# Report of the 2005 RSPCA/UFAW Rodent Welfare Group meeting

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The RSPCA/UFAW Rodent Welfare Group holds a one-day meeting every autumn for its members to discuss current welfare research and exchange views on rodent welfare issues. A key aim of the group is to encourage people to think about the whole lifetime experience of laboratory rodents, ensuring that every potential impact on their well-being has been reviewed and refined. The 2005 meeting focused on the refinement of techniques used in the creation of GM rodents, especially genotyping and vasectomy.

Speakers at the 2005 RSPCA/UFAW [Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare] Rodent Welfare Group discussed an array of topics related to laboratory rodent welfare; one group presented the idea that disruptions to burrowing behavior could be used as an early indicator that health or welfare may be compromised, while another group reviewed current progress in research identifying ways to refine the adjuvant arthritis model in rats. In a special session on refining the methods used to produced genetically modified (GM) rodents, speakers described switching from tail-tipping to ear-notching as a refinement of biopsy procedures and how vasectomy, which is used to produce sterile males for induction of pseudopregnancy in embryo recipients, can be refined by using the scrotal sac rather than the abdominal route. One speaker from the UK Home Office Inspectorate also provided her view on how techniques can be effectively modified to achieve benefits for the animals with minimal risks to welfare or to scientific validity.

## CHANGES IN BURROWING AND NESTING BEHAVIOR AS EARLY INDICATORS OF SICKNESS IN MICE

**Leigh M. Felton, BSc, PhD & V. Hugh Perry, MA, PhD (University of Southampton, Southampton, UK)**

Formal testing of species-stereotyped behaviors, such as burrowing, provides a useful indicator of 'illness' in mammals that can be used for sensitive, noninvasive assessment of disease progression and more effective implementation of humane endpoints, both of which reduce animal numbers and suffering. The technique described below is easy to use, requires minimal training, and could help in assessing animal well-being and refining endpoints in many different fields of research.

When living tissue is exposed to physical damage, infection, or disease, the animal mounts an immune or inflammatory response. Inflammatory responses can give rise to complex changes in metabolism and behavior; 'sickness behaviors' help the immune system to fight infection and promote recovery. Examples of sickness behaviors include locomotor impairment, lethargy, loss of interest in social and self-care behaviors, mood suppression (evidenced by symptoms of depression and/or anxiety), and diminished appetite[1].

As an example, bacterial infections are often modeled in mice by administering lipopolysaccharides (LPS). Following intraperitoneal injection with LPS, mice show many sickness behaviors that are also observed in humans, such as fever, reduced locomotion, anxiety, and anorexia[2]. There are also changes in instinctive species-specific behaviors, such as burrowing and nesting. Spontaneous burrowing behavior

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FIGURE 1 | Burrowing behavior can be assessed by providing mice with tubes filled with food pellets. (Photo courtesy of Leigh Felton, University of Southampton.)

is highly sensitive to LPS dosing and can be assessed objectively by providing a mouse with a burrowing tube filled with food pellets. Burrowing is quantified by weighing the tubes; the average mouse will consume the pellets in a tube in two hours. The burrowing substrate does not have to be food pellets, but we have found that these work best with mice. The grey plastic tubes, 20 cm long and 6.8 cm in diameter, are propped up at one end by a 3-cm-high wooden support to prevent the pellets falling out[3] (**Fig. 1**).

A preliminary investigation has suggested that there are strain differences in mouse burrowing behaviour[4]. C57 mice, which are derived from burrowing populations, are efficient in this task. By contrast, BALB/c mice, who are natural surface nesters, are not so reliable. Mice also appear to benefit from training in which they are first exposed to burrowing tubes as a group.

Burrowing can also be used to study the behavioral impact of other disease models. For example, burrowing behavior is impaired early in the course of murine models of prion diseases, such as scrapie[3,5,6]. Novel pharmacological targets for treating prion diseases have typically been investigated in mice by initially looking for changes in survival time, and then carrying out *ex vivo* pathology studies. There are ethical and welfare issues associated with this approach because the mice must progress to the terminal stages of the disease and because large numbers of animals are typically used in *ex vivo* studies.

An alternative approach is to use more subtle indicators of behavioral dysfunction to assess disease progression. For example, in one model of prion disease, mice demonstrate impaired burrowing behavior at 12 weeks, symptoms of depression or anhedonia (measured by loss of interest in a highly palatable sucrose solution) at 13–14 weeks, and a loss of motor strength and coordination at 16–17 weeks. Many of these subtle behavioral impairments appear well before the onset of clinical or terminal disease, which typically presents at weeks 18–20 (ref. 3). Thus, formal behavioral testing has allowed for the real-time identification of specific pathological aspects that might be alleviated by pharmacological intervention. Furthermore, this approach has allowed for a reduction in the number of animals used in *ex vivo* studies and has aided the identification of a humane endpoint that does not require mice to reach the terminal stages of disease (thereby reducing animal suffering).

## ALLEVIATION OF PAIN AND DISCOMFORT IN ADJUVANT ARTHRITIS RATS

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There are two critically important aims in arthritis research using animals: more objective, noninvasive techniques for assessing discomfort and pain and the ability to provide adequate and appropriate pain relief. The pursuit of these aims should lead to less variable experimental results, reductions in research animal numbers, and the induction of milder forms of arthritis that lead to equally good or better scientific results.

A common animal model used for the study of rheumatoid arthritis (RA) is adjuvant arthritis (AA). In this model, rats are injected intradermally in the base of the tail with Freund's complete adjuvant, resulting in an inflammatory response characterized by necrosis at the injection site and a systemic immune reaction that produces swollen joints and severe pain. Attempts to reduce pain and discomfort in the animals currently include providing extra bedding material and long-spouted water bottles and housing no more than two animals in each cage (thus reducing the chance that the animals might tread on one another's feet). There is clearly a pressing need to reduce both animal discomfort and the number of animals used.

One approach would be to improve the ability to detect the clinical signs associated with adjuvant arthritis, which include swollen joints, abnormal gait, decreased activity, and decreased grooming. The traditional way of assessing animal condition involves scoring the rats' paws from 0 to 4 for swelling and redness, but this is a crude and subjective methodology that resists reliable statistical analysis.

We are investigating the potential for using the CatWalk™ automated gait analysis system (Noldus Information Technology, Wageningen, The Netherlands) and infrared thermography to refine data acquisition

and analysis, and thus improve the efficiency of animal assessment. The CatWalk system comprises a walkway with a glass floor that is illuminated along one edge. When a rat or mouse walks across the glass, the light is reflected downwards and all of the points of contact are clearly visible. This can be recorded and analyzed by the CatWalk program. Paw temperature is measured using a noncontact infrared thermometer (C-1600, Linear Laboratories, Freemont, CA) which is held 0.6 cm from the animal's ankle, foot, or toes.

We trialed the CatWalk and thermography systems using twelve Lewis rats, six injected with the conventional dose of adjuvant and six with a lower dose. There were problems with CatWalk in that some of the rats dragged their bellies as they walked, making paw print analysis impossible, but the infrared paw temperature measurements showed a significant positive correlation with clinical scores. The best correlation was achieved with temperatures from the mid-section and ankles of the hind paws. Rats that received the lower dose of adjuvant experienced disease ranging from mild to severe. Therefore, because of the larger standard deviation in data, lowering the dose of adjuvant would require the use of additional animals compared to using the higher dose. As more animals would be necessary to achieve significant results, and some would still be experiencing severe effects, there is nothing to gain by lowering the dose of adjuvant.

Another way of reducing discomfort in AA rats would be to introduce new analgesic regimes. Most analgesics are anti-inflammatory and therefore not used in arthritis or other autoimmune disease research because there is a fear that they may interfere with immunological parameters. However, since untreated pain will also interfere with the immune response, providing analgesics might make for a more predictive model, corresponding to humans with arthritis who are given pain-relief medications.

An ideal analgesic for AA would achieve an effective decrease in pain and discomfort, have relatively little influence on the immune system (in order to gain acceptance from researchers), and be practical to use in routine arthritis research. We set up a study design using four groups of three AA rats, a saline control, and three injectable analgesic agents: gabapentin (a calcium channel blocker), memantine (an NMDA receptor antagonist), and mexiletin (a sodium channel blocker). These are conventional drugs for the treatment of epilepsy and psychiatric disorders, but have also been found to have an analgesic effect[7,8]. Data were obtained using the conventional AA scoring, infrared thermography, behavior, and the tail flick test; immunological parameters were also collected and analyzed. Preliminary findings to date show that the analgesics tested do not affect the immune response, but the pain-relieving effects of all three compounds are questionable. However, the behavioral data still needs to be analyzed and it may be that we need to test higher doses of the potential analgesics, possibly against a proven analgesic. Another possible change could be to administer the analgesics continuously in water rather than by subcutaneous injection.

In summary, there unfortunately does not appear to be any benefit in reducing the dose of adjuvant in the AA model, but infrared paw temperature may be a variable that could be used to implement refinement and reductions in animal numbers. Further studies are necessary to find an effective analgesic agent and dose to help alleviate discomfort and gain acceptance for use in animal experiments that involve immunological parameters. In the meantime, we would encourage all those involved in arthritis research to have an open mind regarding the provision of pain relief.

## SPECIAL SESSION: REFINING TECHNIQUES USED IN THE CREATION OF GM RODENTS

There are a number of techniques associated with the creation of GM rodents that have the potential to cause discomfort, pain, or distress, including vasectomy, embryo transfer, and biopsy for genotyping. Our discussion focused especially on vasectomy and biopsy, since refinements have been suggested and defined for both of these techniques. For biopsy, ear notching has been proposed to replace tail tipping, and for vasectomy, the less invasive scrotal sac route instead of the abdominal route. However, these potential refinements are not always implemented when they could be. There are a number of reasons for this; for example, people may be daunted by the thought of learning new techniques, concerned about any potential impact of new methods on the animals, or even unaware that particular refinements exist.

There are two main steps to overcoming these issues and refining techniques associated with GM rodent creation, care, and use. The first step is to find out which refinements are available (**Table 1**) and discuss these with the veterinarian and other scientific or animal care staff, as appropriate. The second step is to research and plan how changes can be made without negatively impacting animal welfare or scientific integrity. Good communication with representatives of the body that regulates animal research and testing (*e.g.*, the UK Home Office Inspectorate) is an essential part of the process. Local inspectors can advise on licensing issues and help ensure smooth transitions in which animal welfare is not compromised. The Rodent Welfare Group therefore asked two scientists and a Home Office Inspector to discuss their experiences in refining techniques.

### MOVING FROM TAIL TIPPING TO EAR NOTCHING FOR GENOTYPING

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The development of techniques that permit the

**TABLE 1** | Refinement resources relating to GM rodent creation, care, and use

| Type of resource | What is currently available |
|---|---|
| Refinements in performing biopsies and vasectomies and other procedures such as superovulation, embryo transfer, and identification | **Refinement and reduction in production of genetically modified mice.** Sixth report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. Vicky Robinson *et al. Laboratory Animals* **37(Suppl. 1)**, 1–51 (2003). http://www.lal.org.uk/pdffiles/Transgenic.pdf. |
| Refining rodent housing and care | **Refining rodent husbandry: the mouse.** Third report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. Maggy Jennings *et al. Laboratory Animals* **32(3)**, 233–259 (1998). http://www.lal.org.uk/pdffiles/lab1566.pdf.<br>***The Rat: Good Practice for Housing and Care.*** RSPCA Research Animals Department (2005). Use in conjunction with *Good Practice for Laboratory Animal Housing and Care.* RSPCA Research Animals Department (2004). http://www.rspca.org.uk/laymembers (click on 'Supplementary resources' and scroll down).<br>***Comfortable Quarters for Laboratory Animals***, 9th edition. Edited by Viktor & Annie Reinhardt (Animal Welfare Institute, 2002). http://www.awionline.org/pubs/cq02/cqindex.html. |
| Information to help the lay members of your ethics or animal care and use committee assess projects involving GM animals | ***Supplementary Resources for Lay Members of Local Ethical Review Processes: Projects Involving Genetically Modified Animals.*** Natasha Lane and Maggy Jennings. RSPCA Research Animals Department (2004). http://www.rspca.org.uk/laymembers (click on 'Supplementary resources' and scroll down). |

alteration of the mouse genome via transgenesis has led to a rapid expansion in the use of GM mice. Genetic traits are, however, often recessive, so the genotype must be determined for every individual animal from a breeding colony for that colony to be of scientific value. Quick, early, and reproducible identification of individual genotypes is required for multiple reasons: to ensure that experiments are only carried out on the appropriate GM mice and control animals; to maintain and minimize colony size; and to minimize the welfare burden by identifying those individuals possibly requiring special observation and/or intervention.

Different techniques can be used to obtain tissue for genotyping, including tail tipping, ear notching, or sampling from blood, saliva (cheek cell), or fecal matter. DNA is routinely prepared from a mouse tail biopsy in the majority of labs, and the amount of tail removed is decreasing due to advances in the sensitivity of analytical techniques, such that it is currently not necessary to take more than 0.5 cm. However, removing even this amount of tail is likely to be painful. Work carried out at Imperial College by Jo Dharia and Dominic Wells has demonstrated that the last 5 mm of the tail has tendons, nerves, and coccygeal vertebra that have partly ossified by the time the mice are two weeks old. Histological studies have shown that cutting through nerves may cause hypersensitivity, a finding corroborated by thermal stress (tail immersion) studies. Removing the tail tip causes acute hyperalgesia in the rest of the tail in all strains tested, and some strains show chronic hyperalgesia that can persist for many months. Such evidence (originating from Dominic Wells and colleagues) compels us to define a less invasive tissue or sample biopsy method that remains robust and reproducible for routine genotype analysis.

Ear notching appears to be a good option for genotyping, not only because it is likely to be less painful than tail tipping, but also because ear notching is already frequently used in coding systems for identification purposes. Thus, tissue is by necessity already being removed in such cases, and this could potentially be used for genotype determination as well. Similarly, if ear notching were not currently used for identification purposes, then this would represent an added bonus when using ear notching for genotyping. However, the central issue is whether an ear notch provides sufficient material for routine genotyping.

At the DNA level, our laboratory, along with many others, have changed from Southern assays to more sensitive polymerase chain reaction (PCR) assays for routine genotyping. To test whether ear notch material could be a reliable tissue source for our routine genotyping over a protracted period, we set up a study to compare tail versus ear tissue when carrying out genotyping assays of eight different genes over several hundred mice.

We found that ear DNA yielded results that were at least as good as, or better than, tail DNA (**Figure 2** shows a typical comparison). Specifically, there were fewer negative results, cleaner (and often stronger) PCR bands, as well as a high degree of reproducibility when comparing tests on ear DNA to tail DNA. This may be because the quality of DNA prepared from ear tissue is better than that from the tail, which is perhaps unsurprising in light of the recent histological research on the composition of the last 5 mm of a mouse tail (Dharia, J., Wells, D. personal communication). Furthermore, if the ear DNA preparation yields a negative result, it is clearly less of a welfare issue to take more ear tissue than it is to retip a tail.

It is possible to further refine ear notching to reduce pain or discomfort. Our initial experiments used a 2.0-mm diameter ear punch to recover ear tissue, but we have now successfully moved to using a 0.5-mm

ear punch, which is likely to cause less tissue trauma. In our experience, a 0.5-mm ear punch provides sufficient material for over 150 independent PCR assays. It would be interesting to address whether sufficient material is available for Southern assays from larger ear notch samples.

During our discussion, Rodent Group members explained how they had performed ear notching on mice younger than the three-week-old mice we routinely sample. Group members' experiences were that ear notching was difficult to perform before mice are two weeks old, but some could be ear-snipped with scissors at one week old. This is particularly useful for instances when early genotype analysis is required for experimental and/or welfare considerations. However, such early tissue sampling should not be undertaken without justification, since neonatal rodents have low pain thresholds and lack the inhibitory mechanisms that partially block noxious stimuli in older animals[9]. Overall, our studies therefore allayed all of our concerns and misgivings about moving to ear notching for use in routine genotyping. The results are summarized in **Table 2**.

During our transition from tail to ear tissue biopsy, we have also optimized our laboratory protocols. With ear tissue, there are fewer tissue types (*e.g.*, no bone or connective tissue), so the DNA extraction recipe is simpler and quicker. In our hands, tail tissue commonly took two days to prepare for PCR, and the DNA often needed to be further purified using phenol and chloroform extractions and/or ethanol precipitation, whereas ear tissue only requires two hours of proteinase K incubation and no further purification other than dilution. Preparation time is thus greatly reduced and there is no need to use hazardous organic solvents. It is also much cheaper to use fewer reagents.

All of this has led us to completely abandon tail tipping for routine genotyping, and we now use ear tissue only. Should there ever be a need to verify or extend the PCR assays with Southern assays (for quality assurance/verification or transgene copy-number analysis), then there is no reason why tissue cannot be taken postmortem and analyzed retrospectively at the termination of the experiment. There may be some instances when PCR analysis is not available for genotype identification; however, where PCR assays are or could be used for genotype analysis, ear notch material is the ethical and practical sample of choice.

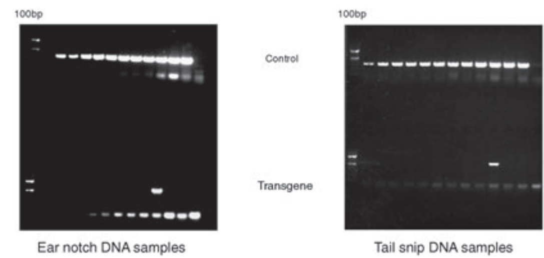


**FIGURE 2** | PCR-based genotyping of transgenic mice using DNA extracted from ear tissue yields comparable results to assays using tail tip tissue. (Photo courtesy of Mark Maconochie, University of Sussex.)

### MOVING FROM THE ABDOMINAL TO THE SCROTAL SAC ROUTE FOR VASECTOMY

**Dominic Wells, MA, VetMB, PhD (Imperial College, London, UK)**

The generation of transgenic mice involves a number of procedures that have the potential to cause pain, suffering, distress, or lasting harm. It is therefore important that every possible effort is made to replace the use of transgenic animals, reduce the number of animals used, or refine the procedures involved. The method used to vasectomize male mice for induction of pseudopregnancy in recipients of GM embryos is one area suited for procedural refinement.

The most common vasectomy procedure involves accessing the vas deferens by performing a ventral laparotomy and then exposing the tube by gently pulling on the fat pad above the testis. However, this involves substantial manipulation of the abdominal contents (which can lead to postsurgical infection) and subjects

**TABLE 2** | Allaying concerns about ear notching

| Perceived disadvantage | Reality |
|---|---|
| There will not be enough DNA. | There is more than enough to do many PCR assays. |
| Samples will be cross contaminated. | This has not proved to be a problem in practice, and moreover, in our experience there is no need to clean the punch or scissors after every ear. |
| It will be too tricky. | The whole process is tricky—a little practice is all that is required. |
| It will not be applicable where single base-pair changes are the basis for genotype differentiation (*e.g.*, for spontaneous or radiation/chemically induced mutants). | PCR-based assays can be developed to discriminate single base-pair changes. |
| Southern analysis is the routine method for genotyping this strain in the laboratory. | This may be for historic reasons rather than a scientific basis for determining genotype in this manner. Once the lesion/gene is known, PCR based assays using ear notch samples can normally be retrospectively designed. These will support faster and cheaper genotype identification. |

the wound to mechanical stress following recovery due to the weight of the gut. The procedure requires pain relief (possibly for more than 24 hours) and is not always performed aseptically.

An alternative approach is to access the vas deferens through a scrotal incision. Although this still theoretically involves opening the peritoneum, the abdominal contents need not be manipulated, diminishing the risk of infection. Importantly, the scrotal incision does not affect the muscles that support the weight of the gut, assuring that the operated area is not weight-bearing in the immediate postoperative period. The scrotal incision may, however, lead to swelling and potential pain that should be controlled by the use of non-steroidal anti-inflammatory drugs (NSAIDs) or buprenorphine. The scrotal approach thus offers an improved technique for vasectomy and should replace the ventral laparotomy procedure.

Vasectomy surgery is carried out on males 7–8 weeks old. Either fentanyl/fluanisone (Hypnorm, Janssen Animal Health, Buckinghamshire, UK) and midazolam (Hypnovel, Roche, Basel, Switzerland) injectable anesthesia (10 ml/kg of a mix of Hypnorm/Hypnovel/$H_20$ at 1:1:2 by volume). Alternatively, isoflurane inhalation anesthesia allows the mice to recover more quickly. In the scrotal procedure, the area is clipped, a small incision slightly left or right of the midline is made high in the scrotum (**Fig. 3**), the vas deferens is located and accessed via another small incision in the overlying peritoneum, and a section of the vas deferens is removed using thermal cautery. The pain-relief protocol we currently employ at Imperial College is a twice-daily regimen of buprenorphine given subcutaneously (s.c.) or intraperitoneally (i.p.) at 0.1 mg/kg or a once-a-day treatment of 10 mg/kg carprofen given s.c., orally, or in water. Other commonly used NSAIDs include s.c. administration of 5 mg/kg meloxicam or 10 mg/kg ketoprofen. Mice can 'mate' two weeks after vasectomy. There is no need to test-mate the mice, but sterility can be assured by using recipient females and vasectomized males that have a different coat color (*e.g.*, albino CD-1 or MF-1 mice) than the donor females.

Once researchers decide to use the scrotal incision approach, it is important for those performing this new technique to become competent in the procedure as quickly as possible to ensure that animals do not feel any avoidable discomfort or pain. Because the scrotal approach requires more technical expertise, it is a good idea to begin by revising anatomical knowledge using a euthanized mouse, preferably killed for another purpose. When mice are euthanized by cervical dislocation, however, the blood empties from the landmark blood vessel that runs along the vas deferens, making it more difficult to identify during the procedure (for more details of the technique, see ref. 10). It is also essential to consider how you will apply aseptic technique (transparent drapes are a good idea) and which analgesic you will use. Allowing

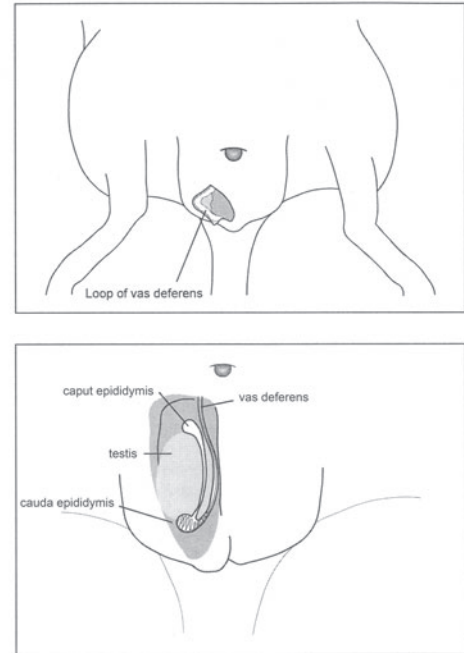


**FIGURE 3** | The scrotal approach to vasectomy is an alternative to the commonly used, and more invasive, abdominal approach. (Reprinted with permission from Raffery, J.A. *Methods in Experimental Embryology of the Mouse* (Johns Hopkins Press, Baltimore, MD, 1970).)

sufficient training time is essential before starting to use the scrotal sac approach routinely.

If naturally sterile males are used in inducing pseudopregnancy in GM embryo recipient females, then there is no need to carry out vasectomies. For example, males of the HsdOla: T145H-Re strain, resulting from a cross between a mutant female and a wild-type male, are sterile. Plugging in GM embryo recipient females occurred following 21% of the matings with HsdOla: T145H-Re males, as compared to 22 % when vasectomized males were used. Thus, naturally sterile males, which can be used for a year or more, are a viable alternative to vasectomized males that remove the need for surgery, although the excess production (and waste) of heterozygous female mice (produced when breeding the sterile males) is an important ethical concern. There may also be biosecurity issues with importing animals into a facility.

In addition to refining vasectomy surgery, it is important to refine the laparotomy surgery that provides access to the oviducts or uterus (*e.g.*, for embryo transfer). This also requires good aseptic technique, transparent drapes, and the use of the least invasive surgical approach. We use a single midline incision followed by bilateral muscle incisions to access each oviduct. It is sometimes erroneously suggested that embryos can redistribute if they are implanted via a unilateral incision; not only is that untrue,

but unilateral transfers will overcrowd a single horn of the uterus. We therefore always carry out bilateral transfers and routinely use hypnorm/hypnovel injectable anesthesia (10–15 ml/kg of a mix of Hypnorm/Hypnovel/$H_20$ at 1:1:2 by volume). For all surgery, minimizing stress during recovery is important; male and female mice need a thermostatically controlled heat pad and appropriate perioperative analgesia. There have been concerns about the possible effects of analgesics on pregnancy, but experience in our group and many others demonstrates that buprenorphine, meloxicam, and ketoprofen do not cause embryo loss.

### MODIFYING TECHNIQUES AND MOVING ON: A UK HOME OFFICE INSPECTOR'S VIEW

**Ngaire Dennison, MA, VetMB**
**(Home Office Inspectorate, Dundee, UK)**

Council of Europe Directive 86/609, which regulates animal research and testing within the European Community, requires that all animal experiments are designed so as to cause the least pain, suffering, distress, or lasting harm while being most likely to produce satisfactory results[11]. Besides this legal obligation to minimize suffering, there are also sound ethical reasons for striving to reduce potential harm to animals and improve their welfare.

If the aims of the Directive are to be achieved, techniques must be regularly reviewed to keep up with current rapid developments in laboratory animal science and welfare. This will help ensure that appropriate refinements or new, improved techniques are introduced as soon as possible after they become available. This applies to even small refinements, as these can have a significant impact on animal welfare, particularly if they involve common techniques that are used in large numbers of animals.

It is understandable that researchers are sometimes reluctant to change techniques, especially if they have been used for many years, fearing that the scientific outcomes might be altered. However, these concerns can be addressed within the establishment by setting up a system for planning how changes will be implemented. The first step is to identify the best contemporary practices. 'Best,' however, can be difficult to agree upon, as these situations are rarely straightforward. The 'best practice' in any one situation will depend on a number of factors, including the previous experience of researchers, care staff, and veterinarians, the specific aims of the study, relevant local factors, and, where applicable, regulatory requirements. Once these factors have been identified, a constructive next step is to set up pilot studies or transitional phases, during which refinements are implemented one step at a time.

The following example, taken from my personal experience as a laboratory animal veterinarian, describes efforts to modify regulatory tests for the detection of shellfish toxins. The regulatory test for paralytic shellfish poison (PSP) is a mouse bioassay of substantial severity that involves injecting mice i.p. with a large volume of highly acidic shellfish extract and then observing the animals for a specified time. If animals die, the time of death is used to calculate the level of toxin present in the sample. Obviously, there was concern about performing this assay and a desire to reduce the suffering caused to the mice. The first stage was to identify the key causes of suffering, which were the acidic nature of the extract, the duration of the assay, and the volume of the injection. We then researched the literature to find out more about the properties of the toxin. This led to a small increase in the pH range of the extract (this apparently minor change decreased acidity by 50–100 times because the pH scale is logarithmic) and a two-thirds reduction in the observation time, thus reducing potential animal suffering.

The protocol was reviewed by researchers and the local ethical review process (ERP) to see whether the use of anesthesia might be feasible. The ERP is a mandatory committee that reviews every research, testing, and breeding facility in the UK, ensuring that the justification for each project is critically assessed, that alternatives are used wherever possible, and that both animal numbers and suffering are reduced and animal welfare is improved within each study and throughout the establishment. We developed a plan with input from the veterinarian (who had knowledge of anesthesia), the researchers (who had knowledge of the practicalities of the test and its scientific aims), a statistician, the ERP (for input from lay persons and scientists with different types of expertise), and the local Home Office Inspector. The regulatory body that required the data from the PSP monitoring program then funded a pilot study.

The pilot study showed that an injectable anesthetic agent was the most practical option. Although the mice still received an i.p. injection, the volume of the anesthetic was only one-fifth that of the shellfish extract and its pH was neutral. This was a significant improvement for the mice, though there were concerns that anesthetic in the peritoneal cavity might interfere with the assay results. The pilot study found that time to death was extended by the presence of anesthesia, but a calibration study using a range of toxin doses confirmed that the alteration in time of death caused by anesthesia was predictable. The new times could therefore be built into the calculation of toxicity, so it was feasible to use anesthesia without compromising scientific validity. A procedure of substantial severity was thus replaced with a procedure carried out under terminal anesthesia, making this case a good example of a refinement that changed experimental results but still allowed the overall scientific objective to be achievable.

When implementing refinements, it may be necessary to strike a balance between scientific requirements and

welfare benefits. Some changes that provide welfare benefits may not affect scientific outcomes. For example, the scientific outcomes for abdominal and scrotal sac vasectomy route are the same. In other situations, however, there may be clear benefits to animals, but possible negative impact on experimental outcomes; the introduction of a palatable carrier to dose animals with a drug rather than using gavage or injection is one such example. It is important for the potential effects of procedural changes on scientific validity to be quantified, as above, to see whether they can be overcome, allowing equivalent results to be achieved with less animal suffering, as required by the Directive.

In conclusion, for refinements to be successfully introduced, it is essential to have an establishment culture that promotes the '3Rs'—replacement of animal experiments where possible, reduction in numbers, and refinement of husbandry and other procedures to reduce suffering and improve welfare. This requires all parties concerned with animal use to 'buy-in' to the Directive, work constructively to address any scientific issues, and recognize the welfare benefits and their importance. A team approach will help to ensure that positive change is achieved. The local ERP or animal care and use committee is an appropriate forum for team building and discussion, as it provides a focus for good communication between researchers, veterinarians, and animal technicians. Provisions for appropriate training, including how to search for and identify refinements, and good supervision of trainee researchers are also critical. Finally, obtaining advice from the body responsible for implementing the national laws regulating animal research and testing, such as the UK Home Office Inspectorate, can be important in ensuring that the best practices are implemented and that changes are made as smoothly as possible.

The key points for implementing refinements are as follows:

- Review currently used techniques regularly
- Consider the 3Rs in their broadest sense, including how refinement applies to the whole lifetime experience of every animal, and not just procedures
- Identify and use the best practices whenever possible
- Take local factors into account
- Use a step-wise approach to making changes
- Identify and use appropriate expertise; make sure people are working as a team
- Be prepared for set-backs; work to overcome them
- Make sure that training programs address the identification and implementation of new refinements
- Discuss changes with regulatory bodies, such as the UK Home Office Inspectorate

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**COMPETING INTERESTS STATEMENT**
The authors declare that they have no competing financial interests.

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