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Hang on: an evaluation of the hemp rope as environmental enrichment in C57BL/6 mice

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Abstract

When introducing environmental enrichment in laboratory animals, positive and/or negative effects on behavioural and physiological parameters should be evaluated. This three-step randomised controlled trial in male C57BL/6 mice investigated the effect of supplementing the environment with one or more hemp ropes. In part 1, the effect of a hemp rope on aggressive and social behaviour, stress and anxiety levels was assessed by social interaction test, elevated plus maze behaviour, and faecal corticosterone metabolites, respectively (n = 224 mice). In part 2, the effect of 1, 2 or 7 hemp ropes on aggressive behaviour in mice subjected to routine handling was evaluated by assessing the number of wounded companion animals and wounds per animal (n = 224). In part 3, climbing activity in the rope and amount of material shredded from the rope was assessed (n = 56). Mice housed with one hemp rope engaged in social behaviour for longer time than mice housed without a hemp rope, while no difference was detected in stress and anxiety levels. No difference was seen in the number of wounded animals or wounds per animal when adding 1, 2 or 7 hemp ropes to the existing environment in mice undergoing minimal human handling. The mice continuously shredded and climbed the rope, even when provided with a new rope, although time spent climbing decreased slightly over time. Thus, a hemp rope can be used as additional environmental enrichment amongst male C57BL/6 mice.

Keywords: *aggression, animal welfare, environmental enrichment, mice, stress, wounds*

Introduction

Environmental enrichment (EE) is increasingly being used to enhance well-being among laboratory animals (Olsson & Dahlborn 2002; Baumans 2005; Baumans & Van Loo 2013), and is incorporated into European legislation (Directive 2010/63/EU 2010). The cage itself is a restricting element and a lack of environmental complexity deprives the mice of the possibility of controlling their physical and social environment (Sørensen *et al* 2004; Balcombe 2006; Fox *et al* 2006). This can possibly cause stress and suppression of natural behaviours that the mice are highly motivated to perform (Olsson & Dahlborn 2002; Sørensen *et al* 2004). Therefore, EE is used to counteract the suppression of natural behaviours. However, changes in caging are not always beneficial, since animals may not respond to the enrichment item as intended and fail to perceive enrichment as being meaningful. The introduction of EE has been shown to increase and, in few cases, reduce agonistic behaviour (Haemisch & Gärtner 1997; Ambrose & Morton 2000, Marashi *et al* 2003; Kaliste *et al* 2006; Abou-Ismail 2011; Akre *et al* 2011; McQuaid *et al* 2012; Mesa-Gresa *et al* 2013). The increase in agonistic behaviour is most commonly related to territorial defence (Olsson & Dahlborn 2002; Hutchinson 2005) and, in such cases, the provision of EE seems to counteract the goal of improving welfare (Haemisch & Gärtner 1994; Haemisch *et al* 1994a). It is therefore important to evaluate the effects of EE on both behaviour and physiological parameters (Van de Weerd *et al* 2002; Baumans 2005; Baumans & Van Loo 2013).

To optimise the welfare of laboratory mice at our research unit, we have introduced a hemp rope hanging from the lid in some cages to see if this would create an opportunity for the mice to access the extra space available in cages with raised lids. The caretakers have observed that the mice use the hemp rope as an escape route from aggressive cagemates. Moreover, the caretakers have not noticed a rise in aggression after introduction of the rope, which may indicate that a hemp robe as EE can be a means of reducing agonistic behaviour. Therefore, the aim of the current study was to investigate: i) the effect of supplementing the environment with a hemp rope on aggressive and social behaviour, stress and anxiety levels; ii) the effect of supplementing the environment with 1, 2 or 7 hemp ropes per cage on aggressive behaviour in mice only subjected to routine handling; and iii) the duration and frequency of climbing a hemp rope and material shredded from it.

Table 1 Groups of mice.

Experiment	Group	Strain	Sex	Total number of mice per group	Number of cages (seven mice per cage)	Number of ropes added
Part 1	B6(1)-C	C57BL/6	Male	112	16	0
	B6(1)-1R	C57BL/6	Male	112	16	1
Part 2	B6(2)-C	C57BL/6	Male	56	8	0
	B6(2)-1R	C57BL/6	Male	56	8	1
	B6(2)-2R	C57BL/6	Male	56	8	2
	B6(2)-7R	C57BL/6	Male	56	8	7
	B6F(2)-1R	C57BL/6	Female	56	8	1
Part 3	B6(3)-1R	C47BL/6	Male	56	8	1

C: Control; 1R: 1 rope; 2R: 2 ropes; 7R: 7 ropes. For the groups of mice, the number in parenthesis denotes either part 1, part 2 or part 3, eg B6(1)-C represents control mice in part 1 and B6(2)-2R represents the intervention group with 2 ropes in part 2.

We hypothesised that mice housed with a hemp rope would display less agonistic behaviour, lower levels of stress and anxiety and more social behaviour than mice housed without this enrichment. Moreover, we also hypothesised that the mice would use the rope for climbing and gnawing.

Materials and methods

All experimental procedures were approved by the Danish Animal Experiments Inspectorate, and carried out in accordance with EU Directives (Directive 2010/63/EU 2010) by the same experimenter. The reporting of the study follows the ARRIVE guidelines for reporting animal research (Kilkenny *et al* 2010). The study is a three-part, randomised controlled trial; part 1 was performed from December 2013 to January 2014, part 2 in September and October 2014 and part 3 September and October 2015. All three studies had a duration of eight weeks.

Housing

All mice were housed in transparent standard makrolon type 4 cages (Techniplast, Italy) (540 × 320 × 180 mm; length × width × height) with a 70-mm raised lid giving a total cage height of 25 cm. All cages were provided with Aspen bedding (Tapvei, Finland), Enviro-Dri paper nesting material (Tapvei, Finland), a cardboard tube (LBS serving Biotechnology, UK), a dark-coloured acrylic hiding element (BachVent, Denmark), biting blocks (50 × 10 × 10 mm) in aspen wood (Tapvei, Finland) and food enrichment in the bedding provided twice a week (0.5 dl irradiated oats in part 1 and 2 ml irradiated mix of oats and corn and 2 ml Trio Munch Grains, SDS (Essex, UK), in parts 2 and 3). The mice were provided with *ad libitum* Altromin type O 1324FF in part 1, and *ad libitum* Altromin type 1324, Maintenance Diet Rats/Mice in parts 2 and 3 (Altromin, Germany). An automated watering system was used. Throughout the study, cages were cleaned once weekly by transferring the mice and all structural elements to a new cage if usable; otherwise, new identical items were added. During testing in part 1, the cages were cleaned two days prior to testing in the elevated plus maze (EPM) and two days prior to the social interaction test (SI) so no cleaning occurred during test periods. The housing room (parts 1, 2 and 3) and the test room (part 1) were maintained at a temperature of 20–22°C with a relative humidity of 45–65%. The mice were housed under a 12-h dark/light cycle with lights on between 0600 and 1800h.

Part 1

Study animals

Two hundred and twenty-four male mice (4–6 weeks old) from C57BL/6 (B6) inbred strains from Charles River Laboratories, Germany, were randomly housed in 32 cages in groups of seven. The 32 cages were randomised into an intervention group and a control group (16 cages in each group) with different housing conditions (Table 1). The control group (B6[1]-C) was provided with the environment previously described and the intervention group (B6[1]-1R) provided with the described environment plus a hemp rope (length 30 cm, diameter 6 mm) hanging from the lid in the centre of the cage. The rope was secured in the lid by making a simple knot in the upper end of the rope before lowering the rope into the cage through the lid. The mice were checked twice daily during the entire study, and mice too injured to proceed with testing were excluded from the study. At 10–12 weeks of age three mice from each of the 32 cages (48 mice from each group) were randomly allocated as test individuals, individually marked on the tail with colour markers and tested in the EPM and the SI. The remaining four mice in each cage served as companion mice. To ensure random pair testing in the SI and equal recovery of seven days between the EPM and the SI, cages within each group were randomly paired and tested in a randomised order. The 16 cages in each group were paired resulting in eight pairs of cages. Each cage contained three test mice. For the SI, each of the three test mice was randomly paired with a test mouse from the paired cage. This resulted in a total of 24 encounters per group. Computer-generated randomisation lists were used in the three parts.

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Handling

The traditional tail-handling method was used during cage cleaning by animal care staff and for tail-marking by the experimenter. During testing in the EPM, tail-handling was used while supporting the mouse with the contralateral hand when placing it on the EPM and tunnel-handling used when taking the mouse off the maze. Handling of the mice at any other point was done by tunnel to reduce anxiety associated with handling (Hurst & West 2010; Gouveia & Hurst 2013).

Faecal corticosterone metabolites (FCM)

Prior to conducting the EPM and the SI, bedding was collected during routine cage cleaning and approximately 10 g faecal boli was sorted from each cage, containing faeces from the preceding six days, and stored at room temperature for later analysis. Concentration of faecal corticosterone metabolites (FCM) was quantified as described by Sundbom *et al* (2011), with the exception of samples being evaporated and dissolved in buffer after extraction instead of being diluted in ethanol. Briefly, faecal samples were weighed and submerged in 96% ethanol (3 ml g^{-1} faeces), after which they were vortexed and incubated on a shaking table overnight. A Scenspeed 1236p centrifuge (LaboGene Aps, Lynge, Denmark) was used to centrifuge the homogenate for 20 min at 3,134 rcf, and the supernatant decanted and the pellets discarded. A table-top centrifuge (Eppendorf 5415D, Eppendorf AG, Hamburg, Germany) centrifuged a 1 ml aliquot of the supernatant for 15 min at 9,300 rcf after which 200 µl of the supernatant was recovered while cautiously avoiding aspirating any pelleted material. The final samples were evaporated in a Genevac EZ-2 personal evaporator (Stone Ridge, NY, USA) for 2 h, dissolved in 300 µl phosphate-buffered saline (PBS) and analysed using the DRG Diagnostics corticosterone (competitive) ELISA (EIA-4164; DRG Instruments GmbH, Maburg, Germany) according to the manufacturer's instructions. B6(1)-1R mice were compared with B6(1)-C mice (serving as a baseline value).

Elevated plus maze (EPM)

At age 10–12 weeks, the mice (48 in each group) were tested during the light phase between 0900 and 1500h for five successive days. Thirty minutes prior to testing, the mice were transported to the experimental room in their home cages for acclimatisation. The maze had a grey acrylic surface and consisted of four arms; two closed (21 × 7 × 30 cm) and two open (21 × 7 cm; length × width) placed across each other and connected by a square centre platform (7 × 7 cm). The maze was elevated 60 cm above the floor and illuminated by 700–800 lux (measured at the centre platform). A video camera placed above the maze was used to record the mice's behaviour for later analysis. Testing was carried out as described by Walf and Frye (2007) and behavioural parameters assessed using EthoVision XT 8 (Wageningen, The Netherlands). Briefly, the mouse was placed in the centre platform facing the open arm opposite the experimenter, and the video camera immediately turned on for 5 min of behavioural recording. At the end of the 5 min test, the mouse was removed from the maze and the maze cleaned before testing the subsequent mouse. Entry into an arm was defined as the moment when the centre of the mouse (defined by EthoVision) crossed the lines defining the centre platform. Parameters assessed were: number of entries made into and time spent in open and closed arms; percentage of open arm entries relative to total entries; percentage of time spent in open arms relative to total time; and time spent on centre platform.

Table 2 Ethogram of behavioural categories used in the social interaction (SI) test.

Main behavioural category	Elements of behavioural categories	Brief description of elements
Agonistic	Latency to first attack (s)	Time to first attack
	Tail rattle (n)	Rapid lateral quivering or thrashing of the tail
	Attacks (n, s)	Biting the opponent
Social	Sniffing (n, s)	Sniffing the opponent at any region of the body
	Following (n, s)	Moving close to the opponent in the same direction
	Mounting behaviour (n)	Placing both forepaws on the opponents

Social interaction test (SI)

Exactly one week after testing in the EPM, the mice (48 in each group) were tested in the SI. The test was carried out in an open field box (49 × 49 × 30 cm) with a grey acrylic surface and illuminated by 700–800 lux (measured at the centre of the box). Testing occurred during the light phase between 0900 and 1500h for five successive days.

Thirty minutes prior to testing, the mice were transported to the experimental room in their home cages for acclimatisation. Before each encounter, the pair of mice to be tested was allowed one minute of habituation in the open field box while separated by a grey plastic barrier. Thereafter, the two mice were confronted with each other for 4 min in total to minimise serious injuries. All encounters were recorded by a video camera placed above the open field box and saved for later analysis. The same experimenter manually scored all the videos and blinding was ensured by concealing information about group allocation. Two behavioural categories were assessed: agonistic behaviour and social behaviour. Agonistic behaviour encompassed latency to first attack (duration), tail rattle (number) and attacks (number and duration). Social behaviour encompassed sniffing (number and duration), following (number and duration) and mounting behaviour (number) (see Table 2 for an ethogram). Behavioural parameters lasting under 1 s were noted as 1 s. For both agonistic behaviour and social behaviour, total time and total number were analysed separately for each pair of mice, as the behaviour of one mouse in the SI depended on that of the other (File & Seth 2003).

Registration of wounds

Two days after finishing the SI, all companion mice (64 in each group) were euthanased by an experimenter using carbon dioxide. Each mouse was investigated for the presence of wounds on the body by another experimenter to ensure blinding.

Part 2

Two hundred and twenty-four male and 56 female mice (4–6 weeks old) from the B6 inbred strain from Charles River Laboratories, Germany, were included in the study. All mice were randomly housed in groups of seven (within the same sex) and kept under the housing conditions previously described (Table 1). The female mice were kept in the same room as the males, but in a separate rack. The 224 male mice were randomly allocated into four groups (eight cages in each group) provided with a varying number of hemp ropes in addition to the environment described in part 1; 0 ropes (B6[2]-C, serving as a control), one rope (B6[2]-1R), two ropes (B6[2]-2R) and seven ropes (B6[2]-7R), respectively. The female mice, eight cages of seven mice, were provided with one hemp rope. All mice were left undisturbed for eight weeks with only cage cleaning and daily health monitoring being performed. Mice that were too injured to proceed with the study were excluded, euthanased and investigated for wounds.

Registration of wounds

At 12–14 weeks of age, all mice were euthanased by carbon dioxide and completely denuded of hair using a shaver. Each mouse was investigated for the presence of wounds on the body. Wounded mice were stored at –180°C and saved for later wound analysis while non-wounded individuals were discarded. One experimenter euthanased the mice and another experimenter investigated for wounds and performed the subsequent analysis. A wound was characterised as a penetration of the skin. After thawing, the number, location (dorsal anterior, dorsal posterior, ventrum, tail, limbs, scrotum, head) and size in diameter (0.00–2.00 mm; 2.01–3.00 mm; 3.01–4.00 mm; 4.01–5.00 mm; 5.01–6.00 mm; 6.01–7.00 mm; 7.01–8.00 mm; > 8.01 mm) of wounds were assessed. Euthanasia and analysis of wounds were performed while keeping cages separated in order to detect possible cage difference within groups. Groups B6(2)-1R, B6(2)-2R and B6(2)-7R were compared with B6(2)-C in order to evaluate the effect of a different number of hemp ropes; and B6F(2)-1R was compared with B6(2)-1R in order to evaluate sex differences.

Part 3

Fifty-six male mice (eight weeks old) from the B6 inbred strain from Charles River Laboratories, Germany, were included in the study. At arrival, the mice were randomly allocated into eight cages (housed in groups of seven) and kept under the housing conditions previously described (Table 1). In addition to the enrichment described in part 1, the mice were provided with a hemp rope of the same type as in parts 1 and 2.

All cages were randomly placed in the same rack and the mice allowed two weeks of habituation prior to any observations being performed. After arrival (week 0), the mice were observed in weeks 3, 6 and 7. A new hemp rope was provided to each cage at the end of weeks 2 and 6. Observations were performed by installing two video cameras inside the rack at the end of week 2. The cameras recorded the behaviour of mice in two cages. Random testing of the cages was ensured by randomly positioning the cages in view of the cameras over four days (eight cages). The two cameras were left in the same position throughout the study. The cameras were turned on for periods of 30 min at 1530, 1700, 1830, 1930 and 2030h providing behavioural observations in both the light and the dark phase (1 h during the light phase and 1.5 h during the dark). At 2100h, the position of the eight cages was changed so that two new cages were placed in the camera angle for observations the following day. The hemp rope was weighed when introduced into the cage and weighed weekly during cage cleaning throughout the study. Thus, it was possible to determine if the mice continued to shred material from the hemp rope. Parameters assessed were: frequency and duration of climbing in the hemp rope (climbing was defined as a mouse 'hanging' in the rope); frequency and duration of biting in the hemp rope (biting was defined as a mouse biting or trying to shred the rope); and weight of the hemp rope in each cage in weeks 3, 4, 5, 6 and 7. At the end of week 6, all hemp ropes were replaced and weighed, and the weight of the ropes re-assessed at the end of week 7.

Statistical analysis

All statistical analyses were performed using the statistical analysis system, SAS 9.3, (SAS Institute Inc, Cary, NC, USA) using a 5% significance level. Data from FCM, the EPM and the SI were analysed using an analysis of variance. In FCM and EPM, the cage was the unit being treated and analyses of the SI were performed per pair of mice (part 1). Goodness-of-fit (linearity, variance homogeneity and normal distribution of residuals) was investigated by visual inspection of plots and variables were log-transformed (log2) where necessary. In SI, the total number of social behaviour was transformed and transformation was successful in achieving a normal distribution. Results from this model are given as a ratio (back-transformed 2^{β}–coefficients). The analysis of wounds (parts 1 and 2) was performed per mouse. The χ^2 test was used to analyse differences in number of wounded mice in each group (B6[1]-C vs B6[1]-1R in part 1 and B6[2]-C vs B6[2]-1R, B6[2]-2R and B6[2]-7R, respectively, and B6[2]-1R vs B6F[2]-1R in part 2). Part 3 was performed as an observational study and no statistical analyses were applied.

Results

Part 1

Two hundred and twenty-four mice were included in the study. Ninety-six mice (n = 48 in each group) served as test mice and the remaining 128 (n = 64 in each group) served as companions.

Faecal corticosterone metabolites (FCM)

As depicted in Figure 1, no difference in FCM concentrations between B6(1)-C and B6(1)-1R ($\beta = 4.56$; CI = [–0.30; 9.42]; $F_{1,30} = 3.67$; $P = 0.065$), indicating no difference in stress hormone levels between the two groups.

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Figure 1

Comparison of faecal corticosterone concentrations in intervention and control mice. B6(1)-C: control group; B6(1)-1R: intervention group with 1 hemp rope. There are n = 16 cages in both groups; the error bars represent standard deviation.

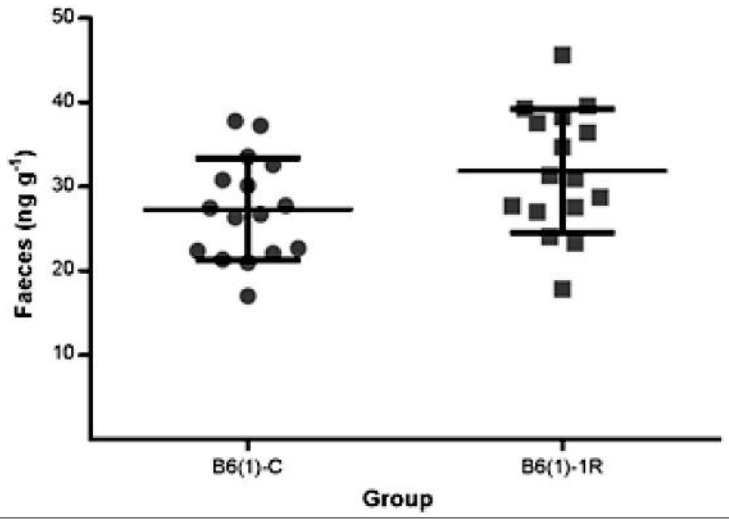


Table 3 Difference between mice housed without a hemp rope and mice housed with 1 hemp rope in the social interaction test.

Parameter	Group	Mean (± SEM)	$\beta/2^{\beta}$ (CI)	*F*-value	*P*-value
Agonistic behaviour, duration (s)		–	–	–	–
Agonistic behaviour, number (n)		–	–	–	–
Social behaviour, duration (s)	B6(1)-C	26.83 (± 1.70)	8.63 (1.03; 16.22)	5.23	0.027*
	B6(1)-R	35.46 (± 3.37)			
Social behaviour, number (n)	B6(1)-C	23.08 (± 1.40)	1.14 (0.93; 1.40)#	1.62	0.21**
	B6(1)-R	27.21 (± 2.20)			

For both groups, n = 24 pairs; no agonistic behaviour was observed.
Number of social behaviour were log2-transformed; the result is given as a 2^{β}-coefficient and is hence a ratio.
CI: 95% confidence interval.
* Significant at $P \leq 0.05$.
** Non-significant.

Elevated plus maze (EPM)

Five mice from B6(1)-C and ten from B6(1)-1R were excluded from the analysis because they fell from the maze during testing. Three mice from B6(1)-1R were excluded because of technical problems with the video camera. This resulted in 43 B6(1)-C mice and 35 B6(1)-1R mice being included in the analysis. The groups did not differ in any of the categories measured (results not shown).

Social interaction test (SI)

Twenty-four pairs in both groups were tested. No mice in any of the groups engaged in agonistic behaviour during the 4 min of testing. Mice from B6(1)-C spent a significantly smaller amount of total time engaged in social behaviour ($\beta = 8.63$; CI = [1.03;16.22]; $F_{1,46} = 5.23$; $P = 0.027$) than mice kept in B6(1)-1R. B6(1)-1R engaged in social behaviour 14% more frequently than B6(1)-C, although this was not found to be significant ($2^{\beta} = 1.14$; CI = [0.93;1.40]; $F_{1,46} = 1.62$; $P = 0.21$). Data are summarised in Table 3.

Table 4 Number of wounded mice in part 1 and part 2.

Part	Study	Mice assessed (n)	Mice with wounds (n)
Part 1	B6(1)-C	63	6
	B6(1)-1R	63	19
Part 2	B6(2)-C	55	9
	B6(2)-1R	55	10
	B6(2)-2R	56	12
	B6(2)-7R	56	7
	B6F(2)-1R	56	0

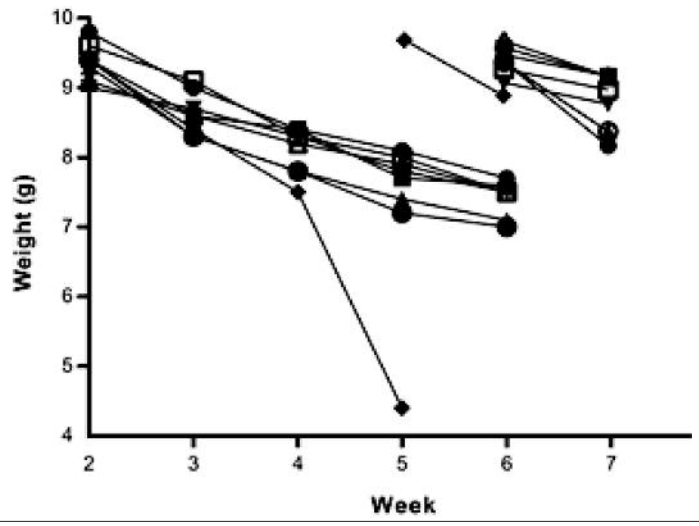
C: Control; 1R: 1 rope; 2R: 2 ropes; 7R: 7 ropes. For the groups of mice, the number in parenthesis denotes either part 1 or part 2, eg B6(1)-C represents control mice in part 1 and B6(2)-2R represents the intervention group with 2 ropes in part 2. In part 1 companion mice were assessed.

Table 5 Target sites (percentage of total wounds) for conspecific offensive attacks in control mice and mice housed with 1, 2 and 7 hemp ropes, respectively.

Target sites	B6(2)-C	B6(2)-1R	B6(2)-2R	B6(2)-7R
Total wounds (n)	79	105	86	24
Attack site (%)				
Dorsal anterior	8.86	10.48	12.79	4.17
Dorsal posterior	49.37	22.86	23.26	12.5
Ventrum	2.53	24.76	19.77	29.17
Right flank	26.58	22.86	24.42	41.67
Left flank	10.13	16.19	18.60	4.17
Tail	–	–	–	–
Limbs	1.27	2.86	1.16	8.33
Scrotum	–	–	–	–
Head	1.27	–	–	–

C: Control; 1R: 1 rope; 2R: 2 ropes; 7R: 7 ropes. For the groups of mice, the number in parenthesis denotes part 2, eg B6(2)-C represents control mice and B6(2)-2R represents the intervention group with 2 ropes. N = 55 in group B6(2)-C and B6(1)-1R, n = 56 in group B6(2)-2R and B6(2)-7R; B6F(2)-1R is not represented as no female mice had wounds.

Figure 2



Weight of hemp rope during study period in mice housed with one hemp rope. Weekly weighing of the hemp ropes (g) in each cage (n = 8). The rope was replaced in one cage (diamond-shaped icon) and a new rope was provided in week 5. After weighing in week 6 a new hemp rope was provided in every cage. A broken line represents a new hemp rope and the different shapes represent different cages.

Wounds

One mouse from each group died during the test period leaving 63 companion mice in each group to be examined for wounds. One mouse (B6[1]-1R) was found hanging from the lid by the tail and one (B6[1]-C) showed clear signs of discomfort and was euthanased and autopsied revealing possible back problems. In B6(1)-C six mice had wounds on the body while 19 in B6(1)-1R were wounded. B6(1)-C differed significantly from B6(1)-1R (χ^2 (1, n = 126) = 8.43; $P < 0.01$) (see Table 4).

Part 2

Two hundred and twenty-four mice were included in the study. One B6(2)-C and one B6(2)-1R mouse were excluded due to injury.

Nine mice in B6(2)-C, ten in B6(2)-1R, 12 in B6(2)-2R and seven in B6(2)-7R had wounds on the body — only seven out of 222 mice had more than ten wounds on the body regardless of the number of hemp ropes provided (data not shown). B6(2)-C did not differ significantly from any of the compared groups. No mice in B6F(2)-1R had wounds on

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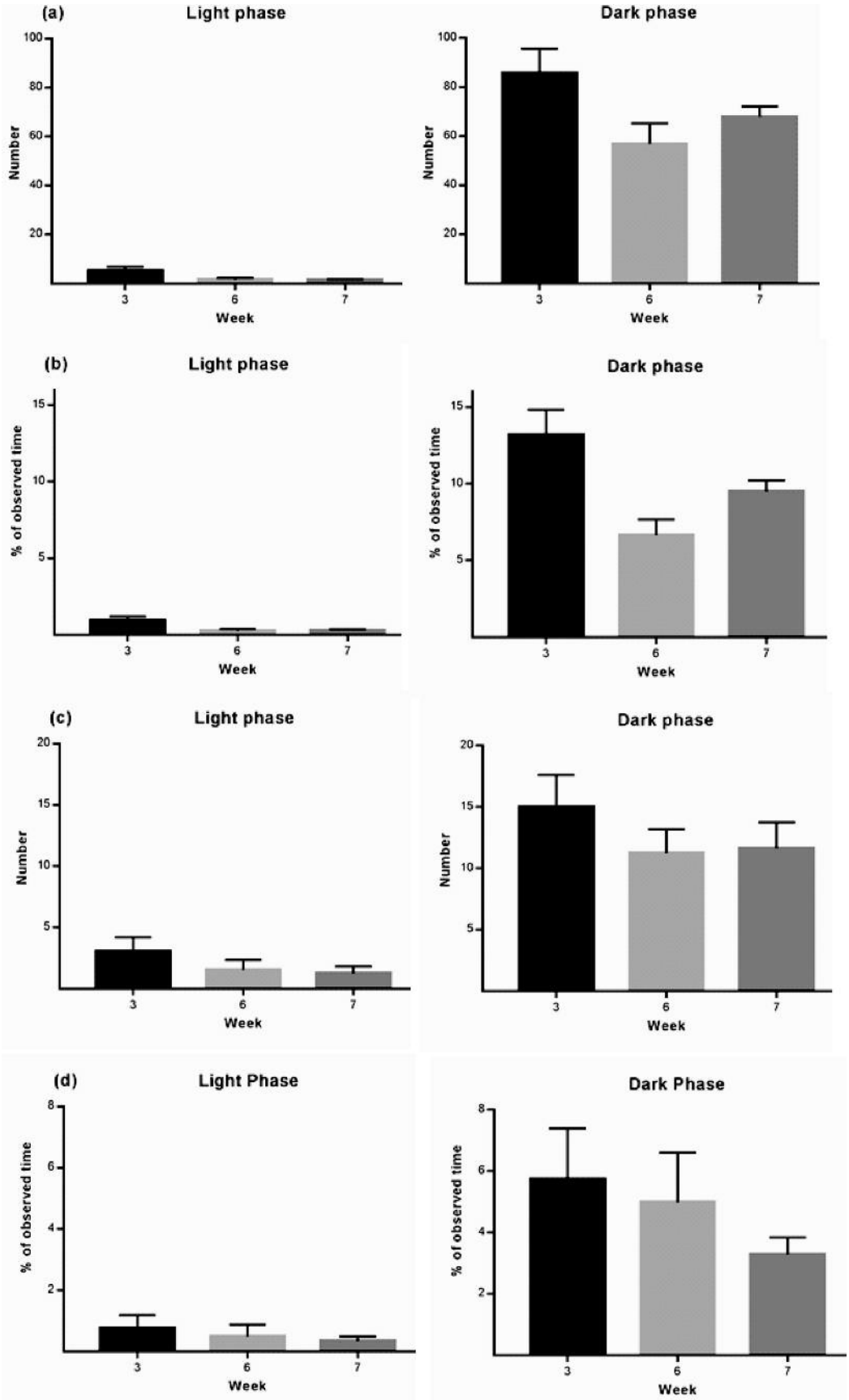
Figure 3



Activity using the hemp rope during light and dark phase observations in mice housed with one hemp rope for (a) frequency of climbing the hemp rope, (b) percentage of total observed time spent climbing the hemp rope, (c) frequency of biting the hemp rope and (d) percentage of total observed time spent biting the hemp rope. All data are given as mean values (n = 8 cages); the error bars represent standard error of the mean.

the body and no statistical analyses were performed on this group. Data are summarised in Table 4.

The difference in number of wounded mice between cages within each group was significant in B6(2)-C (χ^2 [7, n = 55] = 16.03; P = 0.0248) and B6(2)-7R (χ^2 [7, n = 56] = 19.43; P = 0.0069) indicating a variation in aggression between cages within the same group. Table 5 presents the distribution of bites at different body locations as a percentage of total wounds within each group. There was a tendency for mice to be attacked on the lower back (12–50%) and flanks (10–42%). Eighty-seven percent of all wounds (all groups) were noted as being small 0.00–2.00 mm (diameter) in size, 10.88% were noted as 2.01–3.00 mm and the rest (1.71%) were of varying size greater than 3.01 mm. These wounds (> 3.01 mm) were primarily located on the lower back (dorsal posterior). No further statistical analyses were performed.

Part 3

Fifty-six mice were included and no mice were excluded during the study.

The mean weight of the hemp rope in the cages was reduced weekly and when provided with a new hemp rope (week 6), the weight was reduced again (week 7) as seen in Figure 2. At the end of week 5, the rope in one of the cages (represented by the diamond-shaped icon) was almost completely shredded and provision of a new hemp rope had to be made (and again one week later with the rest of the cages). Apart from this cage, the hemp rope was shredded in the same manner in all cages.

During the dark phase, the mice both climbed and bit the hemp rope in all three observation periods (weeks 3, 6 and 7), although a slight decrease was seen over time (Figure 3). The mice also climbed and bit the hemp rope during the light phase with a slight decrease seen over time (results not shown), but since mice are nocturnal, the results from the dark phase provide a more realistic result.

Discussion

In this randomised controlled study investigating positive and negative effects of adding a hemp rope as environmental enrichment in male B6 mice, we found that the number of wounded male B6 mice was significantly higher in frequently handled mice housed with a hemp rope compared with frequently handled mice housed without a hemp rope (part 1). However, in mice undergoing minimal human handling this difference was not seen when comparing male B6 mice provided with 1, 2, or 7 hemp ropes to mice housed without a hemp rope (part 2). Furthermore, male B6 mice housed with a hemp rope (part 1) spent a significantly greater amount of time engaged in social behaviour compared with male B6 mice housed without a hemp rope. No difference was seen in stress or anxiety levels between the groups. The hemp rope was used for both climbing and biting (part 3), and the mice kept using the hemp rope over time, although a slight decrease was seen.

Contrary to our hypothesis, in part 1 we found a significantly higher number of wounded male B6 mice provided with a hemp rope compared to those housed without a hemp rope. To further investigate the level of aggression in the hemp rope group, we conducted part 2 to examine the exact extent and severity of aggression-induced injuries. Our findings in part 2 did not confirm the results from part 1, nor was there any significant difference between the number of wounds per mouse between the male B6 groups. We saw a tendency of mice being wounded on the lower back and flanks, in accordance with previous studies, demonstrating that the attacker places the strongest bite towards the back and the weakest bite at the ventrum (Blanchard *et al* 1979; Blanchard & Blanchard 2005; Litvin *et al* 2007). As expected, no aggression or sign of aggression was observed among female mice (Berry 1970). An increase in agonistic behaviour between male mice kept in groups may be caused by the structural elements encouraging territorial tendencies (Haemisch *et al* 1994a; Haemisch & Gärtner 1997; Marashi *et al* 2004), as these elements can act as a visual landmark for territorial boundaries (Mackintosh 1973). An increase can also be caused by a lack of manipulation of the structural objects, which reduces the mice's possibility of controlling their environment (Olsson & Dahlborn 2002; Van Loo *et al* 2002; Baumans & Van Loo 2013) or, as this study indicates, the handling of the mice. The most obvious aggression-modulating factor between the two studies is the level of disturbance. Part 2 was stripped from external stimuli, eg frequent fixation for tail-remarking and handling during behavioural testing, and only routine handling during cage cleaning was performed. Since the provision of 1, 2, or 7 hemp ropes did not affect the level of aggressive behaviour among the male B6 mice, it is likely that the human interaction in part 1 has resulted in increased inter-male fighting. In part 1, the mice were frequently handled, which was partly done by tail-handling. Tail-handling has been shown to induce increased levels of anxiety in BALB/c and B6 mice (Hurst & West 2010). Therefore, it would be interesting to evaluate if, and how often, the extent, type and severity of different disturbances affect the behaviour of mice. Attention should be given to the refinement of study procedures to diminish the number and duration of potentially stressful situations, hence enhancing the well-being of the mice. As mentioned, we did not see a difference in the level of aggression in mice provided with 1, 2 and 7 hemp ropes, respectively. However, for practical reasons, the provision of one rope is likely preferable.

Corticosterone is considered a sensitive and reliable indicator of chronic or repeated stress (von Holst 1998; Whitten *et al* 1998; Möstl & Palme 2002; Palme *et al* 2005). A rise in aggression in parallel with a rise in corticosterone levels in enriched environments indicates that the enrichment is counteracting the goal of enhancing the welfare of the animals (Haemisch *et al* 1994b, van Loo *et al* 2002; Marashi *et al* 2003; Hutchinson *et al* 2012; McQuaid *et al* 2012). The fact that aggression is increased is not necessarily a sign of reduced welfare as aggression is a normal part of murine behaviour (Berry 1970). As pointed out by Würbel and Garner (2007) it is whether or not the animals can cope with the situation that matters. In this study, we

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found no difference in faecal corticosterone levels between the group housed with and without a hemp rope (part 1). This result proposes that providing a hemp rope through the lid does not change the stress hormone levels in B6 mice. Similarly to our study, van de Weerd *et al* (1997) and Roy *et al* (2001) showed unchanged corticosterone levels in environmentally enriched mice compared with their non-enriched counterparts. Although this was measured in plasma corticosterone values and therefore cannot be directly compared with faecal corticosterone values, it has been shown that some types of environmental enrichment do not cause a change in stress levels in mice.

Social contact is an important element of murine behaviour (Berry 1970; Latham & Mason 2004; Balcombe 2010). Our results in the social interaction test (part 1) showed that mice housed with a hemp rope did not engage more frequently in social behaviours but engaged herein for longer time periods compared with mice housed without a hemp rope. Our results are in accordance with a study by Mesa-Gresa *et al* (2013) showing that mice housed with environmental enrichment spend more time engaged in social behaviour. Thus, our results indicate that the introduction of a hemp rope enhances time spent on social encounters. Nevertheless, whether this difference is of biological relevance is debatable. An increase in social encounters in the social interaction test has also been interpreted as a sign of reduced anxiety (File & Seth 2003). In the present study, though, the elevated plus maze did not reveal any difference in anxiety levels between the groups. Perhaps the anxiety brought on by the tail-handling was large enough to overshadow a more subtle effect of rope-provision (Hurst & West 2010). Additionally, when investigating positive effects of the hemp rope (part 3), we found that male B6 mice used the hemp rope repeatedly, although a slight decrease of duration and frequency of climbing and biting the rope was seen over time. The mice did not only climb and bite the hemp rope, we also found that the mice shredded material from the hemp rope, and when provided with a new rope, started shredding it all over again. We also observed that, besides climbing and biting the hemp rope, the mice used the hemp rope as an escape route when attacked by an aggressive cage-mate and the shredded material was furthermore incorporated into the nests throughout the study.

Animal welfare implications and conclusion

In conclusion, enriched mice display social behaviours for longer time-periods than non-enriched mice while no difference is seen in stress and anxiety levels. Adding 1, 2, or 7 hemp ropes to the existing environment does not influence the level of aggression in male B6 mice undergoing minimal human handling, ie only handling during routine cage cleaning. In addition, female B6 mice do not show signs of aggressive behaviour. Furthermore, part 2 of the study demonstrates a pronounced negative effect of human handling and disturbances; an effect that should not be underestimated or ignored and calls for further investigations. Finally, when adding a hemp rope to the existing environment, mice will climb and bite the rope, and shred it over time — also when provided with a new hemp rope. The mice use the rope as an escape route when attacked by an aggressive cage-mate. We therefore conclude that a hemp rope can be used as additional environmental enrichment amongst male C57BL/6 mice.

Enhancing the complexity of the environment by the use of environmental enrichment is generally acknowledged as a way of improving the welfare of the animals. During this study, the B6 mice used the hemp rope extensively for climbing, biting and manipulation — even after a replacement of the hemp rope. Therefore, it is likely that the positive effects of adding a hemp rope, namely the possibility of mice expressing natural behaviours, such as climbing, shredding and nesting using multiple materials, counteract and even exceed a possible negative effect of increased levels of aggression.

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