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Specialist information

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Breeding planning for laboratory mice

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1. Introduction

The aim of this specialist information is to provide basic concepts for the standardized planning and implementation of the breeding of mice for scientific purposes. Careful breeding planning is essential to comply with legal requirements and to minimize the production of unusable animals (1). Only as many animals may be bred as are required to carry out an animal experiment or to maintain the line. The number of animals must be determined considering the approved experimental objective, the selected animal model and the underlying genetics.

To structure breeding planning and make it clearer, we discriminate between different breeding objectives and strategies, which may become necessary one after the other or in parallel over the course of a project (Fig. 1). The required number of breeders for each type of breeding are determined with the help of calculation tools and can be added up to obtain the total animal numbers to be bred during a project. The practical implementation can then be checked for plausibility using these animal numbers.

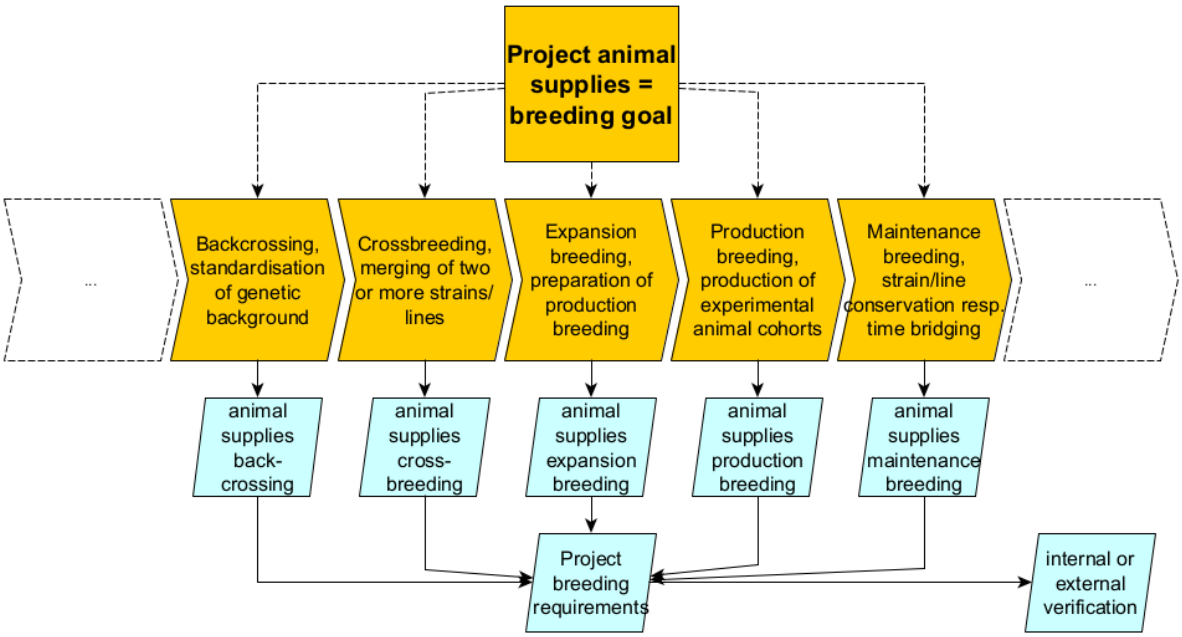

Fig. 1: Flow chart of breeding planning/determination of breeding requirements - Based on the biometrically determined number of animals needed for a project, breeding objectives are derived and assigned to the specified breeding strategies. The breeding requirements for the various breeding strategies are then calculated separately. Depending on the course of the project and the number of lines/strains used, this may be necessary once or several times. The required experimental animals are only generated with the production breeding, the other breeding strategies are to be considered as required. The summarized total number of breeding and delivered animals can be used for internal statistical evaluation and verification purposes or for supplementing an application for an animal experimentation project.

When **crossing existing lines**, the respective alleles are combined to generate complex genotypes for a research question. Such a combination of genetic traits over several breeding steps requires detailed planning. **Backcrossing** is used to transfer alleles to a defined genetic background or to restore this background. **Expansion breeding** is usually used to prepare for **production breeding** and, for example, from maintenance breeding to produce the necessary colony of breeding animals. Finally, production breeding is used to obtain groups of a defined size . It is usually used to generate animals for planned experiments. Production breeding can be **colony-** or **cohort-based** (see sections 3.1 and 3.2). The aim of **maintenance breeding** is to preserve a line that will be required for experiments in the foreseeable future or that cannot be cryopreserved safely or at too great an animal cost (e.g. cryopreservation of embryos).

This specialist information is aimed at individuals involved in breeding planning and implementation. It provides recommendations and practical advice on how to proceed and explains the use of aids.

In summary, the following can be said about breeding:

- The breeding objective is determined by the intended use of the animals and must be defined: maintenance, crossbreeding, expansion or production.
- Each breeding plan includes a mathematically defined number of animals with specified characteristics, usually sex, age and genotype.
- Breeding of burdened and new lines requires official approval (DE, AT, CH).

1.1. Qualification for the breeding of laboratory animals

Due to the large number and complexity of genetic and other parameters, breeding planning should only be carried out by appropriately trained and qualified personnel. This usually includes animal care, technical and scientific personnel. In particular, the varying complexity of different breeding programs must be considered. While the maintenance or expansion breeding of genetically defined strains or lines can be planned and carried out independently by animal care or technical staff, the planning of complex breeding schemes in crossbreeding programs (e.g. several genetic loci to be considered, linkage or X-linked inheritance) requires highly specialized genetic and biological expertise. An expert commission of the EU recommends using regularly trained 'colony managers' for centrally organized and optimized breeding management (2). Persons planning breeding must be qualified according to the complexity of a specific breeding program.

The following points are important for the qualification of staff:

- All those involved in breeding management must undergo regular training in accordance with local regulations.
- In larger facilities, the employment of 'colony managers' is strongly recommended.

1.2. Legitimacy of breeding laboratory animals

In general, the breeding of laboratory animals may only take place if there is an acceptable reason for doing so within the scope of the Animal Welfare Act, e.g. a connection to a planned animal experiment (for Germany Sections 7 or 4 TierSchG) or related, preparatory or breeding-related measures (transgenic techniques, preservation of the genetic background, etc.). Any

unrelated breeding activities must be avoided (see also cryopreservation, Section 1.6). In the same way, the inevitability of maintenance breeding should be questioned, and such breeding should be limited to the indispensable minimum.

Parallel breeding of the same line within a facility without particular reason should be avoided. Exceptions may be, for example, experimentally necessary circumstances such as microbiome, hygiene levels, day/night rhythm, or time-limited breeding . Therefore, before purchasing or importing animals, it must be ensured that the strain or line in question is not already present. If several research groups have parallel requirements for a specific line, this must be coordinated by the husbandry management through central animal allocation. The objective must be to optimize overall animal numbers. Even in the case of decentralized animal facilities, an exchange (of animals) between research groups should take place whenever possible.

Breeding planning must be carried out according to the needs for each strain, line and allele combination. The breeding plan must be adjusted as soon as an increased or reduced demand arises, e.g. due to a change in the number of animals in experimental projects, culling for scientific purposes (Germany: in accordance with Section 4 TierSchG) or export.

In addition to careful breeding planning, forward-looking and needs-based breeding management is required, which must be continuously reviewed and, if necessary, optimized so that a breeding surplus is prevented as far as possible. Matings must be timed so that the further use of the offspring is ensured. Unforeseen breeding events must be responded to immediately in order to avoid a breeding surplus and unusable offspring. The extent to which animals that are not suitable for the original purpose due to the lack of target characteristics (such as genotype or sex) can be used for other purposes must be examined (second use test). The use or breeding of commercially acquired lines or animals or those provided by third parties is generally subject to restrictions imposed by the respective source of supply (e.g. general terms and conditions or material transfer agreement). To not violate these, the respective institution or animal owner should obtain prior approval for secondary use from the source of supply, e.g. the commercial breeder.

Considerations to ensure the permissibility of breeding:

- Breeding without reference to a specific scientific project should be avoided.
- Breeding should be coordinated within a facility.
- A possible secondary use should already be considered and legally clarified when a line is acquired.

1.3. Nomenclature, genotyping and documentation

Given the multitude of existing and newly emerging strains, lines and crosses of laboratory animals, it is essential that they are recorded according to the internationally recognized nomenclature (3-5) to avoid confusion. A correct nomenclature always includes the genetic background as well as any mutations that may be present. It must be documented whether it is pure, fully backcrossed (congenic), or in a mixed form. The presence of mutations (or other markers) should generally be checked and documented for each animal using appropriate methods (e.g. PCR, Southern blot, sequencing). For lines bred exclusively homozygous, this should be done by sampling every two to three generations at the latest. This should be done

up to weaning, but no later than before reaching sexual maturity, to allow early decisions on the use of animals for further breeding and to form stable cage groups. The results of genotyping should be documented according to the published standard as much as possible (6).

Documentation is best carried out in a specialized husbandry database to record data securely and uniformly. The database should also continuously capture and provide the necessary breeding parameters (see Table 1) for each strain and line in an up-to-date form. For newly generated or freshly imported lines, standard values or data from the breeding of origin must be used until own specific line values are available. It is recommended that this documentation, along with other important line-specific information, is documented in the form of a report, e.g., a "mouse passport" (7) or according to documents recommended by the Federal Institute for Risk Assessment (8).

It must also be taken into account that breeding parameters can change over generations when lines are backcrossed to a different genetic background (see section 2.3).

The following should be considered for documentation and genotyping:

- Genotypes must be determined regularly.
- The correct international nomenclature must be used for all lines.
- Newly created lines or sublines must bear the abbreviation of the corresponding laboratory (registration on the ILAR platform; (9)).
- Records should be kept in an animal husbandry database program.
- Documentation of breeding includes the number of generations as a separately managed colony.
- The documented data should lead to automated provision of breeding parameters.
- Documentation should automatically indicate deviations from expected genotype occurrence probabilities (e.g. signs of lethality).

Tab. 1: Breeding parameters

Parameters	**Description**
Fertility	Percentage of productive breeding pairs
Litter size	Number of offspring at a) birth or b) weaning
Productivity (Production Efficiency Index, Colony Index)	Number of young animals (at birth or weaning) per female per week in mating
Partition interval (Parturition interval)	Number of days between litters
Sexual maturity	Earliest possible time for mating. Not optimal, as usually not very successful
Breeding maturity	Optimal (earliest) start time for breeding. The animal is physiologically ready for breeding.

1.4. Genetic background and drift

The maintenance of a certain precisely defined genetic (often inbred) background is essential for the reproducibility and comparability of animal models. Genetic drift changes the genetic background of the animals in a colony over time. The reason for the genetic changes can be spontaneous mutations and the loss of alleles. After 20 generations of independent breeding, the establishment of sublines is assumed. The generations of two independently bred sublines add up. A genetic stability program can avoid this development of sublines. Also, when fixed phenotypic deviations are detected, the establishment of a new subline is assumed.

A genetic stability program can either include the regular introduction of (purchased) genetically defined animals (e.g. the wild-type strain) of both sexes (or at least the males due to the Y chromosome) or the restart of breeding by using cryopreserved embryos. Both should be done after every five breeding generations (10). Partial introduction/partial restarting leads to a correspondingly proportional reduction in drifting generations and must be documented.

To reduce the probability of homozygosity of a spontaneously occurring mutation, brother-sister matings (pedigree breeding) should be avoided as much as possible between the two refreshes. In the past, pedigree breeding was preferred to quickly detect phenotypic changes in a line in the homozygous state and potentially discontinue the corresponding breeding line. However, this leads to a false sense of security, as it is never possible to capture all potential phenotypic parameters. This approach should therefore be avoided from today's perspective.

The continuous breeding of an initial cross of lines with various genetic backgrounds is generally not recommended. Such crossbreedings lead to animals with mixed genetic backgrounds. The background is not sufficiently defined and fixed but gradually leads to a new sub-strain during further breeding with a high inbreeding coefficient. Ideally, lines should have the same genetic background before crossing. The background can be verified by SNP or STR analysis. Adjusting a genetic background is done via backcrossing (see section 2.3).

For genetic stability, the following should be considered:

- Brother-sister matings should be avoided.
- Backcrossing with the original background strain should take place every five breeding generations.
- Cryopreserved material should be used to genetically refresh strains every five generations.
- Crosses between lines with different genetic backgrounds should be avoided.

1.5. Cage quotas

The prerequisites for optimized breeding include ensuring the availability of appropriate housing possibilities in the form of cage space. The institution must guarantee this before breeding begins. After determining the number of breeding animals and before the start of breeding, it must be verified whether there are enough cages and other necessary resources available for both the breeding animals and the weaned offspring, as well as the subsequent experiments. The killing of animals due to poor planning and the resulting lack of housing capacity must be avoided.

Laboratory animal facilities should factor peak demand into their housing capacities. It has proven effective not to allocate more than 80 - 85% of the total available cage spaces in order always to have a sufficient capacity for weaning young animals. Breeding capacities should be allocated in a structured manner. Compliance with allocated animal housing quotas should be automatically monitored (traffic light system) or regularly checked and sanctioned if they are consistently exceeded.

Please note regarding cage quotas:

- Before breeding begins, sufficient cage capacity must be ensured.
- Facilities should have an appropriate management system for space quotas.

1.6. Cryopreservation

Mouse lines and strains are usually cryopreserved in the form of preimplantation embryos or sperm. Due to the inevitable genetic drift, together with the risk of losing unique lines due to natural events, accidents, or infection outbreaks, all lines that cannot be retrieved in good quality from third parties at a reasonable cost should be cryopreserved. Cryopreservation is also an effective means of securing a mutant in its original version without the need for breeding. Since in vitro fertilization usually involves the use of wild-type oocytes, which means that any homozygosity is lost in the next generation, complex lines (e.g., multiple mutants) should be preserved as embryos. Accordingly, lines where individual alleles are mutated should be preserved in the form of sperm. Deviations from this rule may arise due to specific phenotypes.

In the case of hygienic sanitation of a line, it is advisable to also perform cryopreservation, as this combined approach saves animals and avoids a second animal-intensive process. Repeatedly used "tool lines" (e.g. certain Cre, Flp, Rox or Vox recombinase lines) should be centrally maintained in cryopreserved form. In breeding planning, cryopreservation should be weighed against the planned duration of maintenance breeding, as the latter requires more animals, at least in the longer term.

Important considerations for cryopreservation include:

- Simple lines should be cryopreserved as sperm.
- Complex lines should be cryopreserved as embryos.
- The decision between maintenance breeding and cryopreservation must be made on an individual basis.

2. Breeding basics

2.1. General information

The minimum age of female mice for productive breeding (breeding maturity) is typically reached at ten to twelve weeks, even if sexual maturity is reached as early as at six (C3H/HeOuJ) or eight weeks (BALB/c). However, both types of maturity depend on the strain/lineage. Mating before breeding maturity is not very effective. Litter sizes also vary greatly between different strains and (transgenic) lines. Breeding animals should be neither sick nor underweight.

Breeding is usually setup either monogamous as duos (one female and one male) or polygynous, usually as trios (two females, one male), with negligible influence on breeding success (11-14). If breeding is set up with one male and multiple (>2) females (harem), the pregnant females should be separated depending on the cage size used, which has implications for the required cage quota. In cases where polygynous breedings produce asynchronous litters, the dams should be separated, as the offspring in the second litter generally have a lower chance of survival (15). Females that are not visibly pregnant after at least 14 days of mating can be mated with a different male. However, the possibility of pregnancy should be considered. Pregnancy is usually only blocked (Bruce effect) if there is sufficient genetic difference (different strain) between the males (16). In the case of pregnancy, however, there is a risk that the new male will kill the offspring after delivery(17). Weaning cages for the offspring must be provided in due time.

The breeding principles are summarized below:

- Breeding should be started as soon as the animals reach breeding maturity.
- Cages must be provided for weaned animals and, if necessary, for pregnant animals from polygynous matings.
- Only healthy, normal-weight animals without physical abnormalities should be used for breeding.

2.2. Maintenance breeding

Maintenance breeding includes lines that are currently not or no longer required to produce animals for experiments. Typically, this involves the maintenance of wild-type and genetically modified lines. The breeding schemes generally correspond to:

- wild type x wild type strain
- heterozygous mutant x wild type background strain
- homozygous mutant x homozygous mutant

Maintenance breeding should be limited in time for scientific (drift), financial and ethical reasons (see also section 1.4). It is therefore recommended to evaluate the need for maintenance breeding of a line at least once a year. If the line is not used for experimental purposes within one year, it should be cryopreserved. The size of a maintenance breeding program, including the animals kept, depends on the line/strain-specific parameters of fertility, litter size and frequency of the required genotype. This allows for calculating the necessary number of animals (see section 3). In situations with large breeding populations, the productivity of the strain can be used (see section 3.2).

Maintenance breeding should always be carried out with at least two breeding pairs to avoid brother-sister mating in the next generation. To avoid unnecessary offspring, maintenance breeding should be carried out intermittently, i.e. only once every twelve to eighteen weeks (18). To maintain animals of different ages, it may also be useful to stagger the pairing of individual breeding pairs (e.g. after six to nine weeks for two breeding pairs). If the breeding parameters from the line statistics (see Table 1) are used for a new mating approach, the desired number of offspring for this generation should be achieved in nine out of ten cases, for example. If not, the most productive breeding pairs should remain together until the required number of pups is delivered. For expanding a line, e.g. for later production breeding, the

procedure is as for cohort-based breeding (see 3.1) including remating (see 2.7) until the required number of female breeding animals is available for production.

Regarding maintenance breeding, the following should be noted:

- The necessity for maintenance breeding should be regularly evaluated.
- Maintenance breeding should be carried out for a limited period.
- At least two breeding pairs should be maintained
- Intermittent breeding can reduce the number of animals.

Mouse strain	Number ♀ required to obtain 10 ♀ offspring
C57BL/6J	5
BALB/cJ	12
C3H/HeJ	6
DBA/2J	7
129SvJa	7
FVB/N	4

Table 2: Required breeding females for the maintenance of different wild-type strains, assuming that ten females are to be bred simultaneously. The five males required also occur naturally but are not considered here. The values indicate how many female breeding animals are required to achieve the desired number with an 80% probability of success and 100% occurrence of the required genotypes (see section 3 and https://www.ltk.uzh.ch/en/Breeding.html). The strain parameters were obtained from The Jackson Laboratory (19) . It should be noticed that for BALB/c, for example, more than ten future female breeding animals must be kept in stock.

2.3. Back-crossing

In a backcross, one or more alleles are "backcrossed" from an undesired genetic donor background to a desired inbred genetic recipient background. The genetic background of the backcrossed line is referred to as "congenic" after ten generations (N10) (20). To reduce the number of animals required and the time needed for complete backcrossing to another genetic background, this should be done using marker assistance (speed congenics) (21). With this method, successful backcrosses can usually be reduced to five generations (N5) (see also (22)).

In summary, the following should be considered when backcrossing:

- A complete backcross requires ten generations.
- Marker-assisted crossing should be considered, as it allows for a reduction to about five generations.

2.4. Crossbreeding existing lines (polyhybrid inheritance)

Creating new genetic mouse models with multiple genetic modifications is traditionally done by intercrossing mouse strains that already carry the desired alleles. This way, animals with

multiple combined target alleles at different loci can be created. According to Mendelian inheritance rules, many animals that only represent an intermediate breeding stage are produced because they do not yet have the desired allele combination. Sometimes it takes several generations before animals are produced that can then be used for the production breeding of the required experimental animals. Furthermore, due to the rules of inheritance and associated stochastic processes, animals are produced that can neither be used for experiments nor for further breeding. This is unavoidable but can be optimized through good planning.

Before different gene loci and their alleles are combined, it must first be checked whether the genes of interest are located on the same chromosome, i.e., whether or not they are linked (23). If they are not linked, Mendel's rules of inheritance can be applied (24) and the breeding be planned (2.4.1); otherwise, it maybe be possible to proceed with crossover (23) (2.4.2).

2.4.1. Unlinked polyhybrid crossbreeding

In an unlinked cross, the frequencies of occurrence of the various genotypes in the subsequent generation are usually calculated using a Punnett square. It is not necessary to create complicated Punnett squares beyond the 2x2 format. The frequencies can be determined separately for each gene locus and then calculated for the combination of gene loci by multiplication (Fig. 2). Punnett square calculators available on the Internet can also be used (25,26).

Breeding planning for combining different alleles of multiple gene loci represents a cohort-based breeding strategy (Section 3.1). Planning over several generations can currently only be done based on experience, as appropriate software solutions are lacking. Depending on availability, combinations of parent animals should be selected that produce the least number of surplus animals in the respective breeding.

2.4.2. Coupled polyhybrid cross

If genetic characteristics are located on the same chromosome, they are linked and hence inherited together. During meiosis, pieces of chromosomes can be exchanged between homologous pairs (crossover) leading to recombined outcomes in the resulting gametes. If the traits are far enough apart, breeding with the goal of achieving a crossover can be initiated. The probability of a crossover can be calculated. Two megabase pairs (Mbp) of distance correspond to approximately one centimorgan (cM), which equates to a crossover probability of 1% (27). Under these conditions, one out of a hundred offspring is expected to have the desired recombination. This example illustrates that, from the perspective of the 3R reduction principle, it must be evaluated whether the new generation of a double mutant using current transgenic techniques (e.g., CRISPR/Cas9) is preferable to an animal-intensive crossover breeding. The chromosomal positions of genes from different laboratory animal species required for the calculation can be looked up in several databases, such as Ensembl, UCSC Genome Browser, NCBI-Gene (28-30). The position of a gene is represented by the chromosome number followed by two usually nine-digit numbers. One of the two numbers can be subtracted from that of the second gene to determine the distance between the genes. As a rule, in the first step heterozygous animals are created for both gene loci. By backcrossing with wildtype, animals can be identified in the next generation that carry the alleles of interest

for both genes. Such animals are products of a crossover and can be used for further breeding (Fig. 2).

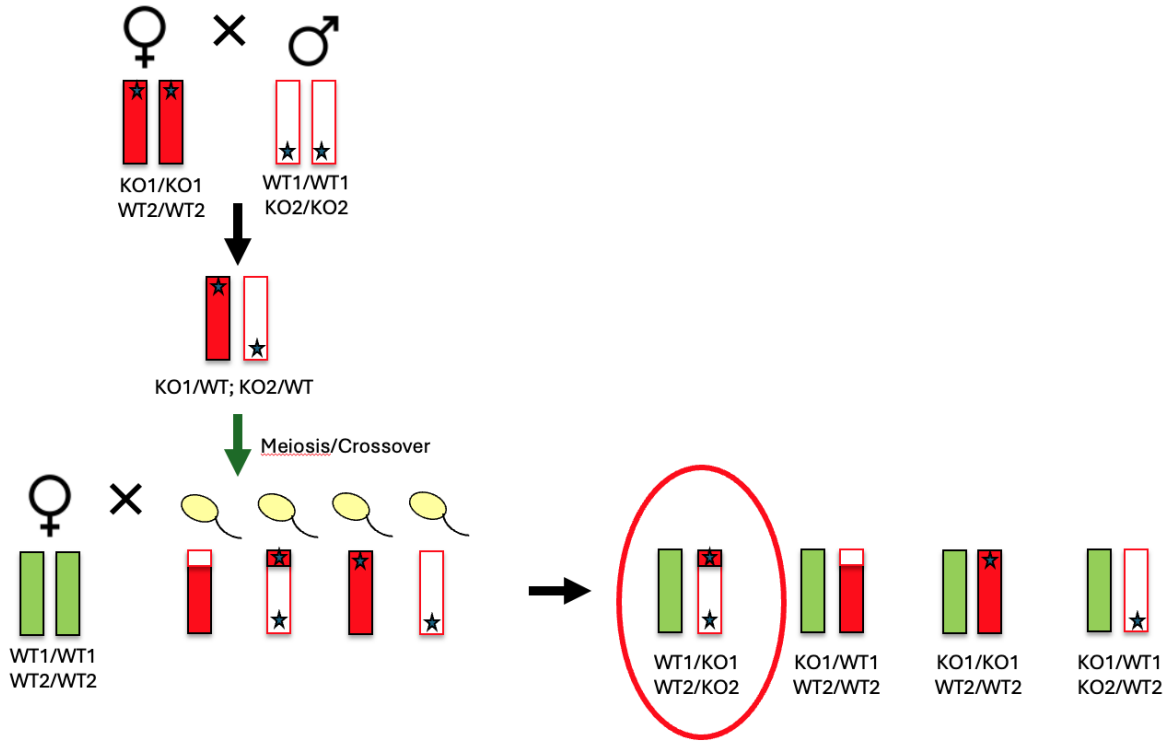

Fig. 2: Example for the creation of a line that has, after crossover, two mutations on the same chromosome. First, the two lines (red and white) are intercrossed. In the next generation, both mutations are present once each on the sister chromosomes in heterozygous animals. These animals are then mated with wildtype animals. In the subsequent generation, offspring are screened for animals that carry both mutations. If this is the case, a crossover has occurred and breeding can continue accordingly.

When crossing different lines, the genetic background must also be considered (see sections 1.4 and 2.3).

2.5. Expansion breeding

Expansion breeding is necessary to provide the required number of breeding animals for first-time production breeding and their replacement. This means that it is based on the actual number of animals available and the number of animals required for production breeding. Often, an existing intermittent maintenance breeding is converted into continuous breeding. For faster expansion, additional animals can be included in the breeding program. Expansion breeding may require several generations before it is transferred to a production breeding. In many cases, it overlaps with the crossing of existing lines and the planning encompasses both processes.

In summary:

Expansion breeding generates the breeding pairs needed for a planned production breeding from the few available breeding animals.

2.6. Production breeding for experiments or transfer

Breedings that directly produce animals for the experimental groups of animal experiment projects are conducted as production breedings. The requirements of the planned experiments determine the necessary number of breeding animals. Calculations can be carried out as cohort-based (Section 3.1) or colony-based (Section 3.2) breeding, depending on demands and colony size. If production is required for more than one generation of breeding, the renewal of breeding pairs must be considered. This can be done either through a replacement rate (typically in colony-based breeding) or by calculating the next replacement cohort (cohort-based breeding). In the latter case, it should be noted that production and replacement breeding pairs are calculated together, as this leads to a lower number of matings for stochastic reasons. If the line is to be used further, animals are transferred to maintenance breeding (Section 2.2) or cryopreserved (Section 1.6).

Production breeding serves the purpose of breeding animals as needed for animal experiments or for killing for scientific purposes or for transfer. The following should be considered:

- A pre-calculated maximum number of animals of the desired age, sex and genotype must not be exceeded according to the demands.
- Replacement of breeders must also be considered.

2.7. Replacement of breeders

For optimal breeding success, the breeding span should be limited to the period with the highest fertility, litter size, and rearing rate. However, breeding animals should also not be replaced too early, as this leads to an increased need for new breeders, more genetic drift, and the production and hence use of additional animals. While the useful breeding range is known for most genetically non-modified background strains (31) it is sometimes influenced by genetic modification. Without line-specific data, the replacement of breeders should be calculated based on the respective background strain, including a percentage safety margin, e.g., 10%.

Male and female animals are usually replaced after the same breeding period to avoid negative influences on breeding success from the new male animals and thereby lowering the the overall productivity of the breeding.

Wild-type breeding females of the common background lines usually show high productivity over seven to nine months, and often acceptable productivity up to eleven months and beyond (32-35)

General guidelines for the replacement of breeding animals are:

- Breeding should be terminated after a breeding period depending on the strain/lineage.
- If there is no visible pregnancy or no litter after 35 days, the breeding pair should be replaced if possible.
- If the number of animals for breeding is low, a second gestation period should be waited for if necessary if there is no pregnancy in the first determined period.

- If no animal from a litter reaches weaning age (rearing result = 0), another litter should also be waited for.
- At the end of the productive period, females and males are usually exchanged simultaneously.

3. Influences of breeding strategies on the number of required breeding females

In principle, animals can be generated in three different ways: by cohort-based breeding, by colony-based breeding, and by in vitro fertilization (IVF) / the revitalization of cryopreserved embryos. Different numbers of females are required in all three cases. For calculating the number of required breeding females, it must also be considered how many animals are needed at what point in time. This can vary considerably depending on the experimental question. For experiments where a large group of animals is required simultaneously at a specific time, different calculations are needed compared to experiments where few animals are used at various times over a longer period.

Cohort-based breeding is characterized by the aim to obtain a defined number of animals of similar or the same age. This is achieved through simultaneous mating. In this way, an experimental cohort of a predefined size can be bred a few times or even just once. The recommended procedure is described in section 3.1.

Colony-based breeding is not synchronized in terms of the mating time point and consequently the age of the offspring. A larger group of breeding animals continuously produces offspring. Such a production colony regularly generates animals for planned experiments but must also produce animals to replace breeders. Details on this can be found in section 3.2.

As biological processes, cohort and colony-based breeding follow stochastic distributions, e.g. the average litter size fluctuates around an average value depending on the background strain and age. In the context of breeding planning, it must therefore be decided with what probability the desired breeding result should be achieved in terms of animal number, genotype or sex. The higher the desired confidence level, the more breeding pairs must be used, which may result in a surplus of animals. One strategy to reduce this number is to divide the experiment into several consecutive groups of animals instead of testing the complete or a predetermined number of animals at one point in time. For example, it is conceivable that an experiment can be carried out simultaneously with fifteen animals or in three groups of five animals each. In this case, the breeding is set so that there is a 90% probability that five animals will be delivered. By chance, more suitable animals are also used, which improves the ratio of animals delivered to animals used in experiments.

Another strategy may be to generate the required number of experimental animals in a targeted manner using reproductive techniques, e.g. IVF. In this procedure (see section 3.3.), oocytes are fertilized in vitro using sperm obtained in the same way and implanted in apparently pregnant foster mothers. Hence, a larger cohort of animals of the same age can be generated on demand.

The following procedure is generally recommended for creating a standardized and documented breeding plan:

- Definition of breeding objectives
- Number of animals required per experimental group
  - Sex
  - Genotype
  - Zygosity (heterozygous, hemizygous, homozygous)
- Decision whether
  - temporally and numerically determined cohorts or
  - breeding of a colony with a small but fixed number of breeding pairs over a longer period or
  - a cohort is to be generated by one or more IVF.
- Carrying out the calculations (see sections 3.1, 3.2, 3.3)
- Documentation of the calculations including the required male animals
- Coordination with any allocated cage quotas

3.1. Cohort-based (production) breeding

In cohort-based breeding, a defined number of animals of a defined genotype, age, and sex are to be produced within a defined period. For this purpose, a specific number of breeding animals of suitable genotypes of the relevant line are mated at one or more time points. Parameters influencing the required number of breeding pairs are the probability of occurrence of the target genotype, the fertility, and the litter size after weaning (36). The probabilities of occurrence of the target genotypes can be calculated using a Punnett square (possibly using an online tool (25,26). Fertility, litter size, and the number of weaned young animals (see 2.4.1) should be made available as relevant breeding statistics data from the in-house husbandry database. Only in the case of new lines should earlier data from the previous breeder or reference values from the background strain be used.

The number of breeding females required to achieve the defined number of offspring can be calculated using the available breeding calculation tools (as an approximation), whether in duos or trios (12-14) can be calculated. The consideration of a probability of success, e.g. 90%, is currently only possible with the program "R Package BreedingCalculator" (37,38) program.

If deviations from the Mendelian distribution are to be expected, adjusted values should be used for the calculations (see 2.4.2). In the same way, litter sizes before weaning/after litter (for experiments with young animals) or even litter sizes in utero (for experiments with fetuses) can be used. In these cases, the probability of occurrence of the different genotypes can change!

The procedures of cohort-based breeding are as follows:

- Definition of breeding objectives (see above).
- Determination of breeding parameters
  - Litter size (usually at weaning of the litter)
  - Fertility (proportion of productive breeding pairs per female breeding animal)
  - Genotype frequency according to Mendel (25,26)
  - Deviations from genotype distributions according to Mendel
  - Absence of linkage (see section 2.4)

- Calculation of the required female breeding animals (37,38)

3.2. Colony-based breeding/continuous production breeding

In colony-based breeding, breeding animals are not synchronized. The number of breeding females required to obtain the number of animals for a defined goal, e.g. an experiment, can be calculated by considering the average weekly productivity of a breeding female (Production (efficiency) Index, PEI). The PEI indicates the number of offspring weaned per female per week (36). The problem with this system is that the average production must first be set in relation to the assumed demand. Several factors make this difficult. Firstly, there are natural biological fluctuations in the productivity of the colony. These are random, and their effect highly depends on the colony's size to be bred. Also, the weekly demand for animals can fluctuate greatly, depending on the researchers' plans, which in turn often depend on the results of ongoing experiments. Flexibility in the age of the animals that can be used in each experiment is another factor to consider (e.g., whether animals can only enter the experiment at ten to twelve weeks of age or at ten to sixteen weeks of age). Greater flexibility can better compensate for fluctuations in production. However, this is a scientific decision that can also change between experiments for one mouse strain. It is clear from these considerations that production must be higher than the number of animals calculated by simply applying the PEI to compensate for random fluctuations. A minimum colony size is also required. The consequences of not being able to meet the demand for animals in time should also be considered. Further, it should not be forgotten to add animals for the exchange of breeders. Also, the genotype frequency needs to be considered.

The Jackson Laboratory's Breeding Colony Size Planning Worksheet (39) can help to plan colony sizes. However, it only includes random fluctuations in the calculation using a so-called "fudge factor" (best described as a "correction factor"). This leads to a dramatic underestimation of the necessary number of breeding animals, especially for the usual animal numbers in academic research (Fig. 3; (36)).

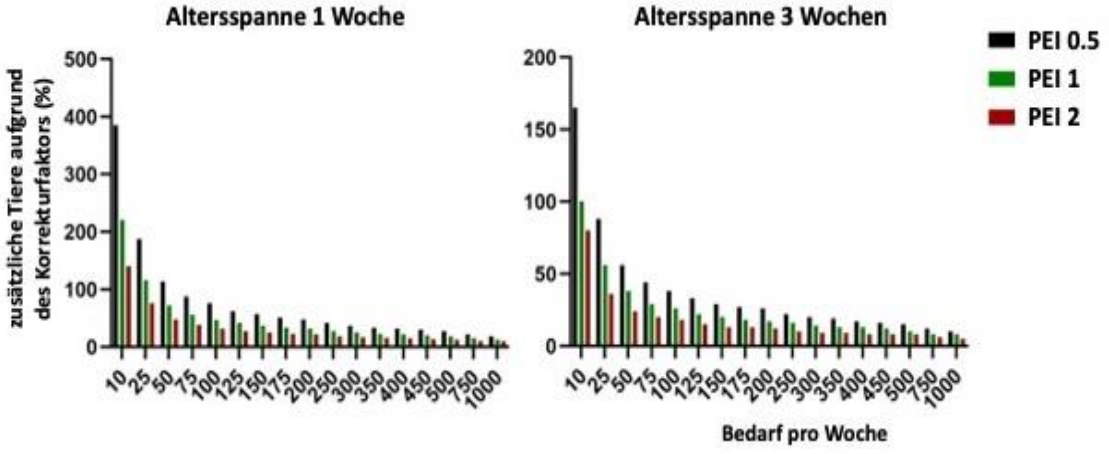

Fig. 3: Correction factor - Add-ons for colony-based breeding. The smaller the allowed age span of the offspring used in the experiment, the higher the add-on must be (left graph age range max. one week, right graph max. three weeks). A probability of success of 90% is assumed, i.e., in nine out of ten weeks, at least as many pups are born as required. The figure shows for three PEIs (0.5, 1, 2) how high the percentage of add-ons must be.

This represents **the so-called "fudge factor"** in the Breeding Colony Size Planning Worksheet of the Jackson Laboratory. An add-on of 200%, for example, corresponds to a total of 300%, i.e., a correction factor of three. To obtain ten offspring with an age range of one week with a success probability of 90%, a PEI of one requires an add-on200%, i.e. three times as many breeding females as calculated without a correction factor.

The procedure is as follows:

- Definition of breeding objectives (see above).
- Determination of breeding parameters
  - Production/colony index PEI
  - Genotype frequency according to Mendel (24)
  - Deviations from genotype distributions according to Mendel
  - Absence of linkage (see section 2.4)
- Calculation of the necessary breeding females (39).
- Documentation and reconciliation (see above).

3.3. Cohorts through *in vitro* fertilization or revitalization after cryopreservation

To generate a larger number of animals of the same age for an experiment, IVF offers an alternative to cohort-based production breeding (40). One advantage is better controllability. The same applies to the revitalization of cryopreserved embryos. A drawback is the distress and harm caused by vasectomy, superovulation and embryo transfer. In addition, this technique can only be applied if the facility has the appropriate expertise. However, it is also conceivable to have such cohorts generated by commercial providers. The calculation of required animal numbers is analogous to cohort-based production breeding. Superovulation and survival rates of the genetically modified animals may be lower than those of the wild-type background strain. As long as no data is available, we recommend a safety margin of 20% compared to the background strain.

The procedure is as follows:

- Definition of breeding objectives (see above).
  - Number of animals
  - Sex
  - Genotype
  - Zygosity
- Determination of breeding parameters
  - Litter size of recipient animals (usually at weaning)
  - Number of oocytes per donor
  - Fertilization rate in IVF
  - Distribution of genotypes according to Mendel
  - Deviations from Mendelian genotype distributions
  - Absence of linkage (see section 2.4)
- Calculation of the necessary donor and recipient animals
- Documentation of calculations
- Coordination with any allocated cage contingents

4. Summary

This specialist information is intended as a tool for appropriately trained individuals to plan breeding programs based on specific requirements, with the goal of optimizing and ideally minimizing the overall number of experimental mice needed and surplus animals generated. To limit the number of animals required for breeding, sensible breeding planning is therefore a top priority. However, efficient breeding of mice for biomedical research is a complex undertaking and mouse strains and lines required for experimentation often must be generated through elaborate breeding programs.

A prerequisite for sensible breeding planning is an understanding of the genetic characteristics and other specific parameters that influence breeding success. In addition, breeding can be roughly divided into categories that facilitate the standardization of the breeding process. The need for required breeding animals and bred offspring can thus be calculated within the chosen confidence framework.

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