Reporting the Implementation of the Three Rs in European Primate and Mouse Research Papers: Are We Making Progress?

Katy Taylor

British Union for the Abolition of Vivisection, London, UK

Summary — It is now more than 20 years since both *Council of Europe Convention ETS123* and *EU Directive 86/609?EEC* were introduced, to promote the implementation of the Three Rs in animal experimentation and to provide guidance on animal housing and care. It might therefore be expected that reports of the implementation of the Three Rs in animal research papers would have increased during this period. In order to test this hypothesis, a literature survey of animal-based research was conducted. A randomly-selected sample from 16 high-profile medical journals, of original research papers arising from European institutions that featured experiments which involved either mice or primates, were identified for the years 1986 and 2006 (Total sample = 250 papers). Each paper was scored out of 10 for the incidence of reporting on the implementation of Three Rs-related factors corresponding to *Replacement* (justification of non-use of non-animal methods), *Reduction* (statistical analysis of the number of animals needed) and *Refinement* (housing aspects, i.e. increased cage size, social housing, enrichment of cage environment and food; and procedural aspects, i.e. the use of anaesthesia, analgesia, humane endpoints, and training for procedures with positive reinforcement). There was no significant increase in overall reporting score over time, for either mouse or primate research. By 2006, mouse research papers scored an average of 0 out of a possible 10, and primate research papers scored an average of 1.5. This review provides systematic evidence that animal research is still not properly reported, and supports the call within the scientific community for action to be taken by journals to update their policies.

Key words: *animal experiments, journal policy, mice, primates, reduction, refinement, replacement, Three Rs.*

Address for correspondence: *Katy Taylor, British Union for the Abolition of Vivisection, 16a Crane Grove, London N7 8NN, UK.*
E-mail: *katy.taylor@buav.org*

Introduction

Russell and Burch outlined the principles of the Three Rs (*Replacement*, *Reduction* and *Refinement*) as the key to humane research involving animals, in 1959 (1). They defined *replacement* as "any scientific method employing a non-sentient material which may in the history of animal experimentation, replace methods which use conscious living vertebrates", *reduction* as "reducing the number of animals used to obtain information of a given amount and precision", and *refinement* as "simply reducing to an absolute minimum the amount of stress on those animals that are still used".

Since the publication of their book and its subsequent reprinting in 1992, awareness and acceptance of the concept of the Three Rs has grown throughout the laboratory animal research community. As a consequence, in more recent times there has been an interest in assessing the impact of the uptake of Three Rs principles on achieving significant replacement, reduction and refinement of laboratory animal use (e.g. 2, 3). However, assessing the implementation of the Three Rs (and its impact on animal use) is not an easy task. The most obvious measure of progress in replacing and reducing animal experiments is the extent of the decrease of the number of animals used in experiments. Unfortunately, however, animal experiments are increasing across Europe; the latest available statistics show a rise in the number of animals used from roughly 10 million per year in 2002, to 12 million in 2005 (4). As a measure of the implementation of the Three Rs, reporting of the actual numbers of animals used has also been criticised, because it does not permit an assessment of the extent to which experiments are being 'refined', nor does it provide information on the 'relative' increase compared to the overall increase in biomedical research in general and any increase in the use of non-animal procedures (5). Other, finer, measures of the uptake and impact of the Three Rs are therefore clearly needed.

Unfortunately, the details of animal experiments are rarely publicly available, either prospectively or retrospectively, but one way to assess whether the Three Rs are being implemented by animal researchers is to review their experiments in pub-

lished papers. For example, it is possible to identify whether the experiments themselves represent Three Rs improvements to established techniques, such as a reduction in the numbers of animals used, or the use of lower species as a 'refinement'. Such an assessment was attempted by Carlsson *et al.* in 2004 (2). However, experimental technique can be repeatedly refined, and therefore becomes a 'moving target'. What might be seen as a refinement in one year (e.g. the use of the Local Lymph Node Assay [LLNA] as a refinement of the Guinea-pig Maximisation Test) may not be in another (e.g. the rLLNA represents further *refinement* and *reduction* compared with the LLNA). It is arguably easier, and less subjective, to assess whether the authors actually report in their paper that they have applied Three Rs principles and, specifically, to what experimental factor. For example, authors could report that they improved the housing conditions of the animals, or that they used analgesia. Whilst the reporting of the implementation of the Three Rs can be taken as evidence that the Three Rs have indeed been implemented, there is always going to be a discrepancy between actual implementation and its reporting. Therefore, reporting of the implementation of the Three Rs can only be taken as an *indicator* of actual implementation, and should be treated as a distinct measure in its own right.

A number of surveys have been undertaken to assess the reporting of experimental factors that are relevant to the Three Rs, and these are usually *refinement*-related. One of the earliest was in 1988, when the RSPCA and FRAME collaborated to conduct a study on the reporting of welfare (i.e. *refinement*-related) parameters within primate research papers originating from UK scientists (6). They reviewed 289 papers published from 1983 to 1988, and unfortunately reported that "It was impossible to tell from many of the published papers precisely what had been done to the animals, why they had been used, how many animals had been involved, where they had come from, what post-operative care was given, how they had been generally cared for, or what had eventually happened to them."

However, this study predated the implementation of legislation on the use of animals in experiments in Europe. In 1986, *Council of Europe Convention ETS123* (7) and EU *Directive 86/609/EEC* (8) were introduced, to provide legislation on animal experiments in Europe. Both documents enshrined aspects of the Three Rs in considerations to be made prior to conducting any animal experiments. For example, Article 7 of *Directive 86/609/EEC* (8) states that:

2. *An experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available.* [i.e. replacement]
3. *When an experiment has to be performed, the choice of species shall be carefully considered and, where necessary, explained to the authority. In a choice between experiments, those which use the minimum number of animals, involve animals with the lowest degree of neurophysiological sensitivity, cause the least pain, suffering, distress or lasting harm and which are most likely to provide satisfactory results shall be selected.* [i.e. reduction and refinement]

In addition, Appendix A to *Convention ETS123* (now revised; 9) provided guidelines for the care and accommodation of laboratory animals. Other national legislations, such as the UK's *Animals (Scientific Procedures) Act 1986* (ASPA; 10), not only repeated these concepts, but also contained mandatory ethical review and licensing as mechanisms by which to implement their use.

Since researchers who use animals are expected to be aware of this legislation and its associated guidelines when designing their studies, it might be expected that this would have had an indirect, if not direct, impact on their implementation of the Three Rs, which, one might assume, would be reported in any subsequent papers on the outcomes of the research.

Unfortunately, only a small number of surveys have looked at whether there has been an improvement, over time, in the reporting in scientific papers of implementation of the Three Rs in experiments. In 2004, Carlsson *et al.* surveyed 2800 articles published between 1970 and 2000 (2). In addition to measuring actual reduction in animal numbers within papers, they also reviewed the occurrence of reports of *refinement*-related experimental factors. However, they did not look at whether these factors were also refined, i.e. they scored the occurrence of reports of cage size, but not whether the cages were defined as being large or small. In 2005, Richardson and Flecknell looked specifically at reports of (only) analgesia in 100 laboratory animal science papers published in the time-periods of 1990–1992 and 2000–2002 (11). The survey was repeated in 2009, to investigate the change between 2000–2001 and 2005–2006 (12). Other studies have focused on only a few aspects of the Three Rs, e.g. pain relief (13) and the reporting of statistical methods or other factors, such as the sex, age and weight of the animals used (14, 15), but have not looked at change over time.

Primate use is a particularly controversial area of experimentation (16), so one might expect that this would be the area where most emphasis on *replacement*, *reduction* and *refinement* has been made. However, there has been no review of whether reports of implementing the Three Rs in specifically primate-based research has improved since the 1988 RSPCA/FRAME review.

A literature survey was therefore designed to answer the following two questions: *Has there been an increase in reporting of the implementation of the Three Rs in primate research papers from Europe since 1986?,* and *Is this extent of reporting greater than in papers featuring less controversial research, such as that on mice?*

Methods

Three Rs-related experimental factors that one might expect to see reported in a scientific paper which features the use of animal models, were selected from key papers on this issue and collated into a checklist. These key papers were two reviews on the welfare of primates (17) and mice (18), and a FRAME review of what factors should be reported in the UK Home Office abstracts of animal research (19). Eight key *refinement*-related factors were chosen (housing-related factors — i.e. increased cage size, social housing, enrichment of the cage environment and food; and procedural-related factors — i.e. the use of anaesthesia, analgesia and humane endpoints, and training for procedures with positive reinforcement; Table 1). In addition, a factor related to *replacement* was chosen (justification of non-use of non-animal methods), and also a factor related to *reduction* (statistical analysis of the number of animals needed). It was decided that papers would then receive a score of 1, if they reported the implementation of a particular Three Rs-related factor (or explained why it was not possible or relevant to do so, as evidence that it had at least been considered), resulting in a maximum total score of 10 per paper. Additionally, it was recorded whether the paper reported the conduct of an ethical review, or referred to ethical committees ('ethical review') and/or adherence to national or regional legislation or codes of practice ('regulations').

In order to review the change since the introduction of *Convention ETS123 and Directive 86/609/EEC* in 1986, a Scopus® (http://www.scopus.com/home.url) search was conducted to find the numbers of papers featuring the use of mice or primates, arising from European countries in 1986 and 2006. Search terms were limited to 'articles' under 'life and health sciences' journals. For non-human primates, papers were sought by using the keywords 'macaque or marmoset or monkey or primate or tamarin or baboon', and for mice, the keyword 'mouse'. To capture European articles, the search was further restricted to 'UK, United Kingdom, England, France, Belgium, Germany, Netherlands or Italy' under 'affiliation', since these are the main laboratory animal-using countries in Europe (see 20).

A total of 3200 papers featuring the use of mice were found in 1986, and 8806 in 2006. A total of

Table 1: Three Rs-related experimental factors and explanation of when a score for implementation was given

	Relevant factor	Score given if Three Rs principles were implemented by:
Replacement	Replacement considered	Giving justification for why **non-animal methods** could not be used instead
Reduction	Numbers of animals used	Giving justification for the **numbers of animals used**
Refinement		
Housing aspects:	Size of cage	Providing **enlarged cages** (or outdoor access in the case of primates)
	Social aspects of cage	Providing **social housing**
	Cage furnishing	Providing **furnishings** such as bedding, toys, refuges, platforms, perches, climbing materials; the terms 'enriched' or 'enrichment' were also accepted
	Food	Providing an **enriched diet**, e.g. treats, fresh fruit and veg, variable diet, foraging devices, gnawing materials
Procedural aspects:	Anaesthesia	Providing **anaesthetic** or justification for non-use
	Analgesia	Providing **analgesia** or justification for non-use
	Training for procedures	Practising **positive reinforcement** (i.e. rewards)
	Humane endpoints	Describing the use of **humane endpoints** or giving justification for non-use

410 papers featuring the use of non-human primates were found in 1986, and 1048 in 2006. In order to control for differences occurring more as a consequence of different journal policies and style, rather than elapsed time, it was decided to review the changes *within* a limited number of journals. In order to have a good sample of papers within a restricted number of journals, the time-frame was widened to 1985–1987 (referred to throughout for ease as '1986') and 2005–2007 (referred to throughout for ease as '2006'), in a similar manner to that proposed by Richardson and Flecknell (11).

For this study, eight journals that were in the top 30 publishers in both years were selected for analysis, for each type of animal. The journals and the numbers of papers they published on mouse or primate research, from European institutions, are shown in Table 2. The journals *Nature* and *Journal of General Virology* featured for both types. The broad areas of research covered by these journals were neurology, dentistry and virology in non-human primates, and cancer, pharmacology, genetic manipulation and immunology in mice.

The abstracts of all the papers from the eight journals were briefly reviewed, in order to identify whether the experiment had been appropriately selected by Scopus, i.e. that it was an article (not a review), had European affiliations, and was conducted on the type of animal in question. Papers were also rejected if they did not describe an experiment that would fall within the definition given under *Directive 86/609/EEC*. This is defined as any animal experiment which "may cause it pain, suffering, distress or lasting harm, including any course of action intended, or liable, to result in the birth of an animal in any such condition, but excluding the least painful methods accepted in modern practice (i.e. 'humane' methods) of killing or marking an animal" (8). In practice, the lowest threshold of this definition is the entry of a needle, or a behaviour test likely to cause distress. Also, experiments where the animal had simply been killed to supply tissue were rejected (unless the animal had been genetically modified first), as were observational studies on free-ranging animals.

Out of this refined list, it was possible to attempt to review all of the papers on primate research over both periods, but for mice, a power calculation had to be performed, to identify the number of

Table 2: Journals reviewed in the survey, and the numbers of primate and mouse research papers arising from European institutions they included, between the years 1986 and 2006

Journal	Mouse research 1986	Mouse research 2006	Primate research 1986	Primate research 2006
Total number of papers	3200	8806	410	1048
Proceedings of the National Academy of Sciences of the USA	29	246	n/a	n/a
Cancer Research	39	140	n/a	n/a
Immunology	133	45	n/a	n/a
Biochemical Journal	42	71	n/a	n/a
British Journal of Pharmacology	30	62	n/a	n/a
European Journal of Immunology	39	131	n/a	n/a
Nature	82	74	10	14
Journal of General Virology	51	57	7	11
Brain Research	n/a	n/a	13	5
Journal of Neurophysiology	n/a	n/a	4	24
Experimental Brain Research	n/a	n/a	7	15
Behavioural Brain Research	n/a	n/a	8	8
Neuroscience	n/a	n/a	6	7
Infection and Immunity	n/a	n/a	3	8
Total number of papers retrieved from the above journals	445	826	58	92
Total number of relevant papers in the above journals	302	514	36	44
Total number of papers reviewed	85	85	36	44

Data from the year '1986' actually refer to data from the years 1985–1987, inclusive. Similarly, '2006' data refer to data from 2005–2007. 'n/a' indicates that the journal was not reviewed for that type of animal.

papers required to ensure that a representative sample had been reviewed. The same calculation was used by Knight in his review of chimpanzee research papers (21). To obtain an accuracy of ±10%, the number of mouse papers calculated to be required (out of 514 identified as relevant) for the largest sample period (2006), was 84. Therefore, 85 papers were selected for review for both years, meaning that the sample size was at least representative for both years. Papers are recalled from Scopus in date order, so a random number generator (www.randomizer.org) was used to randomly select the 85 papers for review.

The chosen papers were then reviewed, focusing on their *Introduction* and *Methods* sections only, and scored for the occurrence of reports of each of the ten factors. (It was not possible to review the papers 'blinded' by year, as the 1986 papers had to be obtained in paper form from the British Library.) The percentage of papers reporting implementation of each of the ten listed Three Rs-related factors, was calculated and compared between time-periods (1986 and 2006) and type of animal (mice and primates). Due to the low scores, it was not deemed valid to perform multiple chi-squared statistical analysis between year and type of animal on these percentages. However, a comparison of total scores at both time-points, for both mice and primates, and also a comparison of median total scores for primate and mouse in the year 2006, was performed by using a Mann-Whitney test for non-parametric data (Analyse-it®; Analyse-it Software Ltd, Leeds, UK).

Results

The number of papers retrieved

The number of papers reporting mouse research nearly tripled between 1986 and 2006, from 3200 mouse research papers in 1986, to 8806 in 2006 (Table 2). This increase was also seen within the sub-sample of eight journals. However, the increase was not explained by a simple increase of mouse papers within each journal. Rather, the number of mouse research papers either stayed fairly similar, dramatically increased (for example the number of mouse research papers increased from 29 to 246 in *PNAS* and also increased disproportionately within *Cancer Research* and the *European Journal of Immunology*) or actually decreased (e.g. mouse research papers in *Immunology* decreased from 133 to 45). A similar increase was seen in the number of primate research papers, increasing from 410 papers in 1986, to 1048 in 2006. The numbers of primate and mouse research papers within the two journals reviewed for both types of animal (*Nature* and *Journal of General Virology*) actually stayed rather similar across the two time-points.

There were 445 papers on mouse research retrieved from the eight journals for the 1986 period. Of these, 302 were considered relevant, and of these, 85 were reviewed (22–106; Table 2). These papers reported studies that included the early production of genetically-modified mice, the ascites method of antibody production, immunology studies involving the injection of cancer cells or drugs into inbred and/or immunocompromised animals, and virology studies in which mice were infected with viruses and monitored. In 2006, the number of papers retrieved within the same eight journals increased to 826, of which 514 were considered relevant, and of which 85 were reviewed (107–191). The studies remained similarly focused on genetics and a more-sophisticated generation of genetically-modified mice, immunology studies, and cancer studies where the use of xenograft models (in which cancer tissue is implanted and grown) was more common. The ascites method of antibody production was rarely reported.

There were 58 papers on non-human primate research retrieved from the Scopus search in the eight journals listed in Table 2, in 1986. Of these, only 36 were reviewed (192–227), as the remainder were not relevant. These studies included: vision research involving the implantation of electrophysiological recording devices in the brains of monkeys and subsequent behavioural tests; neurology experiments, in which monkeys were brain-damaged under surgery and then had to perform tests; Parkinson's disease studies, in which monkeys were injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to induce symptoms; and virology studies, in which monkeys were infected with various viruses and subsequently monitored. In 2006, the number of papers retrieved within the same eight journals increased to 92, of which 44 were reviewed (228–271). The studies remained similar in design to those of 1986, with similar vision studies present, but more emphasis was given to virology research, including work on SIV.

Reports of ethical review and regulations

The number of papers that reported ethical review and adherence to legislation or codes of practice increased significantly for both mice and primates between 1986 and 2006. For both mouse-based and primate-based research in 1986, there were no reports of ethical review. In 2006, for mice, this increased to 28% of the papers reviewed, and to 27% for primates. No mouse research papers reported adherence to regulations in 1986, but this increased to 48% of the papers reviewed from 2006. For primates, only 3% of the papers (i.e. one paper)

reported adherence to regulations in 1986, but by 2006 this had increased to 61%.

Reports of *replacement*-related and *reduction*-related parameters

The increase in reporting adherence to legislation or ethical review did not correspond to an increase in reports of (the lack of) alternative methods or justification for the numbers of animals required for either mouse or primate research (Table 3). Only one mouse research paper in 1986 (less than 1% of the total number reviewed) reported that *in vitro* techniques were not sufficient, as did only 6% in 2006. No mouse research papers reported the justification for the numbers of animals required, in either year. In fact, only 16% and 20% of the mouse papers reviewed, in 1986 and 2006, respectively, reported the numbers used at all in the methods section.

None of the primate research papers explained the inability to use other non-animal methods in either time-period. Also, none of them reported their justification for the numbers of primates used, although the proportion of papers reviewed which actually reported the numbers of primates used was almost 100% in both time-periods (Table 3).

Reports of *refinement*-related parameters

There was no appreciable change in the reporting of the implementation of any *refinement*-related factors in mouse research papers between 1986 and 2006. Overall, the reporting of each factor was extremely low, let alone whether it had been refined. In 1986, reports of the implementation of housing-related refinements ranged from 0% for cage size and food to 5% for the provision of social housing, and there had been little increase by 2006 (Table 3 and Figure 1). Reporting of the refinement of procedural aspects was also extremely low in 1986, from 0% reporting the use of analgesia, training or humane endpoints, to 12% reporting the use of anaesthesia (see Figure 2). The reporting of the use of analgesia and humane endpoints only rose to 4% and 5%, respectively, which represents literally three or four papers out of the sample selected for review.

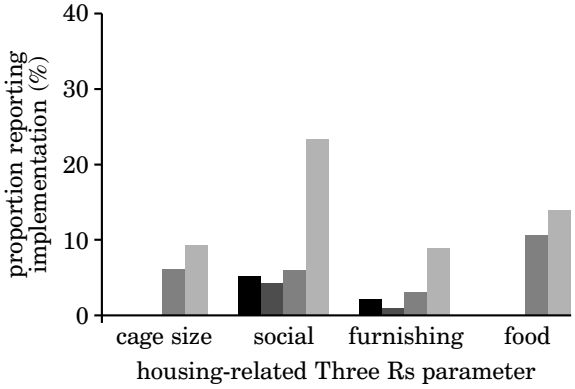
Reports on the refinement of cage size and food were slightly more frequent for primate research than for mouse research, but all the housing-related aspects were poorly reported. There was little appreciable change in the reporting on cage size, provision of enriched cages (furnishing) and food, at 9%, 9% and 14%, respectively, of the papers reviewed from 2006. However, there was an increase in reporting on social housing, from 6% in 1986 to 23% of the papers reviewed from 2006 (Table 3 and Figure 1). Reports of the use of anaesthesia, analgesia and training were greater for primate research than for mouse research, but there was no clear difference in reports of the use of humane endpoints, which remained at less than 5% of the papers. Reports on the use of anaesthetic and positive reinforcement during training, if anything, decreased slightly in 2006, but reports on the use of analgesia increased from 11% of the papers reviewed from 1986 to 27% from the year 2006 (Table 3 and Figure 2).

Table 3: The percentage of papers reviewed that reported implementation of the Three Rs in ten chosen experimental factors

Factor	Mouse research 1986	Mouse research 2006	Primate research 1986	Primate research 2006
1. **Replacement**	< 1%	6%	0%	0%
2. **Reduction**	0% (16%)	0% (20%)	0% (97%)	0% (93%)
3. **Refinement** (cage size)	0% (< 1%)	0% (4%)	6% (6%)	9% (11%)
4. **Refinement** (social cage)	5% (7%)	4% (6%)	6% (14%)	23% (25%)
5. **Refinement** (furnished cage)	2%	< 1%	3%	9%
6. **Refinement** (food)	0% (15%)	0% (19%)	11% (14%)	14% (20%)
7. **Refinement** (anaesthetic)	12%	12%	56%	43%
8. **Refinement** (analgesia)	0%	4%	11%	27%
9. **Refinement** (training)	0% (0%)	0% (< 1)	39% (56%)	30% (48%)
10. **Refinement** (humane endpoint)	0%	5%	0%	2%
Total reviewed	**85**	**85**	**36**	**44**

The numbers in parentheses are the percentages that mentioned the relevant factor at all.

Figure 1: The percentage of mouse and primate research papers reviewed that reported the provision of enlarged cages, social housing, enriched cages (furnishing) and enriched diets (food), in papers over the period 1986 and 2006



■ = Mouse 1986 (N = 85); ■ = mouse 2006 (N = 85); ■ = primate 1986 (N = 36); ■ = primate 2006 (N = 44).

Differences in reporting across time and between types of animal

Each paper could score a maximum of 10 points for reporting implementation of the ten Three Rs-related factors listed in Tables 1 and 3. The median score for mice was 0 in both 1986 and in 2006, and 1 for primates in 1986 and 1.5 in 2006 (see Table 4). There was no significant difference between the median score for mouse research papers between 1986 and 2006 (Mann-Whitney test, $Z = -0.73$, $p = 0.467$, $N = 85, 85$). There was also no significant difference between the median score for primate research papers between 1986 and 2006 (Mann-Whitney test, $Z = 0.05$, $p = 0.961$, $N = 36, 44$). However, there was a significant difference in score between primate and mouse research papers in 2006 (Mann-Whitney test, $Z = -6.37$, $p < 0.001$, $N = 44, 85$), with mice scoring a median average of 0 out of 10 and primates scoring a median average of 1.5 out of 10.

Discussion

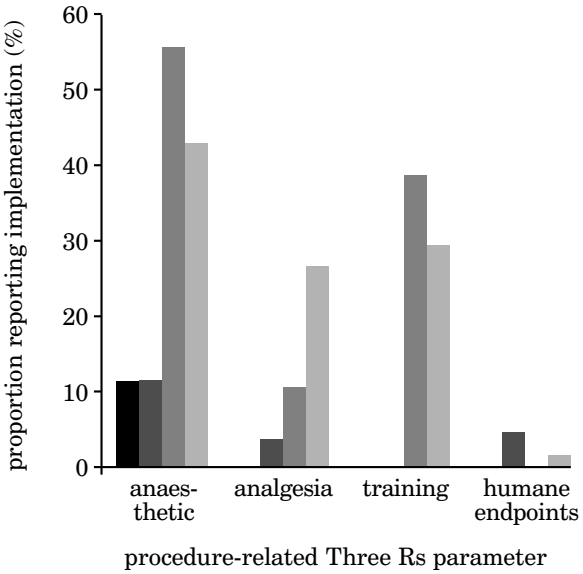
The number of papers

Between 1986 and 2006, there was a two-fold to three-fold increase in the numbers of papers arising from European institutions that reported research on non-human primates and mice. The number of journals did not appear to increase over the time-period analysed (data not shown), so the increase might be explained by an increase within one or more journals (either due to increased size of the journal or increased preference for publishing animal research), or a change in the number of journals reporting such research. The difficulty in selecting journals that were high reporters in both the time-periods analysed supports the latter suggestion, and could reflect an increased diversification of journals, if not an increase in their overall number.

Reporting of ethical review and adherence to regulations

As might be expected since the publication of legislation and guidance in 1986, there was a significant increase between 1986 and 2006 in the reporting of approval by ethics committees and adherence to codes of practice/legislation. There

Figure 2: The percentage of mouse and primate research papers reviewed that reported the provision of anaesthesia, analgesia, positive training for procedures, and the use of humane endpoints, in papers over the period 1986 and 2006



■ = Mouse 1986 (N = 85); ■ = mouse 2006 (N = 85); ■ = primate 1986 (N = 36); ■ = primate 2006 (N = 44).

Table 4: Average scores for reporting the implementation of the Three Rs in ten chosen experimental factors, in European mouse and primate research papers reviewed from 1986 and 2006

Animal	Date	N	Mean score	Standard deviation	Median	Range
Mouse	1986	85	0.2	0.4	0	0–1
Mouse	2006	85	0.2	0.6	0	0–3
Primate	1986	36	1.3	1.1	1.0	0–4
Primate	2006	44	1.6	1.4	1.5	0–5

The maximum possible score was 10. N = number of papers reviewed.

were no reports of either ethical review or regulation adherence in mouse research papers in 1986, but by 2006 this had increased to 28% of papers reviewed reporting ethical review and 48% reporting adherence to regulations. Similarly, only one primate paper reported either ethical review or regulation adherence in 1986, but by 2006 this had increased to 27% reporting ethical review and 61% of the papers reviewed reporting adherence to regulations.

These increases support the premise of the study that, since 1986, when these systems were put in place with the implementation of *Directive 86/609/EEC* and related national legislation, the reporting of ethical review and adherence to legislation has improved. This is a positive finding. However, not only were the percentages still rather low in 2006, but this should not be seen as a proxy for provision of specific information. Simply reporting 'adherence to legislation' does not necessarily tell other researchers whether the animals were socially housed, or even if they received pain relief, since under the legislation, both can be omitted if they are considered scientifically justified. One can only assume from statements such as these that the minimum standards were upheld, which is not always the same as implementing the Three Rs. For example, one paper stated "all animals were maintained using husbandry and housing in accordance with local and national legal regulations" (185). This sentence is virtually meaningless. Not only would one expect the experiment to be legally conducted as a matter of course, but it does not say which regulations they were in accord with, nor whether they exceeded the requirements of these regulations.

A recent survey conducted by animal welfare scientists supports this view (272). In their survey of 137 animal research papers, they found vague reference to approval by either an ethical review body or a regulatory body in 64% of papers they reviewed. They concluded that even the reporting of the extent of ethical review and adherence to regulations could be improved.

The reporting of *replacement* and *reduction*

It is disappointing that increased reporting of adherence to regulations and ethical review did not seem to correspond to an increase in reporting of actual implementation of the Three Rs. An extremely low number of papers reported justification for the use of animals or showed evidence that the researchers had considered alternatives. Indeed, the highest proportion that did so was in the mouse research papers of 2006 (6%). None of the primate papers did so. Reporting the necessity of using animals (as opposed to non-sentient material) should not be confused with justification for the need for the study, or explanation of the hypothesis to be tested. For example, papers did not score, if they reported the use of *in vitro* methods in the *Introduction*, but failed to explain why they then felt the need to move toward the use of *in vivo* methods. Absence of the reporting of a search for alternative methods may reflect a failure on the part of the author to fully evaluate whether the hypothesis could be tested by other means, and what further information was expected to come from the *in vivo* step that could not be found in another way.

Consideration of the use of alternative methods should be an integral part of the research process. In this way, not only would researchers be performing an important part of their own ethical evaluation, but they would be demonstrating their legal obligation not to use animals if an alternative existed. Reporting this consideration would also help to highlight to others what kinds of replacement methods are required, and why researchers do not use those that are available. A recent survey of a large Dutch animal research facility found that none of the scientists had a budget for searching for alternatives, and only 50% reported spending an average of two hours searching for alternatives per research project (273). This survey raises concerns that animal researchers do not reconsider the use of other methods every time they start a new project. Showing evidence that they have done so in their publications would be an

excellent way of demonstrating that indeed they had, which would also serve as a good example and a reminder to other researchers.

Somewhat more surprisingly, authors completely failed to justify the numbers of animals required. None of the papers reviewed in either type of animal or time-period described an assessment made, before the study, of the required numbers of animals. Justification of sample size is not only seen as a basic part of experimental design (274), it is a fundamental requirement in other areas, such as clinical trials (275). A staggering proportion of mouse research papers (80% in 2006) did not even state clearly in their methods sections the numbers of mice used.

Other researchers have also complained about poor justification in animal research papers of the numbers of animals used (14, 276). In their survey in 1997, Smith *et al.* (14) found that only 52% of the papers they considered gave the number of animals in their methods sections. Therefore, it is disappointing to find that this important aspect has not yet filtered down to become a feature of all scientific papers, even though it is claimed to be required by funding bodies and regulators (277). A very recent survey, organised by the UK's NC3Rs, found that 34% of studies did not have exact numbers stated in the methods, and none of the 48 papers they looked at in detail justified these numbers (15). They found higher reporting of the numbers of animals used, if they also extended their review to the *Results* section. However, this is not an ideal situation. Firstly, reporting this information in the results section is not conducive to clearly demonstrating the sample size required, and secondly, it allows the author to discard mention of unusable animals, making it very hard for a third party to establish exactly how many animals were used in total, and whether the conclusions from the results are statistically and scientifically correct. In other areas of research, such as clinical trials, the number of participants entering the study, and the number completing it, are considered to be important methodological parameters (275).

The reporting of *refinement*

There was extremely little reporting of the implementation of *refinement*-related experimental factors in mouse research. With the exception of reports on anaesthesia (12%), reports of all the other experimental factors of this type which were chosen for analysis, were 5% or less, regardless of year. Reports of *refinement* were also low for primates, but, by 2006, reporting was significantly greater than for mice for the provision of social housing (23%), enriched food (14%), anaesthesia (43%), analgesia (27%) and the use of positive rewards during training (30%). Greater reporting of the refinement of procedural factors was probably explained by a difference in the types of experiments to which primates were subjected, as compared to mice. A higher proportion of the papers reported surgical procedures and behavioural experiments in primates than in mice, and, in particular, the use of electrophysiological devices implanted under surgery, followed by behavioural testing. Reporting of the use of humane endpoints, cage size and enriched cages was, however, also rare, as it was for mice, with less than 10% of the papers reporting these in either time-period.

There was no appreciable difference in the reporting of the refinement of individual housing or procedural factors over time, for either mice or primates. The only significant increases seen were reports of the use of analgesia (from 11% of the 1986 papers reviewed, to 27% in 2006) and of the provision of social housing (from 6%, to 23% in 2006) for primates.

The results here compare with those of Carlsson *et al.* (2), who also found very low reporting of the *refinement*-related experimental factors they had looked at. For example, number of animals per cage (7.5%), cage size (15.1%), bedding material (3.8%) and conditioning (6.7%), were among the lowest factors reported, even in 2000. This survey also agrees with the findings of other surveys that have focused more specifically on reports of pain relief. In 1997, Boisvert (278) found reports of analgesia in approximately 10% of papers. Gomez and Conlee found reports of anaesthesia or analgesia in only 39% of papers (13). In their recent study, Stokes *et al.* (12) did find more reporting of anaesthesia and analgesia in rodent studies, and an increase in reports between 2000–2001 and 2005–2006, but they focused on papers reporting surgical procedures. Nonetheless, reports of systemic analgesia remained low for mice (only 11% of the studies in both time-periods).

However, some of these surveys (e.g. 11, 13) have predominately considered laboratory animal science papers, so it is not surprising that they found more-frequent reporting of the implementation of the Three Rs. This is because, for laboratory animal science and welfare research, the use of pain relief and enrichment is often an integral aspect of the research focus (16, 18). Nonetheless, Richardson and Flecknell (11) found that reports of pain relief only increased from 2.7% in 1990 to 19.8% in 2000; this is in line with the results reported in the present study.

The factor of positive reinforcement during training was intended to measure the practice of training or conditioning animals to accept procedures, or to perform for procedures in advance of the actual taking place of the procedure. Conditioning and training can significantly

decrease the stress response toward the procedure, as well as make handling much easier. It has been recommended for primates (18, 279), but it can also be applied to mice (e.g. conditioning to handling [17]). In reality, reports of training were most frequent for primates and were related to training in order to permit the performance of the procedure itself, e.g. training for behavioural responses during vision experiments. Many of the primate papers which featured behavioural testing did report the use of food or drink, and this was recorded as a positive reward. However this does not negate the distinct possibility that, within these studies, the monkeys were actually food and/or water deprived as well (18). Very few of the studies reviewed here, which involved the use of training, reported whether the primates were food or water deprived (*personal observation*).

Less than 5% of the papers reviewed reported the implementation of humane endpoints. This is of particular concern, since the use of humane endpoints is now considered a well-known, if not established, practice. Humane endpoints have been included in guidelines on the care of laboratory animals since at least the 1980s; the UK Coordinating Committee on Cancer Research included them in their guideline on animals used in cancer studies as far back as 1988 (280). The principle is that the animals are monitored and killed if they are found to show signs that comply with a set of predetermined indicators of suffering. Only a few other studies have looked at reports of humane endpoints in published papers, but they also agree with the results of the present study. Gomez and Conlee found reports of humane endpoints in only 25% of papers, which did include animal welfare science papers (13). In a small survey of Huntingdon's disease models, which can cause significant suffering, Olsson *et al.* (281) found that 6 out of 14 studies reported the implementation of humane endpoints. Failure to report the use of humane endpoints not only represents incomplete reporting of the experimental procedure, but is a clear example of where good practice could have easily been shared.

The total change over time

There was no significant increase in median total scores for reporting on the implementation of the Three Rs over time, for either mouse or primate research. Mouse research papers failed to score at all on average, even in 2006, and primate research papers generally scored very low, less than two reports out of a possible 10 on average. However, due in part to the remarkable consistency with which the papers scored (poorly), statistically there was a significant difference in total score between mouse and primate research in 2006, with primate research scoring, in general, one point more than mouse research.

Limitations of the survey

Reviewing the reporting of the use of humane endpoints, anaesthesia or analgesia, may be viewed as only relevant for certain types of experiment. However, given that all the papers featured potentially harmful research (see the definition in the *Methods* section), it could be argued that these types of *refinement* should at least have been considered. In reality, for both mice and primates, many of the reviewed studies involved the creation of sick animals with disease symptoms that could conceivably require analgesia and also a protocol for when to terminate the experiment. Indeed, particularly for mice, several of the studies were lethal challenge tests, where a significant proportion of the mice were reported to have died. Mice were also subjected to procedures where it is conceivable that anaesthesia would have been appropriate, such as the conduct of retro-orbital eye punctures for blood sampling and the harvesting of antibodies by using the ascites method. However, it could be argued that it is equally important that authors explain why they did not apply anaesthesia or analgesia, or humane endpoints. In the survey, if they did this, then they would have scored (although none actually did). It should be noted that it was the author's impression that the types of experiments for both mice and primates did not appear to reduce in severity between 1986 and 2006. Had the severity of experiments decreased, this might have explained the failure of an increase in reports of the use of analgesia, particularly for mice. A more quantitative survey of the severity of experiments in papers has recently been conducted, which supports the observations made here; the authors found that there was no real change between 1998 and 2003 in the proportion of papers from *Nature Medicine* that were subjectively assessed as falling into one of four categories of severity (3).

The present study looked solely at the reporting in research papers of the implementation of the Three Rs, and not at whether the Three Rs had, in fact, been implemented by the researchers. This distinction must be clear — this survey is about *reporting*, rather than implementing. However, following their survey, Richardson and Flecknell (11) contacted those researchers who had not specified the use of pain relief, in order to determine whether absence of reporting might equate to absence of use. Only 28 of the 101 researchers they contacted replied to their question, and out of those that did, 79% said they had not reported the use of analgesia, because they had not used it. It might therefore be reasonable to assume that, in the

absence of reporting, implementation of the Three Rs may not, in fact, have occurred. The onus should therefore be on authors to report the positive Three Rs-related steps that they have taken; otherwise the public, and indeed, other researchers, could be forgiven for assuming they have not been implemented.

The importance of transparency in research

Even though, until now, there have been few actual surveys on reporting on the Three Rs within papers, poor reporting of animal research has been raised as a specific concern for some time (14, 274, 282). Indeed, there was a debate in *Nature*, in 2007, on the extent to which experimental factors relevant to the Three Rs should be reported in research papers (282–285). There are at least three strong arguments for including such information.

Firstly, it is argued that inclusion of these factors is an important part of the methodology, and that not including them can have a negative impact on the interpretation or replication of the results (282), or even on the complete understanding of the methodology (286). It is well established that the conditions in which the animals are kept can have significant effects on physiological and behavioural parameters (287, 288). In addition, Hooijmans *et al.* (288) argue that studies need to be more completely reported, so that systematic reviews can be performed on them. The NC3Rs have recently stated (289) that: "Such information ought to be included from a scientific standpoint, since it is part of the experimental design and therefore necessary for evaluating, replicating or building upon the research results, and because good science is very often dependent on good animal welfare."

Secondly, it is important that researchers who do conduct better practice in their animal research, or have refined their techniques, should make this clear to other researchers; otherwise, such information is confined to a limited number of journals that explicitly report these improvements (282). The UK's advisory body on animal experimentation, the Animal Procedures Committee, noted in its report on the cost:benefit of animal research in 2003 that, "it is vital that animal procedures are reported in sufficient detail… to allow advances in application of the Three Rs [to be] documented and highlighted" (290).

Thirdly, complete reporting is important for reasons of transparency and accountability. Animal experiments are still shrouded in secrecy, and it is very difficult for independent observers or the general public to assess what was actually done to the animals, and why, and whether all steps were taken to ameliorate suffering (see the RSPCA/FRAME quote above, and Thomas [291]). There are other sources of information regarding the experiments themselves, such as the ethical review report and/or the project licence. The easiest solution would be to make the licence itself public, with confidential details omitted, or to publish the outcomes of the ethical reviews. Currently both these aspects remain highly confidential in the UK, with a prohibition on release of this information under Section 24 of the ASPA (10). In the UK, researchers write brief 1–2-page abstracts of their proposed work for public digest. However, an assessment of the first publications of these abstracts, by FRAME in 2005, not only found that not all the abstracts were submitted, but that those that were provided insufficient information for members of the public to judge the details of the experiment and the likely welfare costs to the animals (19). Abstracts scored 56% on average for the amount of information they could have given, and only 13% of them provided evidence of a search for (Three Rs) alternatives, despite this being a requirement of the UK Home Office guidance. Recently, a group of animal researchers have suggested that journals should make the free access to project licences part of their conditions for publishing, or that these are at least made public in the form of a database, as is the case for clinical trials (272). However, it is our experience that animal researchers are nervous about full transparency of this kind, so these suggestions are unlikely to be voluntarily taken up, at least in the near future. Therefore, in the absence of full access to information about licensed projects, published papers are often the only source of this kind of information.

Journal policies as the solution

One of the most obvious ways in which reporting within journals can be improved, would be if the journals themselves insisted on it. Indeed, Hooijmans *et al.* (288) reported that, in interviews, animal scientists expressed reluctance to report experimental parameters unless they were compelled to do so. Gomez and Conlee found that the reporting of provision of pain relief did increase, if the journal had a policy specifying that this should be reported (13). Animal welfare scientists have been pressing for clarification and adoption of Three Rs polices in journals for years (292), and this call has recently been taken up by scientific institutions as well. For example, the UK Nuffield Council on Bioethics, in their 2005 publication, *The Ethics of Research Involving Animals* (293), recommended that: "…all journals publishing results of research involving animals consider the inclusion of a category on the Three Rs in the methodology section". More recently, the Weatherall report on *The Use of Non-human Primates in Research* (294) had, among its recommendations,

that "Scientific journals should include details of animal welfare and steps taken to ameliorate suffering in all published papers that involve non-human primate research."

Unfortunately, these calls have not yet been answered, and in the meantime, there have been surveys which demonstrated that, in fact, journals often have no policy on animal research, let alone on how it should be reported. For example, Boisvert *et al.* (278) conducted a survey of 80 international journals, and found that over 60% had no ethical statements about animal research in their editorial policies. Gomez and Conlee found that only 15% of the journals they reviewed specifically asked authors to report on pain relief (13). Hooijmans *et al.* (288) have show that key journals, including some of the ones reviewed here, such as *PNAS*, *Journal of Immunology*, *Nature* and *Science*, do not require the authors to report on key experimental parameters. A more recent RSPCA survey of journal policies found that 53% of 236 journals had no meaningful policy on the use of animals, and have therefore reiterated their call that journals should construct one (295).

Most recently, in an attempt to stimulate progress, there have been attempts to provide lists of what information journals could demand to see. Following their survey of methodological reporting, which focused more on statistics, randomisation and blinding rather than on welfare related parameters, the NC3Rs published an extensive list of parameters that journals should insist on seeing — this is called the ARRIVE guideline. This list includes welfare parameters, such as whether the animals are provided with social housing, bedding and environmental enrichment (296). However, the list prepared by Hooijmans *et al.* (288) is more complete. Their list is called the Gold Standard Publication Checklist (GSPC), and includes experimental factors such as provision of bedding, cage size, social housing, analgesia and anaesthesia, together with references explaining why these are scientifically relevant.

In the era of online publishing, it should be more, rather than less, possible to include a complete methods section which pays full regard to the Three Rs (283, 286, 288). In fact, although many of the journals reviewed are already making use of 'online supplementary information or methods' (*Nature* and *PNAS*, in particular), in practice this is often used to provide additional results and not further details of methods (*personal observation*). This issue was recently debated in *Nature* itself, where animal welfare scientists called for journals to include a Three Rs section in their methods (283–284). However, some oppose a specific Three Rs section, not because it could be conceived as a burden (285), but because implementation of the Three Rs constitutes an important part of the methodology and should not necessarily be separated (282). To separate the Three Rs into a distinct section may serve to alienate researchers against Three Rs concepts (see 285), and thus not help in the promotion of the Three Rs as basic requirements for good science, as well as ethical science.

While there is continuing debate about improving the transparency of animal experiments (272, 291), it is imperative that journals have Three Rs policies that require that research is conducted according to current best practice and that the papers themselves contain information on parameters that can have key scientific and Three Rs bearing. This would mean that details of the provision of pain relief, housing type, training, justification of numbers, and consideration of alternatives, would be included in all research papers.

Conclusions

This has been the first survey of the extent of the reporting of a range of Three Rs-related experimental factors in European medical research papers. The results show that, despite apparent increases in references to the legislation and codes of practice, there has been little increase in the reporting of specific implementation of the Three Rs between the year 1986 and the year 2006.

Justification of the numbers of animals used and reports of searches for alternative methods remained extremely unsatisfactory, as did the reporting of the care of the animals, both prior to, and during, procedures. Sadly, there appears to have been little change in the quality of reporting since the RSPCA/FRAME study, and it still remains largely impossible to determine from papers exactly how pain and suffering were kept to a minimum. Not only are such experimental factors key parts of the methodology that may affect research results, but also, if researchers value the Three Rs, then one might expect that they might report them in their publications. This finding suggests that there has been little change in reporting style in the journals reviewed. In the interest of good science, as well as the promotion of best practice, journals need to adopt a much more proactive stance, and should insist on the reporting of such measures in the papers they publish.

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