Theme 2
Laboratory animal welfare and refinement

Workshop 2.1
Environmental enrichment and housing standards

Refinement Alternative for Animal Housing – Enrichment

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Summary
Enrichment is a refinement as it should promote animal wellbeing. Any refinement needs to be beneficial to animals and should not interfere with the study. While refinement aims can be connected to research data, reduction alternative suffers from lack of research. Instead of trying to assess the impact of enrichment on variance of every determination, it could be more productive to look at effects on variance of welfare indicators; low variance there is likely to reflect as low variance in other determinations. And at the same time aim at most uniform welfare of the animals in the study.

Keywords: environmental enrichment, refinement alternative, reduction alternative

Background

The use and welfare of laboratory animals in research are major issues in modern society. Both public concern on welfare of animals and continuously increasing demands on quality of both animals and of biomedical research make the issues urgent and complex. As a result the use of laboratory animals is experiencing the turmoil of rapidly changing regulations both at European and national levels.

Environmental enrichment is a term increasingly used in connection with laboratory animal care and housing. Baumans (1999) defines enrichment as any modification in the environment of the captive animal that seeks to enhance its physical and psychological well-being by providing stimuli meeting the animals' species-specific needs. This is a performance based definition, as are the definitions for both refinement and reduction alternatives.

Unfortunately the performance of an enrichment item or program has not always been shown before it has been taken into use. Indeed, various items may be placed into the animal enclosure and called enrichment without verification of their efficacy. Some elements in routine care, like use of bedding and group housing can be considered enrichment, and these have been in use before the term was even introduced.

Revised Appendix A of the Council of Europe Convention calls for environmental enrichment and group housing for all gregarious species unless there are scientific or veterinary reasons not to do so (Council of Europe, 2002). Enrichment is considered as refinement as it should promote animal wellbeing. Interference with an experimental outcome could be a scientific reason, and fighting between incompatible animals a veterinary reason for not implementing enrichment.

A closer look at the European regulations (The Convention and The Directive, 1986) reveals that these are tailored to primarily ensure animal welfare. Yet, these must be regarded as minimum standards, as spelled out in the revised documents of Appendix A of the Council of Europe (2002). Indeed, they address only in passing and superficially natural concerns of the scientific community, i.e. whether certain regulations may interfere with the studies.

Optimal enrichment

It would be naïve to believe that all enrichment programs have only positive effects on animals, and never interfere with research. Enrichment can be evaluated in terms of refinement and reduction. Refinement is essentially welfare status of the animal, and enrichment should improve the status by definition. reduction potential of enrichment should be assessed at the same time, and we should prefer those, which decrease the number of animals needed or at least cause no change.

Any refinement to improve animal welfare requires scientific validation to ensure it is truly beneficial to animals (efficacy) and does not detract from the scientific integrity of the study (safety). When enrichment fails to fulfill one of these criteria, animal welfare may be challenged or the study should not have been done at all. The effect of enrichment on safety may simply be a change in the mean of the determination of interest, and this may not matter as it should affect all groups, but changes in variance will lead to more animals being used, itself an ethical issue. The opposite could also happen and results could be improved leading to fewer animals being used.

While refinement aims can mostly be connected to research data, reduction suffers from lack of research to base regulations on. It is obvious that changes in variance may be strain-, facility- and enrichment-specific, which makes overall guidelines difficult. Indeed, instead of trying to assess impact of enrichment on every determination, it could be more productive to look at effects on variance of welfare indicators with the understanding that low variance there is likely to show as low variance in other determinations. And at the same time aim at most uniform welfare of the animals in the study.

Discussion of enrichment in connection to GLP-studies emphasizes lowest possible levels of pesticides and heavy metals in enrichment items because they can be toxic to the animals. This is just part of the problem – safety of enrichment has to be understood wider; it is also compromised when any compounds that could change the results are brought into the enclosure. Volatile compounds, especially pinenes, which cause induction of liver microsomal enzymes, are a challenge to safety as well. Recent study showed that there still are pinenes present in beddings and that the same is true for enrichment items (Meller et al., 2004)

Pair-housing of rabbits and variance

For social animals, such as rabbits, another animal in the same enclosure is the most challenging enrichment factor. While objects intended for enrichment are static and may have only novelty value, a partner poses continuous and unpredictable challenges to which the individual must react (Stauffacher et al., 2001).

Rabbit as a species is difficult to house in groups, and group housed rabbits show preference for group sizes of one to three animals (Whary et al., 1993), and when housed in pairs, proximity of each other (Huls et al., 1991). Group size of two rabbits – pair-housing – is indeed the simplest form of social enrichment and possible in reasonably sized cages. In order to enhance social enrichment in rabbits, the European regulations will make it possible to have two rabbits in a cage space considered adequate for one (Council of Europe, 2002).

A study designed to simultaneously assess both compatibility and reduction outcome of rabbit pair-housing was done following the revised Council of Europe Appendix A (2002). Variances in growth and serum alkaline phosphatase were significantly smaller in pair-housed as compared to single housed rabbits. If growth is the resulting parameter, then with change from single housing to pair-housing in rabbits one could halve the number of animals needed, perhaps even more. The large decrease in variance can be due to continuous interaction between the pair keeping them busy in social activities at the expense of eating and resting. The point estimate for alkaline phosphatase shows that reduction of at least half of the female rabbit numbers can be considered as anticipated effect of pair-housing (Nevalainen et al., submitted).

Work for food and enrichment

Enrichment could also be used as "a cure" for excessive variation when the source can be identified. *Ad libitum* feeding is considered a poorly controlled factor, likely to result in large result variance and hence require unnecessarily many animals in order to obtain results of the specified significance and power (Keenan et al., 1999). Virtually all rodents are on *ad libitum* feeding. Refinement of the feeding practice of laboratory rodents is likely to improve scientific quality, animal health and welfare. However, in order to implement restricted feeding regimes in rodents, research is needed to achieve systems avoiding the negative effects and ensuring that all group-housed rodents have access to their share of the food provided.

An approach to solve the problems of *ad libitum* feeding is work for a food and enrichment hypothesis. Food is an indispensable item, without which animals cannot survive and endure. It is suggested that rodents will work only for the amount of food they necessarily need, but not beyond that, provided the intensity of the work is set correctly. A simple system has been invented, and a preliminary pilot study shows that it indeed decreases weigh gain of laboratory rats (Nevalainen et al., unpublished data). Full scale studies are needed to ultimately verify efficacy, and to set the dose of work. Safety evaluation is also a necessity, e.g. to assess whether animals are too lean, whether their time budget for working is too hard and what happens to variance of results.

Furthermore, animal well-being is important when assessing any novel feeding regimen. We hypothesize that determination of faecal corticosterone and IgA combined with telemetric cardiovascular parameters and behavior assessment the same animals will give a comprehensive picture of any change and its impact and so help to optimize animal welfare.

COST Action B24

COST Action B24 "Laboratory Animal Science and Welfare" is a new scientists' network focusing on both efficacy and safety of animal housing, including environmental enrichment, and designed to look for answers to the concerns of both the public and the scientists. The approach chosen is to increase knowledge necessary for both ethically sustainable and scientifically valid use of laboratory animals in research. The Action believes that these two aims are not only possible, but indeed an absolute necessity. The Action has planned for five working groups, and enrichment is included into the remit of Working Group 1, Housing of animals and scientific integrity.

The Action serves as an interaction podium and idea generator for scientists and civil servants and paves the way for European research consortia. Furthermore, it aims at the production of research results and collection of technical data based on scientific studies, and ultimately seeks tools needed for real life implementation. Delivery of the processed data is done through harmonizing of training of persons working with animals and as guidelines and recommendations, which should go beyond regulatory minimum standards. This aim can be realized only if a strong and active network is present. A special aim of the Action, in order to further implementation, is a compilation of relevant guidelines and recommendations produced prior to the Action and by the Action into a format, which would allow all interested groups within the field to become familiar with the recommendations in a convenient, but efficient way.

The two Rs initiative

The Replacement alternative is simply not always possible, sometimes not even desirable, and hence there should be more research on the remaining Two Rs to help those animals still being used and the scientists. Replacement alternative has been studied far more than the other two. The COST Action B24 believes that all 3Rs should have equal weight in funding, and calls for full implementation of both the European Convention and in the Directive. To further this goal, the Action has prepared the Two Rs Initiative and submitted it to be included in the European Union's 7th Framework program.

Studies on alternative methods for applications in fundamental biomedical research receives too low funding, even though there is high potential for the 3Rs, and especially for reduction and refinement. Every scientist using animals can and should actively seek implementation of one or more of the alternatives.

The document "Science and technology, the key to Europe's future – Guidelines for future European Union policy to support research" (2004) states that the Commission has made strengthening European research a major objective…is proposing to increase the European Union's research budget….the budget should be doubled. If this is to happen, it will inevitably mean more laboratory animals used in fundamental research, and acute and urgent need for funding of studies of how best to apply the Two Rs methods.

Investing in research of the Two Rs alternatives at the same pace with funding of basic research enables Europe to maintain or even increase the lead it has. All research carried out should simply be ethically sustainable and scientifically valid. Yet, this is not simple nor straightforward, and it can only be achieved through tailor made, considerable funding granted on a competitive basis.

Any considerable funding to research on the Two Rs is likely to generate new knowledge enabling better welfare for fewer animals in research and consequently ease the concerns of the society. It can also be foreseen that this very same research, if carefully planned and executed, is crucial in avoiding practices and procedures compromising the scientific validity of research.

Standardisation of enrichment

The FELASA-working group on "Standardisation of Enrichment" was established with the goal to provide guidance of how to standardise enrichment in laboratory animal enclosures such that essential species-specific needs and individual needs of gender and life stage are fulfilled to guarantee animal welfare and to minimise interference with experimental results. It is expected that once the document will be published, the recommendations given will further the implementation of enrichment so that animal welfare and good science are promoted.

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Importance and Effects of Enrichment on Physiology, Behaviour and Breeding Performance in Mice

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Summary
Sixty DBA/2 breeding pairs were used to understand the influence of enrichment on breeding performance. Four inbred strains of mice (A/J, BALB/c, C57BL/6 and DBA/2) were used for sequential experiments, in a total number of 240 (half for each sex), to collect information about the effect of enrichment on physiological traits and behavioural tests. Non-enriched and enriched groups were always provided with the same cage type to avoid the influence of floor space on agonistic behaviour, which can lead to different inter-individual distances.
According to the presented results environmental enrichment can affect experimental results and will not automatically improve well-being. Furthermore "Reduction" and "Refinement" may conflict with each other. Thus it will be necessary to evaluate the effects of environmental enrichment before an enrichment design is applied for a given experiment.

Keywords: mice, enrichment, variation, refinement and reduction

Introduction

In recent decades, there have been significant developments in laboratory animal science in the field of health monitoring (hygiene control), genetics and environment. This led to the possibility of standardisation in laboratory animal science. Reduction and refinement have been implemented in laboratory animal science in the last decades. Due to that the high level of standardisation has enhanced animal well-being by improving physiological health (refinement) and is largely responsible for the great decrease in the number of laboratory animals used in experimental research, due to the decrease in variation (reduction).

Currently environmental enrichment is intended for further improvement on the laboratory animal housing. Due to that a wide variety of enrichment designs are considered and the interactions between environment and genotype have been reported in many studies. The evaluation of enrichment has mostly been focused on the effects of experimental results (mean values) on the brain functions, behavioural performances and other parameters.

Concerning the reduction of 3Rs, the effects of enrichment on variation also need to be studied. Since the majority of statistical tests are basically comparing the size of the effect (the biological "signal") relative to the amount of variability in the data, the biological effects may be hidden by a large variation in an experiment. However, in contrast to the comparison of group means, only a few studies have aimed at the influence of enrichment on the variation (Eskola et al., 1999; Gärtner, 1999; Nevalainen, 1999; Mering, 2001; Tsai et al., 2002; Tsai et al., 2003b).

In the present study the response to enrichment was measured on the basis of breeding performance (for comparing the well-being status between different housing conditions), physiological variables (such as haematological data, body weight gain and relative organ weights), and behavioural tests (Open Field, Food Drive and Elevated Plus Maze) after long-term enrichment.

Non-enriched and enriched groups were always provided with the same cage type to avoid the influence of floor space on agonistic behaviour, which can lead to different inter-individual distances.

Material and methods

Housing: Two types of enrichment were chosen for the present study to evaluate the effects of enrichment. They contained: 1) a nest box, a wooden climbing bar and nest material according to Scharmann 1993 (E1) and 2) horizontal and vertical dividers, modified from Haemisch and Gärtner 1994 (E2). The purpose of the former was to meet the nest building and climbing behaviour of mice; the later was to offer the animals a burrowing system (see fig. 1 and 2).

Both control (non-enriched, NE) and test groups (enriched, E1 or E2) of each experiment were always provided the same cage size, as cage size can influence the agonistic behaviour and lead to different inter-individual distances.

Experiment 1: Following 4 weeks of adaptation 60 DBA/2 breeding pairs were randomly divided into three rack systems: a ventilated cabinet, a normal open rack and an individually ventilated cage rack (IVC rack) with enriched or non-enriched type II elongated Makrolon cages, half for each housing. Reproduction performance was recorded from 10 to 40 weeks of age for understanding the influence of enrichment (E1) on breeding performance (detail see Tsai et al., 2003a).

Experiment 2: A/J, BALB/c, C57BL/6J and DBA/2 were used for the subsequent experiments, in a total number of 240 (half for each sex). Animals at 3 weeks of age were marked and assigned randomly to non-enriched (NE) or enriched (E1 or E2) type III Makrolon cages with equal numbers of cages in same-sex groups of four. Behavioural tests (Open Field, Food Drive and Elevated Plus Maze) were performed at 9, 10 and 11 weeks of age, respectively. At 14 weeks of age blood samples were collected for haematological analysis. The final body weights and organ weights were measured following euthanasia at 15 weeks of age to determine whether there are strain differences in the reaction to enrichment (detail see Tsai, 2002; Tsai et al., 2002 and Tsai et al., 2003b).

Statistic: The mean values were compared using factorial analysis of variance, followed by the Scheffé test (significance level 5%). To achieve independence from mean values, the coefficients of variation (SD/mean value, CV) were used instead of the variance (SD^2) or mean absolute deviation (MAD) to compare the variation between the non-enriched and enriched groups. As the CVs were not distributed normally, the CV of each variable was compared using the Wilcoxon signed rank test (nonparametric pair t-test).

Fig. 1: Enriched cage in experiment 1

Results

Breeding performance

E1 housing (according to Scharmann 1993) did not improve the reproduction, but the variation of breeding performance increased due to enrichment (see tab. 1).

Physiological traits and behavioural tests

The effects of enrichment designs (E1 and E2) are not consistent, but vary according to the variables studied.

1. E1 housing had significant effects on Elevated Plus Maze performance, while significant differences were found in Open Field and Food Drive tests and in relative organ weights (adrenal, kidney, spleen and liver) due to E2 housing (detail see Tsai, 2002; Tsai et al., 2002 and Tsai et al., 2003b).
2. Strains reacted differently to enrichment (E1 and E2, detail see Tsai, 2002; Tsai et al., 2002 and Tsai et al., 2003b).
3. In comparison with NE groups there was a tendency towards an increased CV in enriched groups, especially in physiological traits and in Open Field and Food Drive tests (see tab. 2).

Conclusion

Our data showed that:

1. Environmental enrichment will not automatically improve well-being.
2. Enrichment may affect experimental results and can cause higher coefficients of variation (CV). Such influences were strain- and test-dependent.
3. The effects of enrichment on physiological traits are more focused on the variance than on the mean values, while enrichment significantly affected behavioural performance (group means and variations).

This indicates that the effects of enrichment designs vary according to strain and the variable studied. Furthermore reduction and refinement may conflict with each other. Thus, it will be necessary to evaluate the effects of environmental enrichment on variation, before an enrichment design is introduced into an experiment.

Fig. 2: Enriched cage used in experiment 2 (E1 left, E2 right)

Tab. 1: Breeding performance of different housing conditions

	NE		E		Housing difference
	Mean	CV	Mean	CV	p
Total No. of litter/dam	5.44	37.9	4.15	57.7	0.0611s
Total No. of pups born/dam	24.9	51.5	15.9	77.1	0.0176s

CV = Coefficient of variation (SD/mean, %)
NE/E = 27 in non-enriched (NE) and 26 in enriched groups (E)
s = significant difference (p<0.1)

Tab. 2: The comparison of CVs between NE and E1/E2 groups

Strain	Physiological traits		Open Field and Food Drive		Elevated Plus Maze		Breeding performance
	NE vs. E1	NE vs. E2	NE vs. E1	NE vs. E2	NE vs. E1	NE vs. E2	NE vs. E1
DBA/2							4/7
A/J	14/22	13/22	3/4	2/4	2/12s	5/12	
BALB/c	8/22	14/22	2/8	4/8	2/12s	5/12	
C57BL/6	16/22s	11/22	6/8	7/8s	6/12	3/12s	
DBA/2	14/22	16/22s	7/8s	7/8s	8/12	6/12	
Total	52/88	54/88s	18/28s-	$20/28s^{+}$	18/48s	19/48	4/7

The CVs of each group were pooled and compared using Wilcoxon signed rank test (nonparametric test).
Data contained 4 strains of mice (A/J, BALB/c, C57BL/6 and DBA/2, both sexes), except A/J data of Food Drive test.
Physiological variables: relative body weight gain, relative organ weights and haematological data.
14/22 means: out of 22 compared variables, enriched groups had higher CV in 14 variables.
s: significant difference (p<0.05); s-: significant difference (p<0.01); s^{+}: significant difference (p<0.001).
For group means, E1 housing had significant effects on Elevated Plus Maze performance, while significant differences were found in Open Field and Food Drive tests and in relative organ weights due to E2 housing.

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