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Long-term effects of husbandry procedures on stress-related parameters in male mice of two strains

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Summary

In socially unstable groups of male laboratory mice, individuals may experience a chronic stress situation. Previous experiments have shown that the transfer of specific olfactory cues during cage cleaning, and the provision of nesting material decrease aggression and stress in group-housed male mice. In this study, the combined effect of these husbandry procedures were tested for their long-term effect on stress in groups of moderately aggressive (BALB/c) and severely aggressive (CD-1) male mice. The physiological and behavioural stress-related parameters used were body weight, food and water intake, spleen and thymus weight, adrenal tyrosine hydroxylase activity, urine corticosterone levels and behaviour in a cage emergence test. Long-term provision of nesting material and its transfer during cage cleaning was found to influence several stress-related physiological parameters. Mice housed in cages enriched with nesting material had lower urine corticosterone levels and heavier thymuses, and they consumed less food and water than standard-housed mice. Furthermore, marked differences were found between strains. CD-1 mice were less anxious in the cage emergence test, weighed more, ate and drank more, and had heavier thymuses but lighter spleens and lower corticosterone levels than BALB/c mice. We conclude that the long-term provision of nesting material, including the transfer of nesting material during cage cleaning, reduces stress and thereby enhances the welfare of laboratory mice.

Keywords Husbandry; environmental enrichment; male mice; stress; welfare

Laboratory mice are generally housed in confined, stimulus-poor environments in which the performance of natural behaviour is impaired. As a result, the animals may show abnormal behaviour such as stereotypies and apathy (Chamove 1989, Cooper & Nicol 1996, Würbel *et al.* 1998). When male mice are housed in groups, their inability to perform normal social behaviour may additionally trigger excessive aggression and, as such lead to a situation of chronic social stress. The use of environmental enrichment to improve the well-being of laboratory mice is promoted widely and is recently incorporated in European legislation (Council of Europe 1997). Its general aim is to enhance species-specific behaviour, promote physical health as far as possible and to decrease abnormal behaviour while keeping a focus on scientific, economic and ergonomic demands (Newberry 1995, Dean 1999,

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Baumans 2000). Before applying any kind of environmental enrichment, its presumed effect on the well-being of the animals, both in the short and in the long term, need to be verified (Shepherdson *et al.* 1998). Several kinds of environmental enrichment have been shown to decrease inter-male aggression (Ward *et al.* 1991, Armstrong *et al.* 1998, Ambrose & Morton 2000), whereas other enrichments appear not to affect inter-male aggression (Eskola & Kaliste-Korhonen 1999), or even increase inter-male aggression (McGregor & Ayling 1990, McGregor *et al.* 1991, Haemisch & Gärtner 1994, Haemisch *et al.* 1994, Van Loo *et al.* 2002). In previous experiments we found that the amount of aggression in group-housed male mice significantly decreased when nesting material was introduced in the cages (Van Loo *et al.* 2002), and when nesting material was transferred during cage cleaning (Van Loo *et al.* 2000). These studies were carried out with an inbred strain (BALB/c), known to be moderately aggressive (Eskola & Kaliste-Korhonen 1999, Van Loo *et al.* 2000) and particularly susceptible to chronic stress exposure (Kopp *et al.* 1999).

The aim of the present study was to investigate the long-term effect on aggression and stress-related parameters of a combination of factors, previously found to decrease aggression in group-housed male mice of the BALB/c strain, and to investigate whether these effects could be extrapolated to a more aggressive mouse strain (CD-1, Parmigiani *et al.* 1999). This paper primarily deals with the housing and husbandry effects on stress-related parameters. To obtain an accurate estimate of the level of stress experienced by the mice, a wide variety of stress-related parameters were measured both during life and post-mortem, and included measures reflecting different bodily responses to challenges. As general physiological parameters, food and water intake and body weight were measured. The level of anxiety of the mice was tested in a cage emergence test. Spleen and thymus were weighed to reveal possible gross immunodepressive effects, and finally two neuro-endocrine measures reflecting HPA axis and sympathetic activation in response to challenges (urine corticosterone levels and adrenal TH-activity, respectively) were measured. Effects on aggression are published elsewhere (Van Loo *et al.* 2003a), as is a thorough review on the housing and management of male mice (Van Loo *et al.* 2003b).

Methods

Animals and husbandry

Sixty male mice of the BALB/cAnNCrlBR (BALB/c) and sixty male mice of the Swiss:CD-1(ICR)BR (CD-1) strain were used. On arrival, all animals were 6 weeks old. Per strain, the animals were randomly divided into 20 groups of three mice, and housed in wire-topped clear Perspex Makrolon® type II cages (375 cm^2, Tecniplast Milan, Italy) provided with 50 g sawdust (Lignocel® 3/4; Rettenmaier & Söhne, Ellwangen-Holzmühle, Germany). Half of the groups received nesting material (two Kleenex tissues, Kimberly-Clark Corporation®, Ede, The Netherlands) in addition to the usual bedding material ('enriched'). The other groups served as controls, without nesting material ('standard'). Pelletted food (RMH-B®, Hope Farms, Woerden, The Netherlands) and tap water were available *ad libitum*. The animal room had a controlled temperature (23–24°C), humidity (60 ± 5%) and ventilation (15–20 air changes/h). The artificial light/dark cycle was 12:12 with lights on at 07:00 h. The mice were marked on the tail as well as on the fur with a black waterproof marker to enable individual identification. Marks were renewed weekly prior to cage cleaning. After arrival the mice were allowed to adapt to their novel housing condition for one week.

Procedure and behavioural data collection

Cages and wire-tops were cleaned weekly. For enriched cages, the nesting material was transferred from the dirty cage into the clean cage and half of a new tissue was added to compensate for loss due to

shredding or eating. Prior to cage cleaning, the mice were weighed and marked, wounds were counted, and food and water were weighed and refreshed. At the age of 9, 12, 15, 18 and 21 weeks, the behaviour of the mice was recorded on videotape for a period of 30 min immediately following cage cleaning. The number of agonistic encounters scored was used to classify individual animals as dominant (dom), most attacked subordinate (sub+) or least attacked subordinate (sub−). Detailed data on aggressive behaviour are published elsewhere (Van Loo *et al.* 2003a). To quantify the individual level of anxiety, mice were subjected to a cage emergence test (described by Van de Weerd *et al.* 1994) at the age of 17 weeks. In short, the cage emergence test measures the latency time for a mouse to escape from a hole in a small barren cage into a larger barren cage, with a maximum of 10 min.

Urine collection and corticosterone analysis

In order to analyse corticosterone levels, urine samples were collected at the ages of 9, 12, 15, 18 and 21 weeks. Samples were taken non-invasively 3 to 4 days after cage cleaning between 09:00 and 10:00 h (method described by Dahlborn *et al.* 1996, and modified by Van Loo *et al.* 2001). Corticosterone levels were measured using a solid phase ^{125}I radioimmunoassay (CAC® Rat Corticosterone TKRC1, Diagnostic Products Corporation, LA), and corrected for creatinine concentrations determined with the use of a commercial test combination (Creatinine, MA-KIT 10 Roche, Roche Diagnostics) on a COBAS-BIO auto-analyser (Hoffmann-La Roche BV, Mijdrecht, The Netherlands).

Physiology

At the age of 22 weeks, the mice were decapitated simultaneously per group between 09:00 and 12:00 h by three animal technicians. Spleen and thymus were dissected and weighed. Adrenals were dissected, individually shock-frozen in 5 mM Tris-HCl-buffer (pH 7.2), and stored at −70°C. Adrenal tyrosine hydroxylase activity (TH) was measured using a tyrosine-^{14}C-assay (method described by Witte & Matthaei 1980).

Statistical analysis

Data on body weight, food and water intake, and organ weights were analysed using a general linear model for repeated measures with multiple comparisons, with age or status as a within-subject factor and strain and housing as between-subject factors. Tyrosine hydroxylase (TH) activity and Co/Cr ratio were analysed using a linear mixed effects analysis with, as fixed factors, housing (TH and Co/Cr), strain, age and status (Co/Cr); and as, random factors, group (TH) or mouse number (Co/Cr). Cage emergence time was analysed using a univariate analysis of variance with strain and housing as between-subject factors. To better conform to the normal distribution, several variables were log-transformed. When multiple comparisons were made in any of the statistical analyses, Bonferroni correction was applied (i.e. P value multiplied by number of comparisons, indicated by P_B). To identify dominant and subordinate animals within each group, the level of individual aggressiveness (as observed on videotape) was used. Five out of the 40 groups showed no or hardly any aggression. As a result the hierarchies of these groups could not be reliably evaluated. When comparisons were made between dominant and subordinate mice, these groups were omitted from the analysis. All statistical tests were carried out with the aid of SPSS for MS Windows, Release 9.0 (SPSS Inc, Chicago, USA) or S-plus 2000 Professional Release 2 (© 1988–1999, MathSoft, Inc.).

Results

Body weight, food and water intake

During the experiment, all mice showed a general increase in body weight ($P < 0.001$, Table 1). Enriched CD-1 mice gained more weight than CD-1 mice housed under

Table 1 Body weight, food and water consumption (mean ± SEM) of BALB/c and CD-1 mice housed under standard or enriched conditions summarized for periods of 4 (body weight) or 5 weeks (food and water)

		BALB/c		CD-1		
	Age (wk)	Standard	Enriched	Standard	Enriched	Overall sign
Body weight (g)	7–10	20.8 ± 0.13	21.0 ± 0.13	32.9 ± 0.24	33.3 ± 0.30	A***
	11–14	23.8 ± 0.10	24.0 ± 0.09	35.8 ± 0.29	36.7 ± 0.37	S***
	15–18	25.2 ± 0.09	25.4 ± 0.09	38.3 ± 0.34	39.2 ± 0.43	SxH*
	19–22	26.3 ± 0.09	26.5 ± 0.09	39.7 ± 0.37	40.5 ± 0.45	AxS***
Food (g/group/wk)	7–11	67.3 ± 0.41	64.9 ± 0.79	95.6 ± 0.90	90.3 ± 1.23	A***
	12–16	65.6 ± 0.64	64.4 ± 0.45	91.5 ± 1.05	84.6 ± 1.13	S***
	17–21	68.4 ± 1.15	65.5 ± 0.84	91.8 ± 1.25	84.9 ± 1.32	H*
						AxS***
Water (ml/group/wk)	7–11	63.7 ± 0.69	62.6 ± 0.72	108.2 ± 2.06	94.2 ± 2.16	A***
	12–16	64.9 ± 0.90	64.0 ± 0.63	98.8 ± 1.74	85.3 ± 1.46	S***
	17–21	65.4 ± 0.80	64.3 ± 0.97	97.2 ± 2.18	88.7 ± 1.74	H*
						AxS***

A = age effect, S = strain effect, H = housing effect
*$P < 0.05$; ***$P < 0.001$

standard conditions ($P_B < 0.05$). No such housing effect on weight gain was found for mice of the BALB/c strain. From the start of the experiment, CD-1 mice were significantly heavier than BALB/c mice ($P < 0.001$) and gained more weight than the BALB/c mice during the experiment ($P < 0.001$). Social status did not affect body weight in either strain.

An overall significant difference in food and water intake between the housing conditions was found (both $P < 0.05$, Table 1). Mice housed in enriched cages consumed less food and water than mice housed in standard cages. In general, food and water consumption showed a parabolic time effect. Initially, both food and water intake decreased, after which it increased again slightly ($P < 0.001$). A strain difference in the total amount of food and water consumed (both $P < 0.001$) and the change in food and water intake over the weeks (both $P < 0.001$) was found, with CD-1 mice consuming significantly more food and water than BALB/c mice.

Urine corticosterone/creatinine (Co/Cr) ratios

For both strains, a significant housing effect on Co/Cr ratios was found (Fig 1a, $P < 0.05$). Mice housed under enriched conditions showed a lower Co/Cr ratio than mice housed under standard conditions. Furthermore, a significant strain effect was apparent. CD-1 mice showed a significantly lower Co/Cr ratio than BALB/c mice (Fig 1a, $P < 0.001$). Co/Cr ratios showed a significant time effect ($P < 0.001$) that differed between strains ($P < 0.05$, Fig 1b: pooled for housing conditions). At the age of 9 weeks, the Co/Cr ratios of the BALB/c mice were quite high, then decreased when the mice were 12 weeks old and started to show an increase again after the age of 15 weeks. The CD-1 mice, on the contrary, showed lower Co/Cr ratios at the age of 9 weeks compared to the BALB/c mice followed by an increase at the age of 12 weeks after which the Co/Cr ratios decreased slightly again. No effect of position in the dominance hierarchy on Co/Cr ratios was found.

Cage emergence test

In the cage emergence test, again a clear strain effect was found. Mice of the BALB/c strain took significantly longer to escape from the small cage than did CD-1 mice (Fig 2, $P < 0.001$). No effect of housing condition could be established.

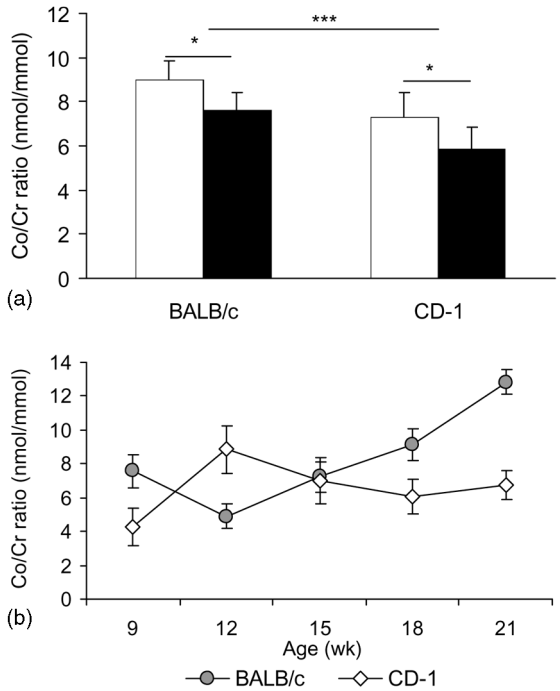

Fig 1 Corticosterone/creatinine ratio (mean ± SEM) of BALB/c mice and CD-1 mice (a) housed under standard or enriched conditions, (b) at five different ages, pooled for housing conditions. $*P < 0.05$; $***P < 0.001$

□ Standard ■ Enriched

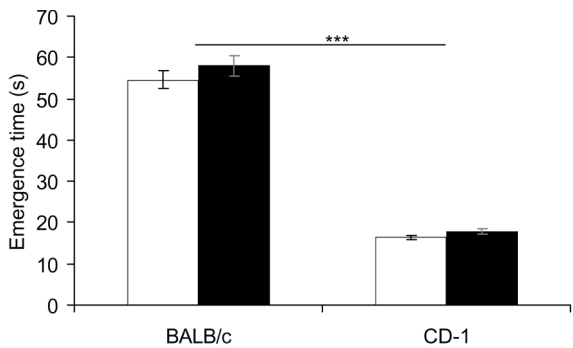

Fig 2 Emergence time (geometric mean ± SEM) of BALB/c mice and CD-1 mice housed under standard or enriched conditions, in the cage emergence test. $***P < 0.001$

□ Standard ■ Enriched

Post-mortem variables

Thymus and spleen weight and TH activity are summarized in Table 2. The thymus weight of enriched-housed mice was significantly higher than thymus weight of standard-housed mice in both strains ($P < 0.05$). Furthermore, CD-1 mice had heavier thymuses than BALB/c mice ($P < 0.05$). In contrast, spleen weight of CD-1 mice was significantly lower than spleen weight of BALB/c mice ($P < 0.05$), and a significant interaction between strain and housing condition was found ($P < 0.01$). For the enriched-housed mice, spleen weight did not differ between the strains, while in the standard housing BALB/c mice showed

Table 2 Organ weights (mg), TH activity (nmol/h/adrenal pair); mean ± SEM

Parameter		BALB/c		CD-1	
		Standard	Enriched	Standard	Enriched
Thymus		30.00 ± 0.90[a]	32.30 ± 1.30[b]	33.30 ± 1.70[c]	35.70 ± 1.70[d]
Spleen		108.70 ± 5.30[e]	100.30 ± 4.50	92.30 ± 3.50[f]	99.70 ± 3.60
TH	dom[g]	8.45 ± 1.10	8.33 ± 1.79	8.38 ± 1.21	8.44 ± 0.87
	sub+	8.46 ± 1.03	8.21 ± 2.03	7.19 ± 0.90	6.87 ± 1.20
	sub-[h]	7.19 ± 0.73	6.79 ± 0.73	6.95 ± 0.61	7.30 ± 1.16
	Unknown status	8.28 ± 1.14	4.40 ± 0.54	–	5.18 ± 0.99

a/b – c/d, a/c – b/d: $P < 0.05$
e–f: $P_B < 0.05$
g–h: $P_B < 0.1$

a heavier spleen weight compared to the CD-1 mice ($P_B < 0.05$).

The TH activity tended to differ for individuals with different positions in the dominance hierarchy (Table 2, $P < 0.1$), and no effects of housing condition or strain were found. Multiple comparisons, pooled for housing and strain, revealed that dominant mice tended to have higher TH activity than least attacked subordinate mice ($P_B < 0.1$), while most attacked subordinate mice were intermediate.

Discussion

Housing condition effects

Housing condition affected several physiological parameters, i.e. food and water intake, body weight, corticosterone levels and thymus weight. Food and water intake for mice housed under enriched conditions were lower than for mice housed under standard conditions while body weight of enriched-housed CD-1 mice was higher than for standard-housed CD-1 mice, and BALB/c mice of different housing conditions gained equal weight. This is in accordance with Dahlborn *et al.* (1996), Van de Weerd *et al.* (1997) and Van Loo *et al.* (2002), who found that mice from cages enriched with nesting material gained equal or more weight than mice from standard housing conditions, although they consumed less food. It was hypothesized that nesting material allows the mice to regulate their body temperature and, as a consequence, might decrease the need for food and water. Others stipulate that laboratory animals kept in standard conditions eat and drink more than animals housed in enriched cages due to boredom (Fiala *et al.* 1977, Van de Weerd *et al.* 1994). Excessive feeding and drinking have been reported as behavioural reactions to prolonged encagement (Wemelsfelder 1993). A discrepancy between food and water intake and body weight gain may also be related to the amount of stress experienced. Many reports have shown that chronic stress can produce a decrease in body weight, or a reduced weight gain in animals that are still growing (Manser 1992).

Corticosterone levels were higher and thymus weight was lower for standard-housed mice compared to mice housed with nesting material that was transferred during cage cleaning. An increase in baseline levels of corticosterone may be an indicator of chronic stress (Manser 1992, Shepherdson *et al.* 1998), and a decreased thymus weight is consistent with higher baseline corticosterone levels (Manser 1992, Moberg & Mench 2000). The lower corticosterone levels of enriched-housed mice found in this experiment are contrary to the results of Haemisch and Gärtner (1994). They found that enriched-housed mice showed increased levels of corticosterone, which they explained by their finding that mice in enriched cages were more aggressive and failed to maintain stable dominance relationships. An important difference between the latter and this experiment is the type of enrichment used. In a previous study

(Van Loo *et al.* 2002) inter-male aggression and corticosterone levels increased in mice housed in cages, structured with a shelter, comparable to the enrichment used by Haemisch and Gärtner (1994), while inter-male aggression decreased in mice housed with nesting material. In another experiment (Van Loo *et al.* 2000) the transfer of nesting material during cage cleaning clearly decreased aggression between male mice. Besides the transfer of familiar odours that may have reduced stress, the provided nesting material (tissues) itself could be used to hide from other mice besides being manipulated for nest building, which gave the mice the possibility of having some control over their environment. Controllability of the environment, next to predictability, has been reported to be an important factor influencing the amount of stress experienced by animals in an environment (Weiss 1972, Manser 1992, Wiepkema & Koolhaas 1993, Shepherdson *et al.* 1998). Moreover, preference tests have shown that both mice and rats clearly prefer nesting material to rigid structures such as a platform, a nest box (Bradshaw & Poling 1991, Van de Weerd *et al.* 1998, Van Loo submitted) or a shelter (unpublished data).

Strain, social status and age effects

Several parameters indicated that BALB/c mice may be more susceptible to social stress than CD-1 mice. BALB/c mice had higher urine corticosterone levels and they were considerably slower to escape in the cage emergence test, indicating more anxiety. In accordance with this, Kopp *et al.* (1999) showed that mice of the BALB/c strain are particularly susceptible to chronic stress exposure compared to several other inbred mouse strains. Although BALB/c mice had lower body weights with accordingly lighter organs, the spleen weight of BALB/c mice was significantly heavier than that of CD-1 mice. The spleen reacts actively to blood-borne antigens, and would thus be expected to increase in weight when mice are wounded (Roitt 1988). Although CD-1 mice showed more aggressive interactions, BALB/c mice were generally more wounded (Van Loo *et al.* 2003a).

Corticosterone levels of BALB/c mice followed a time curve similar to time curves found in previous and other experiments (Bronson 1973, Goldsmith *et al.* 1978, Van Loo *et al.* 2001, 2002): after grouping, levels were quite high due to the social tension associated with establishment of a stable hierarchy. Levels then decreased as the hierarchy within groups remained stable and thereafter started to rise again, parallelling an increase in aggression as the mice became older (Van Loo *et al.* 2003a). For CD-1 mice, on the other hand, corticosterone levels increased from the age of 9 to 12 weeks and declined slightly afterwards. A reason for this is difficult to allege.

The correlation between social status and both corticosterone levels and TH activity were investigated as well. These measures reflect the HPA axis and sympathetic activation in response to challenges, respectively (Manser 1992, Moberg & Mench 2000). For corticosterone levels, no effect of social status could be revealed, although Co/Cr ratios of dominant and most-attacked subordinate mice significantly correlated to the level of aggression (Van Loo *et al.* 2003a). This may indicate that the level of aggression within a group influences corticosterone levels to a greater extent than position in the hierarchy. In general, the TH activity of the dominant mice tended to be higher than for the least attacked subordinate mice, while the TH activity of most attacked subordinate mice was intermediate. Previous findings and those of others (Haemisch & Gärtner 1996, Van Loo *et al.* 2001, 2002) are in agreement with these results. The most obvious explanation being that both maintaining dominance and being defeated is stressful, while accepting a subordinate status without ever challenging the dominant male may be less stressful (Busser *et al.* 1974).

Conclusion and recommendations

Long-term enrichment with nesting material combined with the repeated transfer of

nesting material when cleaning the cages influenced several stress-related parameters. The corticosterone levels of enriched-housed mice were lower, their thymus weight was increased, and they consumed less food and water than standard-housed mice while gaining more or equal weight. Since these results are an indication for reduced levels of stress in enriched-housed conditions, the provision of nesting material combined with its transfer during cage cleaning is recommended for group-housed male laboratory mice.

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