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Facts and Demonstrations: Exploring the Effects of Enrichment on Data Quality

There is widespread support within the scientific community for the concept that better welfare equals better science. It is also broadly accepted that environmental enrichment improves welfare. I have seen much evidence of support for both of these concepts in the literature, at meetings and when I visit animal facilities and see enrichment increasingly becoming the norm. However, this degree of acceptance is still not universal. For example, there are inconsistencies with levels of enrichment that are provided, even within the same facility at times. I still encounter researchers who do not regard enrichment as the default and have to be persuaded to provide it. There are also still, unfortunately, plenty of examples of materials and methods sections in papers that simply say something along the lines of "animals were housed in standard cages with soft wood litter and lab chow *ad libitum*", with no explanation as to why the animals were not provided with anything else.

There are three categories of frequently encountered reasons for resistance to enrichment. First, some individuals and organisations are opposed on economic grounds, as evidenced by some of the responses* to the increased emphasis on enrichment in the most recent US *Guide for the Care and Use of Laboratory Animals* (NRC 2011). Second, some have a perception that the welfare benefits to animals, with respect to particular refinements or enrichment as a whole, are not yet proven. This is often used to try to help justify the first point. For example, the American Psychological Association's (APA 2011) comment to the National Institutes of Health on the new *Guide* stated that "solid bottom cages particularly for rats and mice are based on weak to nonexistent evidence"—yet a classic paper in the animal behaviour literature found that rats housed on grid floors will lift 83% of their body weight to gain access to a solid floor (Manser et al. 1995).

Neither of these objections is sustainable. However, the third reason for resistance to enrichment relates to concerns that experimental variability will increase, or a confound will be introduced, affecting data quality. This does merit further examination. There are three areas of concern:

1. the validity of the science within an individual project, if another variable is introduced;
2. whether or not the data can be compared with those obtained from studies conducted without enrichment; and
3. an ethical issue—whether greater variability will necessitate an increase in animal numbers to ensure that results are significant, trading off refinement and reduction against one another.

Assumptions are often made about all three of these points, hence the title of this paper: "Facts and Demonstrations". This quote from John Ruskin, the nineteenth century artist, social critic and philanthropist, sums up the kind of approach that should be taken when confronted with pre-judgements about enrichment and its effects on the science: **"the work of science is to substitute facts for appearances, and demonstrations for impressions".** This can be applied to all three of the concerns listed next.

* See *http://grants.nih.gov/grants/olaw/2011guidecomments/web_listing.htm*

Issue 1:
The effect on scientific validity within the project

Many international regulations and guidelines do encourage or even mandate enrichment. For example, the US *Guide* presents an enriched environment as the default position. The European Union (EU), Directive 2010/63/EU, which regulates animal use throughout all 28 Member States, requires that restrictions on the animals' abilities to satisfy their physiological and behavioural needs be kept to a minimum (EC 2010). However, both US and EU standards acknowledge that there may be scientific justification for modifying or withholding enrichment. The problem lies in the nature and level of the justification that is accepted by regulators, and/or by ethical or animal care and use committees. This can range from requiring evidence from the literature, or pilot studies, to back up assertions that data quality will be affected, to simply accepting a researcher's assumptions at face value.

So, what is genuine scientific justification for restricting or withholding enrichment? It is essential to ask this question—including asking yourself if you are a scientist who houses animals without enrichment as a matter of tradition. Much can be achieved if assumptions and the status quo are creatively challenged (Fig.1).

Photo: Novo Nordisk

Figure 1: This picture shows a dog used in pharmacokinetic studies who is regularly able to exercise outside in a secure area, with a group of other dogs, even between procedures.

There has always been a steady stream of studies and reviews in the literature that have looked at the effects of refinement, including enrichment, on data variability and standardisation (e.g. Eskola et al. 1999, Baumans et al. 2003, Würbel et al. 2005, Mikkelsen et al. 2010, Toth et al. 2011). It has been found that enrichment has effects on variability or data quality that are significant, or that it has effects that are not significant, or that there are no detectable effects at all. This demonstrates that it is not possible to make sweeping assumptions about the effects of enrichment on data quality. What is really important is making sure that findings of such studies are interpreted, and acted upon, appropriately. That is, even if enrichment does have a significant effect on data, restricting or withholding it may not be the right thing to do.

This is an important point not only for animal welfare reasons, but also because of the current climate of reflection and debate about the translatability and validity of animal "models". Systematic reviews of preclinical studies have indicated that there are significant issues that need to be addressed with respect to translatability, and there is increasing recognition that "standard" housing and care might actually be causing problems. For example, a recent review paper on optimising translation of

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preclinical studies for central nervous system disorders cited the use of sedentary, unstimulated animals as a limitation of current approaches, suggesting that therapies showing promise in standard-housed animals should be retested under conditions of greater environmental complexity, in order to improve construct validity (Burrows & Hannan 2013). From an ethical point of view, it seems to me it would be better to dispense with the "standard housed" stage altogether.

To further illustrate the translatability issue, here are three recent papers that have set a good example with respect to interpreting data from animals provided with enrichment*. If the authors of these three studies had decided to withhold enrichment in the belief that the effects on data quality were negative, one could argue that the models would have been less valid, so that important, relevant results would not have been obtained.

Example 1: fibulin-4+/- mice

Fibulin-4 is a protein that is expressed by vascular smooth muscle cells, and is essential for maintaining arterial integrity. Homozygous fibulin-4+/- knockout mice die before birth from arterial haemorrhage, but heterozygous animals appear outwardly normal—although gaps are present between smooth muscle cells in the aorta and the endothelium also shows signs of damage. Figure 2a depicts gaps (shown by green arrows) and fragments coming away from the endothelium (shown by red arrows). In a controlled study, it was found that housing the heterozygous fibulin-4+/- mice in large cages, with a tunnel and wheel, significantly reduced the gaps and maintained the integrity of the endothelium, as in Figure 2b (Cudilo et al. 2007).

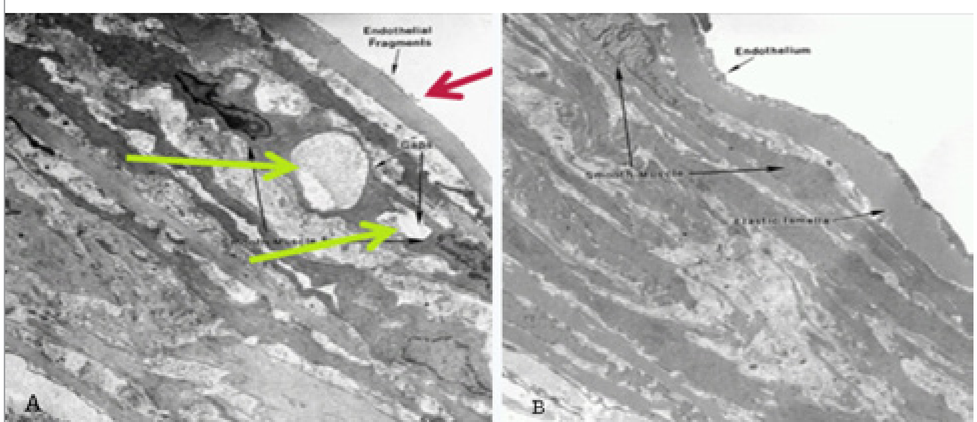

Figure 2: Electron micrographs of sections of aorta from fibulin-4+/- mice housed in (a) standard and (b) enriched cages

The authors' interpretation of these results was that findings assumed to be due simply to genetic differences may have been wrongly interpreted. This is because environmental factors, such as housing conditions, play a more important role in determining this phenotype than was previously thought. Potential mechanisms for this are discussed; for example, it could be that the better housing environment creates conditions in which a surrogate protein could inhibit gap formation. Alternatively, environmental factors could change the methylation patterns of the genes, altering their transcriptional capabilities. Importantly, the authors consider the implications of their findings for human patients with conditions such as the connective tissue disorder Marfan syndrome.

They suggest that programmes of mild exercise could benefit these patients and even help to inform preventative strategies before aneurysms occur. This is a constructive way to interpret differences in animals housed in an enriched environment—accepting that results are different, considering why this might be and exploring ways of increasing translatability and

• Given that the RSPCA's ultimate goal is total replacement with humane alternatives, citing studies that involve animal use as "good examples" does present something of a dilemma. But it is unethical to use animals in protocols that are flawed, as this is causing avoidable suffering and wasting lives. Also, these studies support the case for providing enrichment on scientific grounds, which will lead to welfare benefits for animals while their use continues, an immediate aim of the RSPCA.

benefit. Mice on future studies should also benefit, if they have better quality environments to live in.

Example 2: tumour growth
The second example concerns the effects of an enriched environment on levels of the hormone leptin, on signalling proteins known as eicosanoids, and on the pro-inflammatory mediator COX-2 (Nachat-Kappes et al. 2012). It was hypothesised that these factors would have varying effects on the development and progression of mammary tumours, depending on whether the mice were housed in a standard or enriched environment. The study found that mammary tumour weight was significantly reduced in the enriched environment. In addition, normal mammary glands of mice in the standard environment had more COX-2 positive cells, suggesting an increased inflammatory state of the mammary gland under those conditions. The enriched mice had a marked decrease in intratumoral COX-2 activity and an increase in the plasma ratio of adiponectin/leptin levels, both of which have previously been associated with resistance to tumours.

The authors note that standard housing affects basic biological processes, such as mammary gland development and pathogenesis. They conclude that their study provides "evidence for the need of strong recommendations for future directives on the use of enriched environment in experimental animal models". They also cite Martin et al. (2010), which makes the case that many laboratory rodents are metabolically morbid; that is, sedentary, obese, glucose intolerant and on a trajectory to premature death. In fact, Martin et al.

What do you think? *The Enrichment Record* and RSPCA have put together a survey to gauge people's views on the effects of enrichment on data quality. We would greatly appreciate your input and will feed back and discuss the results in a future edition. Please take part at http://goo.gl/osnClp

suggest that the standard, overfed, sedentary control animal might be a better model for overweight and sedentary human subjects, but may be inadequate for normal weight humans, making the point that patients with cancer, vascular disorders and neurodegenerative diseases are often otherwise fit and cognitively stimulated. Returning to the paper evaluating tumour development, the authors also indicate that their research provides evidence supporting the positive impact of physical and social well-being with respect to managing cancer progression in human patients.

Example 3: amyloid plaques
The last of the three examples relates to $APP^{Swe}/PS1^{L166P}$ mice used in Alzheimer's disease research (Montarolo et al. 2013). In this study, the authors begin by citing reports that humans who undertake enriched social, physical and/or cognitive activities have a reduced risk of developing Alzheimer's disease. One of the aims of this project was to use the effects of enrichment to try to better understand these protective effects of exercise and stimulation. As in the studies mentioned previously, $APP^{Swe}/PS1^{L166P}$ mice in "standard" housing were compared with those in an enriched environment. The authors found that enrichment transiently accelerated the deposition of the amyloid plaques that are diagnostic of Alzheimer's, but there was a protective effect on cognitive deterioration for the enriched mice. The performance deficit in the Morris water maze was significantly reduced for those housed in enriched cages.

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The authors note how enrichment can modulate both symptoms and pathological progression in $APP^{Swe}/PS1^{L166P}$ mice. The results from animals with enrichment confirmed the dissociation between amyloid burden and cognitive deterioration that has been observed in human patients. The authors also review the results of other studies using a variety of different enrichment and exercise protocols, from the aspect of using enrichment to help understand the effects of the environment on pathology.

Addressing animals' needs makes for better science

Considering the above examples, it is hardly surprising that animals in "standard" laboratory housing are not physiologically or psychologically normal. Nor is it surprising that this can affect not only welfare but also scientific quality. To illustrate this, Table 1 provides some examples of the conflicts between the needs of mice and requirements of humans in a laboratory setting.

Sources: Latham and Mason (2004), Burn (2008). Castelhano-Carlos & Baumans (2009), Hurst & West (2010; tail capture), Gaskill et al. (2009; temperature)

Table 1: Conflicts between the needs of laboratory mice and humans

Mice are ...	... but
Nocturnal and crepuscular	they are housed in bright light
Highly dependent on smell and scent markings	their markings are completely destroyed whenever the cage is cleaned
Sensitive to ultrasound	there are many sources of ultrasound in the laboratory, and these are not always checked and minimised
Able to feel more secure when touching objects (thigmotaxis)	they are often housed in barren cages
Master diggers	they have no opportunity to burrow
Highly social (sex and strain dependent)	they are often housed in inappropriate groups or singly
Capable of covering long distances	they are housed in small cages
Omnivorous, trying new foods from different feeding sites	they are fed boring, monotonous diets from hoppers
Made extremely anxious when captured by the tail	most people catch them by the base of the tail
Most comfortable at a temperature of 26° to 34°C	many facilities house them at colder temperatures, and/or do not provide sufficient nesting material

The aspects of mouse behaviour outlined in the table demonstrate just how undesirable a "standard" cage can be from a mouse's point of view. For example, repeated scent markings can actually build up into permanent little "pillars" that are an important feature of the environment for mice, yet these patterns of scent markings are destroyed whenever cages are cleaned. Although there is an obvious need to clean cages for health reasons, some other behaviours can be catered for by altering light regimes, using a bat detector to detect troublesome ultrasound, and providing refuges. The "need" to burrow could perhaps be reduced if appropriately designed tunnels and shelters are provided, but the inability to control social interactions and range over large areas is likely to be a significant stressor. The range sizes of wild mice can be anything from 2 m^2 to 10,000 m^2—but a standard cage can be just 0.035 m^2. Sixty of these cages could fit into 2m^2. Eating plain lab chow from a hopper, with no opportunities to forage, is not only boring but also leads to obesity, with serious implications for validity as mentioned earlier.

Most of the information on mouse behaviour in Table 1 is from Latham and Mason (2004) and Burns (2008), which are well worth reading if you want to understand the mouse and rat better. Castelhano-Carlos and Baumans is an excellent review of the effects of the environment and husbandry procedures on rat well-being and should be required reading for everyone involved in the care and use of any species. For example, the authors cite a study that found rat plasma corticosterone levels doubled as a result of metal cages being banged in the animal room, and remained at these levels for 2 to 4 hours. This would clearly affect data quality if scientific procedures were conducted within this period, to say nothing of the impact on welfare.

It is sometimes argued that strains of laboratory mice have been inbred for generations and such "wild-type" behaviours are no longer relevant, but this is effectively countered by the "Ratlife" documentary and website (*www.ratlife.org*). This is a film that follows a group of laboratory rats released into a semi-wild enclosure, and it demonstrates that natural behaviours are still innate after many generations in the laboratory.

Looking at this another way, rather than worrying about the effects of enrichment on data quality, conditions that cause poor animal welfare should be the prime concern. Inadequate housing, husbandry and care, and unrefined procedures, can lead to stress and abnormal behaviours that are certain to be experimental confounds in themselves (see Box 1).

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Box 1: Effects of timing on anxiety and cognition

DBA mice are thought to be an animal model of anxiety and have also been reported to exhibit cognitive dysfunction in spatial memory tests. However, behavioural inhibition, an indicator of anxiety, was significantly reduced when mice were tested using a modified hole board during the dark (or active) phase as opposed to the light phase. The cognitive performance of the mice was also better at times when they would naturally be awake and active (Roedel et al. 2005). The authors make the point that light conditions can be confounding factors, yet this is often not taken into account. How many papers even state the time and lighting conditions when behavioural or cognitive tests like these were run?

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Although the examples considered so far in this paper relate to positive effects of refining husbandry on data quality, reducing stress during procedures can also benefit the science, especially when aiming to avoid restraint. As an example, retinoic acid, which is used in developmental studies, is usually administered by oral gavage. This involves stressful capture and restraint as well as the distressing gavage procedure. However, after some trial and error with different baits, it has proved possible to give retinoic acid in a chocolate treat which the mouse voluntarily eats when briefly placed in a cage base (Maconochie et al. 2012). This is not only less stressful for the animal but the bioavailability of the retinoic acid is also improved, and there is a good dose-response relationship.

Enrichment as a welfare indicator

Besides the scientific benefits, there are other advantages associated with providing enrichment. Giving animals enrichment items also gives them an additional means of communicating their state of well-being, or whether they are suffering. In Figure 3 (Arras et al. 2007), the mouse in the top two pictures has undergone a surgical procedure but has had adequate perioperative analgesia. He has built a discrete nest and has defaecated outside it, as shown by the circles (arrows). The mouse in the bottom two pictures has not had adequate pain management and has not made a proper nest; his faeces are also deposited within the nesting material. He would clearly benefit from some analgesia, but it would have been much harder to tell if the nesting material had not been present. This is not just about welfare; there could well be an impact on the science too, for example, if further interventions were begun before animals had fully recovered from surgery.

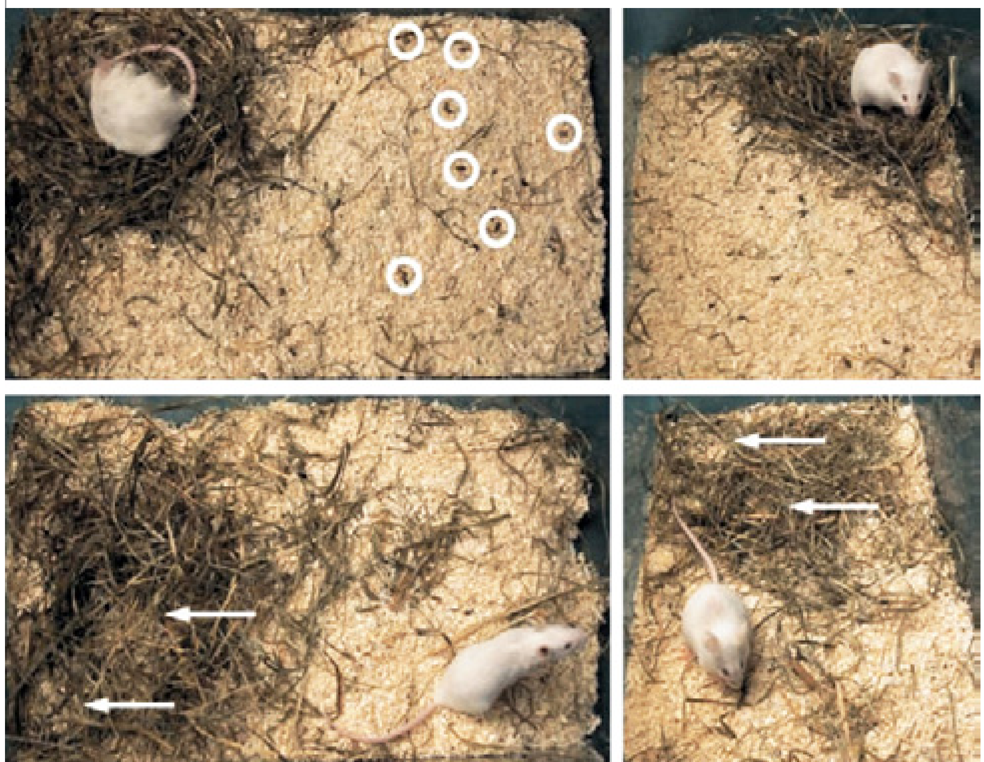

Figure 3: Mouse nest quality as an indicator of postoperative pain

The concept of using enrichment as a welfare indicator is starting to become more widespread. For example, nest scoring is used in Alzheimer's research; in Figure 4, the left hand nest scored 1, and the right hand nest scored 5 out of 5 (Deacon 2012). Burrowing behaviour is a useful and well validated early indicator of pain or sickness behaviour (Jirkof et al. 2010). Although this requires a tube full of burrowing material, which is not often routinely supplied as enrichment, it could be useful in pilot studies as a means of identifying and recognising suffering so that protocols can be refined.

Figure 4: Nest quality as an indicator of the progression of Alzheimer's disease in mice

Issue 2:
Comparability of data with previous studies conducted without enrichment

Whether data obtained from animals provided with enrichment is comparable with data from animals in "standard" conditions is often raised as a concern within regulatory toxicology, although the issue may arise in any field of biomedical research. However, it is essential not to make any assumptions about comparability without conducting objective evaluations to test these. In practice, whatever the research area, data may be comparable, or any differences may be systematic so that it is possible to take them into account, for example, by using appropriate statistical analysis. Alternatively, data quality may be improved by providing enrichment because animals are more physiologically and psychologically normal, as discussed in the previous section. For scientific reasons, data quality should generally be the prime consideration and should take precedence over comparability.

With respect to regulatory toxicology in particular, it is important to look critically at regulatory requirements and what they actually say with respect to enrichment. For example, the OECD states that "proper conditions should be established and maintained for the storage, housing, handling and care of biological test systems", which can be interpreted as providing appropriate housing including enrichment (OECD 1998). The UK Good Laboratory Practice guidelines are more explicit and state that care, housing and containment should "prevent stress and other problems which could affect the test system and the quality of data" (Department of Health 1999). The ICH also recognises that data from unstressed animals will be of better quality (ICH 2000). These statements are borne out by the experience of a major UK Contract Research Organisation, which supplies enrichment as the default and says that most clients readily accept this, provided that enrichment is not abruptly provided or changed during a study.

Issue 3:
Potential for enrichment to lead to increases in animal numbers

This is primarily an ethical issue, as it requires a balance to be struck between animal numbers and suffering. While increasing numbers is diametrically opposed to the aspiration to implement Reduction, it should not be assumed that numbers will have to increase if enrichment is provided. A pilot study can evaluate this one way or the other. Sometimes objections are raised to pilot studies on the grounds that they increase the total number of animals used. However, it is often possible to design pilot studies so that the data can be used as part of the data set for the completed study, and no additional animals will ultimately be needed.

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Even if pilot studies or other analyses demonstrate that more animals would be required, reduction is not necessarily everything. Refinement can "trump" reduction if it means that each individual will suffer less, so increasing numbers but reducing severity may be the right thing to do.

Whatever the final experimental and husbandry protocols, it is essential to explain what has been done to refine housing and care, in papers, posters and talks. Otherwise, in the absence of any explanation, it appears as though no efforts were made to research, consider and implement refinement. Including information on enrichment (and refinement in general) helps to promote good practice amongst colleagues and to reassure the public that efforts are made to minimise suffering, improve welfare and improve science. The ARRIVE guidelines set out useful guidance on the kind of information to include (Kilkenny et al. 2010), and it is also helpful to explain where it has not been possible to refine for justifiable scientific reasons, or where numbers have increased as a result of refinement.

It is good practice to apply this open approach to the public communication strategy relating to animal use at your facility, in the form of information on refinements and how these are considered, evaluated and applied, which can be presented on websites and in other materials such as annual reports and Corporate Social Responsibility statements. Communicating with the public about refinements such as enrichment, and the scientific and animal welfare benefits, is important because (1) most people are concerned about animal use, and the suffering this can cause (Ipsos MORI 2010), and (2) the public directly or indirectly funds research, so is entitled to know whether its donations and taxes are being spent on good quality, well-designed research projects.

Action points:

Facts...

- Do not assume that refinement will adversely affect data or results without actually evaluating whether this is the case, for example by conducting pilot studies.
- Interpret findings of evaluation or pilot studies with an open mind:
    - if the data from animals provided with enrichment are different, this does not mean that they are less relevant, or flawed;
    - it may be that the data from animals in standard housing were flawed; or
    - it may be possible to accommodate the differences by adjusting the experimental design or data analysis.
- Take part in discussion and dialogue among user groups and within the literature on enrichment and its effects, as this is helping to improve communication and openness about the issue.
- If working in a regulatory arena, look critically at regulatory requirements and ensure they have not been misinterpreted with respect to whether enrichment can be provided.

... and demonstrations

- Write up publications according to the principles in the ARRIVE guidelines—which also need to be taken into account at the project design stage, so as to ensure that appropriate information is recorded.
- Include details of refinements in materials and methods sections, challenging any requirements to remove detail. If it really was not possible to refine for justifiable scientific reasons, briefly explain and demonstrate why.
- Keep up with current thinking and approaches to refinement and its impact on both science and animal welfare.
- Actively make sure that there are adequate information channels at your facility for accessing information about enrichment and its implications for experimental design and analysis. This could be via an individual such as the Named Information Officer in the UK, or through an effective ethical review or animal care and use committee that includes one or more members who have the necessary interest and expertise.
- Please fill in our survey at *http://goo.gl/osnClp*

Conclusion

Enrichment should be embraced as essential for humane science, regarded as a requisite for more valid science, taken for granted as the norm unless there is compelling scientific justification, and its application promoted within the scientific community and to the wider public. Things have certainly changed for the better, but there is still much work to be done.

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