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NC3Rs

National Centre for the Replacement, Refinement
and Reduction of Animals in Research

Refinement of rodent research through environmental enrichment and systematic randomization

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Abstract

Recently, conventional housing of laboratory rodents has been criticised for inducing abnormal behaviours and poor well-being, which also questions the validity of many animal experiments. Environmental enrichment may prevent abnormal behaviours and improve animal well-being, but concerns have been raised that it might also disrupt standardisation, thereby reducing the precision and replicability of animal experiments. In this article, we review the logic and evidence surrounding this debate. We show that animal welfare can be improved by beneficial enrichments without disrupting standardization. However, we also argue that standardization is a flawed concept, which entails the risk of obtaining results of poor external validity and therefore needs to be profoundly revised.

Keywords: laboratory rodents, mice, housing, environmental enrichment, animal welfare, refinement, reduction, standardization, replicability, external validity

Introduction

"It is a fallacy [...] to regard experimental animals as inanimate reagents and to make no allowance for the physical responses of these animals to environmental factors which [...] exert their effect through altered behaviour" (1).

Environmental enrichment has been a topic in rodent research for more than 50 years with increasingly diverse applications. Inspired by famous neuropsychologist Donald O. Hebb's incidental finding that the rats he had kept as pets outperformed those from his laboratory in learning and memory tasks (2), scientists began to use environmental enrichment as an experimental variable to study developmental plasticity of brain and behaviour (3). The emerging insights into its effects on brain structure and function led directly to biomedical research on potential therapeutic effects of enrichment on neurodegenerative diseases (e.g. Huntington's, Alzheimer's), aging, and recovery from stroke and other forms of brain damage (4). And in the early 90's, when scientists became aware that the overall well-being of most laboratory rodents may primarily depend on their housing conditions, it did not take long until environmental enrichment became a major subject also in laboratory rodent welfare research.

This research was propelled by findings showing that mice and rats housed in standard 'shoe box' type cages develop a variety of abnormal behaviours, and that they are more fearful and stress-reactive than those housed in enriched cages (5). While some scientists supported enriched housing for these reasons from very early on, claiming that only "happy animals make good science" (6), others were concerned that enriched housing would compromise the validity of animal experiments by disrupting environmental standardization. They argued that it would increase variation in the data, thereby reducing the precision and replicability of experimental findings (7). Although essentially speculative (there was no empirical evidence to support it), this argument turned out to become a major obstacle for the implementation of enriched housing for laboratory rodents. It appeared to beat the welfare advocates with their own cudgel: if enrichment was indeed increasing variation in the data, this would mean that more animals would be needed for each experiment, thereby creating a conflict between refinement and reduction.

In this article, we review the evidence surrounding this debate. We support environmental enrichment because we believe that "good welfare is better science", but at the same time argue that for animal experiments to be truly "good science" the concept of environmental standardization needs to be profoundly revised.

Why environmental enrichment?

Housing systems for laboratory animals have been primarily designed according to hygienic, economic and ergonomic requirements with little or no consideration for animal welfare (8). It is therefore not surprising to find signs of impaired welfare in conventionally housed laboratory animals, including rodents. Most obvious are abnormal repetitive behaviours (9), such as stereotypies (e.g. bar-mouthing, jumping, back-flipping: 10; Figure 1) or compulsive behaviours (e.g. barbering: 11; Figure 2), but fear and stress-related responses have also been demonstrated (8). Abnormal behaviours, fear or stress result when animals are housed in environments where they are chronically exposed to aversive stimuli, where they cannot perform behaviours that would be essential to survival or reproduction in the wild, or where they are unable to perform behaviours that would correct a homeostatic imbalance that they are experiencing (9, 12, 13). In rodents, signs of impaired welfare have been found to be associated with various specific conditions such as social isolation (i.e. in singly housed animals), unavoidable social conflict (e.g. in animals housed in groups but without a means to avoid or terminate social contact), lack of a means to hide from other threatening stimuli (e.g. light, noise, care-takers experienced as potential predators), lack of nesting material (for nest building, to provide a hiding place, and to avoid thermal stress) or sensory and motor deprivation due to a general lack of social and/or inanimate stimulation (5, 8, 9, 12). Thus, there is compelling evidence that some form of environmental enrichment is needed to improve rodent welfare.

Figure 1. *Stereotypic jumping (left side) and bar-mouthing (right) in standard housed mice. Photo by H. Würbel.*

Figure 2. *A mouse that has been barbered by a cage mate. Photo by J.P. Garner.*

Refinement through beneficial enrichment

Unfortunately, the term environmental enrichment is used inconsistently in the scientific literature. For those using enrichment as an experimental variable to study phenotypic plasticity of brain and behaviour, enrichment simply means "a combination of complex inanimate and social stimulation" to increase sensory and motor stimulation, and may even include conditions of overcrowding (4). Obviously, such stimulation does not necessarily need to benefit the animals, and may even induce fear or stress and hence be detrimental to animal welfare. In contrast, animal welfare scientists tend to restrict use of the term enrichment to forms of enrichment that improve animal welfare (12), and sometimes specify those as *beneficial enrichments* (Figure 3). Thus, whether or not a particular form of enrichment improves animal welfare is an empirical question that must involve proper behavioural assessment (9). Debate about whether *enrichment in general* improves animal welfare is obsolete, as it springs from the inconsistent use of the term. For example, in male mice housed in enriched cages several authors found increased aggression associated with elevated stress levels, thus (incorrectly) concluding that environmental enrichment is detrimental to the welfare of male mice (e.g. 14). However, whether or not aggression is increased depends on the form of enrichment – nesting material for instance does not induce aggression both in male and female mice (12). If relevant resources can be monopolized by dominant animals, or if changes in the environment allow animals to establish defensible territories, then social conflict is more likely to occur (15). However properly designed enrichment (e.g. nesting material) can avoid these problems. Moreover, even when agonistic behaviour is increased, this does not necessarily mean that welfare is reduced. After all, agonistic behaviour is part of the animals' natural behavioural repertoire, and particular *kinds* of agonistic behaviour can even strengthen the social relationships within a cage (Howerton, Garner & Mench, unpublished data). Whether welfare is reduced depends on whether the animals are able to cope with such stressors (9, 13).

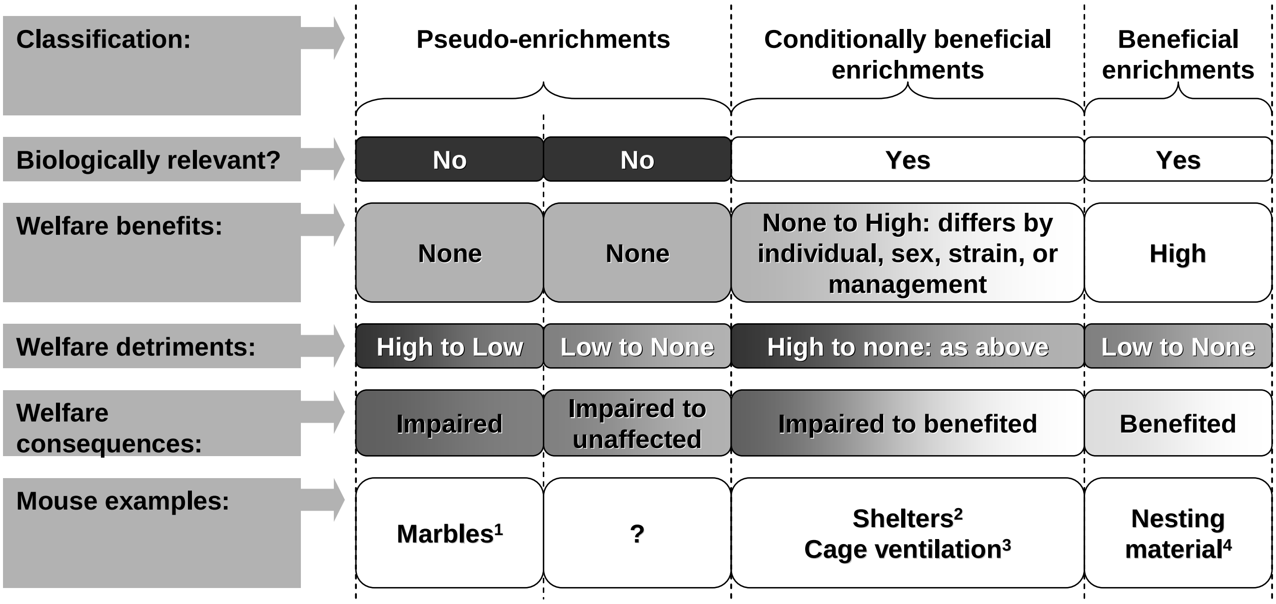

Figure 3. *A classification of enrichments. Enrichments are biologically relevant if they are meaningful to the animal in terms of its natural biology. The shading of each box indicates negative (dark) to positive (light) effects on welfare. Welfare detriments occur when the enrichment introduces a stressor. [1]Marbles are widely used as a stressor in mouse tests of anxiety (17). [2]Shelters sometimes induce territoriality and aggression in group housed mice (18), and thus benefit or detriment mice depending on strain, sex, dominance and management (12). [3]Ventilation reduces ammonia, and therefore benefits physical health, but mice can find the airflow aversive (19). [4]Nesting material is the only mouse enrichment that consistently shows benefits, and does not appear to have associated detriments (12).*

When cages are equipped with structures that allow male mice to avoid or terminate social conflict by hiding or retreat, their stress levels are not increased despite higher levels of agonistic interactions (16).

For these reasons, we distinguish between enrichment as an experimental variable (meaning adding inanimate and/or social stimuli to the environment) and its consequences in terms of animal welfare, and use the term *beneficial enrichment* for cases where enrichment results in improved animal welfare (Figure 3). This distinction is also relevant with respect to the effects of environmental enrichment on the validity of animal experiments. It is clear that a putative enrichment that induces chronic stress is not only detrimental to animal welfare, but also to the validity of experiments with these animals.

However, we are only just beginning to unravel the relationships between housing conditions, animal welfare and the validity of animal experiments. Therefore, the optimal form of enrichment still needs to be worked out for most species, including rodents (12, 20). It may even turn out that there is no single solution for any particular species, as different strains may differ in how they respond to particular enrichments. Furthermore, from research on farm animals we have learned that more extensive housing conditions generally require more sophisticated management, and that their success may critically depend on management practice (21). Animal welfare legislation aims for the individual animal, and the best housing system (i.e. like the natural habitat) may not prevent the occurrence of escalating social conflict, in which case some animals may have to be separated and specifically cared for by animal care staff. This may be the case when shelters are used to enrich the cages of male mice. In addition to providing a hiding place, shelters add a monopolizable resource that can induce increased aggression, resulting in physiological and behavioural signs of decreased welfare (18). Accordingly, the success of shelters as enrichment tools is mixed (12), and male mice especially must be closely monitored if shelters are used. Thus, as illustrated in Figure 3, environmental enrichment can be classified along a spectrum from 'pseudo-enrichments' (that are never biologically relevant, and either neutral or even detrimental to animal welfare) to 'conditionally beneficial enrichments' (that are biologically relevant, but may induce welfare problems if not properly managed) to 'beneficial enrichments' (that are biologically relevant, beneficial to animal welfare, and rarely if ever associated with welfare problems).

Environmental enrichment does not disrupt standardization

Despite its benefits to laboratory animal welfare, implementing environmental enrichment faced (and still faces) great opposition because of concerns that it might disrupt environmental standardization. According to laboratory animal science text books (e.g. 22, 23) environmental standardization essentially serves two distinct goals. First, it is aimed to reduce within-experiment variation, thereby maximising test sensitivity with the welcome effect (economically and ethically) of reducing the number of animals needed for each experiment. Second, it is aimed to reduce between-experiment variation, thereby increasing replicability of results within as well as between laboratories. Based on the assumption that a more complex environment not only produces a higher diversity of behaviour in the home-cage, but also increases inter-individual variability in the animals' responses to experimental treatments, environmental enrichment was thought to reduce the precision and replicability of experimental results and hence their validity (7). Since there was no empirical evidence to support this claim, one of the authors (H.W.) together with colleagues set up a multi-laboratory study to address this question properly. They used 432 female mice of three different inbred strains (C57BL/6J, DBA/2, B6D2F1), half of which were housed from weaning onwards in conventional barren cages, the other half in larger and extensively enriched cages. Enrichments included paper tissue, straw and shredded paper as nesting materials, wooden branches for climbing, and shelters made of wood, cardboard and plastic. These items were gradually added over a 6-week period to expose the mice regularly to novelty also. Three replicates in each of three different laboratories were conducted to study (i) the effect of environmental enrichment on the variance in 20 behavioural response measures (five each from four different standard behavioural tests, including an open field test, an elevated O-maze test, a novel object test and spatial navigation in the Morris water maze) and (ii) the replicability of the test results across the three laboratories and the nine replicates. They found that environmental enrichment increased neither variation in the behavioural test measures, nor the risk of obtaining conflicting data in replicate studies (24). It therefore appears that the housing conditions of laboratory mice (at least for females) can be refined by environmental enrichment to improve animal welfare without adversely affecting standardization.

Standardization and the Red Queen

"For the time being, investigators must be aware of the possibilities that early environmental interactions with genotype may limit the validity of their findings to their own unique laboratory situations" (25).

Public support for animal experimentation is granted on the understanding that any animal suffering involved is outweighed by the contribution of the research to the advancement of relevant human interests (e.g. science, medicine). It is therefore of primary importance that the results of animal experiments be valid with respect to the question being asked. The validity of animal experiments has been questioned on several grounds, one being that barren cages result in abnormal behaviour and physiology, leading to pathological artefacts in the results of animal experiments (5, 9). This is dealt with by environmental enrichment, as discussed above. Another way in which the validity of animal experiments has been challenged is by questioning the external validity of their results, that is whether they are sufficiently robust against minor variation in housing and test conditions for them to be relevant at all (26,27).

Standardization hides poor external validity

Replicability of results is often used as a proxy measure of external validity. Thus, if a result can be replicated in a second experiment, either in the same or a different laboratory, the result is confirmed to be robust, i.e. externally valid. In laboratory animal science, the key concept to increase replicability of results is that of reducing variability by environmental standardization, as discussed above. The rationale is that if the environment is the same for all animals both within and between laboratories, then the results will be highly replicable within as well as between laboratories. However, replicability can only be a proxy measure of external validity if there is variation in housing and test conditions between replicate studies – but this is exactly what environmental standardization is aimed to avoid. Thus, apparent replicability as a result of standardizing otherwise significant environmental effects away can only reflect a false negative effect (due to the loss of the ability to detect such environmental effects). In other words, environmental standardization aims to 'spirit environmental effects away', thereby ignoring that the animals' responses are still affected by that arbitrarily standardized environment in which they have to live (it is as impossible to get rid of the environment as it is impossible not to behave). Moreover, environmental standardization results in pseudo-replication, because it renders the experimental subjects less independent of each other (just as if they were all raised by the same mother), thereby violating the fundamental scientific principle that animals within an experiment need to represent independent entities. This has been referred to previously as the 'standardization fallacy' (26, 27).

More rigorous standardization makes it even worse

Ironically, the standardization fallacy is best illustrated by the poor between-laboratory replicability that environmental standardization causes (against the intentions of its proponents). Although standardization within a laboratory may reduce variation in the data and increase replicability within that laboratory, this 'benefit' is deeply misleading. If a treatment response varies with the exact local constellation of environmental factors, then as different laboratories inherently standardize to different local constellations of environmental factors, results in different laboratories will become more and more distinct, the more rigorously the environment is standardized in each laboratory. This is due to the many environmental factors that resist between-laboratory standardization (e.g. staff, room architecture, noise levels, etc.), as demonstrated in a study by Crabbe et al. (28). The worsening of between-laboratory replicability by attempts to improve it through ever more rigorous standardization has been referred to as 'standardization and the Red Queen' (29; in analogy to Alice running on the spot in Lewis Carroll's *Through the Looking-Glass*).

Implications for research and the 3Rs

The standardization fallacy has serious implications. It may be a primary cause of the many conflicting findings published in the literature. In behavioural genetics studies, for example, non-additive interactions of the experimental treatment (i.e. genotype) with environment have been demonstrated for laboratory (24, 28), feeding (30), experimenter (31), or housing conditions (24, 32). Importantly, if housing conditions affect mutant and wild-type animals differently, as in a study by Rampon et al. (32), or housing conditions mitigate a transgene effect (e.g. 33), then this may lead to fundamentally different conclusions about the function of the mutated gene depending on the conditions under which the animals were housed (5, 26).

The pervasive nature of treatment–by-environment interactions implies (i) that many animal experiments may produce spurious results, the validity of which may be limited to the specific standardized environment employed, and (ii) that there is no chance of knowing whether this is the case, as long as only results from that specific standardized environment are available. Those who argue that environmental standardization reduces animal numbers by reducing variation in the data simply ignore that additional animals will be needed in replicate studies to test whether the results are actually robust against even minor variations in the environment (i.e. if they are externally valid). To sharpen this last point: the benefit of reducing animal numbers per experiment by environmental standardization may amount to the unbearable cost of those animals being wasted for inconclusive research. This cost goes far beyond animal ethics, as it comprises significant scientific and economic costs as well.

Refinement through systematic randomization

In our view, these considerations require profound changes in the design and analysis of laboratory animal research. Thus, systematic environmental variation should become an inherent design feature of experiments, providing not only response values within an arbitrarily standardized environment, but also a measure of their variation across different environments – or at the least a measure of their external validity (27, 29). However, including environment as a factor in the experimental design may easily inflate experiments such that many more animals will be needed for each experiment. So is there any practicable solution to this problem?

The best solution clearly depends on the goal of the research. If the aim is simply to falsify a universal principle, it is of course sufficient to demonstrate violation of that principle in a single genotype in a single standardized environment (27) or even in a single animal. In contrast, if the aim is a comprehensive characterisation of a treatment response, this requires determination of the variation of that response across a range of relevant environments (analogous to dose-response curves in drug research or multiple genetic backgrounds in genetics research).

The vast majority of laboratory animal research is, however, aimed at assessing effects of a particular treatment (e.g. a mutation, a drug, a lesion) on subjects of a particular species or strain of species, and for the results to be relevant they should be reasonably robust against some variation in the environment. For this type of research, simple solutions do indeed exist. These range from running an independent replicate in order to identify spurious results that depend on the exact factor constellation of an experiment (the saying 'never replicate a successful experiment' is very telling in this respect); to systematic environmental randomization using randomized block designs (Figure 4). Randomized block designs allow the introduction of environmental variation in a systematic (i.e. controlled) way without the need for larger sample sizes, while at the same time increasing precision and statistical power (22, 29). Precision and power are higher with randomized block designs because inter-individual variation within each block is normally smaller than overall variation in an unblocked design and because between-block variation is eliminated by comparing treatments always within blocks only (22, 34). Thus, although the underlying shift from standardization to randomization represents a fundamental paradigm shift, the practical implications for animal experimentation in terms of labour, cost and numbers of animals used are surprisingly minute.

Conclusions

Environmental enrichment may or may not improve animal welfare depending on whether or not the enrichments are biologically relevant and beneficial to the animals. In mice (especially males), for example, nesting material may currently be the only enrichment to conventional barren 'shoe-box' type cages that can be recommended unreservedly, although more may be needed to guarantee acceptable well-being. Further research is therefore needed to develop practicable housing systems and enrichments.

Contrary to common claims, there is no evidence that enrichment increases variation in the data of animal experiments, or that it increases the risk of obtaining conflicting results in replicate studies. However, both theoretical considerations and empirical evidence indicate that the concept of environmental standardization (intended to minimize both variation in the data and the risk of obtaining conflicting results in replicate studies) seriously limits external validity of many animal experiments and therefore actually decreases replicability of results. Systematic environmental randomization provides a means to increase the external validity (and hence replicability) of experimental findings without inflating the numbers of animals used. Together, environmental enrichment and systematic environmental randomization therefore contribute to the refinement of animal experiments in the best of meanings of the 3Rs concept.

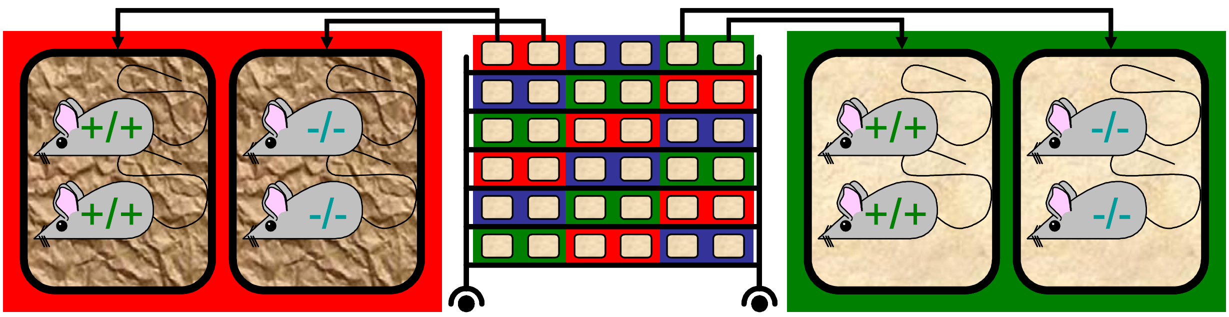

Figure 4. *Example of a randomized block design for an experiment with genotype as treatment and three different housing systems (red, blue, green) to introduce systematic environmental variation. Housing variants red and green may e.g. differ in whether they do (red) or do not (green) contain shelter in addition to nesting material. Variant blue (not shown here) might e.g. be larger cages or involve pre-test handling. The combination of cage and housing variant represents the blocking factor in the statistical design. In principle, however, each coloured cell of two paired cages might represent a slightly different environment. Using cell as blocking factor controls for the environmental variation between the cells and increases external validity of the results without inflating sample size.*

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