Implementation guide ‘Breeding of Laboratory Animals’

Contents

1 Background

These implementation guidelines provide more information about the practical interpretation given by the AWB to the policy document Purchase and breeding of laboratory animals, with a focus on the breeding of mice, rats and zebrafish. The policy and implementation guidelines apply to all persons involved in breeding of laboratory animals at Utrecht University (UU) and University Medical Centre (UMC) Utrecht.

The Animal Welfare Body (AWB) updates this implementation guideline based on new insights, with input from the appointed breeding co-ordinators. These implementation regulations come into force as soon as their effective date is published on the AWB website and in the AWB newsletter.

2 Introduction

In this implementation guide, we outline the practical frameworks. The implementation guide is based, among other things, on the advice 'Genetically modified animals killed in stock' parts 1[1] and 2[2] of the Netherlands National Committee for the Protection of Animals Used for Scientific Purposes (NCad), the revised guide 'The generation, breeding, genotyping, monitoring and keeping of genetically modified animals'[3] of the Central Authority for Scientific Procedures on Animals (CCD), the 'Guidebook on Mouse and Rat Colony Management' by Charles River[4] the 'Handbook on Genetically Standardised Mice' by Jackson Laboratory[7] and 'Colony Management at Zebrafish Facilities'[9]. The right knowledge and training are the basis for proper breeding management. Chapter 13 lists training opportunities.

3 Principles for setting up maintenance and experimental breeding

Chapter 4.6 of the policy on the purchase and breeding of laboratory animals states the considerations that are made for maintaining or cryopreserving a line. If the decision is made to maintain the line, this means that it must be kept genetically pure so that it is future-proof. This maintenance breeding is aimed at keeping the strain alive within the facility, with the smallest possible chance of genetic drift (see 5) and thus maintaining a healthy breeding line. Maintenance breeding is generally set up in small colonies to limit the breeding surplus as much as possible. The breeding co-ordinator, together with the researcher, determines which method is chosen and in what form the genetic modification(s) are maintained.

There are various methods for managing breeding, such as inbreeding, backcross breeding, continuous breeding, temporary breeding, harem breeding, 1:1 breeding [4,7,8,9,11]. If several methods prove to be suitable, with the same degree of discomfort, the route that leads to the least number of animals 'dead or killed before use in breeding or animal experiments'[2] should be chosen. The NC3Rs

has produced a 'best practice handbook' for optimizing the use of breeding and colony management[10].

If timed breeding is used, it is advisable to make use of the Whitten effect, whereby the cycle of the females is synchronised by placing male mice within scent range of the females 48 hours before the start of breeding. In IVC cages, this effect can be achieved by placing sawdust from cages with males in the female cage. The Whitten effect reduces the number of animals required.

Calculation tools are available for mouse breeding:

- colony breeding calculator of the University of Zurich;
- colony planning of The Jackson Laboratory.

3.1 Mice and rats

Several recommendations for maintenance breeding for small rodent colonies[8]:

- A minimum of six breeding pairs is recommended for the management of small colonies. With smaller numbers, the risk of genetic drift (see 5) increases.
- Maintain two generations of the line. Replace breeding reserves when the new generation reaches sexual maturity.
- Try to keep the age of breeding animals between eight (mouse), twelve (rat) and thirty-five weeks. Breeding efficiency in older animals is less predictable.
- The maximum age for mating is nine months for breeding females. After this age gestation and lactation are permitted. For embryonic use, it is permitted to breed females aged between nine and twelve months.
- The maximum age for breeding males is eleven months. After that, the animals may be kept for another month to determine pregnancy in the breeding females or to verify the genotype of the offspring.
- Crossbreed the line back in a timely manner, approximately every seven to ten generations, to prevent genetic drift. (see 5)
- Keep a close eye on breeding performance. If it declines, take immediate corrective measures in consultation with the breeding co-ordinator.

In a few situations, you may deviate from the agreements made in advance with the breeding co-ordinator and the AWB. Deviations are recorded in the appendix to the breeding protocol.

Reasons for increasing the number of breeding pairs	Reasons for reducing the number of breeding pairs:
• regular dead litters; • fewer pups per litter than expected; • breeding animals more often infertile; • abnormalities in offspring (unsuitable for breeding); • breeding with discomfort.	• large litters; • stable breeding line.

After breeding, male mice are more difficult to house socially due to dominant behaviour towards each other. There are several ways to prevent individual housing for male mice. One of these is to set

up the breeding rounds differently. You can choose to use two breeding rounds per breeding pair, with the father being weaned with the male offspring from the first litter. The different forms of preventing individual housing are listed in the policy on preventing individual housing.

Male rats are generally less dominant towards each other and can often be housed back in their old group after breeding. If aggressive behaviour is observed in the rats, it is advisable to replace the entire housing and place the cage mates in the new living environment at the same time. Maintain the old cage composition as much as possible.

3.2 Zebrafish

Number of recommendations for maintenance breeding for small zebrafish colonies[9]:

- A minimum of six breeding pairs is recommended.
- For a genetically healthy wild-type colony (outbred), it is advisable to maintain a larger population to keep the line robust and genetically diverse.
- Keep two generations of the line until the offspring are sexually mature.
- Try to keep the age of the breeding animals between four and twelve months. Efficiency in older breeding animals is less predictable.
- Keep a close eye on breeding performance. Take immediate corrective measures in consultation with the breeding co-ordinator if it declines.

In a few situations, you may deviate from the agreements made in advance with the breeding co-ordinator and the AWB. Deviations are recorded in the appendix to the breeding protocol.

Reasons for increasing the number of breeding pairs:	Reasons for reducing the number of breeding pairs:
• regularly no or poor clutches; • fewer embryos per clutch than expected; • too few founders to create a transgenic fish line; • breeding animals are often infertile; • abnormalities in the offspring; • breeding with discomfort.	• large clutches; • stable breeding line.

4 Recording breeding information

Records must be kept for each **breeding line**, containing at least the following information: average litter size/clutch size, survival rate of pups (rodents), survival rates at the larval stage after switch (zebrafish) and health problems within the breeding line (found dead, humane end point due to clinical problems). If it concerns a new line, this data is not always known. In that case, the line must be monitored during the first two stable generations, see chapter 10.

Records must be kept for each **animal bred** in-house, containing at least the following data: unique animal number, sex, date of birth, genotype, pedigree (traceability), generation, correct naming

(nomenclature), destination of animals (animal experiment, breeding animal, surplus animal) and owner.

4.1 Official naming (nomenclature)

For each genetically modified (GM) line generated, the correct name must be determined in accordance with international nomenclature rules. The breeding co-ordinator records the full strain name together with other strain-specific information in the Utrecht GM lines database. This list can be requested from the breeding co-ordinator.

Online tools for determining the correct nomenclature:

- mouse nomenclature;
- rat nomenclature;
- zebrafish nomenclature.

4.2 Generations

The generations of inbred strains are recorded as 'F' (filial generation). To prevent the creation of a sub-line (twenty generations), backcrossing to the pure background strain is required (see 5.1). If a line is backcrossed to the background strain, this is indicated with an 'N'. However, if brother * sister breeding also takes place during the backcrossing, for example to generate experimental animals, this is indicated as Nx;F1[4,7]. For example: You have already backcrossed three generations to the background strain, but you need experimental animals and you breed with brother * sister, then you indicate the generation of those offspring as N3;F1.

5 Preventing genetic drift

The definition of genetic drift according to Lee Silver's online book 'Mouse Genetics' (1995):

"The constant tendency of genes to evolve even in the absence of selective forces. Genetic drift is fuelled by spontaneous neutral mutations that disappear or become fixed in a population at random."

Lee Silver says here that genes have a continuous tendency to change, regardless of the selection applied within the research. A small colony is more susceptible to genetic drift. This is because in a small colony, the chance of a mouse with a mutation being selected for breeding is greater. The mutation is passed on very quickly. Within four generations, all mice in the colony have the mutation. Genetic changes cannot be stopped, but they can be slowed down.

The following steps can be taken to achieve this:

- Maintain two pedigrees of the same line. That way, you always have a backup available.
- Keep detailed information about the line (see 4).
- Cryopreserve the line (see Ch4.6 breeding policy and 10.4).
- Prevent (unintended) selective pressure (e.g. by selecting the right breeding animals within the line).
- Regularly renew the colony (≈7-10 generations) (see 5.1).

- Single Nucleotide Polymorphism (SNP) analysis. For this, the genome of the F0/1 mouse is analysed and compared every seven to eight generations. However, this is a rather costly method.

Genetic abnormalities in a line pose a risk to the reliability of your research results, but also to the reproducibility of experiments. It is therefore very important to minimise the risk of genetic drift.

Phenotypic abnormalities and reduced fitness within a zebrafish line can be caused by loss of genetic variation (genetic drift) and can compromise both the reliability and the reproducibility of experiments. Therefore, minimizing the risk of genetic drift is essential. In zebrafish facilities, a specific breeding strategy is applied to preserve genetic diversity. Ideally, all individuals in a population should contribute equally to the next generation. In practice, embryos from multiple independent matings are pooled and raised together in petri dishes. This approach increases the effective population size and reduces the impact of genetic drift. Maintaining a high level of genetic variation requires pooling embryos from more than 40 separate matings. When not available, you should at least set-up the highest number of crosses depending on the amount of fish available per strain[9].

5.1 Backcrossing the line

A commonly used method to keep the line pure is to preventively backcross to the background line every ten generations. It is preferable to start this from F7, but no later than F10. Ten generations are required for complete replacement of the background strain of the line (99.9% homogeneity). It is not always necessary to backcross the line, purchasing new breeding animals from the supplier or rederive from available cryo stock is also a possible strategy for genetic stability.

A reliable way to keep the line healthy involves the following steps:

1. Mouse from GM colony (female) * pure background strain (male) → N1 males from this cross have a 'fresh' Y chromosome.
2. N1 male (from cross 1) * pure background strain (female) → N2 males from this cross have 'fresh' X and Y chromosomes and mitochondrial genome.
3. N2 male (from cross 2) * pure background strain (female) → N3 fresh heterozygous line
4. Brother * Sister breeding → Maintain or expand the refreshed line/create homozygous animals.

The breeding protocol specifies in advance at which generation the backcross will start, whether this is the most efficient method, or whether the GM strain can be repurchased.

If you decide to maintain a subline, please check the website of the National Academies of Science to register for a unique lab code.

6 Standard criteria for humane endpoints (HEP)/ unsuitability for breeding

When animals are used for breeding, additional criteria apply for humane endpoints and/or no longer being able to be used for breeding. Deviation from these criteria is only possible approval of the Animal Welfare Body.

6.1 Mice and rats

Breeding animals:

- Breeding animals are replaced if no pregnancy has been detected after six consecutive weeks of breeding.
- Breeding animals are replaced if the pups, or a substantial part of the litter, are dead or unviable at birth or shortly thereafter, unless this is the first litter.
- Female breeding animals are replaced if they are unable to adequately care for their pups in the second litter.
- Breeding animals are euthanised at a maximum age of twelve months.
- Breeding animals are euthanised in the event of visible weight loss (sunken flanks, visible spine); in case of doubt, the animal is weighed weekly (maximum 10% weight loss).
- Breeding animals that experience more than mild discomfort* for more than 24 hours (unless more discomfort is permitted in a project licence for breeding with discomfort) are euthanised.

Pups:

Are euthanized if they:

- visibly lag behind normal development;
- show persistently insufficient activity;
- exhibit morphological abnormalities;
- continue to exhibit abnormal behaviour and/or posture;
- experience more than mild discomfort* for more than 24 hours (see point 5 below; unless more discomfort or line-specific clinical symptoms are permitted in a project licence for breeding with discomfort.)

Once the HEP has been reached, animals may only be kept with the permission of the designated veterinarian or the AWB.

6.2 Zebrafish

Breeding animals:

- Breeding animals are replaced if they fail to lay eggs after three attempts.

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* The following abnormalities are in any case considered to cause more than mild discomfort:

1. Parturition problems are always grounds for euthanasia and are classified as severe discomfort. If these problems (angular bulges, abdomen sinks in and/or becomes harder) are recognized in time, an attempt can be made to save the pups by caesarean section and placing them with a foster mother. Discuss with the breeding co-ordinator whether it is appropriate to do this in this situation (see 8).
2. Clinical symptoms that indicate moderate to severe discomfort: e.g. hunched posture, ears back, abnormal position of the nose or whiskers, reduced mobility, lethargy, hydrocephalus, and elephant teeth (malocclusion).
3. Impairment of the animal's functioning (inability to eat, walk, etc.).
4. Serious injuries caused by fighting: bitten-off body parts, injuries to the penis, thick joints or skin wounds > 1 cm in diameter.
5. Pups: absence of milk spot, rejection by mother (lying outside the nest) and clinical symptoms indicating moderate to severe discomfort, including hydrocephalus and elephant teeth.

- Breeding animals are replaced if they fail to produce offspring with a sufficient survival rate after three attempts or if too many background mutations become visible as phenotypic abnormalities (e.g. scoliosis, short body, motor defects, sideway swimming, etc).
- Breeding animals are euthanized at a maximum age of eighteen months.

Embryos/Larval/Juvenile stage:

Are euthanised if they:

- visibly lag behind normal development;
- show persistently insufficient activity;
- exhibit abnormal swimming behaviour;
- have morphological abnormalities;
- show persistent abnormal behaviour and/or posture.

Once the HEP has been reached, animals may only be retained with the consent of the designated veterinarian and the AWB.

7 Guideline on the killing of animals

7.1 Mice and rats [12, 13]

7.1.1 Neonate rats or mice (< 10 days)

- These may be killed by an overdose of anaesthetic, decapitation, or cervical dislocation. These methods are permitted by Directive 2010/63/EU (Annex IV).
- CO_2 is unsuitable.

7.1.2 Rats and mice (> 10 days)

- These can be killed by an overdose of anaesthesia, CO_2exposure, decapitation under (isoflurane) sedation, or cervical dislocation (for rats up to 150 g). These methods are permitted by Directive 2010/63/EU (Annex IV).

7.1.3 Embryos

- If embryos are needed for further study and must be removed from a mother animal under anaesthesia, a higher dose of anaesthetic than usual must be used, and the anaesthesia must also be maintained for longer. The embryos can then be killed by decapitation. The mother animal can be killed by exsanguination or cervical dislocation.
- Another method is to kill the mother animal by cervical dislocation and place the uterus with embryos in ice water to anaesthetize the embryos before removing them. The embryos can then be killed by decapitation.

7.1.4 Pregnant animals

- CO_2 may not be used.
- If embryos are not needed for further study, the pregnant animals may be killed in any other manner suitable for non-pregnant animals. It should be assumed that it will take at least 30-45 minutes longer (compared to the death of the mother animal) for the embryos to die.

7.2 Zebrafish[14]

7.2.1 Zebrafish embryos 0-5 dpf

- 1% sodium hypochlorite for at least 5 minutes

7.2.2 Zebrafish larvae 5-15 dpf

- Two-step method – step 1. Anaesthesia with MS222. Step 2. At least twenty minutes on ice water (without direct contact with ice), followed by freezing or fixation.

7.2.3 Zebrafish ≥16 dpf

- At least five minutes in ice water (without direct contact with ice), followed by freezing or fixation.

8 Guideline for removing or introducing pups during lactation

Removing the entire litter from the mother during the lactating period carries a risk of engorgement and mastitis (breast inflammation). The mothers must be monitored closely for the first three days after removal of the litter. Mastitis will lead to a clear clinical picture (e.g. swelling and/or redness of the breast tissue, lethargy, hunched posture, etc.). If this is observed, the animal must be euthanized. The procedure may be adjusted based on new insights.

The above does not apply to projects in which, based on the CCD license, a condition has been included regarding leaving one or two pups with the mother or immediately killing the mother after removing the pups.

The introduction of additional rat/mouse pups to a lactating mother, i.e. fostering pups, may occur in the event of unexpected loss of the mother or a mother animal that is not caring for the litter properly. When combining two or more litters, the total litter size may not exceed twelve pups. Larger litters can lead to unnecessary loss of animals because the mother animal is unable to care for all the pups.

Fostering of newborn mice should be done as early as possible and the age difference between the litters should not exceed 24 to 48 hours. The foster mother's acceptance of the pups can be increased by rubbing the pups with nesting material from the foster mother's nest or allowing the female to smell a tissue with perfume on it for a few seconds[8].

9 Additional requirements for animal facilities for the purchase of laboratory animals

The facility where the animals are housed may impose further requirements on the animals to be purchased, related to practical, logistical or zootechnical preconditions, such as microbial status. The following requirements also apply to rodents:

- Purchase is only possible if a particular species, strain, breed, or specific genotype is available from a recognised commercial laboratory animal supplier that has been designated as *a preferred supplier*[†].
- In exceptional cases, after consultation with the designated veterinarian, rodents from other external sources may be obtained. In that case, strict import requirements apply. Depending on their health status, they may sometimes be purchased under strict containment conditions for a short-term, one-off experiment.
- Zebrafish from other external sources can only be admitted after consultation with the manager of the fish facility.
- When importing a rat or mouse line for breeding from a non-preferred supplier, the purchase of sperm or (fertilised) eggs is preferred over the purchase of a breeding pair from which the line will be bred after decontamination. This is done in collaboration with commercial specialist parties.

10 Monitoring animals from breeding with an unknown phenotype

This chapter describes the practical implementation as set out in the Implementing Decision (2020/569/EU)[5] and the associated Working document under the European Directive on genetically modified animals[6].

The following situations are not subject to a project licence requirement with regard to the maintenance of breeding. This only applies if no above-threshold activities are carried out in the maintenance of:

- existing genetically modified lines with a phenotype that does not affect welfare;
- lines in which only reporter genes have been introduced into the genome; these genes do not necessarily lead to a pathological phenotype. even in crossbreeding, this will unlikely lead to a pathological phenotype;
- single Cre or Lox lines with a phenotype that does not affect welfare;
- existing lines with a genetic modification in which the phenotype is only active when treated with an inducer (e.g. tamoxifen). from the moment of induction, a licence is required.

The following situations do require a project licence regarding setting up or maintaining breeding:

- immunodeficient animals;

[†] List of commercial suppliers that meet the facility's requirements with regard to, among other things, microbial status and that are included in the facility's critical supplier audit programme. The management of the facility in question keeps the list up to date and can provide access to it.

- animals from a breeding line in which discomfort only occurs in homozygous animals or a lethal phenotype in homozygous animals. this also applies when a breeding strategy is used in which no homozygous animals are produced, for example by crossing heterozygous animals with wild-type animals;
- strains with physical impairment due to genetic modification. examples include blind mice and overweight animals;
- strains in which a pathological phenotype is age-dependent and has been demonstrated from a certain age onwards. this also applies when the offspring of this line will never reach this age;
- new strains or crossing of two existing lines where it is not yet known whether a pathological phenotype is present (see 10.1).

10.1 Creation of genetically modified lines or the crossing of two existing lines

The working document on genetically modified animals[6] describes the welfare assessment for the creation of genetically modified lines. The aim is to assess the welfare of the animals in a uniform and quantitative manner.

The assessment covers three areas:

A. Relevant information (name of line, genetic modification, assessment data, final severity rating). This is referred to as the animal passport;
B. Welfare assessment based on specific parameters for the species assessed;
C. Information on welfare issues (housing and care requirements, and/or suggestions for refinement strategies).

When should a welfare assessment be carried out?

- if it concerns a newly generated line;
- if it concerns a cross between two existing lines;
- if it cannot be reasonably demonstrated that the line does not have a pathological phenotype due to the genetic modification.

The monitoring consists of:

- animals from the first two stable generations;
- at least seven males and seven females from more than one litter/clutch;
- representative ages. Shortly after birth (e.g. first cage cleaning), during identification (collecting tissue for genotyping), during weaning and again after sexual maturity (during regular care). If the experiment requires older animals, this older age category is also included in the monitoring.;
- animals with relevant genotypes (heterozygous, hemizygous, and homozygous);
- animals with a corresponding genetic background (e.g. wild type) or from a defined reference line should serve as control animals.

Monitoring is carried out in accordance with the DALAS (Dutch Association for Laboratory Animal Science) monitoring forms. These forms can be requested from the breeding co-ordinators, AWB and MyDALAS.

The AWB, designated veterinarian and breeding co-ordinator, together with the breeding manager and researcher, use the welfare monitoring lists to determine whether a breed has a pathological phenotype. The welfare assessment is reviewed and updated as more data becomes available. This is part of the evaluation meetings with the researcher, breeding co-ordinator and AWB.

10.2 Registration of animals

During the monitoring phase:

In the annual statistics, all animals (including parent animals) used to create the line are listed under the purpose of the fundamental/applied research for which the line is being created. The only exception: wild-type offspring are not included in the annual statistics (unless they are used for other procedures, such as invasive genotyping without identification). These animals are included in the five-yearly report.

If pups are found dead (or missing) during monitoring, this must be counted as severe discomfort in the first two generations. There is a chance that this will result in incorrect figures being reported in the annual registration. This is because the wild-type control animals will also have a percentage of losses and premature mortality. The observed discomfort will therefore not be inherent to the discomfort within the line. For this reason, breeding as a whole must be monitored. Only when the percentage of pups found dead or missing is higher than expected, should the breeding programme be recognised as a line with severe discomfort and appropriate measures (discontinuing breeding, requesting change, etc.) be taken. This decision is always made in consultation with the AWB, the designated veterinarian, the breeding co-ordinator and the researcher.

When maintaining a line:

All animals from a line without discomfort are reported annually for the purpose for which they were kept. This concerns animals used for breeding, animals used in experiments (registration on the experimental licence), animals killed for use in experiments or breeding, etc.

Animals from a line with discomfort that are not used under an experimental protocol are only registered under the breeding licence if they have experienced welfare impairment due to the genetic modification. Healthy animals are registered as described for a line without discomfort.

10.3 Genotyping without identification

If surplus tissue is obtained from the identification of an animal, for example by ear clipping, no licence is required. Invasive methods involving the removal of tissue but no identification do require a licence. Examples include genotyping based on blood or tail clipping in rodents, skin swabs or fin clipping in fish. A breeding protocol must be submitted to the AWB for this purpose.

If the initial genotyping has failed and DNA must be collected again.

A licence is also required for 're-clipping' ears for genotyping purposes, as this no longer serves to identify the animals[6]. In that case, the researcher sends an email to the AWB for permission, stating the reason for re-typing and animal-related information. This additional procedure must be recorded and discussed during the annual registration meeting. Animals on which the additional procedure has

been performed also count as animal experiments in the registration, unless the animal has been used in an actual animal experiment. In that case, the animal is only registered for its last use.

10.4 Guidelines for freezing and cleaning lines

Cryopreservation (freezing)

This does not require a licence when frozen semen from killed animals is used. This only applies if these animals were not killed for this purpose. Under Dutch law, the killing of laboratory animals for the purpose of obtaining tissue also counts as an animal experiment. When live animals are used, a licence is required[3].

The continued existence of unique lines must be guaranteed (see policy on the purchase and breeding of laboratory animals H4.7). Back-up of a line can be made by means of cryopreservation. If this strategy is chosen, it is important that sufficient material is stored to restart the line. The general rule applied for rats and mice is a minimum of 3 straws of semen or 120 embryos. More information about this can be obtained from the breeding co-ordinator.

Rederivation (cleaning)

This requires a licence when it is carried out for scientific purposes. For example, when the immune responses can be influenced by the pathogen(s) present, but the health of the animals is not compromized[3].

Rederivation does not require a licence when it is carried out with animal welfare in mind, for example in the case of murine hepatitis virus (MHV), which can make the animals ill[3].

11 Literature

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12 Glossary

Homozygous	Mutation on both alleles
Heterozygous	Mutation on 1 of the 2 chromosomes
Hemizygote	Mutation on the X or Y allele

13 Training opportunities

- The Jackson Laboratory – Online micro- and minicourses: https://education.learning.jax.org/
- Optimising Research Reproducibility by Minimising Genetic Drift https://www.criver.com/resources/webinar-pi-rm-charles-river-hosted-jax-webinar-optimizing-research-reproducibility-minimizing-genetic-drift
- Designing and optimising mouse breeding schemes https://resources.jax.org/jax-on-demand/designing-optimizing-mouse-breeding-schemes

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