Norecopa
c/o National Veterinary Institute
P.O. Box 750 Sentrum
0106 Oslo
Norway
www.norecopa.no
Correspondence to: Adrian Smith (adrian.smith@vetinst.no)
15. December 2008

Toe clipping in mice: an evaluation of the method and alternatives

1. Background

1. On 2 May 2008 the Norwegian Animal Research Authority (Forsøksdyrutvalget, FDU) forwarded to the Norwegian Food Safety Authority (Mattilsynet, MT) a complaint made about their decision of 26 February 2008 to allow toe clipping as a method for the identification and genotyping of genetically modified mice. FDU had set the following conditions for their decision:
    a. toe clipping must be performed before the age of 10 days
    b. only one toe on each hindleg must be clipped
    c. the toe must be anaesthetised with a local anaesthetic (gel or dip method) before clipping
    d. Clipping must be performed before bone tissue and periosteum have been formed.

    In the application for animal research upon which FDU made their decision, it was specified that clipping was to be performed no nearer the body than the first toe joint. The method should therefore be referred to as ***toetip clipping***. FDU considered other methods such as ear marking to be equally severe procedures, at least. FDU were aware of studies on the effect of toe clipping at the University of Zurich and will compare these findings with the results from studies on other marking methods. Based on available knowledge and experience, FDU concluded that they could not see that toe clipping, under the conditions described above, is particularly burdensome compared with other identification techniques.
2. In a letter to Norecopa dated 1 January 2008, FDU requested an evaluation of the different strategies for toe clipping rodents (time for clipping, how far on to the toe should be clipped, the number of feet where toes should be clipped, the number of toes that should be clipped per foot) in relation to other relevant methods for marking and tissue sampling, as regards both animal protection and ease of use. On 11 August 2008 Norecopa replied that it was willing to undertake the task. An early draft of its conclusions were discussed at Norecopa's board meeting on 17 September 2008.
3. In a letter dated 24 September 2008, MT accepted the complaint lodged against toe clipping and reversed the decision made by FDU. MT pointed out that removal of a body part is against the principles embodied in the Norwegian Animal Protection Act, that animals should be given the benefit of the doubt, and that scientific studies are underway to evaluate the methods available. MT questioned the claim that neonatal animals do not feel pain. Until the results of ongoing studies are available, MT wished to be restrictive.

2. Introduction

Reliable and repeatable identification of individuals with a minimum of stress to the animal is an elementary and necessary factor in the quality assurance of most animal experiments. The increasing use of genetically modified animals in laboratory studies, where large numbers of animals are brev in the hope of producing individuals with the desired genotype, has created a need for individual marking and tissues-sampling of as young animals as possible, for

practical and economic reasons. Early humane killing of surplus animals can be claimed to have ethical advantages, because it eliminates the risk of suffering in individuals which will not be used in experiments anyway.

There are few published studies on methods that can be used for tissue sampling in young genetically modified animals, and even fewer behavioural studies of the effects which these methods have on the animals in the longer term.

It is important to differentiate between quantitative and qualitative genotyping. When new transgenic mouse lines are established, it is often desitable to be able to measure quantitatively how the gene of interest has been incorporated into the genome (the number of copies and location). For these measurements a method like Southern blotting is used. In established lines, it is usually sufficient to detect the gene qualitatively (to see if the animal is transgenic or not) and in these cases methods such as PCR, which are based on the amplification of DNA from very small samples, can be used. In other words, the aim of the tissue typing decides how much tissue one needs to take from the animal. A range of relatively non-invasive methods for DNA-sampling for PCR testing have been developed. In the case of quantitative tissue-typing, however, invasive methods have to be used, that in reality consist of amputations: tail clipping and toe clipping.

The decisions made in research communities in Norway and abroad on the choice of method appear largely to have been made without a solid base of comprehensive and complete scientific studies, and are to a large degree a result of the researchers' subjective opinions or asssumptions, based in turn on extrapolations from human experiences. A review of the literature reveals that descriptions of toe clipping are more positive in older publications and guidelines. Norecopa has not found histological or electrophysiological studies of the innervation of the toes in rodents, that could provide an anatomical or physiological basis for assessment of the animal's ability to experience pain when toetip-clipping is performed at the age in question.

It is a generally recandnised concept in modern laboratory animal science that choice of method should be based on an evaluation of several factors:

- the method's scientific quality and reproducibility
- the effect which the method has on the individual on which it is used

Both these two factors should be prioritised before practical and economic considerations.

In Norecopa's opinion there is a need for similar evaluations of the methods used for identifying and tissue-typing wildlife (http://www.nc3rs.org.uk/category.asp?catID=79) and fish (http://oslovet.veths.no/gardermoen.pdf) in research.

3. Terms in use

The Norwegian Regulation on Animal Experimentation §2 gives guidelines for what can be defined as 'simple marking of animals'. Methods for marking animals are not defined as animal experiments as long as there is no 'reason to assume that the experiment will affect the animal's normal way of life, or cause other than slight pain or discomfort of a highly temporary nature'. This type of division into regulated and non-regulated procedures exists in a number of other countries, such as Great Britain:

'Blood or DNA sampling solely to establish the identity or provenance of an animal would not be regulated if the intervention caused no more than momentary discomfort or distress.

Methods of marking or identification, such as toe clipping, which can cause suffering in excess of this threshold, are regulated when carried out for an experimental or other scientific purpose. (www.nc3rs.org.uk)

Toe clipping has been commented upon in the mass media in Norway, in these articles, among others:

1. Researchers want to mutilate mice (Norecopa's translation) (www.nrk.no/nyheter/distrikt/ostlandssendingen/1.5879860).
2. A letter to the editor of Aftenposten 10.06.08 from Bodil Ekerhovd Damsgaard, Oslo, who claims that toe clipping reduces the animal's ability to climb and groom itself.
3. A letter to the editor of Aftenposten 14.06.08 from Janicke Nordgreen and Torunn Fosse, Norwegian School of Veterinary Science, who point out that it is the degree of development of the nervous system that determines whether or not a newborn mouse can feel pain, not the size of the sample taken or the degree of skeletal ossification. The authors also point out that research indicates that the body's mechanisms for reducing painful stimuli are probably not fully developed at birth, such that stimuli may be more painful to newborn animals than they are to adults. In addition, pain experienced in the neonatal period may be "remembered" by the central nervous system, so that the animals show increased sensitivity to pain later in life.

These articles in the media do not always give a precise description of the method for which FDU gave permission, see section 1.1 above.

4. Methods of identification

In this section, the advantages and disadvantages of the different methods for identification and tissue-sampling are briefly described.

1. Toe clipping

Advantages:

- It is a permanent identification method with a low risk of misidentifying individuals (Kumar, 1979)
- It provides enough DNA for quantitative genotyping
- It allows the identification of desirable genotypes before weaning (Nadon & Draeger, 1996)
- It has been reported that the tip of a toe on the forelimb of a mouse will grow out again if the amputation is performed distal to the outermost joint (Borgens, 1982)
- The method is simple and cheap. It must, however, be performed carefully if only the tissue distal to the outermost joint is to be removed
- The method requires minimal restraint of the animal (the animal's hindquarters are lifted up by gripping the base of the tail, or the animal is lifted by the skin of its neck in the same way as young are transported by their dams)
- Local anaesthesia of the foot can easily be carried out using a spray
- Inspection of the toeclip is quick and easy, with small chances for misidentifying two animals

Disadvantages:

- There is reason to believe that the method is painful, regardless of whether the toe contains bone or cartilage at the time, and the animal will experience pain after the procedure, even if analgesics or anaesthetics are used
- There is some behavioural evidence of reduced welfare after toe clipping (Iwaki *et al.*, 1989)
- It may be assumed that mice use all their toes for normal locomotion, including climbing, and that some degree of locomotory impediment arises after toe clipping. Mice in cages appear, however, primarily to use their forelimbs when climbing. The removal of one toetip on one hindleg will probably have limited effect on their ability to climb. On the other hand, the toe stump will be in more or less continuous contact with the cage floor after the procedure, unlike the situation if tail clipping is performed

2. Ear punching

Advantages:

- The method is similar to earmarking used in farm animals, which is a standard practice and therefore generally accepted
- The method is considered to cause less discomfort than tail or toe clipping, because it is performed in an area without bone formation
- The technique provides tissue for qualitative genotyping
- The size of the hole kan be reduced to 0.5 mm and still give enough tissue for PCR (Hawkins *et al.*, 2006)

Disadvantages:

- The method is likely to be painful, even though the ear contains cartilage and not bone. The animal will probably experience pain after the procedure, even if painkillers are used during the procedure
- The method cannot be used for quantitative genotyping
- Problems with reading the ear punches are common, particularly if there has been fighting in the cage, since some management systems require the use of more than one hole per ear. Animals can easily be confused using this technique, which may in turn lead to the use of more animals in a study
- The ear that has been punched may become the target of aggressive behaviour in the cage and the ear may be torn into pieces
- Ear punching requires considerable restraint of the animal, since the operator will want to avoid being bitten in the process
- Animals cannot be ear punched before they are 14 days of age, since the ears are too small to be marked without causing extensive damage to the ear
- The ear is given a permanent perforation, which reduces its thermoregulatory function and the ability to localise sound. The outer ear is an important organ in the mouse for the reduction of body temperature following exertion, since these animals cannot lose water vapour by gasping or sweating

- Local anaesthesia of the ear is difficult because of its proximity to the ear canal and eye

3. Earmarking with metal tags

Advantages:

- The method is the equivalent of earmarking domestic animals, and is therefore generally accepted
- The method is likely to cause less discomfort to the animal than toe clipping as an identification technique, because it takes place in an area without ossification
- It is an unequivocal method of identification

Disadvantages:

- The method is considered to be painful, even though the ear contains cartilage and not bone. The animal will probably experience pain after the procedure, even if painkillers are used during the procedure itself
- Ear tags have to be placed where the cartilage is thickest. This necessitates long experience if they are to be placed correctly in mice
- For this reason, ear tags frequently fall out. If this happens on two animals in the same cage, both must be marked a second time
- Ear tags can result in the ear being caught on cage furniture
- Ear tags can become a target for aggressive behaviour in a cage, with the risk of them being ripped out
- This method does not provide tissue for genotyping
- Animals cannot be tagged before they are 14 days of age, because the ears are not big enough
- The method requires considerable restraint of the animal
- The weight of the tag results in the ear hanging in a lower position than normal, which reduces its function in thermoregulation and the localisation of sound
- Anaesthesia er difficult because of the proximity to the ear canal and eye

4. Tail clipping

Advantages:

- The method provides enough DNA for quantitative genotyping
- It enables animals with a genotype of interest to be identified before weaning
- The method er simple and cheap
- Experience indicates that the method er less painful than ear punching if no more than 5 mm of the tail are removed
- The method is quick and does not require a form of restraint that stresses the animal

Disadvantages:

- The method is likely to cause pain, regardless of whether the tail contains bone or cartilage, and the animal will probably experience pain after the procedure. It is possible that the pain during and after the procedure can be reduced by using

anaesthesia, and this should be investigated more closely. It must also be assumed that suffering after the procedure can be reduced by treatment with painkillers

- The tail is an important organ for balanse and grasping, and a degree of locomotory impairment will arise after this procedure if significant lengths of the tail are removed, for example by serial sectioning
- Removal of only the very tip of the tail will on the other hand have minimal effects on locomotion
- There is a danger of bleeding if the technique is used on older animals. Repeated tail sectioning is therefore not recommended
- The method must be combined with a means of identification

5. Tattooing

Advantages:

- It is a permanent method of identification with little risk of misidentifying individuals
- The method can be used on very young individuals, for example on the palm of the paw (Honma *et al.*, 1986)

Disadvantages:

- The method is difficult to use on animals with pigmented skin
- It does not provide tissue for genotyping
- The effect of tattooing on the toes and palms of small rodents has not been sufficiently investigated
- Tattooing is experienced as painful in humans and the same must be assumed for rodents
- The method can result in activation of the immune system due to uptake and storage of dye particles in tissue macrophages. This is an undesirable factor in many animal experiments

6. ID-chip (transponder)

Advantages:

- This is a permanent method of identification with no risk of misidentifying individuals
- Smaller transponders have been developed in recent years (e.g. Nonatec, www.nonatec.net) which may be assumed to cause a minimum of discomfort. These can be used on young animals
- The transponder is implanted under the skin of the neck, which is an area where the animals are grasped by their dams when they are moved around the cage. It may therefore be assumed that this procedure causes a minimum of fear

Disadvantages:

- The method does not provide tissue for genotyping
- The method requires equipment, including a scanner that supports the system used in the transponder. Differences between systems can cause problems when animals are moved from one laboratory to another

- The larger transponders are likely to be burdensome for small individuals. The needles used to implant the transponder are large and analgesia should be used during implantation

- There is somem evidence of an increased frequency of cancer in rodents that have been implanted with transponders (http://en.wikipedia.org/wiki/VeriChip)
- The transponders can cease to function after a while
- The transponders can wander under the skin, making it difficult or impossible to scan them

7. Blood sampling

Advantages:

- The method does not involve the removal of part of the animal's exterior organs

Disadvantages:

- Blood sampling in small animals is technically demanding and can result in pain and discomfort
- The method must be combined with a means of identification
- The method provides little tissue for genotyping and is most suitable for research where groups of animals are to be separated based on the phenotype of their blood cells
- The method requires some equipment and experienced operators

8. Application of dye to the skin, hair clipping etc.

Advantages:

- There is little risk of misidentifying individuals if the operators use a standard system
- The methods are animal-friendly, simple and cheap

Disadvantages:

- The methods do not provide tissue for genotyping
- Dyes can be rubbed off and frequent handling is necessary to apply more dye or to clip the hair
- In toxicological studies, the use of chemicals that can penetrate the skin should be avoided
- The chemicals may affect the other animals in the cage if they lick each other

9. Saliva/epithelial cells from the buccal cavity

Advantages:

- The method er animal-friendly in individuals that are sufficiently large (Irwin *et al.*, 1996).
- The method provides DNA for qualitative genotyping (Zhang *et al.*, 2006)
- Extraction of DNA from the sample is faster than when using ear punching, tail clipping or toe clipping (Meldgaard *et al.*, 2004; Mitrecić *et al.*, 2008)

Disadvantages:

- The method can only be used for qualitative genotyping (PCR)
- The method must be combined with a means of identification
- There is a risk of contamination by DNA from the mother's milk or mammary glands
- The method is burdensome for young mice
- There is a risk of contamination between animals because they lick each other and consume each others' faeces. The method is therefore not suitable when several animals are housed in the same cage

10. Rectal scrapings/faecal samples

Advantages:

- The methods are animal-friendly and cheap
- Extraction of DNA from the samples is faster than with ear punching or ear/toe clipping (Murgatroyd *et al.*, 2006)

Disadvantages:

- The methods must be combined with a means of identification
- The chance of contamination of faecal samples, or confusion between animals, is great, unless the samples are collected directly from the individual, which is a time-consuming process
- The samples contain DNA from a range of other sources than the animal itself
- Rectal scrapings cannot be performed on very young animals
- The method has not been used extensively, particularly on young animals

11. Removal of hair follicles

Advantages:

- The method er relatively animal-friendly and cheap
- Hair plucking requires minimal restraint

Disadvantages:

- The method must be combined with a means of identification
- Mouse hair easily becomes charged with static electricity, which can result in contamination between individual, either due to hair moulted from other animals or because of insufficient cleaning of equipment between samples. The method is, however, suitable for sampling just a few animals, for example in cases of repeated genotyping
- The method er unsuitable for small animals that have little hair

12. Earprint

This is a new method, inspired by the use of fingerprinting in humans, based upon photography and interpretation of the pattern of blood vessels in the ear of rodents (Cameron *et al.*, http://www.nc3rs.org.uk/news.asp?id=675).

Advantages:

- The method er animal-friendly, rapid and non-invasive

Disadvantages:

- The method is still under development and requires special equipment
- Some readings are of insufficient quality to enable definite conclusions to be reached and the animal has to be re-photographed
- The method does not provide DNA for genotyping

5. Research on toe clipping and other methods

Little research has been published on toe clipping.

1. Vachon (1998) studied the anatomical changes following amputation of the distal end of the first bone at approx. 2 weeks of age. Complete healing of the bone and the overlying skin was observed, but changes in the normal architecture of the bones were observed. Ossification occurred on day 18. No problems were observed after amputation but the author pointed out the need for studies of the innervation of the area and the possible risk of inflammatory reactions.
2. Cinelli *et al.* (2007) did not evaluate toe clipping, but they compared the effects of many biopsy techniques using telemetry of body temperature and heart rate, and locomotory patterns. They concluded that restraint was the most important stress factor for the animals and that hair samples were difficult to handle because of the risk of cross-contamination. Ear marking was the method that gave the greatest and most longlasting effects on the parameters they measured, but all the same it was considerd to be the best method since it functioned both as an identification technique and a biopsy method. The authors pointed out that buccal and rectal scraping often resulted in bleeding and could therefore not be reckoned as non-invasive methods, neither did they give a different stress response than the other methods investigated.
3. Arras *et al.* (2007) studied the effect of tail clipping with or without anaesthesia on a range of physiological parameters in adult mice. They concluded that anaesthesia did not reduce the effects of tail clipping on a range of physiological parameters, and that tail clipping affected these parameters less and for a shorter period than did anaesthesia alone. In a short description of the histology of the tail, they reported that there was little difference between sections taken 2, 6 and 10 mm fra the tip, except for a gradual reduction in the number of nerve fibres further away from the body.
4. Hankenson *et al.* (2008) have recently published a study of ossification, DNA-content and acute behavioural response to tail clipping (various lengths) in 6 mouse lines between 3 and 42 days of age. The authors concluded that the optimal time for harvesting DNA was 14-17 days of age.
5. The European laboratory animal science organisation FELASA (www.felasa.eu) has appointed a working group that is comparing the various methods for identification of rodents. The first phase of their work is a pure literature study. Their conclusions are not available yet.
6. FELASA has also appointed a working group to investigate the possibilities that exist for refining the methods used to genotype genetically modified rodents. The deadline for this report is December 2009.

7. A research group in Utrecht is in the process of comparing tattooing with toe clipping in newborn rodents.
8. Researchers in Uppsala are working on a comparison of toe clipping and earmarking in various mouse strains.

The conclusion that must be drawn is that a number of studies are in progress, but at the present time there are few results of scientific experiments that are of relevance to the current topic.

6. Important considerations when choosing a technique

1. Norecopa is of the opinion that both scientific, legal and ethical considerations should for the basis of judgement when a procedure to be used on an experimental animal is evaluated. As an aid to this process, the "3 R's" of Russell & Burch (1959) and the "3 S's" of Carol Newman (cited in Öbrink & Waller, 1996) may be used:
   Replace, Reduce, Refine
   Good Science, Good Sense, Good Sensibility
2. Any pain or suffering involved is experienced by the individual, regardless of the number of animals used.
3. The total burden placed on the animal in its lifespan should be considered. If a procedure carried out at one phase can eliminate the need for further harmful procedures later, then it should be considered, even if in itself it is a burden to the animal.
4. The advantages of tranquillisers/sedatives or anaesthetics must be weighed against the stress imposed by the handling necessary to administer these drugs, or the drugs themselves. This stress may be greater then the stress of the procedure itself, but optimal use of tranquillisers/sedatives can also contribute to a reduction of the burden on the animals. This must be assessed in each specific case. The Norwegian Regulation on Animal Experimentation §14 states that 'should there not be reason to assume that the intensity of pain experienced in an experiment exceeds the pain intensity of anaesthesia, anaesthesia may be omitted'.
5. The aesthetic aspect of the amputation of parts of a body organ is important for many people, and traditional methods such as earmarking are more generally accepted in general opinion.
6. The Norwegian Animal Protection Act is in general restrictive to amputations. The Act forbids tail docking, beak clipping of chickens and ear cropping of dogs. The present Act is to be replaced in the near future by a new Animal Welfare Act. The restrictive attitude to amputations is present in the draft of the new Act. Although the Norwegian Regulation on Animal Experimentation allows procedures to be performed that are normally forbidden in society's use of animals, in Norecopa's opinion the research community should be especially cautious in employing techniques that fall into that category.
7. Toe clipping is in Norecopa's opinion within the category of identification and sampling techniques where, according to the Norwegian Regulation on Animal Experimentation (§2) 'there is reason to assume that the experiment will affect the

animal's normal way of life, or cause other than slight pain or discomfort of a highly temporary nature.'

8. Toes are more or less in contact with the ground at all times, in contrast to other organs under discussion, such as the tail and ears.
9. A central factor in the evaluation of toe clipping is whether there is a real need for large amounts of tissue for genotyping at the age of 1-10 days. This should be assessed in each case and should be made clear in the application for the animal experiment. Is there really a need for DNA at this age at all, or is the desire for tissue sampling based solely upon practical or economic considerations, or the claim of behavioural advantages by removing unwanted animals from a colony as quickly as possible?
10. Especially when invasive methods are used for sampling, any excess tissue should be stored so that the genotype can if necessary be characterised again without having to take a new sample. The size of the tissue sample should always be adjusted to the test method to be used, so no more tissue than necessary is removed.
11. Colonies of genetically modified animals are often large. It will always be tempting to choose methods (clinical studies, identification techniques, sampling procedures etc.) that are cheap and easy to perform.
12. There are a number of arguments for genotyping the animals in a genetically modified colony as early as possible:
    a. Unwanted animals can be humanely killed before weaning, reducing the number of cages, number of animals, risk of suffering caused by disease outbreaks and the occurrence of fighting/injuries, as well as the work burden on the employees
    b. The removal of some individuals from a litter will result in more milk being available, and will reduce stress, for the remaining young. In this way even the weakest can survive. These are often the genetically modified animals (e.g. homozygote knockouts), that have the greatest need for extra milk in this critical phase. Smaller litters result in larger and more robust young at weaning, and this is a significant argument in the improvement of breeding techniques for genetically modified and mutant animals.
    c. The dam is often pregnant at the time when toe clipping is to be performed, so there is less pressure on her if unwanted young are removed from the home cage (the psychological effects of the loss of these young must however also be taken into consideration). The tissue in the toes and tail tip of mice consists of cartilage, not bone, in the first two weeks of life, so toe and tail clipping at that age are probably comparable to the use of ear punching in older animals as regards av the experience of pain.
    d. Demands for effectivity should not be a main argument for choice of method, particularly if the method is considered to be a burden to the animals. Effective procedures are, however, often of little burden to animals, as they involve the minimum of restraint and therefore reduced stress. The effective management of an research animal unit is also about housing as few animals as possible, for as short a time as possible.
13. Opinions on the experience of pain in newborn animals (and humans) has coloured the debate on whether the more invasive methods are defensible or not. Experience pf pain (and therefore suffering) is conditional on two things: the nervous sytem must be

developed, so pain stimuli reach the brain cortex, and the animal must be conscious. Based upon measurement of the brain's electrical activity (EEG), Diesch *et al.* (2007) divide animal species into three different groups, depending upon how mature their nervous system is at birth. Marsupials (such as the kangaroo) are extremely immature at birth and conscious experience of pain is probably not developed until the animals are 120-180 days old. In other species (e.g. sheep), higher brain activity can be measured approximately 30 days before birth, but the lambs are maintained in a sleep-like state in the uterus by means of a range of substances produced in the brain. Behavioural studies show that these animals develop a full pain response gradually during the first week of life.

Rodents probably occupy a position between these extremes. Diesch *et al.* (2007) cite studies which Ellingsen & Rose (1970) conducted on the elektrical activity in the brain of young rats, and concluded that the animals did not show neurophysiological signs of the conscious experience of pain before 12-18 days after birth. From their own studies on young rats whose tails were clamped under anaesthesia, Diesch *et al.* concluded that conscious experience of pain does not normally occur earlier than 10-12 days after birth, with a gradual maturation process thereafter which lasts about one week.

One should all the same be cautious in exposing animals to painful stimuli during the phase when the nervous system is apparently still under development: studies on boys that were circumcised without anaesthesia revealed that they exhibited oversensitivity to the pain of vaccination up to 6 months later (Fitzgerald & Anand, 1993). In addition, there is evidence that the central nervous system is more (rather than less) sensistive to painful stimuli shortly after birth compared to later in life.

It is unclear how much analgesia is achieved by applying local anaesthesia during toe and tail clipping, or whether (and for how long) painkillers should be given after the procedure. There are practical problems in dosing these preparations to very young animals.

Studies of the brain's electrical activity in young rats cast doubts as to whether animals under 10 days of age are capable of the conscious experience of pain. This does not, however, rule out the possibility that these animals may feel pain later, even if anaesthesia has been used. Insufficient studies have been carried out to be able to assess whether the removal of the outermost joint of one toe affects the animal's normal way of life (e.g. climbing) or creates more than 'slight pain or discomfort of a highly temporary nature' (Norwegian Regulation on Animal Experimentation, §.2), e.g. in the form of the phantom pains that are described in humans following amputations, despite postoperative analgesic treatment. Neither is it possible to conclude whether the removal of a tail tip or a piece of the ear results in less pain than toe clipping. None of the methods are unproblematic. Tail clipping, on the other hand, differs from fra toe clipping in that it does not affect an organ that is in continual contact with the ground. The ears are too small in mice of the age in question to give enough tissue for quantitative genotyping.

7. Practice in other countries

There are few science-based reports from working groups that have evaluated the different methods of identifying and tissue sampling.

The *Joint Working Group on Refinement (JWGR, 2003)* in Great Britain recommended as a matter of principle that consideration should be taken to:

a. The source of the tissue
b. The size of the piece of tissue that is to be removed
c. The age of the animals
d. The need for local or general anaesthesia

The Group pointed out that the least invasive method should be used, and as little tissue as possible should be taken. Many of the least invasive methods are not used because there are traditions for using the more invasive ones, despite the existence of publications describing less invasive methods. Procedures should be reassessed regularly. The method used for genotyping should be discussed: Southern-blot hybridisations need more DNA than PCR techniques, but can be unavoidable if the number of transgenic copies must be identified. PCR should always be considered for routine genotyping of colonies, but it is important to avoid cross-contamination with other DNA.

In cases where both identification and genotyping are needed, the JWGR recommends the following (combined, if necessary, with an identification method):

	<2 weeks	3-4 weeks	>4 weeks
Saliva or faeces	√	√	√
Tail clipping	√/X	√	√/X
Ear punching	X	√	√
Blood	X	√	√
Toe clipping	X*	X	X

X: only in exceptional cases*

The JWGR was of the opinion that toe clipping probably incures pain and can reduce the animal's ability to grasp or groom. The Group concluded that it must not be used as a routine method of identification or tissue sampling for genotyping. In rare cases it may be unavoidable, e.g. where there are good scientific reasons for identifying mice of less than 14 days that are kept in isolators because of infection. In these cases, toe clipping may be the only practical method because of the animals' size and the need for biosecurity. Toe clipping should only be used as a last resort, and only one toe should be removed from one hindleg, under local anaesthesia. The tissue that is removed should be used for genotyping and the animals should not be subjected to further biopsy procedures. The method must not be used on animals older than 14 days, because other methods such as ear punching are then possible.

The JWGR mention several references in their guidelines on tail clipping, which Norecopa considers to be relevant when assessing toe clipping. The tail vertebrae begin to ossify at 2-3 weeks of age. There is evidence in the literature that there is bone substance even in the last millimetre of the tail. The skin and periosteum are rich in nervous tissue, and the JWGR concludes that tail clipping must be considered to be very painful, particularly when bony tissue is cut.

Norecopa's secretary has discussed the matter with the chair of the JWGR committe, Professor David Morton, UK. He recommends tail clipping (3-5 mm of tissue), which should normally be performed on mice before they are 2 weeks old and under full inhalational anaesthesia (e.g. isoflurane, N.B. not ether). Half of the sample DNA should be stored in case the tissue has to be retested, to avoid repeating the procedure (personal communication, cited with permission).

The ILAR Guide for the Care and Use of Laboratory Animals (NRC, 1996), which is used by, among others, the accreditation body AAALAC International (www.aaalac.org) states that toe clipping should only be used as an identification method in small rodents when no other method is possible in practice, and even then only be performed on 'altricial neonates'.

The Canadian organisation CCAC (Canadian Council on Animal Care, www.ccac.ca) have produced guidelines for the care of transgenic animals (1997) but these do not give specific recommendations on techniques for identification or tissue sampling.

An Internet search reveals that many local ethical committees describe the use of toe clipping. Som, such as the *Guidelines for biopsy procedures to facilitate identification and DNA-based molecular genotyping of rodents* from Emory University (2001) allow toe clipping on 8-12 day old animals without anaesthesia. In many cases, however, toe clipping is described as a method for identification alone, and is in such instances often cautioned against, with advice on using less invasive methods like tattooing or the use of transponders. This impression is supported by the replies that Norecopa has received to a posting on an international discussion forum for the laboratory animal community (CompMed) managed by the American laboratory animal science organisation AALAS.

8. Conclusions

1. Quantitative genotyping creates a need for relatively large amounts of DNA and therefore the use of invasive methods of tissue collection. This means in reality that amputations have to be performed on living animals. Amputations should therefore only be performed when tissue for quantitative genotyping is needed to characterise new genetically modified lines. Norecopa's Board believes that this principle should also apply to wild animals and fishk. Amputations should not be accepted as routine methods and special permission should be sought from the regulatory authorities to use them. Less invasive methods that need less DNA should therefore always be used for routine genotyping. It should be emphasised that animal protection organisations are in principle against amputations.

2. There are only two established methods that give sufficient tissue for quantitative genotyping of transgenic lines: tail clipping and toetip clipping. Although toetip clipping has the advantage that it also functions as an identification method, it is assumed to be more of a burden than tail clipping for the animal because it affects the locomotory apparatus which is more or less continually in contact with the ground. There are simple, non-invasive marking methods that can be combined with tail clipping, for example the application of a coloured dye to the skin in the armpits.

3. Norecopa's Board has registered that few institutions use toe clipping for genotyping, and that there is greater resistance to toe clipping than there is to tail clipping, even though both methods are controversial. The majority have replaced toe clipping as an identification method with other procedures, also for animals under 10 days of age, in agreement with the conclusions in the British JWGR report and the American ILAR Guide.

4. **Norecopa's Board is therefore of the opinion that toetip clipping, even with the refinements described in FDU's decision, should not be permitted. In those cases where it is absolutely necessary to undertake quantitative genotyping, tail clipping should be used (3-5 mm performed only once per animal) under anaesthesia, and with post-operative analgesia as long as this does not in itself create a greater burden for the animal. Further studies should be performed to identifiy the optimal anaesthesia and analgesia for the various methods of identification and tissue sampling. In cases where qualitative genotyping is adequate, less invasive methods should be employed, such as the collection of saliva, blood, faecal or hair samples, or (in larger animals) the material removed by ear punching.**

The minority of the Board has expressed their disagreement with this conclusion:

There is no evidence today that amputation of the tip of a toe is worse than amputation of the tip of the tail. Therefore toetip clipping should be permitted in situations where it is necessary to undertake genotyping at an early age. Early genotyping is necessary in cases where weak

offspring will have far better survival rates as soon as the litter size is reduced, in studies where genotyping is necessary before 14 days of age, or in cases where breeding is conducted in an isolator in connection with infection models. If the study design does not permit marking methods such as the use of pens, tattooing or other substances that will affect the study (e.g. toxicological, immunological or carcinogenic studies), toe clipping will function as a combined identification and biopsy method that together results in the least possible burden on the animal.

9. Literature

1. Arras M, Roneich A, Seifert B, Käsermann HP & Rülicke T (2007): Should laboratory mice be anaesthetized for tail biopsy? Laboratory Animals, 41: 30-45.
2. Borgens RB (1982): Mice regrow the tips of their foretoes. Science. 217(4561): 747-50.
3. Cameron J, Jacobson C, Nilsson K & Rögnvaldsson T. Identifying laboratory rodents using earprints. http://www.nc3rs.org.uk/news.asp?id=675
4. CCAC Guidelines on transgenic animals (1997). www.ccac.ca
5. Cinelli P, Roneich A, Seifert B, Bürki K, and Arras M (2007): Comparative analysis and physiolandical impact of different tissue biopsy methodolandies used for the genotyping of laboratory mice. Lab Anim 41(2): 174-184;
6. Diesch TJ, Mellor DJ, Johnson CB & Lentle RG (2007) Responsiveness to painful stimuli in anaesthetised newborn and young animals of varying neurolandical maturity (wallaby joeys, rat pups and lambs) AATEX 14, Special Issue, 549-552. Proc. 6th World Congress on Alternatives & Animal Use in the Life Sciences, August 21-25, 2007, Tokyo, Japan
7. Ellingsen RJ & Rose GH (1970): Ontandenesis of the electroencephalandram. I Developmental Neurobiolandy, s. 441-474. Red. WA Himwich. Illinois: Charles C Thomas Publisher
8. Emory University Guidelines for Biopsy Procedures to Facilitate Identification and DNA-Based Molecular Genotyping of Rodents. http://www.emory.edu/IACUC/pdfs/BiopsyPolicy.pdf
9. FELASA working group on Rodent Identification. www.felasa.eu
10. Fitzgerald M & Anand KJS (1993): Developmental neuroanatomy and neurophysiolandy of pain. I Schecter NL, Berde CB & Yatser M (red.): Pain in infants, children and adolescents, sider 11-32. Baltimore: William and Wilkins.
11. Hankenson FC, Garzel LM, Fischer DD, Nolan B & Hankenson KD (2008): Evaluation of tail biopsy collection in laboratory mice (*Mus musculus*): vertebral ossification, DNA quantity, and acute behavioral responses. J. Amer. Assoc. Lab. Anim. Sci. 47:10-18.
12. Hawkins P, Felton LM, van Loo P, Maconochie M, Wells DJ, Itnison N, Hubrecht R & Jennings M (2006): Report of the 2005 RSPCA/UFAW Rodent Welfare Group meeting. Lab Animal 35(9): 29-38.
13. Honma M, Iwaki S, Kast A, Kreuzer H. (1986): Experiences with the identification of small rodents. Jikken Dobutsu. 35(3): 347-52.

14. Irwin MH, Moffatt RJ, Pinkert CA. (1996): Identification of transgenic mice by PCR analysis of saliva. Nat Biotechnol. 1996, 1094.
15. Iwaki S, Matsuo A, Kast A. (1989): Identification of newborn rats by tattooing. Lab Anim. 361-4.
16. BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement (2003): Refinement and reduction in production of genetically modified mice. Lab.Anim. 37 (Supplement 1), S1-51. http://www.lal.org.uk/pdffiles/Transgenic.pdf.
17. Kumar RK (1979): Lab Anim Sci. 679-80.
18. Meldgaard M, Bollen PJ, Finsen B. (2004): Non-invasive method for sampling and extraction of mouse DNA for PCR. Lab Anim. 38(4), 413-7.
19. Mitrecić D, Mavrić S, Branica BV & Gajović S. (2008): Mice genotyping using buccal swab samples: an improved method. Biochem Genet. 2008 Apr;46(3-4):105-12. Epub 2008 Jan 23.
20. Murgatroyd C., Bilko D. & Spengler D. (2006): Isolation of high-quality DNA for genotyping from feces of rodents. Analytical Biochemistry, 348, 160-162.
21. Nadon NL, Draeger K. (1996): Genomic DNA analysis from mouse toe lysates. Transgenic Res. 5(3):209-11.
22. National Centre for the 3Rs, Storbritannia. Wildlife research. http://www.nc3rs.org.uk/category.asp?catID=79
23. National Research Council (1996): ILAR Guide for the Care and Use of Laboratory Animals, side 46. Washington DC: National Academy Press.
24. Nonatec microtransponder (www.nonatec.net)
25. Russell WMS & Burch RL (1959): The Principles of Humane Experimental Technique. UFAW, Wheathampstead, 238 sider. http://altweb.jhsph.edu/publications/humane_exp/het-toc.htm
26. Vachon P (1998): Anatomical and histolandical observations of fore- and hind limb toes in adult mice after amputations performed at the age of two weeks. Can J Vet Res. 62(4):311-3.
27. VeriChip (http://en.wikipedia.org/wiki/VeriChip)
28. Zhang Y-H, Huang B-L, Eastman K, McCabe LL, MacLennan NK & McCabe ERB (2006): Mouth collection device for newborn mice. Molecular Genetics and Metabolism 89: 164-167.
29. Öbrink KJ & Waller M (1996): Försöksdjurs-kunskap: Refinement, Reduction, Replacement. Studentlitteratur AB, Lund, 393 sider.