The role of bedding depth in the husbandry of group-housed laboratory mice: Analysis of the impact of different bedding volumes on animal welfare and within-group variation of physiological and behavioural parameters

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**Publications**


**Presentations at conferences**


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Abbreviations

Alb    Albumin
ALP    Alkaline phosphatase
ALT    Alanine aminotransferase
AST    Aspartate aminotransferase
Ca**   Calcium
Ca total Total calcium
Crea   Creatinine
Chol   Cholesterol
Cl`    Chloride
CV     Coefficient of variation
BAT    Brown adipose tissue
HGB    Haemoglobin
HTC    Haematocrit
HME    Hepatic microsomal enzyme
IL     Interleukin
IVC    Individually ventilated cage
K+     Potassium
LDH    Lactate dehydrogenase
Na+    Sodium
RBC    Red blood cells
SD     Standard deviation
TNZ    Thermoneutral zone
TP     Total protein
Tri    Triglycerides
UCP 1  Uncoupling protein 1
WBC    White blood cells

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Summary

Jennifer Freymann

The role of bedding depth in the husbandry of group-housed laboratory mice: Analysis of the impact of different bedding volumes on animal welfare and within-group variation of physiological and behavioural parameters

The aim of “environmental refinement” is to improve the husbandry of laboratory animals by allowing them to perform more elements of their natural behaviour. Digging, burrowing and burying are important components of mice’s behavioural repertoire, but the animals need a sufficient amount of bedding to engage in these behaviours. Deep bedding can provide mice with a more natural environment and might also help to ameliorate thermal stress for the animals. The standard housing temperature (≈ 22 °C) is likely to cause cold stress, as it is substantially below mice’s preferred temperature (> 25 °C). Previous preference tests have already revealed that female BALB/c and C57BL/6 mice prefer a larger bedding volume in comparison to shallow bedding. Any changes in housing conditions can increase the variation of experimental results, which leads to more animals being needed for significance. This may counteract the aim of reducing the number of animals used in scientific research (“reduction”). This PhD project used three bedding volumes (0.5 l, 1.5 l or 6 l per Type III cage) to investigate the influence of different bedding depths on mice’s well-being, their physiology and behaviour, as well as on variation of these experimental results.

By the means of an automatic detecting system, the preferences of group-housed male BALB/c and C57BL/6 mice were assessed for the three volumes. Video analysis was used to identify whether larger bedding volumes promote different behavioural patterns, such as increased digging behaviour. In an additional experiment, BALB/c and C57BL/6 mice were housed on 0.5 l, 1.5 l or 6 l to assess the impact of bedding depths on mean values and variation of anatomical (organs weights, body and tail lengths), physiological (blood parameters, body temperature, food intake, pentobarbital sleeping time) and behavioural parameters (open field test, novel object recognition test).

The preference test demonstrated that male mice prefer larger bedding volumes over smaller volumes, underlining the importance of a sufficient amount of bedding for laboratory mice. While mice performed slightly more digging behaviour on 6 l in comparison to 1.5 l and 0.5 l, animals housed on shallow bedding (0.5 l) engaged in more nest-building behaviour (arranging, pulling in, fraying the bedding material) compared to groups on larger volumes (1.5 l or 6 l). The bedding volume had profound effects on mouse physiology and physical appearance. BALB/c and C57BL/6 mice kept on deeper bedding had a higher body temperature during resting phase and showed indicators for warm adaptation and reduced metabolic demands (increased body and tail lengths, reduced liver, kidney and heart weights, lower food intake as well as a higher food conversion efficiency). All mice housed on shallow bedding showed enlarged adrenals weights, suggesting an increased stress response in these animals; higher corticosterone levels were particularly observed in female BALB/c mice. The results strongly indicate that deep bedding can improve animal welfare, as
it enables mice to create a more insulated environment that can reduce heat loss and thereby energetic demands for thermoregulation. Pentobarbital sleeping time and performance in behavioural tests was influenced by strain and gender, but not by the amount of cage bedding provided. Except for pentobarbital narcosis, variation of experimental parameters was reduced in groups housed on larger volumes compared to groups on shallow bedding. Thus, deeper bedding appears to be a sensible way to refine husbandry conditions for laboratory mice without raising the number of animals needed for significance in experiments.
Zusammenfassung

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Mit Hilfe eines automatischen Systems wurden die Präferenzen männlicher BALB/c und C57BL/6 Mäuse für die verschiedenen Einstreumengen untersucht. Mittels Videoanalyse wurde überprüft, ob größere Einstreuvolumina Mäuse zu unterschiedlichen Verhaltensweisen, wie etwa intensiverem Grabeverhalten animieren. In einem weiteren Experiment wurden BALB/c und C57BL/6 Mäuse auf 0,5 L, 1,5 L oder 6 L gehalten, um die Auswirkung der Einstreutiefe auf Mittelwerte und Streuung von anatomischen (Organgewichte, Körper- und Schwanzlänge), physiologischen (Blutwerte, Körpertemperatur, Futteraufnahme, Pentobarbialschlafzeiten) und Verhaltensparametern (Open Field Test, Novel Object Recognition Test) zu analysieren.

Die Präferenztests zeigten, dass männliche Mäuse größere Einstreumengen kleineren Mengen vorziehen. Dies unterstreicht die Bedeutung eines adäquaten Einstreuvolumens für Labormäuse. Während Mäuse auf 6 L geringfügig mehr graben als auf 1,5 L und 0,5 L, zeigten Tiere auf 0,5 L mehr Nestbauverhalten (Herantragen und Zerspleißien von Einstreumaterial) verglichen mit größeren Einstreumengen (1,5 L oder 6 L). Das Einstreuvolumen hatte starke Auswirkungen auf die Physiologie und physische Erscheinung der Tiere. BALB/c und C57BL/6 Mäuse, die auf einem größeren Einstreuvolumen gehalten wurden, hatten eine höhere Körpertemperatur während der Ruhephase und zeigten typische Anzeichen für Wärmeanpassung und einen reduzierten Energiebedarf (größere Körper- und Schwanzlängen, reduzierte Leber, Nieren und Herzgewichte, geringere Futteraufnahme sowie eine bessere Futterverwertung). Mäuse in Käfigen mit einem
1 Introduction

1.1 Animal welfare in laboratory animal science
The concept of “five freedoms” is a well-known and widely accepted definition of animal welfare. It implies the freedom from (1) hunger and thirst, (2) discomfort, (3) pain, injury and disease, (4) fear and distress as well as (5) the freedom to express normal behaviour (Brambell 1965). The author originally developed this concept for farm animals, it was later rephrased by the UK Farm Animal Welfare Council (FAWC 2009). When applied to laboratory animals, it might be inevitable to restrict some of the freedoms due to the experimental protocol (Baumans et al. 2011). Fear has an important fitness value, thus it is disputable whether complete freedom is actually desirable (Korte et al. 2007). For this reason Korte et al. (2007) introduced “the concept of animal welfare based on allostasis”, which focuses on the animals’ ability to adapt and anticipate environmental challenges. According to the authors, change “is crucial to good health and good animal welfare” (Korte et al. 2007).

The major aim of “environmental enrichment” is to improve animal housing by providing stimuli that encourage species-typical behaviour (Newberry 1995). A variety of positive effects of cage enrichment have been discovered, particularly nesting material has been proven to be very beneficial for laboratory mice (Olsson and Dahlborn 2002). Animals from enriched housing showed improved cognitive functions (Kulesskaya et al. 2011), increased variety of behavioural patterns, reduced stereotypies (Olsson and Sherwin 2006), were calmer and less reactive to experimental procedures and human contact (Van de Weerd et al. 2002). To assess whether a housing condition is beneficial for animals, strain as well as gender differences need to be taken into consideration. Especially in male mice, a complex cage environment can evoke aggressive interactions (Haemisch et al. 1994). In view of this problem, the use of the term “environmental refinement “ instead of “environmental enrichment” has been suggested. While refinement implies an actual improvement in animal welfare, enrichment might also be used whenever a housing condition is “enriched” without demonstrable benefit to the animals (Baumans et al. 2011). To avoid confusion or misinterpretation, the term “enrichment” is used in the following, whenever it corresponds to the wording in the references.

Hawkins et al. (2011) published general principles for an effective welfare assessment in laboratory animals. The “ideal” welfare state, includes three key components:

- Physical state (indicators related to physical conditions)
- Physiological/ biochemical state (physiological parameters)
- Psychological state (changes in behaviour)

These three components need to be assessed carefully to analyse animal welfare. The authors pointed out that profound knowledge of reliable indications of well-being is needed, as the signs differ between species or strains (Hawkins et al. 2011). Table 1 shows parameters that have been used to assess physical, physiological and psychological well-being of laboratory rodents. Such information is needed to decide whether a specific housing condition can improve animal welfare.
Table 1: Signs of impaired physical, physiological and psychological welfare of laboratory rodents.

<table>
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<th>Physical state</th>
<th>Parameter</th>
<th>Reference</th>
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<tr>
<td></td>
<td>Spleen weight involution due to stress-related immunosuppression</td>
<td>Manser 1992, Tuli et al. 1995, Van Loo et al. 2004</td>
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<td></td>
<td>Increased faecal corticosterone metabolites</td>
<td>Touma et al. 2003, Touma et al. 2004</td>
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<td></td>
<td>Increased adrenocorticotropic hormone levels</td>
<td>Gadek-Michalska and Bugajski 2003</td>
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<td></td>
<td>Increased thyroxine levels</td>
<td>Tsai et al. 2003b</td>
</tr>
<tr>
<td></td>
<td>Increased mean arterial blood pressure</td>
<td>Sharp et al. 2002, Sharp et al. 2003</td>
</tr>
<tr>
<td>Psychological</td>
<td>Increased aggressive interactions, such as attacking behaviour (in bite wounds), reduced latency until first aggressive encounter</td>
<td>Haemisch et al. 1994, Van Loo et al. 2002, Marashi et al. 2003, Hutchinson et al. 2012</td>
</tr>
<tr>
<td>state</td>
<td>Reduced play behaviour</td>
<td>Marashi et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Reduced species specific behaviour</td>
<td>Rettich et al. 2006, Jirkof et al. 2010, Jirkof et al. 2013b, Jirkof 2014</td>
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<tr>
<td></td>
<td>Reduced reproductive success</td>
<td>Chernoff et al. 1988, Colomina et al. 1997, Tsai et al. 2003a</td>
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Preference tests allow animals to choose between different housing or environmental conditions and their dwelling time in the different test compartments is used as an indicator for their preferences, respectively aversions (Blom et al. 1992). Choice tests have been successfully used to assess mice’s preferences for different nesting (Van de Weerd et al. 1997a) and bedding materials (Kirchner et al. 2012), thus contributing to improved husbandry conditions for laboratory animals. Observation of behavioural patterns evoked by the different test compartments can provide valuable information for interpreting the test results (Blom et al. 1992).

Performance in behavioural tests, such as open field tests (Rasmussen et al. 2011) or cage emergence tests (Van Loo et al. 2004) are commonly used experimental setups to assess animals’ reaction to novel environments. Reduced anxiety-like behaviour in these tests has been associated with improved well-being (Van de Weerd et al. 1997b). Stress is known to affect cognitive functions and learning ability in animals (Larsson et al. 2002, Lin et al. 2016). Housing conditions can influence this interaction and alter the animals’ reaction to a stressor. Performance in water maze tests after psychological stress was more affected in animals housed in impoverished environments compared to animals kept in enriched cages (Larsson et al. 2002).

The impact of poor animal welfare on scientific research should not be underestimated. Behavioural and physiological changes in laboratory animals can influence scientific results and thereby lead to skewed data and inappropriate conclusions. To guarantee high quality research, animal welfare needs to have top priority on ethical as well as scientific reasons (Poole 1997).

### 1.2 Species-specific behaviour: Importance of digging, burrowing and burying for laboratory mice

The behavioural traits digging, burrowing and burying are essential components of mice’s behaviour (Berry 1970, Adams and Boice 1981), they are employed for food storage and to create nesting sites protected from ambient temperatures and predators (Webster et al. 1981, Harper and Batzli 1996). Digging (Van Oortmerssen 1971, Deacon 2006a) and burrowing (Deacon 2006b) are defined as coordinated alternating movements of fore and/or hind paws that displace substrate. While the term digging is used whenever bedding is displaced, burrowing is particularly employed whenever substrate or food pellets are removed from a tube or container, as it is closely related to burrow cleaning behaviour (Schmid- Holmes et al. 2001). Burying is considered a forward pushing movement with forepaws displacing substrate towards a fearful stimulus or predator (“defensive burying”) (De Boer and Koolhaas 2003) or an object (Deacon 2006a). There are good and poor burrowers among mice strains (Van Oortmerssen 1971, Deacon 2006b), nevertheless these behavioural patterns are species-specific for mice in general (Webster et al. 1981). Laboratory mice built complex burrows when suitable substrate, such as soil, was provided (Adams and Boice 1981). The animals showed digging and burrowing even when they had access to readily constructed burrows (Sherwin et al. 2004). This indicated that engaging in these behaviours per se is essential for the animals, most likely due to a self-rewarding
effect (Teeling et al. 2007). Burrowing was therefore considered a “behavioural need” in mice (Sherwin et al. 2004).

Although different protocols, such as the burrowing test (substrate or food pellets need to be removed from a tube or container) (Deacon 2009), digging test (latency to start digging, number of digging bouts) or marble burying test (marbles have to be buried with bedding) (Deacon 2006a) have been established, they presumably measure the same or at least a very similar behaviour (Deacon 2006a). Digging, burrowing and burying are particularly susceptible to hippocampal lesions (Deacon and Rawlins 2005), a reduction in the behaviour was observed with progression of neurological diseases (Deacon et al. 2001, Deacon 2006a, Deacon et al. 2008). The burrowing paradigm has successfully been implied to assess impaired well-being due to post surgical pain (Jirkof 2014).

1.3 BALB/c and C57BL/6 in experimental research

The strains BALB/c and C57BL/6 belong to the most commonly used inbred strains in laboratory animal science. BALB/c and C57BL/6 have been established independently from each other, the different genetic background (Figure 1) makes them feasible to achieve a certain general validity in research (Festing 1979, Fox and Witham 1997). The two strains differ in various behavioural as well as physiological traits. Van Oortmerssen (1971) analysed the genetic and evolutionary origin of behavioural differences in four inbred mice strains, including C57BL/6 and BALB/c. The author discovered constant differences in nest-building behaviour between BALB/c and C57BL/6 mice. BALB/c mice employed special and genetically determined fraying behaviour to build spherical nests at the surface. C57BL/6 mice showed a clear preference for digging and were only able to create a proper nest after they dug a suitable hole. According to Van Oortmerssen (1971), these differences resulted from the strain-specific adaptation to habitats in which their wild ancestors lived: BALB/c most likely originated from surface living, commensal mice and C57BL/6 from hole living, non-commensal mice. These experiments demonstrated that the behavioural difference and resulting habitat preferences are still present in the inbred strains (Van Oortmerssen 1971, Sluyter and Van Oortmerssen 2000). BALB/c and C57BL/6 are also known to differ in behavioural performance (Bothe et al. 2005), blood parameters (Kile et al. 2003) and barbiturate metabolism, due to differences in activity of hepatic microsomal enzymes (HME) (Vesell 1968). Bedding is used in husbandry of all laboratory mice, therefore it is indispensable to know precisely whether the potential impact of bedding volume on the experimental results is strain-specific or can be applied to mice in general. Using two strains that show different responses to routine experimental procedures can help to answer this question.

BALB/c originated from a stock of albino mice acquired by H. Bagg in 1913 (Bagg albino strain = Balb). MacDowell conducted inbreeding in 1923, continued by Snell in 1932, who added a “c” to indicate the genotype at the colour locus was c/c (albinism). The strain was sold to different facilities, including the Jackson Laboratory (Bar Harbour, USA), where the sub-strain BALB/cByJ was established in 1974. BALB/c mice are widely used in various disciplines in biomedical research (Festing 1979, JanvierLabs 2017a), such as toxicology,
immunology, oncology or virology. According to JanvierLabs (2017a) female BALB/cBYJRj mice have an average weight of 22 g, males of 27 g at 70 days of age.

The strain C57BL originated in 1921 from A. Lathrop’s stock. The strain was divided into two sub-strains C57BL/6 and C57BL/10 before 1937. The C57BL/6 strain was transferred to the Jackson Laboratory (C57BL/6J) and later to the National Institute of Health where C57BL/6N was established (Festing 1979, JanvierLabs 2017b). C57BL/6 is one of the most commonly used inbred strains, with many different applications, including metabolism studies, toxicology, immunology or cardiovascular research (JanvierLabs 2017b). At 70 days of age female C57BL/6NRj mice weigh 20 g, males 25 g (JanvierLabs 2017b).

Figure 1: The origins of commonly used inbred mice strains (Fox and Witham 1997).
1.4 Bedding volume in the husbandry of laboratory mice

Laboratory mice should be provided with an appropriate bedding depth to allow them to engage in digging behaviour (Jennings et al. 1998, Deacon 2009). A bedding depth of approximately 1 cm is common practice, however up to 5 cm are needed to evoke intensive digging and burrowing (Deacon 2006a). According to Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123) bedding material should “(…) allow the animal to perform certain species-specific behaviours, such as foraging, digging or burrowing (…)”. The report of the rodent refinement working party states that “bedding should be provided in sufficient quantities to allow the animals to manipulate their environment and microclimate. A thin layer of substrate as a base is not adequate on its own” (Jennings et al. 1998).

The positive effect of a larger bedding depth on other rodents has been described previously, e.g. golden hamsters kept in 80 cm deep bedding showed significantly less wire-gnawing than those kept in cages with 10 cm bedding (Hauzenberger et al. 2006). In female BALB/c and C57BL/6 the preference for a larger bedding volume compared to shallow bedding has been confirmed recently (Freymann et al. 2015). A larger bedding volume reduced intra-cage ammonia levels (Rosenbaum et al. 2009) and therefore allows to increase the cage change interval, which can be especially beneficial for breeding animals (Reeb-Whitaker et al. 2001). Increased ammonia levels led to pathological changes particularly in the nasal region (Vogelweid et al. 2011, Ferrecchia et al. 2014) and also affected the activity of hepatic microsomal enzymes, thereby interacting with the metabolic rate of barbiturates (Vesell et al. 1976). The authors demonstrated that animals exposed to high ammonia levels show a prolonged sleeping time after barbiturate narcosis, as a result of reduced drug metabolism due to inhibited HME activity (Vesell et al. 1976).

1.5 Mice’s thermal preferences and the implication for biomedical research

There has been a dispute regarding the optimal housing temperature for laboratory mice to mimic the thermal environment in humans. While Speakman and Keijer (2012) stated that humans occupy an environment slightly below their thermoneutral zone (TNZ), Feldmann et al. (2009) and Karp (2012) pointed out that people in a modern society have a variety of options to adjust the surroundings according to their individual needs and therefore do not experience metabolic cold stress. TNZ is considered the range of ambient temperature where metabolic heat production and evaporative heat loss are at minimal levels (Gordon 1985, 2012). Laboratory mice are usually housed at ambient temperatures 22 ± 2 °C (ETS 123), which are quite substantially below their thermoneutral zone (26 °C - 34 °C (Gordon 1993)). A major concern is that an animal constantly subjected to cold stress can jeopardise biomedical research and will not be an adequate model for humans (Karp 2012). Ambient temperature influenced cardiovascular parameters in rats and mice, with a greater impact on mice (Swoap et al. 2004). Comparable to the different thermal comfort zone of females and males (Cannon and Nedergaard 2011), mice have higher metabolic demands, resulting from a detrimental surface to volume ratio, compared to rats. Thus, especially mice are likely to suffer from cold stress at standard housing temperatures (Swoap et al. 2004). Mice housed at 20 °C showed physiological and biochemical indicators of cold stress, including increased heart rate, blood pressure as well as changes in brown adipose tissue (BAT).
compared to animals housed at 30 °C (Maher et al. 2015). BAT is an essential organ for non-shivering thermogenesis (heat production not involving contraction of skeletal muscles) in small mammals (Gordon 1993). Kokolus et al. (2013) revealed a reduced antitumor immune response and increased tumor growth and metastasis for mice housed at 22 - 23 °C compared to 30 - 31°C. Temperatures up to 28 °C did not have negative effects on early reproductive fitness of mice, this supports the idea of raising the temperature in holding rooms (Helppi et al. 2016).

Thermal preferences of laboratory mice varied depending on sex, age, strain, social housing, time of day and behaviour (Gordon 2012). Mice showed significant preferences for 25 °C and 30 °C over 20 °C during maintenance (feeding, nesting, grooming) and inactive behaviours, but not during active phase (Gaskill et al. 2009). The preference for ambient temperatures close to 30 °C were especially pronounced in females, aged or single-housed mice (Gordon et al. 1998, Gaskill et al. 2009). Gaskill et al. (2012) reported a preference between 23 °C and 26 °C for male mice and approximately 29 °C for females. These variations make it difficult to define one optimal housing temperature for laboratory mice (Gaskill et al. 2009). Provisions of nesting and bedding material can accommodate the difference in thermal needs and ameliorate cold stress for laboratory mice (Gaskill et al. 2011, Gordon 2012). Gaskill and Garner (2014) pointed out that thermal preferences were assessed in static cages, however with the increased use of individually ventilated cages (IVCs), mice are likely to face more cold stress. In line with this hypothesis, mice were only willing to accept ventilated cages when nesting material was provided (Baumans et al. 2002), suggesting higher thermal stress in these cages.

Mice use behavioural (huddling, nesting, thermotaxis) as well as physiological (heat generation via BAT, reduction of periphery blood flow) strategies in response to a cold environment (Maher et al. 2015). Apart from varying thermal preferences mice also differ in their behavioural and physiological response to cold ambient temperatures (Gaskill et al. 2013). While C57BL/6 preferred to use thermotaxis and relocated to warmer environments, BALB/c first impulse was to increase nesting behaviour. Overall, BALB/c mice were willing to accept colder ambient temperatures when nesting material was provided compared to C57BL/6 (Gaskill et al. 2012). When thermotaxis was not possible, all mice adjusted shape and quality of the nest to reduce heat loss (Gaskill et al. 2013). It is important that the amount of nesting material matches the numbers of animals per cage to ensure that sufficient nests can be built (Baumans et al. 2002).
1.6 Variation of experimental results

Two forms of variability, random variability and fixed effects, can be distinguished. Random variability refers to the variability within a group and is caused by the genotype of individuals, environment as well as an innate, intangible variance, which quite likely results from epigenetic mechanisms. Fixed effects are primarily caused by age, sex and differences between genetic lines as well as environmental conditions, leading to variations between groups of animals that differ in precisely these characteristics. In contrast to random variability, fixed effects can particularly be reduced by standardization (Gärtner 1990).

In order to analyse within-group variability, the coefficient of variation (CV), the ratio of the standard deviation (SD) to the mean, is a valuable statistical tool. The CV is unitless and allows to compare degrees of variation between groups (Gärtner 1990). The variability of traits in laboratory animals can endanger reproducibility and comparability of experimental results and can increase the number of animals needed for significance (Hutchinson et al. 2005). While a CV of 5 % requires three animals per group (mean difference 20 %, α = 0.05, power = 0.90), a CV of 15 % triples the number of animals (Beynen et al. 2001).

To restrain variability, standardization of genotypes, health and husbandry of laboratory animals has been one of the major objectives in laboratory animal science (Hutchinson et al. 2005). However, it is controversial to which extend housing conditions should be standardized to guarantee reproducible results (Wolfer et al. 2004, Hutchinson et al. 2005). It has been argued that highly standardized housing conditions might compromise external validity of experimental research and that controlled environmental heterogenization might lead to more robust results and reduces the chance of discovering a “local truth” (Richter et al. 2009). Hutchinson et al. (2005) pointed out that housing design must be considered as an experimental variable that can not only distort results, but also affect variability and reproducibility of experiments. Scientists need to be aware that housing conditions can result in a conflict between “reduction” and “refinement” as improved housing might increase within-group variability and thus the number of animals needed for an experiment. Different studies have assessed the influence of housing conditions on experimental results (Eskola et al. 1999, Mering et al. 2001, Tsai et al. 2002, Van de Weerd et al. 2002, Tsai et al. 2003a, Tsai et al. 2003b, Hutchinson et al. 2005, Mikkelsen et al. 2010). Table 2 shows a summary of the results. It becomes clear that housing conditions can increase variation, however it is not possible to make a general statement as the impact varies depending on animals, housing design and parameter studied (Tsai et al. 2002). Therefore, variation needs to be analysed whenever changes in the husbandry are intended, to be aware of the potential impact it might have on the experimental parameters and animals (Toth et al. 2011).

Table 2
<table>
<thead>
<tr>
<th>Reference</th>
<th>Animals</th>
<th>Housing</th>
<th>Statistical parameter used to assess variation</th>
<th>Outcome measures</th>
<th>Impact of enriched housing on variation compared to standard housing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eskola et al. 1999</td>
<td>Rats, male and female</td>
<td>Enriched (E) = bedding, aspen block or aspen tube Standard (S) = bedding</td>
<td>SOLO power analysis to calculate the smallest number of animals (n) needed to detect an arbitrarily chosen 20% effect size, (P &lt; 0.05, power at 0.90)</td>
<td>N-ratio (n E / n S) indicates how many times more (&gt;1) or less (&lt;1) animals are needed in the enrichment group</td>
<td>N-ratios &lt; 1.5</td>
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<tr>
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<td>Wistar</td>
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<td>Final body weight</td>
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<td>Growth</td>
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<td>Adrenals</td>
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<td>Brown adipose tissue</td>
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<td>Alanine aminotransferase</td>
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<td>Aspartate aminotransferase</td>
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<td>Gamma- glutamyltransferase</td>
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<td>Outcome measures</td>
<td>Impact of enriched housing on variation compared to standard housing</td>
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<tr>
<td>Hutchinson et al. 2005</td>
<td>Mice, breeding pairs</td>
<td>Enriched (E) = bedding, ladder and jar, nesting materials</td>
<td>Standard deviation</td>
<td>Newborns / litter</td>
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<td></td>
<td>CB17-Prkdc&lt;sup&gt;scid&lt;/sup&gt;, B10.D2/nSnJ</td>
<td>Standard (S) = bedding</td>
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<td>Pups weaned / Litter</td>
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<td></td>
<td>Mice, female and male BALB/c</td>
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<td>Coefficient of variation</td>
<td>Cytokines (IL-2, IL-4, IFN-γ, IL-10)</td>
<td>no trend</td>
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<tr>
<td>Mering et al. 2001</td>
<td>Rats, male Wistar</td>
<td>Enriched (E) = gnawing blocks</td>
<td>SOLO power analysis to calculate the smallest number of animals (n) needed to detect an arbitrarily chosen 20% effect size, (P = 0.05, power at 0.90)</td>
<td>Final body weight</td>
<td>1, 3</td>
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<tr>
<td></td>
<td></td>
<td>Standard (S) = no blocks</td>
<td></td>
<td>Growth</td>
<td>1, 2, 3, 4</td>
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<td>Cages: solid bottom cages with bedding (SBC) or grid floor cages (GFC)</td>
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<td>Thymus weight</td>
<td>2, 4</td>
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<td>Experiment 1</td>
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<td>Adrenal weight</td>
<td>1, 3</td>
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<td></td>
<td></td>
<td>- transfers from SBC to GFC for all animals</td>
<td></td>
<td>Spleen weight</td>
<td>1, 2</td>
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<td></td>
<td></td>
<td>- group size (1, 2, 3 or 4 rats/cage)</td>
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<td>3, 4</td>
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<td></td>
<td></td>
<td>- SBC (E and S), GFC (E and S)</td>
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<td>Experiment 2</td>
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<td>Final body weight</td>
<td>all</td>
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<td></td>
<td></td>
<td>- SBC (E and S), GFC (E and S) or transfers (Trans) from SBC to GFC (E and S)</td>
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<td>Growth</td>
<td>Trans</td>
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<td></td>
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<td>- same group size</td>
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<td>Thymus weight</td>
<td>SBC, GFC</td>
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<td>Adrenals weight</td>
<td>SBC, Trans</td>
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<td>Spleen weight</td>
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<td>Brown adipose tissue</td>
<td>SBC</td>
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<td>Epididymal adipose tissue</td>
<td>GFC</td>
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<td>Corticosterone</td>
<td>GFC, Trans</td>
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*Table:* A comparison of studies on the impact of enriched housing on variation compared to standard housing. The table includes details on the animals used, housing conditions, statistical parameters, outcome measures, and the impact on variation.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Animals</th>
<th>Housing</th>
<th>Statistical parameter used to assess variation</th>
<th>Outcome measures</th>
<th>Impact of enriched housing on variation compared to standard housing</th>
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<tbody>
<tr>
<td>Mering et al. 2001</td>
<td>Rats, male Wistar</td>
<td>Experiment 3 - SBC (E and S), Trans (E and S) - same group size</td>
<td>SOLO power analysis to calculate the smallest number of animals (n) needed to detect an arbitrarily chosen 20% effect size, (P &lt; 0.05, power at 0.90)</td>
<td>Final body weight</td>
<td>↑ SBC, ↓ Trans</td>
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<td>Growth</td>
<td>↑ SBC, ↓ Trans</td>
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<td>Thymus weight</td>
<td>↑ SBC, ↓ Trans</td>
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<td>Adrenal weight</td>
<td>↑ SBC, ↓ Trans</td>
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<td>Spleen weight</td>
<td>↑ SBC, ↓ Trans</td>
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<td>Brown adipose tissue</td>
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<td>Epididymal adipose tissue</td>
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<td>Adrenal weight</td>
<td>↑ SBC, ↓ Trans</td>
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<td>Brown adipose tissue</td>
<td>↑ SBC, ↓ Trans</td>
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<td>Enriched (E) = type IV macrolon cage (595 x 380 x 200 mm), bedding, nesting material, Novo Nordisk hide, aspen brick</td>
<td>Coefficient of variation</td>
<td>Cholesterol</td>
<td>↑ E, SE</td>
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<td>Superenriched (SE) = scantainer Novo type IV cage (595 x 380 x 325 mm), bedding, built-in shelf, nesting material, a Novo Nordisk hide, aspen brick</td>
<td></td>
<td>Albumin</td>
<td>↑ E, SE</td>
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<td>Standard (S) = type IV macrolon cage (595 x 380 x 200 mm), bedding</td>
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<td>Total protein</td>
<td>↑ E, SE</td>
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<td>Phosphorus</td>
<td>↑ E, SE</td>
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<td>Urea</td>
<td>↑ E, SE</td>
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<td>Fibrinogen C</td>
<td>↑ E, SE</td>
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<td>Thrombin time</td>
<td>↑ SE</td>
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<td>White blood cells</td>
<td>↑ SE</td>
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<td>Red blood cells</td>
<td>↑ E, SE</td>
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<td>Haemoglobin</td>
<td>↑ E, SE</td>
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<td>Haematocrit</td>
<td>↑ E, SE</td>
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<td>MCV</td>
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<td>MCH</td>
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<td>MCHC</td>
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<td>Platelets</td>
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<td>Lymphocytes</td>
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<td>Statistical parameter used to assess variation</td>
<td>Outcome measures</td>
<td>Impact of enriched housing on variation compared to standard housing</td>
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<tr>
<td>Tsai et al. 2002</td>
<td>Mice, female BALB/c C57BL/6 A/J</td>
<td>Enriched (E) = type III macrolon cage (375 x 215 x 150 mm), bedding, nest box, wood bar, nesting material Standard (S) = type III macrolon cage (375 x 215 x 150 mm), bedding</td>
<td>Coefficient of variation</td>
<td>Final body weight</td>
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<td>White blood cells</td>
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<td>Red blood cells</td>
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<td>Haemoglobin</td>
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<td>Haematocrit</td>
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<td>Liver weight</td>
<td>A/J, BALB/c C57BL/6</td>
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<td>Kidney weight</td>
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<td>Adrenal weight</td>
<td>BALB/c C57BL/6</td>
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<td>Spleen weight</td>
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<td>Uterus weight</td>
<td>A/J</td>
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<td>Heart weight</td>
<td>BALB/c C57BL/6</td>
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<tr>
<td>Tsai et al. 2003a</td>
<td>Mice, breeding pairs DBA</td>
<td>Enriched (E) = type II elongated macrolon cage (325 x 165 x 140 mm), bedding, nest box, wood bar, nesting material Standard (S) = type II elongated macrolon cage (325 x 165 x 140 mm), bedding Housing: scantainer (SC), open rack (OR) or individually ventilated cage (IVC); all E and S</td>
<td>Coefficient of variation</td>
<td>Total nr. of litters / dam</td>
<td>all</td>
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<td>Total nr. pups born /dam</td>
<td>all</td>
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<td>Breeding index (young weaned / female / week)</td>
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<td>Body weight of pups weaned (18 days)</td>
<td>SC</td>
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<td>Age of dam at first birth</td>
<td>SC, OR</td>
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<td>Age of dam at first weaned</td>
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<td>Litters interval</td>
<td>SC, OR</td>
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<tr>
<td>Tsai et al. 2003b</td>
<td>Mice, female and male DBA</td>
<td>Enriched 1 (E1) = type III macrolon cage (375 x 215 x 150 mm), bedding, nest box, wooden climbing bar, nesting material</td>
<td>Coefficient of variation</td>
<td>Final body weight</td>
<td>all</td>
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<td>Enriched 2 (E2) = type III macrolon cage (375 x 215 x 150 mm), bedding, horizontal and vertical clear safety glass dividers</td>
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<td>Travel distance (Open Field Test)</td>
<td>Female, E1 male</td>
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<td>Standard (S) = type III macrolon cage (375 x 215 x 150 mm) with bedding</td>
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<td>Freezing (Open Field Test)</td>
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<td>Frist touch (Food Drive Test)</td>
<td>Male, E2 female</td>
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<td>Eating (Food Drive Test)</td>
<td>all</td>
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<td>Red blood cells</td>
<td>Female, E1 male</td>
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<td>Haemoglobin</td>
<td>Male, E2 female</td>
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<td>Haematocrit</td>
<td>E2</td>
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<td>Final body weight</td>
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<td>Liver weight</td>
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<td>Kidney weight</td>
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<td>Adrenal weight</td>
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<td>Spleen weight</td>
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<td>Uterus weight</td>
<td>E1</td>
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<td></td>
<td>Heart weight</td>
<td>E1 female, Male, E2 female</td>
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<td>Testis weight</td>
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</tbody>
</table>
| Van de Weerd et al. 2002 | Mice, male N: NIH     | Enriched (E) = type II macrolon cages (375 cm²), bedding, one tissue, nest box with climbing grid  
Superenriched (SE) = type III macrolon cages (840 cm²), bedding, two tissues, nest box with climbing grid, wood gnawing blocks, plastic tube, wood wool, stainless steel wire grid floor under the food hopper  
Tissue (T) = type II macrolon cages (375 cm²), bedding and one tissue  
*T used as control = standard* | Mean absolute deviations | Open field test: Frequency and duration of locomotion, climbing, rearing, interaction behaviours  
Frequency of behaviours (= T< E, SE) | Duration of behaviours |
|                    | Mice, male  BALB/c     | Superenriched (SE)                           |                                                | Body weight x (= T< E, SE), except for first measurement                        |                                                                     |
|                    |                        | Standard (S) = type III cages with bedding    |                                                | Immune response x (= T< E< SE)                                                  |                                                                     |
1.7 Objectives
Despite the fact that bedding is invariably used in the husbandry of laboratory mice, the current state of research reveals a lack of knowledge regarding the potential impact of different bedding volumes on the outcome of scientific research.

This PhD project assessed how different bedding volumes affect mice’s preferences and behavioural patterns, their anatomical (body and tail lengths, organ weights) and physiological parameters (body temperature, food intake, pentobarbital sleeping time, blood values) as well as their behavioural performance (open field test, novel object recognition test).

The aim of this study was to provide a comprehensive overview of the influence of different bedding volumes on animal welfare and experimental results. Two key questions shall be answered:

(1) *Can deep bedding be considered a refinement in the husbandry of laboratory mice?*

(2) *Does deeper bedding interfere with experimental results, including variation?*
The impact of bedding volumes on laboratory mice

J. Freymann, P.-P. Tsai, H. Stelzer, H. Hackbarth


doi: 10.1016/j.applanim.2016.11.004

Contribution to the manuscript
I was involved in the study design, performed all the experiments (except for the corticosterone ELISA) and analysed the data. I wrote the manuscript.
Abstract
Environmental refinement is considered to be an improvement in housing conditions for laboratory animals. Previous preference tests showed that female BALB/c and C57BL/6 mice prefer deeper bedding in comparison to shallow bedding (Freymann et al. 2015). In order to give a comprehensive insight into the impact of bedding depths on laboratory mice, we continued to examine the influence of three different bedding volumes (0.5 l, 1.5 l, 6 l) on the preference of male mice (experiment 1), home cage behaviour (experiment 2) as well as body temperature, food intake, food conversion efficiency (gram food intake per gram weight gain), intra-cage ammonia and corticosterone levels (experiment 3) of females and males. Experiment 1 used an automatic system to assess the preferences of male BALB/c and C57BL/6 mice. The bedding volumes were tested in pairs, which resulted in three test conditions (A = 0.5 l vs. 1.5 l; B = 0.5 l vs. 6 l; C = 1.5 l vs. 6 l). The results revealed significant preferences for cages containing large bedding volumes (test conditions A, B: p < 0.0001 for both strains; C: p = 0.0110 (BALB/c), p = 0.0511 (C57BL/6)). The second experiment analysed the home cage behaviour of female and male BALB/c mice between 18:00 - 20:00 (CET) using instantaneous sampling. No significant differences regarding the behavioural patterns locomotion, grooming, agonistic interaction, feeding, drinking, nest-building, resting, digging and burrowing were detected. However, animals housed on shallow bedding (0.5 l) engaged more in nest-building behaviour compared to groups housed larger volumes (1.5 l or 6 l). Experiment 3 demonstrated that bedding volumes (0.5 l, 1.5 l or 6 l) have profound effects on mouse physiology. BALB/c and C57BL/6 mice kept on deep bedding showed higher body temperatures (p < 0.05 (0.5 l compared to 1.5 l, or 6 l)), lower food intake (p < 0.01 (6 l compared to 0.5 l, or 1.5 l) as well as reduced intra-cage ammonia levels compared to groups on shallow bedding. In addition a larger bedding volume increased food conversion efficiency and reduced corticosterone levels in female mice. The trend became particularly obvious in female BALB/c mice (p < 0.05 (0.5 l compared to 1.5 l, or 6 l) for both parameters). Our results underline the importance of a sufficient amount of cage bedding in the husbandry of laboratory mice.
Impact of bedding volumes on physiological and behavioural parameters in laboratory mice

J. Freymann, P.-P. Tsai, H. Stelzer, R. Mischke, H. Hackbarth

Accepted by Laboratory Animals (2017)

doi: 10.1177/0023677217694400

Contribution to the manuscript
I was involved in the study design and performed all the experiments (except for calculation of organs weights and analysis of blood samples). I analysed the data statistically and wrote the manuscript.
Abstract
The standard housing temperature in animal facilities is substantially below the lower critical temperature of mice. This does not only endanger animal welfare, it can also jeopardize scientific research as cold stress has a major impact on mouse physiology. There is some evidence that deep bedding, comparable to nesting material, can help mice to reduce heat loss. Whenever changes are applied to the cage environment, the potential impact on experimental results, including variation, needs to be assessed. An increased variation can result in a conflict between reduction of experimental animals and refinement, when more animals are needed for significance due to the housing design. The aim of this study was to assess the impact of different bedding volumes (0.5 L, 1.5 L and 6 L per type III cage) on mean values and coefficient of variation (CV) of physiological (pentobarbital sleeping time, blood and anatomical parameters) and behavioural parameters (open-field and novel object recognition tests) of group-housed female and male BALB/c and C57BL/6 mice. A larger bedding volume did not interfere with the CVs, but influenced mean values of organ weights and tail lengths. Mice housed on deeper bedding showed a significant reduction in adrenal, liver, kidney and heart weights as well as an increase in tail lengths; these anatomical changes are akin to warm adaptation, and were previously observed for mice housed under warmer environments. A larger bedding volume appears to be a sensible way to reduce cold stress for laboratory mice without increasing variation in experimental results.
4 Discussion

4.1 Impact of bedding volume on anatomical and physiological parameters

4.1.1 Anatomical parameters

Ambient temperature impacts mice’s metabolic demands, which leads to profound morphological changes (Yamauchi et al. 1983, Hammond et al. 2001, Zhao et al. 2010, Gordon 2012, Gordon et al. 2014). To compensate higher energy expenditures with decreasing temperatures, mechanical as well as metabolic performance of organs needs to increase (Gordon et al. 2014). Heart, liver and kidney contribute a substantial part to the animal’s metabolic rate, therefore changes are particularly prominent in those organs. (Gordon 1993). Enlargement of the heart at cold temperatures appears to correspond with increased blood perfusion to ensure higher levels of heat production (Gordon 2012). The present study was able to document a reduction in heart, liver and kidney weight for mice housed on larger bedding volumes, strongly suggesting that these morphological changes are the result of reduced energetic demands. Examination of lungs (Hammond et al. 2001, Krol et al. 2003, Gordon 2004), digestive organs (Hammond et al. 2001, Krol et al. 2003) or pancreas (Krol et al. 2003) could have provided further evidence for the insulating effects of deep bedding. Zhao et al. (2010) housed male Swiss mice at different ambient temperatures (23 °C, 15 °C, 0 °C, −8 °C, −15 °C), transferring the animals to a colder environment after 2 weeks each. Enlargement of different organs, including liver, heart, kidney and BAT was observed at every housing temperature, indicating that anatomical changes can already be visible after a short period of time. In accordance with these observations, female BALB/c mice showed changes in liver and kidney weights after a three-week exposure to different bedding volumes, underlining the profound impact of bedding volume on heat loss. While CD-1 mice housed with nesting material at 20 °C revealed reduced heart weights after 4 weeks compared to controls without nesting material, no changes were observed in BALB/c and C57BL/6 mice (Gaskill et al. 2013). Since heat loss is highly dependent on body size, the authors chose to control for body weight instead of age: at the start of the experiment CD-1 mice were 7 weeks, BALB/c mice 14 weeks and C57BL/6 mice 12 weeks of age (Gaskill et al. 2013). Apart from possible strain differences, the age difference between the animals might have contributed to the discrepancies. Anatomical changes were more prominent in mice housed on different bedding volumes from weaning through 15 weeks of age compared to animals that arrived at 12 weeks of age. Comparable to changes in tail growth rate (Al-Hilli and Wright 1983), particularly young animals might be able to respond and adapt to changes in the environment. Further studies regarding the ability to provide laboratory mice with insulation from the ambient temperature are needed to allow a more elaborate comparison between deep bedding and nesting material.

Assessment of BAT and thyroid hormones has been used to estimate thermal properties of different housing conditions in laboratory mice (Zhao et al. 2010, Gaskill et al. 2013). A decline in housing temperature corresponded to a significant increase in BAT in single-housed mice (Zhao et al. 2010). Lower BAT weight and reduced expression of uncoupling protein 1 (UCP 1) mRNA was also observed in BALB/c mice when housed with nesting material (Gaskill et al. 2013). UCP 1 (Cannon and Nedergaard 2011) as well as thyroid hormones (T3, T4) play an important role in thermogenesis and impact BAT’s thermogenic capacity (Gordon 1993). A reduction in T4 blood concentration was visible in male mice
housed with nesting material, but not in females, indicating that the amount of nesting material used (8 g) might not be enough for females to alleviate thermal stress (Gaskill et al. 2013). Analysis of BAT and thyroid hormones of mice kept on different bedding volumes could provide further valuable insight into the thermal properties of deep bedding compared to nesting material and warmer ambient temperatures.

Different publications have reported changes in tail lengths that coincided with changes in ambient temperature (Al-Hilli and Wright 1983, Gordon et al. 2014). Comparable to housing temperature, bedding volume affected appendage development in laboratory mice: reduced tail lengths were noticed in mice on shallow bedding compared to deep bedding. These observations closely reflect Allen’s rule that protruding body parts, including tail, ears, limbs decrease in size at lower temperatures to reduce the animals’ surface area and thereby heat loss. Barnett (1965) bred three mice strains at 21 °C or 3 °C over multiple generations and discovered profound impacts on tail morphology. Tail vertebrae of mice housed at 3 °C were not only shorter and narrower, but also reduced in number. Animals reared at 33 °C had longer tails (approximately 20 %) compared to mice housed at 8 °C (Al-Hilli and Wright 1983). The effect was also prominent in mice kept at less extreme temperatures (21 °C compared to 27 °C) (Serrat et al. 2008). Recent data suggests that extremity growth is caused by changes in cartilaginous tissue due to higher appendage temperatures (Serrat et al. 2008). The impact of temperature on tail growth was particular prominent in younger mice (Al-Hilli and Wright 1983), respectively in animals that had been housed at different temperatures from weaning through maturity (Serrat et al. 2008). This could explain why changes in tail lengths were only significant in mice housed on the different bedding volume from three weeks of age.

Body lengths also increased in mice housed on larger bedding volumes, a correlation of body lengths with ambient temperature has been reported, but the effect varied between strain and gender and was not as distinct as tail growth (Barnett 1965).

4.1.2 Body weight and food consumption

Animals that live in colder climates tend to be larger compared to individuals in warmer regions (Bergmann’s rule). However, the bedding volume did not change mice’s body weight, which is in accordance with observations by Gordon et al. (2014) and Serrat et al. (2008), who were not able to document an impact of different housing temperatures on mice’s weight. Additionally, wild mice did not strictly adhere to Bergmann’s rule, no clear trend in body size was visible in mice occupying cold or warm regions (Jakobson 1981). Provision of nesting material resulted in increased body weight at standard ambient temperatures (Van de Weerd et al. 1997b). Stress is known to cause weight loss (Van Loo et al. 2002, Retana-Marquez et al. 2003), this effect might have interfered with weight development.

Rising metabolic demands due to increased heat loss result in higher food consumption (Cannon and Nedergaard 2009). Mice consumed less food when housed on larger bedding volume, these findings are in line with Gaskill et al. (2013) and Yamauchi et al. (1983), who reported lower food intake in mice housed with nesting material (Gaskill et al. 2013), respectively housed at warmer ambient temperatures (Yamauchi et al. 1983). This provides further support for the hypothesis that deeper bedding can reduce mice’s metabolic
demands due to reduced heat loss. Food conversion efficiency was only affected in female but not in male mice. The lower food conversion efficiency on shallow bedding could be an indication for females’ higher susceptibility to cold temperatures (Cannon and Nedergaard 2011).

4.1.3 Haematological parameters
Haematological values are known to vary depending on ambient temperature (Sealander 1960, Jakobson 1981). Feral house mice had higher hematocrit, haemoglobin and a larger blood volume in winter compared to summer, these changes are the result of increased oxygen demands associated with a temperature dependent increase in metabolic rate (Maclean and Lee 1973). Although the bedding volume presumably helped mice to create a warmer place for resting, the differences were clearly too small to have resulted in haematological changes. In line with this assumption, haemoglobin and hematocrit values were significantly increased in deer mice housed at 5 °C compared to animals at 30 °C, but only small differences were detected between groups on 20 °C and 30 °C (Sealander 1960). Yamauchi et al. (1983) estimated the effect of room temperature on haematology in laboratory mice. Mice were housed and bred at different ambient temperatures (range between 12 °C and 32 °C) to compare erythrocytes, leukocytes, haemoglobin and hematocrit values. Erythrocytes decreased significantly in warm ambient temperature of 30 °C and 32 °C in first- and second-generation mice. There was no clear trend regarding leukocytes count and haemoglobin, changes occurred either at temperatures below 20 °C or above 26 °C. The second-generation mice showed increased hematocrit values at colder temperatures, but there was no clear tendency for the first-generation. These results showed that haematological values of laboratory mice are more likely to be affected by age (Mazzaccara et al. 2008), gender (Yamauchi et al. 1983) or strain (Kile et al. 2003) than ambient temperature under conventional husbandry conditions.

4.1.4 Body temperature
Conventional methods like rectal measurements of body temperature (Selman et al. 2001) require handling and restraint of the animals. This causes arousal and leads to stress responses, such as an immediate raise in body temperature (Watanabe et al. 1999), thus hampering the interpretation of study results. The implantation of transponders either in the abdominal cavity (Gordon 2004, Leon et al. 2004, Johnston et al. 2007, Gaskill et al. 2013) or subcutaneously (Kort et al. 1998, Vlach et al. 2000, Warn et al. 2003) allows continuous measurement of body temperature without disturbing the animals. This study used the subcutaneous approach to obtain the body temperature of mice housed on different bedding volumes. Consequently, the obtained measurements did not reflect the animals’ core body temperature as accurately as intraperitoneal implantation would have done (Kort et al. 1998). The implantation into the abdominal cavity requires surgery with the potential risk of postoperative complications, such as abdominal adhesions or intestinal obstructions (Johnston et al. 2007). Changes in body weight and behaviour indicated increased stress levels after intraperitoneal implantation (Baumans et al. 2001). The subcutaneous approach is likely to also have had some effects on the animals’ well-being, however the impact was presumably less profound, due to the considerably faster and less invasive procedure. Previous studies did not detect significant differences between the subcutaneous and intraperitoneal measurements (Kort et al. 1998, Vlach et al. 2000). The temperatures
obtained in this study were lower compared to intraperitoneal (Gordon 2004, Gaskill et al. 2013) and rectal measurements (Selman et al. 2001), however the subcutaneous readings confirmed gender (Selman et al. 2001, Gaskill et al. 2013) as well as housing differences (Gordon 2004). In accordance with previous findings (Gordon 2004), the bedding volume had significant effects on body temperature during resting phase. Gordon (2004) compared body temperature and metabolic rate of mice housed on a thin (just sufficient to cover the cage floor) and deep layer of bedding (approximately 7 – 10 cm depths). Core body temperature of mice housed on shallow bedding decreased by 1 °C during the light phase (inactive phase), while the core temperature of mice housed on a deep layer of shavings remained constant. These differences vanished during the dark phase as the animals became active (Gordon 2004). The author discovered that mice on shallow bedding either maintained their metabolic rate, which resulted in a decrease in core temperature (strategy 1) or increased metabolic rate to ensure a stable core temperature (strategy 2). During the day the animals followed strategy 1, during night strategy 2, this led to a decrease in body temperature during inactive phase and an increased activity during active phase compared to animals on deep bedding. A continuous measurement of body temperature and metabolic rate in the present study would have provided valuable insight regarding the impact of bedding volume on mice with respects to their circadian rhythm. Since Gordon (2004) only obtained data from two rather extreme housing conditions - comparable to 0.5 l and 6 l as used in this study - a continuous recording of physiological parameters of mice housed on intermediate bedding volumes, such as 1.5 l, would be of particular interest.

4.1.5 Pentobarbital narcosis

Pentobarbital sodium is a widely used anaesthetic in cardiovascular research (Liu et al. 2009, Jiang et al. 2011, Shekarforoush et al. 2016). Age has been known to interact with pentobarbital metabolism due to different levels of HME in different age groups (Vesell 1968, Canada et al. 1986, Lovell 1986a). To avoid this impact, narcosis was conducted at the same age (15 weeks) in this experiment. Previous studies (Vesell 1968, Cunliffe-Beamer et al. 1981, Jeon et al. 2015) performed barbiturate narcosis in younger animals, thus a comparison to the present data is rather difficult. Further information on the impact of bedding volume on pentobarbital sleeping time of different age groups is therefore still needed. The experiments confirmed the impact of strain and gender (Lovell 1986b) on pentobarbital sleeping time. Long-term as well as short-term effects of bedding volumes on mice ought to be analysed, therefore the animals were housed either a short or long period of time before the narcosis was performed. An adequate acclimatization prior to the experiments is crucial to avoid that transportation stress influences outcome measures. Most physiological parameters take at least up to 7 days to normalize (Obernier and Baldwin 2006). Although the present study used a short adaptation period of 2 weeks, physiological changes caused by transportation stress should not have influenced the pentobarbital narcosis. Particularly after a cage change interval of 2 weeks, the bedding volume affected ammonia levels within the cage, but no significant impact on pentobarbital metabolism was detected. A strong impact of ammonia on microsomal drug metabolism was observed in rats housed in mesh wire cages with a pan under the cage to collect urine and faeces (Vesell et al. 1976), thus it is likely that the ammonia levels in the present study were too small to impact the activity of HME.
A further factor known to influence barbiturate metabolism is ambient temperature, mice kept at 20 °C slept longer with corresponding lower levels of HME than animals housed at 25 °C and 30 °C (Vesell 1968). Although the present study provided evidence that a larger bedding volume is likely to affect the microclimate within the cage, the narcosis was performed at the same ambient temperature and all mice were placed on a heating plate, therefore observations compared to Vesell (1968) were not expected.

4.2 Impact of bedding volumes on mice’s well-being

4.2.1 Physical and physiological signs of stress
According to the present results, deeper cage bedding can increase mice’s welfare. Reduced adrenal weights were observed in mice housed on larger bedding volumes, strongly indicating lower stress levels in these animals (McCarty and Richardson 1974, Manser 1992). Cold stress has been successfully used to evoke adrenal hypertrophy in rats (Woods 1957) and mice (Wilson et al. 1972). Especially female mice housed at 12 °C, 21 °C or 30 °C showed enlarged adrenals with decreasing temperatures (Wilson et al. 1972), probably due to their higher vulnerability to thermal stress (Cannon and Nedergaard 2011). The impact of bedding volume on adrenal weights was present regardless of strain or gender, however only female BALB/c mice showed a significant increase in corticosterone levels when housed on small bedding volumes. This indicated that shallow bedding is more likely to trigger chronic stress responses than acute (Manser 1992) and that particularly female BALB/c mice might have been stressed by this housing condition. Assessment of thymus weight (Marin et al. 2007) and adrenal tyrosine hydroxylase (Marashi et al. 2003) would have provided further valuable evidence for the long-term impact of shallow bedding on laboratory mice.

In line with Yamauchi et al. (1983) no clear impact on spleen mass could be determined. Reduced spleen weights were observed in female BALB/c housed on larger bedding volumes, but neither in males nor C57BL/6 mice. While chronic stress-related immunosuppression can cause a decrease in spleen mass (Manser 1992), lower temperature can increase spleen weight (Krol et al. 2003), thus two factors might have impinged the spleen weight, leading to these discrepancies.

It is unlikely that the only reason for mice’s stress response was the insufficient insulation against the ambient temperature on shallow bedding. Comparable to nesting material (Gaskill et al. 2012), deep bedding probably fulfils various purposes, not only thermoregulatory ones. Consequential, mice might also prefer deep bedding even when their thermal needs are met. In accordance with this hypothesis, Gaskill et al. (2012) demonstrated that mice prefer a combination of nesting material and warmer ambient temperatures. The authors used a behavioural titration experiment to analyse the interaction between thermotaxis and nesting behaviour. Animals were able to choose between a standard environment (20 °C) with nesting material and ambient temperatures up to 35 °C without nesting material. Especially C57BL/6 and female mice carried nesting material to the warmer cage environment.
4.2.2 Impact on behaviour

Regardless of the different genetic backgrounds and resulting behavioural differences (Van Oortmerssen 1971), BALB/c as well as C57BL/6 mice preferred cages containing larger bedding volumes. Mice’s preference for deep cage bedding was more pronounced when 0.5 l was provided as shallow bedding. Even though the difference in bedding depths between 0.5 l and 1.5 l (test combination A) was a lot smaller than between 1.5 l and 6 l (test combination C), the preference was more pronounced in combination A compared to C. Moreover, dwelling time on the larger volume did not differ between test combination A (0.5 l vs. 1.5 l) and B (0.5 l vs. 6 l), although the difference between the volumes varied greatly (1 l for combination A, respectively 5.5 l for combination B). This strongly suggests that 0.5 l per Type III cage is not an adequate bedding volume for laboratory mice and that even a small increase in bedding depths can improve mice’s well-being. Workload and financial costs are limiting factors for large bedding volumes, underlining the importance of this observation.

Different studies have underlined the importance of burrowing and digging behaviour and used them as a reliable indicator for well-being in laboratory mice (Sherwin et al. 2004, Jirkof 2014). Despite the fact that 1.5 l and 6 l provided mice with a better opportunity to dig and burrow, the video analysis was not able to confirm the assumption that burrowing and digging behaviour increases with bedding volume. Comparable to Gross et al. (2012), the first 2 h of the dark phase were analysed (18:00 - 20:00 CET). This period of time was chosen because a previous scan of the videos revealed that the animals were particularly active during the onset of the dark phase. It is conceivable that mice showed more intensive digging behaviour at a different time period. A circadian rhythm has already been described for nest-building behaviour (Jirkof et al. 2013a). More video footage needs to be analysed, to allow an unambiguous statement regarding the impact of bedding volume on behavioural patterns in laboratory mice. Observation of home cage behaviour of C57BL/6 mice could reveal whether strain differences regarding the intensity of digging behaviour (Van Oortmerssen 1971) are visible on different bedding volumes. Digging has been described as a form of redirected nest-building behaviour (Van de Weerd et al. 1997a), this could explain why less nest-building was observed on larger volumes.

The video analysis did not show an impact of bedding depths on aggressive behaviour. Increased agonistic interactions have been described in male mice housed under enriched conditions (Haemisch et al. 1994). Deep bedding is a resource that is available to all animals and not a rigid housing component that needs to be defended. Comparable to nesting material (Van Loo et al. 2002) a larger bedding volume allows the mice to manipulate their surroundings. The opportunity to have some control over the cage environment is very likely to have a stress-reducing effect (Van Loo et al. 2002).

Breeding performance is a crucial factor in laboratory animal husbandry. There have been opposing results regarding the impact of housing designs on reproductive success. While a reduced number of pups born per dam have been reported for mice housed in enriched cages (nesting material, wood bar and nest box) (Tsai et al. 2003a), beneficial effects (Whitaker et al. 2009) or neutral effects (Shair et al. 2012) were also documented. According to the Directive 2010/63/EU “nesting materials or structures for breeding animals” need to be provided, underlining the importance of nesting material within the scope of rearing (Bult
and Lynch 1997). Due to their very limited ability to thermoregulate, especially newborns are dependent on nests, which protect them from heat loss. Mice have been observed to adjust the “maternal nest” according to the ambient temperature and a correlation between nest quality and offspring survival has been demonstrated (Lynch and Possidente 1978). It is easier to build an enclosed nest when it is possible to dig a small hollow in the bedding, which is then covered with nesting material (Van Oortmerssen 1971). A larger bedding volume can therefore facilitate nest-building for breeding mice and thus help to minimize heat loss for newborns.

The behavioural tests did not reveal signs of improved psychological well-being or reduced anxiety in mice on larger bedding volumes. Apart from strain and gender differences, the tests did not show a clear impact of the bedding volume. Van de Weerd et al. (1997b) observed a small impact of nesting material on open field activity in C57BL/6 mice, but not in BALB/c. The authors pointed out that major differences in behavioural tests were only detected between animals housed with or without objects. This could explain why no effect of the bedding volume on performance in open field and novel object recognition test was noticed.

### 4.3 Implications for mice’s husbandry

Whenever possible, mice create enclosed nesting sites to protect themselves from environmental conditions (Hess et al. 2008). Gordon et al. (2014) reported that mice housed at 22 °C used shredded nesting material to cover the bottom of the nest, avoiding contact with the cage floor. The results of the preference test strongly indicated that 1.5 l and 6 l provide a more similar environment than 1.5 l and 0.5 l do, despite their larger difference in bedding depths. The bedding material used in this project did not allow mice to dig tunnels and create burrows, however 1.5 l – and certainly 6 l – provided mice with enough material to easily cover the bottom of the nesting site with bedding. Significant differences regarding the body temperature were noticed either between 0.5 l and 1.5 l or 0.5 l and 6 l. This observation supports the hypothesis that mice housed on 1.5 l and 6 l could reduce heat loss more sufficiently than animals on 0.5 l. Consumer demands studies could help to assess the strengths of motivation for different bedding volumes. This would facilitate establishing a threshold for bedding volume used in the husbandry of laboratory mice.

The in-cage temperature has been analysed with opposing results, while Rosenbaum et al. (2009) did not detect an impact of bedding, Smith et al. (2004) discovered lower in-cage temperatures for corncob bedding compared to other bedding materials, including shaving bedding. As the materials were not presented in the same volume, it is not possible to determine whether the material, the amount or a combination of both was responsible for the results. The two studies used different methods to obtain in-cage temperature, impeding a comparison. A difference between bedding volumes or materials is most likely to be detected if the temperature is measured inside the nest (ideally with mice inside) rather than on the surface of the bedding.

Using a “phantom” mouse to mimic heat loss, Gordon et al. (1998) demonstrated that bedding material (wood shavings or beta chips (a material comparable to the bedding used in the present study)), cage tops (wire or filter) and cage bottoms (acrylic or wire) had a
strong impact on the thermal cage environmental. Heat loss was lowest when the phantom was covered with wood shavings in a cage with filter tops. Preference test confirmed that mice prefer softwood shaving compared to a chip structure (Kirchner et al. 2012). It is very likely that thermal preferences played an important role in this study. In contrast to wood shaving, a chip bedding does not allow mice to create burrows and hide under the bedding, thus increasing their heat loss. Consequentially, different bedding material might not provide laboratory mice with the same thermal comfort even when they are present in the same amount. Since only chip bedding was used in this study, further studies are needed to assess whether a softwood bedding leads to similar anatomical, physiological and behavioural changes.

4.4 Impact of bedding volume on variation

Previous data suggested that cage enrichment can increase variation, thus raising the numbers of animals needed in experiments (Eskola et al. 1999, Mering et al. 2001, Tsai et al. 2002, Tsai et al. 2003a). Mice housed on 0.5 l showed a tendency of increased variation compared to groups on deeper bedding, except for pentobarbital narcosis. Vesell (1968) reported that SD rose in direct proportion to an increase in hexobarbital sleeping time, thus CV was not affected by the duration of the narcosis. The present data was not able to confirm a correlation between SD and sleeping time. Organ weights were normalized (Gaskill et al. 2013, Gordon et al. 2014), before the CVs were calculated. When actual organ weights were used to analyse the variability, the trend 0.5 l > 1.5 l > 6 l was not changed. BALB/c and C57BL/6 mice in enriched housing revealed higher CVs for the majority of haematological values and organs weights (Tsai et al. 2002). Comparable to the present study, Tsai et al. (2003b) pooled the data of each parameter and discovered a tendency towards a higher CV in enriched groups compared to standard housing in behavioural tests (food drive test and open field test) and haematological values in DBA mice. Variation in organ weights was not only influenced by housing design, parameter studied and strain (Tsai et al. 2002), but also by group size (Mering et al. 2001). Previous studies showed that variation of clinical chemistry was affected by cage enrichment, but there was no clear trend towards an increase or decrease (Eskola et al. 1999, Mikkelsen et al. 2010). The current experiments discovered significant differences between the bedding volumes for pooled clinical chemistry values, with lower CVs on deeper bedding. The overall positive impact of deep bedding on variation provides further arguments in favour of increasing the bedding volume in the husbandry of laboratory mice. The cited studies used various objects (such nest boxes or wood bars) to enrich the husbandry. Unlike deep bedding, these resources were not available to all the animals at all times, possibly contributing to the different effects on variation. Barnett (1965) reported increased CVs for tail lengths in mice housed at -3 °C compared to animals kept at 21 °C, however the trend was not persistent in all strains. It would be sensible to analyse further data of mice housed at different ambient temperatures, to assess whether cold stress is a potential factor that influences variation.
4.5 Conclusions
This project was able to characterize the importance of bedding volume in the husbandry of laboratory mice and provided vital information on the impact of different bedding volumes on animals and experimental results.

Can deep bedding be considered a refinement in the husbandry of laboratory mice?

A rise in the standard housing temperature is the most obvious solution to ameliorate thermal stress for laboratory mice, thus making them more predictive and reliable models in biomedical research (Karp 2012). However, there is not one optimal housing temperature for mice, as their thermal preferences are dependent on various factors (Gaskill et al. 2009). Increased ambient temperatures over 25 °C will result in an uncomfortable working environment for humans (Helppi et al. 2016), higher financial costs and also bear the danger of increased aggressive behaviours in male mice (Greenberg 1972). Instead of the housing temperature, the housing environment should be changed. Deeper bedding provides mice with more control over their cage environment, they are able to adjust their surroundings (dig hollows in the bedding, etc.) according to their specific thermal needs. The present data demonstrated that an increased bedding volume can be considered as an environmental refinement, it matched mice’s preferences and the animals showed clear signs of improved well-being. Deeper cage bedding is a sensible adjustment in mice’s husbandry, not only in terms of animal welfare, but also for scientific research. Even though BALB/c and C57BL/6 have a different genetic background (Festing 1979), with resulting behavioural differences, they showed similar preferences and physiological responses to different bedding volumes. This strongly suggests that the results have a certain general validity and that deeper bedding can refine housing conditions for other mice strains as well as.

Does deeper bedding interfere with experimental results, including variation?

According to the present data, the bedding volume had a major impact on mean values, but not on variation. Significant physiological and morphological changes were most likely the results of reduced heat loss and metabolic demands on deeper bedding. Variation of experimental parameters were mainly reduced rather than increased, therefore a larger bedding volume is not likely to raise the number of animals needed for significance. Even small changes in the bedding volume, such as an increase from 0.5 l to 1.5 l, had a strong impact on the animals and thereby on experimental parameters. This emphasizes the importance to adhere to guidelines, such as the ARRIVE guideline (Kilkenny et al. 2010) or the Gold Standard Publication Checklist (Hooijmans et al. 2010) and mention bedding material as well as the amount used in scientific studies. Otherwise reproducibility of experiments can be compromised and a differentiation between effects caused by the bedding volume and experimental procedures is hampered.
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