Aus dem Institut für Tierschutz, Tierverhalten und Versuchstierkunde
des Fachbereiches Veterinärmedizin
der Freien Universität Berlin
und
der Fachgruppe für Versuchstierkunde
der Abteilung Experimentelle Toxikologie und ZEBET
des Bundesinstituts für Risikobewertung

Recording and Reducing Boredom Symptoms in Laboratory Mice - the Semi Naturalistic Environment

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
an der
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vorgelegt von
Paul Mieske
aus Frankfurt (Oder)

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Abbreviations

CON	conventional housing condition/control
EE	environmental enrichment
ENR	enriched housing condition
RFID	radio frequency identification
SNE	semi naturalistic environment

1 Introduction

Mice are the most commonly used animal model for biomedical and behavioral research in Europe (*Summary Report on the statistics on the use of animals for scientific purposes in the Member States of the European Union and Norway in 2019,* 2019). The use of mice in experiments, especially inbred strains of mice, has many advantages. Mice have a short reproduction cycle (Brust et al., 2015). In the recommendations for keeping mice, not much space for housing is required (*Directive 2010/63/EU of the European Parliament and of the Council off 22 September 2010 on the protection of animals used for scientific purpose* 2010). Also, mice are low in cost of care. For maximum comparability of experimental conditions, inbred strains provide unlimited access to individuals that are in theory genetically identical.

In laboratory studies, mice are usually kept in plastic cages, approximately the size of a shoebox. Depending on the stocking density, these cages offer between 330 and 820 cm^2 of floor space. The cages have transparent plastic walls and are closed off at the top with a grid lid. Usually, there is another plastic cage cover on the grid lid, which allows circulation with ambient air through a filter mat. Newer and more advanced versions of these cages are specifically ventilated and are stored in racks where the environmental conditions are controlled, and an automated regulation of the access to food and water is integrated.

As social animals, mice must be kept in groups unless the specific study design or animal welfare reasons (e.g. aggression) contradict this (*Directive 2010/63/EU*). The directive states that the home cage environment must be complex enough for animals to develop and act out natural behaviors. Despite this requirement, cages in animal studies often feature a minimum amount of bedding and nesting material. It is usually up to the experimenter and the study design wether additional shelter in form of a small house made of cardboard or plastic is provided in the cage. In most experimental designs, this minimal equipment represents conventional cage housing.

By expanding the cage space, keeping larger groups of animals than usual, and adding structural or interactive elements, conventional caging can be made more complex. This expansion of housing conditions is called environmental enrichment (EE). It is generally assumed that EE promotes the development and expression of natural behaviors and reduces stereotypical behavior in mice (Marashi, Barnekow, and Sachser, 2004; Gaskill and Pritchett-Corning, 2015; Bailoo et al., 2018). Thus, EE increases the welfare of laboratory mice being kept. Despite the advantages of EE, a frequently voiced criticism is that greater complexity in housing conditions compromises the standardization of housing. Additional enrichment elements must be affordable and considered in the daily care of the animals and cages. However, there are no standardized specifications for EE, so a variety of elements from a wide range of providers are used.

Since 1960, EE has been used to study or achieve a vast number of effects. Much of this research relates to the behavior of laboratory animals. However, EE may also have effects on physiological parameters. One emotional state that can take on both ethological and physiological alterations and can be caused by a monotonous housing environment is boredom. Humans are able to describe

and communicate boredom quite clearly. In animals, due to the lack of language, their behavior is the most obvious expression of boredom.
This doctoral project investigated boredom in laboratory mice. Methods were developed and evaluated to record and reduce symptoms of boredom. Groups of female mice were kept in three different housing conditions and studied through experiments that recorded both behavior and physiological parameters. A controlled and replicable enrichment protocol extended standard cage housing. In addition, an alternative housing system was used that provided a semi naturalistic environment (SNE) for a large group of animals. The SNE provided more cage space per individual animal. Levels at different heights enabled vertical exploration. Access to food, water, shelter, and structural enrichment elements in every part of the enclosure resulted in a complex environment, as it might be found in nature. The focus of this thesis was to evaluate the suitability of the SNE as a housing system. It was investigated whether individual behavior could be observed and whether data collected from animals housed in the SNE produced higher variances than data from animals in standard housing.

2 Literature

This section is covered by a systematic review that was done during the doctoral studies. The use of EE in behavioral studies on mice and rats were reviewed. The study focused on whether boredom is taken into account in these studies. It was found that boredom itself was greatly underrepresented, but many parameters associated with boredom were examined. In addition, the study provides an overview of the methods used to investigate boredom-associated parameters.

Title:
Bored at home?—A systematic review on the effect of environmental enrichment on the welfare of laboratory rats and mice

Authors: Paul Mieske*, Ute Hobbiesiefken*, Carola Fischer-Tenhagen, Céline Heinl, Katharina Hohlbaum, Pia Kahnau, Jennifer Meier, Jenny Wilzopolski, Daniel Butzke, Juliane Rudeck, Lars Lewejohann and Kai Diederich

* shared first authorship - These authors have contributed equally to this work

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Bored at home?—A systematic review on the effect of environmental enrichment on the welfare of laboratory rats and mice

Paul Mieske[1†], Ute Hobbiesiefken[1†], Carola Fischer-Tenhagen[1], Céline Heinl[1], Katharina Hohlbaum[1], Pia Kahnau[1], Jennifer Meier[1], Jenny Wilzopolski[1], Daniel Butzke[1], Juliane Rudeck[1], Lars Lewejohann[1,2] and Kai Diederich[1*]

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Boredom is an emotional state that occurs when an individual has nothing to do, is not interested in the surrounding, and feels dreary and in a monotony. While this condition is usually defined for humans, it may very well describe the lives of many laboratory animals housed in small, barren cages. To make the cages less monotonous, environmental enrichment is often proposed. Although housing in a stimulating environment is still used predominantly as a luxury good and for treatment in preclinical research, enrichment is increasingly recognized to improve animal welfare. To gain insight into how stimulating environments influence the welfare of laboratory rodents, we conducted a systematic review of studies that analyzed the effect of enriched environment on behavioral parameters of animal well–being. Remarkably, a considerable number of these parameters can be associated with symptoms of boredom. Our findings show that a stimulating living environment is essential for the development of natural behavior and animal welfare of laboratory rats and mice alike, regardless of age and sex. Conversely, confinement and understimulation has potentially detrimental effects on the mental and physical health of laboratory rodents. We show that boredom in experimental animals is measurable and does not have to be accepted as inevitable.

KEYWORDS

animal behavior, animal welfare, enriched environment, boredom, abnormal behavior, impoverished environment, laboratory animals (mouse and rat)

Introduction

Recommendations for the husbandry of laboratory animals have been developed primarily with a view to standardizing experimental conditions and providing basic needs like water and food (1, 2). While satisfying basic needs helps avoid obvious pain and suffering in laboratory animals, in modern animal husbandry, saving resources

and personnel costs is certainly also an important factor. For the planning of animal experiments, compromises are made between the various interests of researchers, animal caretakers, animal house managers, and animal welfare advocates. The guidelines of the EU-directive for example contains basic recommendations including that social animals should be kept in groups and that all laboratory animals should be given the opportunity to develop a wide range of normal behavior by providing a housing condition with sufficient complexity (Directive 2010/63/EU). Moreover, species-specific recommendations for rats and mice call for the provision of environmental enrichment to make laboratory animal housing more diverse (e.g., https://www.nc3rs.org.uk/3rs-resources/housing-and-husbandry-mouse). However, the type of housing referred to as "enriched environment" has changed significantly in the last decades (3, 4). For example, some of what was described as enriched animal husbandry 25 years ago nowadays just meets the basic recommendations [i.e., a cardboard tube (5, 6)]. Moreover, not only has the concept of enrichment changed over time, but so has the related conventional housing, which usually reflects the actual state of housing and legal requirements at the time of publication. Still, the current housing of most laboratory animals reflects an impoverished environment compared to truly species-specific housing. More specifically, one must assume that the lack of stimuli has far-reaching consequences for the well-being and health status of laboratory animals. In fact, Cait et al. (7) showed in a meta-analysis of 214 studies that conventional housing increases morbidity and mortality in research rodents. This is backed up by the here reviewed research on comparing laboratory conventional housing to a more varied enriched housing using more space, social contact, and/or physical items, which conclusively describe positive effects on well–being and behavior of mice provided with enrichment.

Environmental enrichment was initially introduced to laboratory animals for studies investigating the effect of environment on neurobiological parameters and learning behavior (8). For this very purpose it is still being used, for example, enrichment has been proven to be an effective therapeutic intervention in animal models of various diseases including stroke (9) and neurodegenerative diseases like Alzheimer's disease (10). Moreover, a stimulating environment improves learning and memory formation and is a potent trigger for neuroplastic events in the adult brain—a process originally thought to occur only in the young developing brain (11). In addition to disease models and neurobiological studies, increasing focus has been placed on the effect of stimulating environments on animal welfare. Stress-responses were mitigated under enriched housing conditions and the activity of natural-killer cells was enhanced (12). Expression of abnormal repetitive behaviors (i.e., stereotypies) were reduced in mice living in an enrichment environment (13–16) as were behavioral measures related to anxiety (13, 17). In summary, most publications indicate that enriched and varied housing conditions improve the well–being of laboratory animals. However, due to the low stimuli of conventional housing systems compared to a species-appropriate environment, this conclusion might be validly expressed in the opposite sense, that confined housing of laboratory animals compromises animal welfare and health.

Conventional husbandry of laboratory animals in research laboratories is characterized by confinement, monotony, and lack of challenge. In humans, such conditions are usually accompanied by a condition known as boredom. Boredom is an emotional state that usually relates to individuals having nothing to do, are not interested in their surroundings, and feel that life is dull and tedious (18, 19). This state could also very aptly describe the life of many laboratory animals housed in small barren cages. Few studies have directly addressed the issue of animal boredom so far. However, based on the findings from human studies (20), some behavioral abnormalities observed in captive animals can be readily linked to boredom (21).

For example, barbering behavior in animals has recently been related to Trichotillomania ("hair-pulling disorder"), a human disorder reportedly triggered by boredom (22, 23). Common abnormalities in captive animals are stereotypies, which are often related to a lack of stimulation in laboratory animals. Stereotypic behavior in mice like wire gnawing/bar-mouthing (6), circling at the cage lid, back-flipping, route tracing, and twirling (13, 14) was shown to be decreased under more stimulating enriched housing conditions. Another symptom of human boredom is an altered perception of time, in which time does not seem to pass in monotonous situations (24). In animals, this phenomenon can be measured objectively by training them to expect a specific event or reward after a predictable period and measuring their anticipatory behavior after being exposed to monotonous tasks or environments (21). This method was successfully trained in starlings using pecking a key as an anticipatory behavior (25). It is reasonable to assume that laboratory rodents also experience such a perceptual shift, but as far as we know this has not been investigated until now. Overall, it is not unfounded to speculate that the great overlap between human symptoms of boredom and similar phenomena in rodents indeed indicates that boredom in animals is both real and underestimated in laboratory animals.

Since a sufficient form of stimulation is lacking in boring situations, sensation-seeking or stimulus-seeking behavior also occurs in animals (21). This is seen as a form of escape from the unpleasant, boring situation. Indeed, it has been described that it is sometimes of little importance whether the stimulus has a positive or negative valence if interaction is possible at all (26). Burn et al. (27) showed stimulus seeking in ferrets as increased contact to negative and ambiguous stimuli compared to a control group which were provided a 1 h daily play time. Furthermore, ferrets without playtime spent more time lying awake with their eyes open, screeched

more but sat and stood less, than after playtime (27). This form of awake inactivity as a form of suboptimal arousal can be seen as an indicator of bored animals as well and was also more apparent under non–stimulating housing conditions in mink (26, 28) and mice (29). Moreover, Meagher et al. (28) found increased interest in different external stimuli in mink in non-enriched environments as a form of sensation seeking of potentially bored animals. These two almost opposite extremes of boredom symptomatology—sensation seeking vs. awake inactivity—illustrate the multifaceted nature of the expression of boredom and thus the difficult search for a fixed definition for this distressing and damaging emotional condition. In psychology and medicine, boredom is gaining increasing recognition as a potentially harmful emotional state and as a field of research for translational studies (19, 30). Regarding animal welfare, boredom becomes a serious concern with an urgent need for research. In this systematic review, we therefore examined the literature on enriched environment with specific regard to the effects of housing conditions on well-being in laboratory mice and rats. Moreover, we examine the existing body of literature specifically related to boredom symptoms. By identifying measures of boredom as well as clues to potential cures for boredom in laboratory rodents, we aim to lay the groundwork for addressing this pressing issue in the context of modern animal research.

Materials and methods

Search strategy

In accordance with PRISMA guidelines, we searched the database Web of Science on July 5th, 2019, and again on February 24th, 2021, before data analysis commenced. We performed a supplementary search on Web of Science, Embase, and PubMed on March 29th, 2022. In terms of population, we focused on mice and rats, the most widely used laboratory animals in experimental research. Enriched housing conditions were included as intervention and a corresponding non-enriched/conventional housing as a comparator. At least one behavioral observation or test should have been performed as an outcome parameter for animal welfare. For further specialization of the resulting search string boredom and its synonyms were as well–included as their respective counterpart. To achieve a high outcome of relevant research papers in the final search, truncations with wildcards and synonyms were used in the search string establishment.

Searchstring:

TS = (boredom OR tedium OR ennui OR tediousness OR stuffiness OR dullness OR boringness OR monotony OR bor OR monoton* OR motivat* OR stimulat* OR excit* OR activ* OR "affective state*")*

AND TS = (hous OR husbandry OR "animal keeping" OR environment*)*

AND TS = (mice OR mouse OR rat OR rats)

AND TS = (behavior OR behavior*)*

AND TS = (standard OR conventional OR barren OR restricted OR impoverished)

AND TS = (enrich OR seminatural OR semi-naturalistic)*

Selection of studies and information extraction

Abstract screening was done by nine reviewers (PM, UH, CF-T, CH, KH, PK, JM, JW, and KD) using the systematic reviewing online tool SyRF (http://www.syrf.org.uk/). Exclusion criteria included the use of other animals than rats and mice, no behavioral observation or experiment, use of only one housing condition, use of psychoactive drugs, use of a disease or transgenic models. We excluded editorials, conference abstracts, and review papers.

Ten reviewers (PM, UH, CF-T, CH, KH, PK, JM, JW, LL, and KD) independently screened full text and extracted information from eligible studies into a standardized form. Extracted parameters included species, strain, sex, age at the start of the housing period and the beginning of the behavioral experiment, the presence of a focus on animal welfare, the disease/lesion model, genetic modification, psychoactive substances/stimulations, enrichment category (social, object, space of home cage) and description, number of groups including control group and their housing, the mean behavioral outcome parameter and the used behavioral test. Compliance with scientific quality criteria in the included studies was assessed by ascertaining whether the allocation of animals to experimental groups was randomized and the assessment of outcomes was blinded. Any discrepancies were resolved by consensus. Randomization was done with the sample() function in the statistical computing software R (https://www.r-project.org/).

Categorization and classification of age and durations of housing

Outcome parameters were categorized as follows: social behavior, aggressive behavior, abnormal behavior, affective well–being, activity, cognition, nociception, motor function, circadian rhythm, and exploratory behavior. An overview of the behavioral tests used in the studies and the assignment to the categories is shown in Supplementary Table 1. In addition, we extracted information about glucocorticoid hormones to evaluate effects of housing on stress. However, determination of stress hormones regarding sample source, number, and

sampling-time was very heterogeneous. We therefore included glucocorticoids only in the main overview.

For a detailed examination of the effects of enrichment on animal welfare, the results of each study were considered in terms of sex of experimental animals, age of experimental animals, and duration of housing in the respective housing environments. For age classification, animals were designated as postnatal from 0 to 21 days of age, adolescent from 21 to 60 days of age, adult from 60 to 750 days of age, and post reproductive from more than 750 days of age (31). Duration of husbandry was classified in short, mid, and long-term housing duration with short defined as 0 to 30 days, mid with 30–90 days and long-term with more than 90 days.

For an in-depth investigation of boredom, all selected publications were screened again for boredom-specific parameters. Because few studies have explicitly examined boredom in animals, especially laboratory animals, the classification of boredom parameters was based on the symptomatology of human boredom and relevant translatable phenomena in mice and rats. The sources for these parameters were literature on human boredom (20, 32) and Charlotte Burn's pioneering review article on animal boredom (21). All studies selected in this systematic review were examined regarding these parameters. For the examination of the parameter "drug seeking behavior", the studies related to the use of psychoactive substances that were excluded for the main analysis were re-integrated into this single analysis. Results of this part of the analysis are summarized in Table 1.

Analysis

Analysis and illustrations were done using the software environment R (version 3.6.3, https://www.r-project.org/, R Foundation for Statistical Computing, Vienna, Austria) and the development software and graphical user interface RStudio (version 1.2.1,335, RStudio, Inc., Boston, MA, United States).

To assess the impact of enrichment on the defined categories, it was determined whether the selected studies reported an increase or a decrease in the respective categories; if no change was found, the result was classified as neutral. In the figures, the bars represent the studies that reported an increase, a decrease, or no change in the respective parameter in the corresponding category. The thickness of the bars reflects the amount of identified and investigated studies for this category. The numbers indicate the observed effect of the enrichment as a decimal number. If this value reaches 1, all studies in this category have observed an increase; correspondingly, a decrease if the value reaches−1. A bar located further to the right of the scale thus indicates an increasing effect of the applied enrichment on the category under consideration. The numbers correspond to the principle of a Likert scale.

Results

Study inclusion and study characteristics

Search strategy and study selection results are presented in Figure 1. After removal of duplicates, 884 titles/abstracts were screened, of which 438 were excluded. Full texts of the remaining 446 records were then screened, and 228 did not meet the eligibility criteria. This left 186 articles for qualitative synthesis.

71.6% of studies reported randomization of animals to treatment groups and only 24.3% of studies indicated blinding of outcome assessors.

Figure 2 shows the parameters that were examined in the context of environmental enrichment. The figure also shows the parameters that were defined as indicators of boredom and explicitly searched for in the publications. There is a large overlap between the factors examined in the studies and the boredom-related parameters.

Increasing number of publications about home cage enrichment

The number of studies examining the effects of enriched housing on mouse and rat behavior has steadily increased, particularly over the past decade, with peaks in 2013, 2015, and 2018 (Figure 3). In 2022, three papers were included in the parameter extraction with one of them focusing on animal welfare. All studies that explicitly aimed to improve the housing conditions of laboratory animals and thus were dedicated to refining animal experiments were categorized as "Focus on animal welfare". Although the absolute number of publications with a focus on animal welfare was slightly increasing over time, its overall proportion is still low.

Results on reviewed methods and experimental designs

Rats have been used more frequently than mice to study the effects of housing conditions on behavior and for both species, mainly males were examined (Figure 4). The most frequently used rat strain was Sprague-Dawley (48 studies) followed by Wistar (44 studies). Twenty-two studies housed Long-Evans rats as experimental animals. Eighteen different strains of mice were studied in the context of environmental enrichment. The most used strains were C57BL/6 (39 studies), BALB/C (13 references) and CD-1 mice (11 references).

The enrichment applied in the examined studies was divided into three categories. "Social enrichment" was defined as being housed in a group or provided with a cage partner. When additional space by increasing the home cage size was used to provide enrichment, the category

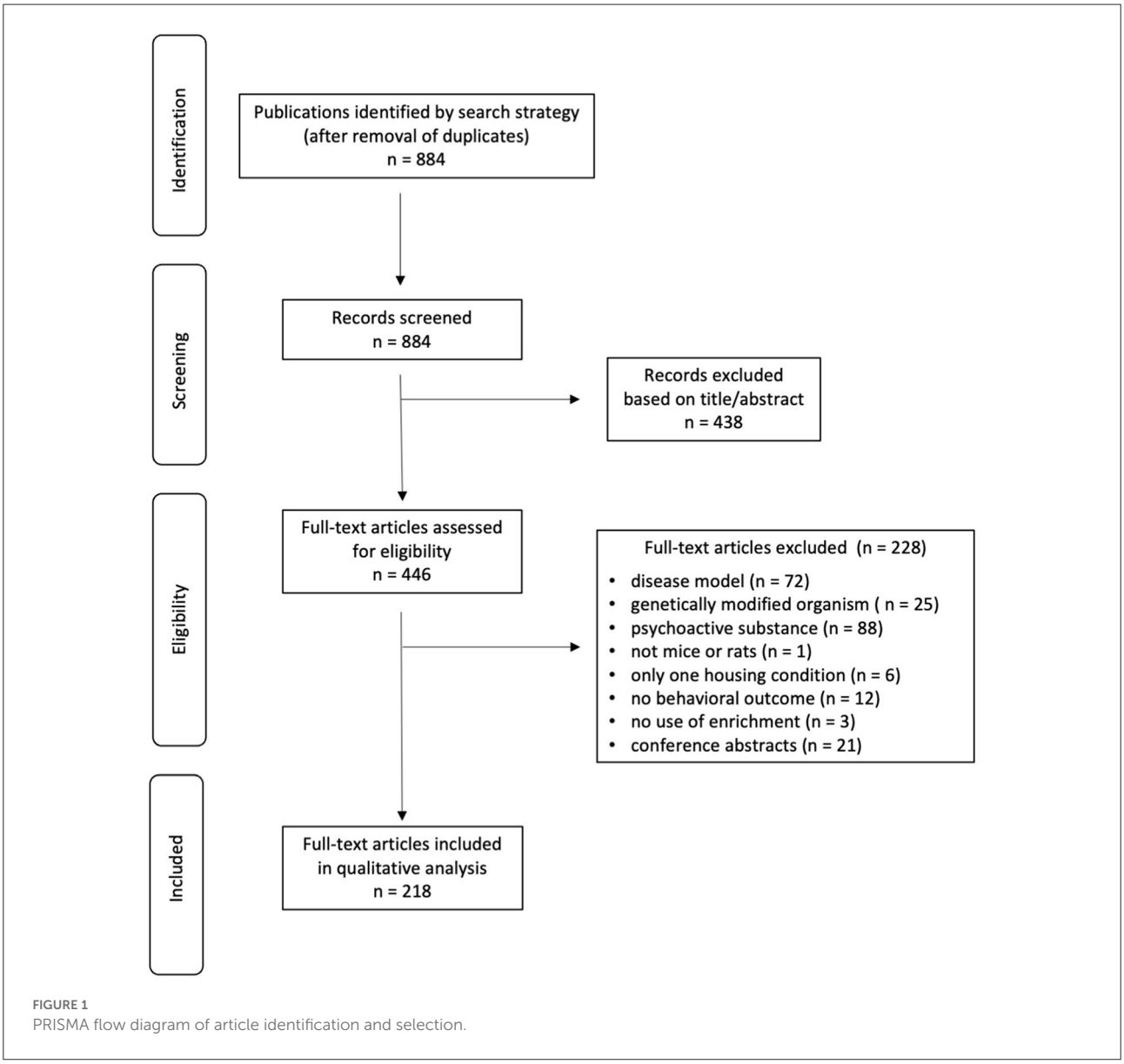

FIGURE 1
PRISMA flow diagram of article identification and selection.

'size enrichment' was indicated. The "object enrichment" category was assigned when the environment was changed by the introduction of objects of any kind (toys, climbing opportunities, structural elements).

Most studies used a combination of all three types of enrichment in their experiments (104 studies). This was followed by a combination of object enrichment and size enrichment of the home cage (55 studies). Social enrichment alone (6 studies), enrichment of home cage space alone (3 studies) and the combination of social and spatial enrichment (3 studies) were the least used types of enrichment. Three studies used environmental enrichment in their experiments but did not mention the type.

A stimulating environment is essential for the development of natural behavior and animal welfare

Providing animals with an enriched environment substantially improves cognitive skills. Motor function, social behaviors and affective state were positively affected, and abnormal behaviors were considerably decreased compared to conventional or barren housed animals, also indicating a positive protective effect. The effects of enrichment on the categories aggressive behavior and activity though remain inconclusive. There is no clear tendency for stress hormones to increase or decrease in relation to housing conditions (Figure 5).

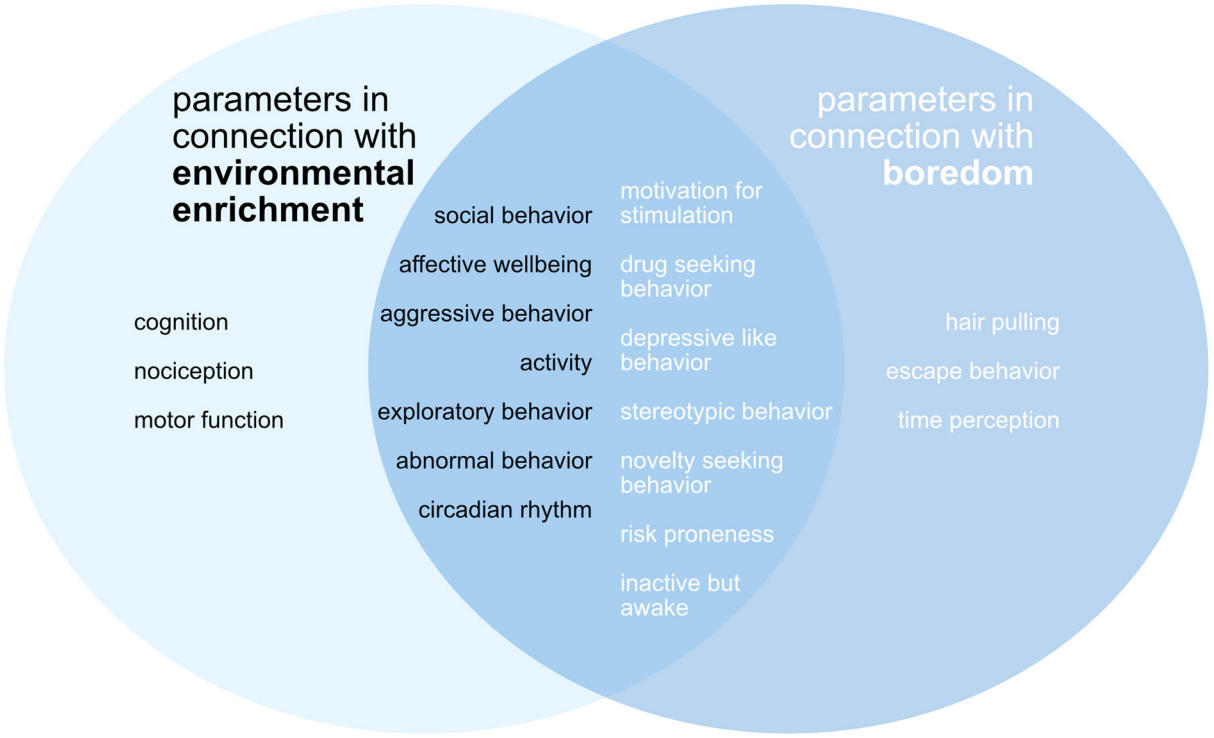

FIGURE 2
Scheme of the assessed outcome parameters with reference to enriched environment and boredom. Left in black letters: parameters in connection with environmental enrichment. Right in white letters: parameters in connection with boredom. There is a considerable overlap between the categorized parameters in the examined publications and the parameters associated with boredom.

Enriched housing promotes well–being in mice and rats, and regardless of sex and age

The reported effects of environmental enrichment on animal welfare are largely independent of the animal species compared in this study. Mice and rats benefit similarly from enrichment of their living environment (Figure 6A).

Most of the studies examined were performed on males (123 studies, Figure 6B). Fifty-eight studies examined both sexes whereas only 31 studies did experiments on female animals. Enrichment increases cognition, social behavior and motor function and decreases abnormal behavior in females and males, with these effects being more pronounced in females. Regardless of sex, a similar number of studies reported an impairment, a reduction, or no effect on activity. Exploration and aggressive behavior in females increased with the provision of enrichment. Eight studies examined the effect of enrichment on aggressive behavior in male animals. In four of these studies, an increase in aggressive behavior was observed.

Most of the studies reviewed were conducted with adolescent animals (117 studies, Figure 6C). Seventy studies used adult animals and 29 studies used postnatal animals. Two studies used post-reproductive animals. Apart from this discrepancy in the use of animals of different ages, the effects of enrichment on cognition, affective well-being, social behavior, and the development of abnormal behavior proved generally positive for all age groups. Motor function was positively affected by enrichment but data in postnatal and adult animals are lacking here as well as in post-reproductive animals. Ambiguous results of the effect of enrichment on aggressive behavior, exploratory behavior, and activity with an increase, decrease as well as a neutral or no effect could be detected.

The longer the period of housing in an enriched environment, the higher the benefit to welfare

Most of the included studies applied a medium housing period (30–90 days, 124 studies). The most beneficial effect of enrichment was obtained with a long housing duration (more than 90 days, 33 studies) but all durations could improve motor function, cognition and affective well–being and exert a protective effect against the development of abnormal behavior (Figure 7). The effect of enrichment duration on aggressive

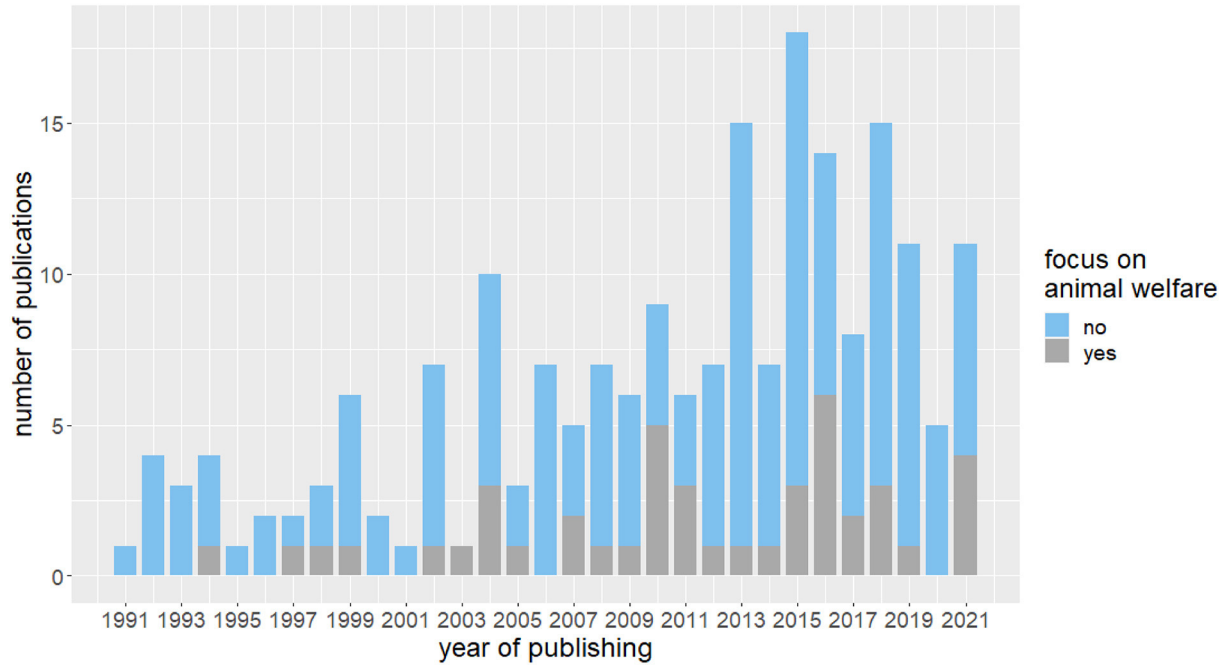

FIGURE 3
Absolute number of included publications in the years 1991 to 2022. Indicated is the number of publications with and without explicit focus on animal welfare in the publications.

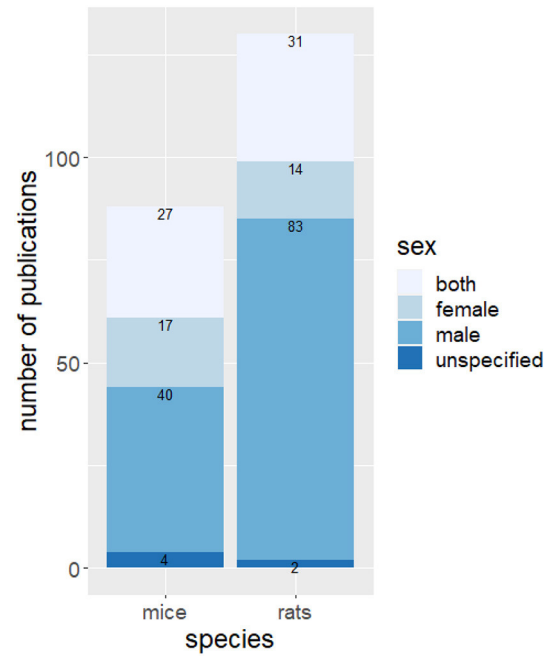

FIGURE 4
Number of publications using rats and mice and sex bias. Indicated is the absolute number of publications with the specified species and sexes.

behavior and activity remained inconclusive with a tendency to an increase in aggressive behavior and activity in a long-term provision of enrichment.

Discussion

Environmental enrichment has been a popular research topic for some time, not excessively but continually researched. Neuroscience research has provided some fundamental results in this field, elucidating the close relationship of animal housing conditions on the structure and function of the central nervous system. Most published studies use enrichment as an intervention in animal models of various diseases, including stroke (127, 128), traumatic brain injury (129), and Alzheimer's disease (10). Although this is a highly exciting field of research, these studies were deliberately not included in this systematic review. This systematic review instead focuses on enriched environment as a means of preventing boredom-like symptoms and improving the welfare of laboratory animals.

While research activity on enriched environments has increased steadily over the years, only a small fraction of the investigated studies dealt specifically with animal welfare. This is perhaps not surprising, since there are various definitions of animal welfare (130), and no consensus on how to improve it.

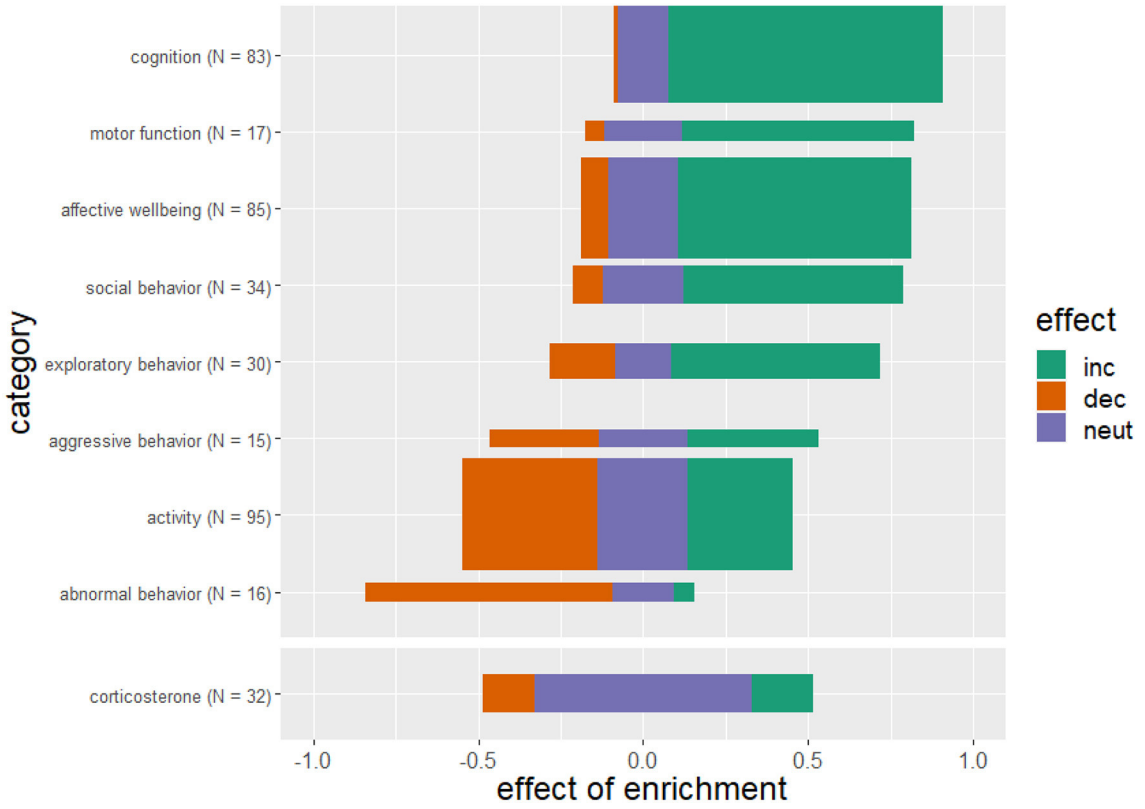

FIGURE 5
Effects of enriched housing on behavioral outcome and corticosterone level. Bars represent the studies that reported an increase (inc), a decrease (dec) or no change (neut) in the parameters of the corresponding category. Values indicate the observed effect of the enrichment as a decimal number. The thickness of the bars reflects the number of investigated studies for this category. The sum of references per category is greater than $N = 218$ studies because some studies examined more than one outcome parameter.

However, our data show that the proportion of studies with a specific focus on improving the living conditions of laboratory animals in enrichment research is slightly rising. As animal welfare research gains increasing recognition as an established research discipline, the number of research papers in the field will likely continue to grow. For example, recent research shows that tunnel handling can improve physiological well-being and often the handling tunnel is used as an additional enrichment item (131).

Our analysis shows that rats are used more frequently than mice in enrichment research and that different strains of both species are used. Nevertheless, rats and mice benefit similarly from an enriched living environment and there is no evidence that housing conditions affect the welfare of strains differently. Females are underrepresented in studies with mice and even more so in studies with rats. Among the studies using mice, 31% reported the use of both sexes, 46% the use of male, and 19% the use of female mice. In the rat studies, 24% used both sexes, 64% used male, and only 11% used female rats. A similar bias toward the use of male subjects has been found in preclinical animal research (132). The underrepresentation of female subjects in animal research is based on the belief that females are more variable than males due to their estrous cycle. However, for most applications including behavioral measures, female rodents display no more variation than males do; and female estrus cycles therefore need not necessarily be given special consideration (133). The underrepresentation of females in animal research is still pervasive, and the scientific understanding of female biology is compromised by these persistent disparities. To address the inadequate inclusion of female animals, the US National Institutes of Health has implemented policies in 2014 that require applicants to indicate their plans for a balance of males and females in preclinical studies in all future applications, unless sex inclusion is not warranted due to strictly defined exceptions (134). The bias toward male subjects in animal research is receiving additional attention due to a plausible implication in the much-discussed translational crisis. Less consideration has so far been devoted to the obvious ethical implications of this sex imbalance. Since no fewer females than males are born in breeding facilities for laboratory animals, the question inevitably arises as to what happens to the "surplus" females (130).

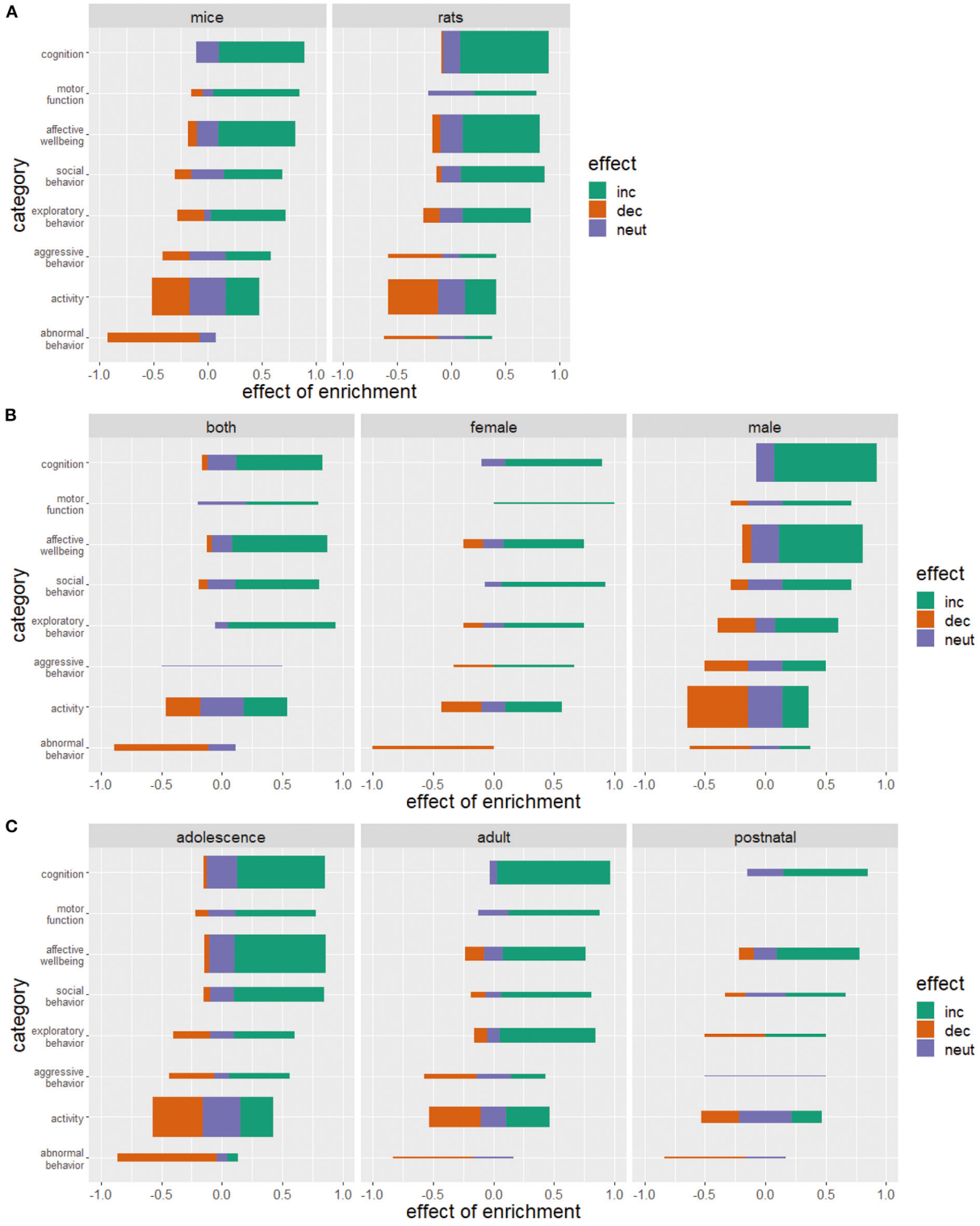

FIGURE 6
Effects of enriched housing on mice and rats **(A)**, in relation to sex **(B)**, and age **(C)**. Bars represent the studies that reported an increase (inc), a decrease (dec) or no change (neut) in the parameters of the corresponding category. Values indicate the observed effect of the enrichment as a decimal number. The thickness of the bars reflects the number of investigated studies for this category. **(C)** Animals were considered postnatal at the age of 0–21 days, adolescent at the age of 21–60 days, adult at the age of 60–750 days and post reproductive at the age of more than 750 days.

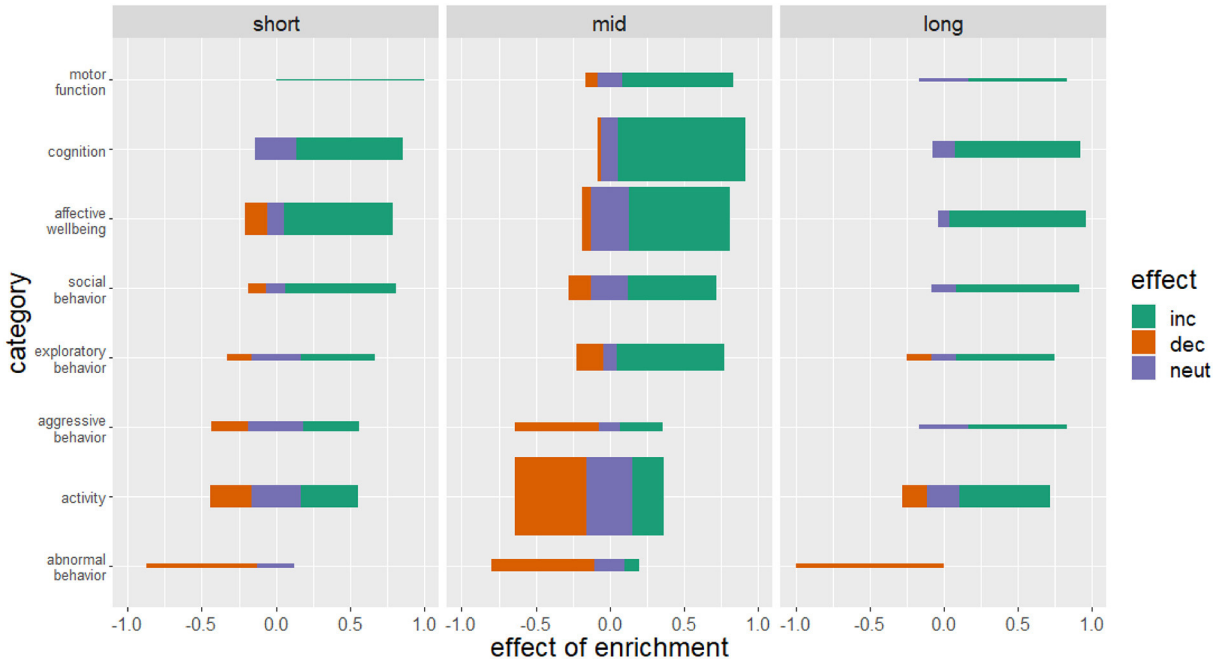

FIGURE 7
Effects of enriched housing in relation to housing duration. Bars represent the studies that reported an increase (inc), a decrease (dec) or no change (neut) in the parameters of the corresponding category. Values indicate the observed effect of the enrichment as a decimal number. The thickness of the bars reflects the number of investigated studies for this category. Duration of enriched housing was classified in short housing duration of 0–30 days, mid housing duration of 30–90 days and long-term housing duration of more than 90 days.

Age is another important experimental factor in animal research that is often inadequately considered in experimental design and poorly reported in publications. Animals used in the examined enrichment studies tend to be young. In most of the studies, the housing phase in the enriched cages started at 0–4 weeks of age. In the behavioral tests, many of the animals were then tested at 6–14 weeks of age. This corresponds to the average age of 8–12 weeks at which laboratory animals are usually used in animal research (135). At this age, many developmental processes are not yet complete. It is therefore important to note that age-related physiological changes can have a major influence on experimental outcomes.

The positive effects of a diversified housing on physical, cognitive, and affective health of laboratory animals have been demonstrated by numerous publications analyzed in this review. Motor function, cognition, affective well–being, and social behavior benefited most from enriched housing. A reduction in abnormal behavior was also frequently reported with enriched housing. The effect of enrichment on activity remains inconclusive. One possible reason for the ambiguous results on the activity parameter is the broad definition of the parameter, which might limit the interpretability. Another reason could be the observed decrease in abnormal behaviors (stereotypies) due to housing in an enriched environment, which are usually accompanied by a significant level of activity. Since an enriched environment is often associated with more space and/or the provision of a running wheel, animals in these housing conditions clearly have more opportunity for physical activity than animals in confined housing. Mice housed in enriched cage systems outperformed conventionally housed animals on the rotarod, indicating that enrichment stimulates motor coordination and presumably fitness, even when no running wheel or disc is provided (136). Numerous studies on animals and humans have evidenced the beneficial influence of physical activity on the musculoskeletal system (137, 138). It is therefore a reasonable assumption that keeping laboratory animals in confined cages can harm the bone structure and musculature of laboratory animals.

Interestingly, we did not detect a clear increase or decrease in glucocorticoid stress hormones associated with housing conditions. In a recent review, however, it was suggested that conventional laboratory housing was found to be associated with chronic stress (7). Instead of a chronic increase in stress hormones, we suggest that conventional housing may rather reduce the capacity of the stress axis to cope with environmental challenges and that the health impairments result from constant under-stimulation. This would be in line with the proposed non-linear relation of stress and welfare as proposed by Korte et al. (139). However, it should be noted that the determination of stress hormones in the included publications was very

TABLE 1 Overview of publications addressing boredom related parameters, the respective outcome, and the behavioral test used.

Boredom related parameter	Publications	Outcome	Behavioral test
novelty seeking	(33)	increase	open field, behavioral observation
behavior	(34)	increase	Y-maze
	(35)	increase	open field, light-dark test
	(36)	increase	open field, behavioral observation
	(37)	increase	open field, object recognition test
	(38)	decrease	elevated plus maze, open field
	(39)	decrease	open field
	(40)	decrease	activity cage
	(41)	decrease	open field, behavioral observation
	(42)	decrease	two-lever operant conditioning chamber
	(43)	increase	object recognition test, open field
	(44)	increase	behavioral observation
	(45)	increase	radial-arm maze
	(46)	increase	open field, Y-maze
	(47)	increase	open field, Y-maze, light-dark test
	(48)	increase	Y-maze, object recognition test
	(49)	increase	barrier test, group test, intruder test
	(50)	decrease	open field, light-dark test
	(51)	increase	elevated plus maze, light-dark test, concentric square field test
	(52)	increase	Y-maze, light-dark test
	(53)	increase	object recognition test
	(54)	increase	corridor field task
	(55)	decrease	behavioral observation, elevated plus maze
	(56)	increase	open field
	(57)	increase	open field elevated plus maze, light-dark test
	(58)	increase	hole board test
	(59)	increase	light-dark test, concentric square field test
	(60)	decrease	open field, elevated plus maze
	(61)	increase	open field, light-dark test, hole board test
	(62)	decrease	novelty place preference
	(63)	increase	object recognition, passive avoidance test
	(64)	decrease	elevated plus maze, open field
	(65)	decrease	lever-responding task
depressive like behavior	(66)	decrease	forced swim test
	(67)	increase	forced swim test
	(68)	neutral	forced swim test
	(69)	decrease	forced swim test
	(70)	decrease	forced swim test
	(71)	decrease	forced swim test
	(72)	neutral	tail suspension test
	(73)	decrease	forced swim test
	(74)	neutral	forced swim test
	(75)	increase	tail suspension test
	(76)	increase	forced swim test, sucrose preference
	(77)	increase	forced swim test
	(78)	neutral	forced swim test

(Continued)

TABLE 1 Continued

Boredom related parameter	Publications	Outcome	Behavioral test
	(79)	decrease	forced swim test
	(50)	decrease	forced swim test, sucrose preference
	(80)	decrease	tail suspension test
	(81)	decrease	forced swim test
	(82)	decrease	forced swim test
	(83)	decrease	forced swim test
	(29)	decrease	forced swim test
	(84)	decrease	forced swim test
	(85)	decrease	sucrose preference
	(86)	decrease	forced swim test
drug-seeking behavior	(87)	decrease	conditioned place preference
	(88)	decrease	conditioned place preference
	(89)	decrease	conditioned place preference
	(90)	decrease	conditioned place preference
	(91)	decrease	cocaine context renewal test
	(92)	decrease	operant conditioning chamber
	(93)	decrease	conditioned place preference
	(94)	decrease	conditioned place preference
	(95)	decrease	operant conditioning chamber
	(96)	decrease	conditioned place preference
	(97)	decrease	drinking in the dark test
	(98)	decrease	operant conditioning chamber
	(84)	decrease	two-bottle choice test
	(99)	decrease	operant conditioning chamber
	(100)	decrease	conditioned place preference
	(101)	decrease	operant conditioning chamber
	(102)	neutral	operant conditioning chamber
	(103)	decrease	context induced relapse test
	(104)	decrease	conditioned place preference
	(105)	decrease	operant conditioning chamber
	(106)	decrease	liquid consumption
	(107)	neutral	sign tracking
	(108)	decrease	conditioned place preference
	(65)	decrease	alcohol self-administration
stereotypic behavior	(6)	decrease	behavioral observation
	(109)	decrease	behavioral observation
	(110)	decrease	behavioral observation
	(44)	neutral	behavioral observation
	(111)	decrease	behavioral observation
	(112)	decrease	behavioral observation
	(113)	neutral	behavioral observation
	(114)	decrease	behavioral observation
	(76)	decrease	activitymeter, behavioral observation
	(115)	neutral	behavioral observation
	(116)	decrease	behavioral observation

(Continued)

TABLE 1 Continued

Boredom related parameter	Publications	Outcome	Behavioral test
	(29)	decrease	behavioral observation
	(117)	decrease	activity testing chamber
	(118)	neutral	behavioral observation
	(13)	decrease	behavioral observation
	(16)	decrease	behavioral observation
motivation for	(119)	increase	running wheel, open field
stimulation	(120)	increase	operant training
	(121)	decrease	operant conditioning test
	(50)	increase	open field, hole board
	(122)	decrease	behavioral observation
Inactive but awake	(110)	decrease	behavioral observation
	(123)	decrease	open field, behavioral observation
	(29)	decrease	behavioral observation
	(124)	decrease	behavioral observation, open field
risk proneness	(125)	increase	open field, radial water maze
	(51)	increase	elevated plus maze, light-dark test
	(126)	neutral	open field, elevated plus maze, inhibitory avoidance

The table is sorted showing the boredom related behaviors with the largest number of publications first. The publications investigating the specific behaviors are sorted by year ascending in order to show actual trends in this field of research. The boredom related parameters escape behavior, hair pulling and time perception were not investigated in the reviewed publications.

heterogeneous in terms of sample source, number, and timing and that these parameters were not assessed. This evaluation was not a central topic of this work, and measurement of glucocorticoid stress hormones was not a part of the search strategy. However, our preliminary data suggest that a more thorough analysis of this parameter may be warranted.

The effects of a stimulus-rich environment on cognition and affective well–being are well–documented and there is accumulating evidence for potential underlying brain structures and neurophysiological mechanisms. These extend from brain region volume and morphology to neuron complexity and excitability, adult neurogenesis, synaptic plasticity, and a plethora of molecular responses including gene-environment interactions, inflammation, and trophic factors (140–143). Many of these effects are likely linked to the increased physical activity associated with an enriched housing. However, there are processes that are directly attributable to the stimulative elements of enrichment. These include the successful differentiation and long-term survival of newly formed neurons during neurogenesis, processes that can be clearly distinguished from the proliferation of neural cells, which in turn is facilitated in particular by physical activity (144).

In the studies reviewed, a variety of housing, bedding, and nesting materials, as well as various items or any combination thereof, were used as enrichment. It is worth noting that pre-build shelters can have different effects than providing material for building their own nests (145). Historically, all additions to housing cages were considered enrichment. In this way, "enrichment" became an umbrella term for a variety of shelters, bedding and nesting materials, and miscellaneous items, or any combination thereof, and lacked a general theoretical framework for what should be considered enrichment (4). This is also reflected in the studies reviewed. In most publications, a combination of social, object and spatial enrichment was used (Supplementary Table 2). Because of the widespread simultaneous use of all types of enrichment, there is no clear consensus on which form is most effective in preventing housing-specific behavioral disorders.

Enriched environment alleviates boredom-like symptoms in laboratory animals

Some of the outcomes extracted in this review may be directly related to boredom in laboratory animals. These included abnormal behaviors like stereotypic, hyperactivity, and inactive-but-awake behavior, as well as novelty-seeking, drug-seeking, and depressive like behavior. Thirty-three publications dealt with novelty-seeking behavior in the broadest sense (Table 1). This parameter is often investigated with the open field test or elevated plus maze, but also by observing the behavior or activity in newly presented home cages. While novelty seeking is assumed to be an indicator of boredom, the measurement

of novelty seeking is often linked to activity and exploration in a range of different tests. This makes it difficult to clearly attribute the results of tests classified as novelty seeking in terms of boredom. Therefore, there is no unequivocal effect of environmental enrichment on novelty seeking behavior.

Twenty-three of the included publications investigated depressive like behavior in connection with environmental enrichment. This was mostly done with the forced swim test and tail suspension test. Fifteen studies (65%) describe a decrease and four (17, 4%) an increase in symptomatology in animals housed in an enriched environment. The 10 most recent studies published since 2014 uniformly show a decrease in depressive-like behavior in animals housed in enriched environments.

Twenty-four publications were identified as studies on drug-seeking behavior. Here, a consistently positive effect of environmental enrichment was reported.

Sixteen studies examined stereotypic behavior in mice and rats were. There was an overall decrease of stereotypic behavior under enriched housing conditions. Although the occurrence of stereotypic behavior appears to be a multifactorial event in animals (6, 15), it can be observed more frequently under barren restrictive housing conditions and has been shown to be reduced by the use of enrichment in zoo animals (146). Burn (21) argued that stereotypic behaviors increase under monotonous situations and identified abnormal repetitive behaviors as a potential measurable boredom parameter in captive animals.

Very poorly represented are the boredom parameters motivation for stimulation, inactive but awake and risk proneness with 12 publications in total. These characteristics, which closely relate to human boredom, are also influenced by environmental enrichment. Motivation for stimulation is a parameter that has been reported to be both increased and decreased by an enriched environment. This parameter is usually derived from the activity behavior of the animals and determined by a variety of tests that lead to inconclusive results. Awake inactivity was reduced by enriched environment in every included publication. Two studies found increased, and one found unchanged risk proneness, in animals living in an enriched environment. However, with only three publications related to risk proneness in our body of literature, this statement should be viewed with caution.

Escape behavior, hair pulling, or a possible shift of time perception were not examined by any publication. Overall, it must be noted that in only a few cases boredom was specifically mentioned at all.

Methodological considerations

Although boredom is resonant in many enrichment studies, it is almost never directly examined and rarely mentioned at all. Due to limited data availability, conducting a meta-analysis on this particular topic is not feasible. Nevertheless, to approach the topic, we developed a systematic review in which we investigate the effect of animal husbandry on the welfare of laboratory animals and assign some of the extracted welfare parameters to typical symptoms of boredom. Since boredom and animal welfare are multifaceted conditions, this work is not based on the investigation of a single outcome, as considered in the classical PICO scheme but examines a set of parameters related to welfare and potentially boredom of laboratory animals.

The evaluation of the compliance with the established scientific quality criteria in the examined studies revealed a common lack of reported blinding. The percentage of about 25% of studies reporting blinding seems to be relatively low especially compared to preclinical biomedical studies (147) and also compared with a recent meta-analysis of the effects of housing on mortality in animal models of disease (7). One possible reason for this could be that behavioral studies are increasingly automated and/or conducted in the home cage without any required intervention with a (blinded) experimenter. In the case of behavioral observations in the (enriched) home cage, blinding of the observer is difficult to implement; in the case of automated behavioral analyses, it may not be necessary. This was not explored in this work; however, a systematic review of the use of automated and home cage-based systems for behavior analysis would be intriguing.

Although the study protocol was determined a priori, the protocol of this systematic review was not pre-registered. While this was not done in this work, it should be emphasized here that prospective registration of systematic reviews and meta-analyses reduces the potential for bias and fosters transparency (148).

Conclusion

Our findings show that a stimulating environment can be considered essential for the development of natural behavior and animal welfare of research rodents. Although boredom is almost never studied directly and rarely mentioned, this theme clearly resonates in many studies of the effects of improved housing conditions. Chronic boredom as a consequence of living in a barren and confined environment can pose a health risk to laboratory animals, limiting their validity as model organisms for biomedical research. A stimulating living environment sustains the well–being of laboratory rats and mice alike, regardless of age and sex. Although a longer period of housing might be more beneficial, even a short period in a stimulating environment improves essential parameters of animal welfare. Providing animals with adequate space, social contact, and a stimulating environment should not be considered a luxury or a treatment, but a necessity to ensure mental and physical health and a foundation for the expression of natural behaviors.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

PM, UH, DB, LL, and KD: developed the study concept and design. PM, UH, CF-T, CH, KH, PK, JM, JR, JW, LL, and KD: were involved in data acquisition and analysis. PM, UH, LL, and KD: drafted the manuscript and figures. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.899219/full#supplementary-material

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3 First Publication

Title:

Roaming in a Land of Milk and Honey: Life Trajectories and Metabolic Rate of Female Inbred Mice Living in a Semi Naturalistic Environment

Authors: Paul Mieske, Kai Diederich and Lars Lewejohann

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Roaming in a Land of Milk and Honey: Life Trajectories and Metabolic Rate of Female Inbred Mice Living in a Semi Naturalistic Environment

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Simple Summary: Different forms of environmental enrichment are used to increase the wellbeing of laboratory animals. These forms include extending the available cage space, housing a large group of animals within the same unit and adding stimulating physical objects. The semi naturalistic environment (SNE) used in this study implements all of these enhancements. However, there is debate as to whether such variation in housing standards increases the variability of experimental data. Indeed, it has been shown that mice living in the SNE developed individual differences in activity and behavioral parameters. Here, we investigated whether housing in the SNE enhances individual differences in aged animals and whether these differences are reflected in certain physiological parameters. These aspects were considered to assess the suitability of the SNE as a reference system in future studies. We found that the individual-level activity patterns of the animals stabilized during the housing period in the SNE. These behavioral characteristics did not correlate with the measured physiological parameters. Considering the variance of the measured data, which is comparable to the literature, the SNE seems to be a suitable system for studies comparing different housing systems in terms of animal welfare.

Abstract: Despite tremendous efforts at standardization, the results of scientific studies can vary greatly, especially when considering animal research. It is important to emphasize that consistent different personality-like traits emerge and accumulate over time in laboratory mice despite genetic and environmental standardization. To understand to what extent variability can unfold over time, we conducted a long-term study using inbred mice living in an exceptionally complex environment comprising an area of 4.6 m^2 spread over five levels. In this semi-naturalistic environment (SNE) the activity and spatial distribution of 20 female C57Bl/6J was recorded by radio-frequency identification (RFID). All individuals were monitored from an age of 11 months to 22 months and their individual pattern of spatial movement in time is described as roaming entropy. Overall, we detected an increase of diversification in roaming behavior over time with stabilizing activity patterns at the individual level. However, spontaneous behavior of the animals as well as physiological parameters did not correlate with cumulative roaming entropy. Moreover, the amount of variability did not exceed the literature data derived from mice living in restricted conventional laboratory conditions. We conclude that even taking quantum leaps towards improving animal welfare does not inevitably mean a setback in terms of data quality.

Keywords: laboratory mice; animal welfare; environmental enrichment; behavior; physiology; individuality; housing condition; home cage monitoring; semi naturalistic environment

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1. Introduction

Even in most well-planned and optimized studies using laboratory mice, the animals usually spend less time in the experiment than in everyday animal housing [1]. This should bring into focus the question of the suitability of the housing systems used. In Europe, minimal standards for animal housing are regulated by guidelines at national and international level (i.e., directive 2010/63/EU). Such guidelines stipulate, that mice should be kept in pairs or groups, taking into account sex-specific aspects to avoid undesirable reproduction or overly aggressive behavior. With regard to the size of cages the appendix of the EU directive gives minimum requirements of 330 cm^2 in area (60 cm^2 per mouse) and 12 cm in height. For reasons of costs and efficiency, most laboratory animal housing is based on these minimum criteria rather than on the space requirements that would be desirable from the animals' point of view. Indeed, studies indicate that mice prefer to explore larger territories than provided in standard sized cages [2]. In addition, the EU Directive 2010/63 states that animals should be provided with 'space of sufficient complexity to allow expression of a wide range of normal behavior'. In fact, it is questionable whether the minimum requirements meet this overall objective.

Although not always feasible in terms of maintenance and available laboratory space, a larger habitat would certainly mimic much more appropriately the ecological niche for which the mouse genome has evolved. Since mice are social animals, group housing during laboratory experiments is also important to promote animal behavior that comes as close as possible to their natural state. In addition, studies have shown that isolation of mice causes stress and lasting damage [3–5]. As a consequence, for biomedical research, a more natural setting might even strengthen translational research with regard to understanding how certain treatments affect mice without constraints imposed by barren housing conditions. In fact, natural enclosures have been successfully used to measure different kinds of behavioral, physiological and morphological parameters [6–8]. An enriched environment presented to a bigger group of animals may, however, increase the agonistic behavior within the group [9]. For example, in a study using a semi natural enclosure to characterize a transgenic model of Alzheimer's disease [8,10], considerable numbers of agonistic interactions occurred between male mice. However, it is of note that the social structure and the behavioral patterns to establish such a mouse typical despotic social hierarchy could only be observed to the full extent precisely because the animals were kept in a large enclosure.

However, even when housed in small and barren cages, mice tend to exhibit individual personality-like traits leading to variability, which is seemingly resistant to even rigorous standardization [11]. In fact, the concept of animal personality has proved to be a viable way to explore variability in experimental data [12]. In order to understand the emergence of such individual differences, it has been a fruitful approach to observe the animals over long periods. In a previous study using a semi-naturalistic environment, it was shown that differences in exploratory behavior became more pronounced over time and correlated with adult neurogenesis and behavioral patterns [13,14]. In addition, repeatability of activity measures became increasingly stable during the early life of mice indicating that individual behavioral phenotypes were more predictable after adolescence [12].

In this explorative study, we focus on analyzing adult female mice regarding their activity, spontaneous behavior, and physiological parameters. Measurement of activity in our semi naturalistic environment is fully automated by the use of RFID antennas [8,10,13–15]. This creates a huge amount of continuous data without the need for handling or placing the animals into a novel environment. We examined the development of individual differences in a large group of twenty female C57Bl/6 mice with a special focus on the late adult phase of life. In contrast to previous studies, the mice were older (434 days) at the start of the activity measurement and the observation period also was longer (8 months). The unique dataset also allows the correlation of physiological and behavioral

parameters as well as their activity patterns. Increased variability in the data due to housing the experimental animals in an enriched environment has already been addressed [16]. We aimed to determine whether a super-enriched environment increases the individuality of the mice across a variety of behavioral and physiological parameters. Thereby, we evaluate the suitability of the SNE as a housing system for comparative experiments possibly improving animal welfare in future experiments. Thus, we report on the life of these mice, which, in contrast to standard housing conditions, lived in a "land of milk and honey" with generous amounts of space to roam while having access to food and water *ad libitum*.

2. Materials and Methods

2.1. Animal Husbandry

When housed together with females, males are known to defend their territorial boundaries in such an enclosure [10]. In order to avoid pronounced aggression, we started by studying only females. Twenty female C57Bl/6J mice were purchased from Charles River (Charles River, Sulzfeld, Germany) at an age of eight weeks. At arrival the animals were special pathogen free and were then kept in standard cages in an open rack system. Our mouse facility conducts an annual health check in order to maintain the status. After seven days, the mice were tagged individually with a radio frequency identification (RFID) transponder. This was followed by another seven days of monitored recovery. During these two weeks, animals were kept in a home cage system consisting of two type IV cages connected by a plastic tube. At an age of 10 weeks, the animals were transferred to the semi naturalistic environment. Animals were habituated and kept at a 12/12 h light cycle (summertime lights on 8:00 a.m.–lights off 8:00 p.m., wintertime lights on 7:00 a.m.–lights off 7:00 p.m.), at 22.0 ± 2.0 °C, and 50.0 ± 5.0% humidity. Once a week, animals were weighed and handled to check for their health status. In the course of the study, one mouse died at an age of 404 days due to causes unrelated to this study, therefore the number of mice was reduced to 19 except for the weight development data. All experiments were conducted in accordance with the applicable European and national regulations and were approved by the State Office for Health and Social Affairs Berlin (G 0069/18).

2.2. Transponder Injection

All animals were marked individually for identification and activity measurement with an RFID transponder (ID 100, diameter: 2.12 mm; length: 11.5 mm, Trovan, Ltd., Douglas, UK). For pain treatment, 60 min before the injection, the animals received the non-opioid analgesic meloxicam (0.1 mg/kg, Meloxydyl, Ceva Tiergesundheit GmbH, Düsseldorf, Germany) orally. The transponder was subcutaneously injected between the shoulder blades under inhalation anesthesia with isoflurane according to established procedures (1.0–1.5% in 30% O_2 with 70% N_2O). The wound was then closed with tissue adhesive. The waking of the animals was monitored.

2.3. Semi Naturalistic Environment

The semi naturalistic environment (SNE) is a large wire mesh cage of 1.7 × 1.7 × 2.1 m (length × width × height, Figure 1). The two-part base level and three elevated levels together create an area of 4.6 m^2. The whole area was filled with aspen bedding (Polar Granulate 3–5 mm, Altromin, Lage, Germany) about 3 cm high. The different levels are connected with plastic tubes. Every level provides food (autoclaved pellet diet, LAS QCDiet, Rod 16, LASvendi, Soest, Germany) and water *ad libitum* and shelter for the animals. The two upper levels additionally provide two nesting boxes built from inverted type II cages with drilled-in air holes.

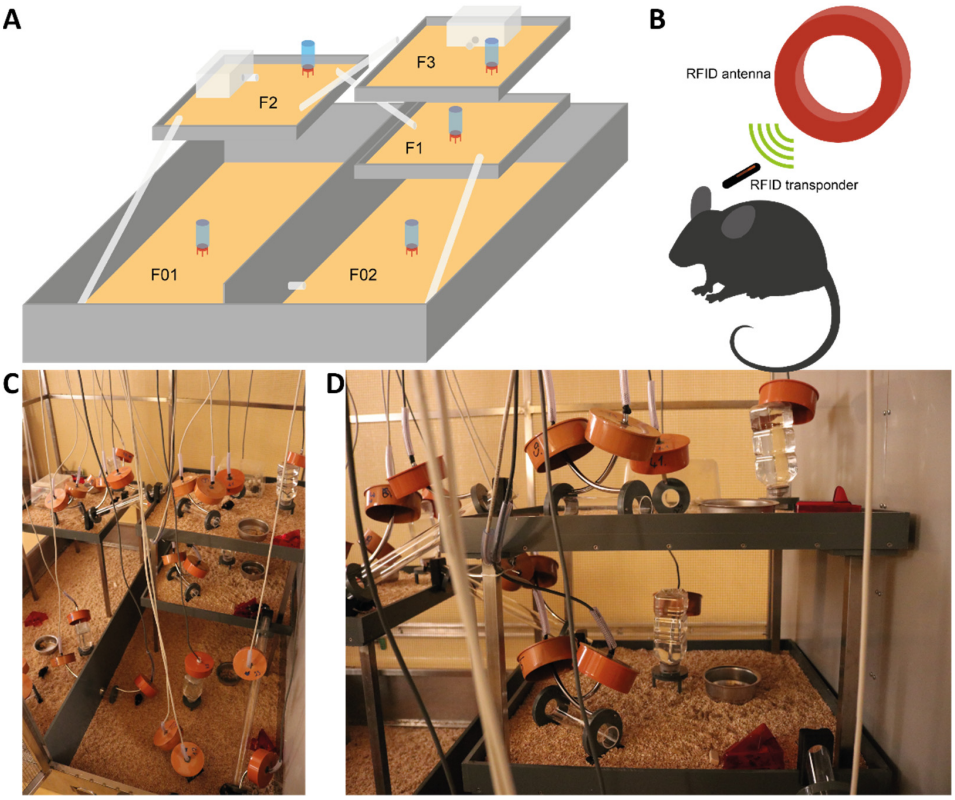

Figure 1. (**A**) Semi naturalistic environment (SNE) divided into five different levels (F01–F3). (**B**) Schematic illustration of the principle of tracking the mice through the RFID antennas. (**C**) Picture of the entire cage interior. (**D**) Zoomed in picture of the attachment of the antennas to enrichment tubes, drinking bottles and connecting tubes.

Throughout the SNE, 27 RFID ring antennas were placed systematically so that any change between the levels as well as the use of water sources and nesting boxes could be detected (Figure 1C,D). The arrangement of the antennas and the structure of the SNE is based on earlier work with a similar enclosure [15,17]. The antennas receive the signal of the transponder when a mouse passes through. The SNE was cleaned weekly by exchanging food, water, and soiled parts of the bedding. Every four weeks the whole cage was emptied and all surfaces as well as the nesting boxes and tubes were cleaned with hot water. During cleaning, animals were kept in a standard type IV cage with access to food, water and shelter as well as nesting material.

2.4. Measurement of Activity

Activity was measured as the number of contacts an animal had with the RFID antennas within the SNE. When a mouse passes an antenna, it receives a signal from the transponder of the animal. The antenna number as well as the unique ID of the animal is stored in a database with a corresponding timestamp. This was done with the Jerry 2 Recorder software used previously [15]. Thereby data of the movement and activity of every individual in the SNE was recorded continuously. To compare the spatial exploratory behavior, activity data was converted into the roaming entropy (RE). RE depicts a distribution value that sums the probabilities of every antenna being contacted by an animal at a certain time point (see Equation (1)). Thus, an animal that contacted many antennas on a broad range during an observed time obtains a higher RE than an animal that only passed a few antennas on a small area during the same time [13,14]. RE ranges between 0 and 1.

By cumulatively adding up the RE for every observed time period (e.g., day, week), the cumulative roaming entropy (cRE) is gained (see Equation (2)).

RE and thus cRE have been calculated daily for the activity during the dark phase (12 h), since activity during the light phases was influenced by the possible presence of caregivers or the performance of experiments. It can be assumed that during the dark phase undisturbed behavior is shown and thus a higher comparability between the individual, observed time periods arises.

Equation (1): roaming entropy RE, i—individual, t—observed point of time or period of time, j—antenna, k—maximal number of antennas

$$RE_{i,t} = -\sum_{j=1}^{k} (p_{i,j,t} \log p_{i,j,t}) / \log(k).$$

Equation (2): cumulative roaming entropy cRE, i—individual, t—observed point of time or period of time

$$cRE = RE_{i,t_1} + RE_{i,t_2} + RE_{i,t_3} + \cdots + RE_{i,t_n}.$$

2.5. Behavioral Observation

Spontaneous behavior of individually marked animals in the SNE was recorded by live focal animal observation. At the start of the observations, animals were 74 weeks old (524 days). Observations ended at the age of 80 weeks (565 days). Behavior was recorded in seven sessions of two days each scheduled between 7:30 and 9:00 a.m. Every individual animal was observed for 5 min per session. The order of observed animals was randomized. Behavior was recorded using the "Behavioral Observation Research Interactive Software" (BORIS) [18]. Short keys on a keyboard were pressed whenever a behavior occurred for frequency of behavior or pressed two times (start–stop) for duration of behavior. The time an animal spent resting (more than 10 s no movement) and time spent out of sight of the observer was subtracted from total observation time. The method for behavioral observations as well as the ethogram of the behavioral categories were based on Freund et al. (2015) [14].

2.6. Metabolic Rate

The principle of indirect calorimetry (TSE phenomaster, TSE Systems GmbH, Bad Homburg, Germany) was used to evaluate the metabolic rate. The calorimetry system was situated at a separate room at a 12/12 h light cycle, 22.0 ± 2.0 °C, and 50.0 ± 5.0% humidity. Animals were tested at the age of 82–84 weeks (584–594 days) in a randomized order. Following habituation to the experimental room (12 h), mice were weighed and placed individually in measurement cages equipped with bedding, shelter and nesting material. Food and water were accessible *ad libitum* during the entire measurement and were weighed before and after the experiment. Measurement cages and an empty reference cage were perfused with air. In the measurement cage, oxygen was lowered, and carbon dioxide was increased by the respiration of the animals during the measuring period (12 h light period). After flowing through both cages, the composition of air was compared between measurement cage and reference cage. By calculating the difference between air compositions, the metabolic rate of the examined animal was assessed. After the measurement the mice, food, and water were weighed, and the animals were placed back into their home cage. The resting metabolic rate (RMR) was measured as oxygen consumption rate ($\dot{V}O_2$) during the resting phases of the animals. To separate resting phases from active phases, the cumulative frequency percentage was plotted against the measured $\dot{V}O_2$. With a segmented linear regression, the threshold between $\dot{V}O_2$ of the resting phase and the active phase could be calculated. Data below the threshold were used to determine the RMR ([19]; R package 'segmented').

2.7. Timeline of Experiments

After habituation, the experimental animals were kept for 364 days in the SNE before data acquisition was started. During this time the RFID setup was build, developed, and tested. Figure 2 shows the sequence of experiments performed during the housing in the SNE. At an age of 501 and 508 days, additional measurements of physiological parameters (bone density and grip strength of mice) were done as part of another study with a different focus. This also included a blood sampling on day 510. These data were not discussed in the present article but the fact that measures were conducted must be considered for the discussion of activity profiles.

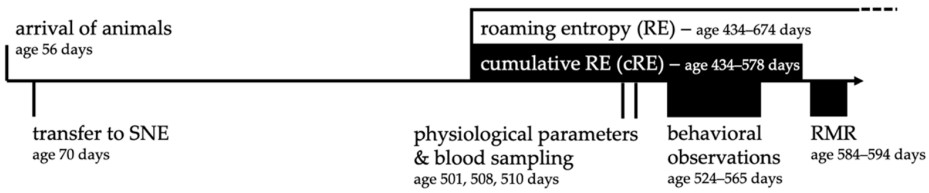

Figure 2. Schematic timeline of the housing of the experimental animals and the different experiments done.

2.8. Statistical Analysis and Calculations

Unless describes otherwise, all measured data is presented as mean ± standard deviation. In addition, the coefficient of variation (CV), the maximum value, the minimum value and the number of measured animals is given. The CV is calculated by dividing the standard deviation to the mean and is expressed as a percentage. It is used as an accepted parameter to evaluate variability within and across studies [20,21].

Analysis and illustration of data were done with the software environment R (v 3.6.3, R Foundation for Statistical Computing, Vienna, Austria, R Studio v 1.2.1335, RStudio, Inc., Boston, MA, USA). Continuous data (body weight and RE) were analyzed using linear models ('lm()' function). Related predictors were added as mixed effects to the regression models (package 'lme4' [22], 'lmer()' function). Subsequent statistical comparison of different models ('anova()' function) identified the factors affecting the continuous data. Observed behavior was counted and analyzed as frequencies. Behavioral categories were compared descriptively. The physiological parameters were measured once and therefore also evaluated descriptively and considered in a final correlation analysis. Correlation analysis was done with the function 'cor.test()'. Multiple testing of individual parameters in a correlation analysis is a known source of alpha inflation and therefore a cautious approach for interpreting findings is recommendable. One approach would be to follow the Bonferroni method or the extended Bonferroni–Holm procedure to correct for multiple testing. However, a large number of correlations as it is common in exploratory research would lead to an overly conservative corrected alpha. Due to the exploratory design of the correlation analysis, we refrained from controlling for type I error by alpha correction but advice to interpret the calculated *p*-values with caution. To show a possible stabilization of the animals' behavior in the SNE, the animals' activity data in the form of RE were subjected to a repeatability analysis. The repeatability value R was calculated over a time period of four weeks (week 1–4). The following repeatability value R for direct comparison was also calculated over four weeks starting one week offset (week 2–5). This principle was continued until the end of the entire measurement period of RE. The repeatability was estimated with a linear model analyzing the mean RE depending on the individual animal as an influencing factor. The calculation of the repeatability was done with the R package 'rptR' and the function 'rpt()'.

3. Results

Animals weighed 19.8 ± 0.7 g (CV = 3.5%, max = 20.8 g, min = 17.2 g, n = 20) at the time of arrival (Figure 3B). The weight of the animals increased significantly (F (1|1668) = 7.441, $p < 0.0001$, adj. $R^2 = 0.82$). A mixed model applied to the data confirmed that weight increased over time and is influenced by the individual animal as a random effect and by time as a nested random effect. The applied model explains the variance of the data significantly better than a model that ignores time as a predictor ($p < 0.0001$). With 12–13 weeks of age experimental animals moved into the SNE. They stayed in this housing condition until the age of 96 weeks (670 days) and at this age a mean weight of 34.6 ± 3.0 g (CV = 8.7%, max = 40.0 g, min = 28.4 g, n = 18, Figure 3B) was measured.

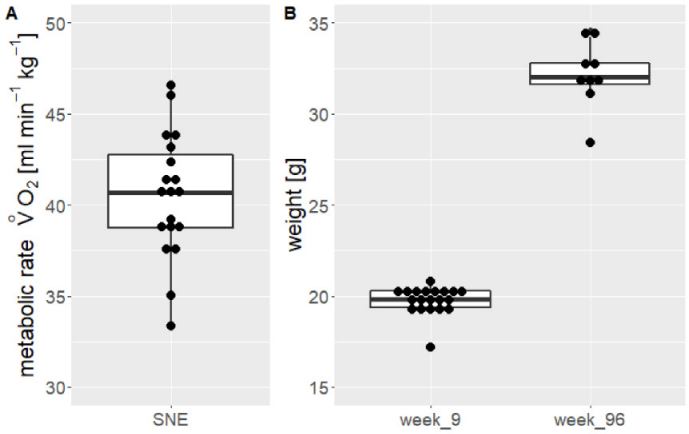

Figure 3. Summarizing boxplot of the resting metabolic rate (**A**) and the weight (**B**) of 19 female mice in the SNE. Metabolic rate was recorded at an age of 82–84 weeks. The weight is displayed at the animal age of 9 weeks and 96 weeks (start and end value).

3.1. Activity

Movement and activity data were recorded for 34 consecutive weeks, starting when the animals were 62 weeks (434 days) old. Therefore, the collected results reflect the condition and behavior of aged mice.

Daily activity was comparable for all mice in the SNE. The activity pattern over the day is shown in Figure 4 on 136 consecutive days. During the night a constant high activity is shown until 3–4 h prior to the light phase. One hour prior to the light phase activity increases again until the resting phase of the animals that starts with the lights going on. The resting phase was interrupted by the presence of an animal caretaker, the weekly cleaning of the SNE, or experiments during the light phase. The rest of the light phase shows a low level of activity until it slowly increases again 1–3 h prior to the dark phase. The highest levels of activity are shown at the beginning and the end of the dark phase.

During the housing of mice in the SNE the roaming entropy could be measured for 236 days. The RE of the animals averaged 0.079 ± 0.014 (CV = 18.0%, max = 0.113, min = 0.059, n = 19, Figure 5A) on the first day and decreased over time (F (1|134) = 59.46, $p < 0.0001$, adj. $R^2 = 0.30$) being influenced by the individual animal and time of measurement as random effects (mixed model). Including time as a predictor explains the variance of data significantly better than other applied models ($p < 0.0001$). The cumulative roaming entropy was calculated for 136 consecutive days (age 62–81.5 weeks or 434–570 days, Figure 6). At the end of the observed time period RE was 0.057 ± 0.007 (CV = 12.0%, max = 0.066, min = 0.042, n = 19, Figure 5A). The variance of the RE also decreased over time (F (1|134) = 29.76, $p < 0.0001$, adj. $R^2 = 0.13$, Figure 5B). Although there was a decreasing range of RE for the animals, the cRE showed different slopes for different animals and thus seems to indicate individual differences in long-term activity (Figure 6).

The cRE increased to an average 8.654 ± 0.928 (CV = 10.7%, max = 10.716, min = 7.359, n = 19) over the time of 136 days (F (1|134) = 129100, $p < 0.0001$, adj. $R^2 = 0.99$). In contrast to the variance of the RE, the variance of the cRE also increased over time (F (1|134) = 3342, $p < 0.0001$, adj. $R^2 = 0.96$).

Figure 5A also showed outliers of the RE on the recording days 502, 509, 510 and 511. These minimum values were recorded in the nights after the measurement of bone density (day 501) and grip strength/blood sampling (day 508 and 510).

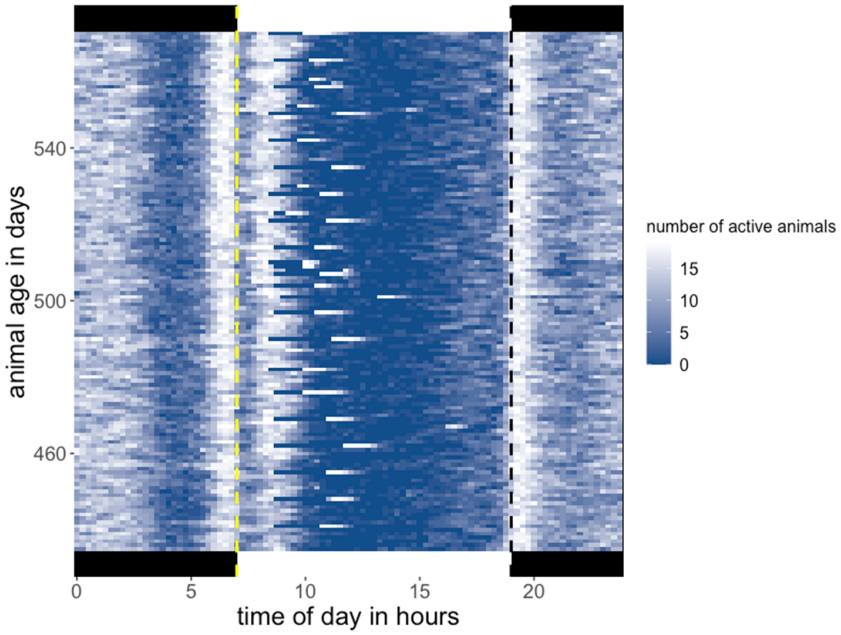

Figure 4. Heatmap of active animals in the SNE. Thy Y-axis shows the age of the animals in 136 consecutive days (day 434–day 570). Animals are recorded as active if there were at least a contact with two different antennas within a 15 min time period. The brighter a 15 min area, the more animals were recorded as active. The changing of the light/dark phase is marked by the dashed lines (yellow line = lights on, grey line = lights off).

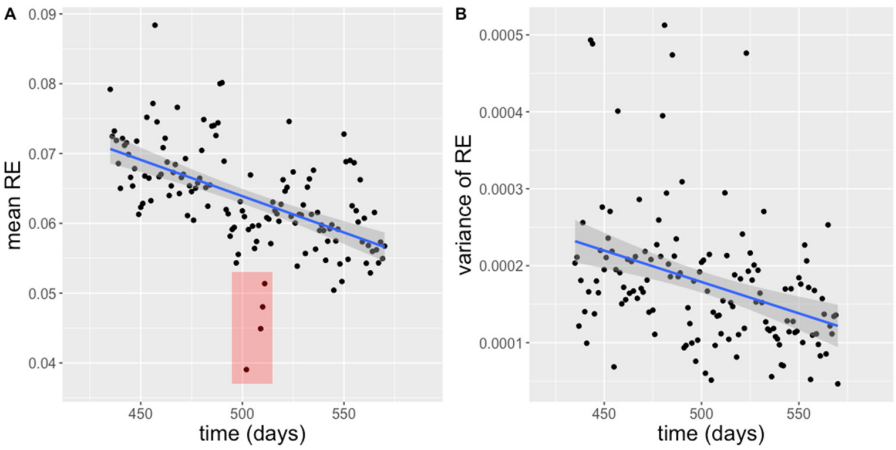

Figure 5. (**A**) Mean roaming entropy (RE) for 19 mice in the SNE over time with the change of the corresponding variance (**B**). The red area marks outliers in the values of the RE.

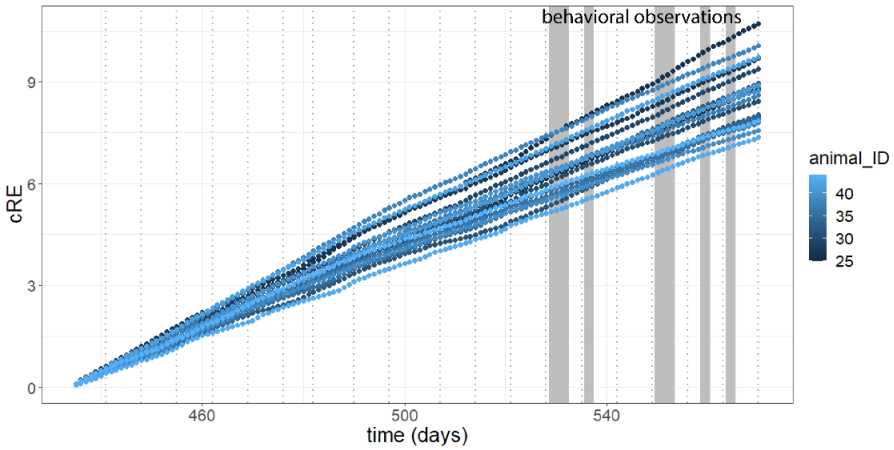

Figure 6. Cumulative roaming entropy (cRE) for 19 mice in the SNE over time. Vertical dotted lines indicate the weekly cleaning of the cage. Vertical grey areas indicate behavioral observations.

3.2. Spontaneous Behavior

Behavioral observations were completed with 19 animals. During the different observation periods some individuals were at rest or outside the field of the observer's view. Therefore, seven behavioral observation sessions were conducted in total. Overall, no stereotypic behavior was observed (Figure 7). The highest frequency was shown in social exploratory behavior including approaching and sniffing a cage mate. Non-social exploratory behavior was observed at one-third of the frequency of social exploratory behavior. Socio positive, agonistic, and play behavior reached similar low frequencies. The second most observed behavior was maintenance behavior including drinking and feeding. If the categories are examined combined as self-related and social behavior, the animals showed more social behavior (83.26 ± 13.91 per 30 min) than self-related behavior (71.00 ± 17.99 per 30 min).

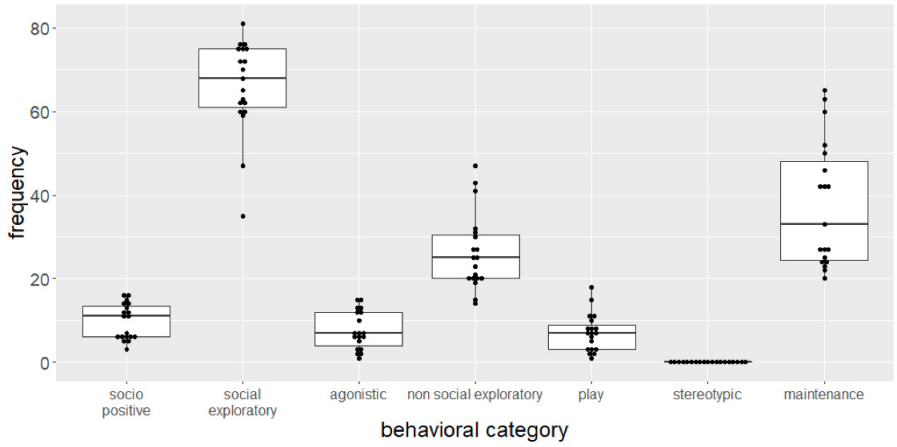

Figure 7. Mean frequency of behavior shown by female mice (n = 19) in the SNE during a cumulated 30 min observation period. Behavioral categories are combined from 28 possible behaviors to be observed.

3.3. Metabolic Rate

The resting metabolic rate was measured for 19 animals. The mean weight of the animals at the time of the measurement was 34.7 ± 2.8 g (CV = 8.1%, max = 41.3 g, min = 29.4 g, n = 19, Figure 3). During the first 2–3 h of the measurement period, the metabolic rate

fluctuated due to the animals exploring the cages and nearly reached maximum values for each individual. Since the measurement was performed during the light phase, the experimental animals eventually entered the resting phase. Therefore, 9–10 h of $\dot{V}O_2$ could be determined for each individual, interrupted only by brief increases in activity during which the animals ingested food or water or explored the cage before continuing to sleep. The mean $\dot{V}O_2$ was 40.5 ± 3.3 mL min^{-1} kg^{-1} (CV = 8.2%, max = 46.6 mL min^{-1} kg^{-1}, min = 33.3 mL min^{-1} kg^{-1}, n = 19).

3.4. Correlation Analysis

The results of the correlation analysis are summarized in Table A1 listed in the Appendix A. The metabolic rate did not correlate with the cRE. Weight showed a significant correlation with the cumulated activity at one of four examined time points (day 565, p = 0.0177). The overall weight change correlated with the decrease of RE over time (days 434–578, p = 0.00270).

Furthermore, all observed behavior had negative correlation factors with the cRE. The more an animal interacted with a cagemate, operated maintenance or showed exploratory behavior the less antennas were passed. Only one significant negative correlation between socio positive behavior and roaming behavior was identified (p = 0.0297). All significant findings should be interpreted with caution as no correction for multiple testing was done.

4. Discussion

In the present study, behavioral observation, automated activity measurement and measuring physiological parameters provided full insight into the lifestyle and development of a group of female mice living in an SNE. The enriched housing system was suitable for keeping the test animals under laboratory conditions. All measurements could be performed as planned and provided results for an analysis of the emergence of individual differences in a large group of mice living in an SNE.

The overall activity of animals in the SNE decreased over time, which is common for aged mice [23]. Activity patterns during day and night were as expected [24] with higher activity during the dark phase compared to the light phase [25] and activity peeks around the time of daylight change. Previous studies using the SNE showed comparable behavioral patterns in female mice kept in a similar enclosure. The increasing variability in the cRE over time is also comparable to data from Freund et al. (2013) [13]. While in the beginning of the observation period cRE was similar between individual animals, individual differences in the daily RE led to increasingly divergent values of the cRE (Figure 6). After 136 days of observation, some animals displayed persistently higher spatial activity than other individuals of the group. This shows that these animals explored more enclosure space than others, or at least included more antennas in their routes through the enclosure. In contrast to the findings of Freund et al. (2015) [14] variance of cRE was found to be increasing, although variance of RE was decreasing over time. An increase in variability of overall cRE while the variability of the level of activity and roaming within the homecage was getting lesser indicates a stabilizing behavior of the individual animals. Thus, we conclude that aged female mice each show individual routine roaming behavior.

This stabilization of behavior over time was also shown by repeatability analysis of the RE (Figure 8). The repeatability value R was 0.34 at the beginning of the observation period and increased to 0.62 over time ($F(1|15) = 37.08$, $p < 0.0001$, adj. $R^2 = 0.69$). This indicates that the behavior in the last four weeks is more stable on an individual level than in the first four weeks.

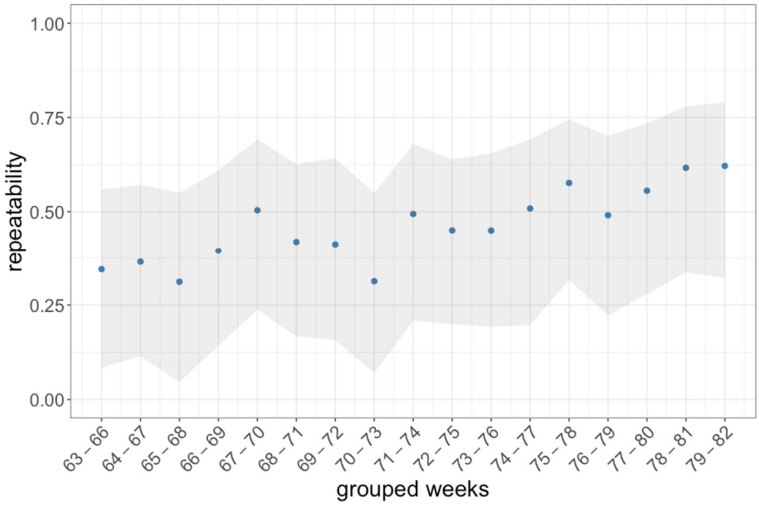

Figure 8. Calculated repeatability for the RE with the animal ID as a random factor. Each repeatability value (R, blue dots) was calculated over time periods of four weeks resulting in 17 groupings. The repeatability was estimated with a linear model. The grey areas depict the confidence intervals (2.5% and 97.5%) resulting from 500 bootstrapping runs and 100 permutations (R package 'rptR', function 'rpt()').

Figure 4 shows repeatedly a shift of the active phase that usually occurs in the morning to later in the day around early afternoon. This shift in activity could be attributed to the cleaning of the cage and handling of the animals on those days. Therefore, we checked whether the cleaning days cause effects on the activity in the respective following nights, which represent the determination periods for the RE. This was not the case, as the development of the cRE did not show a different course, if the nights following cleaning days were excluded from the calculation. Neither was the repeatability of the RE affected by the disturbance introduced through cleaning of the SNE.

It is of note that, unlike the unchanged cumulative activity values, the measurement system is sensitive enough to detect behavioral changes following potentially aversive experiments and interventions. In the RE, global minima were shown after measurements of physiological parameters associated with removal of the animals from the housing system and potentially aversive treatments. The reduced activity in the nights following these treatments was probably due to anesthesia during bone density measurement and the blood sampling that was conducted after the grip strength measurement. Just removing the mice from the SNE as it was done routinely during the cleaning did not lead to a reduced RE in the following night.

The second experimental focus was on spontaneous behavior of the animals. During the observation of spontaneous behavior, no stereotypical behavior was detected. The housing of 20 female animals within the SNE led to more social behavior than self-related behavior. Besides maintenance behavior, exploration of the environment and behavior directed to the cagemates were observed the most. The large area of the enclosure as well as the ability to keep a large group of animals allows the mice to perform a wide range of different behaviors. This is in contrast to small standard cages, where for example observing exploratory behavior would be challenging. The observed behaviors also correspond quite well to the natural behavior of mice and thus clearly shows the advantages of the SNE for the expression of the full range of the behavioral repertoire [10]. The observed behavior for a group of female mice might change if the SNE would house a group of male mice or a mixed sex group. Male mice show increased aggression, the bigger the available area and the bigger the group of animals [26]. Aggression between individuals would also increase with potentially pregnant animals [27].

The observations were also consistent with the findings of Freund et al. (2015) [14], where exploration was observed more frequently than other behaviors. However, non-social exploratory and play behavior occurred more often than social exploratory behavior in their data. Higher values for play behavior may be due to the younger age of the animals. The mice were observed at an age of six to twenty weeks. Play behavior is more common in such young animals compared to old mice [28]. In summary, in contrast to the results shown in the present study, Freund and colleagues observed more self-related behavior than social behavior. This difference could have been also caused by the different social structure in the group. Much older animals were examined in our study and a longer cohabitation could also favor a more pronounced social behavior.

Nevertheless, it is worth noting that the aged mice in the SNE showed any play behavior at all. Each individual performed sudden movements in vertical and horizontal directions with no apparent reason or interaction with a cagemate (e.g., chasing or being chased). These movement patterns are considered typical solitary play behavior. Play behavior in mice is normally found primarily in young animals, but play is enhanced by extremely enriched environments [28–30]. It is known that play behavior is most likely carried out in a positive life context while it is suppressed within unpleasant situations. Therefore, play behavior is deemed a suitable indicator of positive welfare that also applies to aged mice [31,32].

Since the SNE is a non-standard housing condition, we provide a detailed analysis of the data and a comparison with the literature in order to estimate potential flaws with regard to generalizability of results to more common housing conditions. The metabolic rate was obtained outside the home-cage and the described methods are widely used regardless of housing conditions.

Measuring the resting metabolic rate is dependent on a number of factors that strongly influence the results. Literature data vary to a considerable extent, and it is not always clear which factors have to be considered when comparing absolute values. To add to the confusion, there is no established standard with regard to the unit RMR is measured in. In order to evaluate our data against common findings from the literature we recalculated the measures given to the same units of measurement. Literature data on RMR ranges from 21.6 ± 1.7 mL min^{-1} kg^{-1} (CV = 7.9%) for female C57BL/6J mice [33] to 77.0 ± 6.3 mL min^{-1} kg^{-1} (CV = 15.4%) [34]. For male C57BL/6J mice in a study on the correlation of oxygen consumption to exercise values of up to 87.0 ± 4 mL min^{-1} kg^{-1} (CV = 4.6%) [35] were measured. Thus, our oxygen consumption rate of 40.5 ± 3.3 mL min^{-1} kg^{-1} is well within established findings, taking into account the differences in weight and age (Table 1). On the other hand, there are factors in the applied method that call comparability into question. The transport and measurement cages were typical transparent plastic cages and were therefore very different from the SNE. Due to the open mesh system, the animals were probably accustomed to different smells, temperature dynamics and ventilation characteristics. However, one major factor was probably the changed social constellation of the group during habituation and measurement. Since the calorimetry system does not allow measuring all animals at the same time, there is a division of the group for this time. Considering the sensitive social structure of these group animals, the isolation of a group member for the measurement must be considered as an important influence on the resting metabolic rate. Resting alone during the measurement also leads to increased thermoregulation and therefore also to an increased metabolic rate [36,37]. To evaluate the quality of our measurement further, we calculated the coefficient of variability (CV) as a way to compare the measures with regard to their prospective reproducibility. This analysis resulted in a CV of 8.1% for our RMR measurement, which is not at all conspicuous, compared to data from the literature. It is noticeable that measurements on the mouse group in the SNE generally led to low CV values, in some cases lower than in studies with standardized husbandry. Thus, if with regard to variability, comparable results can be

obtained in a large and richly structured environment, the SNE could be used as a reference system in comparative studies. This is especially true if one aims at evaluating the physiological potential under more naturalistic conditions.

Table 1. Overview of the measured resting metabolic rate (RMR) with the associated coefficient of variability (CV) and the comparison to the literature values.

Source of Data	Sex of Animals	RMR in mL min^{-1} kg^{-1}	CV in %
this article	f	40.5 ± 3.3	8.1
M. Konarzewski and J. Diamond, 1995	f	21.6 ± 1.7	7.9
T. D. Williams et al., 2002	f	77.0 ± 6.3	15.4
V. Schefer and M. I. Talan, 1996	m	87.0 ± 4	4.6

To further investigate the development of individual differences in the group, the results of the respectively performed measurements were correlated with the activity of the animals. Only few significant correlations were found. Roaming activity and weight of the animals correlated negatively. The less active an animal was within the SNE, the heavier it was. The correlation between body weight and activity in rodents is also suggested by studies on voluntary exercise [38–40]. Thus, although no alpha correction was made, we deem this effect as biologically meaningful. In addition, roaming entropy negatively correlated with socio-positive behavior. This may be explained by constraints of their time budget which would render actively roaming mice less likely to spent time interacting with conspecifics. However, Freund et al. (2015) did not observe significant effects of roaming and socio-positive behavior. Thus, we advise interpreting the relationship we observed with caution.

The metabolic rate did not correlate to the activity within the SNE or the weight of the animals. Overall, although our results show the development and stabilization of individual roaming behavior, these individual differences were not strongly reflected in the physiological parameters studied.

We are aware that the approach of studying a large group in a single SNE also comes with limitations. The aim was to determine whether the SNE would produce individual differences in experimental animals. If such individual differences exceed the variability found in conventional housing systems, it would be difficult to compare results in future studies using the SNE. We did not include a direct comparison of the SNE housed mice with mice from other types of housing but relate to literature data. However, we could demonstrate that the individual differences measured as CV did not exceed literature data. In addition, it is debated whether or not a single mouse or a group of mice housed in the same cage are to be considered as a statistical unit [41]. Some argue that individual results of all animals in one housing unit shall be averaged to only one statistical unit due to being mutually influenced by the same environment. However, here we analyze the effects of living in an SNE on the variability within the group. Living in an SNE deliberately includes living in a social environment. We therefore assume that any differences were caused by the influence of the housing and social system on the individual. Consequently, each experimental animal was treated as a separate data point.

In summary, the SNE represents a suitable housing system for a large group of female mice. The daily routine, such as the control and care of the animals, can be performed in a reasonable time compared to controlling conventional cage systems. In order to conduct physiological measurements, the mice have to be taken out of their familiar environment. Catching the mice was manageable in a reasonable time, however, still this procedure takes longer than just opening a standard sized cage. Thus, one concern might be that capturing, and transport affect the measured results more compared with standard

housed mice. However, the results of this study do not confirm this assumption for the most part. Overall, the variability of the data is comparable to the literature. The handling of the animals outside the SNE was outweighed by the benefit of permanent monitoring of the home cage. The RFID system was able to record activity and thus behavioral data independently of light/dark cycles and experimenters and therefore enabled an unbiased understanding of mouse behavior in the SNE. These properties constitute the SNE as a powerful tool for phenotyping mice [42,43]. Finally, undisturbed long-term monitoring presents the opportunity to enhance reproducibility, as the most common cause of experimental variation is the experimenter.

5. Conclusions

In this study, the SNE was successfully used as an enriched housing system for a large group of female mice. Home cage monitoring was used to record the activity and observe the behavior of the animals. The semi naturalistic environment, as used and monitored here, promoted the emergence of individual differences with regard to the activity of the animals in the available space. The extensive recording and high sensitivity to external influences makes the roaming entropy the most important parameter of the SNE. The animals showed natural social and exploratory behavior, and even play behavior was observed in mice at a high age. Therefore, this housing truly follows the European directive (2010/63/EU) in providing 'space of sufficient complexity to allow expression of a wide range of normal behavior'. The automated SNE is able to detect behavioral changes and did not prevent the taking of consistent measurements with sufficient accuracy. All in all, the SNE can be developed into an important tool for enhancing animal welfare in comparative studies.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results".

Appendix A

Table A1. Correlation results between measured parameters during the housing in the SNE and the recorded activity of 19 mice. The table shows the correlation factor r and the respective p-value below of each comparison made. Depending on an existing normal distribution the factor was calculated by the correlation methods according to Pearson or Spearman. Grey areas = *p*-values, that indicate a significant correlation between two parameters (uncorrected). If a parameter was used more than once for correlation analysis, the alpha mistake decreased by a n^{-1} according to Bonferroni correction.

Correlated Parameters	cRE (d578)	Weight (d502)	Weight (d510)	Weight (d565)	Weight (d578)	Weight Change (d434–d578)
cRE (d502)		−0.452 0.0520				
cRE (d510)			−0.418 0.0746			
cRE (d565)				−0.537 0.0177		
metabolic rate	0.0283 0.9086				0.158 0.518	
cRE (d578)					−0,45 0.0535	
slope RE (d434–d578)						−0.647 0.0027

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4 Second Publication

Title:

Effects of more natural housing conditions on the muscular and skeletal characteristics of female C57BL/6J mice

Authors: Paul Mieske, Julia Scheinpflug, Timur Alexander Yorgan, Laura Brylka, Rupert Palme, Ute Hobbiesiefken, Juliane Preikschat, Lars Lewejohann*, Kai Diederich*

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Effects of more natural housing conditions on the muscular and skeletal characteristics of female C57BL/6J mice

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Abstract

Background Enrichment of home cages in laboratory experiments offers clear advantages, but has been criticized in some respects. First, there is a lack of definition, which makes methodological uniformity difficult. Second, there is concern that the enrichment of home cages may increase the variance of results in experiments. Here, the influence of more natural housing conditions on physiological parameters of female C57BL/6J mice was investigated from an animal welfare point of view. For this purpose, the animals were kept in three different housing conditions: conventional cage housing, enriched housing and the semi naturalistic environment. The focus was on musculoskeletal changes after long-term environmental enrichment.

Results The housing conditions had a long-term effect on the body weight of the test animals. The more complex and natural the home cage, the heavier the animals. This was associated with increased adipose deposits in the animals. There were no significant changes in muscle and bone characteristics except for single clues (femur diameter, bone resorption marker CTX-1). Additionally, the animals in the semi naturalistic environment (SNE) were found to have the fewest bone anomalies. Housing in the SNE appears to have the least effect on stress hormone concentrations. The lowest oxygen uptake was observed in enriched cage housing.

Conclusions Despite increasing values, observed body weights were in the normal and strain-typical range. Overall, musculoskeletal parameters were slightly improved and age-related effects appear to have been attenuated. The variances in the results were not increased by more natural housing. This confirms the suitability of the applied housing conditions to ensure and increase animal welfare in laboratory experiments.

Keywords Animal welfare, Mice, Physiology, Musculoskeletal characteristics, Body weight, Environmental enrichment, Semi naturalistic environment

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Background

Enrichment of the housing conditions of laboratory animals is not subject to any universal principles and therefore takes different forms [1]. In general, increased provision of stimuli to enable natural behavior and improve animal welfare could be a definition of environmental enrichment (EE). In fact, many comparative studies on the impact of EE on laboratory mice show effects on animal welfare [2]. The majority of studies conclude that the use of EE is beneficial for the animals. Housing in enriched environments can reduce stereotypies [3–7], reduce anxiety [8–10] and promote exploratory behavior [11, 12], as well as promote the development of favorable physiological parameters, especially in the neurological field [13, 14]. It is also important that the enrichment is versatile and easily accessible. Dispersed enrichment using different elements or larger enclosures has the potential to minimize aggression between individuals in a group [15]. Despite the clear advantages of using EE, the minimum legal requirements for standard laboratory husbandry unfortunately remain unchanged. In most experimental studies the cages continue to be only minimally equipped and thus constitute a barren environment. A recent meta-analysis has shown that such barren housing conditions in biomedical translational studies can negatively affect a number of health parameters in the experimental animals, thus substantially compromising the validity of these studies [16].

However, mandating enrichment of laboratory animal cages it is not an easy endeavor, as a number of factors must be considered for implementation. Laboratories and breeding facilities need to gauge which EE elements can be added, minding the applicability and financial feasibility. When introducing EE elements, hygiene standards for the animals must be maintained and standardization between experiments and laboratories must not be compromised [17]. Finally, an increasing individualization of the cage design according to one's own views, experiences, and tastes may affect the comparability of methods and results.

Another major criticism on the use of EE is the fear that the emergence of individual differences of experimental animals promoted by EE results in increased variability. However, previous studies have shown that the use of EE emphasizes individuality without necessarily increasing the variability of physiological parameters [18, 19]. Providing simple enrichments such as additional nesting material and plastic or cardboard tunnels over long periods can change social behavior, but do not necessarily affect physiological parameters [20]. A further advance to enriching conventional housing conditions of laboratory animals is to design an environment that resembles nature. However, even the use of a semi naturalistic environment that promotes individual diversified behavior did not result in greater deviations in the measurement of physiological parameters compared to conventional studies [21, 22].

Only by gaining more knowledge and disclosing additional benefits of EE is it possible to increase awareness and acceptance of the need to use EE, which ultimately also leads to an increase in overall animal welfare.

An example of a suitable method of laboratory cage enrichment was recently presented by Hobbiesiefken et al. [3]. By exchanging enrichment elements in different categories on a weekly basis, an EE concept was developed, that reduced stereotypies and served to evaluate individual enrichment elements through behavioral observations. In the present study, three housing conditions with increasing opportunities to exhibit more natural behavior were used to analyze effects on physiological parameters. In addition to the conventional and enriched housing conditions used by Hobbiesiefken et al. a semi naturalistic environment [21–27] was used to exploit the full potential of the mice's natural behavior as much as possible. Since many previous studies focus more on animal behavior than on uncovering the effects of EE on physiological traits, we here analyze whether the use of objects, social enrichment, or larger enclosure space affects musculoskeletal characteristics of female C57BL/6J mice. Nevertheless, our analysis of musculoskeletal characteristics was conducted within the framework of the animal welfare perspective. Additionally, to be able to monitor changes in animal welfare on a more obvious and holistic level, body weight, resting metabolic rate and stress hormone levels were measured. With regard to biomedical studies it might be under consideration, whether an increase or decrease of the respective parameter is the desired outcome. We admit that it might be questionable whether an altered muscle weight or bone density is ultimately decisive for improved animal welfare. However, one could assume that the physiological parameters we measured under more challenging environmental conditions are a better representation of the natural state. When considering effects on human bone structure, twin-studies are used to distinguish between genetic and environmental influences. It has already been established that in humans up to 70% of individual differences are of genetic origin [28, 29]. In laboratory mice, genetic variability can be controlled. Thus, to pay greater attention to the influence of environmental enrichment on muscle and skeletal properties, inbred mouse strains are particularly suitable, since virtually an unlimited number of genetically identical individuals are available. Nonetheless, it has been shown that individual differences emerge despite genetic uniformity even in strictly standardized and limited housing conditions [30].

Albeit genetics and housing conditions are not exclusive factors that should be considered for the evaluation.

Results

Weight data

On arrival, the experimental animals weighed 19.8 ± 0.9 g (4.5%, 22.5–17.2 g, n = 44). During the experimental period of 88 weeks animal weight increased significantly ($F(1|3604) = 7716$, p-value < 0.001, $R^2 = 0.68$). A mixed model applied to the data confirmed that weight increased over time and is influenced by the individual animal, the housing condition and the time as a random effect and by the individual animal within the different housing condition and the individual animal over time as nested random effects. The applied model explains the variance of the data significantly better than a model that ignores the housing condition and time as predictors ($p < 0.001$). The different influences on the weight resulted in animals living in the SNE being the heaviest and the animals living in the conventional housing (CON) being the lightest (Fig. 1A). At the time of perfusion of the animals they showed a mean animal weight in CON housing of 30.4 ± 3.1 g (10.1%, 36.1–26.1 g, n = 11), in enriched housing (ENR) of 33.5 ± 3.1 g (9.2%,

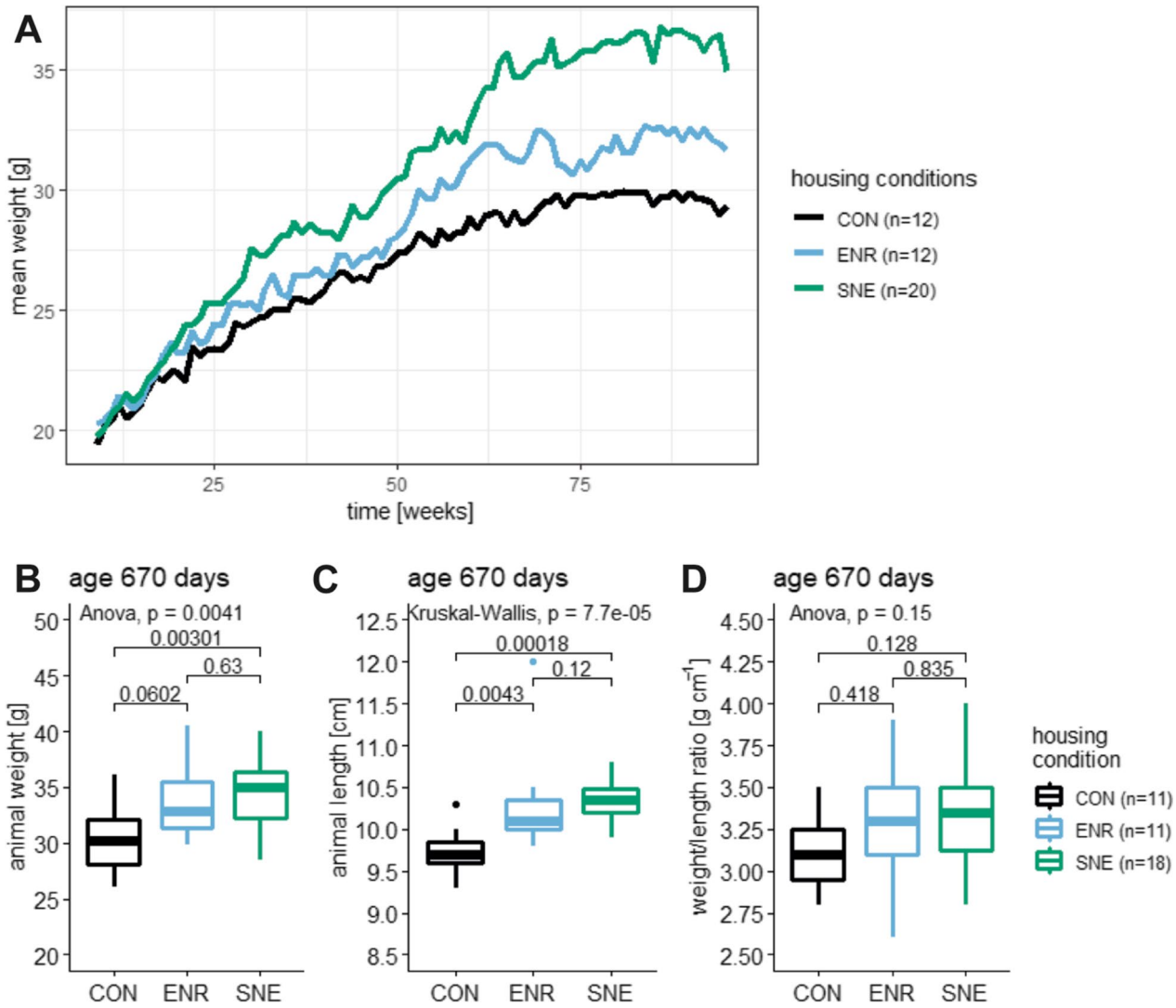

Fig. 1 Body weight and body length of female C57BL/6J mice. **A** mean body weight over time with CON housing in black, ENR housing in light blue and SNE housing in green. **B** mean body weight at the day of perfusion (age 670 days, same coloring scheme as A) with respective p values from a post hoc Tukey test. **C** mean animal length at the day of perfusion (age 670 days, colors equal to A) with respective p values from a post hoc Wilcoxon test. **D** mean ratio of animal weight to animal length at the day of perfusion (age 670 days, same coloring scheme as A) with respective p values from a post hoc Tukey test

40.5–29.8 g, n = 11) and in SNE housing of 34.6 ± 3.1 g (8.9%, 40.0–28.4 g, n = 18, Fig. 1B). Animals in SNE housing were significantly heavier than the animals in CON housing. The weight increase from CON to ENR housing was marginally not significant. There was no significant difference between ENR and SNE housing.

In order to be able to map the physical characteristics more accurately, the length of experimental animals was also measured and placed into relation with the animal weight. The animal length in CON housing was 9.7 ± 0.3 cm (2.8%, 10.3–9.3 cm, n = 11), in ENR housing 10.1 ± 0.2 cm (2.2%, 10.5–9.8 cm, n = 10) and in SNE housing 10.3 ± 0.2 cm (2.1%, 10.8–9.9 cm, n = 18, Fig. 1B). Comparable to the body weight ENR and SNE animals were significantly longer than CON animals with no significant difference between them. The ratio of animal weight to animal length in CON housing resulted to 3.1 ± 0.3 g cm^{-1} (8.2%, 3.5–2.8 g cm^{-1}, n = 11), in ENR housing to 3.3 ± 0.4 g cm^{-1} (10.9%, 3.9–2.6 g cm^{-1}, n = 11) and in SNE housing to 3.3 ± 0.3 g cm^{-1} (8.7%, 4–2.8 g cm^{-1}, n = 18, Fig. 1B). Overall, the same trend as in body weight and length was revealed, but no significant differences.

Adipose tissue weight

At the time of perfusion, retroperitoneal adipose tissue weight for animals in the CON housing was 0.057 ± 0.032 g (56.8%, 0.127–0.030 g, n = 11), for ENR housing 0.076 ± 0.019 g (25.3%, 0.092–0.037 g, n = 10) and for SNE housing 0.112 ± 0.048 g (43.1%, 0.201–0.020 g, n = 18, Fig. 2A). For periovarian adipose tissue weight animals in CON housing showed 0.276 ± 0.157 g (56.9%, 0.593–0.020 g, n = 11), for ENR housing 0.409 ± 0.182 g (44.5%, 0.798–0.184 g, n = 11) and for SNE housing 0.506 ± 0.236 g (46.6%, 1.053–0.127 g, n = 18, Fig. 2B). For both tissues SNE animals showed

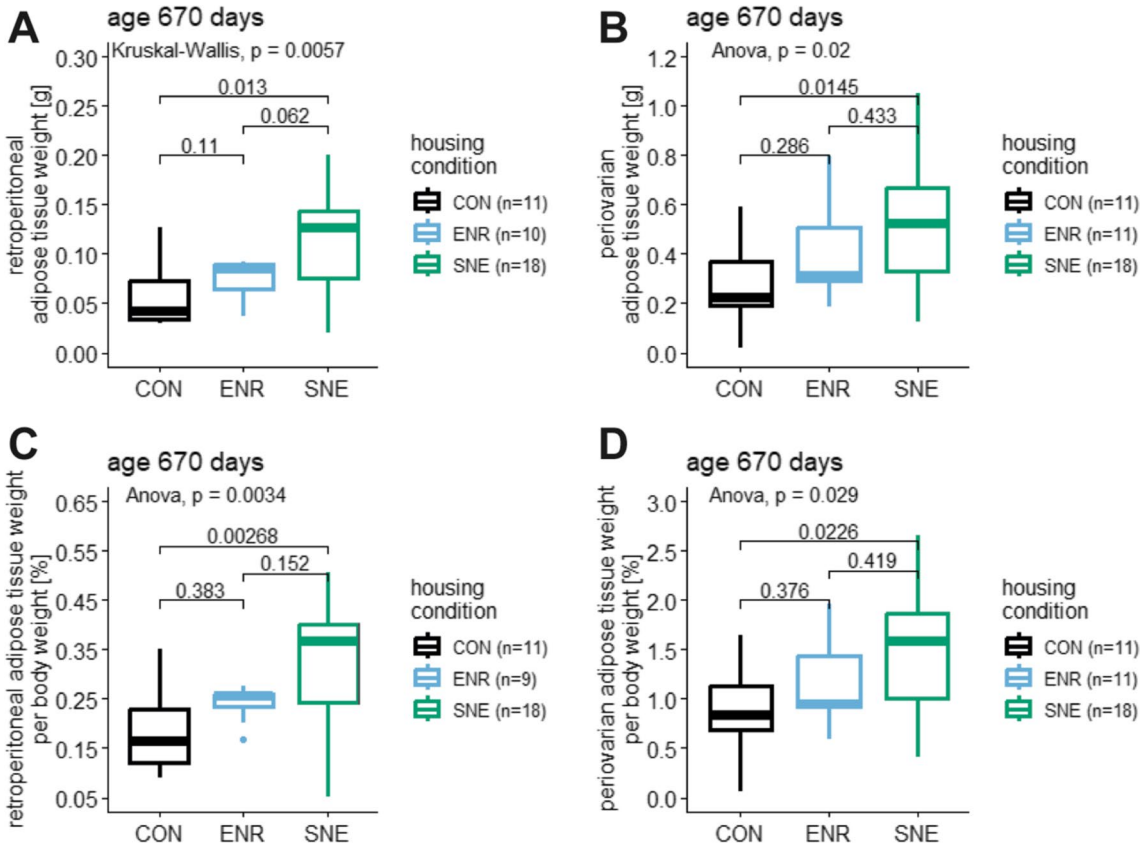

Fig. 2 Adipose tissue weight and adipose tissue weight relative to body weight of female C57BL/6J mice. Data is shown at the time of perfusion (age 670 days). **A** retroperitoneal adipose tissue weights of animals in CON housing (black), ENR housing (light blue) and SNE housing (green) after perfusion at an age of 670 days with respective p values from post hoc Wilcoxon test. **B** periovarian adipose tissue weights (same coloring scheme) after perfusion at an age of 670 days with the respective p values from post hoc Tukey test. **C** retroperitoneal adipose tissue weights relative to animal body weight (same coloring scheme) after perfusion at an age of 670 days with respective p values from post hoc Tukey test. **D** periovarian adipose tissue weights relative to animal body weight (same coloring scheme) after perfusion at an age of 670 days with the respective p values from post hoc Tukey test

significant heavier adipose tissue weights than CON animals. There was no statistical difference between CON and ENR animals nor ENR and SNE animals. The weights of the adipose tissues in relation to the body weight of the mice were in the same proportion to each other. The percentage of retroperitoneal and periovarian adipose tissue increased significantly from the CON housing to the SNE housing.

Bone density and structural properties data

To observe the change of the bone density over time, it was measured at three different ages of female C57BL/6J mice during housing in different conditions. At an age of 340 days, the mice showed a bone density in CON housing of 1.51 ± 0.24 g cm^{-3} (15.6%, 1.81–0.98 g cm^{-3}, n = 11), in ENR housing of 1.69 ± 0.17 g cm^{-3} (16.5%, 1.90–1.42 g cm^{-3}, n = 12) and in SNE housing of 1.72 ± 0.16 g cm^{-3} (16.0%, 1.92–1.35 g cm^{-3}, n = 20, Fig. 3A). Both animals in ENR and SNE housing had a significantly higher bone density than the control animals, but showed no statistical difference between the two enriched housing conditions. At an age of 501 days the bone density values decreased to 1.44 ± 0.15 g cm^{-3} (10.6%, 1.63–1.16 g cm^{-3}, n = 11) in CON housing, 1.58 ± 0.19 g cm^{-3} (12.0%, 1.87–1.25 g cm^{-3}, n = 10) in ENR housing and 1.57 ± 0.23 g cm^{-3} (14.4%, 2.09–1.36 g cm^{-3}, n = 19, Fig. 3A) in SNE housing. The difference between the three housing conditions was not statistically significant anymore. Bone density showed further degression at an age of 664 days with 1.21 ± 0.13 g cm^{-3} (10.8%, 1.44–1.02 g cm^{-3}, n = 11) for CON animals, 1.38 ± 0.25 g cm^{-3} (17.8%, 1.92–1.06 g cm^{-3}, n = 11) for ENR animals and 1.35 ± 0.16 g cm^{-3} (12.2%, 1.68–1.05 g cm^{-3}, n = 17, Fig. 3) for SNE animals. Analysis showed no significant difference although the relation between values showed a trend comparable to the first measurement at 340 days of age.

A linear model confirmed the significant decrease of bone density over time ($F(2|120) = 27.41$, p-value < 0.001, $R^2 = 0.30$). The observation of a lower bone density at a higher age of female C57BL/6J mice was mostly influenced by the housing condition as a random factor (model 1). A mixed effect model without housing condition as a random factor (model 2) was significantly less adequate to describe the data ($p = 0.016$, model 1 AIC = −44.35, model 2 AIC = −40.62)). However, a comparison with housing condition alone as a random factor (model 3) showed no significant difference (p = 0.24).

In addition to the bone density, detailed properties of the bone structure were determined via µCT analysis. The examination of the macroscopic compartments of the bone revealed a cortical thickness of the femur for female C57BL/6J mice in CON housing of 164.4 ± 23.2 µm (14.1%, 197.4–132.1 µm, n = 9), in ENR housing 169.5 ± 19.7 µm (11.6%, 205.3–136.7 µm, n = 9) and in SNE housing 185.5 ± 24.5 µm (13.2%, 216.2–134.6 µm, n = 12, Fig. 3B). SNE animals showed the highest cortical thickness but the difference between the housing conditions was not significant. The ratio of trabecular bone volume to tissue volume was the lowest in CON housing with 1.32 ± 0.78% (59.0%, 2.81–0.57%, n = 8). The highest bv/tv was measured for ENR housing with 1.78 ± 1.09% (61.1%, 3.52–0.55%, n = 9). Animals in SNE housing showed bv/tv of 1.53 ± 1.10% (71.8%, 4.01–0.51%, n = 18, Fig. 3C). The differences between the groups were not significant.

The femur length for the experimental animals in CON housing was 16.55 ± 0.15 mm (0.9%, 16.72–16.28 mm, n = 9), was increased in ENR housing with 16.67 ± 0.32 mm (1.9%, 17.34–16.25 mm, n = 9) and reached the highest value in SNE housing with 16.79 ± 0.28 mm (1.7%, 17.29–16.25 mm, n = 11, Fig. 3D). The increase in length with the rising level of enrichment was not significant.

Comparable to the femur length, the femur midshaft outer diameter was also increased in ENR and SNE housing. In CON housing the diameter was 1.76 ± 0.04 mm (2.3%, 1.83–1.69 mm, n = 9), in ENR housing 1.77 ± 0.03 mm (1.8%, 1.81–1.73 mm, n = 9) and in SNE housing 1.83 ± 0.04 mm (2.3%, 1.90–1.74 mm, n = 12, Fig. 3E). The mean diameter in SNE housing was significantly higher than the diameter in animals in CON and ENR housing.

Additional structural properties are mentioned in table (see 3.9. data summary). Respective figures are shown in Additional file 1: Fig. S1. In summary, no significant differences were found in the femora for cortical porosity, trabecular thickness, number and separation.

Structural bone anomalies were found in animals of every housing condition. In percentage terms, SNE housing showed the lowest number of animals with anomalies (Fig. 4D). In CON housing every individual showed at least one atypical feature (Fig. 4B and C). The effect of housing condition on the percentage of animals with anomalies was not significant. On average, the animals in ENR housing had 2.2 anomalies per individual animal. CON housing showed only slightly less with 1.9 anomalies per animal whereas animals in SNE housing on average showed 1.0 anomaly per individuum (Fig. 4E). Relative to the number of individuals in the housing conditions, SNE housing resulted in the fewest anomalies.

Grip strength data

The grip strength of female C57BL/6J mice was measured at two times during their housing period. At an

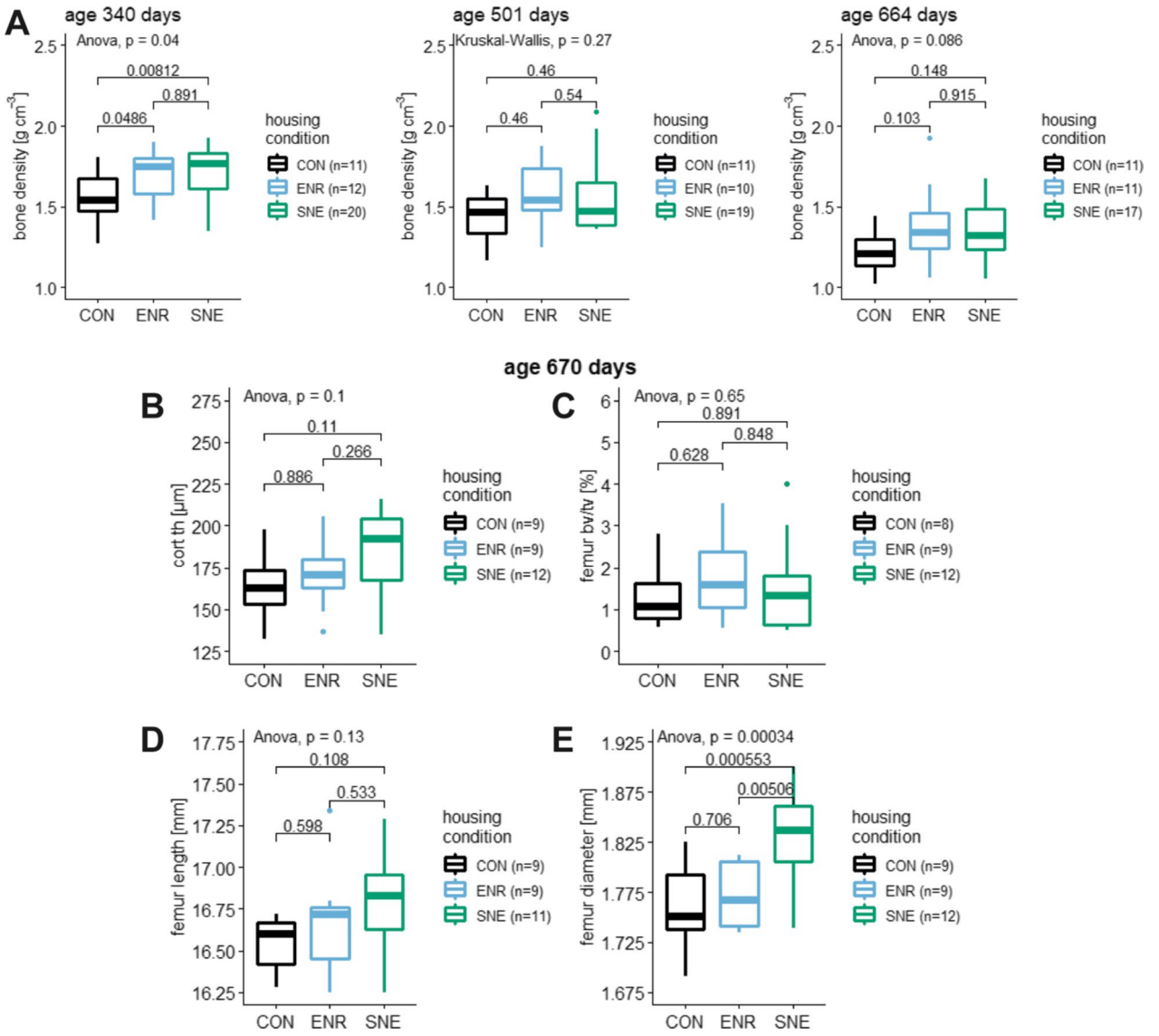

Fig. 3 Bone density at three different ages and structural femur properties of female C57BL/6J mice. **A** bone density in g cm^{-3} at the age of 340, 501 and 664 days of animals in CON housing (black), ENR housing (light blue) and SNE housing (green) with respective p values from post hoc Tukey (A left and right) and Wilcoxon (A middle) test. B—E–parameters were measured after perfusion at an age of 670 days in CON housing (black, ENR housing (light blue) and SNE housing (green). **B** cortical thickness in µm with respective p values from post hoc Tukey test. **C** femur bone volume per tissue volume in % with respective p values from post hoc Tukey test. femur midshaft outer diameter in mm with respective p values from post hoc Tukey test. **D** femur length in mm with respective p values from post hoc Tukey test. **E** femur midshaft outer diameter in mm with respective p values from post hoc Tukey test

age of 508—510 days the mice showed a grip strength in CON housing of 2.29 ± 0.39 N (17.0%, 3.26–1.78 N, n = 10), in ENR housing 2.60 ± 0.41 N (15.6%, 3.23–1.99 N, n = 12) and in SNE housing 2.38 ± 0.35 N (14.5%, 3.29–1.89 N, n = 19, Fig. 5). Mice in ENR housing showed the highest grip strength in comparison to the animals of the other housing conditions. The difference is not significant. In the second measurement at an age of 664 days animals showed in CON housing 2.34 ± 0.43 N (18.4%, 3.00–1.71 N, n = 11), in ENR housing 2.65 ± 0.26 N (9.8%, 3.04–2.21 N, n = 12) and in SNE housing 2.40 ± 0.40 N (16.6%, 3.09–1.85 N, n = 18, Fig. 5). The relationship between values has not changed and was still not significant. Also a linear model showed no significant change of the grip strength between the two measurements ($F(1|79) = 0.095$, p-value = 0.76, $R^2 = -0.01$).

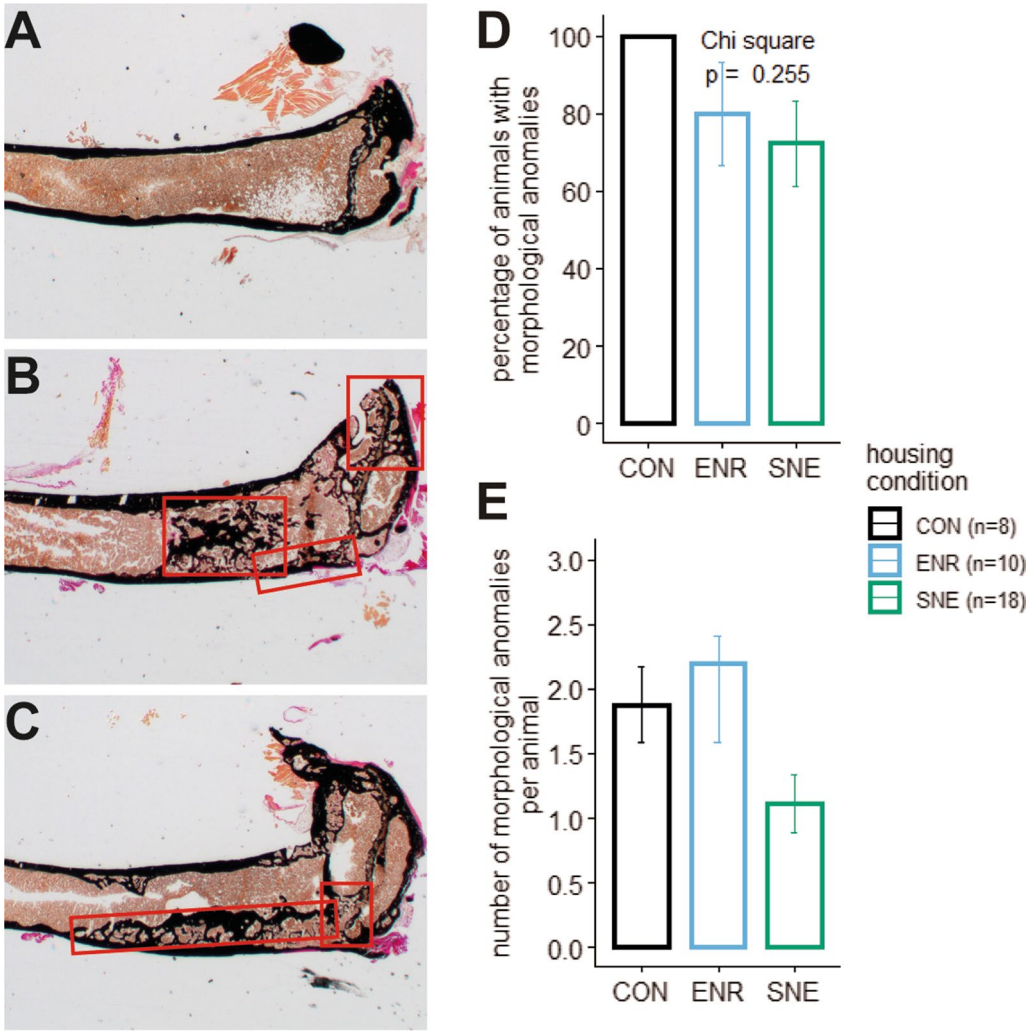

Fig. 4 Kossa stained tibia sections of female C57BL/6J mice and the occurrence of bone structure anomalies. Sections were magnified × 2.5. **A** example for no anomalies in the tibia section. **B** example for trabecularized cortical bone, mineralized lesions and anomalous trabecular localization in the tibia (here: epiphysis). **C** example for pseudocortical structures within the medullar cavity and the partial disruption of the growth plate. **D** bar plot of the percentage of animals within each housing condition CON (black), ENR (light blue) and SNE (green), that showed morphological anomalies with the respective p value from a χ^2 test. **E** bar plot of the number of morphological anomalies per animal within the three housing conditions (same coloring scheme)

Muscle weight data

After perfusion of the animals at an age of 670 days the muscle weight of the musculus biceps femoris was measured. For CON housing animals showed a muscle weight of 0.123 ± 0.023 g (19.0%, 0.161–0.097 g, n = 11), for ENR housing 0.127 ± 0.021 g (16.5%, 0.166–0.097 g, n = 11) and for SNE housing 0.120 ± 0.020 g (16.3%, 0.161–0.087 g, n = 18, Fig. 6). Animals in the ENR housing showed the highest muscle weight. There was no significant difference between housing conditions. Muscle weight in relation to body weight decreased significantly from the CON housing to the ENR housing to the SNE housing. However, the individual comparison between the housing conditions did not show any singificant differences.

Bone and muscle turnover parameter data

Three musculoskeletal turnover parameters were measured in blood samples after the perfusion of female C57BL/6J mice at an age of 670 days. Myostatin is an endogenous protein that inhibits muscle growth. The myostatin concentration for animals in CON housing was 692.26 ± 132.87 pg ml^{-1} (19.2%, 952.38–466.68 pg ml^{-1}, n = 11), in ENR housing 714.96 ± 107.04 pg ml^{-1} (15.0%, 796.48–584.37 pg ml^{-1}, n = 5) and in SNE housing 692.26 ± 132.87 pg ml^{-1} (19.2%, 952.38–466.68 pg ml^{-1},

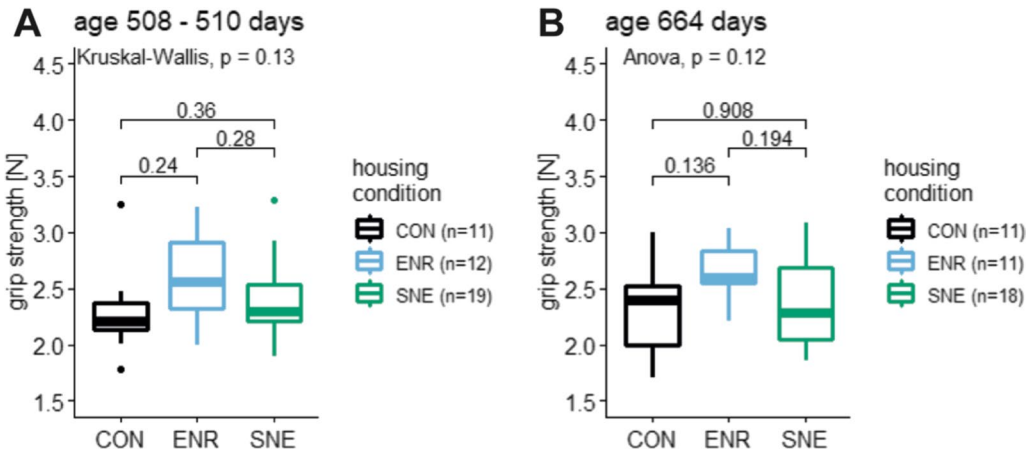

Fig. 5 Grip strength of female C57BL/6J mice at two different ages. **A** grip strength at an age of 508—510 days of animals in CON housing (black), ENR housing (light blue) and SNE housing (green) with respective p values from post hoc Wilcoxon test. **B** grip strength at an age of 664 days (same coloring scheme) with respective p values from post hoc Tukey test

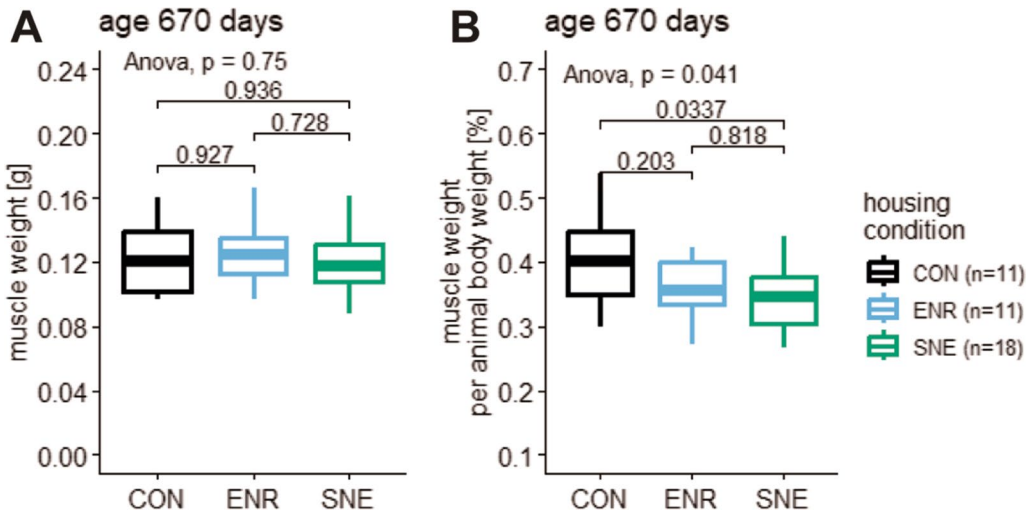

Fig. 6 Muscle weight and muscle weight relative to animal body weight of female C57BL/6J mice. **A** muscle weight in [g] and **B** relative muscle weight in [%] in the three different housing conditions CON housing in black, ENR housing in light blue and SNE housing in green. The parameter was measured after the perfusion at the age of 670 days and is displayed with the respective p values from a post hoc Tukey test

n = 18, Fig. 7). There was no significant difference in the myostatin concentration between the three housing conditions.

Osteocalcin is a component of the extracellular non-collagenous bone matrix and serves to mineralize bone during new bone formation or healing. In mice osteocalcin also stimulates insulin secretion and thus lipolysis. The osteocalcin concentration in CON housing was 4.77 ± 3.67 ng ml^{-1} (77.0%, 14.15–1.90 ng ml^{-1}, n = 11), lower in ENR housing with 4.48 ± 2.96 ng ml^{-1} (66.1%, 8.95–1.70 ng ml^{-1}, n = 9) and slightly increased in SNE housing with $5.18.26 \pm 2.79$ ng ml^{-1} (53.8%, 9.91–1.47 ng ml^{-1}, n = 17, Fig. 7). Comparable to the relation in the values for the myostatin concentration, no significant difference was found.

C-terminal telopeptides are metabolic products of collagen and represent a suitable marker for bone resorption. An elevated CTX-1 level indicates a reduced bone turnover. The results for the CTX-1 concentration showed a more distinct relationship. In CON housing CTX-1 concentration was the highest with 32.97 ± 7.20 ng ml^{-1} (21.8%, 43.08–23.15 ng ml^{-1}, n = 10). With no significant difference to CON housing, concentration for animals in ENR housing was 31.00 ± 7.80 ng ml^{-1} (25.2%, 41.41–18.14 ng ml^{-1}, n = 9). SNE housing lead to a significantly lower CTX-1 concentration in comparison to CON and

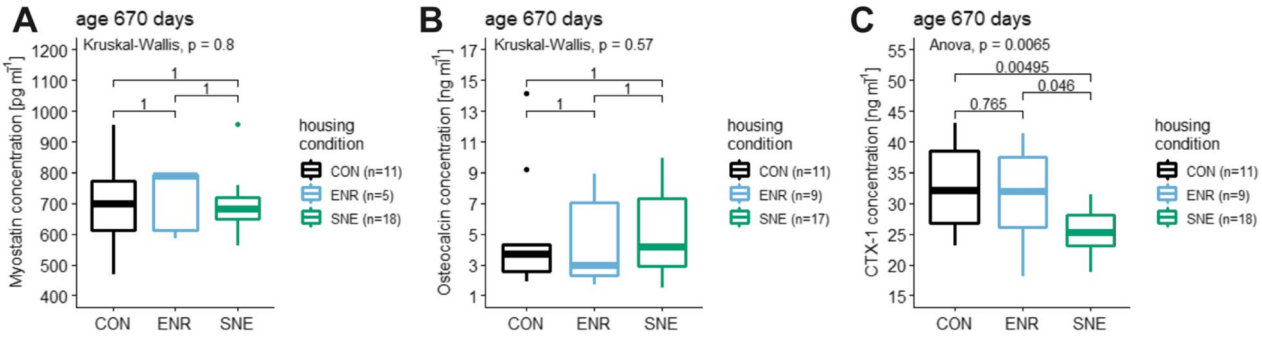

Fig. 7 Blood serum concentration of three muscle and bone turnover markers of female C57BL/6J mice. **A** muscle turnover marker myostatin, **B** bone turnover marker osteocalcin and **C** CTX-1 for three different housing conditions CON housing in black, ENR housing in light blue and SNE housing in green. The parameters were measured after the perfusion at an age of 670 days and are displayed with the respective p values from post hoc Wilcoxon (A and B) and Tukey (C) tests

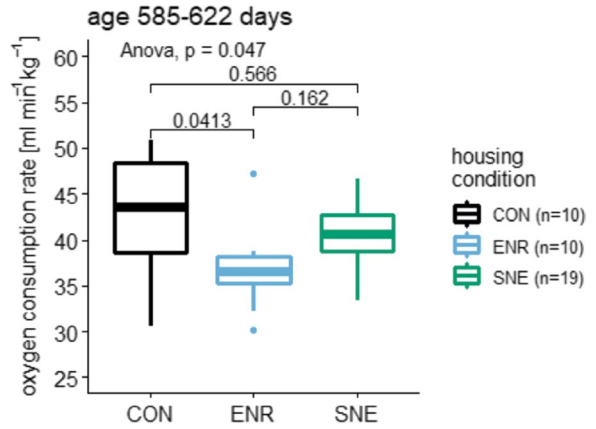

Fig. 8 Resting metabolic rate in mmol min-1 kg-1 for female C57BL/6J mice. Data is shown for three different housing conditions CON housing in black, ENR housing in light blue and SNE housing in green. The rates were measured at an age of 585—622 days and are displayed with the respective p values from a post hoc Tukey test

ENR housing with 24.77 ± 4.38 ng ml^{-1} (17.7%, 31.49–14.71 ng ml^{-1}, n = 18, Fig. 7).

Resting metabolic rate

Resting metabolic rate (RMR) data for animals in SNE housing were already published [21]. Here the values were compared with those of the other housing conditions. The resting metabolic rates were measured at an age of 585–622 days. For animals in CON housing a rate of 42.5 ± 7.4 ml min^{-1} kg^{-1} (17.4%, 50.9–30.6 ml min^{-1} kg^{-1}, n = 10) was observed. In ENR housing oxygen consumption was at 36.9 ± 4.5 ml min^{-1} kg^{-1} (12.2%, 47.3–30.2 ml min^{-1} kg^{-1}, n = 10) and in SNE housing at 40.5 ± 3.4 ml min^{-1} kg^{-1} (8.4%, 46.6–33.3 ml min^{-1} kg^{-1}, n = 19, Fig. 8). The housing condition has a significant effect on the metabolic rate of experimental animals. In ENR housing the RMR is significantly lower than in CON housing. Although also being lower than in CON housing, the decreased oxygen consumption in SNE housing is not significantly different to the rates in the other housing conditions. Animals in the CON housing showed the highest variance in the RMR.

Corticosterone and corticosterone metabolite concentration

Fecal corticosterone metabolite (FCM) concentration of animals in CON housing was 2.53 ± 0.77 ng mg^{-1} (30.5%, 3.90–1.26 ng mg^{-1}, n = 9) and 2.73 ± 0.78 ng mg^{-1} (28.5%, 3.85–1.26 ng mg^{-1}, n = 11) for animals in ENR housing. Concentration was lower for animals in SNE housing with 1.94 ± 0.65 ng mg^{-1} (33.6%, 3.66–0.86 ng mg^{-1}, n = 19, Fig. 9). There was an overall effect of housing condition with SNE mice showing significant lower fecal corticosterone metabolite concentrations than ENR animals, but not animals in CON housing. FCM concentration was not significantly different between CON and ENR housing.

Blood corticosterone concentration was measured following two different methods of blood sampling at two different time points. At 508–510 days of age, facial vein blood of animals in CON housing had 142.32 ± 93.33 ng ml^{-1} (65.5%, 303.55–37.42 ng ml^{-1}, n = 10) corticosterone. ENR housing showed 132.44 ± 81.47 ng ml^{-1} (61.5%, 276.49–44.35 ng ml^{-1}, n = 9). These concentration values doubled for the animals in SNE housing with 321.50 ± 139.66 ng ml^{-1} (43.4%, 564.08–155.03 ng ml^{-1}, n = 15, Fig. 9). Post hoc analysis showed that corticosterone concentration in SNE housing was significantly higher than in animals from CON and ENR housing.

After the perfusion of the animals, the measurement was done with perfusion blood samples. Corticosterone

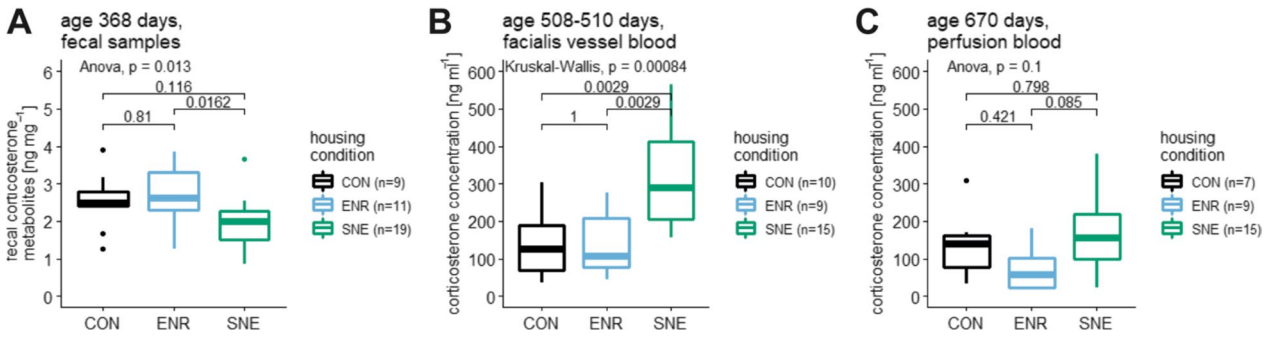

Fig. 9 Concentrations of fecal corticosterone metabolites (FCM) and blood corticosterone for female C57BL/6J mice. **A** fecal corticosterone metabolite concentration in ng mg^{-1} in CON housing (black), ENR housing (light blue) and SNE housing (green) at an age of 368 days with respective p value from a post hoc Tukey test. **B** facial vein blood corticosterone concentration in ng ml^{-1} in the housing conditions (colors equal) at an age of 508—510 days with respective p value from a post hoc Wilcoxon test. **C** perfusion blood corticosterone concentration in ng ml^{-1} in the housing conditions (colors equal) after the perfusion with respective p value from a post hoc Tukey test

concentration was 136.95 ± 90.79 ng ml^{-1} (66.3%, 308.13–34.85 ng ml^{-1}, n = 7) in CON housing, 78.95 ± 61.60 ng ml^{-1} (78.0%, 179.75–19.26 ng ml^{-1}, n = 9) in ENR housing and 163.47 ± 102.92 ng ml^{-1} (63.0%, 379.90–21.75 ng ml^{-1}, n = 15, Fig. 9) in SNE housing. These values gave the same trend as the measurement on facial vein blood, but could not reach statistically significant differences.

Data summary and data correlation

Table 1.

Discussion

Housing female C57BL/6J mice under conventional, enriched, and semi naturalistic conditions for a period of 588 days affected the examined parameters in this study in several ways. Physiological parameters body weight and body length both reached higher values with higher complexity of the housing condition. This correlated with the effect of housing condition on the examined retroperitoneal and periovarian adipose tissues. The adipose tissue weight was also higher in animals of ENR housing in comparison to CON housing and increased significantly in SNE housing. In contrast, no effect was observed in muscle weight. Comparable to the lack of differences in muscle weight, mice from different housing conditions also showed no differences in grip strength and only a weak effect on the metabolic rate was detected. Animals of the ENR housing showed the lowest oxygen demand.

The bone characteristics of the mice seem to have been influenced in part by the different housing condition. However, no uniform effect emerged. There was a clear trend of SNE housing inducing a higher bone density with a significant effect on one year old animals. This trend was also observed for the cortical thickness of the femur bones, the femur length and the femur diameter during post mortem analysis one year later. A more detailed look into bone formation and degradation parameters showed only the effect of CTX-1 being lower in blood samples of SNE animals than in CON and ENR animals indicating reduced osteoclast-mediated bone resorption. Surprisingly, all animals showed anomalies in the bone structures. However, these anomalies seemed to be reduced by the SNE housing. Finally, the stress hormones of the animals were examined according to different methods. Here, too, opposite effects were observed. In the fecal samples of the animals, the lowest corticosterone concentrations were shown in semi naturalistic environment compared to the other two housing systems. In blood samples, however, SNE animals showed the highest concentrations. The individual results are discussed in more detail below.

The conditions and experiments listed here suggest that laboratory mice achieve greater body lengths and weights with increasing complexity of housing. For body weight, this was also observed by Augustsson et al. in an experimental design with increasing EE [19]. The difference in body weight was due to either different feed intake or different levels of exercise. Since environmental enrichment usually prevents altered feed intake [31–33] and the same type of feed was available ad libitum in all housing conditions, it could not be due to the feed itself but to the amount consumed and maybe to the way the feed was consumed. In cage housing, the feed was available at the feeding racks of the cage lid. In SNE, the feed was in small bowls on the bottom of the cage surface. In conventional and enriched cage housing, the animals thus reared up to get to the feed. In SNE, they eat from the floor as in nature and thereby not having to rear up. This different way of feeding might conserve energy and potentially shift energy turnover and thereby promote adipose

Table 1 Summarized results of examined parameters and correlation analysis

Animal age	Parameter	Housing condition	Mean ± SD	CV	Correlating parameter	Corr. factor r	*p* Value
Days				%			
340	Body weight	CON	26.8 ± g	7.7			
		ENR	27.2 ± g	**5.5**			
		SNE	29.8 ± g	8.1			
	Bone density	CON	1.51 ± 0.24 g cm^{-3}	**15.6**	Body weight	0.447	0.168
		ENR	1.69 ± 0.17 g cm^{-3}	16.5		0.475	0.119
		SNE	1.72 ± 0.16 g cm^{-3}	16.0		0.553	0.011
501	Body weight	CON	29.5 ± 3.4 g	11.6			
		ENR	32.1 ± 4.1 g	12.7			
		SNE	36.3 ± 3.2 g	**8.9**			
	Bone density	CON	1.44 ± 0.15 g cm^{-3}	**10.6**	Body weight	−0.027	0.937
		ENR	1.58 ± 0.19 g cm^{-3}	12.0		0.391	0.234
		SNE	1.57 ± 0.23 g cm^{-3}	14.4		0.460	0.047
508/510	Body weight	CON	29.8 ± 3.7 g	12.3			
		ENR	31.1 ± 3.6 g	11.4			
		SNE	35.2 ± 3.0 g	**8.6**			
	Grip strength	CON	2.29 ± 0.39 N	17.0	Bone density 501 days	−0.513	0.106
		ENR	2.60 ± 0.41 N	15.6		0.795	0.003
		SNE	2.38 ± 0.35 N	**14.5**		0.323	0.177
664	Body weight	CON	29.4 ± 3.0 g	9.8			
		ENR	31.7 ± 2.9 g	**9.0**			
		SNE	35.0 ± 3.0 g	9.3			
	Bone density	CON	1.21 ± 0.13 g cm^{-3}	**10.8**	Body weight	0.493	0.123
		ENR	1.38 ± 0.25 g cm^{-3}	17.8		0.214	0.528
		SNE	1.35 ± 0.16 g cm^{-3}	12.2		0.413	0.100
	Grip strength	CON	2.34 ± 0.43 N	18.4	Bone density	0.240	0.476
		ENR	2.65 ± 0.26 N	**9.8**		−0.071	0.836
		SNE	2.40 ± 0.40 N	16.6		−0.196	0.451
585 — 622	Resting metabolic rate	CON	42.5 ± 7.4 ml min^{-1} kg^{-1}	17.4	Grip str. 664 d	0.018	0.959
		ENR	36.9 ± 4.5 ml min^{-1} kg^{-1}	12.2		0.221	0.514
		SNE	40.5 ± 3.4 ml min^{-1} kg^{-1}	**8.4**		0.565	0.015
670	Body weight	CON	30.4 ± 3.1 g	10.1	RMR 585–622 d	0.326	0.329
		ENR	33.5 ± 3.1 g	9.2		0.490	0.126
		SNE	34.6 ± 3.1 g	**8.9**		−0.301	0.224
	Body length	CON	9.7 ± 0.3 cm	2.8	Body weight	0.838	0.001
		ENR	10.1 ± 0.2 cm	2.2		−0.182	0.592
		SNE	10.3 ± 0.2 cm	**2.1**		0.685	0.002
	Weight/length	CON	3.1 ± 0.3 g cm^{-1}	**8.2**			
		ENR	3.3 ± 0.4 g cm^{-1}	10.9			
		SNE	3.3 ± 0.3 g cm^{-1}	8.7			
	Retroperitoneal adipose tissue weight	CON	0.057 ± 0.032 g	56.8	Body weight	0.729	0.011
		ENR	0.075 ± 0.019 g	**25.3**		0.874	> 0.001
		SNE	0.112 ± 0.048 g	43.1		0.539	0.021
	Periovarian adipose tissue weight	CON	0.276 ± 0.157 g	56.9	Body weight	0.535	0.090
		ENR	0.409 ± 0.182 g	**44.5**		0.953	> 0.001
		SNE	0.506 ± 0.236 g	46.6		0.582	0.011
	Cortical thickness	CON	164.4 ± 23.2 µm	14.1			
		ENR	169.5 ± 19.7 µm	**11.6**			
		SNE	185.5 ± 24.5 µm	13.2			

Table 1 (continued)

Animal age	Parameter	Housing condition	Mean ± SD	CV	Correlating parameter	Corr. factor r	*p* Value
	Femur length	CON	16.55 ± 0.15 mm	**0.9**			
		ENR	16.67 ± 0.32 mm	1.9			
		SNE	16.79 ± 0.28 mm	1.7			
	Femur midshaft outer diameter	CON	1.76 ± 0.04 mm	2.3	Bone density 664 days	0.409	0.275
		ENR	1.77 ± 0.03 mm	**1.8**		0.127	0.745
		SNE	1.83 ± 0.04 mm	2.3		0.478	0.116
	Femur bone volume/tissue volume	CON	1.32 ± 0.78%	**59.0**			
		ENR	1.78 ± 1.09%	61.1			
		SNE	1.53 ± 1.10%	71.8			
	Muscle weight	CON	0.123 ± 0.023 g	19.0	Body weight	0.221	0.515
					grip str. 664 d	−0.136	0.590
					RMR 585–622 d	0.313	0.348
		ENR	0.127 ± 0.021 g	16.5	Body weight	0.055	0.872
					grip str. 664 d	0.077	0.823
					RMR 585–622 d	−0.284	0.398
		SNE	0.120 ± 0.020 g	**16.3**	Body weight	0.435	0.071
					grip str. 664 d	0.296	0.377
					RMR 585–622 d	−0.409	0.092
	Myostatin concentration	CON	692.26 ± 132.87 pg ml^{-1}	19.2	muscle weight	0.026	0.191
		ENR	714.96 ± 107.04 pg ml^{-1}	**15.0**		0.711	0.178
		SNE	692.26 ± 132.87 pg ml^{-1}	19.2		−0.323	0.939
	Osteocalcin concentration	CON	4.77 ± 3.67 ng ml^{-1}	77.0	Cortical thickness	−0.180	0.642
					Cortical porosity	0.376	0.319
		ENR	4.48 ± 2.96 ng ml^{-1}	66.1	Cortical thickness	−0.016	0.969
					Cortical porosity	−0.113	0.790
		SNE	5.18.26 ± 2.79 ng ml^{-1}	**53.8**	Cortical thickness	−0.721	0.008
					Cortical porosity	0.685	0.014
	CTX-1 concentration	CON	32.97 ± 7.20 ng ml^{-1}	21.8	Cortical thickness	−0.470	0.240
					Cortical porosity	0.201	0.634
		ENR	31.00 ± 7.80 ng ml^{-1}	25.2	Cortical thickness	0.278	0.505
					Cortical porosity	−0.213	0.613
		SNE	24.77 ± 4.38 ng ml^{-1}	**17.7**	Cortical thickness	−0.562	0.057
					Cortical porosity	0.602	0.038
	FCMs	CON	2.53 ± 0.77 ng mg^{-1}	30.5			
		ENR	2.73 ± 0.78 ng mg^{-1}	**28.5**			
		SNE	1.94 ± 0.65 ng mg^{-1}	33.6			
	Corticosterone (*V. facialis*)	CON	142.32 ± 93.33 ng ml^{-1}	65.5			
		ENR	132.44 ± 81.47 ng ml^{-1}	61.5			
		SNE	321.50 ± 139.66 ng ml^{-1}	**43.3**			
	Corticosterone (perfusion)	CON	136.95 ± 90.79 ng ml^{-1}	66.3			
		ENR	78.95 ± 61.60 ng ml^{-1}	78.0			
		SNE	163.47 ± 102.92 ng ml^{-1}	**63.0**			

Shown is the age of the animals at the respective time of measurement and the value of the parameter for the animals from the three housing conditions CON, ENR and SNE housing. Values are shown as the mean with the standard deviation (SD) and the coefficient of variance (CV). The housing condition showing the lowest CV per measured parameter is marked Bold. If a correlation analysis with another measured value was made, it is marked as the correlating parameter and the results are shown as the correlation factor r and the respective *p* value. If a correlation lead to a *p* value lower than 0.05, the correlation is marked underline

tissue storage in the animals. However, longer distances to food sources most likely negate such an effect. The retroperitoneal adipose tissue weight did correlate with the body weight in all three housing conditions. The correlation between periovarian adipose tissue weight and body weight of the animals was not found for the CON housing condition. All in all, this indicates that feeding in enriched environments might lead to greater adipose tissue deposits and thereby higher body weight. However, this conclusion only applies under the assumption that environmental enrichment does not affect the time spend feeding [6]. In order to better understand how the increased weight in SNE and ENR conditions came about a more detailed analysis of feeding behavior is warranted. It is worth noting that none of the animals showed severe signs of obesity under any of the housing conditions.

Another reason for the physical change were more and different possibilities of movement. The ability to run longer and farther or climb more in an enriched or semi naturalistic environment suggest that more muscle is developed and therefore higher body weights are achieved [34]. However, no significant difference in muscle weight was observed in relation to different body weights. One reason for this could be the advanced age of the animals at the time of the investigation. The older the animals become, the lower the muscle mass and strength of the animals [35]. Regular exercise and stressing of the muscles slow down this process, but it cannot be prevented.

The lack of difference in muscle weight is consistent with the findings in the myostatin concentration of the animals. There were no significant differences in the myostatin concentration between the animals in the different housing conditions. There was also no correlation between the measured concentration of myostatin and the muscle weight of the animals in all three housing conditions. Since the muscle mass of mice is directly linked to the levels of myostatin [36, 37], an effect of housing should be recognizable on the basis of myostatin concentration. Also corresponding to the consistent muscle weight in the three housing conditions, no significant difference was detected in the grip strength of the animals. There was a comparable trend between the two measurements, in which animals of the ENR housing had the highest values of grip strength while CON and SNE had similar values. In addition, no effect of the age of the animals in relation to grip strength was observed. Muscle strength is expected to diminish with age [35, 38]. However, at 558 days of age, when the grip strength was first measured the animals must already be considered old. Therefore, any effect of progressively increasing age on muscle strength could probably not have been detected.

The increasing body weight of experimental animals in this study could also have been influenced by the bone skeleton properties. This is supported by the, in some cases, significantly larger dimensions of the femora of the animals in the enriched environments. Increasing length and diameter of the femora in ENR and SNE housing were also accompanied by increasing cortical thickness. The cortical thickness and body weight did correlate in CON animals – the heavier the animals were, the thicker the femur cortical bone. It is possible, that increased opportunity for exercise in ENR and SNE housing has stimulated longitudinal growth, especially while the animals were still younger [39].

Besides the mere dimensions of femora, at two different time points during the housing of the animals under different housing conditions, there was a correlation between the bone density and body weight of the animals. The higher the bone density the higher the animal's weight. There was also an indirect correlation of cortical porosity with body weight in all three housing conditions. In CON housing even significantly before alpha correction – the heavier an animal, the less porous the femur cortical bone. However, it is likely that the underlying causality is not that mouse body weight is determined by bone density, but that higher body weight leads to higher bone density. In fact it is well established that increased body mass leads to increased bone density [40]. Moreover, the correlation between body weight and bone density could not be found for CON and ENR animals.

The causes of differences in bone characteristics are multifactorial and might be influenced by movement behavior, food intake, body weight and regular forces (e.g., high jumps) experienced by the skeletal system. The increased bone density in more complex environments can on the one hand probably be explained by the larger bones in the two dimensions recorded. The SNE animals tended to have longer femora and significantly larger femora in diameter. A consistent cortical thickness was observed between the housing conditions. An equally thick bone wall with larger bones means a higher bone mass, which yields higher values in the applied bone density method. In ENR housing femur length did correlate with cortical thickness and did negatively correlate with cortical porosity. The longer the femur, the thicker and less porous the cortical bone. This correlation could not be shown for CON and SNE animals but might explain the effect at 340 days of age and the trends at the two later measurement times.

When looking at the parameters regarding the composition and structure of the bone skeleton, only few correlations were discovered. For ENR animals a strong correlation between bone density and grip strength was found. Animals with higher muscle loading, as it for

example occurs during climbing in enriched environments, could have higher grip strength. It is known that sustained muscular loading also increases the dimensions and strength of bones [41, 42]. However, contrary to our data it would be expected that in the SNE, where there was climbing at the grid and where the animals covered a lot of distance, this effect would have been confirmed.

The concentration of CTX-1 as a factor of bone resorption was significantly influenced by housing conditions. Animals living in the SNE showed less CTX-1 than the animals of CON and ENR housing. In studies on humans, exercise has been found to reduce CTX-1 even in older participants [43, 44]. Therefore, it can be assumed for mice as well that the larger range of motion in the SNE reduces bone resorption. This also seems to be reflected in the frequency of bone anomalies. Although the proportion of animals exhibiting anomalies was generally high, the enriched housing had fewer animals with anomalies. These also had fewer anomalies per animal, at least in the SNE, than in the CON housing. This observation is potentially linked to the increased opportunity and necessity for movement under SNE housing conditions. It is well established that regular exercise improves skeletal metabolism by inhibiting osteoclast activity among other effects [45–47]. Our results regarding CTX-1 appear to be in line with this pattern. Overall, however, only minor effects on total bone density and structural parameters were observed. It is therefore likely that other confounding factors, especially the age of the animals, mask a stronger skeletal manifestation of the observed biochemical changes.

Besides the body weight, the body length also increased in ENR and SNE housing compared to CON housing. This was examined when measuring the anesthetized animals before their perfusion at 670 days of age. A correlation was also found for body length and body weight. Except for ENR housing, all animals showed, that the longer their bodies, the higher was their body weight. This might indicate a leaner "athletic" body type under ENR conditions. The enrichment elements used in the ENR housing promotes vertical movement and the running disc should create an incentive for activity compared to the CON housing. There is indication that stereotypies, which can occur in caged mice, are compensated by increased use of running wheels [48]. Therefore, increased exercise on the running disc may have favored a leaner body type. On the other hand, it remains unclear why the unequally larger exploration area in the SNE did not cause the same effect.

In general, there is a close biological relationship between resting metabolic rate and body weight. As body weight increases, greater metabolic rates are achieved within the same species. The alteration of the resting metabolic rate depends on the energy needs of all organs and body components [49]. It is reasonable to assume that the possibility of more outlet and activity increases the proportional demand of muscle mass. However, in our study, muscle weight was not affected by housing conditions and the metabolic rate of mice from the SNE was not increased despite of larger cage space and possibility of movement. There was also no correlation found between body weight of the animals and their respective resting metabolic rate nor specifically between muscle weight and resting metabolic rate. To further specify the cause of the results of the present study, the activity of the animals would have to be recorded. Due to the diversity of the housing conditions, a comparable method for determining activity was not feasible in this experiment.

Besides the activity of the animals, thermoregulation could be another reason for the differences in resting metabolic rate. The SNE housed twenty animals. This usually leads to a grouping of all or large groups of the mice in common nests during the resting periods. More animals within the same nest allow for less thermoregulation during resting and therefore a lower metabolic rate [50, 51]. ENR housing promotes the same effect by providing additional nesting material and clearly more delineated and narrower nesting areas. On the other hand, animals in SNE housing showed no significant difference in metabolic rate compared to control animals. Lower metabolic rate due to a long-term effect of lower thermoregulation could be explained by increased stress level during calorimetry. The differences in housing conditions between SNE and single housing in a type II Makrolon measuring cage may be associated with higher stress for the SNE mice. The animals are not accustomed to a confined cage and may show increased resting metabolic rate due to increased fear.

To determine the effects of housing conditions on the stress level of the experimental animals, adrenocortical activity was measured using different samples with opposing results. Fecal samples showed a significantly lower concentration for SNE housing and equally high concentrations in CON and ENR housing. In contrast, blood samples obtained from the facial vein showed significantly higher concentrations for SNE housing. Again, CON and ENR housing did not cause statistically relevant differences in corticosterone concentration. Results from perfusion blood samples showed no significant effect but a similar trend. SNE housing caused the highest concentration. In this measurement, values for CON housing were closer to values for SNE housing than to values for ENR housing.

The fact that different results are obtained with different samples matrices is not unusual. Whereas measuring fecal corticosterone metabolites is noninvasive

and of sufficient accuracy [52, 53], concentrations in fecal samples reflect a more pooled and temporally deferred sample of stress hormonal activity. FCMs are pooled due to the mixing of feces in the intestine during metabolism. The temporal delay results from the length of time the feces remain in the animals' bodies between the measured state and the actual sampling [54, 55]. In contrast, measuring corticosterone concentrations in blood always requires taking a blood sample within a few minutes. In fact, the sampling method itself introduces a potentially more stressful situation for the animals than everyday housing can cause [56–58]. For efficient sampling of all individuals, the animals were removed from the SNE and placed in a separate cage. From this cage, the individuals were taken one by one, scanned for identification, then the blood sample was taken and the mouse was placed back into the enclosure. This procedure could take a total of 30–60 min for 20 animals. The literature shows that the concentration of stress hormones in mice can be significantly increased after only 15 min by an experimental situation [59]. Compared to the direct removal of the animal from the housing environment and immediate blood sampling in the CON and ENR animals, the time from picking a mouse out of the SNE until obtaining the blood sampling might have been too long to still reflect baseline corticosterone level. All in all the lack of statistical different concentrations in trunk blood suggest that the stress level at the time of perfusion was the same for animals from all housing conditions. The results from the fecal samples on the other hand indicate that an enriched semi naturalistic environment reduces baseline stress levels and is in line with previous results [5]. However, it should be emphasized that the different samples were also collected at different times during the housing of the mice. Thus, an effect of age as a cause for the contrasting results cannot be excluded without doubt. Given this relationship, EE seems to have more of a stress-reducing effect in this study.

Comparable to a previous analysis [21] and literature data [5], no indication for increased variability was found. To the contrary, in this study a total of 20 parameters were evaluated in 30 different measurements. In 22 measurements, lower variances of the measured values were measured for one of the two enriched housing conditions (ENR or SNE) compared to the conventional housing. In 14 cases this was true in both enriched housing conditions. This indicates that the general concern of increased variability due to improved housing conditions does not hold true.

Limitations

Although a significant difference between the SNE housing and the other housing conditions was found in the weights of both adipose tissues examined (retroperitoneal and periovarian), these results should be viewed with caution. The variance of adipose tissue weight is strongly dependent on the execution of the section. Dissecting the adipose tissue requires skill and a clear differentiation between the target tissue and surrounding tissue. Although the preparation was always performed by the same person, methodological error cannot be ruled out. In addition, no insights into the type or exact composition of adipose tissue were obtained in this study. This will require histological examination in possible follow-up studies. Therefore, the pure weight of adipose tissue should not be unreservedly related to the activity or body weights of the experimental animals in the different housing conditions.

The method to measure grip strength applied here might not have been ideal, since the steady pulling of the animals on the holding device is motorically demanding and requires training and experience of the animal as well as of the experimenter. In addition, it has to be taken into account, that during the process of pulling the animals, the motivation to hold on rather than the actual strength of the animals is measured.

One argument against the generalizability of the data collected could be the duration of the housing itself. There are a few indications in the literature that effects of EE can be reduced or weakened by long housing periods [60]. Many of the parameters in our study were measured at a high age of two years. Effects of EE on the development of the animals could therefore hardly be shown. Indeed, it is likely that ageing related degenerative effects partially mask the environmental effects at this age. This is quite well indicated by the clear and significant difference of bone density at the age of one year (generally healthy, middle-aged animals) that vanished throughout the subsequent year. Also, the high prevalence of skeletal anomalies indicates that skeletal degeneration has progressed quite far in these older mice.

On a sidenote, it can be concluded from this data that the investigated housing conditions do not prevent ageing-related bone loss.

The correlation analyses used to discuss the physiological parameters among themselves were also subject to a prior test for normal distribution. Nevertheless, these analyses involve different group sizes. In particular, the number of individuals in the SNE housing can add weight to the results. The correlations were therefore considered with caution.

Conclusions

Overall, female C57BL/6J mice in all housing conditions exhibited strain-typical values for body weight development throughout the lifespan [61, 62]. Within this range, housing them in conditions that are more natural, increased weight and length of the animals. It is worth noting, that none of the studied parameters was negatively affected by more enriched housing. All in all, bone properties appear to be slightly improved by more natural housing and age-related increased bone resorption was reduced. We confirmed previous studies, showing that the variance of the data was not increased by more natural housing conditions. This indicates that more natural housing conditions are a feasible way for housing and testing laboratory mice.

Methods

Animals

For this study 44 female C57BL/6J mice were purchased from Charles River (Charles River, Sulzfeld, Germany). Social housing of male mice in large enclosures may promote increased aggression due to territorial behavior in male animals [27]. To minimize possible adverse effects of aggressive behavior, only female animals were used in this study. At arrival, animals were eight to nine weeks old. The mice were special pathogen free, were checked for their health status, weighed and then randomly assigned to one of seven groups in three different housing conditions (3×4 animals in conventional housing CON, 3×4 animals in enriched housing ENR and one group of 20 animals in a semi naturalistic environment SNE). Prior to the experiment, the groups were kept in standard Type III Makrolon cages in an open rack system. After seven days of habituation and daily handling training, animals were tagged individually with a radio frequency identification (RFID) transponder. After another two weeks of monitored recovery and handling training, animals were transferred to their respective housing conditions in a special laboratory area for animal keeping. During habituation and experimental housing animals were kept at a 12/12 h light cycle (summertime lights on 8:00 a.m.– lights off 8:00 p.m., wintertime lights on 7:00 a.m.– lights off 7:00 p.m.), at 22.0 ± 2.0 °C, and 50.0 ± 5.0% humidity. The animals were kept in the experimental housing conditions from 82 days of age to 670 days (approx. 2 years). At different points during this time, physiological parameters were measured. Once a week, animals were weighed and handled to check for their health status. At the end of the experimental phase, the animals were put under anesthesia with a mixture of ketamine and xylazine and were transcardially perfused. Body length was measured, blood was collected, and adipose tissue, muscles, and bones were removed and weighed. Adipose tissue and muscle weights were analyzed and plotted both as actual weights and relative to animal body weight. No further histological examinations were performed with the adipose and muscle tissue samples collected. During the study, four of the 44 animals died prior to the planned perfusion of the experimental animals due to causes unrelated to this study. One animal each died from the housing conditions CON and ENR, and two animals from SNE. There was no indication that the housing condition had an influence on the death of the animals. The reduced animal numbers are marked in the respective parts of the results. All experiments were conducted in accordance with the applicable European and national regulations and were approved by the State Office for Health and Social Affairs Berlin (G 0069/18).

Transponder injection

All animals were marked individually for identification with a RFID transponder of two types (Type 1–FDX-B transponder according to ISO 11784/85; Planet-ID, Essen, Germany/Euro I.D., Köln, Germany or Type 2–ID 100, diameter: 2.12 mm; length: 11.5 mm, Trovan, Ltd., Douglas, UK). For analgesia the animals received the non opioid analgesic meloxicam (0.1 mg kg^{-1}, Meloxydyl, Ceva Tiergesundheit GmbH, Düsseldorf, Germany) orally 60 min before the injection. The transponder was injected subcutaneously between the shoulder blades (scapulae) under inhalation anesthesia with isoflurane according to established procedures (1.0–1.5% in 30% O_2 with 70% N_2O). The wound was then manually closed and fixed for a few seconds to initiate natural wound closure or closed with tissue adhesive when necessary. The awakening of the animals was monitored in a separate cage.

Housing conditions

Conventional housing CON

This housing condition served as the control condition during the experiments. It meets the minimal standards for animal housing, regulated by guidelines at national and international level (i.e., directive 2010/63/EU). Similar to the standard caging during habituation CON housing (Fig. 10A) consisted of a Type III Makrolon cage with a floor area of 840 cm^2 and 153 mm height. It was filled with approx. 3 cm aspen bedding (Polar Granulate 3–5 mm, Altromin, Lage, Germany). The cage contained a red triangle plastic house, a wooden gnaw stick, a small cotton roll of nesting material and two pieces of paper towel. Mice had ad libitum access to tap water and food (autoclaved pellet diet, LAS QCDiet, Rod 16, LASvendi, Soest, Germany).

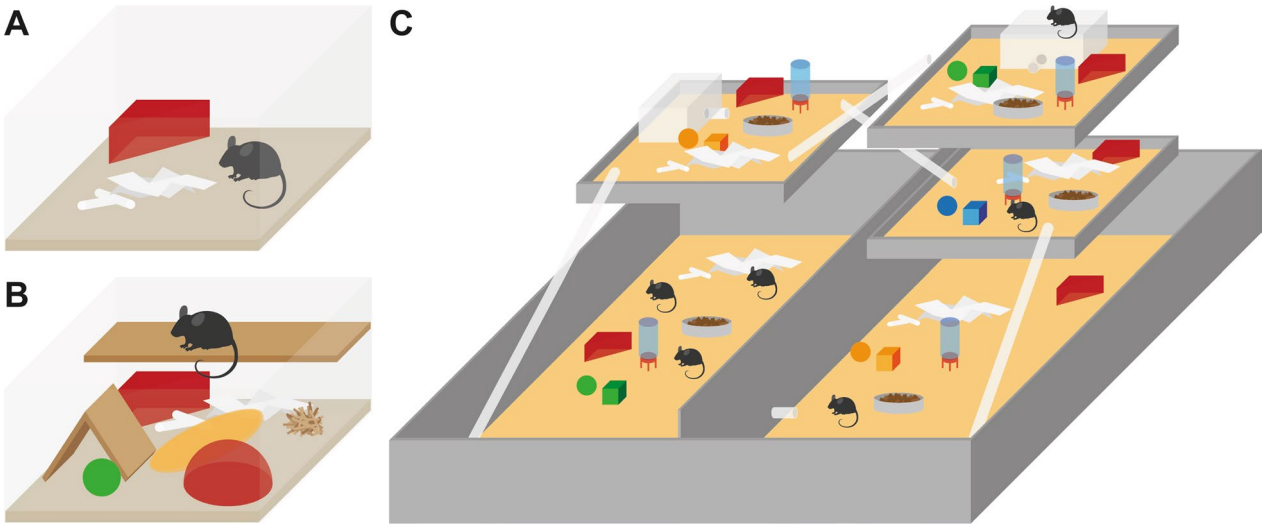

Fig. 10 Illustrative representation of the three housing conditions. **A** conventional housing (CON), **B** enriched housing (ENR) and **C** semi naturalistic environment (SNE). **B** shows the extension of the conventional cage housing (**A**) with additional shelter, nesting and structural elements. **C** shows the five levels of the semi naturalistic environment with access to water, food and shelter on every level as well as enrichment elements

Enriched housing ENR

The enriched cage housing (Fig. 10B) was set up identically to the conventional housing but was extended with different kinds of enrichment. These enrichment elements were assigned to categories regarding their prospective function and placed in the cages additionally or as an alternative to the conventional housing features. The equipment of a single cage consisted of a mouse house with a running disc, an alternative house, a wooden platform clamped between the walls of the cage, one structural element hanging from the cage lid, an interactive enrichment element and alternative nesting material in addition to the cotton roll and pieces of paper. This combination of the enrichment elements was changed every week. The only permanent element within the cage was the running disc. Elements of the other categories were combined randomly. In addition to regular food, the interactive enrichment element daily offered approx. 3.5 g millet seeds as a treatment to facilitate interaction with the interactive enrichment. The same amount of millet seeds was offered to animals in the other housing conditions (CON and SNE) by spreading it in the bedding. For a detailed description of the enrichment elements and their combination, see Hobbiesiefken et al. [3].

Semi naturalistic environment SNE

The SNE (Fig. 10C) was set up and operated as described in Mieske et al. [21]. Briefly, the SNE consists of a large mesh wired enclosure with an area of 4.6 m^2 spread over five different levels in different heights. On each level of the SNE there was access to water, food and shelter in form of a red triangle plastic house. The two upper levels also provided upside down Type I Makrolon cages as nesting boxes. Plexiglas tubes connected the different levels. Similar to the CON and ENR housing conditions, the SNE was also filled with 3–5 cm of aspen bedding. Every level provided two wooden gnawing sticks, two cotton rolls and two pieces of cellulose paper as nesting material. The two nesting boxes also provided two cotton rolls and two pieces of paper. For additional enrichment, the animals had access to Plexiglas tubes as structural enrichment elements and a small selection of self-designed toys of different shape and color.

Bone density and structural properties

X-ray images for determination of bone density were obtained on a Bruker InVivo Xtreme II (Bruker, Billerica, MA USA). The animals were picked in a randomized order, were anesthetized with isoflurane (1.0–1.5% in 30% O_2 with 70% N_2O) and placed on the platform for X-ray acquisition. Anesthesia was maintained during the whole procedure. Eyes of the animals were protected with dexpanthenol creme. The awakening of the animals was monitored before the animals were placed back into the home cage. On the x-ray images a region of interest (ROI) was selected on the right femur of the animals. Bone density in g cm^{-3} was then determined by the Bruker Molecular Imaging Software. Bone density was measured three times during the housing of the animals at 340 days of age, 501 days and 664 days.

After perfusion of the animals, the leg bones of the animals were dissected. The samples were fixed for 24 h in paraformaldehyde (4% PFA), washed three times with phosphate-buffered saline (PBS) and afterwards stored in 30% sucrose solution. Length and diameter of the right femur and characteristics of the cortical and trabecular bone were analyzed using x-ray micro-computed tomography (μCT). The μCT scanning and analysis were performed as described by Zhao et al. [63]. Briefly, the right femur of each mouse was fixed and placed into a radiotranslucent sample holder. Samples were scanned and analyzed with a voxel resolution of 10 μm using a μCT 40 desktop cone-beam microCT (Scanco Medical, Switzerland) according to standard guidelines [64]. Trabecular bone was analyzed in the distal metaphysis in a volume situated 2500–500 μm proximal to the distal growth plate. Cortical bone was analyzed in a 1000 μm long volume situated in the middle of the diaphysis. Cortical bone evaluation was performed with a threshold of 300, whereas for trabecular bone, a threshold of 250 was used. The length of the femora was determined by the number of slices containing the bone.

For histology, tibiae were embedded in Poly(methyl methacrylate)(PMMA) and sectioned at 4 μm thickness in the sagittal plane. Sections were stained by the von Kossa/van Gieson or Toluidine blue staining procedure [65]. Structural anomalies in the tibia bones were characterized by microscopic inspection and their occurrence was counted. For biomechanical testing, a three-point bending test was performed on dissected femora using a Z2.5/TN1S universal testing machine and testXpert software (both Zwick Roell, Germany) as described previously [66].

Grip strength

Animals were tested separately and in a randomized order. Grip strength was measured with a computerized grip strength meter (TSE Systems GmbH, Bad Homburg, Germany). The apparatus consisted of a T-shaped metal bar connected to a force transducer. To measure the grip strength in the hind paws of the mice, the mice were carefully held at the base of the tail and guided towards the metal bar with their hind paws. Their front paws were placed on a wire mesh cylinder to prevent the mice from grasping the bar with their front paws. The animal was then gently pulled backwards until the grip was lost. The peak force applied to by the hind legs was recorded in ponds (p) and converted to Newton (N). This measurement was done three times per animals on one day and the mean peak value was recorded. After the procedure, animal ware placed back into their home cage. The grip strength was measured two times during the housing of the animals at the ages of 508–510 days and 664 days.

Bone and muscle turnover markers

The blood serum concentration of the three following bone and muscle turnover parameters were analyzed with enzyme-linked immunosorbent assays (ELISA). All used ELISA kits were performed according to the manufacturer's instructions.

C-terminal telopeptides (CTX-1)—Serum CTX-1 concentration was detected with the RatLaps^{TM} (CTX-1) ELISA kit (competitive ELISA) (Immunodiagnostic Systems Holdings Ltd., Boldon, UK).

Osteocalcin—Osteocalcin concentration in the blood serum was detected with the Mouse Osteocalcin (OC) ELISA kit (competetive ELISA) (MBS275134, MyBioSource, Inc., San Diego, CA USA). The serum was diluted 1:10 before analysis.

Myostatin—Serum myostatin concentration was analyzed with the Mouse Myostatin ELISA kit (quantitative sandwich ELISA) (MBS166373, MyBioSource, Inc., San Diego, CA USA).

Resting metabolic rate

The principle of indirect calorimetry (TSE phenomaster, TSE Systems GmbH, Bad Homburg, Germany) was used to evaluate the metabolic rate. The calorimetry system measures differences in the composition of air passed individually through four measurement cages and an empty reference cage. The system was situated at a separate room at a 12/12 h light cycle, 22.0 ± 2.0 °C, and and 50.0 ± 5.0% humidity. Animals were tested at 584–594 days of age in a randomized order. Following habituation to the experimental room (12 h), mice were weighed and placed individually in measurement cages equipped with bedding, shelter and nesting material. Food and water were accessible ad libitum during the entire measurement and were weighed before and after the experiment. Measurement cages and the reference cage were perfused with air. In the measurement cage oxygen was lowered and carbon dioxide was increased by the respiration of the animals during the measuring period (12 h light period). After flowing through both cages, the composition of air was compared between measurement cage and reference cage. By calculating the difference between air compositions, the metabolic rate of the examined animal was assessed. After the measurement the mice, food, and water were weighed and the animals were placed back into their home cage. The resting metabolic rate (RMR) was measured as oxygen consumption rate $\dot{V}_{O_2}$ during the resting phases of the animals. To separate resting phases from active phases, the cumulative frequency percentage was plotted against the measured $\dot{V}_{O_2}$. With a segmented linear regression, the threshold between $\dot{V}_{O_2}$ of the resting phase and the active phase

could be calculated. Data below the threshold was used to determine the RMR ([67]; R package 'segmented').

Corticosterone and corticosterone metabolite concentration

The concentration of corticosterone or corticosterone metabolites was measured two times during the housing of experimental animals and one time after the perfusion of the animals. The first measurement was done at an age of 368 days. At 8:00 to 10:00 am animals were individually placed in a random order in Type II Makrolon cages. The cages were just equipped with flatly spread paper towels. After a minimum period of 20 min and maximum of 30 min animals were placed back into their home cage. The fecal boli that the animals had deposited in isolation were collected and used for analyzing corticosterone metabolites (fecal corticosterone metabolites—FCM) as described before [52, 54].

At an age of 508–510 days on three consecutive days animals were individually fixated and blood was taken from the *Vena facialis* after puncture with a lancet needle. For the CON and ENR animals, blood sampling was performed immediately after the animals were removed from the cage (within 1 min). The SNE animals were first removed from the large enclosure and held collectively in a type 4 cage for a short time (30–45 min). The blood samples were collected in 0.2 ml reaction tubes and stored at -80 °C for further analysis. Serum corticosterone concentration was determined with a DRG Corticosterone ELISA (EIA-4164, DRG International Inc., Springfield, NJ USA).

The third measurement was done with trunk blood samples collected directly from the mouse`s heart before perfusion of the animals at 670 days of age. Concentration of corticosterone was determined as described before.

Statistical analysis

Unless described otherwise, all measured data is presented as mean ± standard deviation. In addition, the coefficient of variation (CV), the maximum value, the minimum value and the number of measured animals is given ($\bar{x} \pm$ SD (CV, max–min, n)).

Analysis and illustration of data was done with the software environment R (v 3.6.3, R Foundation for Statistical Computing, Vienna, Austria, R Studio v 1.2.1335, RStudio, Inc., Boston, MA, USA). When preparing the data for statistical analysis, they were first examined for normal distribution ('shapiro.test()' function) within the different groups (housing conditions). Possible outliers were identified using the 'boxplot. stats()$out' function and excluded for the presentation and statistical analysis of the respective data set. If normal distribution was given, an ANOVA with a Tukey post hoc analysis was used to compare the data between the housing conditions ('aov()' and 'tukey_hsd()' function). If data were not distributed normally, the Kruskal–Wallis test with a Wilcoxon post hoc analysis was applied ('kruskal.test()' and 'compare_means(method = "wilcox.test")' function in 'ggpubr' package). Unless described otherwise, boxplot figures show the adjusted *p*-value after Bonferroni correction.

Continuous data were analyzed using linear models ('lm()' function). Related predictors were added as mixed effects to the regression models (package 'lme4' [68], 'lmer()' function). Subsequent statistical comparison of different models ('anova()' function) identified the factors affecting the continuous data.

Possible statistical differences in discrete data were analyzed using the χ^2 test (chi square test). This was performed by using the 'chisq.test()' function.

Abbreviations

CON	Control/conventional housing
CV	Coefficient of variation
EE	Environmental enrichment
ELISA	Enzyme-linked immunosorbent assays
ENR	Enriched housing
FCM	Fecal corticosterone metabolites
µCT	Micro computed tomography
PBS	Phosphate-buffered saline
RFID	Radio frequency identification
RMR	Resting metabolic rate
SNE	Semi naturalistic environment

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42826-023-00160-9.

Additional file 1: Fig. S1. Structural femur properties of female C57BL/6J mice in three different housing conditions. **A** cortical porosity in % with respective p values from post hoc Wilcoxon test. **B** femur trabecular thickness in mm with respective p values from post hoc Tukey test. **C** number of femur trabecular bones in mm-1 with respective p values from post hoc Tukey test. **D** separation between femur trabecular bones in mm with respective p values from post hoc Tukey test. **Table S1.** Summarized results of examined parameters in addition to 3.3 Bone density and structural properties data and Table 1. Shown is the age of the animals at the respective time of measurement and the value of the parameter for the animals from the three housing conditions CON, ENR and SNE housing. Values are shown as the mean with the standard deviation (SD) and the coefficient of variance (CV). The housing condition showing the lowest CV is marked in the CV column in respective to the used color scheme (CON black, SNE green).

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Author contributions
Conceptualization: PM, UH, LL and KD; methodology: PM, UH, LL and KD; formal analysis: PM, JS, JP, LB, TY, RP; data curation: PM; writing—original draft preparation: PM; writing—review and editing: PM, UH, JS, JP, LB, TY, RP, LL and KD; visualization: PM; supervision: KD and LL; project administration: KD and LL. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
The datasets generated and/or analysed during the current study are available in the RefinementReferenceCenter/musculoskel2022_mieskep_available_data repository, https://github.com/RefinementReferenceCenter/musculoskel2022_mieskep_available_data.

Declarations

Ethics approval and consent to participate
All experiments were approved by the Berlin state authority, Landesamt für Gesundheit und Soziales, under license No. G 0069/18 and were in accordance with the German Animal Protection Law (TierSchG, TierSchVersV).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

5 Discussion

The two publications combined in this cumulative dissertation provide an overview of how the project approached recording and reducing boredom-related symptoms in mice. In the research project, a graded enrichment paradigm was used for the reduction of boredom. First, standard laboratory housing cages (CON housing) were enriched. This was done by providing alternative and additional elements to the home cage. The elaborated enrichment elements were categorized and presented in randomized combinations on a weekly basis (ENR housing). The design of the laboratory cages, according to the controlled protocol, was able to reduce stereotypies and allowed the evaluation of each element with respect to the preferences of mice (Hobbiesiefken et al., 2021).
For an increase in environmental conditions to a semi naturalistic environment (SNE housing), a large enclosure was used in which a group of 20 mice could be kept in a complex environment. The SNE provided a total area of 4.6 m² spread over five levels in different heights and of different sizes. All levels were connected with plastic tubes functioning as tunnels and provided access to food, water, and shelter. The maintenance and operation of the SNE as well as the measurement of various parameters on the SNE, were focus of this PhD project. Since the collected results have already been put into scientific context in the publications mentioned above, the everyday handling of the housing conditions and the recording of results will be discussed here as a matter of priority.

5.1 The SNE in comparison to other naturalistic housing concepts

Considering the advantages of EE, there are numerous approaches to provide larger and more natural environments in experimental designs. Especially if natural behavior is to be observed, the housing environment should allow the recording of behavioral parameters without affecting the animals. For this, setting up a separate area or pen in nature is a suitable concept (Boice and Adams, 1980). Depending on the application, these outdoor enclosures can have different dimensions and shapes (Lidicker, 1976; Dell'Omo et al., 2000; Schmid-Holmes et al., 2001; Vyssotski et al., 2002; Ilmonen et al., 2008; Landers et al., 2011). Certain monitoring methods, such as RFID technology, may require a more controlled environment than outdoors. In a study with house mice, an entire barn was used to create the animals' natural habitat while providing a controllable environment for experimental purposes (B. König et al., 2015).
If parameters are to be collected that require regular contact with the animal, it is more feasable if the housing system is of smaller dimensions (Arnesen et al., 2021). The enclosure should be accessible to allow the capture of an individual. If it is then additionally necessary to control the environmental conditions to allow comparison between different housing conditions or even to be able to analyze biomedical parameters, it must be possible to set up the housing system in laboratory conditions. For applications in animal facilities or laboratories there are concepts that use the enrichment of large cages. There are numerous individual approaches (e.g. Kempermann et al., 1998, Caston et al., 1999, Mora et al., 2007) as well as efforts to develop and establish standardized

systems (Fares et al., 2012). These cages are usually slightly larger than standard type 4 cages. The dimensions still allow the cages to be placed on shelves or regular tables. To achieve a large housing area under laboratory conditions, it is also possible to combine individual cages into one enclosure (Körholz et al., 2018).

Naturalistic housing systems are versatile. The SNE uses aspects of both of the concepts noted above (Lewejohann et al., 2009). Compared to the mentioned outdoor enclosures, the SNE could not directly provide natural habitat. In this study, the SNE was enclosed by a wire mesh cage. The large cage was located in a room of an animal facility where a certain level of hygiene had to be maintained (Figure 1 A, page 68). Nevertheless, the interior of the cage, with different areas in a vertical arrangement, created a housing area that is atypical for indoor animal housing. Connections between levels were designed in a way that mice could use different routes within the cage. Numerous hiding places and structural elements created a complex environment (Figure 1 B, page 68). Mice were able to move long distances and explore in various ways (e.g. digging, climbing). Nesting boxes (inverted standard cages) provided the equivalent of underground nesting cavities. In addition, housing a large group of animals enabled the development of social networks, which cannot be guaranteed by standard cage housing. At the same time, the equipment of the SNE consisted entirely of materials that could be cleaned and reused. Bedding and nesting materials were the same as those used in standard cages. Therefore, the SNE could be used for laboratory housing while providing nature-resembling conditions.

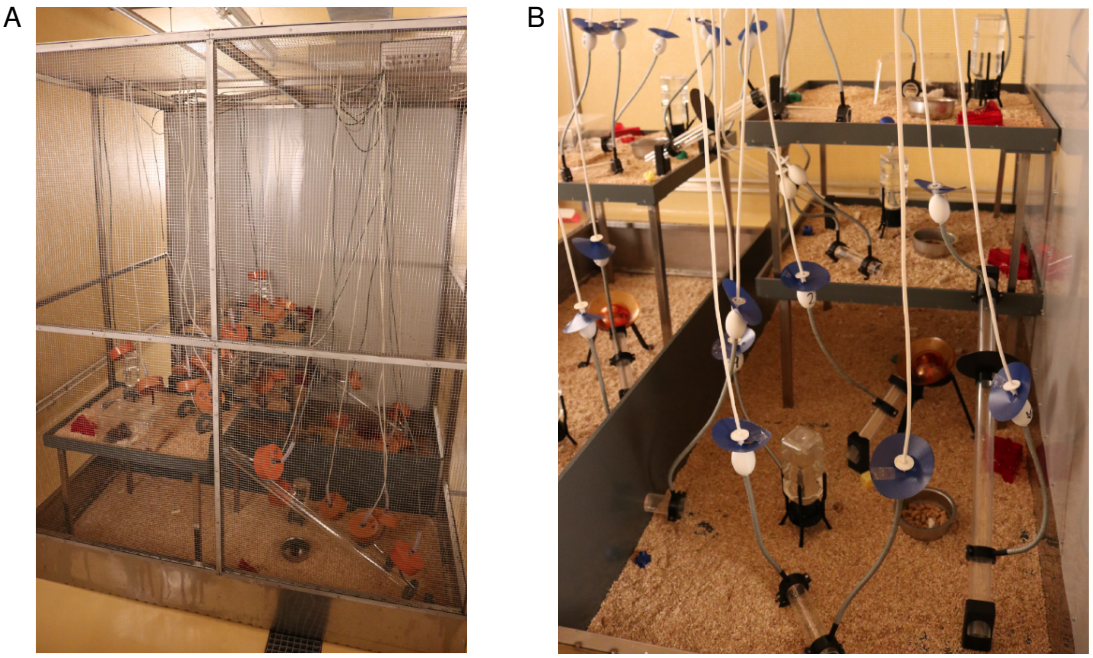

Figure 1: (A) Exterior view of the SNE set up in a room of the animal facility. The enclosure was positioned in the center of the room to be accessible from all sides. (B) Interior view of the SNE. Five housing areas were arranged at different height.

5.2 The SNE as a suitable housing condition

The enriched large enclosure, as used here, provided the opportunity for mice to be housed under laboratory conditions in a semi naturalistic environment. The cage was located in a light-controlled room, which provided adjustment and monitoring of temperature and humidity. The cage was set up in the center of the room, allowing access from all sides. Autoclaved food, as well as the equipment of the enclosure with autoclaved bedding and enrichment, allowed a very high comparability of the housing conditions over time. Access from all sides made it possible to perform a daily visual inspection of the animals. Mice were marked on the base of the tail and thus were easy to distinguish visually from each other in an uninterrupted field of view. For the daily inspection, mice did not have to be taken out of the enclosure. Additional handling of the animals to inspect them outside of the enclosure might have caused more stress and would have affected the natural behavior to a greater extent (Tuli et al., 1995).
At the beginning of housing, a transponder was injected subcutaneously in the neck area of the experimental animals. Within the enclosure, animals then were tracked with a radio frequency identification (RFID) system. A dense network of ring antennas was set up at various points in the enclosure. The signal of the transponder was received whenever an animal would pass an antenna. Movement data were recorded in a database on a computer. This system made it possible to collect detailed information about the animals as individuals and as a group without interfering with their routine behavior. Data collection was easy to use (software "JerryTS", see Lewejohann et al., 2009). Data were downloaded from the database once a week and were integrated into analyses as a .csv file.
As part of the weekly health check and weighing of the experimental animals, a basic cleaning of the enclosure was performed. This required the interruption of RFID tracking. The antenna design of the RFID system made it possible to hang the ring cases from the enclosure ceiling and thus clear the enclosure during checking the animals. Cleaning and maintenance included the replacement of all nesting material and parts of the bedding. In particular, the bedding in the nesting boxes and increasingly used areas of the enclosure (e.g. increased excretions, uneaten food) was replaced. Food and water were also renewed. Due to the use of PVC and Plexiglas for the cage interior, all surfaces could be rinsed with hot water. After cleaning, the enrichment elements were again distributed in the enclosure, and the RFID antennas were placed back into position. The functioning of the tracking system was checked and then restarted for the upcoming data collection. The animals were then returned to the enclosure. The entire process took 2 to 2.5 h (see table 1, page 70). Once a month (every fourth basic cleaning), all bedding in the enclosure was replaced. Collecting all bedding and cleaning the surfaces of the housing area prolonged the maintenance process to 2.5 to 3 h (see table 1, page 70).

With a stocking rate of four mice per cage, the SNE would be equivalent to five standard cages. In this study, each standard cage (CON and ENR housing) was processed separately. First, the

Table 1: Average time for cleaning the SNE (mean, SD, max value, min value) in hours based on 55 total recordings (N) of the cleaning time. Data is also shown for the weekly basic cleaning and the monthly complete cleaning seperately. The cleaning time is based on the RFID data collected and therefore not available for all cleanings performed during the study. Cleaning time additionally varied with the help of another animal caretaker.

	mean (h)	SD	max (h)	min (h)	N
all	2.37	0.55	3.37	1.17	55
all basic (weekly)	2.22	0.51	2.97	1.17	43
all complete (monthly)	2.91	0.35	3.37	2.20	12

weighing and checking of the animals took place here as well. Then, the entire cage was exchanged for a newly enriched cage. The equipment of the cages was prepared separately, but must still be considered in the maintenance time. Overall, the time required to maintain housing in the SNE (weekly basic cleaning) was comparable to standard cage housing. This observation is valid under the assumption of normal operation and no failures of the measurement system.

5.3 Disadvantages of the SNE

There were some details in the design of the cage and the measurement system that led to disadvantages of the SNE. The assembly of the different cage components (wire mesh walls, floor plates) inevitably led to hard-to-reach spaces where urine and feces piled up. If cleaning had only been superficial, odor signals may not be completely eliminated, and animal behavior could remain affected (Hurst, 1987; Liu et al., 2020).

The antennas of the RFID system were connected to the recording device with cables due to their power requirements. These cables represent the greatest vulnerability of the measurement system. They should not be susceptible to biting and at the same time should be flexible. Protective devices that prevent animals from gnawing and climbing on the cables should not affect the receptivity of the ring antennas and should restrict the experimenter's view into the enclosure as little as possible. After trying different methods, sections of shower hoses were used to protect the cables (Figure 2 A, page 71). These were placed 2 - 3 cm away from the ring antenna so as not to interfere with the antenna's reading range. Climbing of the cables by mice was prevented by a round, egg-like plastic body (3D printed) at the end of the shower hose (Figure 2 B, page 71).

5.4 The validity of SNE measurement results

Through direct behavioral observations at the enclosure, it became clear that the semi naturalistic environment favors behaviors that are normally reduced or not exhibited by older animals at all in CON housing. This primarily included play behaviors such as jumping on the spot for no apparent reason and chasing each other without recognizable aggressive behavior. Mice showed prominent social and individual exploration behavior. This included interacting with the objects in the enclosure,

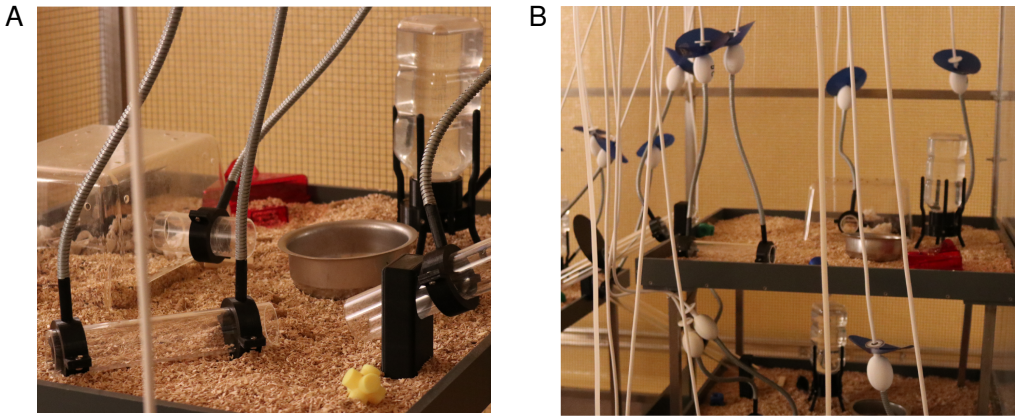

Figure 2: Sections from the equipped SNE showing the various protective measures on the cables of the antennas. (A) shows the shower hoses that attach to the antenna casing and protect the lower part of the cable from biting. Prevention from climbing the cables is provided by white spherical plastic bodies (B).

rooting in the bedding, and roaming the boundaries of the enclosure. Furthermore, no stereotypies were observed. Although a strict protocol was followed during the behavioral observations, the results should be viewed with caution. Influences based on experimental design that may have affected animal behavior are discussed below.

Ideally, the daily visual inspection was always done in the same time period. However, this involved the presence of an animal caretaker. Even if the animals could not see the person, the presence led to changing light conditions in the enclosure. Since the cage was made of wire mesh, the presence of a person also induced odor changes. These changes in the environment and forced proximity to humans may have caused stress (Morgan and Tromborg, 2007) and influenced the behavior of the animals (Roy et al., 2001; Rabadan et al., 2019).

The nocturnality of the animals resulted in a decreasing activity after the room lights were turned on. It is known that this decrease can be influenced by disruptive activities, such as cage cleaning or feed deprivation (Hut et al., 2011; Pernold et al., 2019). However, in order to visually distinguish the animals, the behavioral observations had to be performed with light. This was therefore done at the same time as the daily visual inspection. Thus, during this period, the animals finish their activity phase and at the same time are accustomed to the daily appearance of an animal caretaker. Despite this habituation, the observation period does not reflect the natural activity of the animals. Even if observed calmly and with as little movement as possible, atypical behavior of the animals during this time cannot be ruled out.

All experimental procedures required the animals being taken out of the SNE, except for measuring the activity and observing individual behavior. The removal of the animals from the SNE was not comparable to that from standard laboratory cages. Holding a plastic tube in front of the animal, as is common in tube handling, worked only to a limited extent with the large and distributed area of the SNE. If an animal was out of reach from the cage door or did not enter the plastic tube voluntarily,

the experimenter had to enter the enclosure to actively retrieve or even chase the animal. Even with careful movements, the intrusion into the habitat of the experimental animals represented a significant stressor. In addition, a new environment was presented whenever the animals were transferred from the SNE to a standard cage for measurement or transport purposes. This most likely meant an increase in the stress level of the experimental animals before each survey of a physiological parameter. For the first group of mice kept to establish the housing system and methods, this stress was minimized by training. Using food rewards (white chocolate, *Schogetten*, *Ludwig Schokolade GmbH & Co. KG*, Bergisch Gladbach, Germany), the animals were accustomed over a period of two weeks to visit the area close to the cage door when opening the enclosure and placing a plastic tube in the bedding. This facilitated the removal of the animals with the plastic tube. Although simple handling is less stressfull than restraining or isolating an animal (Bowers et al., 2008) and habituation and training to procedures reduce potential stress (Juczewski et al., 2020), the increased stress level of the animals might have influenced the results.

There are several references in literature on the effects of increased stress levels on ethological and physiological parameters. Activity, heart rate, and body temperature of small rodents are influenced by stress (Clement et al., 1989; Kramer et al., 1993). Corticosterone, in particular, as a stress hormone, can affect other measures. In rats, there is evidence for increased corticosterone levels impairing learning abilities (Foy et al., 1987). Mice also appear to respond with prolonged weight gain to elevated stress hormone levels (Karatsoreos et al., 2010).

Since the measurement method of any parameter always represents a deviation from the experienced everyday life of the test animals, it is not only the measurements themselves that need to be considered but also the stress caused by the situation. Habituation could promote normalization. Nevertheless, in SNE housing, it would be advisable if the test apparatus could be directly connected to the enclosure or integrated into the enclosure. This would allow independent exploration by the animals. Then an individual can decide for itself when to enter and leave the test apparatus. For example, a radial arm maze equipped with a RFID tag controlled automatic door could be used to study spatial learning independent of an experimenter or strict experimental times (Mei et al., 2020). In addition, there were already concepts of a weighing chamber where animals could individually enter a scale to determine body weight. Of course, this does not work with every physiological parameter. Certain parameters require a certain movement of the body or complete rest of the body for their measurement (grip strength, x-rays). More passive metrics (behavior, body weight, metabolic rate) could, however, be determined by adjusting the measurement method under less stressful conditions. This consideration is, of course, subject to a cost-benefit balance and the adaptability of certain measurement devices.

The two publications of this thesis already dealt with the comparison of data to the literature. In addition, causes for the possible deviation of measurement data were discussed. In consideration of this, housing in the SNE did not seem to influence data to such extent that an application of the SNE would be contradictory.

5.5 Limitations of the study

The experiments in this project were performed only on C57BL/6J females. The results obtained can therefore not necessarily be regarded as universally valid. There are many studies on differences in behavioral and physiological parameters between different mouse strains (Beamer et al., 1996; Võikar et al., 2001; Crabbe et al., 2003; Reed et al., 2007; C. König et al., 2020). Therefore, it would be useful to conduct the study on the suitability of housing conditions to reduce boredom on other strains and even species.

In general, it is desirable to keep the sex ratio in studies as balanced as possible to avoid bias in the results. However, there are several indications that the provision of environmental enrichment in group housing can lead to increased territorial behavior and aggression, especially in male mice (Marashi, Barnekow, Ossendorf, et al., 2003; Lewejohann et al., 2009; Mesa-Gresa et al., 2013). Since this project was designed to test new enrichment methods, it therefore seemed appropriate to minimize these possible aggressions by keeping only females.

For future applications of the SNE or comparable highly enriched housing systems, it would be desirable to enable the housing of males without the occurrence of aggressive behavior. Suitable control mechanisms would have to be found for this purpose. Janus et al., 1995 observed increased fighting behavior and bite injuries when offering running wheels. This was reduced by regularly replacing the wheels with plastic tubes. In other studies, animals that showed particularly aggressive behavior were separated from the common housing (McQuaid et al., 2012). In a version of the SNE divided into separate areas or modules, aggressive animals could be separated from each other. This way, the animals would not have to be removed from the group or enrichment elements would not have to be exchanged. Such subdivision would also allow setups in which animals must work to access certain areas (Olsson and Dahlborn, 2002). It would be worth investigating whether working for access to a resource could reduce aggressive behavior in male mice.

In the course of the literature search and work on the systematic review, it became clear that there is no definition of boredom in laboratory animals. Rather, in studies examining the effects of EE, fourteen parameters were identified that may also be associated with boredom symptoms. The EE applied in these studies followed a similar concept and could be well categorized (e.g. social EE - larger groups, spatial EE - larger cages). The parameters studied, however, were assigned to detailed research questions. It gives the impression that there is a general understanding that stereotypic behavior, negative affective state, and severe weight gain, for example, are signs of boredom. Nevertheless these parameters are not explicitly assigned to boredom. Conversely, this means that the studies in this project also cannot clearly prove how to reduce boredom, as an emotional state described by humans. Although accepted methods were used and the results can be compared with literature, there is no classification to an explicit symptomatology.

5.6 Conclusions and outlook

The present work has made an important contribution to the treatment of symptoms in mice that could be associated with boredom. Environmental enrichment - specifically the semi naturalistic environment - has been successfully used to prevent stereotypies and to promote natural behavior in female C57BL/6J mice. It has been shown that the SNE can be used beneficially for long-term housing of large groups of animals. Behaviors were observed that mice do not normally exhibit at an advanced age. Individualization in the behavior of genetically identical animals could also be observed. The examination of physiological parameters showed that the characteristics of the bone skeleton seem to be slightly improved in semi naturalistic environments. The age-related degradation of bone substance was reduced. Above all, and in agreement with previous observations (Marashi, Barnekow, and Sachser, 2004; Rozman et al., 2018) it was shown that the anticipated increase in the variance of the measured data did not occur.
Considering the drawbacks identified for the SNE, some changes regarding the design are conceivable. First, a wireless concept for the ring antennas of the RFID system would be useful. The energy supply could be ensured via a battery and the signal could be transmitted wirelessly (WLAN module). However, such a concept takes up a certain amount of space, which would not always be compatible with the design of the enclosure.
The second change to the SNE concept could improve human-animal interaction and thus minimize potential stress to the animals. The individual levels that the enclosure currently offers could be constructed as individual cage modules, which could then be connected with plastic tubes to form a large overall area (Figure 3, page 74).

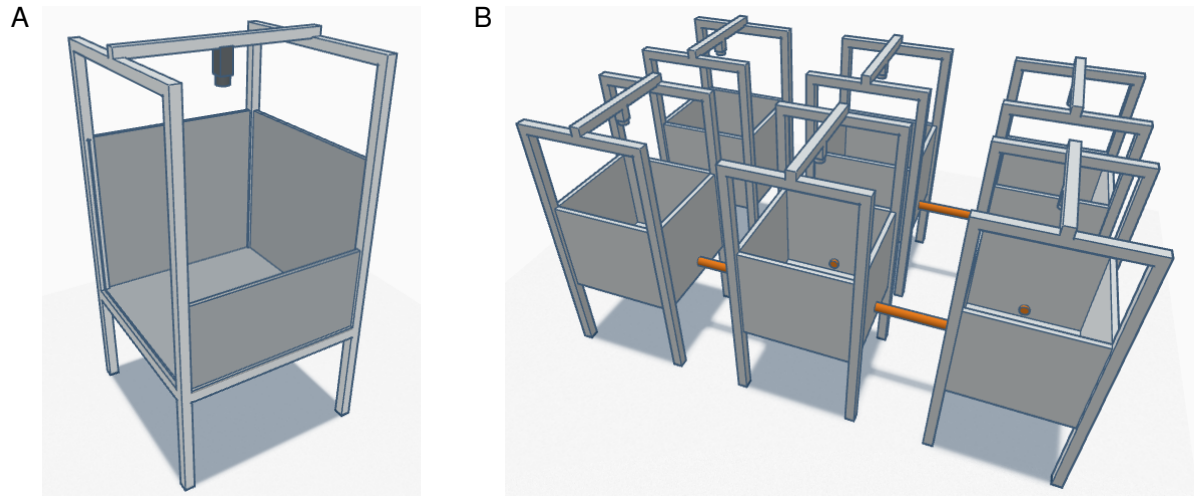

Figure 3: 3D model of a possible housing module for mice (A) and a concept for a large housing system of several combined modules connected with plastic tubes (B).

First, a modular system simplifies the separation of the enclosure. If areas can be separated, escapes over long distances can be prevented when animals are removed. This reduces time and

stress. This also applies when repairs or the replacement of enrichment elements must be carried out. It is then possible to simply separate a module without removing all animals from the home cage environment. For modules that are individually rather small compared to the SNE, the enclosure also does not have to be entered, but can be operated from the outside. In addition, the modular design increases comparability between areas of the enclosure. Lastly, if the modules are built with a distance to the ground, most of the antennas can be placed outside the enclosure, preventing access by the animals.

With a few optimizations, the SNE delivers a scientific added value and an important contribution to animal welfare. I believe that its high suitability for laboratory mice housing should definitely find an application in future projects.

6 Zusammenfassung

Messung und Reduzierung von Langeweile-Symptomen bei Labormäusen - die halbnatürliche Haltungsumgebung

Mäuse, die zu Versuchszwecken in Laborumgebungen gehalten werden, erfahren oft eine standardisierte und geringe Komplexität ihrer Haltungsumgebung. In Tierversuchen verbringen die Versuchstiere jedoch die meiste Zeit in diesen Haltungsumgebungen. Unter Umständen kommt es zu einem monotonen Haltungsalltag und zur Ausprägung von Langeweile. Dies kann sich in Verhaltensauffälligkeiten oder sogar physiologischen Defiziten äußern.
Um diesen Effekt zu verhindern, kann die Käfighaltung angereichert werden. Verschiedenste Anreicherungsmethoden umfassen die Bereitstellung zusätzlicher Haltungsfläche, die Haltung in großen Tiergruppen und die Ausgestaltung der Haltungsumgebung durch Gegenstände. All diese Mechanismen sorgen für die Annäherung der Haltungsumgebung an naturnahe Bedingungen.
In diesem Forschungsprojekt wurden drei Haltungsformen in verschiedener Komplexität hinsichtlich ihres Einflusses auf die Langeweile-Symptomatik bei Labormäusen untersucht. Augenmerk lag dabei auf einer halbnatürlichen Umgebung (semi naturalistic environment, SNE). Dabei handelt es sich um ein großes Gehege, in dem die Haltungsfläche auf fünf Ebenen in unterschiedlicher Höhe aufgeteilt ist. Die Ebenen sind miteinander über Plastiktunnel verbunden und bieten jeweils den Zugang zu Wasser, Futter und einem Unterschlupf. Im SNE wurden im Verlauf des Projektes zwei Gruppen von jeweils 20 weiblichen C57BL6/J Mäusen gehalten. Die Mäuse bekamen zum Beginn der Haltung einen Transponder implantiert, der mittels radio frequency identification Technologie (RFID) über Antennen im SNE ausgelesen wurde. So konnten Aktivität und Explorationsverhalten aller Individuen dauerhaft direkt im Haltungssystem gemessen werden. Zusätzlich wurden ethologische und physiologische Parameter erhoben. Der zeitliche Verlauf einzelner Parameter und die Korrelationen zwischen den Parametern ließen eine möglichst umfangreiche Auswertung des Effekts der Haltungsformen zu.
Die halbnatürliche Umgebung hatte verschiedene Effekte auf die weiblichen Mäuse. Die kumulierten Bewegungsdaten zeigten die Ausprägung individueller Unterschiede im Explorationsverhalten der Tiere. Stereotype Verhaltensauffälligkeiten, die in konventioneller Käfighaltung auftraten, konnten bei Tieren im SNE nicht beobachtet werden. Stattdessen wurde im SNE auch bei 1,5 Jahre alten Tieren noch Spielverhalten beobachtet, was in diesem fortgeschrittenen Alter bei konventioneller Haltung untypisch ist. Im Vergleich zwischen den Haltungsformen erreichten die Tiere aus dem SNE das höchste Körpergewicht und die größte Körperlänge. Die Knocheneigenschaften der Mäuse scheinen sich durch das SNE leicht zu verbessern und die altersbedingte erhöhte Knochenresorption wurde verringert. Weiterhin erhöhten die angereicherten Haltungsbedingungen nicht die Varianz der Messdaten. Insgesamt konnte die Eignung der halbnatürlichen Umgebung zur Verringerung der Langeweile-Symptome gezeigt werden. Eine Anwendung dieses alternativen Haltungssystems in vergleichenden Laborstudien ist ebenfalls möglich.

7 Summary

Recording and Reducing Boredom Symptoms in Laboratory Mice - the Semi Naturalistic Environment

Mice kept in laboratories for experimental purposes often experience standardized and low complexity in their housing environments. However, in animal studies, laboratory animals spend most of their time in these housing environments. In some circumstances, this results in monotonous conditions and the expression of boredom. This can manifest itself in behavioral abnormalities or even physiological deficits.

To prevent this effect, caging can be enriched. A wide variety of enrichment methods include providing additional enclosure space, keeping animals in large groups, and adding objects to the housing environment. All of these mechanisms provide for the approximation of the housing environment to near-natural conditions.

In this research project, three housing conditions of varying complexity were investigated with respect to their influence on boredom symptoms in laboratory mice. The focus was on a semi naturalistic environment (SNE). This is a large enclosure in which the housing area is divided into five levels at different heights. The levels are connected to each other via plastic tunnels and each provides access to water, food and shelter. Two groups of 20 female C57BL6/J mice each were housed in the SNE during the project. The mice were implanted with a transponder at the beginning of the housing period. The transponder information was read via antennas in the SNE using radio frequency identification technology (RFID). Thus, activity and exploration behavior of all individuals could be measured permanently directly in the housing system. Additionally, ethological and physiological parameters were collected. The time course of individual parameters and correlations between parameters allowed for the most comprehensive evaluation of the effect of the enclosure.

The semi naturalistic environment had various effects on the female mice. The cumulative movement data showed the emergence of individual differences in the exploration behavior of the animals. Stereotypic behavior that occurred in conventional cage housing was not observed in animals in the SNE. Instead, play behavior was still observed in SNE even in 1.5 year old animals, which is atypical at this advanced age when housed conventionally.

In comparison between housing types, animals from SNE achieved the highest body weight and body length. Bone characteristics of the mice appeared to be slightly improved by the SNE and age-related increased bone resorption was reduced. Furthermore, neither of the enriched housing conditions did increase the variance of the measured data.

Overall, the suitability of the semi naturalistic environment to reduce boredom symptoms was demonstrated. Application of this alternative housing system in comparative laboratory studies is also possible.

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9 Formalities

9.1 Authors contribution

Bored at home?—A systematic review on the effect of environmental enrichment on the welfare of laboratory rats and mice

Paul Mieske: development of study concept and design, data acquisition and analysis, writing of the original draft, figure preparation, review and editing, correspondence with reviewers
co-authors: development of study concept and design, data acquisition and analysis, writing of the original draft, cooperation with figure preparation, review and editing, correspondence with reviewers

Roaming in a Land of Milk and Honey: Life Trajectories and Metabolic Rate of Female Inbred Mice Living in a Semi Naturalistic Environment

Paul Mieske: experimental planning and conducting, literature research, animal care taking, maintenance and cleaning of housing system, data analysis and visualization, article conceptualization, writing, figure preparation, review and editing, correspondence with reviewers
co-authors: experimental planning and conducting, literature research, animal care taking, cooperation with article conceptualization, review and editing, cooperation with correspondence with reviewers, supervision

Effects of more natural housing conditions on the muscular and skeletal characteristics of female C57BL/6J mice

Paul Mieske: experimental planning and conducting, literature research, animal care taking, maintenance and cleaning of housing system, data analysis and visualization, article conceptualization, writing, figure preparation, review and editing, correspondence with reviewers
co-authors: experimental planning and conducting, literature research, animal care taking, cooperation with article conceptualization, coopertaion with writing, review and editing, cooperation with correspondence with reviewers, supervision

9.2 Publications index

Original articles

Mieske, P.; Diederich, K.; Lewejohann, L. Roaming in a Land of Milk and Honey: Life Trajectories and Metabolic Rate of Female Inbred Mice Living in a Semi Naturalistic Environment. Animals 2021, 11, 3002. https://doi.org/10.3390/ani11103002

Hobbiesiefken U., Mieske P., Lewejohann L., Diederich K. (2021) Evaluation of different types of enrichment - their usage and effect on homecage behavior in female mice. PLoSONE 16(12):e0261876.

https://doi.org/10.1371/journal.pone.0261876

Mieske, P., Scheinpflug, J., Yorgan, T.A. et al. Effects of more natural housing conditions on the muscular and skeletal characteristics of female C57BL/6J mice. Lab Anim Res 39, 9 (2023). https://doi.org/10.1186/s42826-023-00160-9

Review articles

Mieske P., Hobbiesiefken U., Fischer-Tenhagen C., Heinl C., Hohlbaum K., Kahnau P., Meier J., Wilzopolski J., Butzke D., Rudeck J., Lewejohann L. and Diederich K. (2022) Bored at home?—A systematic review on the effect of environmental enrichment on the welfare of laboratory rats and mice. Front. Vet. Sci. 9:899219. doi: 10.3389/fvets.2022.899219

Lang, B., Kahnau, P., Hohlbaum, K., Mieske, P., Andresen, N.P., Boon, M.N., Thöne-Reineke, C., Lewejohann, L., Diederich, K. (2023). Challenges and advanced concepts for the assessment of learning and memory function in mice. Front Behav Neurosci. 2023 Sep 21;17:1230082. doi: 10.3389/fnbeh.2023.1230082

Kahnau, P., Mieske, P., Wilzopolski, J. et al. A systematic review of the development and application of home cage monitoring in laboratory mice and rats. BMC Biol 21, 256 (2023). https://doi.org/10.1186/s12915-023-01751-7

Surveys

Kahnau, P., Jaap, A., Hobbiesiefken, U., Mieske, P., Diederich, K., Thöne-Reineke, C., Lewejohann L. and Hohlbaum, K. (2022). A preliminary survey on the occurrence of barbering in laboratory mice in Germany. Animal Welfare, 31(4), 433-436. doi:10.7120/09627286.31.4.009

Talks

Paul Mieske (Jan 2019) Strategies for recording and reducing boredom symptoms in experimental animals. BB3R-PhD-Symposium, Berlin, Germany

Paul Mieske (Nov 2019) Strategies for recording and reducing boredom symptoms in laboratory animals – semi naturalistic environment as an alternative housing condition. PreDocSymposium German Federal Institute for Risk Assessment, Berlin, Germany

Paul Mieske (Sep 2020) Die semi-naturalistische Umgebung als alternatives Haltungssystem für Versuchstiere. 58. Tagung der GV-SOLAS und 20. Fortbildungsveranstaltung der IGTP, online

Paul Mieske (Jun 2021) Mice roaming in a semi-naturalistic environment. EUSAAT Virtual Seminar Series, online

Paul Mieske (Oct 2022) The semi naturalistic environment as an alternative to laboratory cage housing. BB3R Autumn School 2022, Alternatives to Animal Testing, Berlin, Germany

Posters

Paul Mieske (Feb 2020) A semi-naturalistic environment as an alternative housing condition for laboratory animals? 15th Annual Meeting of the Ethological Society, Tübingen, Germany

Paul Mieske (Feb 2023) Effects of long-term housing in a semi naturalistic environment on female C57Bl/6J mice. Joint meeting of the BfR and the Ethological Society, Berlin, Germany

Paul Mieske (Sep 2023) Effects of a semi naturalistic environment on the musculoskeletal properties of female C57BL6/J mice. 60. Wissenschaftliche Tagung der Gesellschaft für Versuchstierkunde GV-SOLAS und 21. Fortbildungsveranstaltung der IGTP, Mainz, Germany.

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9.5 Conflict of interest

The author declares no competing interests.

9.6 Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit erstmalig einreiche, selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Berlin, 19.02.2024 Paul Mieske

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