

# BETWEEN THE SPECIES

## Does Lack of Enrichment Invalidate Scientific Data Obtained from Rodents by Compromising their Welfare?

### ABSTRACT

In countries where major animal research is conducted, comprehensive laws exist to ensure that the animals' physical needs are satisfactorily met. However, as animals also require an environment that allows them to fulfill their behavioral needs, this will be the focus of the article. Two studies, which were performed by the author to compare the effects of enriched and un-enriched cage environments on rodent physiology, are described in detail, one on rats and the other on genetically modified mice. There is presented evidence showing that if research rodents are housed in cages lacking structures that allow them to perform their normal behaviors, this can lead to significant changes in their physiology and pathology, possibly leading to erroneous and/or oversimplified interpretations of scientific data. The question of whether lack of enrichment impairs the wellbeing of research rodents is also discussed.

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## 1. Introduction

Most people who support the use of animals in medical research are adamant that these animals should not lack essential physical needs such as food, water, veterinary care and clean, well-ventilated accommodation that is kept at a temperature, humidity, and light level appropriate for the animal species. In countries where a great deal of animal research is conducted, such as the United States, United Kingdom and Australia, comprehensive laws mandate that institutional animal care and use committees monitor animal facilities within research institutions in order to ensure that they maintain rigorous standards. However, animals also have behavioral needs, and those requirements will be the focus of this paper. According to the U.S. Department of Agriculture (USDA), rodents comprise approximately 90% of all animals used in research today and for this reason I will limit this discussion to rodents. The *Guide for the Care and Use of Laboratory Animals* addresses the existence of behavioral needs as follows:

All animals should be housed under conditions that provide sufficient space as well as supplementary structures and resources required to meet physical, physiologic, and behavioral needs. Environments that fail to meet the animals' needs may result in abnormal brain development, physiologic dysfunction, and behavioral disorders (Garner 2005; van Praag et al., 2000; Würbel 2001) that may compromise both animal wellbeing and scientific validity. The primary enclosure or space may need to be enriched to prevent such effects. (ILAR 1996, 50-51)

Well-conceived enrichment provides animals with choices and a degree of control over their environment,

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which allows them to better cope with environmental stressors (Newberry 1995). For example... elevated shelves for rabbits and shelters for rodents allow them to retreat in case of disturbances (Baumans 1997; Chmiel and Noonan 1996; Stauffacher 1992). (ILAR 1996, 53)

More generally, enrichment in the case of research animals can be defined as items or structures that stimulate animals to interact with their environment, increase species-specific behaviors and decrease abnormal repetitive behaviors. Species-specific behaviors are those that an animal is strongly motivated to perform in a given set of circumstances as a result of stimulating factors from its external environment and/or internal physiology. If such behaviors are prevented, the welfare of the animal is compromised and detrimental effects on physiology and/or behavior can be seen. For example, lack of enrichment can impose a host of adverse physiological consequences on rodents, including an increase in corticosterone levels (Kant et al. 1987; Dunn et al. 1987) and the development of repetitive behaviors (e.g., excessive grooming, digging, rearing, yawning, and fighting/biting) (Wurbel and Stauffacher 1996; Wurbel 2001; Olsson and Dahlborn 2002; Moyaho and Valencia 2002; Wurbel and Garner 2007).

Although the *Guide for the Care and Use of Laboratory Animals* recommends the provision of enrichment in the cages of research animals, it also takes into account the wishes of researchers as illustrated by the following two quotations:

Housing should provide for the animals' health and well-being while being consistent with the intended objectives of animal use. (ILAR 1996, 52)

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Some scientists have raised concerns that environmental enrichment may compromise experimental standardization by introducing variability, adding not only diversity to the animals' behavioral repertoire but also variation to their responses to experimental treatments (e.g., Eskola et al. 1999; Gärtner 1999; Tsai et al. 2003; Bayne 2005). (ILAR 1996, 54)

Since it is critical that experimental outcomes are accurate and reproducible, it is important that the data obtained from rodents correctly represent the actual (average) response(s) to whatever perturbation is under investigation. As a result of scientists' concerns that environmental enrichment may confound their experiments, many research animals, particularly rodents, are sometimes housed in small plastic cages that lack items common to their natural environment and limit their opportunities to perform their natural behaviors, resulting in stress-related repetitive behaviors and activation of the stress response (Wurbel 2001; Olsson and Dahlborn 2002; Sherwin 2004; Wolfer et al. 2004). Stress adversely affects every physiological system, thereby introducing another confounding variable into experimental designs and compromising experimental outcomes. In this paper I will present experimental evidence to support the argument that inclusion of enrichment items is necessary in order to obtain valid and meaningful experimental results from rodents. I will describe two studies on which I was an author, one on rats (Brauner et al. 2010) and one on genetically-modified mice (Cudilo et al. 2007), showing that inclusion of enrichment allows rodents to provide us with a much more valuable insight into their physiology by imposing fewer restrictions on their behavior than does housing them in a sterile environment.

## **2. Experiment 1: Effects of Enrichment on Heart Rate Variability in Rats**

### **Background**

Heart rate variability (HRV) is how the heart rate varies with time. This variability results from a variety of factors including neural input from the parasympathetic and sympathetic nervous systems and is affected by stress and emotions. Sympathetic activation tends to produce low frequency (LF: 0.05 - 0.15 Hz) oscillations in heart rate, whereas parasympathetic activation produces higher frequency (HF: 0.15 - 0.40 Hz) oscillations. By comparing the relative contributions of the two types of oscillations (LF/HF) to HRV, it is possible to determine whether sympathetic or parasympathetic activation is dominant in an animal at a given time. A significant increase in the ratio of sympathetic to parasympathetic nervous activation (LF/HF) is representative of an increased stress and this results in a low overall HRV. In humans, low HRV is associated with individuals who have increased hostility and anxiety levels and a low capacity to deal with these emotions. Using HRV as a measure of stress has the advantage that it is non-invasive and the measurement process itself does not affect the data. Some measures of the stress response, such as taking blood samples to measure concentrations of the stress hormone, corticosterone, actually produce stress themselves.

Heart rate variability is commonly used to predict clinical outcomes in trials involving treatment of heart disease in humans. People with higher HRV tend to have lower mortality rates (Kleiger et al. 1987). This phenomenon is relevant to rodents because rats are considered a good model for cardiovascular disease and are used in trials for drugs intended to

treat heart disease (Dillmann 2008). For this reason, it is important that potential factors that can change HRV in research rats, independent of the effects of the drug being tested, are identified; otherwise a drug may mistakenly be believed to be changing HRV leading to a faulty prediction of clinical outcome. It could be argued that if the results from the experimental group are compared with those from the placebo group, this will cancel out the effects of confounding variables. However, if those effects are large, they may overwhelm outcomes from the drug itself, and so vital information will be lost. One potential confounding factor is emotional stress, such as might result from lack of enrichment. Cage size may also affect an animal's degree of stress. Research studies on the effects of cage size are relatively few in number and the results are divergent (Galef and Durlach 1993; Patterson-Kane 2002). In most animal research facilities, cage size is determined more by cost and space availability than by its effect on animal psychology. There may also be an interactive effect of cage size and cage enrichment on animal wellbeing. For example, a cage may be well equipped with enrichment items, but to an extent that they crowd the cage so the animal's movement is over-restricted.

The goal of this study was to evaluate the effects of enrichment and cage size on LF/HF of rats housed in one of two standard sizes of rodent cages and provided with or without two enrichment items (tube and shelf). Physical activity of the rats was also evaluated by analyzing videotapes taken at regular intervals, day and night because activity also affects HRV (Sherwin 2004; Miki & Yoshimoto 2005) and so it is important to evaluate any changes in activity of the rats when they are housed under different conditions.

## Experiment

Before the experiment, the 10 rats were housed in pairs in large, enriched cages because our previous preliminary studies (Baldwin et al. 2005) showed that rats housed in large, enriched cages demonstrated less aggressive nocturnal behavior than those housed in small, un-enriched cages. The cages were located in a university animal facility with a 12 hour light-dark cycle. One of each pair of rats was pre-implanted with a telemetric transducer to allow remote measurement of electrocardiogram (ECG) from which LF/HF was derived. At the start of the experiment the rats were housed in the small un-enriched cage (SU) and (after the first 3 week assessment) were randomly assigned to each of the other three cage conditions, small enriched (SE), large un-enriched (LU), and large enriched (LE), until they had experienced each condition once. All of the cages contained a layer of pine shavings as bedding. Large cages provided a floor area of 3.5 cm<sup>2</sup> per gram weight (350 g rats) or 4.0 cm<sup>2</sup>/g (500 g rats), and small cages provided the rats with a floor area of 2.5 cm<sup>2</sup> per gram weight. The enrichment items consisted of a polyvinyl chloride tube and a wire mesh shelf to increase the complexity of the cage while stimulating the rodent's natural species-specific behaviors (nesting behaviors and subordinate rat escape behaviors). Our previous observations on seven pairs of rats videotaped for ten 10-minute periods each in the morning and evening (Baldwin et al. 2005) showed that on average each rat spent 51% ± 20% of the observation time interacting with either of the items. In addition, these items were chosen because of their widespread accessibility at most university animal facilities (Institute of Laboratory Animal Research 1996). During the first week of each cage condition, the rats acclimated to their new surroundings. For the next two weeks ECG data were collected and the rats were videotaped for behavioral analysis twice a day (8 am and

8 pm) for 10 minutes, three days a week. Rat behaviors involving activity were classified from video recordings by means of an established Rat Ethogram. Activities were subdivided into non stress-associated locomotive activities, such as moving on the cage floor, moving in and out of the tunnel and climbing onto and off of the shelf, and stress-associated activities such as excessive grooming of self and/or cage-mate, digging and scratching the floor, rearing and fighting or biting cage-mate. The percentage of total time each rat spent performing active non-stressed and stressed behaviors was evaluated.

### **Hypothesis**

It was hypothesized that providing rats with larger cages and enrichment would reduce their stress levels assessed by decreased sympathetic nervous activation, increased locomotive activity, and decreased stress-associated activity, resulting in reduced LF/HF.

### **Results**

There was no difference in LF/HF between the four cage conditions when considered independent of sleep/wake cycle but LF/HF increased when the rats were awake and active ( $p < 0.05$ ,  $F = 32.3$ ) (Figure 1a). Since the HF component (primarily parasympathetic nervous activity) was not different, regardless of cage condition or time of day the increase in LF/HF ratio reflects an increase in sympathetic nervous activity (SNA). The amount of time spent in the active state increased during the evening ( $p < 0.05$ ,  $F = 80.47$ ) (Figure 1b). The increase in LF/HF seen when the rats were awake compared to asleep was driven by the un-enriched cage condition ( $p < 0.05$ ,  $F = 5.63$ ) as no significant change in LF/HF between morning and evening was observed in the enriched environment (Figure 2). On the other hand, the increases in total activity levels observed when the

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rats were awake compared to asleep were seen in both enriched and un-enriched conditions ( $p < 0.05$ ). However, stress-related activity only increased when the rats were awake compared to asleep in the un-enriched cage condition ( $p < 0.05$ ). Increases in heart rate and blood pressure were also observed in the evening compared with the morning, but these changes did not differ between cage conditions. In summary, the data suggest that enrichment significantly reduces the difference in LF/HF experienced by the rats throughout the sleep/wake cycle in the un-enriched cage condition and that this effect cannot be explained by a reduced variation in total activity levels.

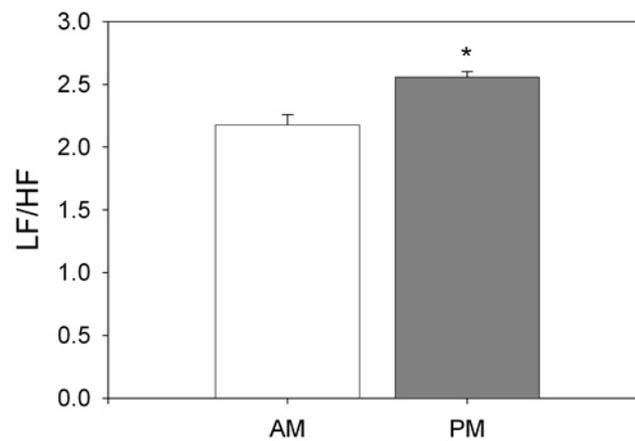


Figure 1a. Averaged over all cage conditions, LF/HF increased in the PM.

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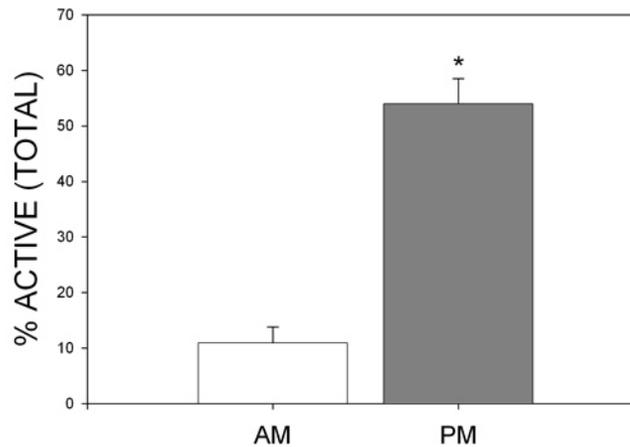


Figure 1b. Averaged over all cage conditions, activity increased in the PM

The increases in LF/HF and activity seen in the evening when the rats were awake occurred for both the small and large un-enriched cage conditions (Figure 2). Thus, increasing cage size above the recommended minimum, without adding enrichment was insufficient to minimize diurnal changes in LF/HF experienced by the rats. However, there was a significant decrease in locomotive behavior, averaged over day and night, of rats in the small, enriched cages compared to those in the large enriched cages, suggesting that the presence of the enrichment items in the small cages restricted the rats' normal locomotive activity.

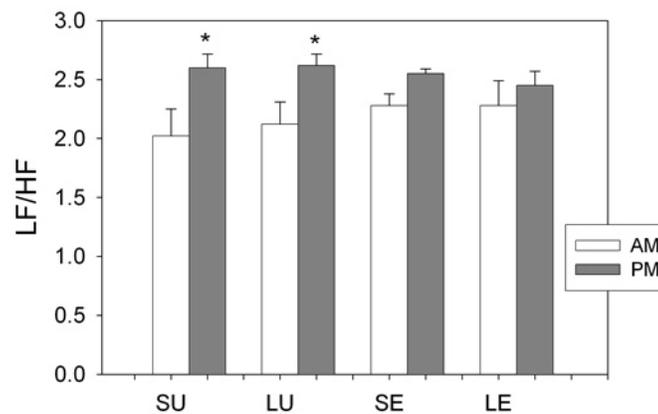


Figure 2. LF/HF, AM vs PM for all four cage conditions.

### 3. What does this study tell us about effects of lack of enrichment on validity of data?

Contrary to our hypothesis, increasing cage size and adding a tunnel and a shelf to the cages did not result in an overall reduction in sympathetic nervous activation of the rats (reduced LF/HF) nor did it increase their locomotive activity. Enrichment did, however, reduce the time they spent performing stress-related activities. In addition, provision of enrichment, regardless of cage size, significantly reduced the apparent diurnal rhythm of LF/HF. The fact that the circadian rhythms for heart rate and blood pressure were conserved regardless of cage condition, but the circadian rhythm for HRV was not, implies that the latter rhythm is more subtle and only manifests in the absence of external environmental stimuli. Interestingly, another study showed that when miniature swine were housed together in pairs instead of in isolation, the diurnal rhythm of LF/HF also disappeared (Kuwahara et al. 2004). These results suggest that the apparent diurnal rhythm of LF/HF is an artifact

in caged animals, only seen when animals are restricted from performing species-specific behaviors. Such an artifact could easily confound the interpretation of results from clinical trials. Investigators who discount the more subtle consequences of housing rodents in un-enriched cages are in danger of oversimplifying the interpretation of experimental data and its relevance to the human condition.

One problem with optimizing housing for research animals is that both increased cage size and/or the addition of an enrichment item raises the initial cost of experimentation. However, when the small, standardized cages are used, there is a subsequent increase in the cost of the experiment due to the variability of the size of the invoked stress responses between animals, leading to a need to use more animals. This factor, together with the risk of false interpretation of data arising from the confounding effects of un-enriched environments, argues strongly for providing rodents with accommodation that allows for species-specific behaviors.

#### **4. Experiment 2: Arterial Pathology in Knockout Mice**

##### **Background**

Fibulin proteins play an important role in maintaining the mechanical properties of artery walls. Fibulin-4 is an extracellular matrix protein expressed by vascular smooth muscle cells and is essential for maintaining arterial integrity. In humans, low levels of a related matrix protein, fibrillin-1, is linked to Marfan syndrome in which the walls of major arteries are weakened, leading to aneurysm or arterial rupture. Fibulin-4<sup>-/-</sup> mice, in which both fibulin-4 genes are knocked out, die just before birth due to arterial hemorrhage, but fibulin-4<sup>+/-</sup> mice,

in which only one gene is knocked out, appear to be outwardly normal. One of my colleagues, Dr. Lihua Marmostein, asked me whether I would perform experiments to determine if the fibulin-4<sup>+/-</sup> mice showed normal arterial structure on a microscopic scale.

### Experiment and Results

First I performed experiments on fibulin-4<sup>+/-</sup> mice housed in the usual way (four mice per cage in standard cages (26 cm (length) x 16 cm (width) x 12 cm (height)) containing bedding but no other enrichment). Similar experiments were performed on mice with both fibulin-4 genes intact (wild-type mice). Each mouse was anesthetized and the aorta prepared for preservation for microscopy. The mouse was then sacrificed with an overdose of anesthetic and the aorta preserved, excised and processed for microscopy. In the fibulin-4<sup>+/-</sup> mice, electron microscopy showed localized regions of loosely packed, disorganized arterial tissue or “gaps” between some of the medial smooth muscle cells in the aortas. On the other hand, in the wild-type mice the smooth muscle cells of the aorta were closely connected to each other and the tissue was more compact. The number of gaps per square mm of arterial tissue was more than ten times greater for fibulin-4<sup>+/-</sup> mice ( $172 \pm 43$  (SEM)) than for wild-type mice ( $15 \pm 8$ ) ( $p < 0.01$ ,  $n=8$ ).

I was rather disturbed by the sterile, un-stimulating conditions in which the mice were housed and decided to repeat the experiments on mice housed, two per cage, in larger cages (33 cm (length) x 25 cm (width) x 25 cm (height)) that contained a shelf, ladder, exercise wheel and a tunnel. When the mice were housed in these enriched cages where they could run in the wheel and climb up and down from the shelf and nestle in the tunnel, they weighed significantly less than the mice in the

standard cages, even though they were the same age, and also showed very little fat in their abdomen. Microscopic examination of the aortas of the fibulin-4<sup>+/-</sup> mice revealed that the number of gaps per unit area of tissue ( $35 \pm 12$ ) was only slightly greater than those seen in wild-type mice and was significantly lower than for fibulin-4<sup>+/-</sup> mice in the standard cages ( $p < 0.05$ ,  $n = 8$ ).

### **5. What does this study tell us about effects of lack of enrichment on validity of data?**

This experiment demonstrates for the first time that the environment in which rats live can influence the degree of manifestation of the symptoms of a genetically determined vascular disease. If mice missing a particular gene (knock-out mice) are housed in an enriched environment, their bodies may be able to compensate for genetic deficiencies in some way. For example, it is possible that interactions between the animal and the environment may stimulate the nervous system to promote the release of hormones that bind to factors influencing gene expression and/or gene capabilities (Gilbert 2005). This finding would not have been revealed had the mice been housed in the standard way, as is the case in un-enriched cages. These results illustrate the importance of paying careful attention to the housing conditions of research animals and bearing in mind that different environments and lifestyles, as well as genetic modifications, can alter experimental results.

Genetically modified animals were used in a total of 764,000 regulated procedures in 2003, accounting for 27 percent of all procedures for 2003 (Home Office (2004) Statistics of Scientific Procedures on Living Animals Great Britain 2003 (London: HMSO)), and that number is steadily growing. Therefore it is urgent that interpretation of the data obtained from these

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animals is not oversimplified by neglecting factors that are unrelated to genetic modification. As stated by Animal Aid:

Even when scientists think they have a “good model” it is difficult to determine how much its attributes are due to its genes or to environmental factors. Wildly differing results have been found to occur in different laboratories using the same strains of animal in the same procedures (Crabbe et al. 1999). (Animal Aid 2012)

In summary, the two studies described in this article demonstrate that lack of enrichment in accommodation of rodents used in research can compromise the scientific data obtained from the animals. Apart from the deleterious effects of inappropriate housing of research animals on experimental data, a more basic issue is the effect on their wellbeing as discussed below.

## **6. Does lack of enrichment compromise the welfare of research rodents?**

Welfare can be considered both in terms of physiological welfare, and psychological welfare. In terms of physiological welfare, in the experiment described (Brauner et al. 2010) providing the rats with larger cages containing a shelf and tunnel, did not make any significant difference to their average heart rate, blood pressure or LF/HF. On the other hand, the genetically modified mice did benefit physiologically from being housed in larger cages with a shelf, tunnel and exercise wheel (Cudilo et al. 2007). Not only were they leaner, they were able to compensate for their genetic deficiency regarding the pathology of their aortic structure. It is possible that had the rats in the first experiment also been provided with a wheel, that they might have also shown a physiological improvement.

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Psychological welfare of animals is much harder to evaluate than physical welfare, but it is generally thought that animals are happier if they are not restricted from performing behaviors similar to those they choose to perform in the wild. As long ago as 1965 the importance of providing housing situations that enabled animals to perform species-specific behaviors was recognized in the case of farm animals. The United Kingdom Farm Animal Welfare Council sets forth the following basic requirements for farm animals in its Welfare Code in terms of five freedoms:

1. Freedom from hunger and thirst
2. Freedom from discomfort by providing an appropriate environment including shelter and a comfortable resting area
3. Freedom from pain, injury and disease
4. Freedom to express normal behavior by providing sufficient space, proper facilities, and company of the animal's own kind
5. Freedom from fear and distress by ensuring conditions and treatments that avoid mental suffering

The concept of Five Freedoms originated with the *Report of the Technical Committee to Enquire into the Welfare of Animals kept under Intensive Livestock Husbandry Systems*, the Brambell Report, December 1965.

Many researchers believe that we are also required to ensure that these freedoms are also provided to laboratory animals. The British Society of Animal Science believes that provision

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of these freedoms is applicable to other types of animal use by humans, besides farm animals, but recognizes some difficulty in finding agreement on the interpretation of the fifth freedom and states that, “A scientist’s interpretation of the fifth Freedom implies a requirement to meet the ‘behavioral needs’ of the species”. While it is relatively easy to assess physical welfare, since poor welfare results in characteristic changes in physiology and pathology of the body’s regulatory systems, the ability to assess mental welfare is still at an early stage in scientific terms.

According to the Universities Federation for Animal Welfare web site:

Ensuring good welfare is about more than ensuring good health. Animal welfare is about the quality of animals’ lives: their feelings. It is now widely agreed, although it was not always so, that many species are sentient—they have the capacity to feel pain and distress, they can suffer and, conversely, be aware of pleasant feelings—and that this matters morally. But how do we assess, from the animal’s point of view, what matters to them and how much?

The experiments described in this paper show that monitoring an animal’s behavior throughout the day, particularly its interaction with the environment, is one way of evaluating which environmental conditions in the laboratory encourage behaviors similar to those seen in the wild. In addition, using established species-specific ethograms to evaluate the percentage of time that research animals spend in stress-associated activities is a useful way to assess psychological welfare. In the case of the rats observed in the first experiment (Brauner et al, 2010),

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provision of a tunnel and a shelf in the cage significantly reduced the amount of time they spent in stress-associated behaviors. One possible reason for this result may have been that the tunnel and shelf could provide a refuge for one of the pair, should the other become aggressive during their waking period. On the other hand, during the day when the animals were sleeping, the tunnel offered a cozy, enclosed space that encouraged proximity and contact between the pairs, as confirmed by the video-recordings. Since the rats spent significantly more of their waking hours interacting with the tunnel and shelf than not (Baldwin et al, 2005), this suggests that the enriched cage condition is indeed beneficial to psychological animal welfare. In fact, according to the Institute of Laboratory Animal Research 1996, the observation of “increasing animal-to-habitat interactions” is defined as a sign of enhanced animal welfare.

The experiments described in this article, along with the experiments and observations of other investigators provide strong evidence that housing rodents used for research in unenriched cages can impair the animals’ welfare and also compromise the validity of the scientific data obtained from them.

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