



Faecal corticosterone concentrations indicate that separately housed male mice are not more stressed than group housed males

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Abstract

Mice account for over 80% of all animals used in experimentation. This study investigated how different housing conditions affected stress levels by measuring both corticosterone levels, using non-invasive faecal collection, and behaviour. Sixty outbred MF1 male mice were used which were separated into five different housing conditions at the beginning of the study, (A) individually housed, floor area 490 cm² per individual, (B) groups of three mice, floor area 163 cm² per individual, (C) groups of three mice, floor area 320 cm² per individual, (D) groups of six mice, floor area 160 cm² per individual, (E) groups of six mice, floor area 230 cm² with extra height per individual to allow visual contact. Mice in all housing conditions were provided with a basic enrichment of paper bedding and a plastic house. The results from this study showed that singly housed mice reduced their corticosterone levels over time after separation reaching a minimum from 14 days onwards. Groups of 6 mice housed together showed no difference over time. Also there was no significant difference in corticosterone levels between the different housing densities, with no differences for aggression or stereotypical behaviour suggesting that there is no ideal group density for this strain and sex of mouse. Providing additional enrichment to the cages caused a significant decrease in corticosterone levels for group housed mice, but individually housed mice remained unaffected by increasing their enrichment level. They spent significantly more time sleeping in the enhanced cage but without any reduction in stereotypical behaviour. For group housed mice, additional enrichment should be mandatory to reduce stress levels and therefore increase their welfare standards, while singly housed mice required only basic levels of enrichment and should be separated from their group for a minimum of 2 weeks before measurements are taken.

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1. Introduction

Over 2.73 million animals are used in biomedical research each year in the UK and Home Office figures state that in 2002, 84% of these were laboratory rodents. Most rodents are nocturnal, which means that they have a very different sensory perception to humans. They use their olfactory sense to a much greater degree such as to identify each other and recognise social status through urinary deposits [1]; however this is compromised in inbred strains which may lead to greater aggression [2]. If they are not exposed to stimulation during their early life, they can develop behavioural problems such as

increased aggression [3] or fear [4]. Some of the earliest studies on rodents were carried out with the animal living in a barren environment because the regulations stated that housing need only provide features necessary to maintain good physical and reproductive health and meet husbandry protocols, and barren environments were thought to minimise uncontrollable factors [5]. Recent studies however, have suggested that barren environments are detrimental to normal brain development and behaviour (e.g. [7,8]), and are therefore stressful for the rodent [6]. If stress is increased, it can lead to a depression of the immune system, which could also be detrimental for research studies [9].

Laboratory cages should provide satisfaction for both the physiological and behavioural needs of the animal [10]. Enhancing housing conditions to improve stress levels is a method that has been used since the early 1980s and is widely

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promoted across Europe [11,12]. The degree of the enrichment level is fundamental to obtaining a significant change in the behaviour and physiology of the animal [13]. Sherwin and Nicol [14] and Sherwin [15], found that mice also regarded additional space as an extremely important enrichment that allowed them to express their highly motivated exploratory behaviour.

Assessing the stress levels of animals in research can be difficult. Early research into stress of laboratory animals was carried out by assessing the relative adrenal gland weight [16]. This was an accurate technique, however it was terminal and therefore it was not possible to continually monitor the animals [17]. Recent studies have shown that stress hormones are an accurate indicator of an animal's condition [18], which can be used in conjunction with traditional methods like behaviour analysis. Corticosterone is a metabolite secreted by the adrenal cortex when an animal is faced with a stressful situation [19,20]. Blood sampling to analyse corticosterone levels is the most sensitive and accurate method of continual stress analysis of an individual [21]. Blood sampling, however, is itself an invasive sampling technique [20], which obviously raises welfare issues, but in addition, capture and physical restraint during this procedure could increase corticosterone levels in the blood sample, therefore giving an elevated result [18–22]. Corticosterone levels can also be measured in saliva, urine and faeces. Saliva and urine are seen as less practical for use in rodents, as sample volume is small and difficult to obtain [23]. Analysis of the faeces may be the most practical alternative to blood sampling as it allows repeated sampling from the same individual. However, this non-invasive technique of faecal sampling has only recently been established in certain species, including domestic, farm, zoo and wild animals when investigating health and welfare issues [23].

Touma et al. [20] found male mice produced significantly more corticosterone than females and corticosterone levels were affected by the time of day the sample was collected depending on when the animal was more active. Mostl and Palme [18] investigated excretion of metabolites, explaining that understanding the species specific gut transit time of the animal is essential when calculating an accurate lag time, which they recommended for mice is 6 to 12 h [18,20]. However, Harper and Austad [21] and Good et al. [22] found the lag time in certain strains of mice to be as little as 4 h.

There has been much research on improving laboratory rodent welfare to reduce stress levels, using both behavioural observations and physiological indicators. There have been no stress studies investigating ideal housing conditions for laboratory mice using the new faecal corticosterone analysis technique. Faecal corticosterone analysis may be an efficient, non-invasive method of evaluating the stress caused to an animal by its environment [20] and therefore, may be the ideal method of analysing welfare in laboratory animals. In this study, we asked 3 questions:

1. Do singly housed mice suffer from elevated corticosterone levels, and do these levels reduce when they become more accustomed to their housing condition?

2. How do different group densities affect corticosterone concentrations and behaviour in mice?
3. Do additional enrichments reduce corticosterone levels in individually and group housed mice?

2. Methods

2.1. Animals and husbandry

Sixty male outbred MF1 mice, aged 9 weeks, entered the protocol. These mice had been housed in large groups of 12 from weaning. The mice were divided into ten groups of six for a weeklong 'settlement' period. Throughout the study the animal room had a controlled photoperiod (12 h light/dark cycle with lights on at 6:00 am and a light intensity of approximately 200 Lux), humidity ($46\% \pm 1\%$), and temperature ($22 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$). Also, water and food pellets (pelleted rat and mouse, breeder and grower diet, Special Diets Services, BP Nutrition, UK) were provided ad libitum. Cage cleaning can increase aggression in male groups due to the disruption of odours that normally define their social environment [11]. Hence, no cleaning was carried out the day before a faecal sample was collected.

After acclimatisation the mice were separated into the different housing conditions (Table 1) for the duration of the 6-week study period and each mouse was given a unique identification mark. When faecal sampling occurred each mouse from all 5 housing conditions was treated in the same manner to maintain the same protocol for each individual throughout the study. Three different questions were addressed.

2.2. Do singly housed mice suffer from elevated corticosterone levels, and do these levels reduce when they become more accustomed to their housing condition?

Two different group densities were used to address this question, singly housed mice (A), and groups of six (D) as a control. In total five faecal samples were collected over a 28-day period (Days 1, 3, 7, 14 and 28) (Fig. 1) from the same 6 mice from each density (all 6 individual mice and 2 from each cage of 6). For this and subsequent faecal sampling all mice were separated into individual cages with standard hard floors

Table 1
Housing conditions for each group

Housing condition	Number of cages	Number of animals per cage	Total floor area cm ² (cage type, NKP Kent)	Floor area per animal cm ²
A	6	1	490 (M3)	490
B	3	3	490 (M3)	163
C	3	3	960 (MB1)	320
D	3	6	960 (MB1)	160
E	3	6	960 (RB3)+396 (shelf)	160+66

Groups, A–D allowed mice olfactory and audible contact but no visual contact with other cages. Group E had additional height and a shelf, which allowed them to also have visual contact with other cages. All cages were obtained from North Kent Plastics (Kent) and had standard hard floors.

(M3 Macrolon type III NKP, Kent), and enrichments of paper bedding and one polycarbonate house, for a period of 2 h to enable the appropriate volume of faeces to be collected (0.1–0.2 g). The samples were always collected at the same time of day (9:00 am), which meant that the corticosterone level measured accounted for stress levels experienced over the previous 4–12 h depending on the lag time for this strain and was therefore during the active period of the night when there was no human disturbance. The samples were weighed (to 4 decimal places: Sartorius, BL403) and 4 ml 95% ethanol was added to each vial. They were then placed in –20 °C freezer in preparation for hormone extraction.

2.3. How do different group densities affect corticosterone concentrations and behaviour in mice?

Faecal samples collected from 6 individuals from all 5 housing conditions on Day 28 (two randomly selected samples from each group housed cage). Behavioural analysis was also performed for each group. Mice were observed during hours of darkness (for minimal human disturbance) using a video camera (Sony: Hi 8) and red lamp. The sample mice used for the corticosterone study were the same mice observed for the behavioural study. The videos were viewed, 10 min after lights out, and the behaviour of the mice noted on the minute for 100 min. Behaviour was separated into eight different categories – sleeping, grooming, eating, drinking, normal exploratory behaviour, aggressive behaviour towards cage mates, positive behaviour (interaction with cage mates that did not result in aggression) and stereotypical behaviour (repetitive action). This analysis produced a percentage of each type of behaviour for the six individuals in each housing density.

2.4. Do additional enrichments reduce corticosterone levels in individually and group housed mice?

A basic level of enrichment was given to the mice for Days 1–28 – with one polycarbonate house (per three mice), sawdust and paper bedding. On Days 28–42 they had high level enrichment of sawdust, polycarbonate house (per three mice), bedding squares which they had to shred and make into a nest themselves, and one large cardboard tube (per three

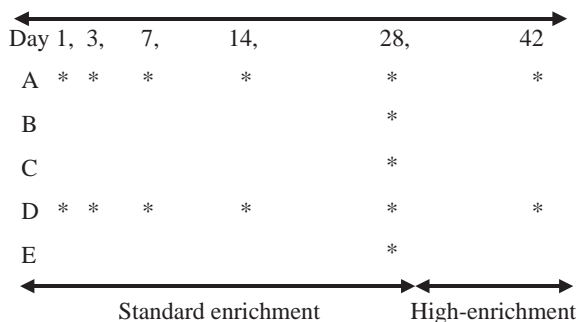


Fig. 1. Protocol. Housing conditions A–E (see Table 1 for explanation). *Faeces were collected for analysis.

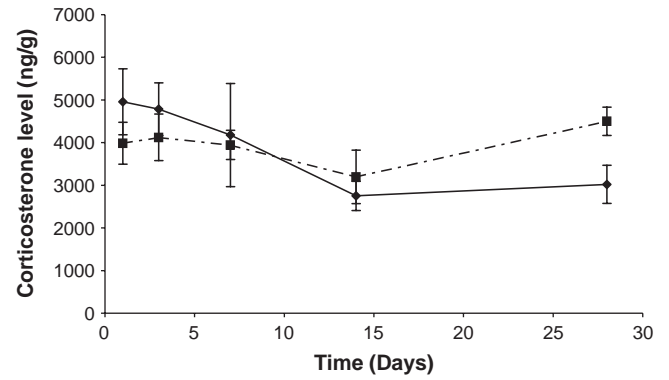


Fig. 2. The average faecal corticosterone level in singly and group housed MF1 mice measured over a 28-day period. The solid line shows average corticosterone levels for individually housed mice, while the broken line shows the average corticosterone levels for groups of 6 mice. Singly housed mice reduced their stress levels until day 14 as they became more used to their housing conditions while stress levels in group housed mice did not alter.

mice). Faecal samples were collected from group A (n=6) and group D (n=6) prior to and during the increased enrichment phase, on Day 28, to show stress levels with basic enrichment, and Day 42, to show stress levels with high enrichment. Behaviour was also recorded and analysed as previously described.

2.5. Hormone extraction and corticosterone radioimmunoassay

The extraction procedure was modified from that of Creel et al [24]. Faecal samples and storage ethanol were transferred into a conical flask. The volume was made up to 10 ml 95% ethanol. The conical flask was placed onto a hotplate and left at a rolling boil for approximately 20 min, or until half the ethanol has evaporated. The remaining ethanol was then removed and placed in a test tube. Another 10 ml 95% ethanol was added to the conical flask. Again, it was boiled for 20 min and remaining ethanol pipetted into test tube. The test tube was placed in 70 °C water bath to evaporate the ethanol off and nitrogen was passed through the ethanol to quicken this process. The hormone remaining in the test tube was

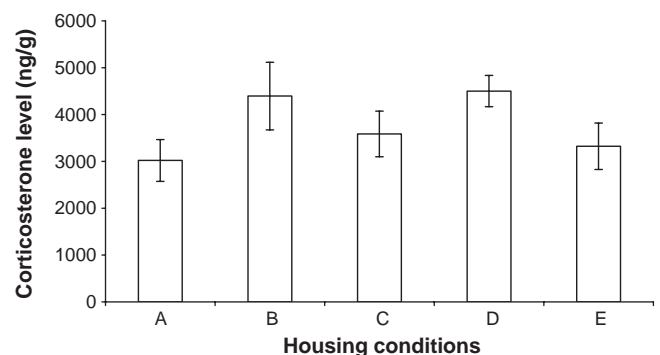


Fig. 3. The effect of different group densities on average faecal corticosterone levels in male MF1 mice after 28 days of acclimation. No statistical significances were found between the groups.

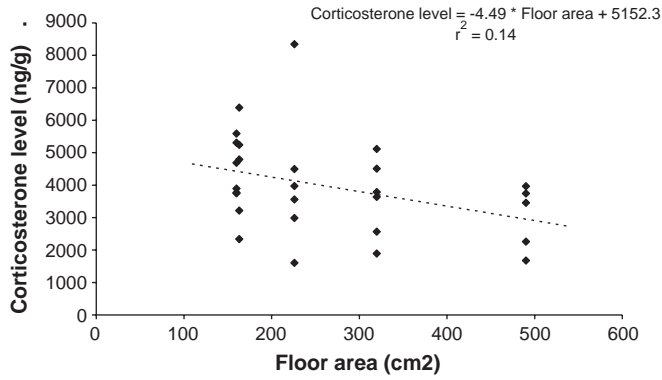


Fig. 4. The effect of individual floor area on average faecal corticosterone levels after 28 days of acclimation for each group. The linear regression did not reach significance.

reconstituted with 3 ml ethyl acetate/hexane (3:2, v/v) and vortexed for 30 s. The ethyl acetate/hexane was then evaporated off in the water bath, again with nitrogen passing through it. The hormone was finally reconstituted with 4 ml 95% ethanol and stored at -20°C freezer until assay could be carried out.

Samples were assayed using a double-antibody corticosterone ^{125}I radioimmunoassay kit for rats and mice (ICN Biomedicals, Inc.) in duplicate, following manufacturer instructions at half volumes. Isotope levels were then counted on a gamma counter (Packard Instrument Company, Bucks, UK), before correction per gram of faeces.

2.6. Statistical analysis

The data was analysed using a repeated two-way ANOVA (Sigma Stat Version 1 Jandel Corporation, Ergath, Germany), to determine differences between group and individually housed mice over time and group densities. One-way ANOVA was used to determine differences between different group densities and Paired *t*-tests (Minitab 14.12) were used to

determine differences between basic and high level enrichment. Results are shown with standard errors.

3. Results

3.1. Do mice have reduced corticosterone concentrations when they become more accustomed to their housing condition?

Faecal samples were collected, from both individually (A) and group (D) housed mice, on five different days within a 28-day period (Fig. 2). Initially, corticosterone levels averaged 4958.3 ± 678.5 ng/g faeces in the singly housed mice on Day 1 and 3985.8 ± 491.7 ng/g faeces in the group housed mice. There was, however, no significant difference between the group densities at any time (repeated measures two-way ANOVA: $F_{1,10}=0.001$, $P=0.99$). There was a significant density/time interaction (repeated measures two-way ANOVA: $F_{1,10}=4.91$, $P=0.004$). The statistics indicated that stress in single housed mice only was significantly higher on Days 1 and 3 compared to Days 14 and 28 while time had no significant affect on stress endured by group housed mice.

3.2. How do different group densities affect corticosterone concentrations and behaviour in mice?

On Day 28 of the study, faecal samples were collected from all five housing conditions (Fig. 3). This experiment shows that the number of male mice in the group and housing density had no significant effect on corticosterone levels (ANOVA: $F_{4,27}=0.93$, $P=0.47$). This also shows that the group that had visual contact, due to the presence of a shelf, did not have reduced stress levels. Corticosterone levels measured were also used to compare stress with allocated individual floor area (Fig. 4). The data approached but did not reach significant differences between different floor areas, however, a strong trend was observed where the greater the floor area available to each individual the less stressed they are with their environ-

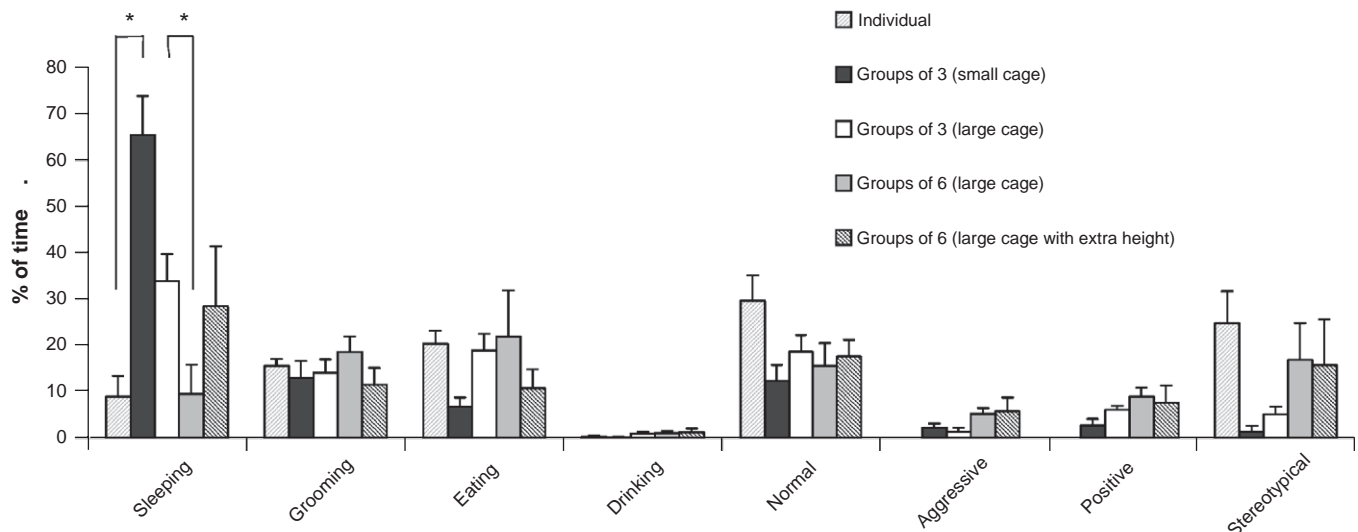


Fig. 5. The effect of different group densities on average time spent on different behaviours in male MF1 mice after 28 days of acclimation. Statistical significances were only found between the groups for time spent sleeping.

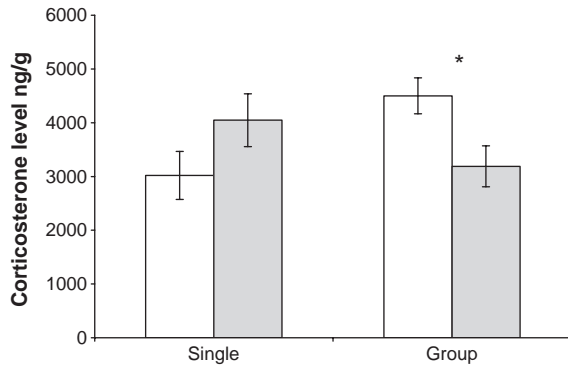


Fig. 6. Comparison of average corticosterone levels in mice at basic (day 28) and high (day 42) level enrichment (basic=non-shaded columns, high=shaded columns). There was no significant difference in corticosterone level in the singly housed mice while the group housed mice significantly reduced their stress levels when provided with additional enrichments.

ment (Linear Regression: $F_{1,27}=4.10$, $P=0.053$; $r^2=0.14$). The behavioural analysis was conducted only for the six mice in each group that provided faecal samples for the corticosterone analysis (Fig. 5). The only difference between the groups was for time spent sleeping. Individually housed and groups of six in MBI cages sleep for significantly less time than groups of three in small cages (ANOVA: $F_{4,25}=7.97$, $P<0.001$).

3.3. Do additional enrichments reduce corticosterone concentrations of individual and group housed mice?

Corticosterone levels measured on Day 28 represent the stress experienced by the mice at basic level enrichment, while measurements at Day 42 (from the same mice) were used to represent the stress experienced by the mice at high-level enrichment (Fig. 6). Faecal samples from A and D were analysed. Additional enrichment had no significant effect on the stress levels of individually housed MF1 male mice (Paired t -test: $T=0.91$, $P=0.45$). However, it did significantly reduce the stress levels of group housed MF1 males (Paired t -test: $T=5.84$, $P=0.004$). Their behaviour was once again separated into categories (i.e. sleeping, grooming, eating, drinking, normal, aggressive, positive and stereotypical) (Fig. 7), and the mice that had their behaviour analysed were the same mice that had their corticosterone levels measured. For individually housed mice, additional enrichment significantly increased sleeping (Paired t -test: $T=8.01$, $P<0.001$) and drinking (Paired t -test: $T=2.72$, $P<0.042$); however, it had no significant effect on any other behaviour recorded. Additional enrichment in groups of six, only caused a significant decrease in positive interaction with cage mates (Paired t -test: $T=3.64$, $P=0.015$).

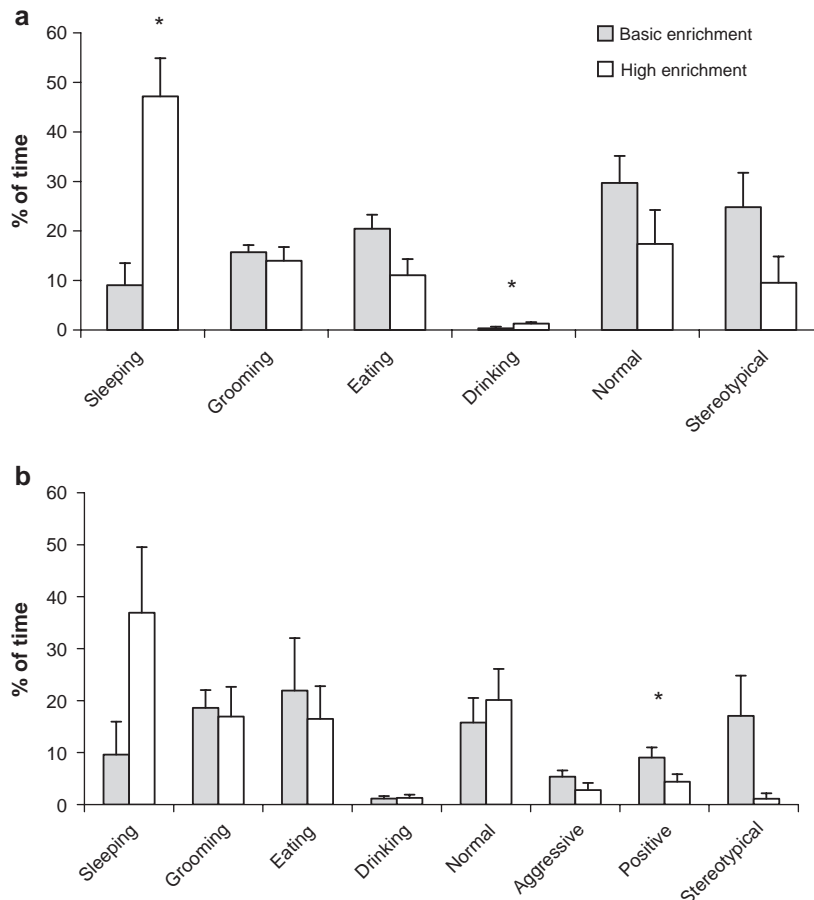


Fig. 7. Behavioural differences between basic and high levels of cage enrichments for a) singly housed mice and b) groups of six mice. *Significant differences (Paired t -test: $P<0.05$).

4. Discussion

The suffering and distress of a laboratory animal are usually associated with experimental procedures carried out during a study. However, this particular stress only lasts for a short period of time. When an animal is kept in an unsuitable environment during a study that is not specific to their needs, the stress caused by the poor housing conditions is prolonged.

We studied corticosterone levels in male mice housed under different conditions at 9 am, which was thought to account for stress experienced by the mice over the previous 4–12 h (active phase). Unfortunately the lag time from a stressful event until it is represented in the faeces can vary depending on the time that the event occurred. This depends on levels of activity, which influence the excretion rate [20]. Touma et al. [20] found that if the event was to occur during an inactive phase it may take as long as 10 h to be detected in the faeces while during an active phase, the peak in corticosterones may appear after only 4 h. There is also a naturally occurring large diurnal variation in corticosterone levels in the faeces [19,25] with levels being lower in the evening following the inactive phase, and almost double in the morning following the active phase [19]. As mice are nocturnal, our measurements reflected the active period when the majority of interactions and aggressive encounters with conspecifics may occur, but when the animals had no human disturbance.

Many research studies, such as those that monitor food intake, require mice to be individually housed. Results obtained from this study, through faecal corticosterone analysis, suggest that MF1 male mice used in research that requires individual housing should be allowed an acclimatisation period of at least 14 days to ensure stress caused by housing conditions does not affect results. In an earlier study, male TO albino mice were measured over a longer period of time, from 7 to 98 days of individual or group housing [26]. Mice were culled to collect blood samples and like our study there was no difference found in the plasma corticosterone levels of individually and group housed (groups of 6), although there was a reduction with age [27]. These mice also did not induce any major immuno-endocrine effects in groups compared to individual housing showing that they do not suffer from increased health implications [28]. It is possible, however, that there could be longer-lasting stress affects which can be measured after elevated corticosterone levels have receded, such as changes in adrenocorticotrophic hormone (ACTH) or in the circadian rhythm of ACTH or corticosterone [29,30], but as we also saw very little change in behaviour at 28 days between the different housing densities it is unlikely that the individually housed mice had long lasting stress affects.

The ideal group size for male mice has been suggested from behavioural assays to be three individuals [7,9]. Groups of three were found to have the most stable dominance hierarchy and show the least aggression between cage mates [9]. Our study, however, showed no differences

in the levels of aggression or corticosterone levels of individually and group housed mice. In addition, the provision of visual contact in group housed mice with other cages (housing condition E) did not cause any difference in stress levels. Where aggression occurs, both dominant and subordinate individuals suffer increased stress as subordinates have more frequent attacks and dominants have elevated corticosterone secretion as they are constantly trying to maintain their elevated position [28]. This is backed up by corticosterone and immune function measurements of dominants and subordinates in groups of 3 individuals housed together, as there were no differences between the social status position [27].

The faecal corticosterone levels observed in this study tentatively suggest that there might be an effect of floor area. Although not reaching significance, there was an indication that there were reduced stress levels with increased floor area. This is supported by studies that found mice regard additional space as an extremely important enrichment [13,14]. There is, however, some conflict in the literature because BALB/cAnNCrIbR mice showed a reduction in aggressive bouts with decreased floor area per individual [11]. The mice in this latter study did not receive any enrichment and increased aggression may have occurred because the dominant male found it difficult to maintain his social status in a large barren environment. However, another possibility is that there are significant strain effects of housing conditions on stress responses especially between inbred and outbred strains. As the BALB/cAnNCrIbR strain is inbred, they may have more difficulty maintaining the dominance hierarchy in a larger area because their genetic similarity prevents the use of normal olfactory identification leading to increased aggression during encounters with cage mates.

Faecal corticosterone levels for individually housed MF1 male mice show that increasing the level of enrichment has little or no effect on their stress levels as it increased the amount of time spent sleeping but showed no reduction in stereotypical behaviour. A reduction in the level of activity may be expected to reduce corticosterone levels [31], but although the mice spent less time being active, they may have been conducting more vigorous activities, utilising their greatly enriched environment. The corticosterone level obtained from groups of six mice shows in contrast, that they experience significantly less stress at a higher-level of enrichment compared to basic level enrichment without changing the amount of sleeping they conducted or the amount of stereotypical or aggressive behaviour. These findings contrast suggestions that increasing enrichment levels may cause male mice to become more stressed due to increased aggression between cage mates [1,29]. Marashi et al. [31] suggested that this might only be a factor if there is limited space that prevents the mice from avoiding conflicts. In contrast to our study, Marashi et al. [31] found that there was no difference between stress levels in groups of four housed in basic or super enriched cages. It is likely that this is because the strain of mouse used in this study was inbred,

and like the previous study where inbred mice were used [11], the lack of variation in olfactory cues caused an unstable dominance hierarchy as they tried to defend the additional enrichment. It is also possible that as the group housed mice with high enrichment levels in our study had significantly fewer encounters with other mice than those under basic enrichment (combined positive and aggressive behaviour), this could have caused the reduced stress. A recent study carried out on group housed male mice concluded that providing nesting material for males leads to reduced aggression which is probably the easiest and most cost efficient method of improving the welfare of group housed male mice [32] although increasing floor area may be equally important. Our data suggest that providing more than a basic level of enrichment to reduce stress for individually housed mice may be a waste of resources.

More understanding is needed of the species specific characteristics of the laboratory mouse, including sensory perceptions and motivations, to ensure the ideal living conditions for this animal, become standard in all laboratories. There appears to be no additional stress caused by individually housing male outbred mice, however, an acclimatisation period should be increased to a minimum of 14 days. Providing enrichment, especially for all group housed outbred mice, should be mandatory to increase welfare standards and to allow a range of behaviours to be expressed.

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