

# Effects of Litter Origin and Weight on Behaviour of Outbred NIH/S Mice in Plus-maze and Staircase Tests

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## Summary

The objective of this study was to investigate the effects of litter and weight on the behavior of mice. Male outbred NIH/S mice from 8 litters were randomly distributed among 6 cages and subjected to the plus-maze and staircase tests. The litter from which the animals had originated had a significant effect on the behavior of mice in the plus-maze test; furthermore addition of the covariates final weight and weight gain had no effect on significance or explanatory value. It is proposed that litter origin might influence the adaptation processes, the development of social status and consequently, the behavior of mice. Differences attributable to litter were not observed in the staircase test, but when both weight parameters were added as covariates this proved to be significant. Though the source of these litter-related differences remains to be clarified, these differences do have a significant effect on the behavior of mice. Therefore they need to be considered since knowledge of the litter where the outbred mice originated can partly explain differences in the behavior of the animals. The comparison of models showed that incorporation of the natural features of the animals (as derived from their biological origin) into a calculation can help rationalise the results; and provide ample opportunities for discussion and understanding of this complex issue.

## Introduction

For almost five decades, the 3Rs, *i.e.* Replacement, Reduction and Refinement alternatives have had a central role in laboratory animal experiments (Russell & Burch, 1959). Of these, the Reduction alternative is the most poorly understood, and hence rarely implemented in research. However if reduction was feasible, it should be possible to obtain the same amount of information from fewer animals or to gain better quality from the same number of animals.

A logical approach to Reduction is to use the litter as a natural feature of a group and individual features of animals in statistical analyses (Festing *et al.*, 2002). If litter has a significant effect on the behavior of animals, litter coding would decrease noise and consequently reduce the number of animals needed. This may also be the case for an easily measurable parameter which can be used as a covariate – animal weight. Even if no significant effects are detected, no results would be lost, and the lack of a family or weight effect could be reported. Theoretically this should work better with outbred stocks because of their inherent large variance in phenotype.

In a previous study (Õkva *et al.*, 2004) male outbred NIH/S mice were evenly distributed among experimental groups and subjected to treatment

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with either ethanol or a nitric oxide synthase (NOS) inhibitor N<sup>G</sup>-nitro-L-arginine (L-NOARG). After drug administration, the behavior of mice in the plus-maze test was observed. It was noted that litter was a significant determinant in the behavior of outbred mice, and the use of the litter in statistical analyses could serve as a way to reduce the numbers of animals needed to obtain a statistically significant difference.

The aim of this present work was to study in greater detail the effect of litter alone and combined with the animal weight on the behavior of mice in two exploratory models of anxiety – the plus-maze and the staircase tests. Both of these models combine indices of locomotor activity (total number of entries, number of steps taken) and of the level of anxiety (entries onto the open arms, number of rearings made). It has also been demonstrated that both of these models of anxiety depend on locomotor activity (Dawson *et al.*, 1995; Lister, 1990).

These behavioral models, especially the plus-maze test, are widely used in evaluation of anxiolytic and anxiogenic drugs. Since the validation of the plus-maze test in rats (Pellow *et al.*, 1985) and mice (Lister, 1987), it has been repeatedly shown that anxiolytic drugs increase the percentage of entries made onto, and the percentage of time spent on, the open arms of the plus-maze whereas anxiogenic drugs decrease these measures. In the staircase test, anxiolytic drugs decrease the number of rearings at doses which do not reduce the number of steps climbed (Simiand *et al.*, 1984). Since both the plus-maze test and the staircase test are in widespread use it was decided to study the effect of litter and weight in these two models.

## Materials and Methods

### Ethics

This study protocol was reviewed and approved by the Animal Ethics Committee of the University of Tartu.

### Animals

Naive male outbred albino mice (NIH/S, National

Public Health Institute, Kuopio, Finland) were used. Animals were bred under barrier conditions by the vendor and came with a health monitoring report according to the FELASA guidelines (Nicklas *et al.*, 2002). In the present experiments, the mice were chosen from 8 litters that included at least 6 male mice. Mice used in experiments were identified by the breeder using ear notching. They were housed in litter groups until the 5<sup>th</sup> week of life. Then the mice were evenly and randomly distributed between new cages – *i.e.* each cage included one member from litter 1, one from member from litter 2 *etc.*

The mice were housed in polycarbonate cages (Tecniplast, Italy) measuring 42.5 x 26.6 x 15.0 cm (Eurostandard type III) and were maintained under SPF conditions – water, cages, lids and bedding were autoclaved. The room temperature was 20 ± 2 °C and relative humidity was 50 ± 5 %. Food (Labfor R70, Lactamin, Sweden) and autoclaved water were available *ad libitum*. The mice were exposed to a 12 h: 12 h light/dark cycle. Lights were on from 08:00 to 20:00. Autoclaved aspen chips (chip size 4 × 4 × 1 mm, Estap, Estonia) were used as bedding. At the time of the experiments, the mice were 9 weeks old and weighed 34.5 ± 0.36 g (mean ± SEM). There were significant weight differences between mice from different litters. However, the weight mean and variation were equal in the cage groups established at five weeks of age. The cages were changed on each Monday.

### The plus-maze test

The animals were transported from a familiar animal room to the study room one hour before the plus-maze test in order to allow a period of habituation. The mice could not see the plus-maze apparatus. The plus-maze test was carried out with a minimal amount of background noise from the ventilation system and in dim light. No other activities were taking place in the room.

The plus-maze test was carried out according to Lister (1987). The plus-maze was made of polystyrene and consisted of two open (8.0 × 17.0 cm) and two closed arms (8.0 × 17.0 × 30.0 cm),

which were connected by a central platform (8.0 × 8.0 cm). The plus-maze was elevated 30 cm above the surface level. Mice were placed on the central platform facing an open arm. For the next five minutes, the number of entries made into the open and into the closed arms, and the time spent on the open arms, were recorded. After each mouse, the plus-maze was thoroughly cleaned with antiseptic solution of 1% Virkon®S (Antec™ International, England). From this data, the percentages of entries made onto the open arms, and the percentages of time spent on the open arms, were calculated.

#### *The staircase test*

The staircase test was carried out according to a method slightly modified from those described by Simiand *et al.* (1984) and Thiebot *et al.* (1973). The staircase was made of polystyrene and consisted of five identical steps 2.5 cm high, 10.0 cm wide and 7.5 cm deep. The staircase was surrounded by walls, the height of which was constant along the whole length of the staircase. The mouse was placed on the floor of the box with its head facing the staircase. The number of steps climbed and the numbers of rearings made during a three min period were recorded. Similarly to the plus-maze test, the staircase was thoroughly cleaned with antiseptic solution after each test.

#### *Statistical analysis*

The behavioral data of animals in the plus-maze test and staircase tests and weight changes were analyzed using analysis of variance (ANOVA) using litter as the main effect and weight gain during four weeks acclimatization and final weight as covariates. The two latter models test whether weight is a significant factor within each litter. Only parameters with significant changes were considered for closer inspection.

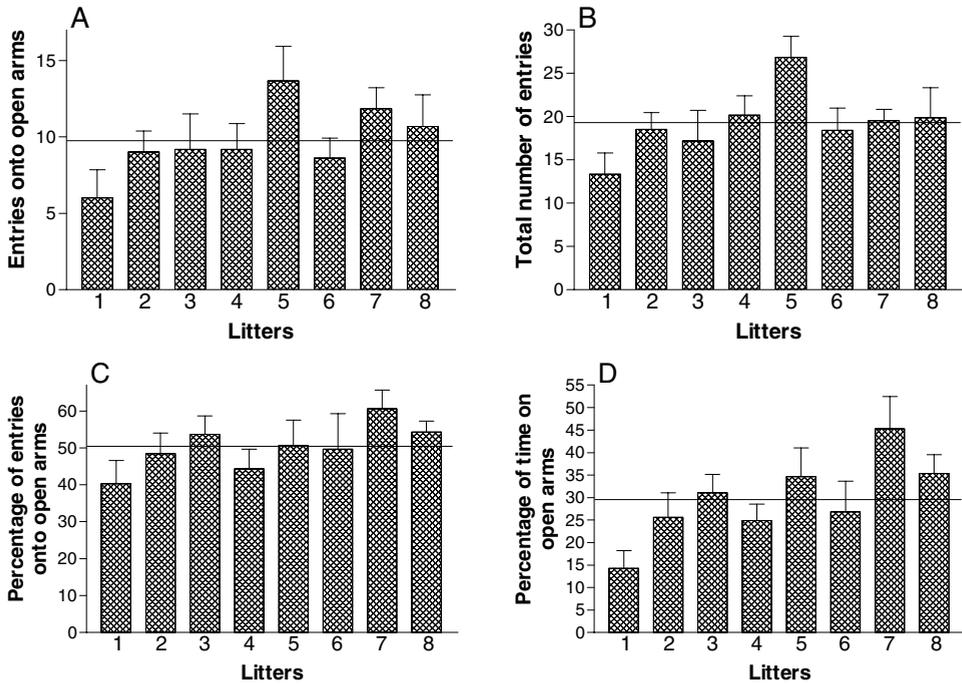
#### **Results**

In the plus-maze test the mean number of entries made into the open arms was  $9.8 \pm 0.7$  (mean  $\pm$  SEM), into the closed arms  $9.4 \pm 0.6$  and the total number of entries was  $19.2 \pm 1.0$ ; the percentage of entries made into the open arms was  $50.2 \pm 2.1$  and percentage of time spent on the open arms was  $29.8 \pm 2.2$ . These data are similar to the values reported in the literature in the classic works of Lister (1987) and File *et al.* (1989).

The cage in which mice were housed had no effect on the behaviour of animals in the plus-maze or staircase test (Table 1). Litter was a significant ( $0.006 < p < 0.010$ ) factor in all open arm parameters (Figure 1), and inclusion of weight gain or final weight did not increase the explanatory value (Table 2).

**Table 1.** The behaviour of mice from different cages in the plus-maze and staircase tests. Data are presented as mean  $\pm$  SEM from groups of 8 mice.

Cage	Entries onto the open arms	Entries into the closed arms	Total number of entries	% Entries into the open arms	% Time on the open arms	Steps	Rearings
Cage 1	7.7 $\pm$ 2.0	6.4 $\pm$ 1.3	14.1 $\pm$ 2.7	52.8 $\pm$ 6.7	28.9 $\pm$ 7.9	50.9 $\pm$ 5.1	18.9 $\pm$ 2.2
Cage 2	9.9 $\pm$ 1.2	9.4 $\pm$ 0.9	19.2 $\pm$ 1.3	50.4 $\pm$ 5.0	27.8 $\pm$ 3.6	34.9 $\pm$ 5.9	15.6 $\pm$ 1.5
Cage 3	13.2 $\pm$ 1.1	9.2 $\pm$ 0.9	22.5 $\pm$ 1.6	58.9 $\pm$ 2.9	39.2 $\pm$ 4.4	45.0 $\pm$ 3.4	18.2 $\pm$ 1.6
Cage 4	9.4 $\pm$ 0.9	10.9 $\pm$ 1.6	20.2 $\pm$ 2.3	47.5 $\pm$ 2.6	28.4 $\pm$ 3.8	45.7 $\pm$ 4.3	18.1 $\pm$ 1.5
Cage 5	9.2 $\pm$ 2.1	10.1 $\pm$ 1.9	19.4 $\pm$ 3.5	47.1 $\pm$ 6.3	27.7 $\pm$ 5.7	42.2 $\pm$ 4.4	15.12 $\pm$ 1.3
Cage 6	9.1 $\pm$ 2.1	10.9 $\pm$ 1.4	20.0 $\pm$ 2.3	43.9 $\pm$ 6.1	26.4 $\pm$ 5.7	46.7 $\pm$ 8.7	19.6 $\pm$ 2.2



**Figure 1.** The effect of litter on the behavior of mice in the plus-maze test. Data are presented as mean ± SEM from litters of 6 mice. This figure shows the number of entries made into the open arms (A), the total number of entries (B), the percentage of entries made into the open arms (C) and the percentage of time spent on the open arms (D) in the plus-maze test. Lines across the bars show overall means of all mice for the corresponding parameter of exploratory behavior.

**Table 2.** Significances and explanatory values (R squared) of litter alone or when combined with final weight and weight gain as covariate in behavioral tests. Abbreviations: NS = not significant

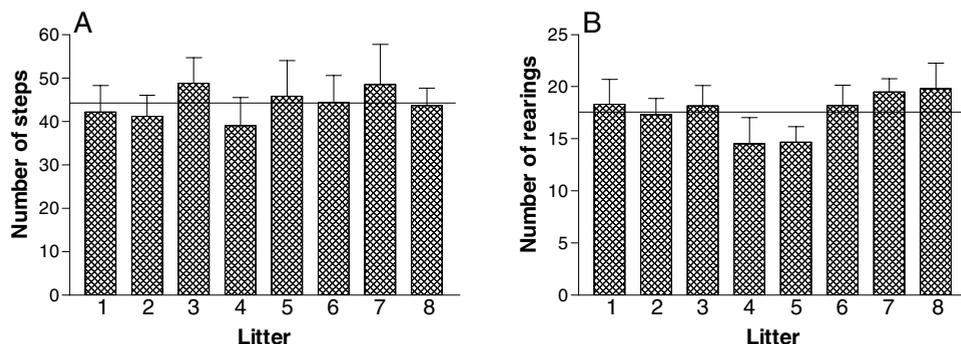
	Litter as main effect	Litter as main effect and final weight as covariate	Litter as main effect and weight gain as covariate
	p-value/R squared	p-value/R squared	p-value/R squared
Time -open	Litter-0.006 / 0.456	Litter - 0.010 / 0.461	Litter-0.009 / 0.457
Entries-closed	Litter-0.051 / 0.351	Litter -0.045 / 0.373	Litter-0.055 / 0.367
Steps	Litter- NS / 0.111	Weight-0.049 / 0.224	Weight-0.033 / 0.243
Rearings	Litter -NS / 0.281	Litter-NS / 0.284	Litter-0.048 / 0.366

Litter alone as the main effect did not reach significance in the closed arm parameter, but statistical significance was achieved by inclusion of final weight of the individual mouse ( $p = 0.045$ , Table 2).

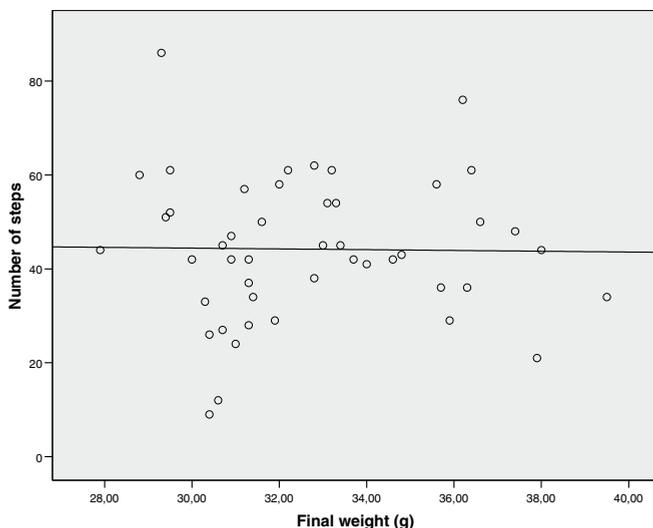
The number of steps made in the staircase was  $44.2 \pm 2.2$  and the number of rearings made in the staircase was  $17.5 \pm 0.7$ . Litter had no effect on the behaviour of mice in the staircase test (Figure 2). Both weight gain ( $p = 0.049$ ) and final weight ( $p <$

$0.033$ ) were significant covariates when the number of steps taken was assessed, and a slightly extensive standard deviation coefficient was achieved by inclusion of weight gain.

In the number of rearings neither litter alone nor combined with final weight showed any significance, but inclusion of weight gain converted litter into a significant ( $p = 0.048$ ) main effect (Table 2). The contributions of the two covariates, final weight and weight gain, are shown in Figures 3 and 4.



**Figure 2.** The effect of litter on the behavior of mice in the staircase test. Data are presented as mean  $\pm$  SEM from litters of 6 mice. This figure shows differences in the number of steps (A) and rearings (B) made in the staircase test. Lines across the bars show overall means of all mice for the corresponding parameter of exploratory behavior.

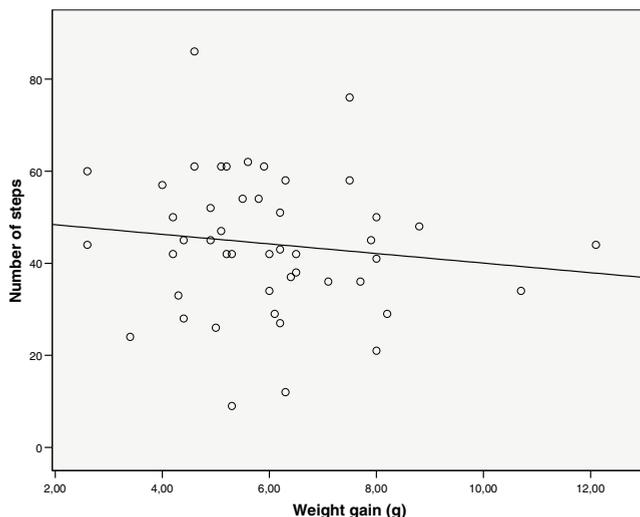


**Figure 3.** Direction of significant ( $p = 0.049$ ) covariate final weight on number of steps taken.

**Discussion**

It is a common practice in outbred large animal studies to pay attention to kinship of individual animals. This does not seem to be case in studies with outbred rodents and rabbits. In safety

that the behavior of mice in the plus-maze test is influenced by several factors, including strain of animals and the specific laboratory where the test is carried out (for review see *Wahlsten et al., 2003*). Whenever outbred stocks are used, it may be possible



**Figure 4.** Direction of significant ( $p = 0.033$ ) covariate weight gain on number of steps taken

evaluation studies the numbers of large animals used are much lower than those of small animals. When the numbers of animals used are high, the chance of bias is low due to an uneven distribution of animals into groups during the randomization.

In academic research, the numbers of rodents in a group may range from five animals upwards, which is indeed similar to the situation in safety evaluation studies. Accounting for litters is really a question of number of animals to be used, not the size of animal.

By definition outbred stocks exhibit heterozygosity, which results in extensive between-animal variation. A litter, *i.e.* group of offspring born at the same time to the same mother, is a natural group, *i.e.* one would expect less within-litter variance than between all animals of the stock (*Festing et al., 2002*).

The results of behavioral studies typically are subject to high variance. It has been demonstrated

to achieve true reduction by taking accounting of the litter. The major finding of this study is that the litter of animals had a significant effect on the behavior of mice in the plus-maze test, but addition of covariates final weight and weight gain had no effect on significance or explanatory value

The two parameters registered in the plus-maze, reflect locomotor activity (total number of entries) and the level of anxiety (open-arm activity). It has been repeatedly demonstrated that compounds that decrease anxiety in man increase the percentage of entries into, and the time spent on, the open arms; and compounds that evoke anxiety in man, in contrast significantly reduce the percentage of entries into and time spent on the open arms (*Pellow et al., 1985*). Therefore it can be concluded that litter of origin has a significant impact on the exploratory behavior and on the level of anxiety.

At the present, one can only speculate on the origin

of litter differences. It has been demonstrated that several factors *i.e.* genetic, developmental and environmental as well as previous social experience, influence the behavior of mice. For example, it has been demonstrated that maternal stress affects the behavior of mice in the plus-maze test (*Palermo-Neto, 2001*) and learning ability in the water-maze (*Nishio et al., 2001*).

It has been repeatedly demonstrated that the re-grouping of rats and mice causes aggressive attack toward unfamiliar animals, social reorganization and stress (*Avitsur et al., 2001; Marrow et al., 1999*). It has also been shown that weight development reflects stress and adaptation processes (*Keeney & Hogg, 1999*) and that social hierarchy of mice has an effect on the behavior of mice in the exploratory models (*Hilakivi-Clarke, 1992; Keeney et al., 2001*). Therefore it can be speculated that litter might have some effect on the adaptation processes, on the development of social status and consequently, on the behavior of mice.

Differences caused by litter were not observed in the staircase test, but addition of a covariate did reveal a significant effect with both weight parameters. Therefore it is possible that different behavioral models differ in their sensitivity to the effect of litter, while larger animals or animals undergoing the greatest weight gain during the adaptation period seem to take fewer steps.

Irrespective of the mechanism of litter differences, it must be recognized that they not only exist but that they also have a significant effect on the behavior of mice. Therefore it makes sense to include knowledge of the litter of outbred mice in attempts to explain differences in the behavior of animals. Furthermore, inclusion of simple data such as body weights as covariates may help in achieving statistical significance for certain main effects, such as rearings in this study.

The litter appears to be a significant determinant of some behaviors in an outbred mouse stock, and use of indices of weight as covariate may improve significances and explanatory values. It is proposed that the use of litter and weight information could

serve the purposes of either Reduction or may increase the precision of the experiment, and should be considered as a key element of good experimental design.

In conclusion the comparison of models show that incorporation of the biological origin (litter) of the animals used into the calculation can improve the explanatory value of the results; and provide new perspectives for discussion and understanding of this complex issue.

#### **Acknowledgements**

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