

RECOMMENDATIONS AND GUIDELINES FOR APPLIED REPRODUCTION TRIALS WITH RABBIT DOES

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ABSTRACT: This paper consists of a set of recommendations for applied reproduction trials with rabbit does. In the first part, the preparation of the experimental design is particularly developed (animals, size of the sample, housing conditions, breeding and feeding systems, establishing groups, insemination conditions, duration of the experiment). The second part defines some reproductive performances and how to measure them (sexual receptivity, fertility, prolificacy, mortality and growth of young, overall productivity). The last part provides a tool for data analysis. In the final considerations, it is emphasised that nowadays experiments have to take into account the recommendations related to animal welfare as well as the increasing sensitivity of consumers to considerations of meat quality. Reproduction traits are defined in an appendix.

Key words: reproduction, rabbit does, guidelines.

INTRODUCTION

As for most mammalian species, reproduction experiments with rabbit does are time consuming and expensive. Consequently, there have been several instances of an insufficient number of animals being employed by researchers to obtain consistent data. Moreover, slightly different methodologies are introduced to achieve exactly the same objectives, so that results are often difficult to interpret or to link to previous work. A deficient description of the experimental conditions and methods can sometimes undermine the claimed conclusions.

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With the aim of making experiments with reproducing does as efficient and comparable as possible, the International Rabbit Reproduction Group (I.R.R.G.; BOITI 1998) decided to harmonise the methodology used within its own context and to establish general guidelines for reproduction trials designed to help the whole research community. However, they do not aim to provide a fixed scheme, but are expected to serve as a useful tool in the preparation of the experimental design, data analysis, and publication of results. The present guidelines have been discussed and approved by a panel of experts (see footnote) in the course of various meetings in the context of COST action 848, sponsored by the EU.

GENERAL GUIDELINES

These guidelines have been designed for research experiments under temperate climatic conditions, whatever the breeding system (intensive or extensive). Experiments can be performed either in experimental research facilities, under strictly controlled conditions, or in commercial rabbitries, but special attention has to be given to the experimental treatments being carefully executed and to the correct handling of data collection. Both types of experimental conditions can provide complementary information. Commercial rabbitries can validate results obtained in experimental farms on a larger number of animals and under real farm conditions.

Animals

For applied reproduction trials, preference has to be given to strains commonly used for commercial purposes. Hybrids, New Zealand White or other performant breeds are recommended. However, particular strains can be used when required for specific reasons. In all cases relevant information on genotype should be provided or references cited.

Experiments can start with young does or with productive does. In the latter case, attention must be given to a homogenous distribution (physiological status, genealogy...) over the experimental treatments (see Establishment of groups section).

Relevant information concerning age and pre-experimental rearing conditions is highly recommended.

Since rabbits are a species very sensitive to diseases, special efforts have to be taken to include only healthy does throughout the experiment. Unhealthy conditions and diseases should be avoided due to their undesirable side effects, which may greatly bias the results obtained.

Besides the experimental groups (one or more), a control group with the same standard protocol and the same number of does is recommended. The only differences between the groups should be in the factors under test.

Size of the sample

The number of experimental does depends on the variability of the trait studied, the expected difference and the level of significance required. Table 1 shows coefficients of variation of various production traits. All these traits have a high variability. Consequently, a large number of observations is necessary to prove that relevant differences observed are statistically significant.

Table 1: Coefficient of variation (CV) of various productive traits in rabbits, calculated for different experiments.

Parameter	CV (%)
Fertility	35 - 50
Litter size (total) at birth	25 - 30
Litter size (alive)	20 - 25
Birth weight of young	15 - 20
Litter size at weaning	
If not <i>equalised</i> at birth	20 - 25
If <i>equalised</i> at birth	7 - 10

Table 2: Number of observations necessary in each group to expect 90 % certainty of detecting a real difference (significance level = 5%) between 2 means according to the variation and the magnitude of the difference (adapted from LEBAS, 1986).

CV (%)	Relative difference (%) between 2 means ¹				
	5	10	15	20	25
10	84	21	9	6	4
15	189	47	21	12	8
20	336	84	37	21	13
25	525	131	58	33	21
30	756	189	84	47	30
40	1344	336	149	84	54
50	2100	525	233	131	84

¹Relative difference = difference between 2 means as a percentage of the reference mean.

In the elementary case of the comparison of two treatments, the experimental design should allow real differences to be detected with a high degree of success. This percentage is called the “power function” of the design. It depends on sample size, trait variability, real differences between treatments and on the choice of the significance level (generally between 0.1% and 5%). If CV is the coefficient of variation, d the relative difference between two means, the number of observations is: $21 CV^2/d^2$. For example, for a 5% relative difference in fertility between 2 treatments, a 20% coefficient of variation, in order to have 90% certainty in determining the efficiency of the tested treatment at a 5% significance level, 336 inseminations are needed per group. For an *a priori* fixed power, the number of observations necessary to assess the efficiency of the tested treatment is shown in Table 2 (LEBAS, 1986). These concepts are fully described in DAGNELIE *et al.* (1973).

More information concerning the number of replicates required to detect a significant difference can be found in a recent paper by GARCIA *et al.* (2001). Different examples based on their rabbit experimental data are presented for various traits.

Housing conditions

The different experimental groups have to be housed in the same compartment of the building or have to be homogeneously distributed over the different experimental rooms and cage rows. Temperature and relative humidity values have to be controlled and a daily record is recommended. The use of a fixed artificial light schedule is recommended for indoor experiments. For outside experiments, daily natural light duration and season must be recorded. A nest-box with sufficient nest material should be provided for pregnant does, 3-4 days before the expected parturition.

All housing conditions are to be precisely described in the Material and Methods section of the paper: lighting program (natural or artificial), season, ventilation system (static or dynamic), heating and cooling systems as well as the type of cage (size, material, layout, etc).

Breeding system

It is preferable that a fixed reproduction rhythm should be chosen; in most cases a 42 day interval between successive matings or inseminations should be adopted. All other breeding systems as well as the remating intervals must be well defined.

Adoptions of young during the early live stage are well accepted by the doe and minimise the initial high variability of the litter size (Table 1), but the number of young at birth before and after homogenisation and at weaning must be specified. Except for experimental purposes, adoptions must take place within experimental groups. Controlled lactation (one suckling per day at the scheduled time) can be used, but the time of application and the duration are to be specified. A distinction must be made between litters following the intended rhythm and those corresponding to re-inseminations.

Unless it is the subject of research, the use of hormonal treatment to synchronise oestrus is not advised. However, if does are subjected to this treatment, a detailed description should be given in the Material and Methods section. If eCG (PMSG) is

used, it should be given as an injection only to lactating does at the lowest efficacious doses, which seem to be 8 IU for a 35 day reproduction rhythm (THEAU-CLÉMENT *et al.*, 1998) and 12-25 IU for a 42 day reproduction rhythm (THEAU-CLÉMENT and LEBAS, 1996, VIUDES *et al.* 1998, THEAU-CLÉMENT and MERCIER, 2003). In the case of physiological necessity, GnRH is used to induce ovulation in artificially inseminated does. Generally, 0.2 mL of Gonadorelin (containing 20 mg GnRH) or 0.2 mL of a GnRH analogue (e.g. containing 0.8 mg of Buserelin) are intra muscularly injected. The GnRH type, dose and form of injection should be defined. The use of hCG is not recommended since antibodies anti-hCG appear after 3 to 4 injections (ADAMS, 1961).

Feeding system

If not a research topic, a balanced pelleted diet satisfying the nutritional requirements should be fed in accordance with current recommendations (DE BLAS and MATEOS, 1998). At least the calculated chemical composition should be mentioned and care should be taken that changes in composition and distribution during the experimental period are minimal. Although reproducing rabbits are generally fed *ad libitum*, a fixed feeding system is required during the different reproduction phases. Since rabbits eat dry food, free access to drinking water is necessary, preferably with an automatic system.

All treatments administered (e.g. antibiotics, coccidiostatics, etc.) or water supplements (e.g. vitamins) should be controlled and mentioned in the report.

Establishment of groups

Does must be distributed in comparable groups between the different experimental treatments at the beginning of the trial. For a new herd, does have to be distributed by age and/or weight and genealogy (sisters in different groups). For productive does, they should be distributed according to physiological status (lactating or otherwise, sexual receptivity when possible, current litter size and parity) or by considering their previous performance (POUJARDIEU and THEAU-CLÉMENT, 1995)

If it is consistent with the experimental design, a doe should be definitively assigned to a group; this allows her to be in line with field conditions and to judge long term effects. If a doe passes from one group to another, eventual residual effects could be difficult to estimate and would necessitate a greater number of animals. Designs with changes are not advisable for reproduction trials.

General insemination conditions

If natural mating is used, information concerning the number of does/male, mating frequency of males, mating technique (single, double or multiple) is required. If artificial insemination is practised, evaluation and treatment of the semen should be precisely described (levels of semen selection, solvent, preservation temperature and duration, semen conditioning, number of spermatozoa per insemination dose...). If not a research topic, in order to limit variation in the biological characteristics of inseminated semen, heterospermic pools should preferably be used. On a given day, the same mixed semen diluted in the same solvent should be used for all the does of the different treatments. More information about the adequate management of males can be found in a recent paper by IRRG (2005).

Care should be taken to avoid inter-treatment effects due to the inseminator or insemination technique, or different time intervals between collection and insemination.

For health reasons, a single use of the insemination pipette is strongly advised. Re-used insemination material must be carefully washed and sterilised.

Experiment duration

The duration of the experiment depends mainly on the number of does available for the experiment and the number of required observations (see Size of the sample section). In large farm conditions, short-term effects can be measured, but finally, treatments have to be studied over the whole reproductive life of does to measure global productivity, long-term effects and to point out any eventual secondary effects.

RECORDED PERFORMANCES

Although records are primarily dependent on the experiment, some recommendations should be considered. Some definitions are given in the appendix.

The *sexual receptivity* of the doe is easy to judge when natural mating is applied. In fact, all the does that accept mating are sexually receptive. When artificial insemination is performed, two methods can be used to judge the receptivity of the does; by a sexual behaviour test or by scoring the vulva colour and turgidity.

A sexual behaviour test consists of presenting each doe first to one and later to a second buck. Does are considered as receptive if they take a lordosis position. Undesired mating should be avoided. In order to be sure of detecting an extremely rare natural mating, which may happen inadvertently, it is suggested that receptivity should be tested with coloured bucks. Crossbred Californian x New Zealand young rabbits are white and abnormally coloured litters are discarded from analysis. Another alternative is to use a vasectomized buck.

When judging the colour of the vulva; a distinction is made between white, pink, red and purple. However, this subjective method does not totally reflect the physiological conditions, and is therefore not recommended. A better distinction between receptive and non-receptive does is based on the colour and turgency of the vulva. Two categories are distinguished (RODRIGUEZ and UBILLA, 1988): does showing a pink, purple or red and turgid vulva are considered as receptive (acceptation rate of mating = 76 %), while does with a pale or purple non-turgid vulva are considered as non-receptive (acceptance rate of mating = 26 %). Since a number of intermediate does may also be detected by this subjective method, a third group can also be formed of doubtful does.

Fertility: measuring aptitude for reproduction is usually defined by the *kindling rate*. Nevertheless, fertility can be estimated for the *pregnancy rate* by abdominal palpation (from the 10th day of pregnancy) or by ultrasonography (from the 8th day of pregnancy, YPSILANTIS and SARATSI, 1999), laparoscopy, laparotomy or autopsy

at a specified time. But this information may not be considered as true fertility since the embryo viability between observation and littering are not known.

Laparoscopy, laparotomy and autopsy are efficient tools for studying certain productivity components such as *ovulation frequency* and *ovulation rate* and *embryo mortality* at different times during pregnancy. The time of the observation should be recorded. The use of laparoscopy seems not to have unfavourable effects on embryonic survival (THEAU-CLÉMENT and BOLET, 1987; SANTACREU *et al.*, 1990).

Prolificacy is considered as the litter size at birth (total and alive) and should be recorded within a maximal interval of 24 h *post partum*. The difference between alive and total born includes stillborns and early mortality due to low maternal quality or unfavourable conditions (low temperature, out of nest-box, cannibalism, etc).

If standardisation (or cross-fostering) is used, litter size should be recorded before and after homogenisation. Depending on the research protocol, a weekly determination of litter size and weight can be justified.

Mortality and growth of young should be expressed as a percentage of the initial number of young rabbits (after standardisation) and number of total lost litters. It is highly recommended to record the number of young rabbits and their weight at 21 days *post partum* since they are strongly dependent on maternal ability. Number of weaned young rabbits, weaning age and weaning weight are important components, if general productivity is to be judged.

Overall productivity at a specific time (birth, weaning, end of fattening period) is a synthetic criterion of commercial significance which takes into account fertility, litter size and litter weight at the specified time. For example, the overall productivity at weaning is the weight of weaned rabbits / number of mated or inseminated does. This productivity index is a useful tool for determining the efficacy of a treatment (or a method) as it includes not only reproductive performance but also growth and viability of the young.

Other determinations, such as *milk production* (weight difference of the mother before and after the suckling, LEBAS, 1968), feed intake, weight gains of mother and young, should be considered if relevant. The weight of does at different phases of the reproduction cycle can be helpful in explaining certain effects.

Hormone titration can be a valuable help when results have to be interpreted at a physiological level. Depending on the aim, the timing and frequency of blood collection should be relevant. For example, to examine luteal function at the moment of insemination by means of progesterone and/or estradiol-17 β assay, blood samples should be collected just after insemination, but before GnRH injection. On the other hand, to evaluate pituitary response at ovulation, blood should be taken 60-90 minutes after GnRH. To measure metabolic hormones, such as insulin, leptin, GH, as well as metabolic parameters, blood samples should be obtained in the post absorptive phase. Depending on the assay, the blood must be collected with the appropriate anticoagulant to avoid interference.

DATA ANALYSIS

The first step in data computing is to check the quality and coherence of the data. Due to undesired biological reasons (mortality, illness) data should be excluded if relevant. However, if there is no known accidental reason, outliers may be included in the dataset.

In most cases, analysis of variance (ANOVA) methods are applied to the continuous data. The independence of fixed effects, the statistical equality of variances computed for each elementary cell and a normal distribution of the trait are assumed. If there are sufficient data, ANOVA is robust against a non normally distributed trait but not against non equal variances or when effects are linked. Before testing an effect or an interaction, it should be checked that each basic cell comprises a minimum of ten observations. If this is not the case, it is necessary to group cells when there is biological significance. All factors should be expressed simultaneously in the

statistical model. Easily interpreted first order interactions are estimated; if they are non significant, they are taken out of the model. It is better to combine effects (e.g. physiological status combining receptivity, parity and lactating status) rather than to retain an empty or undersized cell.

Receptivity and fertility can be analysed by a chi square test; provided that the assumption of single distribution is true for these traits. They can take the form of a Bernoulli variable and analysed as classical continuous. Applying the limit central theorem, litter sizes can be analysed as continuous variables. Mortality can be studied by analysing litter size at any stage after birth and by introducing birth litter size as a co-variable in the model. The means values indicated for a particular factor are the adjusted means given with their standard error. Nevertheless, for discrete characters (binary characters, counting) a statistical analysis using the generalized linear model is preferable with traditional ANOVA analysis.

In the case of repeated data or for genetic analysis of data, the recourse to more complex models can be considered (for example: the use of a mixed linear model to take into account more elaborate structures of variance-covariance, taking into account correlations between data, relationships between individuals).

Data can be analysed using *for example* the SAS statistics library or any other commercially available software.

The chosen analysis should be precisely described. The statistical significance of any difference is indicated “NS” (non significant) when $P > 0.05$, * when $P < 0.05$, ** when $P < 0.01$ and *** when $P < 0.001$. However, the presentation of the exact P -value of each trait is recommended (rather than merely giving the level).

FINAL CONSIDERATIONS

Nowadays experiments have to take into account the recommendations or legislation concerning the use and care of experimental animals. General recommendations are given by, for example, the American Society of Animal Science (http://jas.fass.org/misc/care_use.shtml). More specific guidelines concerning ethical acceptability can be consulted, such as those from the Federation of European Laboratory Animal Science Association (FELASA: <http://www.felasa.org>) or the “Guide for the Care and Use of Laboratory Animals” of the American Institute of Laboratory Animal Resources (<http://www.nap.edu/readingroom/books/labrats>). It is recommended to approach the experimental design with these guidelines in mind if no ethical recommendations are available in the country where the experiment takes place, or to send the initial protocol to the designated Use and Care Committee for approval. Also, all meat production should consider the increasing sensitivity of consumers to quality related aspects. These factors should be taken into consideration by authors proposing new techniques in order to preserve the “natural” image of rabbit meat.

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APPENDIX

SOME DEFINITIONS

For the rabbit doe, **artificial insemination** is a double operation corresponding to the introduction of the semen in the doe's genital tract combined with a treatment (generally hormonal) to induce ovulation.

Sexual receptivity. A rabbit doe is receptive (or in oestrus) when she has a behaviour of acceptance at mating. A non-receptive doe is said to be in dioestrus.

When using artificial insemination, the frequency of receptive does or "receptivity rate" can be measured by using a sexual behaviour test in the presence of a rabbit buck or estimated by observing the turgescence and the colour of the vulva, immediately before insemination:

$$\text{Receptivity rate (\%)} = \frac{\text{Number of does having an acceptance behaviour or a turgescence purple or red vulva}}{\text{Number of observed does}} \times 100$$

By using natural mating, one speaks more of the acceptance rate than of the receptivity rate.

$$\text{Acceptance rate (\%)} = \frac{\text{Number of does accepting the mating}}{\text{Number of observed does}} \times 100$$

The **ovulation frequency** and **ovulation rate** are estimated by observing the ovaries at the moment of a laparotomy, laparoscopy or after sacrificing the animal at a given time of pregnancy. A doe having at least one *corpus luteum* on one ovary has ovulated. It is considered that the number of *corpora lutea* corresponds to the number of oocytes laid.

$$\text{Ovulation frequency (\%)} = \frac{\text{Number of ovulating does}}{\text{Number of mated or inseminated does}} \times 100$$

$$\text{Ovulation rate} = \text{Number of } \textit{corpora lutea} \textit{ per ovulating doe}$$

Fertilization can be appreciated at the beginning of pregnancy, 24 to 48 hours after mating or insemination. Indeed, at this stage, it is possible to distinguish, after washing of the oviducts, the oocytes from segmented ova (at least 2 blastomeres). A segmented ova is considered as a fertilized egg and a doe is considered as fertile if she has at least one segmented ova.

$$\text{Fertilization rate (\%)} = \frac{\text{Number of segmented ova}}{\text{Number of } \textit{corpora lutea}} \times 100$$

Fertility measures the aptitude to reproduce. At a given time, a rabbit doe can be :

Fertile or ready to be fertilized

Unfertile or temporarily unable to be fertilized,

Sterile or definitively unable to be fertilized.

A variable often used in applied rabbit experiments is the rate of pregnancy. Indeed, for rabbit does, pregnancy can be diagnosed from the 10th day of pregnancy after the mating or the insemination, by abdominal palpation. For an experienced operator, the error rate of palpation is lower than 3%.

$$\text{Pregnancy rate (\%)} = \frac{\text{Number of does palpated as pregnant}}{\text{Number of mated or inseminated does}} \times 100$$

$$\text{Kindling rate (\%)} = \frac{\text{Number of kindling does}}{\text{Number of mated or inseminated does}} \times 100$$

When mortality occurs before the palpation or parturition, these inseminations have to be removed from the data set.

Total embryonic mortality corresponds to the losses between fertilization and kindling. Generally, it is calculated by a comparison with the number of corpora lutea and thus includes the cases of non-fertilization. It is broken up into early mortality (between fertilization and implantation) and foetal mortality (between implantation and kindling). For rabbits, laparotomy, laparoscopy or slaughtering at different moments of pregnancy allow the analysis of embryonic mortality. Implantation occurs about the 7th day after insemination, the observation of ovaries and the implantation sites in the uterine horns permit the study of:

$$\text{Early mortality rate (\%)} = \frac{\text{Number of corpora lutea} - \text{Number of implantation sites}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Implantation rate (\%)} = \frac{\text{Number of implantation sites}}{\text{Number of corpora lutea}} \times 100$$

Observation of uterine horns, at the end of pregnancy, generally around the 28th day of pregnancy makes it possible to determine :

$$\text{Foetal mortality rate (\%)} = \frac{\text{Number of implantation sites} - \text{Number of alive foetuses}}{\text{Number of implantation sites}} \times 100$$

$$\text{Total mortality rate (\%)} = \frac{\text{Number of corpora lutea} - \text{Number of alive foetuses}}{\text{Number of corpora lutea}} \times 100$$

Perinatal mortality corresponds to the mortality around birth (young rabbits found dead at the 1st control after kindling).

$$\text{Perinatal mortality (\%)} = \frac{\text{Number of stillborn}}{\text{Number of total born}} \times 100$$

Birth-weaning mortality corresponds to the mortality of born alive between birth and weaning.

$$\text{Birth-weaning mortality (\%)} = \frac{\text{Number of born alive} - \text{Number of weaned rabbits}}{\text{Number of born alive}} \times 100$$

Prolificacy or litter size at birth, includes the mean number of young rabbits (total, alive and stillborn) per litter.

$$\text{Prolificacy} = \text{Total number of born per delivered doe}$$

Fecundity (combining fertility and prolificacy) measures the aptitude of a doe to be fertile and prolific.

Numerical productivity at a specific time (birth, weaning, marketing) is a global criterion with commercial significance, which takes into account fertility, litter size at birth and young viability.

$$\text{Numerical productivity at birth} = \text{Number of born alive per mated or inseminated doe.}$$

The numerical productivity at weaning takes into account the viability of the young between birth and weaning.

$$\text{Numerical productivity at weaning} = \text{Number of weaned rabbits per mated or inseminated doe}$$

The overall productivity at a specific time which must be indicated (birth, weaning, marketing) is a global criterion with commercial significance which takes into account fertility, litter size and litter weight.

$$\text{Overall productivity at birth} = \text{Weight of born alive per mated or inseminated doe}$$

Overall productivity at weaning takes into account the young viability and growth between birth and weaning.

Overall productivity at weaning = Weight of weaned rabbits per mated or inseminated doe