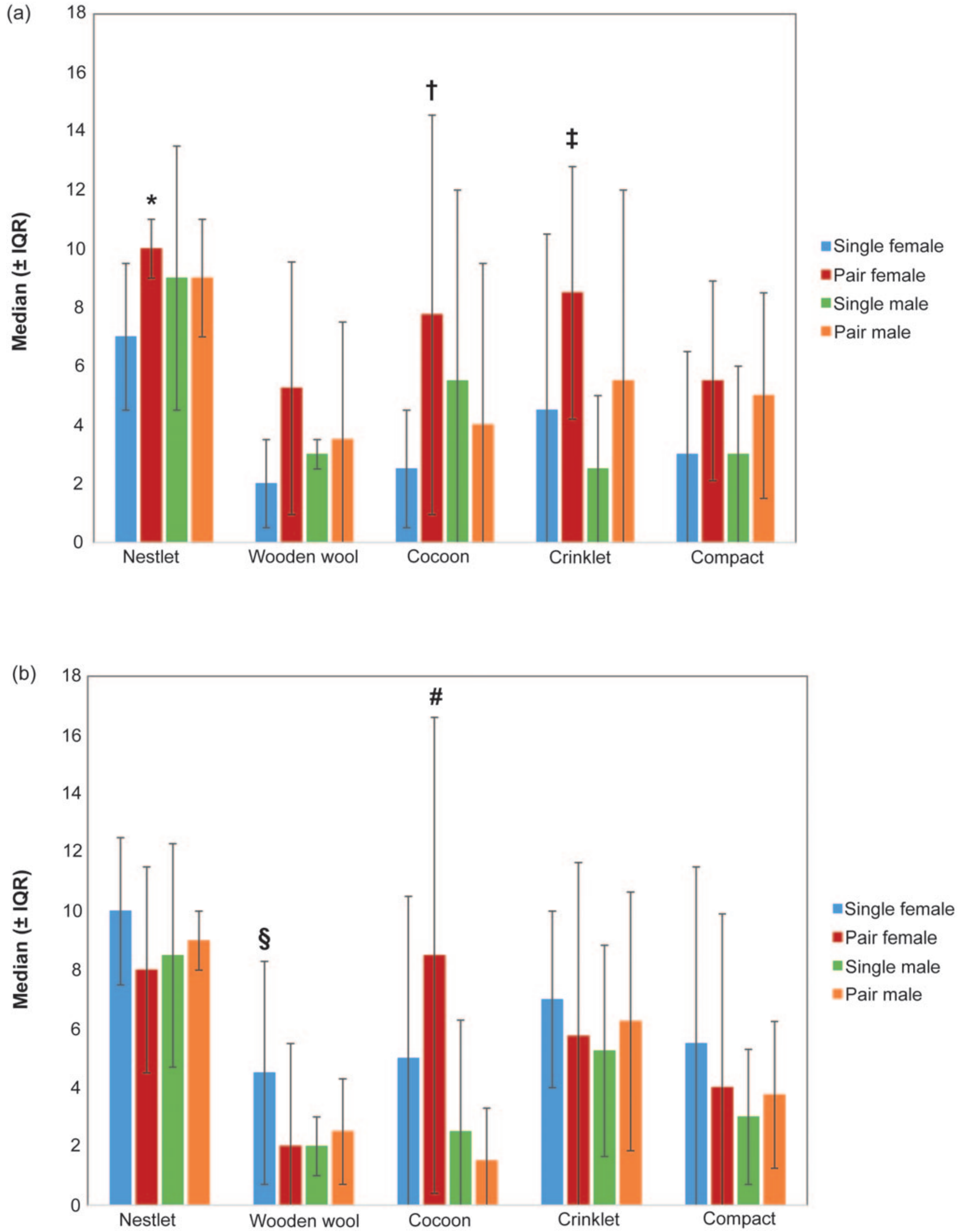
**Figure 5**




Influence of housing on nest-building scores in (a) C57BL/6J and (b) BALB/c mice in the five nest-building materials used; * $P < 0.05$ vs corresponding housing regimen (single- or pair-housed). Data are presented as median (± IQR).

**Figure 6**




Comparison of nest-building scores according to the combination of sex and housing in (a) C57BL/6J and (b) BALB/c mice in the five nest-building materials used; * $P < 0.05$ vs single female and single male; † $P < 0.05$ vs single female; ‡ $P < 0.05$ vs single male; § $P < 0.05$ vs all other housing groups of the same nest-building material; # $P < 0.05$ vs pair-housed male. Data are presented as median (± IQR).



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these demands require a profound understanding of animals' preferences for different nest-building materials (Latham & Mason 2004). This understanding is even more important since there are reports describing negative effects, such as increased fighting with particular murine strains provoked by certain nest-building materials (Kaliste *et al* 2006). Therefore, we have chosen to include five different ubiquitously available nesting materials in our analysis (see Figure 1) and found significant differences concerning their impact on nest-building behaviour. While the animals seemed to prefer nestlets to build more complex nests, wooden wool consistently scored much lower values. Other reports, albeit using different nest-building materials, confirm our results. For instance, Van der Weerd *et al* (1997) examined six different nesting materials (three different paper materials and three different wood materials) in a preference test. The authors used a multiple housing system with interconnected cages equipped with the different nest-building materials and compared dwelling times per cage. All mice showed a clear preference for cages with tissues or towels as compared to paper strips or no nesting material and for cages with cotton string or wooden wool as compared to wood-shavings or no nesting material. Another report has examined the location of the nest built with different materials and described a strong tendency for animals to build nests in the same location used for sleeping prior to the nesting material being provided (Sherwin 1997). In our study, we have chosen a different approach and have not provided the different materials simultaneously but consecutively and have assessed the complexity of the nests as a sign of material preference.

Regarding the two strains chosen, it has to be stated that while BALB/c mice are real nest-builders and surface nesters, C57BL/6 are diggers and make holes. As such, the two strains differ in their behaviour (Van Oortmerssen 1971). This can be seen as a potential reason for the different nest-building behaviour with certain materials (cocoon and crinklet). In our study, female BALB/c but not female C57BL/6J mice built more complex nests than males. Other investigations examining potential influences of strain and sex on nest-building performance have yielded conflicting findings. Xiong *et al* (2018) have described lower nest-building scores for male BL/6 mice compared to females. In contrast, other studies performed with C57BL/6J and/or BALB/c mice found no significant differences in preference between the strains and/or sex (Van de Weerd *et al* 1997; Hess *et al* 2008). Likewise, Schwabe *et al* (2020) have assessed nest building in rats in two different facilities and have not found robust and consistent influence of sex. Therefore, the importance of considering variables such as sex and strain on experimental design variables in future work on environmental enrichment has already been underlined (Aujnarain *et al* 2018).

In our investigation, C57BL/6J and not BALB/c pair-housed mice scored higher nest-building scores than single-housed animals of the corresponding strain. In Uba6 brain-specific knock-out mice (NKO), pair-housing significantly improved nest-building behaviour compared to single-housing (Kim *et al* 2019). In contrast, in an investigation by Jirkof *et al* (2012) in C57BL/6J and C57BL/6J-TyrC-Brd female mice, the authors describe post-surgical home cage behaviour including time spent with nest building. They report no distinct negative effects of single- versus pair-housing in the immediate post-operative period.

A variety of different methods have been applied to measure nest-building performance in laboratory animals, with most of them rating the quality of the nests (Deacon 2006). These range from crude systems (nest absent or present), or systems grading 0 for no nesting, 1 for incomplete nesting and 2 for complete nesting, to different Likert Scales (Jirkof *et al* 2013). For example, Deacon (2006) suggested a five-point nest-rating scale. However, their score only includes detailed description for pressed cotton waivers (nestlets). Likewise, Xiong *et al* (2018) described a nest-building score only applicable for white paper used as enrichment. In 2008, Hess *et al* (2008) presented a naturalistic nest-scoring system and describe an ICC of 0.97 without giving further details on how the ICC was derived. They found that C57BL/6J mice built higher quality nests with shredded paper strips than with facial tissues and compressed cotton squares. Furthermore, Van Loo and Baumans (2004) presented a validated scoring system for nest building in rats. This score was used by Schwabe *et al* (2020) resulting in an ICC of 0.54. Therefore, the authors developed a more detailed training and scoring system. Applying this system, the ICC could be raised to 0.79. However, the latter (Van Loo & Baumans as well as Schwabe *et al*) scores were evaluated in rats and therefore may or may not be applicable in mice. Therefore, we chose to use a more complex weighted scale with values ranging from 0 to 10 points (compare Figure 2 and Supplement 1; https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material). We are aware that a different amount of work has to be invested to build nests from different materials. To account for this bias, however, our score is largely based on the percentage of material torn. Moreover, in order to validate our score, we have performed repeatability analyses and were able to describe excellent ICCs for both intra- and inter-rater reliability.

Limitations of the present study include the unbalanced number of cages for the different groups used for our experiments. However, mice were donated for this study by other groups and not specifically ordered for this project. This conforms with the 3Rs policy and explains the heterogenic group sizes. Another limitation is that our study animals might have been familiar with nestlets and cocoons thereby influencing the results. However, at least for cocoons, this did not increase the nest-building scores questioning the influence of its previous use. Moreover, nest-building scoring always has the potential to be relatively subjective. In order to minimise this bias, we have performed additional scorings and have calculated intra- and inter-observer reliability. The ICC for both were excellent. Another interesting issue for future projects would be the use of a combination of different nest-building materials.

## Animal welfare implications

Animal welfare demands the provision of nest-building materials for environmental enrichment of laboratory mice. However, mouse enrichment is not a one-size-fits-all husbandry procedure. The present study sought to examine possible preferences of different mouse strains for different nesting materials and examine possible gender- and housing-specific effects. In adult male and female mice of two strains, we have examined nest-building behaviour using five different nest-building materials and two different housing conditions. We have found significant strain-, gender- and housing-related influences on the complexity of constructed nests using different standardised building materials. Such observations need to be taken into account when planning the optimal enrichment programme for laboratory animals.

## Conclusion

Our results give a thorough insight revealing significant strain-, sex- and housing-related influences on the complexity of constructed nests using different standardised building materials. Such observations need to be taken into account when planning the optimal enrichment programme for laboratory animals. Nevertheless, larger studies should be performed to deepen our knowledge concerning appropriate environmental enrichment

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