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The application of traditional behavioural and physiological methods for monitoring of the welfare impact of different flooring conditions in rodents

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Summary

In this study, observations on traditional methods, such as open field test, corticosterone assays and monitoring of barbiturate sleeping time, were performed to validate the impact of housing conditions on the welfare of rats and mice in order to compare the outcome with observations previously achieved by preference tests and telemetry. These traditional methods failed to demonstrate the impact on the animals caused by grid housing previously shown by telemetry and preference studies, and it is, therefore, concluded that these traditional tests may be less sensitive for monitoring subtle small environmental impacts on laboratory rodents.

Although, the methods were not able to reveal any differences in corticosterone level and open field test between housing conditions, there were significant differences between mouse strains and sexes. These differences between strains should be taken into account when choosing the best suitable strain for a study.

Introduction

Evaluating welfare has become increasingly important when using animals for research. Therefore, it is of importance to be able to (a) define welfare and (b) develop methods for its current monitoring.

Welfare may be defined in different ways (*Broom and Johnson, 1993*; *Gonyou, 1993*; *Mench, 1993*; *McGlone, 1993*), but a direct and precise definition of welfare is still missing. Welfare may be defined from a hedonism point of view, i.e. welfare is the net sum of the good and the bad feelings experienced during an animal's life. Alternatively, welfare may be defined from a perfectionism point of view, i.e. welfare is correlated to the animal's attempts to cope with its environment and maintain homeostasis. Finally, welfare may be defined from a preference point of view, i.e. welfare is correlated to the animal's ability to make its own free choices in life and do whatever it likes to do. No matter which definition is used, monitoring stress becomes an important part of monitoring the welfare of an animal. Stress might be defined as *an environmental effect on an individual which overtaxes its control systems and reduces its fitness or appears likely to do so* (*Broom and Johnson, 1993*), i.e. the more stressed the animal, the poorer its welfare. Therefore, a variety of tools has been developed for evaluating stress levels in laboratory animals (*Krohn et al., 2001*). Some of these tools are based upon direct measurements on the animal's physiology, e.g. heart rate, body temperature, blood pressure, levels of corticosterone (*Hennesey and Foy, 1987*), and others on hormones and cells of the immune system (*Bohus et al., 1991*; *Barnett and Hemsworth, 1990*; *Khansari et al., 1990*; *Friend, 1980*). In addition, the activity of liver cytochromes P-450 and other liver enzymes may

be increased as a cause of stress in the animal. An indirect way of evaluating this is by measuring the barbiturate sleeping time, which may be inversely correlated to stress levels (*Lovell, 1986*; *Dairman and Balazs, 1970*). Studies have shown that the effects on the barbiturate sleeping time occur shortly after exposure to the unpleasant condition (*Nielsen et al., 1984*; *Cunliffe-Beamer et al., 1981*).

Other tests monitor the behaviour of the animal, either in the natural environment e.g. home-cage observations (*Hurst et al., 1997*; *Saibaba et al., 1996*), or in a test environment, e.g. as done by the widely used open field test (*Walsh and Cummins, 1976*), in which stress is known to reduce ambulation, i.e. numbers of segments crossed, and rearing, i.e. raising on hind legs (*Bateson, 1991*). Many variations of the open field test have been described (*Dahlborn et al., 1996*; *Prior and Sachser, 1995*; *van-de-Weerd et al., 1994*).

The preference test, which is often used to survey housing environments (*Blom, 1993*; *Baumans et al., 1987*), is the only test in which the animal can give information about its present preference. It is, however, problematic that the animal must choose between a limited number of options, and that only the animal's here-and-now preference can be observed.

Not all the different tests or measurements may be equally sensitive, or may monitor the same impact on the animal. It is, therefore, important to compare these tests and measurements when used for monitoring the same experimental parameters. As an example, we have studied the impact that cage flooring has on the animal. Preference tests have shown that animals given the opportunity avoid sleeping on grid floors (*Krohn and Hansen, 2001*; *Manser et al., 1995*; *Blom, 1993*), and telemetry studies have shown that housing rats on grid floors increases their blood pressure and heart rate (*Krohn et al., 2003*). Therefore, we find a clear indication that grid floor housing has an impact on the animal and this impact may be due to stress. The question is, whether this impact can also be shown by traditional methods for measuring stress, such as corticosterone monitoring, barbiturate sleeping time and the open field test. If this is not the case, these methods may not be sensitive enough to register and reveal small impacts on the animal, which would be needed for studying the impact of less stressful parameters, such as ventilation and levels of different residuals in the environment.

It was, therefore, the aim of the present study to compare observations from traditional welfare methods with observations achieved by preference tests and telemetry when studying the effects of different housing conditions for mice and rats. The study was carried out in two different set-ups, one using rats housed on grid or bedding, and another using mice housed on either grid, plast or bedding. The hypotheses tested were, that animals placed on other flooring than bedding have a shorter barbiturate sleeping time, a higher level of plasma corticosterone, a reduced growth rate, and are less active compared to animals placed on bedding.

Materials and Methods

Barbiturate sleeping time

Thirty-two male rats (LEW/Mol, M&B, Denmark) weighing 150-199 gram were used. The animals were, when not used in the experimental set-up, housed in type III cages, 810 cm^2 floor area (Tecniplast, Italy) with aspen wood bedding (Tapvei, Finland), changed twice a week (due to normal routine in the animal facility), and offered food (Altromin 1324, Brogården, Denmark) and water ad libitum. The rats were housed pairwise and in the same pairs as at the breeder to reduce eventual aggression.

The animals were divided into two groups; one with a test period of four days (n=16) and one with a test period of seven days (n=16). The study was carried out as a crossover design for each test (Table 1 and 2). Between each test, there was a recovery period twice the duration of the test period to eliminate carrying-over effects between each test i.e. eight days or 14 days. During the test, animals were housed either on bedding or on a

Table 1: The experimental set-up for a four day study using barbiturate sleeping time for evaluating the impact of two types of cage flooring on Lew/Mol rats.

	4 days	8 days	4 days
Group 1 (n=8)	Bedding	Standard	Grid
Group 2 (n=8)	Grid	Standard	Bedding

Table 2: The experimental set-up for a seven day study using barbiturate sleeping time for evaluating the impact of two types of cage flooring on Lew/Mol rats.

	7 days	14 days	7 days
Group 1 (n=8)	Bedding	Standard	Grid
Group 2 (n=8)	Grid	Standard	Bedding

grid inlet (Tecniplast, Italy) placed on a small layer of bedding. At the end of each test period, the animals were weighed, and pentobarbitone (Mebumal®, 65 mg kg^{-1}) was given intraperitonally between 0900h and 1000h. After injection, the animals were placed in their home cage for one hour, to ensure steady anaesthesia. (Any animal still showing reflex within an hour after injection of the barbiturate in the home cage was excluded from the test). Hereafter, the duration of the sleeping time was tested by checking interdigital reflexes every five minutes. When the first animal showed reflexes the rest of the animals were checked every two minutes for the rest of the period. The reflex checks were shifted between the two hind legs in order to keep sensitivity (and prevent numbness).

The sleeping time was defined as the time from the injection of mebumal until the return of interdigital reflexes.

All data were tested for normality by the use of normality test (Test: Ryan-Joiner, Minitabs ver 12.1, Minitab Inc). The groups were tested statistically against each other by a two-way-test ($\alpha = 0.05$), as the results were normaly distributed.

The open field test and corticosterone levels

Twenty-four mice, six male BALB/cA/Bom, six female BALB/cA/Bom, six male C57BL/6J/Bom and six female C57BL/6J/Bom (M&B, Denmark), eight weeks of age, were used in a multifactorial design. The mice were divided randomly into six groups, three female groups and three male groups (Table 3). By using multifactorial design, the numbers of animals in each group were reduced as the groups can be pooled afterwards to analyse effects of the different conditions.

The animals were housed in type III cages (Tecniplast, Italy). Food (Altromin 1324, Brogården, Denmark) and water were given ad libitum. The groups on bedding were housed on aspen wood bedding (Tapvei, Finland). The

Table 3: The multifactorial design for evaluating the impact of three types of cage flooring on mice tested in open field and by corticosterone monitoring.

Group	Sex	Strain	Housing condition
1	♀	C57BL/6J/Bom (n=2)	Bedding
		BALB/cA/Bom (n=2)	
2	♂	C57BL/6J/Bom (n=2)	Bedding
		BALB/cA/Bom (n=2)	
3	♀	C57BL/6J/Bom (n=2)	Plast
		BALB/cA/Bom (n=2)	
4	♂	C57BL/6J/Bom (n=2)	Plast
		BALB/cA/Bom (n=2)	
5	♀	C57BL/6J/Bom (n=2)	Grid
		BALB/cA/Bom (n=2)	
6	♂	C57BL/6J/Bom (n=2)	Grid
		BALB/cA/Bom (n=2)	

groups on plast were housed on a plastic inlet placed on a small layer of aspen wood bedding to absorb the urine. The groups on grid were housed on a grid inlet (Tecniplast, Italy) placed on a small layer of aspen wood bedding. The cages were changed twice a week.

The experimental period lasted for four weeks, and during that period of time, animals were weighed once a week, and at day 4, 14 and 21 blood samples were collected by periorbital puncture with a glass capillary tube after the animals had been sedated by inhalation of Isofluran (IsoFlo™ vet, Schering-Plough Animal Health, Denmark) in one minute, to ensure that the blood samples were taken within two minutes from capture of the mouse. All samples were taken between 0900h and 1000h.

The blood samples were centrifuged and plasma was separated and frozen. The plasma samples were analysed and the level of corticosterone were determined by the use of the ^{125}I RIA Kit (ICN Biomedicals Inc, U.S.).

After four weeks, the animals were tested in an open field test. All animals were tested between 1000h and 1400h. Each animal was tested for five minutes in a circular arena (diameter=90 cm,

height=50 cm) which was divided into an inner circle (diameter=30 cm) and an outer circle divided into twelve subfields. Each mouse was recorded on an ordinary video, which was subsequently studied manually, registering the following parameters: latency (time from being placed in the inner circle until the mouse leaves the inner circle for the first time), ambulation (number of lines crossed in the outer circle), and rearing (number of times the mouse stands on it hind legs).

For both studies, the room had automatic day/light shift (06.00 hr to 18.00 hr), room temperature at 23±1 °C and relative humidity at 45±5 %. The room was ventilated 10-15 times per hour.

All data were tested for normality by the use of normality test (Test: Ryan-Joiner, Minitabs ver 12.1, Minitab Inc). Corticosterone data were analysed by the use of the GLM procedure (SAS ver 6.12, SAS Institute Inc, USA) for differences between sexes, strains and housing conditions (α = 0.05). Open-field test data were tested by the use of Mann-Whitney u-test with confidence interval (α = 0.05) (Minitabs ver 12.1, Minitab Inc), as the results were not normaly distributed.

Results

Barbiturate sleeping time

Different flooring conditions did not lead to significant differences in barbiturate sleeping time (Figure 1). No significant effects were found between the groups neither for the four days nor for the seven days study. No carryover effect between the two test days could be detected. A power analysis showed that the minimum difference, which could be shown, would be 20 minutes difference with a power of 80 per cent and 23 minutes with a power of 90 per cent.

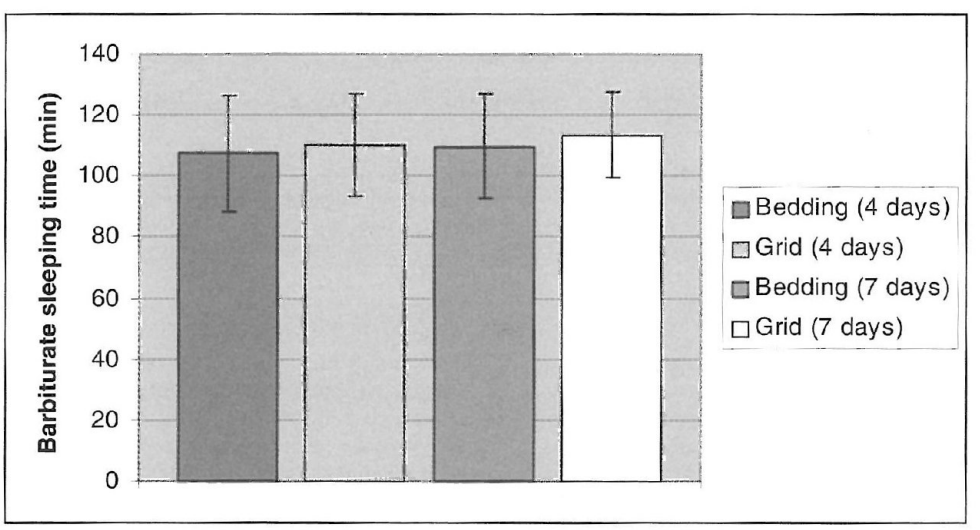

Figure 1: Results of testing barbiturate sleeping time in rats housed on different types of flooring (bedding or grid) in a four and seven day experiment. The sleeping time is shown as minutes from injection of pentobarbitone (65 mg kg^{-1}) to the return of interdigital reflexes. Mean values for the groups ± standard deviation. No significant differences were found neither between bedding and grid, nor between the four days and the seven days study.

Growth curves

The weight scheme for the mice used for the open field test and corticosterone monitoring is shown in Figure 2. Males had a higher growth rate than the females, whereas both sexes housed on bedding showed a higher, but non-significant, growth rate compared to the two other housing conditions. A power analysis showed that the minimum difference, which could be shown in the growth curves, would be a 6 gram (20%) difference with a power of 80 per cent and a 7 gram (23%) difference with a power of 90 per cent.

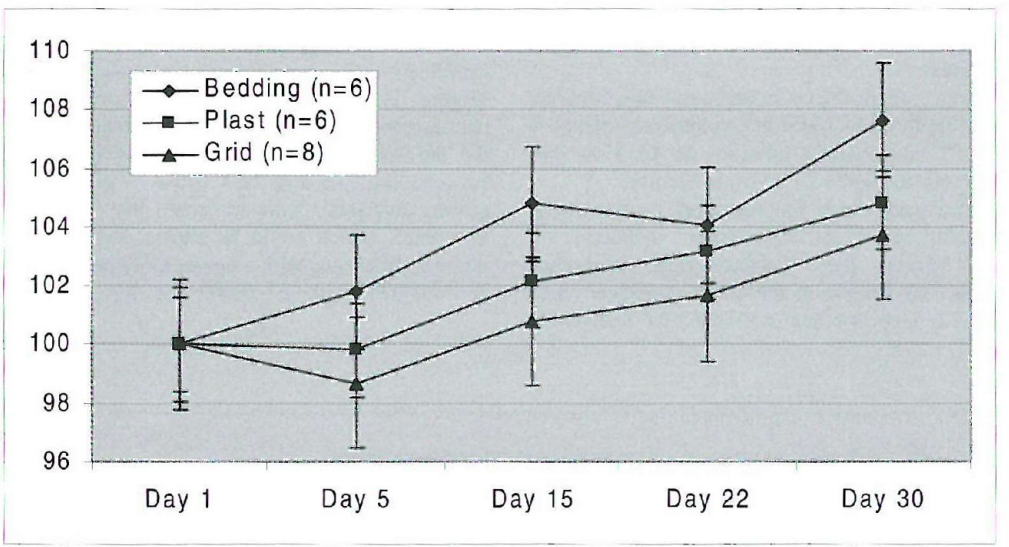

Figure 2: Growth curves for mice housed on three different types of flooring (bedding, plast or grid). The results are mean-values ± standard deviation for each housing condition standardised to day 1 with the value of 100. None of the differences between the three housing conditions were statistically significant.

Corticosterone levels

Figure 3 shows plasma corticosterone levels for mice compared between sexes as well as strains. The results were pooled in different categories and analysed across sex, strain and housing conditions. There were no significant differences between the different housing conditions, but females had significantly higher levels than males and BALB/cA mice had significantly higher levels than C57BL/6J mice. A power analysis showed that the minimum difference, which could be shown between the three housing conditions, would be differences more than 100 ng/ml with a power of 80 per cent.

Open field test

Table 4 shows the results of the open field test. The results were pooled in different categories and analysed across sex, strain and housing conditions. No significant differences between sexes or housing conditions were observed, but BALB/cA mice were significantly less active than C57BL/6J mice. A power analysis showed that the minimum difference in ambulation, which could be shown between the housing conditions, would be 40 lines with a power of 80 per cent and 50 lines with a power of 90 per cent.

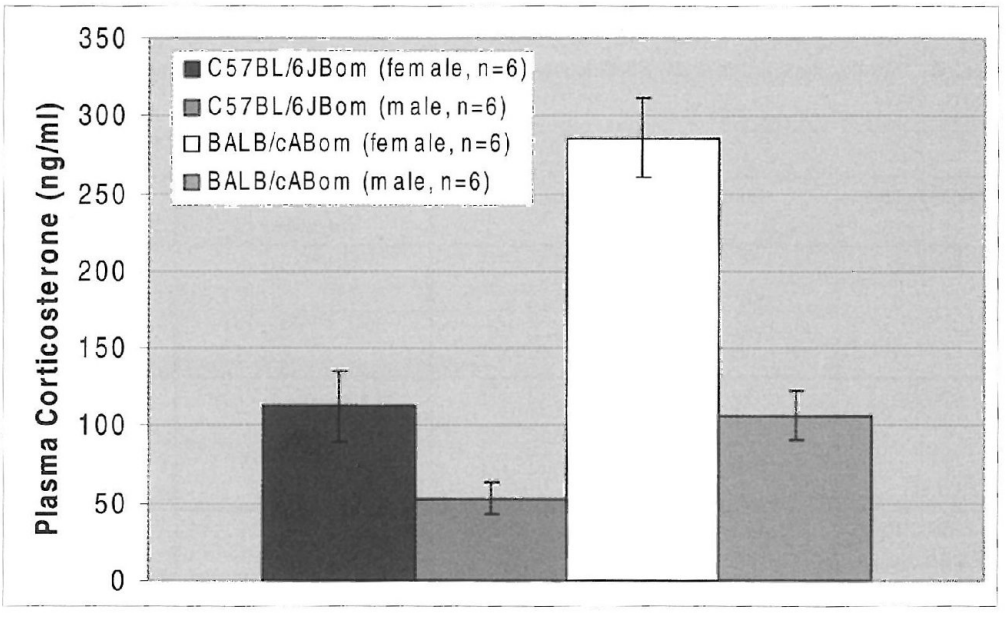

Figure 3: Plasma corticosterone levels for mice and effects of sex and strain. The results are mean-values ± standard error. There was a significant difference between the sexes and between the two strains ($p<0.05$), but no significant differences between the three housing conditions; bedding, plast and grid (not shown).

Discussion

Previous telemetry studies have shown that housing on grid floor has an impact on animals' physiology (*Krohn et al., 2003*), and previous preference studies have shown that rodents will avoid sleeping on grid if possible (*Manser et al., 1995*; *Blom, 1993*; *Krohn and Hansen, 2001*). In the present study the use of the barbiturate sleeping time test, open field test and corticosterone monitoring failed to demonstrate any impact on rodents from difference in flooring conditions. On the other hand, it was possible to show differences in the chosen parameters between the mouse strains used.

In previous studies it has been shown that barbiturate sleeping time is reduced in relation to stress, e.g. as caused by single housing (*Einon et al., 1976*; *Dairman and Balazs, 1970*). In the same way it has been shown that the activity/ambulation in the open field arena is affected by the housing conditions (*Dahlborn et al., 1996*; *Prior and Sachser, 1995*; *van-de-Weerd et al., 1994*). There might be two possible explanations why the results of the barbiturate sleeping time differ from results of telemetry studies (*Krohn et al., 2003*), preference tests studies (*Krohn and Hansen, 2001*; *Blom, 1993*) and the present growth curve monitoring. First, barbiturate-sleeping time may not be a very sensitive method and, therefore, it may only be possible to show differences if the stress level is significant. For a social animal such as the rat, single housing has a significant effect on the animal's physiology and biochemistry (*Perez et al., 1997*; *Viveros et al., 1990*) and these effects are very likely due to stress. Being housed on a grid floor may not be that stressful (*Manser et al.,*

Table 4: The results for mice subjected to three different housing conditions tested in the Open-Field. The results are shown as median with indication of range for the strains, the sexes and the three housing conditions (bedding, plast and grid). Significant differences within the different parameters are marked with* ($p < 0.05$).

Parameters	Details	*Latency (sec)*	Activity/Ambulation (number)	Rearing (number)
Strain	BALB/cA/Bom (n=11)	3 [1-57]	90 [47-107]	6 [0-24]
	C57BL/6J/Bom (n=9)	2 [1-5]	141 * [78-198]	29 * [18-42]
Sex	Male (n=10)	2 [1-57]	113.5 [47-175]	18 [0-42]
	Female (n=10)	3 [1-43]	99.5 [54-198]	16.5 [1-36]
Housing Condition	Bedding (n=6)	2.5 [2-6]	92 [78-175]	21 [6-36]
	Plast (n=6)	2.5 [1-5]	101 [86-198]	9 [1-42]
	Grid (n=8)	2.5 [1-57]	120 [47-179]	18 [0-36]

1995), although it has some impact on the animal. Second, avoidance of a factor, as tested in the preference test, does not necessarily mean that the factor is stressful, but simply that it is less attractive than the alternative. However, changes in blood pressure and pulse seem to support the theory (*Krohn et al., 2003*), that housing on a grid floor has a negative impact on the animal, and that the barbiturate sleeping-time test is simply not sensitive enough to show this impact.

As seen from the power analysis of the present study, a difference in barbiturate sleeping time of 20 minutes or more would be detectable. A difference of 20 minutes in sleeping time is a very small change compared to what has been seen when animals are single-housed compared to housing in groups (*Dairman and Balazs, 1970*). So the impact on the animal when housed on grid floors compared to bedding is either not present or much smaller than the impact from being housed singly. In addition, there do not seem to be any carry-over effects from the barbiturate between the two test periods, as the results are the same whether the period between the two tests are either eight days or 14 days.

Neither could this study support the hypothesis, that animals on a grid floor would have a significantly higher level of corticosterone and be less active in the open-field test compared to animals on bedding. Compared to the results from the barbiturate sleeping time, the lack of impact on the corticosterone is not surprising, as the amount of corticosterone is correlated to the barbiturate sleeping time. The liver cytochromes P-450 complex is linked with corticosterone and other stress hormones, as a high level of hormones in the blood means a high amount of liver enzyme. Hormones are cleared from the blood by the liver and decomposed by the liver cytochromes P-450 complex. The complex also decomposes

barbiturates and other foreign chemical components in the organism. The amount of liver cytochromes P-450 complex can be measured indirectly via the barbiturate sleeping time, which may be inversely correlated to stress (*Lovell, 1986*; *Dairman and Balazs, 1970*).

Using the corticosterone level as an indication of stress is difficult as a wide range of parameters has an effect on the level of corticosterone in the blood (*Mason and Mendl, 1993*). The individual variation is very wide, even when the experimental procedures are standardised as far as possible. This means that in order to detect a small impact on the animal, a very large number of animals have to be used to minimize this natural individual variation.

In contrast to the above, the impact seen in previous studies is consistent with the weight curves generated in this study as the growth of mice housed on grid and plastic flooring compared to bedding is lower, although non-significant. These differences may have been caused by differences in the micro-temperature of the animals, which might, of course, have been stressful, but it should also be considered that animals housed on bedding may have the highest growth rate due to a smaller heat loss, as the bedding conserves the body heat more effectively than plastic or grid. Results from previous studies (*van-de-Weerd et al., 1996*) measuring body weight and growth in mice (*Dahlborn et al., 1996*), when housed under different conditions, singly vs. group, standard vs. enriched, showed a significantly lower growth rate when animals were housed in a stressing environment compared with being housed singly or without enrichment (*Chvédoff et al., 1999*). This indicates that being housed in a stressing environment may influence the body weight of the animal, although in the present study the differences may be caused be different insulation effects of the flooring conditions.

It is surprising that genetics rather than gender seem to have a major impact on both corticosterone and activity in the open field. Corticosterone monitoring as well as the open-field test showed differences between the two strains. In addition, significant differences were shown between the measured corticosterone values of the two sexes. This has previously been shown by others (*Nevison et al., 1999*; *Dahlborn et al., 1996*; *van-de-Weerd et al., 1996*; *van-de-Weerd et al., 1994*). Although it was not the original idea in the present study to show and analyse the differences between strains, it is still of interest. The differences between the mouse strains are much larger than the differences that could have been expected as an effect of the different flooring conditions. So, when using these different methods, the strain used must be chosen very carefully. Some strains, e.g. C57BL, are very active in the open field test as shown in the present study as well as by others (*van de Weerd et al., 1997*; *Dahlborn et al., 1996*), whereas other strains, e.g. BALB/c, show a high basal level of corticosterone (*Nevison et al., 1999*; *van de Weerd et al., 1997*).

For future studies, it could be advantageous to get more information about different strains of mice and rats, and how they react to different welfare measurements methods to get the best and most reliable result, and to prevent unnecessary use of animals, leading to a reduction in the use of laboratory animals (*Russell and Burch, 1959*). If the right strain is used, the largest response to a given test will be achieved, thereby lowering the group-size.

The present study indicates that, barbiturate sleeping time, the open field test, and monitoring of corticosterone seem to have a limited value for monitoring small environmental impacts on laboratory rodents, at least when grid or plastic floor is used as stressor. Another kind of stressor may lead to a different result. This underlines that welfare evaluation should be based upon a range of different tests, and conclusions drawn from any experimental condition should be made with caution. Ideally, parallel tests should indicate the same result before any conclusions are drawn.

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