

Impact of pre-breeding feeding practices on rabbit mammary gland development at mid-pregnancy

ABSTRACT

Line 28: consider changing the word “*performance*” by “*potential*”. The reproductive capacity of an animal is, in part, given by the genetic potential of each individual. By modulating the environmental conditions, such as the feed, the expression of the genetic potential is altered.

Lines 29-31: please revise this sentence. Selected and non-selected rabbits have nutritional needs. What may have changed through selection for high prolificacy (and other reproductive traits) is the nutritional requirement. In respect to the feeding strategy during the rearing period, this sentence introduces the hypothesis that selection for reproductive traits also changed (incremented) the nutritional requirements of young rabbit females. This hypothesis seem to not be sustained by the available literature (e.g. compare the H1M & H2M groups in the paper of Quevedo *et al.*, 2005; <https://doi.org/10.1079/ASC40850161>).

Lines 32-35: please introduce the objective of the study. The feeding strategies are the means to attain the objective. Please consider the following suggestion: “*The aim of this study was to analyse the impact of four different feeding strategies in the early life of rabbit females (**combination** of high or moderate feed restriction from 5 to 9 weeks of age **with** restricted or ad libitum feeding regime from 9 to 12 weeks of age), on their...*”

Lines 37-38: please mention the period of the measurements. Is it only from 3 to 9, from 3 to 12, from 9 to 12 weeks or other periods?

Lines 38-40: same as above.

Line 46: introduce the word “feed” at “...restrictive feed strategies...”

Lines 47-49: Did diets affect milk yield, quality and neonatal survival? If not, please review this recommendation.

INTRODUCTION

Lines 54-55: A reference is required.

Lines 55-56: Dietary restriction is mainly used to reduce the risk of digestive disorders in the post-weaning period (cited review). The reduction of feed costs is a collateral, non-negligible, consequence of this strategy related to the capacity of young rabbits to re-gain the live weight not acquired during the restriction period. Based in this information and to correctly quote the references, please re-structure this paragraph (lines 54-59).

Line 62: consider changing the words “*control*” by “*restricted*”.

Lines 63-64: please specify the periods and mention the values for the reduced fertility compared with the ad libitum strategy. “*Feed restriction **during**...energy deficit **during**..., leading to a reduced fertility (XX vs YY; Pascual et al. 2003)*”. The reference provided (if Pascual JJ, Cervera C, Blas E, Fernández-Carmona J. 2003. High-energy diets for reproductive rabbit does: effect of energy source. *Nutrition Abstracts and Reviews*, 73 (5), 27R-39R) is not the most adequate to sustain this argument. This reference is not listed in the Reference section. Please revise this issue.

Lines 64-65: revise this sentence. Martinez-Paredes *et al.* (2019) observed no effect of a feed restriction plan (CR diet) when compared to the *ad libitum* strategy (CAL diet) on milk yield and composition (1st lactation), blood metabolites (females), kits live weight (1st lactation), kits mortality (1st lactation), kits feed intake (1st lactation), females reproductive interval (1st to 2nd parturition), or in any other measured parameters.

Lines 70-71: consider the following change: “...are long-term process that starts early in life and continues through adulthood...”

Lines 71-73: mention the early life factors that influence the mammary gland development in the pre-pubertal phase. Of the mentioned factors to what extent, does dietary restriction impede the mammary gland development? Provide key references to sustain this argument. The cited reference (Robinson 1995) may not be the most suitable here.

Lines 75-76: please provide a reference. See the comment above.

Line 93: delete the word “still”

Recommendation: authors may restructure and shorten the introduction section. They may focus on the physiological changes in the mammary gland development induced by an unbalanced intake of nutrients (too much and/or too little) in the early life of mammals. Then introduce the potential issue of feed restriction strategy adopted in rabbit production, a strategy that focus on the correct body development and on the reduction of fat deposits (adequate body composition) at first insemination, on the subsequent development of the mammary gland. It is important to mention that although, the available literature does not support the hypothesis that feed restriction impairs the milk yield or quality (Martinez-Paredes *et al.*, 2019), there is a lack on the knowledge on the impacts of a long-term feed restriction, starting as soon as 5 weeks old, on the physiological development of the mammary gland and tissues. Then present the objectives of the study.

MATERIAL AND METHODS

Lines 109-111: Provide the agreement number issue by the French Ministry of Education, Research and Innovation (MESRI).

Lines 113-114: Provide the temperature range and average measured during the entire experimental period. Even in indoor conditions, a constant temperature of 18°C

is the target temperature. Concerning the illumination program, provide the numbers of dark and light hours at each period.

Line 121: Reference not needed.

Lines 124-125: “At 12 weeks of age, rabbits were housed individually and received 150 g/d of diet”. (1) Provide information on the size of the individual cages. (2) No information concerning the size or the number of animals housed from 5 to 12 weeks is provided. Please add this information.

Line 128: change “sacrificed” by “euthanized”. Describe the method used to euthanize the animals.

Lines 127-129: Split the sentence “At...fasting”. Suggestion: In the first phrase talk about the artificial insemination and the method used to declare pregnancy. In the second phrase precisely describe the moment and the method of euthanasia. An addition sentence explaining the reasons of the euthanasia is required. Example: *The 40 pregnant females were euthanized at 14 weeks of gestation to obtain mammary gland tissues...*

Line 129: “...hydrous fasting”. Not clear if feed or water was restricted. Please consider “...after 12 hours of fasting (water provided ad libitum)”

Key question:

How many animals you used in the study, from weaning (5 weeks) to 14 days of gestation to obtain the 40 pregnant females euthanized? Please indicate the total number of animals used, not only the 40 females euthanized. Mortality and infertility are normal outcomes in animal science.

Figure 1. Please change period names:

- 5 to 9 weeks: **fattening period** ;
- 9 to 11 weeks: **pubertal period** ; although it is mainly considered as part of the fattening period
- 12 to 19 weeks: **rearing period** ;

Key question:

Why animals of SRAL group were limited to a daily ration of 150 g of feed per day from 12 weeks to 14 days of pregnancy instead of being fed *ad libitum*?

Feeding restriction programs for young rabbit females may start as early as five weeks old (Rommers, 2004; <https://doi.org/10.1051/rnd:2004037>) or later (Martinez-Paredes et al., 2012; <https://doi.org/10.1017/S1751731111002643>), but in most cases (Pascual et al., 2013 ; <https://doi.org/10.4995/wrs.2013.1236>) feed restriction last for at least 9 weeks to have an effect on reproductive traits. Here, if I understand correctly, animals were subjected to four feed restriction plans during the fattening period (5 to 11 weeks; 11 weeks is the slaughter age in France), followed by a feed restriction plan of 150 g/days during the rearing period (12 to 19 weeks plus during the first 14 days of pregnancy). Please clarify this point in the introduction. To me, the confusion emanates from a lack of

information on the periods of feed restriction in the studies quoted in the introduction (rearing periods for the Pascual *et al.*, 2003 and Martinez-Paredes *et al.*, 2019) and the period aimed here (fattening period).

Lines 137-138: “*then mammary samples were processed and stored for further analyses*”. Please describe the process used to conserve the mammary tissue and storage conditions.

Line 151: Please move this information to the statistical analysis sub-section.

Line 175-179: Please provide information on the model used, as the estimation of the SEM depends on the model used. Provide also information on the distribution of the different variables studied. Are all variables normally distributed? For sample size, is it 30 samples per group? If yes, how do you achieve that as you have 10 samples per group (considering the animal as the statistical unit)? Differences should be declared only at a P-value below 0.05 and not below or equal to 0.05. Please correct it.

Please indicate the statistical software used to perform the statistical analysis.

RESULTS

Lines 187-190: Consider exclude these sentences; no need to recall the experimental plan.

Lines 190-193: This is the real restriction observed, following the restriction method proposed in the Material & Methods. Authors may present this information in the Material & Methods section, as this is the intended restriction rather than an observed result from the restriction applied.

Key question concerning the calculation of feed intake during the feed restriction period (lines 193-194):

How many animals per cage? How many cages? Females and male young rabbits were housed separately or together? There is no issue in presenting results of more animals (or statistical units: cages if group housing) than the 40 females used here to demonstrate the intake level of feed restriction. However, this information must figure in the manuscript. In addition, the real intake on the cages where the 40 females lived (*number of cages, average and standard deviation for each group*) must be presented as the “farm” average may not represent the real restriction the studied animals were subjected.

Lines 194-196: Please indicate the value of the difference between the SR and MR groups. Provide the P-value for the difference observed at week seven. How many samples have been used in this calculation? Only the individual weight of the 40 rabbit does (10 per group) should be presented.

Lines 197-199: Is a difference of +15.7 for the SRAL or +17.5 g/day for the MRAL groups respect to the restricted groups (SRR and SRR) significant (in terms of energy and nutrient ingested by the animals and statistically)? Present only the results concerning the 40 animals used in the study, not the farm average.

Lines 200-203: provide the values of the differences between groups and the p-values of each comparisons. Figure 1 should be constructed only with the live weight of the studied 40 animals, not the farm average per restriction group (the error bars are too small for a sample of 10 individuals per group). Otherwise indicate in the material & methods that 40 females were sampled from four subpopulations of rabbits (n=X rabbits per group) that followed the following restriction plans... The results here presented appears to not reflect what is indicate in the material and methods section.

Lines 203-206: Indicates the values of the differences in the text. Calculated only with the 10 animals per group.

Lines 207-209: same comment as above.

Lines 210-212: comparison with the feed intake between SRR and MRR with SRAL and MRAL groups were not performed because of a lack of feed intake measurement for the restricted groups (140 g/day). Can you ascertain that every single cage in the 140 g/day restriction ended the daily feed allowance? Please indicates the standard deviation for each calculated mean (SRAL and MRAL). Only data on the 40 animals should be consider, see comments above.

Lines 213-217: Please revise this sentence to present the differences and P-values for a pairwise comparison between two groups at a time.

Lines 225-228: Exclude the word “*unexpectedly*”. Start the sentence at “*Unrestricted*”. Present the only difference observed, between SRR and SRAL for cholesterol.

Key question concerning the distribution of metabolic blood parameters measured:

Indicate in the material and methods, section statistical analysis the distribution of these variables. Are they normally distributed?

Lines 230-231: Do not mention the fertility rate, as you focus the samples on the 40 rabbit does being pregnant. Start at prolificacy and foetal viability. Correct Figure 4 A y-axis: “*Average number of foetus per rabbit doe*”.

Lines 241-243: Exclude from this sentences the text: “*although ... respectively*.” The alpha level chose to declare a statistical significance was set to <0.05 . A value equal or above this value indicates a non-significance at the delta expected (*i.e.* the relevant difference between groups). To sustain your argument, a bigger sample size would be required to declare a difference of ~0.8 points of percentage (Figure 5 C). Figure 5. Include a C letter for the graph presenting the “relative percentage of mammary tissue”

Line 252: exclude “*..., and these differences were strongly significant*”.

Figure 6: Include the letters “A” and “B” for the left and right graphics, respectively.

Lines 263-264: please correct this sentence. The effect is not related to the diet, it is related to the feeding strategy.

Figure 7: same comment as for Figure 6. Add here the letter “C”.

DISCUSSION

Line 286-287: please consider excluding “*although ...performances.*”

Line 288: please change “*deciphered*” by “*studied*”. Exclude “*fertility*” (this was not studied – results not shown; lines 230-231).

Lines 292-293: please explain the use of the word “*sensitivity*”. Do you mean, their daily energy and other nutrient requirements for growth are high, making the intake of animals in the MR group as high as 120.75 g/day (+25 g/d compared SR).

Lines 293-298: Which effects? Please describe.

Lines 298-299: These results underline that feed restriction was effective in limiting feed intake and growth. There is no direct relation between early-life being a critical period for nutrition. Which is important is to consider that feed restriction alter the normal development of the animal by delaying it. Please revise this sentence.

Lines 299-301: Discuss the “harmonization” from the two angles treated. For the restricted group (140 g/day) animals gain weight, while for the *ad libitum* group the animals started a mild restriction (155.7 or 157.5 g/day to 150 g/day), but adapted quickly.

Lines 301-303: In your case, growth does not seem to be irregular; no loss of live weight observed on the average curves (Figure 2 B). It is important to mention the negative consequence observed by Neave et al. (2019, dairy cows) and Haschke et al. (2019; humans) when a malnutrition event occur early in life. The present results does not evidence a long-term effect of a feed restriction plan on the measured parameters influencing the reproductive capacity nor a correct development of the mammary tissue. Does the increment on the adipose and epithelial tissues, followed by a reduction in the connective tissue affect the future nursing capacity of the rabbit females in the SRR group respect to the less restrictive feeding strategies?

Lines 304-310: Please introduce in this part of your discussion the notion of the ‘level’ of the feed restriction (nutrient restriction) early in life, and its duration needed to affect the reproductive performance in the first reproductive event. In the present study, and with the different restriction plans adopted, can you conclude that more severe feeding strategy (SRR) impaired? Should a rabbit breeder using the SRR system be concerned?

Line 312: A through revision on the lipid metabolism of restricted mammals is required. For instance, Yang *et al.* (2010; <https://doi.org/10.1016/j.rvsc.2010.04.003>) observed a lower body weight and a high serum total cholesterol in restricted fed broilers compared to *ad libitum* ones.

Lines 313-316: No assessment on the stress was performed in the present study. To avoid speculation, please exclude this sentence.

Lines 319-322: Please provide a reference for the age of development of the mammary tissue in rabbit. The references provided here (Denamur, 1963 & Borellini, 1989) are not listed in the reference section. Please correct this issue. Authors should consider citing their own paper (Hue-Beauvais, 2019; <https://doi.org/10.1002/dvdy.91>).

Lines 334-336: The results does not support this sentence. No statistical differences were detected.

Lines 339-340: The results does not support this sentence. No statistical differences were detected.

Line 368: only discuss statistically significant results.

Line 369: change “(*SR group vs MR group*)” by “(*SRAL vs MRAL groups*)”.

Line 386: change “*count*” by “*cost*”.

Line 396: Is it due to voluntary or involuntary culling? Please precise the reasons. The cause-consequence link is not direct.