animal

Early deviations in performance, metabolic and immunological indicators affect stayability in rabbit females

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The main purpose of this study was to find several early factors affecting stayability in rabbit females. To reach this goal, 203 females were used from their first artificial insemination to their sixth parturition. Throughout that period, 48 traits were recorded, considered to be performance, metabolic and immunological indicators. These traits were initially recorded in females' first reproductive cycle. Later, removed females due to death or culling and those that were non-removed were identified. A first analysis was used to explore whether it was possible to classify females between those reaching and those not reaching up to the mean lifespan of a rabbit female (the fifth reproductive) cycle using information from the first reproductive cycle. The analysis results showed that 97% of the non-removed females were classified correctly, whereas only 60% of the removed females were classified as animals to be removed. The reason for this difference lies in the model's characteristics, which was designed using early traits and was able to classify only the cases in which females would be removed due to performance, metabolic or immunologic imbalances in their early lives. Our results suggest that the model defines the necessary conditions, but not the sufficient ones, for females to remain alive in the herd. The aim of a second analysis was to find out the main early differences between the non-removed and removed females. The live weights records taken in the first cycle indicated that the females removed in their first cycle were lighter, while those removed in their second cycle were heavier with longer stayability (−203 and +202 g on average, respectively; P < 0.05). Non-removed females showed higher glucose and lower beta-hydroxybutyrate concentrations in the first cycle than the removed females (+4.8 and −10.7%, respectively; P < 0.05). The average lymphocytes B counts in the first cycle were 22.7% higher in the non-removed females group (P < 0.05). The females removed in the first reproductive cycle presented a higher granulocytes/lymphocytes ratio in this cycle than those that at least reached the second cycle (4.81 v. 1.66; P < 0.001). Consequently, non-removed females at sixth parturition offered adequate body development and energy levels, less immunological stress and a more mature immune function in the first reproductive cycle. The females that deviated from this pattern were at higher risk of being removed from the herd.

Keywords: early reproduction, metabolism, performance, immune status, rabbit females

Implications

The identification of the main early factors, whose alteration may notably affect females' survival and stayability, is of much interest to develop strategies (for husbandry, nutrition, genetics, etc.) that ensure meeting the targeted values of these traits and to reduce the risk of females being removed from the herd. If these aims are fulfilled, the longevity of females would increase, the replacement ratio would be lower and, consequently, robustness and resilience would also emerge across farms.

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Introduction

Improving productivity of livestock is a lifelong goal in animal science. However, its relative weight is decreasing in the last years in order to search for the sustainability of the production system (Neeteson *et al.*, 2013). Consequently, some authors (ten Napel *et al.*, 2011) have promoted modern livestock production systems so that they become more robust. To fulfil this goal, researchers may work on different production system aspects, such as animals, their housing, farm practices, etc. Here, in this study, we focus on a single component: animals. Indeed, particular interest is

paid to identify those individuals capable of producing at high levels and over a long time period in a wide range of changing environments that they can encounter through life.

Theoretically, an animal would stay longer in the herd (i.e., stayability) if it is able to quickly return to its physiological trajectory (or find a new stable trajectory) in the face of environmental insults that force its organism to deviate from the original trajectory (Martin and Sauvant, 2010). Genetics, among other factors, seems to play a key role in individuals' 'ability' to return to their internal equilibrium. Yet despite artificial selection improving productivity traits, several undesired side effects accompany its progress, such as reduced productive lifespan (Rauw, 2009), which could be related to the aforementioned deviations from the original physiological trajectories.

Environmental disturbances, for example, fewer available resources, changes in climate conditions or exposure to pathogens, may force those trajectories to deviate. Fortunately, not all animals respond equally to disturbances as some are more flexible than others (Martin and Sauvant, 2010). Survival, for example, has been described to be closely associated with an animal's homeostatic capacity to return to its original physiological trajectory (Sauvant et al., 1983). According to Friggens et al. (2013), an animal's homeostatic capacity is determined mainly by its genetics and current physiological state, such as gestation or lactation. Together, these two factors play a key role in defining how an animal may use its soma (i.e., body size and body reserves) to sustain and recover its original physiological trajectory when faced with adverse environmental conditions. Animals seem more sensitive to deviation in their physiological trajectory when their soma is not completely developed. Indeed, many works have related an adequate soma at the beginning of reproductive life to greater stayability in gilts (O'Dowd et al., 1997; Tarrés et al., 2006) and rabbit females (Martínez-Paredes et al., 2018). Other factors, such as feed intake capacity (e.g., resource acquisition ability) and immune status (e.g., white blood cell counts), are likely to help animals to face constraints and return to their physiological equilibrium. A better understanding of how some of these traits, which happen at the beginning of an animal's life and influence farm animals' stayability, is very interesting to construct robust and sustainable livestock production systems.

Rabbits can be considered a suitable animal model to study these traits for several reasons. They have been selected for productive traits for more than 30 years (Baselga, 2004) and the reported stayability is low (Rosell and de la Fuente, 2009). It is a multiparous species characterised by short reproductive cycles, it is easy to handle and its raising costs are low compared with other livestock species. All these characteristics allow a vast amount of data on many traits to be produced within a short time interval. In this study, we explored how performance, metabolic and immunological indicators, measured early in the lives of rabbit females, are associated with the definition of suitable physiological trajectories. The possible influence of these traits on the stayability of reproductive rabbit females was also investigated.

Material and methods

Animals
The life of 203 crossbreed rabbit females (New Zealand × Californian) was followed from their first artificial insemination (**IA**) to their sixth parturition. During this period, females could be removed from the herd for two reasons: (i) death and (ii) culled due to reproductive failure (three consecutive failed conceptions).

Herd management
Females were individually housed in wire mesh cages (Length: 70 × Width: 50 × Height: 32 cm) that included an external nest for litters from 28 gestation days to 30 days post-parturition (**dpp**). Cages were placed inside a closed building with controlled air temperature and a daylight regime. Throughout the experimental period, the average temperature variation ranged from 13°C to 26°C. The daylight regime was set at 16 h of light and 8 h of darkness. Air renewal was assured by a forced ventilation system, set to vary between 0.10 and 0.40 m/s according to the internal air temperature. Rabbit females accessed *ad libitum* a diet formulated for reproductive rabbit females (11.3 MJ of digestible energy and 126 g of digestible protein per kg DM) as of 12 weeks of age. Drinking water was also freely available. Females were artificially inseminated (**AI**) for the first time at 19 weeks of age by being re-inseminated 11 dpp. Non-pregnant females were re-inseminated 21 days later. A maximum of three consecutive attempts was allowed before females were culled due to reproductive failure. After parturition, litter size was adjusted to be 8 offspring for primiparous females (8.24 ± 0.04) and 10 offspring for multiparous ones (10.04 ± 0.03). Litter size adjusted at birth had no effect on the female lifespan. Lactation lasted 30 days, when the offspring were weaned. This management was maintained until the sixth parturition (i.e., the end of the trial).

Traits measured before and throughout the first reproductive cycle
Below are listed several traits related to performance, metabolism and immune system. The role of the traits on these systems has been reported in several works (Guerrero et al., 2011; Savietto et al., 2013; García-Quirós et al., 2014), suggesting that they could also be related to the stability of females at farm.

Performance traits. The acquisition capacity during the first reproductive cycle was monitored by controlling the feed intake of females from 0 to 17 dpp and that of females and their litter from 18 to 30 dpp. Resource allocation and reproductive effort in the first cycle were monitored by recording the live weight (**LW**) and perirenal fat thickness (**PFT**) of females (at first IA, parturition, 18 dpp and weaning), litter weight (at parturition, 18 dpp and weaning) and milk yield (over the first three lactation weeks). Perirenal fat thickness was obtained by ultrasound scans, following the recommendation of Pascual et al. (2000), and milk yield

was achieved by weighing females before and after suckling their litters (four times per week). Performance traits were measured in the whole population.

Plasma metabolites. Blood samples were collected at IA, parturition, 18 dpp and upon weaning in the first reproductive cycle from 86 females. A peripheral venous catheter and a tube with ethylenediaminetetraacetic acid (**EDTA**) were used to obtain 5.0 ml of blood from the median ear artery of each female. All the animals were subjected to a minimum 3-h fasting period before samples were obtained (feed was freely provided after blood sampling). Samples were immediately centrifuged ($3000 \times$ **g** for 10 min at 4°C) after collection. Plasma was separated and frozen at −40°C until further analyses. Plasma samples were analysed for glucose, non-esterified fatty acids (**NEFA**) and beta-hydroxybutyrate (**BOHB**). Glucose was determined by standard procedures (Siemens Diagnostics® Clinical Methods for ADVIA 1650, Tarrytown, NY, USA). Non-esterified fatty acids were established by the NEFA C test by acyl-coenzyme-A-synthetase and acyl-coenzyme-A-oxidase (**ACS-ACOD**) assay method (Wako Chemicals GmbH, Neuss, Germany). Beta-hydroxybutyrate was determined as an increase in absorbance at 340 nm owing to NADH production at a slightly alkaline pH in the presence of BOHB dehydrogenase. Sample blanks were included and the method involved oxamic acid in the media to inhibit lactate dehydrogenase, as proposed by Harano *et al.* (1985). Analyses of glucose, NEFA and BOHB were performed in an auto-analyser, ADVIA 1650® Chemistry System (Siemens Medical Solutions, Tarrytown, NY, USA). In all instances, the intra- and inter-assay CVs were below 2.0% and 4.0%, respectively.

Leukocyte populations. An additional 1.0 ml of blood was also collected from the median ear artery (in EDTA vacuum tubes) to characterise the peripheral-blood leukocytes population of 86 females at the first IA and 30 dpp (the same ones monitored for plasma metabolites). Samples were analysed immediately after sampling and prior to any other procedure. The white blood cells count was determined in a haematology analyser (MEK-6 410; Nihon Kohden, Tokyo, Japan). Blood samples were then transferred to 50.0-ml tubes, in which 40.0 ml of ammonium chloride lysing solution at 4°C

was added to isolate white blood cells. After a 6-min incubation in the dark, samples were centrifuged ($400 \times$ **g** for 5 min at room temperature). The supernatant was eliminated, and the pellet was carefully re-suspended in 1.0 ml of phosphate-buffered saline $1\times$ (**PBS**). The density of the suspension was adjusted to 10^6 cells per ml, counted in a Neubauer chamber. Primary monoclonal antibodies were added (Table 1) and incubated in the dark for 20 min at room temperature. Pellets were washed with 1.0 ml of PBS and centrifuged again ($400 \times$ **g** for 5 min at room temperature). Secondary antibodies (Rat anti-mouse IgG2a+bPhycoerythrin (VMRD, Inc., Exalpha Biologicals, Shirley, MA, USA) and Goat anti-mouse IgM: R-Phycoerythrin-human adsorbed (AbDSerotec, Düsseldorf, Germany)) were added, and the samples were incubated for 20 min at room temperature in the dark. Finally, 1.0 ml of PBS was added before passing the samples to the flow cytometer. The outcome white blood cells suspensions were analysed in a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA, USA). The common leukocyte antigen CD14 and CD45 expressions were used for the 'lymphogate' setup as previously described (Guerrero *et al.*, 2011). The calculations of the total lymphocyte and respective subsets counts were done as the product of the white blood cells count and the specific populations percentages, as described by Guerrero *et al.* (2011).

Statistical analysis

Initially, the data obtained from the animals with information from all the measured 48 traits ($n = 86$ females) were subjected to partial least square regression discriminant analysis (**PLS-DA**; PLS procedure of SAS, Statistical Analysis System Institute Inc., Cary, NC, USA). The PLS-DA method was used to explore whether it was possible to classify females between those reaching and those not reaching up to the mean lifespan of a rabbit female (the fifth reproductive) cycle using information from the first reproductive cycle. The model included stayability up to the fifth parturition as a response variable (binary variable: 1 for the non-removed females that reached the fifth parturition ($n = 61$); 0 for those that did not ($n = 25$)); all the studied variables were measured in the females and their litters as predictors. The PLS-DA method was chosen because it avoids overfitting

Table 1 *The monoclonal antibodies used for the flow cytometry analysis of the rabbit females in this study*

Monoclonal antibody	Iso.	Spec.	Cell labelling	Clone	Ref.	Comp.
Mouse anti-rabbit T-lymphocytes: FITC	IgG1	CD5	T-cell	KEN-5[1]	Kotani *et al.* (1993a)	Abd Serotec
Mouse anti-rabbit α-pan B	IgM	IgM	B-cell	MRB143A	Davis and Hamilton (2008)	VMRD Inc.
Mouse anti-rabbit CD4	IgG2a	CD4	T-cell subset	KEN-4	Kotani *et al.* (1993a)	Abd Serotec
Mouse anti-rabbit α-CD8	IgG2a	CD8	T-cell subset	ISC27A	Davis and Hamilton (2008)	VMRD Inc.
Mouse anti-rabbit CD25	IgG2b	CD25	Activated T-cells	KEI-ALPHA1	Kotani *et al.* (1993b)	Abd Serotec
Mouse anti-human CD14: FITC	IgG2a	CD14	Monocytes & granulocytes	TÜK4	Jacobsen *et al.* (1993)	Abd Serotec
Mouse anti-rabbit α-CD45	IgM	CD45	All leukocytes	ISC76A	Davis and Hamilton (2008)	VMRD Inc.

Iso. = isotype; Spec. = specificity; Ref. = references; Comp. = company; FITC = fluorescein IsoTioCyanate.
[1]Clon KEN-5 recognises rabbit T-lymphocytes and immunoprecipitates. This antibody recognises rabbit CD5, but does not bind to rabbit CD5 transfectants. Known rabbit CD5 antibodies also show binding to most B-lymphocytes, which are not labelled by this clone (information obtained from the datasheet).

by using a smaller set of new independent variables (PLS factors) to make the classification. Partial least square factors are obtained using the linear combinations of the original variables, which maximises the correlation between the PLS factor and the response variable, and PLS1 was the linear combination with the highest correlation. Of all the independent linear combinations for PLS1 (perpendicular eigenvectors), PLS2 had the highest correlation, and so on for the subsequent PLS factors. In this way, the PLS factors are variables that are independent of one another, which were ordered according to their correlation with the dependent variable. To make the classification, the procedure also used a linear model:

$$\text{Stayability} = b1 \cdot \text{PLS1} + b2 \cdot \text{PLS2} + b3 \cdot \text{PLS3} + \cdots$$
$$+ bn \cdot \text{PLS}n$$

where $b1$, $b2$, $b3$, ..., bn represent the estimated regression coefficients for covariates PLS1, PLS2, PLS3, ..., PLSn. Females were classified as being capable of reaching the fifth parturition when the prediction of stayability exceeded 0.5 (class 1) and were classified as incapable (class 0) when it went below 0.5. Consequently, the threshold between the non-removed and removed females was defined as the geometric trace of predictions that equalled 0.5. This threshold was obtained as the optimum balance between sensitivity and specificity from the receiver operating characteristic curve of the method (Supplementary Material Figure S1). The estimated coefficients, $b1$, $b2$, $b3$ ... bn, were back-transformed to obtain standardised coefficients (**SC**) to predict stayability until the fifth parturition based on the original traits.

A different sort of analysis was applied to the whole data set to find out main differences at early reproductive life of females according to their stayability (those reaching mean lifespan and those being removed earlier). In this new analysis, animals were reclassified into two groups according to the last accomplished cycle (**LAC**). The '*non-removed group*' included 109 females that reached at least the fifth parturition. The
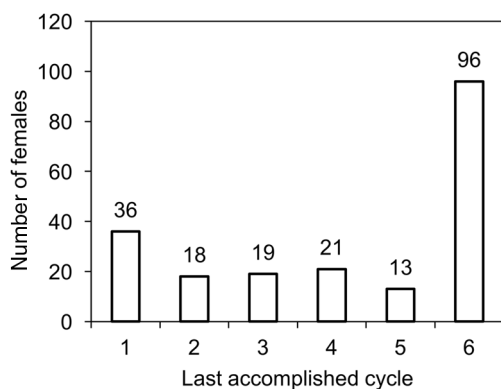
Figure 1 Number of rabbit females removed (culled or died) according to the last accomplished reproductive cycle (first to fifth). Each cycle included the number of females removed between the parturition of cycle n and the day before the parturition of cycle $n + 1$. The data for the sixth accomplished cycle include all the animals that reached the sixth parturition as this parity represents the end point of the experiment.

'*removed group*' comprised the remaining 94 females, which were removed before the fifth parturition (Figure 1). A weight variable was set to weight the data within subgroups, which allows to use all the information, but the data closest to the definition of the group have a higher relative weight. Therefore, weight variable values were set in descending order as the reproductive cycle increased in the '*removed group*' (1.00, 0.75, 0.50 and 0.25 for the females whose last accomplished parturition was the first, second, third and fourth, respectively), and in ascending order in the 'non-removed *group*' (0.75 and 1.00, respectively, for those females whose last accomplished parturition was the fifth, or they reached the sixth, parturition). Two different mixed models were used to determine the early differences between these two subgroups or according to their accomplished reproductive cycle.

$$[\text{Model 1}] \; Y = \text{Sample day} + \text{Group}$$
$$+ \text{Sample day} \cdot \text{Group} + b_1 \cdot T + e$$

$$[\text{Model 2}] \; Y = \text{Sample day} + \text{LAC} + \text{Sample day} \cdot \text{LAC}$$
$$+ b_1 \cdot T + e$$

where Sample day (four levels; IA, 0, 18 and 30 dpp) Group (two levels; removed and non-removed) and LAC (six levels; cycles 1, 2, 3, 4, 5 and 6, except for the plasma metabolites, where none of the analysed animals were removed in the fourth cycle) are the fixed effects; T is the inner room temperature as a covariate and b_1 its regression coefficient; e is the residual random error whose variance–covariance matrix was modelled without assuming any defined structure for its parameters (unstructured matrix; SAS). Weight variable was considered. The linear and quadratic effects of accomplished cycles were also determined by the estimate procedure of SAS for the analysis performed with Model 2.

Results

All 203 females had at least one litter and 96 females reached the end of the experiment (sixth parturition). Figure 1 shows the classification of females by the LAC, which was done by including the data of the females removed between parturitions n and $n + 1$.

Classification ability: Partial least squares analysis
The SC obtained from the PLS regression of stayability up to the fifth parturition from the 48 traits measured in the 86 females are provided in Figure 2. These SC, which ranged from -0.12 to $+0.23$, allowed a direct proportional comparison to be made of the positive or negative contribution to the classification ability of a particular trait at a given time. In general, the plasma metabolites had the highest SC values. The SC values for glucose concentration were negative at first IA (-0.10) and were positive (above $+0.20$) at 18 and 30 dpp. The SC values were always negative for the plasma BOHB concentration (from -0.02 to -0.10) and were positive for NEFA (from $+0.04$ to $+0.12$). In general, the SC
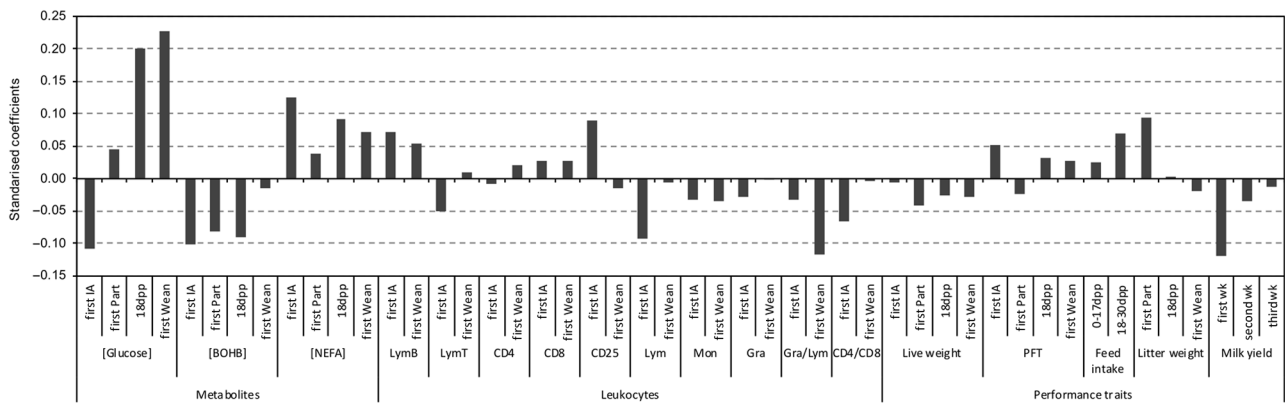
Figure 2 Standardised coefficients for the metabolic, immunological and performance traits measured in the first reproductive cycle when used in a multivariate study (discriminant analysis) to distinguish between the rabbit females reaching the fifth parturition from those that were previously removed from the herd. The variable with values close to zero (small bars) has a low (irrelevant) discriminant value. IA = artificial insemination; Part = parturition; Dpp = days post-parturition; Wean = weaning; BOHB = beta-hydroxybutyrate; NEFA = non-esterified fatty acids; Lym = lymphocyte; Mon = monocyte; Gra = granulocyte; PFT = perirenal fat thickness.

values for the blood leukocyte counts and the performance traits were low. At first IA, the SC values were positive for lymphocytes B (+0.06) and CD25+ (+0.09) and were negative for total lymphocytes (−0.09), T-lymphocytes (−0.05) and the CD4+/CD8+ ratio (−0.07). At 30 dpp, the SC value of the granulocytes/lymphocytes ratio was negative (−0.12). Regarding performance traits, the SC values were positive for PFT at first IA (+0.05), with feed intake between 18 and 30 dpp (+0.07), and LW at the first parturition (+0.09). The SC value was negative for milk yield, especially in the first week of lactation (−0.12).

Figure 3 represents the classification ability of the PLS analysis. Black squares represent the actual non-removed females, and the white rhombus denotes the actual removed females. The dashed line represents the classification threshold between groups. Any points above the dashed line were classified as 'non-removed' and those below it were taken as 'removed'. Of the 61 actually non-removed females up to the fifth reproductive cycle, 59 were classified as correctly, whereas 2 were taken as removed. Of the 25 actually removed females, 15 were classified as removed and 10 as non-removed. These results indicate that 86% (74 of 86) of the females were correctly classified. However, the classification accuracy for both groups was not equal, sensitivity was 97% (59 of the 61 actually non-removed females were classified as non-removed), whereas specificity was only 60% (15 of the 25 actually removed females were classified as removed).

Main differences between non-removed and removed females: mixed model analysis
Table 2 shows the average values for all the traits in the first reproductive cycle for the 109 females that reached the fifth parturition (non-removed) and for the 94 females that left the herd before the fifth parturition (removed). The effects of the LAC on these traits are also shown. We did not observe any significant difference in the average performance traits in the first cycle between the removed and
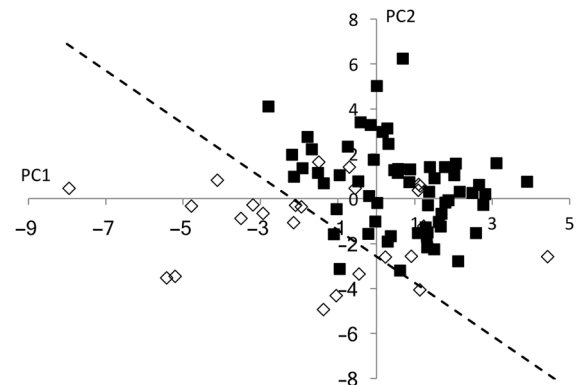
Figure 3 Representation of the two first principal components (PLS1 and PLS2) in the multivariate analyses to discriminate between the non-removed (■, the actual rabbit females reaching the fifth reproduction cycle) and removed rabbit females (◇, the actual rabbit females that were previously culled or died). The dashed line represents the classification limit between both classifications. Points above the dashed line were classified as non-removed and those below it were taken as removed.

non-removed females. However, when comparing the LW in the first cycle, the females removed in this cycle were lighter and those removed in the second cycle were heavier than the females with longer stayability (−203 and +202 g on average, respectively; Figure 4A). These differences came from the LW data measured during lactation as the LAC had no effect on the LW record taken at first IA (interaction in Table 2; *P* = 0.011). Another interesting feature was related to the weight of litters upon first parity: on average, the females with a longer reproductive life seemed to produce heavier offspring at first parturition (linear effect: +5.89 ± 2.89 g/cycle; *P* = 0.043; Figure 4B).

As regards the plasma metabolites, non-removed females had higher glucose and lower BOHB concentrations in the first cycle than the removed females (+4.8 and −10.7%, respectively; *P* < 0.05; Table 2). In fact, the higher the glucose concentration in the first cycle, the higher the last cycle accomplished by females (linear effect: +0.07 mM/cycle;

Table 2 *Differences in performance, metabolic and immunology traits in the first reproductive cycle between the non-removed (those that reached the fifth reproduction cycle) and removed rabbit females (those previously culled or died), and the statistical effect of the LAC on these traits*

Traits in first cycle	Removed		Non-removed		Effect of last accomplished cycle[1]			
					LAC	LAC × Sd	Linear	Quad
Live weight (kg)	4243	(39)	4258	(31)	0.026	0.011		
PFT (mm)	7.61	(0.08)	7.64	(0.06)				
Daily feed intake (g/d)	314	(8)	332	(6)				
Milk yield (g/d)	121	(7)	122	(5)				
Litter weight (g)	1744	(85)	1801	(61)		0.018	0.043	
[Glucose] (mM)	6.291	(0.082)[a]	6.593	(0.052)[b]	0.021	0.027	0.008	
[BOHB] (log10 mM)	−0.731	(0.022)[b]	-0.780	(0.014)[a]	0.015			
[NEFA] (log10 μekv/l)	2.527	(0.019)	2.546	(0.012)				
Total lymphocytes (log10/l)	9.414	(0.018)	9.423	(0.013)				
Lymphocytes B (log10/l)	7.376	(0.036)[a]	7.465	(0.026)[b]	0.049		0.041	0.079
Lymphocytes T CD5$^+$(log10/l)	9.212	(0.019)	9.236	(0.013)				
CD4$^+$(log10/l)	8.987	(0.018)	9.004	(0.013)				
CD8$^+$(log10/l)	8.551	(0.025)	8.596	(0.018)				
CD4$^+$/CD8$^+$	2.951	(0.133)	2.766	(0.094)				
CD25$^+$(log10/l)	7.142	(0.040)	7.189	(0.029)				
Monocytes (log10/l)	8.463	(0.024)	8.463	(0.017)				
Granulocytes (log10/l)	9.485	(0.020)	9.475	(0.015)				
Granulocytes/lymphocytes	1.896	(0.230)[b]	1.255	(0.149)[a]	0.005	0.001	0.003	0.018

PFT = perirenal fat thickness; BOHB = beta-hydroxybutyrate; NEFA = non-esterified fatty acids.
[1]*P*-values: LAC = effect of the last accomplished cycled achieved as a fixed effect; LAC × Sd = Interaction LAC with Sample day time (first artificial insemination, 0, 18 and 30 days post-parturition); Linear = Linear effect of LAC; Quad = Quadratic effect of LAC. Only *P*-values under 0.10 were included in the table.
[a,b]Means not sharing superscript differed significantly (*P* < 0.05).
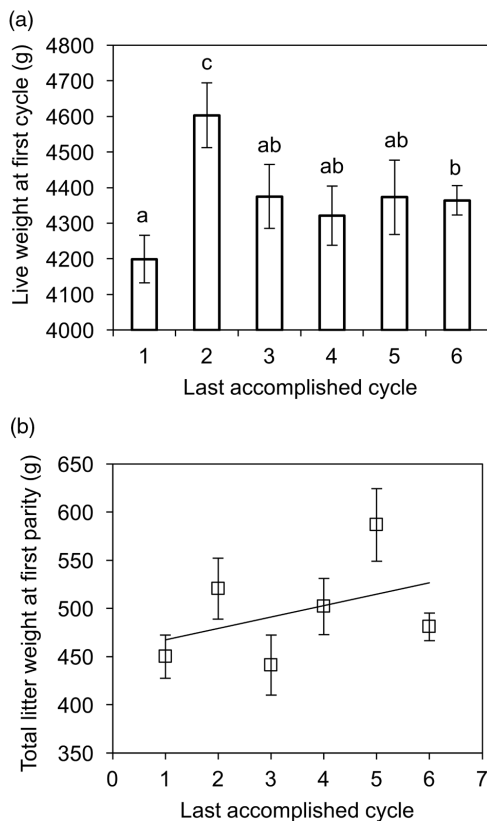
Figure 4 Means and standard errors of the performance traits according to the reproductive cycle they accomplished: the average live weight (LW) that rabbit females had in the first reproductive cycle (A) and the total LW at first parturition (B). The bars not sharing the same letter were significantly different at *P* < 0.05.

P = 0.008; Figure 5A). Although we did not observe any linear or quadratic pattern for the BOHB concentrations (Figure 5B), the females that were either removed in the second cycle or reached the sixth parturition had lower BOHB concentrations in the first cycle than those females removed in the first or third cycles (0.16 and 0.21 mM on average, respectively; *P* < 0.01).

In the average peripheral-blood leucocytes count in the first cycle, only lymphocytes B and the proportion of granulocytes compared with lymphocytes differed between the removed and non-removed females (Table 2). The average lymphocytes B counts in the first cycle were 22.7% higher in the non-removed females group (*P* < 0.05). According to the results graphed in Figure 6A (quadratic effect: *P* = 0.0796), the females reaching at least the third cycle had higher lymphocytes B counts in the first cycle. A lower granulocytes/lymphocytes ratio was observed at the first cycle for the non-removed females (approximately 1.26 for the non-removed females and 1.90 for the removed ones; *P* < 0.005). This result was mainly due to the females removed in the first reproductive cycle, which had a higher granulocytes/lymphocytes ratio in this first cycle than those that reached at least the second cycle (4.81 *v.* 1.66; *P* < 0.001; Figure 6B).
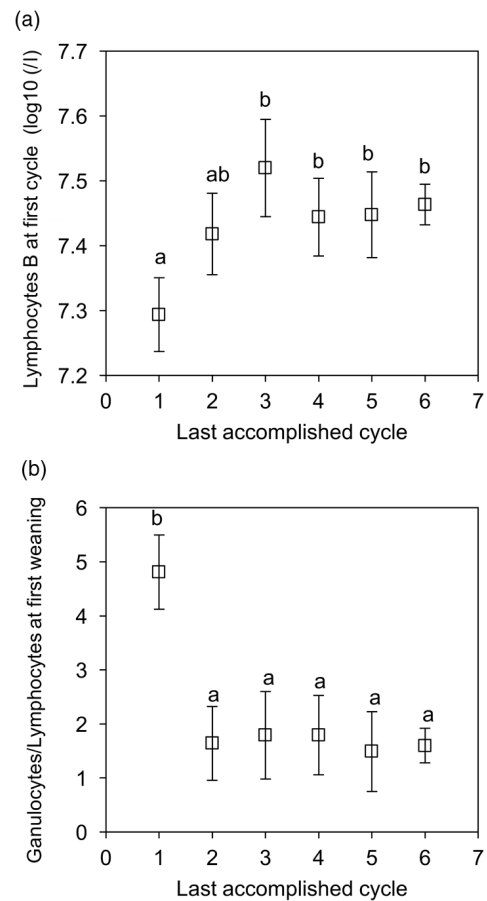
Discussion

Average life of reproductive rabbit females in commercial farms is 5 to 7 reproductive cycles. In the present work, we wanted to search for existing differences between animals that

(a)

(b)
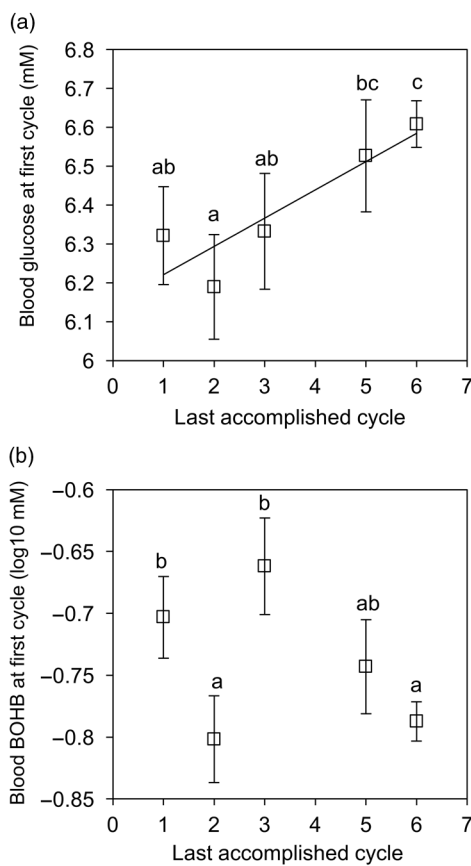
Figure 5 Means and standard errors of blood glucose (A) and blood beta-hydroxybutyrate (B) concentrations in rabbit females in their first reproductive cycle according to the reproductive cycle they accomplished. The means not sharing the same letter were significantly different at $P < 0.05$.

(a)

(b)

Figure 6 Means and standard errors of the blood lymphocytes B counts (A) and the granulocytes/total lymphocytes ratio (B) in rabbit females in their first reproductive cycle according to the reproductive cycle they accomplished. The means not sharing the same letter were significantly different at $P < 0.05$.

complete the fifth reproductive cycle (average lifespan) and those that are removed before this moment. In this sense, we report that 73 of the 107 removed females (approximately 68%) were removed before the fourth reproductive cycle. In line with this finding, Rosell and de la Fuente (2009) worked with a census of 118 832 reproductive rabbit females and observed that of the females that were culled or died between the first and sixth parturitions, 67% occurred in the first three cycles, and the second parturition had the highest incidence. Notwithstanding, there is a wide range of intrinsic and extrinsic factors that ultimately determine whether females die or are culled throughout their reproductive lives. Moreover, the first reproductive cycle of a breeder has some peculiarities that are absent in the following cycles; this is the first time that females are pregnant, which results in the establishment of different priorities to compete for the available resources to be employed for dynamic functions such as body maintenance, body growth, pregnancy, lactation and immunity (Bauman and Currie, 1980). In addition, the female soma and acquisition capacity are not fully established in relation to the next cycles, when adult size and full feed intake capacity are achieved (Xiccato, 1996; Pascual *et al.*, 1998). These two circumstances may limit an animal's capacity to regulate its physiology in

response to both intrinsic (competing priorities) and extrinsic (environmental conditions) constraints.

Classification ability

The fact that we could classify 86% of the females successfully with information from the first reproductive cycle is compelling evidence that early physiological status and females' stayability are related at some degree. The differences observed between the classification accuracies in both groups could also offer an interesting interpretation. On the one hand, 97% of the actually surviving females were correctly classified (sensibility). This means that almost all the actually surviving females presented the necessary physiological indicators to survive according to the model. On the other hand, only 60% of the actually removed females were correctly classified (specificity). This implies that the model failed when classifying a large proportion of the removed females. If we consider that a female may be removed from the herd for many reasons (Rosell and de la Fuente, 2009), our results would suggest that the model defines the necessary conditions, but not the sufficient ones, to remain on the farm. This is because the model only

classifies when females will be removed due to performance, metabolic or immunologic imbalances early in their lives.

Main early average differences between the surviving and removed females

The females who reached the third reproductive cycle onwards had an intermediate live weight (4258 ± 31 g), high blood glucose (6.59 ± 0.052 mM), high B-lymphocytes counts ($7.47 \pm 0.026 \log_{10}$/l) and a low granulocyte/lymphocyte ratio (1.26 ± 0.149) in the their first cycles. Put more simply, they displayed adequate body development and glucose levels, less immunological stress and a more mature immune function, which seem necessary for them to remain on farms. Our B-lymphocytes results indicated a direction that coincides with several authors. Jeklova *et al.* (2007a) recorded a significant increase in B-lymphocytes during immune system maturation. This difference to other species may be related to B-lymphocytes development in rabbits, where short-term B lymphopoiesis occurs early in development, conversely to humans and mice in which B lymphopoiesis takes place throughout their lifetime (Jasper *et al.*, 2003). A relationship has been described between the selection of B-lymphocytes for their development and maturation after B lymphopoiesis and lymphocyte homeostasis and, consequently, immune function (Miller and Cancro, 2007). These facts would justify the importance of the number of B-lymphocytes required to determine stayability in the non-removed females group. There is also a link between B-lymphocytes and glucose, which is also consistent with our results. Dufort (2012) described how B-lymphocytes respond to environmental cues for their survival, growth and differentiation through receptor-mediated signalling pathways. Naïve B-lymphocytes must acquire and metabolise external glucose to support the bioenergetics associated with maintaining cell volume, ion gradients and basal macromolecular synthesis. The upregulation of glycolytic enzyme expression and activity via engaged B-cell receptor-mediated events is glucose-dependent. This suggests the essential role of glucose energy metabolism to promote B-cell growth, survival and proliferation in response to extracellular stimuli.

First artificial insemination. Starting reproductive life with optimal physiological development and an adequate level of reserves is expected to improve the life span (Martínez-Paredes *et al.*, 2018) and reduce the risk of death and culling (Theilgaard *et al.*, 2006). In the present work, the non-removed females (those that reached the sixth parturition) seemed to be characterised by having an appropriate level of body reserves (approximately 7.6 mm of PFT), glucose (above 6.4 mM), BOHB (below 0.165 mM), NEFA (above 351.5 μekv/l) and B-lymphocytes (above 6.4 $\log_{10}$/l) at the beginning of their reproductive life. Recently, Martínez-Paredes *et al.* (2018) identified body reserves at first mating as one of the main factors to influence life span in reproductive rabbit females. In fact, Theilgaard *et al.* (2006) observed a higher risk of culling among the females with very high or very low PFT values, which suggests that an optimum level of fat reserves may

exist. Regarding immunity, newborn rabbits start their lives with a competent, but a still naïve, immune system and specific responses develop with time (Jeklova *et al.*, 2007b). In line with this, protection acquired by innate immune mechanisms and newborn rabbits' ability to adapt their immunological features throughout their lives, according to their needs, play an important role in rabbits' defence against productive, environmental and infectious challenges. Indeed, an increase in the number of B-lymphocytes and CD25+ T-lymphocytes, and a lower CD4+/CD8+ ratio, in peripheral blood are the indicators of greater innate immune maturity in both rabbits (Jeklova *et al.*, 2009; García-Quirós *et al.*, 2014) and dairy cows (Mehrzad and Zhao, 2008). These results suggest that adequate maturity in both physiological development and immunological level terms is apparently necessary at the beginning of reproductive lives to maximise resilience and life spans in rabbit females. Along these lines, according to the data collected from 130 commercial farms with more than 300 000 females over a 6-year period, Rosell and de la Fuente (2009) observed a negative correlation ($r = -0.31$; $P < 0.001$) between age at first mating and the risk of removing a rabbit female from a herd.

First parturition. The females that reached the fifth reproduction cycle were characterised by having slightly heavier litters (+3%). Nonetheless, they presented lower BOHB levels in blood at first parturition (−11%). Other previous works have observed a higher risk of culling for nulliparous females with a small litter size (Piles *et al.*, 2006). In that work, the authors speculated that the fact that females with small litter size presented higher risk of culling could be related to underlying pathological problems. However, our results seemed to indicate that the females capable of good deliveries managed better their body reserves (being capable of coping with such challenges without excessive mobilisation which reduced replaced risk due to poor body condition), which could increase their stayability.

First lactation. From the time lactation begins, a period of intense energy expenditure for females comes into play. This effort is especially important in primiparous rabbit females because: (i) their reproductive level is high (genetic selection programmes for litter size leads to big litter sizes in the first reproductive cycles; Baselga, 2004); (ii) they are still growing (females usually reach their adult weight between the fourth and fifth parturitions; Quevedo *et al.*, 2006); (iii) their acquisition capacity is still not completely developed (Xiccatto, 1996). Thus, insufficient resources acquisition in the first lactation may increase the risk of culling of young reproductive females. In fact, the lactating primiparous females with fewer resources (lower feed intake and blood glucose level) and, at the same time, with sustained high milk yields showed greater reserves mobilisation (higher BOHB and light LW) and were, thus, the females with a higher probability of dying or being removed before the fifth reproductive cycle. These results agree with those observed by Savietto *et al.* (2013), where the rabbit females of a line formed for productive longevity criteria

showed higher feed intake and blood glucose levels in the first lactation than those of a line selected for litter size at weaning. In any case, unsuitable performance indicators (above or below the normal values of a given population) may be generally considered to warn that something is wrong. In fact, the females removed in the first cycle had lighter average LWs, and lower B-lymphocyte counts and granulocyte/lymphocyte ratios. We also observed that an excessive LW in the first cycle (above 4500 g) negatively affected reproductive life, and this was a particular trait of the females removed from the herd in the second reproductive cycle.

As a result of this effort made in the first lactation, and a possible negative energy balance, Rosell and de la Fuente (2009) observed that the main casualties and eliminations of females occurred between the first weaning and the second parturition. Therefore, it would seem normal that the females at low risk of culling which reached the fifth cycle would present at first weaning clearly higher blood glucose levels and a better innate immune state, as characterised by a higher B-lymphocyte count and a lower granulocyte/lymphocyte ratio. This ratio is a chronic immunological stress indicator that is known to increase if several stressors are present, such as starvation (Gross and Siegel, 1983), diseases or infections (Davis et al., 2008). In other words, the more immunologically stressed an animal is, the higher the granulocyte/lymphocyte ratio, which is a valuable biological indicator to predict future problems in both populations and individuals.

Conclusions

Despite the present work has been developed at one single farm in a single breeding system standardising litter size, results reported herein show that deviations in performance, metabolic or immunological indicators exist for 60% of the females that were removed before their sixth parturition. In line with this, it would appear that the non-removed females at sixth parturition present adequate body development and energy levels, less immunological stress and a more mature immune function in the first reproductive cycle. The females that deviate from this pattern are at more risk of being removed from the herd.

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Declaration of interest
The authors declare that they have no competing interests.

Ethics statement
The experimental procedure was approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València and carried out following the recommendations of the European Group on Rabbit Nutrition (Fernández-Carmona et al., 2005) and the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes.

Software and data repository resources
The data belong to the Universitat Politècnica de València and the Universidad Cardenal Herrera-CEU but can be made available by requesting them from the Corresponding Author.

Supplementary material
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