Effect of enrichment on variation and results in the light/dark test

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Summary

Several confounding factors may influence the outcome of an experiment and the extent of inter-individual variation. The aim of this study was to investigate if cage enrichment induces an effect on experimental mean values and on inter-individual variation in the light/dark paradigm using diazepam as the anxiolytic drug. The behaviour of 216 naive adult male mice of two different strains (BALB/c and C57BL/6) was studied. The animals were housed in groups of four in 'non-enriched', 'enriched' (nesting material) or 'super-enriched' (nest-box, nesting material, wooden gnawing stick and PVC tube) cages. After 5 weeks the animals were assigned to one of three treatments: control (no injection), sham (saline injection i.p.) or diazepam (1 mg/kg bw i.p.) and tested in the light/dark test for 5 min. Variation data were analysed using three different methods (mean absolute deviation, coefficient of variation and power analysis). The C57BL/6 mice scored higher than BALB/c mice in activity related measurements and showed a less 'emotional' behaviour profile in the pharmacological control situation of the light/dark test. In this study the anxiolytic effect of diazepam was clear in BALB/c mice but absent in C57BL/6 mice. Mice housed in enriched and super-enriched cages gained more weight than mice in non-enriched cages, although food intake was not affected. Generally, the strain of mouse had the greatest impact on both mean values and variation. However, there was no consistent increase for one particular strain. The choice of statistical method for analysing variation may influence the interpretation of within-group variability, but none of the methods showed any significant differences between standard and enriched conditions on variability in any of the parameters measured.

Keywords Housing; exploration; activity; emotionality; LABORAS; mice

Laboratory animal housing is characterized by being a constant, controlled and standardized environment. Small rodents, like mice, are kept in rectangular plastic or metal cages covered with a wire top. Generally, some kind of bedding material such as wood shavings or sawdust is added. The rationale for this type of housing is that it is practical and cost-effective. However, it is an environment lacking most of the structural features or complexity of the natural habitat of the wild genus from which the laboratory animal is derived, potentially resulting in a detrimental effect on their welfare (Meyerson 1986, Wemelsfelder 1990). Attempts have been carried out to improve the environment of the captive animal by adding artificial

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Accepted 14 April 2003

substitutes to natural features, thereby providing the animal with an opportunity to perform a more species-specific behavioural repertoire (Newberry 1995). By doing so, the animal's ability to cope with and to control stressors in its environment is thought to increase, thereby increasing its welfare (Broom 1991, Newberry 1995, van de Weerd 1996, Clark *et al.* 1997). Numerous studies have investigated the preference for and potential benefits of different types of environmental enrichment for laboratory mice (Manosevitz & Joel 1973, Scharmann 1990, Dahlborn *et al.* 1996, Sherwin 1996, 1998, van de Weerd *et al.* 1994, 1997) and the use of enrichment is generally recommended in guidelines for laboratory rodents (Jennings *et al.* 1998). Even in toxicology studies performed under strict GLP protocols, enrichment is encouraged (Dean 1999).

There are many potential sources of variation between animals in an experiment. Genetic variation may be minimized by using inbred strains, and the increasing use of microbiologically defined animals has reduced the risk of confounding factors due to subclinical diseases. Variations due to physical factors such as temperature and humidity are generally controlled by using automatic regulation. Factors of variation within the cage are, however, more often overlooked. Both cage structure and social environment have effects on the individual animal that are not always equal within the same cage. The animal's reaction to its environment may also vary depending on its position in the hierarchy (Blanchard *et al.* 2001) and on how the cage area and features are divided between individuals. Although there is general agreement on the beneficial value of environmental enrichment on animal welfare, concern has been raised that introducing enrichment into the standardized cages of laboratory animals may increase the inter-individual variability, resulting in an increase in the number of animals needed in order to reach statistical significance (Eskola *et al.* 1999, Gärtner 1999, Mering *et al.* 2001, Tsai *et al.* 2002). Others, however, report no adverse effect on variation, which indicate that these concerns may be overemphasized or valid only under certain circumstances and for certain parameters (van de Weerd *et al.* 2002). It has also been hypothesized that animals kept in enriched cages will respond more uniformly and with less stress to novel situations than non-enriched animals (Baumans 1997, van de Weerd *et al.* 2002). Hence, there is a great need for studies investigating the effects of different types of environmental enrichment on the experimental results and variability between animals in a variety of parameters, and also using different statistical methods. In comparing environmental effects on variation, several different methods have been used, such as the coefficient of variation (CV) (Gärtner 1999, Tsai *et al.* 2002), mean absolute deviation (MAD) (van de Weerd *et al.* 2002), and sample size needed to obtain a pre-specified power (SS) (Eskola *et al.* 1999). As it is complicated to compare conclusions between different studies using different measures of variation, we chose to compare the results of the different methods (MAD, CV, SS) within this study to find out whether the choice of method would influence the outcome and interpretation of the extent of variation.

Several experimental models have been developed to facilitate pre-clinical research on the behavioural pharmacology of anxiety (Belzung & Le Pape 1994, Rodgers 1997). One of those models, which we chose for the present study, is the light/dark test (LD) first described by Crawley and Goodwin (1980) and later further validated and modified by others (Costall *et al.* 1989, Onaivi & Martin 1989, Hascoët & Bourin 1998). The paradigm used in the LD test is the conflict between leaving a familiar dark (safe) area to explore a non-familiar brightly lit (unsafe) area, i.e. a model for the evolutionary established trade-off between exploration for resources (food and mates) and staying in the safe home environment and thereby avoiding exposure to dangers such as predators and competing conspecifics. Benzodiazepine compounds such as diazepam are known to be potent anxiolytic drugs and are therefore commonly used as a reference drug when validating new compounds or tests of anxiety. In the LD test, benzodiazepine dose-dependently increases the number of crossings and the time spent

in the light compartment and decreases the latency to enter the light compartment (Baumans 1997, Griebel *et al.* 2000)

The main aim of this study was to investigate if enrichment induces an effect on experimental results and on inter-individual variation in the behaviour of two different strains of mice (BALB/c and C57BL/6) in the light/dark paradigm and/or on general parameters such as body weight and food/ water intake. These strains were earlier used for the validation of the enrichment used in this study (van de Weerd 1996). A commonly used pharmacological treatment (diazepam) was included to assess whether the enrichment would alter the effect of the drug in the two strains used. The magnitude of the inter strain difference served as a comparison.

Materials and methods

Animals and housing

A total of 216 naive adult male mice of two strains (C57BL/6JOlaHsd and BALB/cOla Hsd, Harlan) was used ($n = 12$ per treatment). The mice arrived at the age of 5 weeks and were housed in groups of four in one of three housing conditions (see 'Experimental design') for another 5 weeks prior to the experiment. The mice were housed in a controlled environment with a 12:12 light cycle and with room illumination at desk level 200 lux. A wire topped Makrolon type III cage (825 cm^2, Techniplast, Italy) with sawdust bedding material (Lignocel 3/4, Rettenmeier & Söhne, Ellwangen-Holzmühle, Germany) was used as the standard cage. Food (RMH-TM 10 mm pellet, Hope Farms, Woerden, The Netherlands) and tap water were available *ad libitum.*

Experimental design

We used a $2 \times 3 \times 3$ factorial design (two strains, three housing conditions and three pharmacological treatments) in this study. The animals arrived in four batches with equal number of animals per treatment group. The first three batches arrived with one-week intervals and the last batch 8 weeks after the first batch. On arrival the mice were randomly assigned to one of three different housing treatments: 'non-enriched' (NE), 'enriched' (E) and 'super-enriched' (SE):

- 'Non-enriched' (NE): no enrichment
- 'Enriched' (E): nesting material (two Kleenex® tissues, Kimberly-Clark Corp, Ede, The Netherlands)
- 'Super-enriched' (SE): nesting material (two Kleenex® tissues, 10 g wood wool, BMI, Helmond, The Netherlands), a perforated metal nest box ($8 \times 10 \times 6$ cm) with an attached metal climbing grid, a black PVC tube (Ø 5 cm) and a small aspen gnawing block (Tapvei Oy, Finland).

One week after arrival the mice were weighed for the first time and individually marked on the tail with a felt-tipped waterproof marker. Food and water were weighed weekly for an estimation of group food and water intake starting one week later than body weight measurements. During cage cleaning the enrichment objects were transferred from the old cage to the new cage and placed at the same location as in the old cage. One new Kleenex tissue was added to the E and SE cages every week to compensate for loss due to shredding. After 3 weeks, new wood wool (10 g) was added to the SE cages.

After five weeks, all animals in each cage were assigned to one of three pharmacological treatments: control (C), sham (S) and diazepam (D) (benzodiazepine):

- Control (C): no treatment
- Sham (S): intraperitoneal injection with 0.1 ml of 0.9% saline solution (B. Braun, Melsungen AG, Germany), 30 min before testing
- Diazepam (D): intraperitoneal injection with 0.1 ml diazepam (Valium®, Centrafarm, 0.25 mg/ml) in a dosage of 1 mg/kg body weight, 30 min before testing.

Within each batch, testing was performed for 3 successive days. The test day was randomized per cage and the test order between individual mice was successively altered. One animal per cage was tested before the second mouse of any cage on that particular day. Testing took place between 13:00–17:00 h during the light period of the day in the same room as the mice were housed.

Test procedure: LD test

The light/dark box consisted of a Makrolon type III cage ($38 \times 22 \times 27$ cm) divided into two equally sized compartments: one light compartment painted white on three sides and the fourth side of transparent plastic (to allow video recording), and an open top and one dark compartment painted black on all four sides with a sliding lid on the top to allow for placement of the mouse. A clear Perspex tunnel ($10 \times 6 \times 5$ cm) connected the two compartments. The illumination in the black compartment was 50 lux, in the white area it was increased to 1000 lux, generated by an extra light source. Before each test the box was cleaned with 70% ethanol and wiped with a paper tissue. The mouse was placed in the middle of the dark compartment and was allowed to explore the test apparatus for 5 min.

Behavioural recordings: manual and LABORAS

The latency to enter the light compartment, number of tunnel crossings and duration time spent in the light compartment was scored manually from video recordings using *The Observer* (version 3.0 for Windows, Noldus Information Technology bv, Wageningen, The Netherlands). A crossing was defined as the mouse moving from one compartment to the other with all four paws. The total distance travelled (cm), duration of locomotory activity (s) as well as the duration of immobility (s) and velocity (cm/s) were recorded automatically during the test using LABORAS (Laboratory Animal Behaviour Observation, Registration and Analysis System, Metris, Hoofddorp, The Netherlands), a system described and validated by van de Weerd *et al.* (2001). No distinction between compartments was made for these measurements. The time resolution for behavioural sampling was 0.25 s and the measures based on changes of gravity of the mouse. Changes in gravity that exceeded 1.45 cm/0.25 s were recorded as 'locomotion'. Immobility was registered when the animal moved less than 0.75 mm/0.25 s. In between no behaviour was registered (behaviours such as grooming and rearing would if recorded fit into this intermediate range).

Statistics

Data were analysed using S-PLUS 2000 Professional Release 3 (MathSoft Inc.). Body weight was analysed using a linear mixed effects model, with correction for first cage and then animal nested in cage as random effects to account for dependencies in the data. Food and water intake were measured per group; they were also analysed using a linear mixed effects model, with batch and cage nested in batch as random effects. Duration and number of crossings were analysed with ANOVA, after being transformed to conform better to the normal distribution (square root transformation for duration, natural logarithm for number of crossings). Where appropriate, significant main effects were further analysed using Tukey's HSD method for multiple comparisons. Dependent variables were modelled using three factors (strain, housing, treatment) plus their interactions. Non significant effects were subsequently dropped to arrive at a parsimonious model.

Latency time was limited to 300 s, therefore Cox proportional hazards regression was used to take this into account using likelihood ratio (LR) and Wald Chi^2.

To analyse whether the experimental factors (strain, housing, treatment) influenced the variation of our variables of interest we quantified the variation within each of the cages in three different ways: using CV, MAD and sample size needed to obtain a pre-specified power 'power analysis' (SS). MAD is calculated within each cage as the mean of the distance that individual mice are removed from their cage mean. As SS is a function of CV, namely $SS = \text{constant} \times CV^2$, this means that when SS data are square-root-transformed, results of these two methods are identical to those of untransformed CV data. ANOVA and Wilcoxon rank-sum test were used to compare variations for manually recorded data, Linear mixed effects model and/or ANOVA were used to compare variations for body weight, food and water intake,

ANOVA and Tukey's *post hoc* test were used for LABORAS data. No statistical test was performed between the different measures for variation (MAD, CV, SS).

Results

Body weight, food and water intake

The means and standard deviations of body weight, food and water intake are presented in Table 1. During all 6 weeks and across all housing conditions the C57BL/6 mice weighed consistently more than the BALB/c mice ($P < 0.001$). Weight gain in time differed significantly for the two strains: within the observed 6 weeks C57BL/6 mice gained weight linearly, while weight gain for BALB/c mice tended to level off at week 6. No significant batch effects were found. In this study housing had a significant effect on weight ($P < 0.05$): for both strains mice housed in the enriched conditions (E or SE) were slightly heavier (on average 0.6 g) than mice in the NE housing condition. At all time points food intake of BALB/c mice was higher than that of C57BL/6 mice ($P < 0.001$). The difference decreased in time from 2.5 to 1.25 grams. C57BL/6 mice consumed more water than BALB/c mice at all time points ($P < 0.001$).

Manually recorded behaviours

In the pharmacological control situation, the C57BL/6 mice crossed more frequently ($P < 0.001$), spent more time in the lit compartment ($P < 0.001$), and had a shorter latency time ($P < 0.001$) than the BALB/c mice. For all of these behaviours, the two strains reacted significantly differently to treatment. After diazepam treatment, BALB/c mice increased crossings frequency ($P < 0.001$), increased duration spent in light ($P < 0.01$), and decreased latency time ($P < 0.001$). Diazepam-treated C57BL/6 mice remained unchanged in their behaviour (not significant) in all parameters. No significant batch effects were found. None of the three housing alternatives induced significantly diverging results in either of the strains (Fig 1).

In all activity-related measurements recorded by LABORAS (Fig 2) the C57BL/6 strain scored higher than the BALB/c strain.

Table 1 Means ± SD of body weight (g), food and water intake (g) presented per strain and week

			Week 1 mean ± SD	Week 2 mean ± SD	Week 3 mean ± SD	Week 4 mean ± SD	Week 5 mean ± SD	Week 6 mean ± SD
Body weight	BALB	NE	19.6 ± 1.2	21.3 ± 1.3	22.5 ± 1.7	23.3 ± 1.4	24.2 ± 1.4	24.6 ± 1.4
		E	20.3 ± 1.4	21.7 ± 1.6	22.9 ± 1.5	24.0 ± 1.6	24.7 ± 1.7	25.1 ± 1.7
		SE	20.3 ± 1.3	21.6 ± 1.4	22.8 ± 1.6	23.7 ± 1.5	24.6 ± 1.5	25.2 ± 1.4
	C57	NE	21.2 ± 1.0	22.5 ± 1.3	23.6 ± 1.8	25.0 ± 2.0	25.9 ± 2.2	26.7 ± 2.0
		E	21.7 ± 1.3	22.9 ± 1.6	24.1 ± 1.9	25.6 ± 2.0	26.7 ± 2.2	27.5 ± 2.2
		SE	21.7 ± 1.6	23.0 ± 1.7	24.1 ± 2.4	25.8 ± 2.0	27.1 ± 2.2	27.7 ± 2.2
Water intake	BALB	NE		13.6 ± 1.7	13.5 ± 1.3	14.0 ± 1.3	14.3 ± 1.5	14.4 ± 1.6
		E		14.1 ± 2.0	13.9 ± 1.3	14.6 ± 2.2	14.5 ± 1.2	14.8 ± 1.7
		SE		12.9 ± 2.1	13.2 ± 0.7	14.6 ± 1.9	14.4 ± 2.1	14.6 ± 1.4
	C57	NE		15.5 ± 1.1	16.3 ± 1.2	17.2 ± 1.2	18.0 ± 2.2	17.7 ± 2.6
		E		16.3 ± 1.2	16.1 ± 1.9	17.2 ± 1.8	18.3 ± 2.0	18.8 ± 3.0
		SE		15.3 ± 2.8	17.1 ± 2.2	19.6 ± 4.2	17.9 ± 2.1	19.8 ± 3.7
Food intake	BALB	NE		18.0 ± 2.1	17.1 ± 1.9	16.6 ± 1.8	17.2 ± 4.1	16.1 ± 1.3
		E		16.6 ± 1.7	16.4 ± 1.5	15.8 ± 2.0	15.7 ± 1.6	15.8 ± 1.1
		SE		16.6 ± 1.9	16.4 ± 1.8	15.6 ± 1.8	15.0 ± 1.3	15.3 ± 1.3
	C57	NE		14.6 ± 1.3	14.2 ± 1.3	14.5 ± 1.1	14.3 ± 0.6	14.5 ± 0.8
		E		14.8 ± 0.7	14.5 ± 1.0	14.0 ± 0.9	14.2 ± 0.4	14.6 ± 0.8
		SE		14.7 ± 0.8	13.7 ± 1.3	14.3 ± 1.3	14.5 ± 1.6	14.5 ± 1.5

NE = non-enriched, E = enriched, SE = super-enriched

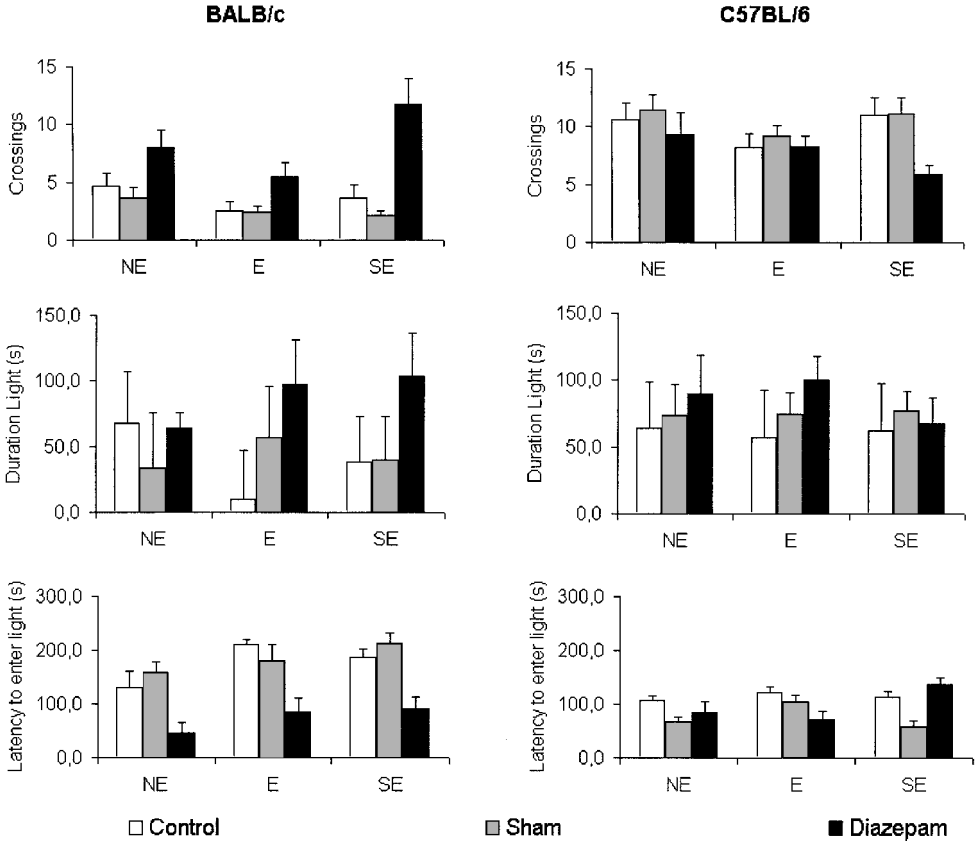

Fig 1 Results of manually recorded behaviours in the light/dark test. Presented as mean ± SE for each strain (in columns), housing (NE = non-enriched, E = enriched, SE = super-enriched) and pharmacological treatment

The duration of locomotion was lower for BALB/c mice than C57BL/6 mice for all treatments ($P < 0.05$). An increased activity was found in BALB/c mice after diazepam treatment compared to the control group ($P < 0.05$) but no effect was found for C57BL/6 mice. For all treatments C57BL/6 mice moved faster (velocity, cm/s) than BALB/c mice ($P < 0.001$). Diazepam-treated BALB/c mice increased their velocity compared to both control and vehicle treatment mice ($P < 0.001$). No treatment effect on velocity was found in C57BL/6 mice. As follows from these results, the distance travelled was higher in C57BL/6 mice than in BALB/c mice ($P < 0.001$). With the LABORAS system no clear effect of housing was found on either locomotory activity or velocity, however a strain × housing interaction ($P < 0.001$) was found in distance travelled. This was caused by the fact that the mean score for BALB/c mice housed in SE cages was higher than for BALB/c mice in the NE and E cages whereas for C57BL/6 mice the mean score for distance travelled was shorter for mice housed in SE cages than for other alternatives. There was a treatment effect of diazepam in BALB/c mice but not in C57BL/6 mice ($P < 0.001$). Time spent in immobility was higher in BALB/c mice than in C57BL/6 mice ($P < 0.001$) for all treatments.

Variation

The results of the three different ways of estimating variation for strain, housing, batch treatment, time and their possible

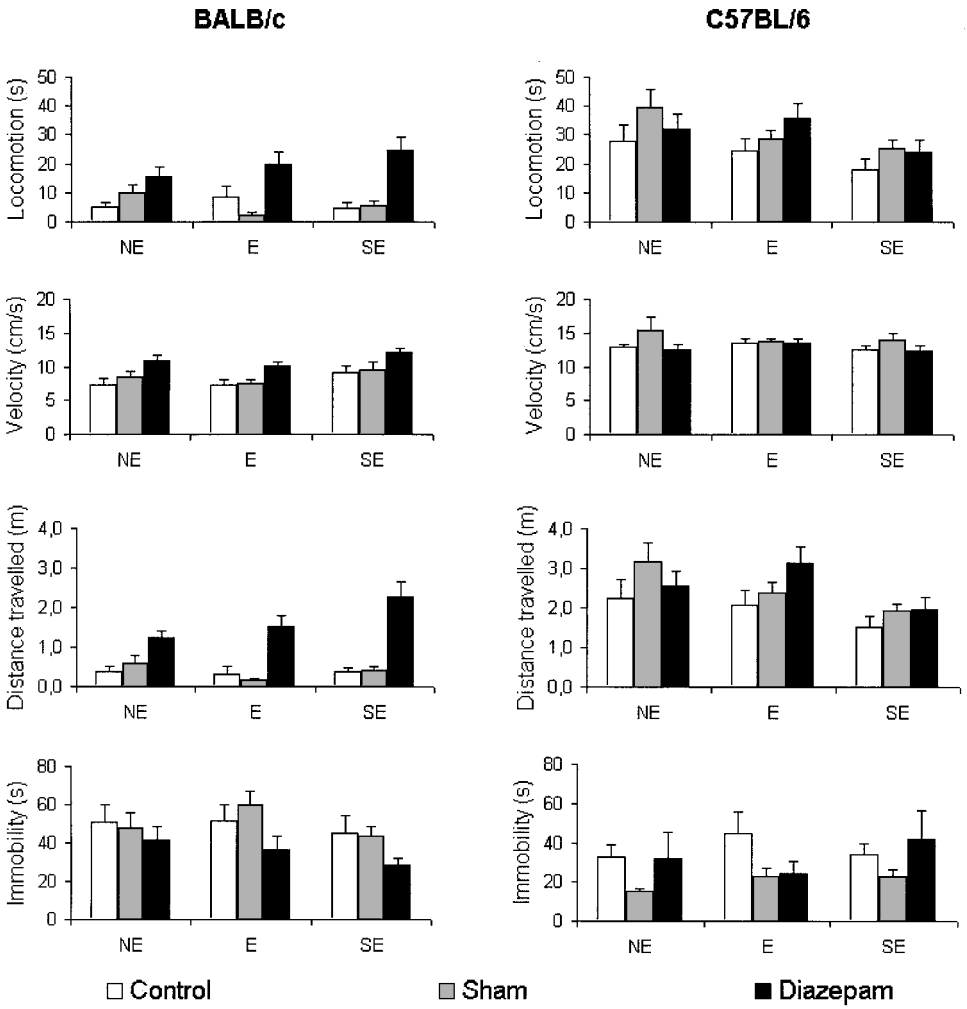

Fig 2 Results of LABORAS behaviour registration system in the light/dark test. Presented as mean ± SE for each strain (in columns), housing (NE = non-enriched, E = enriched, SE = super-enriched) and pharmacological treatment

interactions are presented in Table 2. For variation in water consumption, both MAD and CV had to be transformed using the square root to better conform to the normal distribution. As SS did not conform to normality, even after transformation, the analysis of SS was excluded for both water and food. For the LABORAS data (immobility, locomotion, velocity and distance) SS either had to be transformed using the square root, after which it is equivalent to CV, or did not conform to assumptions even after transformation. Consequently only MAD and CV were used.

Body weight, food and water intake

Variation in body weight was greater in C57BL/6 mice than BALB/c mice using all methods ($P < 0.05$). Looking at all time points simultaneously, significant effects of batch ($P < 0.05$) and strain ($P < 0.05$) were found on food intake using both MAD and CV. At individual time points, the strain effect was

Table 2 Summary of comparisons of variation for each parameter (strain, housing, batch, treatment and interactions) using MAD, CV and SS

Parameter	Method	Strain	Housing	Batch	Treatment	Interactions
Body weight	MAD	* (BALB > C57)	ns	ns	—	* (time × strain)
	CV	* (BALB > C57)	ns	ns	—	* (time × strain)
	SS	* (BALB > C57)	ns	ns	—	* (time × strain)
Water intake	√MAD	ns	ns	ns	ns	ns
	√CV	ns	ns	*	ns	ns
	SS	—	—	—	—	—
Food intake	MAD	* (BALB > C57)	ns	*	ns	ns
	CV	* (BALB > C57)	ns	*	ns	ns
	SS	—	—	—	—	—
Crossings	MAD	ns	ns	ns	* (S < D)	ns
	CV	ns	ns	ns	ns	ns
	SS	* (BALB > C57)	ns	ns	ns	ns
Duration light	√MAD	ns	ns	ns	ns	ns
	CV	*** (BALB > C57)	ns	ns	ns	ns
	SS	*** (BALB > C57)	ns	ns	ns	ns
Latency to enter light	MAD	ns	ns	ns	ns	ns
	CV	ns	ns	ns	ns	ns
	SS	ns	ns	ns	ns	ns
Locomotion	√MAD	*** (BALB < C57)	ns	ns	** (S < D)	* (treatment × housing)
	CV	*** (BALB > C57)	ns	ns	*** (C > S, D)	* (strain × treatment)
	SS	—	—	—	—	—
Velocity	MAD	ns	* (SE > E, NE)	ns	*** (C, S < D)	ns
	CV	** (BALB > C57)	ns	ns	** (C, S > D for BALB)	** (strain × treatment)
	SS	—				
Distance travelled	MAD	*** (BALB < C57)	ns	ns	*** (C, S < D)	ns
	CV	*** (BALB > C57)	ns	ns	*** (C < D for BALB)	* (strain × treatment)
	SS	—	—	—	—	—
Immobility	MAD	ns	ns	ns	* (C > S, D)	ns
	CV	ns	ns	ns	* (C, D > S)	ns
	SS	—	—	—	—	—

Square root transformed data are indicated with √ before the name of the method. Significant differences are presented with direction of differences, non-significant differences with 'ns' and not performed analyses with '—'. *P* values are expressed as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

significant only in week 2 and week 4 using MAD, and in week 4 using CV, where variation in C57BL/6 mice was significantly lower than in BALB/c mice. A batch effect was found also with water intake using CV ($P < 0.05$). Housing had no effect on variability on any of these parameters.

Manually recorded behaviours

For crossings, sham-treated mice had significantly less variation than diazepam-treated mice using MAD. Control-treated mice were intermediate, not different from either sham- or diazepam-treated mice. CV found no significant effects for strain, housing or treatment. For SS there was a significant strain effect ($P < 0.05$), with a higher variation in BALB/c than in C57BL/6. For duration light, clear differences between the three methods were found: MAD found no effects, whereas both CV and SS showed a very clear strain effect ($P < 0.0001$), with BALB/c having considerably larger variation than C57BL/6 mice. A Mann–Whitney test confirmed this: for MAD, not significant, for SS and CV, $P < 0.0001$. The difference between MAD and CV and SS for the strain effect was because, although standard deviations were comparable for both strains, mean values for duration in BALB/c were often low, resulting in high values for CV and SS. For latency to enter light, no significant effects were found

for strain, housing and treatment, using any of the three methods. Housing or treatment effects were not found with any of the three methods.

LABORAS

For *locomotion*, using MAD, effects of strain ($P < 0.001$), treatment ($P < 0.01$) and a treatment × housing interaction ($P < 0.05$) were found. BALB/c mice showed less variation in locomotory activity than C57BL/6 mice. The variation was smallest for sham-treated mice, which was significantly smaller than for diazepam-treated, with control-treated mice intermediate. The treatment × housing effect was due to the fact that for different treatments the variation of housing varied but for none of the treatment groups were these housing effects significant. In contrast to the results using MAD, BALB/c mice were found to have a more variable locomotory activity than C57BL/6 ($P < 0.0001$) using CV. With CV also a differing treatment effect (control > sham and diazepam, $P < 0.01$) and also a strain × treatment interaction (C > S > D for BALB/c and C > D > S for C57BL/6, $P < 0.05$) were found. For *velocity* a significant housing effect was found using MAD ($P < 0.05$), but no strain, batch or treatment effects. Using CV, conclusions were completely different: no housing or batch effects were found, but a strain ($P < 0.01$), and a strain × treatment interaction ($P < 0.01$) were found. For BALB/c a significant treatment effect on variation was found ($P < 0.001$), whereas for C57BL/6 treatment effects were not significant. For *distance*, MAD and CV did not agree at all. Using MAD, BALB/c mice had a lower variation than C57BL/6 ($P < 0.0001$), and a treatment effect ($P < 0.005$) was found where control and sham had less variation than diazepam-treated mice. Using CV, BALB/c showed higher variation than C57BL/6 ($P < 0.0001$), control-treated mice had lower variation than diazepam ($P < 0.01$) and a general strain × treatment effect on variation ($P < 0.05$) was found. On strain level this interaction was only significant in BALB/c mice ($P < 0.01$). No effects of housing or batch were found using either MAD or CV.

A significant treatment effect for immobility could be shown with both MAD and CV. Using MAD, control-treated mice were more variable than sham-treated and diazepam-treated mice. Using CV, diazepam-treated mice and control-treated mice were more variable than sham-treated mice. No strain, housing or batch effects could be detected.

Discussion

In summary, housing had a very limited effect on both mean values and variation on parameters recorded in the LD test in this study. The same was true for variation in body weight, food and water intake. However, mean values in body weight were clearly affected by housing. Strain was a factor of greater influence on the outcome. In the LD test, mice of the C57BL/6 strain had a higher tendency to cross between the two chambers than BALB/c mice and they were also more active in the test. The anxiolytic effect of diazepam was clear in BALB/c mice but absent in C57BL/6 mice with the dose used.

Analysis of variation

The three different statistical measures of variation used in this study: MAD, CV and SS, have all been previously used to describe the effect of enrichment on variation in data (Eskola *et al.*1999, Gärtner 1999, Mering *et al.* 2001, Tsai *et al.* 2002, van de Weerd *et al.* 2002). As these different methods were developed for different purposes and for different types of data there is a risk that choice of method could influence the outcome and interpretation of the extent of variation. CV is defined as standard deviation divided by mean, and is a measure of variation relative to the overall level of response. It is particularly useful in the case of a multiplicative model, i.e. where variation increases with overall mean level. MAD is defined as the mean distance that individual observations have with respects to their group mean. It is independent of overall mean level, and therefore is more appropriate in additive models, i.e. when size of variation is independent of mean value. SS, on the other

hand, directly addresses the issue that in the case of larger variation more animals are needed to retain the same power, i.e. the probability of correctly obtaining a statistically significant test result. SS is a multiple of the square of the CV, and as such is usually not appropriate for either additive or multiplicative models but only as a practical tool to predict the number of animals to be used in a study with a certain predicted variation.

In this study, the factor causing most of the variation was strain. However, which strain caused the largest variation depended on the parameter and method used. Differences in variation resulting from housing were only found for one parameter (velocity using MAD). For weight, water intake, food intake and manually recorded behaviours the three methods mostly agreed and no housing effects were found. For LABORAS data, analyses using MAD and CV differed quite often, not only for significance of effects, but even for direction of effects.

Although no statistical comparison was made between the results of the different methods, it is clear from our results that by choosing one method, the interpretation of the effect on variation caused by housing environment may differ from one method to another. This fact is important to take into account in future studies of variability. Overall, however, the majority of the comparisons showed no differences in estimated variation between the different methods comparing variation. The differences in results between MAD and CV for LABORAS data seem to be caused by the fact that mean values can be close to zero, resulting in high values for CV (=SD/mean), meaning that relative variation (CV) can be quite different from absolute deviation (MAD).

Strain differences

The LD test has been proposed as a model for 'state anxiety', which is defined as anxiety that the subject experiences at a particular moment in time and that is increased by the presence of anxiogenic stimuli, as opposed to 'trait anxiety' which does not vary from moment to moment and is considered to be an enduring feature of an individual or strain (Beuzen & Belzung 1995). The BALB/c strain is often classified as an 'emotional' or high-reactive strain and the C57BL/6 strain as a 'non-emotional' or low-reactive strain (Beuzen & Belzung 1995, Kopp *et al.* 1999). This is in accordance with our study, where untreated controls of the BALB/c mice showed greater 'emotionality' than C57BL/6 mice in all parameters recorded in the LD test. In general, C57BL/6 mice show higher levels of activity than BALB/c mice in tests like the open field (File 2001). This was true also in the present study both with regards to crossings, velocity and distance travelled.

The diazepam dose used in this study did not affect the behaviour of C57BL/6 mice, whereas it had an anxiolytic effect on BALB/c mice. The dose chosen was derived from earlier studies as an intermediate dose resulting in anxiolytic action but not sedation in these strains; however a difference in sensitivity to diazepam between these strains is in accordance with earlier findings (Baumans 1997, Kopp *et al.* 1999, Griebel *et al.* 2000). The lack of effect on C57BL/6 mice of the diazepam dose used in this study makes comparisons between diazepam-treated mice uncertain, and interpretations should be made with this in mind. However, this does not affect the validity of the comparisons between control- and sham-treated mice which show clear strain differences in both 'emotionality' and variation.

Housing effects

In this study housing had no effect on the mean of any of the behavioural parameters measured. Only for body weight, where enriched mice weighed more than non-enriched mice, did housing have an effect. This was also shown by van de Weerd *et al.* (1997). For variation, only one parameter (velocity) was affected, and that with only one of the statistical methods (MAD) used.

The term 'environmental enrichment' is used both in neuroscience and laboratory animal science but with some potentially important difference in meaning. In neuroscience, the enrichment protocol is mainly based on novelty induced stimulation

and the objects used as 'enrichment' items are changed regularly to measure effects on neuronal plasticity. This is a different approach from what is commonly promoted for enhancing the welfare of laboratory animals. In the latter case a standardized set-up of items validated for having a lasting positive effect on parameters related to laboratory animal welfare is used. In neurological studies, 'enriched' environments have proven to affect brain areas such as the amygdala and the hippocampus, compared to rodents housed under standard conditions, with subsequent effects both on emotional reactivity, memory and learning (van Praag *et al.* 2000). For instance, in a study on the effect of rearing environment on later reactivity (Chapillon *et al.* 1999) showed that BALB/c mice reared under enriched conditions (EC) are less fearful in anxiety tests for both 'state anxiety' using the elevated plus maze and 'trait anxiety' using the free exploratory paradigm compared to mice from standard conditions (SC) of the same strain. For C57BL/6 mice the greatest effect was found on 'state anxiety'. It is unclear what the major contributing factor is that promotes these neurological changes, but it may also be hypothesized that mice kept in a constant enriched environment may react differently to non-enriched mice in tests of anxiety.

In this study enrichment was kept constant apart from the unavoidable changes due to the animal's own manipulation of the objects. The same procedure was performed and the same amount of nest building material was provided in all cages by the same person at all times. However, we found no effect of housing on exploration in the LD test in any of the two strains. It could be argued that the period of enrichment occurred during a less sensitive period in the life of the mouse or that the period was too short to produce any effect. Much research is performed on adult animals purchased from a breeding facility and used in studies after only 1–2 weeks of acclimatization, which makes this study design comparable to the conditions in many other studies. In this study, all mice were reared under non-enriched conditions and assigned to one of the three constant housing conditions as adults 5 weeks before the experiment. The aversive elements of the LD test (light) also differ from the elevated plus maze (openness and elevation) and the free exploratory paradigm (emergence from known environment). However, other studies have found effects on exploratory behaviour when constant enrichment was introduced at a later age of the mice (Dahlborn *et al.* 1996). Previous findings (van de Weerd *et al.* 1994) indicate differences between C57BL/6 mice and BALB/c mice after being housed in an enriched environment in their response to different tests of exploration. No differences between the strains were found in this study.

A recent study (van de Weerd *et al.* 2002), investigated the effect of three different types of housing conditions (similar to the ones in this study) on immune response and Open Field behaviour in male mice. They found no effect of enrichment on the mean immune responses or on variation measured by MAD. In a second experiment using BALB/c mice, they found no effect of housing on variation. In the present study, no main effect on variation due to differences in housing alternatives was found.

It is likely that variation varies with different types of housing, enrichment, and strain, and also with different parameters (Mering *et al.* 2001, Tsai *et al.* 2002, van de Weerd *et al.* 2002). The housing treatments used in this study and the terms used to describe them represent different levels of complexity in the environment. The level of complexity necessary to meet the needs of the mouse has not been investigated in this study. Experience show that in many facilities, enrichment items are changed at irregular intervals and that the amount of nesting material, for example, often differs between cages. This practice may result in a higher variation than if enrichment use is standardized, irrespective of complexity. Correctly applied, cage enrichment may improve the animals' ability to cope with other types of interactions such as experimental procedures (Baumans 1997) and thereby also act to reduce variability between individual animals.

Conclusions

No significant differences in mean were found between standard and enriched housing conditions on any of the behavioural parameters measured in the LD test. The strain of mouse had the greatest impact on both mean values and variation but there was no consistent increase in variation for one particular strain. The choice of statistical method to analyse variation may influence the interpretation of inter-individual variability. However, the three methods used in this study revealed no significant differences between standard and enriched conditions on any of the parameters measured in this study.

Acknowledgments This study was supported by a grant from the 3R Foundation (Grant 6-99). H. Augustsson's stay at Utrecht University was supported by the Swedish Medical Research Council. The authors thank Kristina Dahlborn and Bengt J. Meyerson for their contributions during the writing of the manuscript.

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