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DETERMINATION OF THE NUMBER OF REPLICATES REQUIRED TO DETECT A SIGNIFICANT DIFFERENCE BETWEEN TWO MEANS IN RABBIT TRAITS

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ABSTRACT : The number of replicates required to detect differences of variable size at several levels of significance, has been determined for growth, lactation and digestion traits from the equation : $n \geq s^2 \times t^2 \times 2/d^2$. Residual standard deviation (s) values have been derived from results obtained with rabbits in our Research Department. An average value of s = 3.93 g/day has been obtained, for instance, in the case of average daily gain, for the average experimental conditions in our laboratory, and from a model including weaning weight as covariate and litter as block effect. This s value increased up to 5.38 when neither covariate nor block was considered in the model. Based on the s values obtained, the number of replicates needed to detect significant differences between two means for different traits were tabulated for several values of d and levels of significance. These values permit to decide the size of experiment according to the size of the differences to be detected. For example in our average experimental conditions, a sample size of 20 rabbits per treatment would allow to detect as significant differences (P = 0.05) of 3.0 g/day of average daily gain, 20 percentage units of mortality and 2 percentage units of dry matter digestibility during the fattening period.

RESUME : Détermination du nombre de répétitions nécessaires pour détecter une différence significative entre deux moyennes dans les expérimentations sur le lapin.
Les auteurs proposent une méthode pour déterminer n le nombre de répétitions à mettre en place pour espérer détecter comme significative une différence d fixée d'avance entre deux moyennes pour plusieurs niveaux de signification. L'équation utilisée est : $n \geq s^2 \times t^2 \times 2/d^2$n dans laquelle t est la valeur du t de Student pour le seuil de signification retenu et s l'écart-type du critère étudié. Les valeurs proposées pour s sont les écarts-types résiduels provenant des résultats obtenus par les auteurs au Département de Recherches Cunicoles de l'Université de Valence pour les paramètres classiquement utilisés dans les études sur la croissance, la lactation et la digestibilité des aliments, après prise en compte des facteurs contrôlés. Par exemple, une valeur moyenne de s = 3,93 g/jour a été obtenue pour le gain moyen journalier, à partir d'un modèle incluant le poids au sevrage comme covariable et la portée comme effet bloc. Cette valeur de « s » augmente jusqu'à 5,38 lorsque ni la covariable, ni l'effet bloc ne sont pris en compte dans l'analyse de variance servant à déterminer s (écart-type résiduel). Sur la base des valeurs de « s » obtenues, le nombre n de répétitions nécessaires pour détecter comme significative des différences entre deux moyennes ont été calculées pour différentes valeurs de d et différents niveaux de signification. Ces chiffres permettent de déterminer la taille des groupes expérimentaux en accord avec l'ampleur des différences que l'on souhaite détecter. Par exemple dans les conditions moyennes d'expérimentation des auteurs, un essai avec 20 lapins par lot permet d'espérer trouver comme significative (P = 0,05) une différence de 3,0 g/jour de gain de poids moyen entre deux traitements, ou une différence de 20 unités entre deux taux de mortalité ou encore une différence de 2 unités entre deux coefficients de digestibilité de la matière sèche

1.- INTRODUCTION

One of the questions that the scientist would like to know before beginning an experiment is if the number of replicates used is appropriate to detect as significant the difference between means expected. The answer to this question can help to optimise the resources and to improve the experimental design.

Thus, if we want to detect as significant a small difference and we do not have enough replicates available, then probably the experiment will be worthless. On the contrary, if we expect a large difference between means the use of a high number of replicates will be wasteful.

To decide the size of the experiment we need to know the size of difference that is important to us and the variability, measured as the standard deviation, which might be expected in the experiment. Once we have obtained these data, the number of replicates (n) (in some experiments : rabbits, litters or cages) that we need to detect a significant difference of a magnitude of 'd' units between two means is given by the **following equation** (ROBERTS, 1983; MEAD *et al.*, 1993) :

$$n \geq s^2 \times t^2 \times 2/d^2 \qquad \text{equation 1}$$

where 's' is the standard deviation and t is the value of the Student distribution.

2. ESTIMATION OF THE STANDARD DEVIATION

Firstly, we need to know the **mean standard deviation** (s) of the trait studied, which is related to the variability of the animal population used. This value (s) can be estimated as the residual standard deviation (square root of the Mean Square Error), obtained in the variance analysis. As an example, the 's' value obtained for the average daily gain in the whole fattening period (g/day) from several trials done in our Department is shown in table 1. These experiments have been carried out following the procedures described in the appendix.

The model used in the feeding experiments done in our Department was :

Average daily gain = Mean + Diet$_i$ + Litter$_j$ + β (Weaning weight$_{ijk}$ – Mean weaning weight) + ε_{ijk}

Where controlled factor **Litter**, acts as a block, and β **(Weaning weight$_{ijk}$ – Mean weaning weight)** is the effect of the covariate.

Table 1 : Values of 's' observed in the experimental determination of the average daily gain during the whole fattening period (g/day).

References	n	Standard error (SE)	Standard deviation (s)
de Blas *et al.*, 1991. *J. Appl. Rabbit Res.*, 14 :148-150.	45	0.47	3.15
García *et al.*, 1992. *J. Appl. Rabbit Res.*, 15 :1008-1016.	20	0.86	3.85
Taboada *et al.*, 1994. *Livest. Prod. Sci.*, 40 :329-337.	46	0.67	4.54
Taboada *et al.*, 1996. *Reprod. Nutr. Dev.*, 36 :191-203.	39	0.57	3.56
Fernández and Fraga, 1996. *J. Anim. Sci.*, 74 :2088-2094.	29	0.69	3.71
Motta Ferreira *et al.*, 1996. *Anim. Sci.*, 63 :167-174.	8	1.45	4.10
de Blas *et al.*, 1998. *Anim. Feed Sci. Technol.*, 70 :151-160.	38	0.60	3.70
Nicodemus *et al.*, 1998. Unpublished.	36	0.46	2.76
García *et al.*, 1999. *ITEA.*, 20 :469-471.	71	0.41	3.45
Nicodemus *et al.*, 1999. *Anim. Feed Sci. Technol.*, 80 :43-54.	36	0.78	4.68
Nicodemus *et al.*, 2000. *7th World Rabbit Congress,*	35	0.57	3.37
Nicodemus, N., 2000. PhD. Thesis.	40	0.54	3.41
Gutiérrez *et al.*, 2000. *7th World Rabbit Congress,*	35	0.88	5.20
Gutiérrez *et al.*, 2000. Unpublished.	35	0.73	4.32
Gutiérrez *et al.*, 2000. Unpublished.	42	0.79	5.14
Mean value of the standard deviation (s)			**3.93**

The residual standard deviation values depend primarily on the trait studied and on the model used. For instance, in table 2 is shown the change in the residual standard deviation value for average daily gain obtained in our Department when the model of variance analysis is modified.

It is also important to point out that a change in the experimental conditions (e.g. environmental temperature, feeding system, etc.), genetic variability of the animals or other factors, may modify the standard deviation. Their inclusion in the statistical model, when pertinent, may reduce the residual standard deviation used as estimation of 's'. In Figure 1 is shown the frequency distribution of standard deviation values of average daily gain in papers published in *World Rabbit Science*. In those papers in which standard deviation was not specified it was calculated as : s = standard error × √n, where n = number of replicates per treatment. For these published works (n = 21) where 's' values ranged from 2 to 8, the average 's' value was 3.93, which is very close to the average value shown in table 1.

Otherwise, for one trait 's' values are independent of the number of replicates per treatment, of the number of treatments, and of the subject of the experiment.

Table 2 : Effect of statistical model on the 's' value used in the equation 1 for average daily gain studies.

Model includes		Residual standard deviation ('s')
Individual weaning weight used as Covariate	Litter used as Block effect	
no	no	5.38
YES	no	4.99
no	YES	4.17
YES	YES	3.93

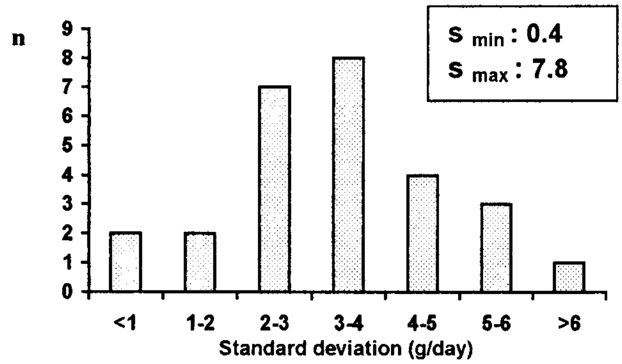

Figure 1 : Frequency distribution of 's' values of average daily gain, in 27 papers published in *World Rabbit Science*

3. CALCULATION OF THE T VALUE AND THE NUMBER OF RABBITS REQUIRED

The t value for a probability level of 0.05 and 20 or 60 degrees of freedom (21 or 61 replicates, respectively) varies only from 2.09 to 2.00, respectively. However, if a low number of animals per treatment is used (i.e. 6, and so, 5 degrees of freedom) the t value increases to 2.57 (table 3).

As an example, it has been considered that the number of replicates (rabbits, cages, ...) used in fattening trials is 61 per diet (to obtain easily the value from the Student distribution). So, the t values for 60 degrees of freedom and a probability level of 0.05, 0.01 and 0.001 are (table3) :

$t_{0.05} = 2.00$ $t_{0.01} = 2.66$ $t_{0.001} = 3.46$

Table 3 : Examples of the t of Student distribution for different degrees of freedom (ddf) and 3 different signification levels.

ddf	Probability		
	0.05	0.01	0.001
5	2.571	4.032	6.859
8	2.306	3.355	5.041
10	2.228	3.169	4.587
13	2.160	3.012	4.221
16	2.120	2.921	4.015
20	2.086	2.845	3.850
25	2.060	2.787	3.725
30	2.042	2.750	3.646
40	2.021	2.704	3.551
60	***2.000***	***2.660***	***3.460***
100	1.984	2.626	3.389
500	1.960	2.586	3.310
∞	1.960	2.576	3.291

Therefore, the number of rabbits required to detect as significant (P = 0.05) a difference of 3.0 g/day between two means for the average daily gain in the whole fattening period is :

$$n \geq (3.93^2 \times 2.00^2 \times 2/3.00^2) = 13.7 \Rightarrow 14 \text{ rabbits}$$

As 14 rabbits is much lower than the 61 used in the just above calculation, it is advisable to recalculate the latter expression using the t value for the same probability but 13 degrees of freedom ($t_{P=0.05;\ df=13}$) instead of that of 60 :

$$n \geq (3.93^2 \times 2.16^2 \times 2/3.00^2) = 16.0 \Rightarrow 16 \text{ rabbits}$$

In the same way, the number of animals required to get different levels of statistical significance is shown in table 4.

Table 4 : Number of rabbits required (n) to detect a significant difference (P = 0.05, 0.01 or 0.001) between two means for the average daily gain in the whole fattening period, if the standard deviation is 3.93 (average value in table 1).

Difference to be detected (g/day)	Number of rabbits required (n)		
	P = 0.05	P = 0.01	P = 0.001
0.5	475	822	1337
1.0	119	206	335
2.0	33	55	84
3.0	16	27	44
4.0	11	18	28
5.0	8	12	19

Using the number of animals calculated in table 4 we will not be sure either to detect as significant the difference expected. Effectively, if the standard deviation in the experiment is higher than the average 's' shown in table 1, it will be necessary to use a higher number of animals to detect as significant the expected difference. For example, for the highest 's' value observed in the table 1 (5.2 g/day), the number of replicates necessary to detect as significant a difference of 3.0 g/day, is increased from 16 up to 28. On the contrary, if the 's' value of the experiment is lower than the average 's', then we will be able to detect as significant even a smaller difference than the one expected. For example, if 's' is only 2.76 g/day, the lowest value of the table 1, an experiment conducted with 16 rabbits per treatment, would consider as significant a difference between means of 2.1 g/day.

Using the same procedure followed to calculate table 4, and from 's' values obtained in our Research Department with the model described later in each table, it has been calculated the number of

Table 5 : Mean standard deviation and number of rabbits required (n) to detect a significant difference (P = 0.05) between two means for fattening traits (degrees of freedom = 60) (Model : Fattening trait = Mean + $Diet_i$ + $Litter_j$ + β ($Weaning\ weight_{ijk}$ – Mean weaning weight) + ε_{ijk}).

Traits[1]	Mean standard deviation (s)	Number of rabbits required (n) to detect with a P = 0.05 a *difference (d)* :					
Units of the difference : g/day		*1*	*2*	*3*	*5*	*8*	*10*
Feed intake two weeks after weaning period (9)	**10.6**	**863**	**216**	**96**	**37**	**17**	**11**
Feed intake in the whole fattening period (12)	**11.1**	**946**	**237**	**106**	**41**	**18**	**12**
Units of the difference : g/day		*0.5*	*1*	*2*	*3*	*4*	*8*
Average daily gain two weeks after weaning (10)	**5.50**	**930**	**233**	**61**	**29**	**18**	**7**
Units of the difference : g daily gain/g feed intake.		*0.005*	*0.01*	*0.02*	*0.03*	*0.04*	*0.07*
Feed efficiency two weeks after weaning period (9)	**0.054**	**897**	**225**	**59**	**28**	**17**	**8**
Feed efficiency in the whole fattening period (12)	**0.036**	**399**	**100**	**28**	**14**	**10**	**6**
Units of the difference : percentage units.		*5*	*10*	-	-	-	-
Mortality in the whole fattening period (10)	**28.65**	**253**	**65**	-	-	-	-

[1] Number in parenthesis indicates number of trials considered for each trait

experimental units (rabbits, does or litters) required to detect a significant difference (P = 0.05) between two means for fattening, lactating, digestibility and caecal traits. The results are shown in tables 5, 6, 7 and 8, respectively.

Otherwise, the proportion of variance explained (R^2) and the significance (P=0.05) of the effect of the traits used as covariates or controlled factor (blocks) in fattening and lactating trials are shown in tables 9 and 10.

4. CONCLUSIONS

In order to establish the minimal number of replicates required to detect a significant difference of a given size between two means, it is advisable :

i) to determine the value of 's' for the trait studied from previous data in similar experimental conditions.
ii) to establish a safety margin (e.g. a 15% increase of the number of replicates) to take into account that the value of 's' in a particular experiment can differ from the average value obtained in previous experiments.
iii) the value of 's' and the number of replicates needed can be lowered by including in the variance model covariates that can explain a part of the total variability (e.g. weaning weight in growth trials).
iv) the value of 's' and then the number of replicates can also be lowered by using for example litter as a controlled effect (block) in growth trials, or parity order in reproduction experiments.

Table 6 : Mean standard deviation and number of experimental units (does or litters) required (n) to detect a significant difference (P = 0.05) between two means for lactating traits (degrees of freedom = 15) (Model : Lactating traits = Mean + Diet$_i$ + β_1 (Day of mating$_{ij}$ – Mean day of mating) + β_2 (Litter size$_{ij}$ – Mean litter size) + ε_{ij}).

Traits[1]	Mean standard deviation (s)	Number of experimental units (n) to detect with a P = 0.05 a *difference (d)* :					
Units of the difference : g/day		*10*	*15*	*20*	*25*	*50*	*75*
Feed intake of rabbit does/lactation (10)	**44.7**	**154**	**69**	**41**	**28**	**9**	**6**
Feed intake of litter from 21 to 30 days (5)	**43.8**	**148**	**66**	**39**	**26**	**9**	**6**
Units of the difference : g/day		*1.0*	*2.0*	*3.0*	*4.0*	*5.0*	*6*
Average daily gain of litter from 21 to 30 days (5)	**4.81**	**178**	**47**	**22**	**14**	**20**	**8**
Units of the difference : kg		*0.1*	*0.2*	*0.3*	*0.4*	*0.6*	*0.8*
Milk production per lactation (9)	**0.76**	**444**	**111**	**52**	**31**	**15**	**10**
Litter weight at 21 days of age (8)	**0.40**	**123**	**33**	**17**	**11**	**6**	**5**
Litter weight at weaning (8)	**0.64**	**315**	**79**	**37**	**22**	**12**	**8**
Units of the difference : kg weaned/kg feed.		*0.02*	*0.03*	*0.04*	*0.05*	*0.06*	*0.07*
Feed efficiency (5)	**0.052**	**55**	**26**	**16**	**11**	**8**	**7**

[1] Number in parenthesis indicates number of trials considered for each trait.

Table 7 : Mean standard deviation and number of rabbits required (n) to detect a significant difference (P = 0.05) between two means for digestibility traits (degrees of freedom = 9) (Model : Digestibility traits = Mean + Diet$_i$ + ε_{ij}).

Traits[1]	Mean standard deviation (s)	Number of rabbits required (n) to detect with a P = 0.05 a *difference (d) :*				
Units of the difference : percentage units.		*2.0*	*3.0*	*4.0*	*5.0*	*6.0*
Dry matter digestibility (20)	**2.58**	**16**	**9**	**7**	**5**	**4**
Gross energy digestibility (20)	**2.73**	**17**	**9**	**6**	**5**	**4**
Crude protein digestibility (23)	**3.32**	**24**	**13**	**8**	**6**	**5**
Neutral detergent fibre digestibility (15)	**4.67**	**45**	**22**	**13**	**10**	**8**
Acid detergent fibre digestibility (12)	**6.08**	**71**	**35**	**21**	**15**	**11**
Crude fibre digestibility (7)	**7.63**	**112**	**50**	**31**	**21**	**15**

[1] Number in parenthesis indicates number of trials considered for each trait.

Table 8 : Mean standard deviation and number of rabbits required (n) to detect a significant difference (P = 0.05) between two means for caecal traits (degrees of freedom = 9) (Model : Caecal traits = Mean + Dieti + εij).

Traits[1]	Mean standard deviation (s)	Number of rabbits required (n) to detect with a P = 0.05 a *difference (d)* :					
Units of the difference : pH units.		*0.05*	*0.10*	*0.15*	*0.20*	*0.25*	*0.30*
Caecal pH (13)	**0.18**	**100**	**27**	**14**	**9**	**7**	**6**
Units of the difference : mmol/liter		*4*	*6*	*8*	*10*	*12*	*14*
Caecal volatile fatty acids concentration (9)	**9.28**	**44**	**21**	**13**	**10**	**8**	**6**
Units of the difference : mmol/liter		*1*	*2*	*3*	*4*	*5*	*6*
Caecal ammonia nitrogen concentration (11)	**3.41**	**89**	**25**	**13**	**9**	**7**	**5**
Units of the difference : % Body weight		*0.2*	*0.4*	*0.6*	*0.8*	*1.0*	*1.2*
Weight of caecal content (14)	**0.87**	**146**	**39**	**19**	**12**	**9**	**7**
Units of the difference : g DM/day		*2*	*3*	*4*	*5*	*6*	*8*
Soft faeces excretion (9)	**5.52**	**60**	**28**	**17**	**12**	**10**	**7**

[1] Number in parenthesis indicates number of trials considered for each trait.

Table 9 : Effect of weaning weight used as covariate and of litter used as controlled effect (block), on fattening traits observed in 8 different experiments conducted in the author's laboratory (R^2 is expressed as percent)

References	Average daily gain				Feed intake				Feed efficiency			
	Weaning weight		Litter		Weaning weight		Litter		Weaning weight		Litter	
	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P
Taboada *et al.*, 1996. *Reprod. Nutr. Dev.*, 36 :191-203.	5.8	0.0001	53.1	0.0001	4.3	0.0001	37.6	0.0001	1.6	0.04	38.7	0.0001
Nicodemus *et al.*, 1998. Unpublished.	5.6	0.0002	62.6	0.0001	2.2	0.03	60.2	0.0001	2.4	0.04	49.3	0.0008
Nicodemus *et al.*, 1999. *Anim. Feed Sci. Technol.*, 80 :43-54.	3.0	0.01	49.7	0.0001	1.0	NS	34.6	0.01	0.9	NS	49.4	0.0001
García *et al.*, 1999. *ITEA.*, 20 :469-471.	4.1	0.007	49.8	0.0001	0.6	NS[1]	42.3	0.0001	2.5	0.04	43.4	0.0006
Gutiérrez *et al.*, 2000. *7th World Rabbit Congress*, In press.	8.8	0.0001	36.0	0.0001	19.2	0.0001	30.6	0.0001	3.6	0.004	27.6	0.03
Nicodemus, N., 2000. PhD Thesis.	3.6	0.003	45.8	0.0001	5.2	0.0005	37.6	0.0001	0.004	NS	44.1	0.0001
Gutiérrez *et al.*, 2000. Unpublished.	3.8	0.005	46.4	0.0003	4.0	0.007	40.9	0.01	3.2	NS	36.0	0.03
Gutiérrez *et al.*, 2000 Unpublished.	14.5	0.0001	24.7	0.0001	14.1	0.0001	31.5	0.0001	3.5	0.002	24.5	0.001

[1] NS : Non significant (P > 0.05).

Table 10 : Effect of day of effective mating and litter size used as covariates on milk production, observed in 3 different experiments conducted in the author's laboratory (R^2 is expressed as percent)

	Total milk production				Milk production 1-10 day				Milk production 11-20 day				Milk production 21-30 day			
	Day of mating		Litter size		Day of mating		Litter size		Day of mating		Litter size		Day of mating		Litter size	
	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P
1[1]	3.7	NS[4]	10.3	0.02	0.4	NS	12.0	0.01	4.8	NS	13.2	0.009	23.8	0.0003	1.1	NS
2[2]	4.9	0.06	22.8	0.0002	0.04	NS	20.3	0.0009	0.4	NS	18.5	0.001	16.5	0.001	12.1	0.006
3[3]	0.44	NS	17.9	0.01	1.3	NS	22.6	0.0002	0.3	NS	22.3	0.0001	9.0	0.03	2.8	NS

[1] de Blas et al., 1999. *World Rabbit Sci.*, 7 :203-207. [2] Nicodemus et al., 1999. *Anim. Feed Sci. Technol.*, 80 :43-54.
[3] Nicodemus, N., 2000. PhD. Thesis. [4] NS : Non significant (P > 0.05).

Appendix : Common material and methods of the experiments used in tables 4 to 9 calculations.

In all the experiments animals (New Zealand White × Californian) were housed in flat-deck cages. Besides, the temperature was maintained between 15 and 25°C using building heating or cooling systems and forced ventilation.

Fattening trials :
- Age at weaning : 25-30 d.
- *Ad libitum* access to the feed
- the experiment was conducted until rabbits reached approximately 2 kg of body weight.
- Individually caged.
- Weaning weight was used as linear covariate and litter as block effect.

Lactating trials :
- *Ad libitum* access to the feed in late pregnancy (from day 28 on) and throughout lactation.
- Adaptation period to the experimental feed 60 days.
- Milk production was estimated daily from weight loss of does during suckling.
- Day of effective mating and litter size at weaning were used as linear covariates.

Digestibility trials :
- Growing rabbits between 1.5-2 kg body weight.
- *Ad libitum* access to the feed.
- Adaptation period to the feed 10-14 d.
- Faecal collections made on four consecutive days.
- No covariates were used.

Caecal trials :
- Growing rabbits between 1.5-2 kg BW. *Ad libitum* access to the feed.
- Adaptation period to the feed 10-14 d.
- Wooden collar to prevent caecotrophy put on at 08.00 and removed 24 h later.
- Animals slaughtered at 18.00 h.
- No covariates were used.

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