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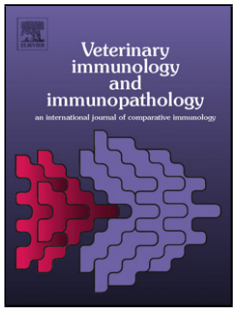

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FULL-LENGTH PAPER (ORIGINAL RESEARCH)

How selection for reproduction or foundation for longevity could have affected blood lymphocyte populations of rabbit does under conventional and heat stress conditions.

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ABSTRACT

The present work characterises how selection for reproduction (by comparing two generations -16th and 36th- of the V line selected for litter size at weaning) or foundation for reproductive longevity (the LP line) can affect the blood lymphocytes populations of reproductive rabbit does under normal [conventional housing, average daily minimum and maximum temperatures of 14ºC and 20ºC, respectively] and heat stress conditions [climatic chamber, 25ºC and 36ºC] from the first to the second parturition. Housing under heat stress conditions significantly reduced the B lymphocytes counts in female rabbits (–34 × 10^6/L; P<0.05). The highest lymphocytes population value in blood (total, T CD5$^+$, CD4$^+$ and CD8$^+$) was noted at the first parturition, while the B lymphocytes count was significantly lower at the second parturition (–61 × 10^6/L; P<0.05). Selection for litter size at weaning (V females) reduced the average counts of total and B lymphocytes in blood (–502 and –60 × 10^6/L, respectively; P<0.01), mainly because these populations in V36 females continuously lowered from the first to the second parturition under normal housing conditions. Thus, more selected females (V36) at the second parturition showed significantly lower counts in blood for total, T CD5$^+$ and CD25$^+$ lymphocytes (–1303, –446 and –33 × 10^6/L, respectively; P<0.05). The main differences in blood counts between V36 and V16 females disappeared when housed under heat stress conditions, except for T CD5$^+$ and CD25$^+$, which significantly increased (T CD5$^+$: +428× 10^6/L; CD25$^+$: +41× 10^6/L; P<0.01) in the V16 *vs.* V36 females on day 10 post-partum. Under normal conditions, no differences between LP and V36 females were found for most lymphocyte populations; only higher counts were noted in CD25$^+$ (+20 × 10^6/L; P<0.05) for LP females. However, the lymphocytes counts [especially total (+1327× 10^6/L; P<0.01) and T CD5$^+$ (+376 × 10^6/L; P<0.10)] of LP females increased under heat *vs.* normal conditions when lymphocytes populations

presented the lowest values (second parturition), while V36 females' counts remained invariable. Positive correlations were found between feed intake (r=+0.51 P<0.001) and females' perirenal fat thickness (r=+0.40; P<0.001) with B lymphocytes counts in the blood of primiparous rabbit females in the week 2 of lactation. These results indicate that selection for litter size at weaning might diminish their immune system's response and adaptation capacity, while the foundation for reproductive longevity criteria leads to more robust rabbit females as they present greater modulation under heat stress conditions when the immune system is affected.

KEYWORDS: rabbit; lymphocyte populations; heat stress; genetic origin; longevity.

INTRODUCTION

In the last three decades, rabbit meat production has evolved from more or less traditional production systems to other more intensive ones due to relevant advances in genetic selection, reproductive management and feeding systems (Pascual, 2010). Genetic selection for reproduction has worked, with programmes resulting in an effective increase of between 0.05 and 0.13 live-born kits per generation of selection (de Rochambeau et al., 1994; Gómez et al., 1996; García and Baselga, 2002a,b). The requirements of reproductive rabbit does have probably increased considerably in recent years, perhaps compromising body condition, lifespan and general health on the farm (Pascual, 2010). In some species, selection for exclusively productive criteria is frequently observed to have some negative associated effects, such as higher disease incidence (Dourmad et al., 1994). In fact, health may be considered one of the main concerns of current rabbit production under commercial conditions, with high replacement rates and the frequent appearance of digestive disorders (Rosell and de la Fuente, 2009).

Long-living animals, which are able to maintain high reproductive performance during successive lactations, are of much interest in animal production as they can help cut the replacement cost of animals and improve animal welfare (Theilgaard et al., 2007). A line (LP) founded for reproductive longevity criteria (an extremely high number of parities and average reproductive performance) was seen to have a longer reproductive life than a well-documented line (the V line) selected during 31 generations solely on litter size at weaning (Sánchez et al., 2008). It has been reported that the LP line delays reproductive senescence and shows less environmental sensitivity than the V line, which might be mediated by greater body energy reserves (Theilgaard et al., 2007).

There is enough evidence for genetic variability to confront heat stress conditions in

85 other species such as pigs (Zumbach et al., 2008) and cattle (Ravagnolo and Misztal,
86 2002). Heat directly affects not only immune system cells (Franci et al., 1996a), but also
87 immunoglobulin and cytokines production (Rodenhiser et al., 1985; Franci et al.,
88 1996b), which might be a concern as far as animals' health is concerned.
89 The aims of this work were to (1) characterise blood lymphocytes and their evolution
90 from the first to the second parturition of rabbit does differing in animal type (by
91 comparing two distant generations of a line selected for litter size at weaning and a line
92 founded with reproductive longevity criteria); (2) to study animals' response to heat
93 stress in terms of animal type.
94

MATERIAL AND METHODS

Animals

A total of 65 female rabbits of two different genetic lines (20 and 45 females from LP and V, respectively) were used, with the participation of females of two generations (16^{th} and 36^{th}) of the V line (23 and 22 females from V16 and V36, respectively). The V line was selected for litter size at weaning using the best linear unbiased prediction (BLUP) as the selection criterion in a single-trait repeatability animal model (Estany et al., 1989; García and Baselga, 2002). The parents of the V16 females were stored as frozen embryos to be thawed and transferred to obtain live adults, which allowed the constitution of the V16 population by reproduction and which were contemporary to the current generation (V36). The LP line was founded according to the longevity and reproductive criteria (selecting females from commercial farms with at least 25 litters and a minimum average litter size of 7.5 live-born kits), as described by Sánchez et al. (2008). Then females were selected by litter size at weaning during six generations (the average prolificacy value in the Spanish commercial rabbit population is approximately nine live-born kits per litter and an average of six parities; Ramón and Rafel, 2002). The Committee of Ethics and Animal Welfare of the Universidad Politécnica de Valencia approved this study. All the animals were handled according to the principles of animal care published by Spanish Royal Decree 1201/2005 (BOE, 2005; BOE = the Official Spanish State Gazette).

Experimental procedure

From 63 days of age until the first parturition, all the female rabbits were housed in conventional housing (with a light-alternating cycle of 16 hours of light and 8 hours of

120 darkness under controlled environmental conditions: average daily minimum and
121 maximum temperatures of 14ºC and 20ºC, respectively), using individual cages
122 (700×500×320 mm) provided with a nest for litters from gestation day 28. After the first
123 parturition, the animals from the three animal types (LP, V16 and V36) were randomly
124 distributed into two different experimental housing systems: CH, where 33 females (10,
125 11 and 12 from LP, V16 and V36, respectively) were maintained in conventional
126 housing at the average daily minimum and maximum temperatures of 14ºC and 20ºC,
127 respectively; CC, where 32 females (10, 12 and 10 from LP, V16 and V36, respectively)
128 and their litters were housed in a climatic chamber and were maintained with a
129 sinusoidal daily curve from 25ºC to 36ºC. Litter size was standardised to 9 and 10 kits
130 at the first and second parturition, respectively, in both environments.
131 The climatic chamber was equipped with a heating/cooling system which scheduled a
132 sine function for the daily environmental temperature, with a minimum temperature of
133 25ºC early in the morning and a maximum one of 36ºC in the afternoon. This system
134 ensured environmental stress with a temperature up to 28ºC for 65% of the day (see the
135 technical details in García-Diego et al., 2011). Briefly, the indoor microclimate was
136 monitored by three probes located 30 cm above the animal cages. Another probe was
137 located outside the farm. Each probe contained a 1-wire protocol integrated circuit
138 (model DS2438, Maxim Integrated Products, Inc.), incorporating a temperature sensor.
139 This integrated circuit was designed for the on-chip measurements of battery
140 temperatures and voltages. Probes were calibrated before being installed, as described in
141 a previous study (García-Diego and Zarzo, 2010). Data were saved at a frequency of
142 one datum per minute.
143 Until the first parturition, all the females received a rearing diet *ad libitum* (9 MJ of
144 digestible energy (DE) and 133 g of digestible protein (DP) per kg, dry matter (DM)).

From this time onwards, females and their litters were fed the same diet as lactating rabbit does (11.5 MJ DE and 120 g DP per kg DM), which was provided *ad libitum* until the end of the experiment (second parturition). Does were artificially inseminated (AI) on day 11 post-partum (dpp) and successive inseminations were carried out every 21 days, when necessary. Litters were standardised at birth to 9-10 kits and weaned on 28 dpp.

To evaluate the possible correlation between the energy balance and the immunological status of females, daily feed intake (DFI) during lactation week 2 and perirenal fat thickness (PFT) on 14 dpp were controlled by ultrasound (Pascual et al., 2000) given the recovery of body reserves in rabbits in the first part of lactation (Quevedo et al., 2006). Thus, feed intake and body reserves during lactation week 2 were expected to be crucial for female rabbits (Theilgaard et al., 2006).

Blood samples were taken from females at the first parturition (at the start of the environmental challenge) on 4 dpp (after a short exposure to the environmental challenge), on 10 dpp (close AI and maximum body condition during lactation), and at the second parturition (end of the experiment). All the blood samples were drawn from the median artery of the ear using vacuum tubes with EDTA. Diurnal variations in haematological parameters were minimised by collecting blood at approximately the same time (9:00–11:00 h).

Flow cytometry analysis

A flow cytometry analysis was carried out as previously described (Guerrero et al., 2011). Blood samples were processed 1 h after sampling. Before performing the flow cytometry studies, a white blood cells (WBC) count and the percentage of lymphocytes were determined with a haematology analyzer (MEK-6410, Nihon Kohden, Japan).

After mixing by inverting the tube, 50 µL of whole blood were pipetted into flow cytometry tubes and primary monoclonal antibodies (**Table 1**) were added, following the manufacturer's recommendations, and incubated for 15 min at room temperature in the dark. WBC were isolated by lysing erythrocytes by adding 1 ml of ammonium chloride lysing solution (8.02 g NH_4Cl, 0.84 g $NaHCO_3$ and 0.37 g EDTA per litre of Millipore water) at 4ºC. After incubating for 5 min in the dark, samples were centrifuged at 400×g for 5 min at room temperature, the supernatant was carefully eliminated and the pellet was washed with 1 ml of phosphate-buffered saline (PBS). After another wash, secondary antibodies (rat anti-mouse IgG2a + b Phycoerythrin [VMRD, Inc. α-exalpha] and goat anti-mouse IgM: R-Phycoerythrin-human adsorbed-[AbD Serotec]) were added. These were incubated for 20 min at room temperature in the dark. Finally, 1 ml of PBS was added before running the flow cytometer. The resulting WBC suspensions were analysed in a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA). Specific data acquisition protocols for rabbit WBC were designed using the CXP software (Beckman Coulter, Brea, CA). The common leukocyte antigen CD14 and the CD45 expression were used for the "lymphogate" setup, as previously described (Jeklova et al., 2007). The gates of each leukocyte type were adjusted with an isotype negative control. All the samples were processed in duplicate. The total lymphocyte count was calculated as the product of the WBC count and the lymphocyte percentage, the lymphocyte subset counts and percentages, as described by Hulstaert et al. (1994).

Ultrasound measurements

The PFT of does was measured on 14 dpp by ultrasounds. Previously, fur was removed from the thoracic and lumbar vertebrae areas by shearing to improve image retrieval.

Animals were placed in an immobilising box (150 mm × 370 mm × 150 mm) while ultrasound measurements were taken and ultrasound gel was applied to the scanning area. The probe was always placed in the same position to obtain a repeatable transversal section of perirenal fat at 3 cm in front of the space between the second and third lumbar vertebrae. Images were obtained with an ultrasound unit (JustVision 200 'SSA-320A' real-time machine; Toshiba; Medical Systems Co., Ltd, Tokyo, Japan) equipped with a micro-convex electronic transducer of multi-frequency (5.0, 6.0 and 7.0 MHz; PVG-681S) and an image analyser software to determine distances. The average of the left- and right-side PFT was used for further calculations.

Statistical analysis

Data about lymphocyte populations in the blood of the rabbit does at first parturition were analysed using a general linear model (PROC GLM; Statistical Analysis System, 2002), with a model including only the animal type as fixed effect. To analyse the evolution of the lymphocyte populations in the blood of rabbit does after the first parturition, a mixed model (PROC MIXED; Statistical Analysis System, 2002) was used according to a repeated measures design, which takes into account the variation between animals and the covariation within them. Covariance structures were objectively compared using the most severe criteria (Schwarz Bayesian criterion), as suggested by Littell et al. (1998). The model included the animal type (AT: LP, V16 and V36), housing (H: CC or CH), the control day (D: first parturition, 4 dpp, 10 dpp and second parturition) and their interactions as fixed effects. The data from the control at the first partum were used as covariates within genetic lines (X_{ijklm}), where β was the regression of Y on the covariate. The random terms in the model included a permanent effect of each animal (p) nested to animal type and housing, and the error term (e).

220 $y_{ijklm} = AT_i + H_j + D_k + AT_i*H_j + AT_i*D_k + H_j*D_k + AT_i*H_j*D_k + \beta X_{ijklm} + p_l + e_{ijklm}$

221 Finally, in order to test the relationship between the lymphocyte populations of rabbit

222 does on 10 dpp with both feed intake during lactation week 2 and PFT on 14 dpp of

223 females, Pearson's correlation coefficients (ρ) were obtained using PROC CORR of the

224 Statistical Analysis System (2002).

225

RESULTS

Table 2 shows the effect of animal type and housing on the lymphocytes populations. As many interactions between the main factors were found, a three-way interaction is represented in Figures 1 and 2.

As seen in **Table 2**, housing under heat stress conditions resulted in only a significant reduction of the B lymphocytes counts in female rabbits ($-34 \pm 14 \times 10^6$/L; $P<0.05$). On the other hand, the blood counts of the majority of lymphocytes populations (total, T $CD5^+$, $CD4^+$ and $CD8^+$) showed the highest values at the first parturition (on average $+443$, $+252$, $+113$ and $+73 \times 10^6$/L if compared to the remaining control days; $P<0.01$), while the B lymphocytes count was significantly lower at the second parturition ($-61 \pm 16 \times 10^6$/L; $P<0.05$).

The V36 population rabbit does presented a lower number of total lymphocytes (**Table 2**) than those of the V16 population ($-502 \pm 173 \times 10^6$/L; $P<0.01$) and the LP line ($-349 \pm 172 \times 10^6$/L; $P<0.05$). This scenario relates mainly to a drop in this cellular population in the V36 females housed in CH on 10 dpp and at the second parturition, when differences between V36 and V16 females were significant (**Figure 1a**). In LP females, the number of total lymphocytes lowered from the first parturition to 4 dpp ($-1205 \pm 421 \times 10^6$ /L, $P<0.001$; **Figure 1a**). If compared to CH, the total counts at the second parturition in CC significantly increased for LP females ($+947 \pm 476 \times 10^6$/L; $P<0.05$), but lowered for V16 females ($-808 \pm 403 \times 10^6$/L; $P<0.05$). The difference between LP and V36 females reached the level of significance (**Figure 1a**).

The V36 population females presented lower B lymphocytes counts than the V16 ones ($-60 \pm 15 \times 10^6$/L; $P<0.001$), while LP females showed intermediate counts (**Table 2**). These results relate mainly with the high counts recorded on 4 dpp and 10 dpp for V16 in CH, which were not detected in CC because of the significant reduction noted (-122

and $-129 \pm 39 \times 10^6$/L on 4 dpp and 10 dpp, respectively, P<0.01; **Figure 1b**). At the second parturition in CC, the LP line animals displayed higher counts than those of the V line (not statistically significant, on average $+46 \pm 40 \times 10^6$/L; P>0.10). A positive relationship was found between the B lymphocytes in blood on 10 dpp with feed intake during lactation week 2 (r = +0.51; P<0.001) and PFT on 14 dpp (r = +0.40; P<0.001). In CH, the T $CD5^+$ lymphocytes counts followed a similar pattern to those of total lymphocytes, showing higher counts for V16 females than those for LP and V36 females at the second parturition (on average +30%, P<0.05; **Figure 1c**). As described for total lymphocytes, the counts recorded at the second parturition in CC, if compared to CH, were higher for LP females, but lower for V16 females; however, differences were not statistically significant (LP line: $+355 \pm 204 \times 10^6$ /L; P<0.10; V16: $-295 \pm 189 \times 10^6$ /L; P>0.10). Similarly to the observations made for B lymphocytes at the second parturition in CC, the LP line animals had higher counts than those from both the V line populations (not statistically significant; on average $+260 \pm 194 \times 10^6$ /L; P>0.10).

The counts of lymphocytes $CD4^+$ and $CD8^+$ fitted the pattern described for the T $CD5^+$ lymphocytes and for the changes relating to animal type or housing (**Figure 2 a, b**). However, the $CD25^+$ lymphocytes counts followed a dissimilar pattern because they were higher in LP females than in V36 females (average $+20.98 \pm 5.80 \times 10^6$ /L; P<0.001; **Table 2**). This was due mainly to the differences noted in CH from 4 dpp onwards (**Figure 2c**). When compared to CH, a significant increase in the $CD25^+$ counts was detected in CC for V16 females on 10 dpp ($+47.6 \pm 15.7 \times 10^6$ /L; P<0.01).

DISCUSSION

Although rabbits have been traditionally used as experimental models for many years, data about their blood lymphocyte populations are scarce. This lack of information is more evident in commercial rabbits (Guerrero et al., 2011) where it is vital to characterise and to evaluate rabbit genetic lines and their responses to different challenges, like heat stress, which is the case in this study.

As reported in a previous study (Guerrero et al., 2011), where a similar methodology was used, several imbalances appear in the results obtained in the present study: (1) the sum of the CD5+ and B lymphocyte percentages is lower than 100 and (2) the sum of the CD4+ and CD8+ lymphocytes is lower than the number of T CD5+ lymphocytes. These discrepancies with previous studies (Jeklova et al., 2007) may relate to the type of animals studied (older commercial rabbit does under stressful conditions) and the antibodies used (Guerrero et al., 2011).

By genetic selection, animals with different useful immunological characteristics in humoral or cellular immune responses can be obtained (Lavi et al., 2005). Differences in the number of lymphocytes have been found between genetic lines of chickens (Cheeseman et al., 2004) and between breeds of pigs (Clapperton et al., 2005). Moreover, it has been proposed that these differences may be implied in resistance to infection by a wide range of pathogens and subsequent disease effects. However, as far as the authors are aware, such information is not available for rabbits. As previously reported (Wells et al., 1999; Kim et al., 2002; Guerrero et al., 2011), the lymphocyte populations in the current study varied throughout the rabbit does' productive cycle, with differences found among the animal types involved.

Thus under conventional housing conditions, V36 females showed lower counts at the second parturition than V16 females for all the studied lymphocyte populations, with

300 significant differences found for total, T $CD5^+$ and CD25, while differences in the B-
301 lymphocytes were significant earlier (on 4 dpp and 10 dpp). We hypothesise that
302 selection for litter size at weaning might have some negative effect on the immune
303 function. In this sense, PFT on 4 dpp was slightly lower in V36 females if compared
304 with V16 or LP females (–0.25 mm, $P<0.10$) and a positive correlation was observed
305 between PFT on 14 dpp and the B lymphocyte counts on 10 dpp. In a previous work
306 done with females of the V line, Theilgaard et al. (2007) observed a higher risk of
307 culling for rabbit does with a low fatness level on 10 dpp. However, litter size selection
308 at weaning during 12 generations did not affect the risk of culling animals (Theilgaard
309 et al, 2006), but even increased the depth of PFT at 3 months of age in the more selected
310 animals (Quevedo et al., 2005). Risk of culling in rabbit does peaks during the two first
311 lactations, especially at the end of pregnancy (Rosell and de la Fuente, 2009);
312 consequently, the possible relationship of the differences found in lymphocyte counts
313 and the culling rate (through illness or death) due to litter size selection at weaning
314 deserves further research.
315 LP females showed similar counts to V36 females during the study period, although a
316 sharp drop in the total, $CD5^+$, $CD4^+$ and $CD8^+$ lymphocytes was observed from
317 parturition to 4 dpp in LP females, but not in V36 females; besides, the CD25+ counts
318 were higher for LP females than for V36 females on 4 dpp, 10 dpp and at the second
319 parturition. A higher level of T-activated cells may evidence a stronger robustness of LP
320 females as opposed to V36 females. Previous studies have demonstrated major
321 robustness for LP rabbit does if compared with the animals from the 31^{st} generation of
322 the V line; this has been related to a more efficient utilisation of their body reserves to
323 successfully confront environmental (heat stress and feed restriction) or productive
324 challenges (Theilgaard et al., 2007). Guerrero et al. (2011) reported that rabbit does with

less physiological wear due to shorter lactations may be more capable of modifying their number of lymphocytes throughout the productive cycle in a less body condition-dependent way. Thus, the differences observed in the current study may relate with the immune system being more capable of adapting to the productive cycle in LP than in V36 rabbit does under normal favourable conditions.

On the other hand, immune cells of different animal species are affected by high temperatures. Thus there have been reports of a fall in splenic NK cell activity in mice (Won and Lin, 1995), a smaller number of lymphocytes in the spleen, mesenteric and peripheral lymph nodes in rats (Krynicki and Olszewski, 1989), and enhanced lymphocyte quantity in the bone marrow of limbs and spine in rats (Krynicki and Olszewski, 1989). Besides, chronic heat stress impairs the expression of contact sensitivity *in vivo* and the proliferation of T lymphocytes *in vitro* in avian species, although the B-cell and T-helper cell functions were not compromised (Regnier and Kelley, 1981). However, Franci et al. (1996a) reported how thermal stress treatments diminished the capacity of rabbits' peripheral blood mononuclear cells to proliferate and inhibit the differentiation of B lymphocytes in antibody-secreting cells, which induce a suppression of either immunoglobulin production or IL-2 synthesis (Franci et al., 1996b). Besides, lymphocytes' resistance to heat stress has been reported to be modified by breed in chickens (Regnier and Kelley, 1981) and bovines (Kamwanja et al., 1994). In the present study, heat stress also differently affected the lymphocyte populations of the compared rabbit does as the differences observed under conventional housing between the V36 and V16 populations (in the B lymphocyte counts on 4 dpp or 10 dpp, and in the total, $CD5^+$ and $CD25^+$ lymphocyte counts at the second parturition) and those observed at the second parturition generally increased to favour LP females if compared to V36 females under heat stress conditions (especially total lymphocyte

counts), except that observed for $CD25^{+}$. So it can be hypothesised that the former finding could be related to V populations being selected in a warm climate (Spanish Mediterranean) and/or to there being no differences in the body condition between V16 and V36 when housed under heat stress conditions. This fact suggests that litter size selection at weaning may not affect females' immune function under heat stress conditions. This last finding might contribute to the above-mentioned robustness of LP line rabbits if compared to V populations, resulting in a lower risk of culling and longer productive life (Sánchez et al., 2008).

In conclusion, these results indicate that, under conventional housing conditions, litter size selection at weaning for 20 generations may affect the immune system since V36 animals had lower lymphocyte counts than V16 animals at a very critical time (e.g., the second parturition), whereas under heat stress conditions, the animals from a line founded by screening for reproductive longevity (the LP line) presented higher lymphocyte counts at this particular stage than those from V36. This scenario could contribute to a greater ability to confront infectious challenges and to confer animals a more robust nature.

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476 **FIGURE CAPTIONS**

477 **Figure 1.** Effect of animal type (LP □, V16 ▪ and V36 ■) on the evolution of (a) Total
478 lymphocytes, (b) Lymphocytes B and (c) Lymphocytes T $CD5^+$ ($\times 10^6$/L) in the
479 peripheral blood of rabbit does when housed in a conventional environment [upper
480 figures] or in a climatic chamber under heat stress conditions [lower figures]. LP, the
481 line constituted by hyperlongevity and reproductive criteria selection; V16 and V36, the
482 populations selected for litter size at weaning for 16 and 36 generations. [a,b,c] The means
483 for each environment, which do not share a superscript in the same figure, were
484 significantly different (P<0.05). Error bars correspond to the standard error for each
485 least square mean.

486

487 **Figure 2.** Effect of animal type (LP □, V16 ▪ and V36 ■) on the evolution of
488 lymphocytes (a) $CD4^+$, (b) $CD8^+$ and (c) $CD25^+$ ($\times 10^6$/L) in the peripheral blood of
489 rabbit does when housed in a conventional environment [upper figures] or in a climatic
490 chamber under heat stress conditions [lower figures]. LP, the line constituted by
491 hyperlongevity and reproductive criteria selection; V16 and V36, the populations
492 selected for litter size at weaning for 16 and 36 generations. [a,b,c] The means for each
493 environment, which do not share a superscript in the same figure, were significantly
494 different (P<0.05). Error bars correspond to the standard error for each least square
495 mean.

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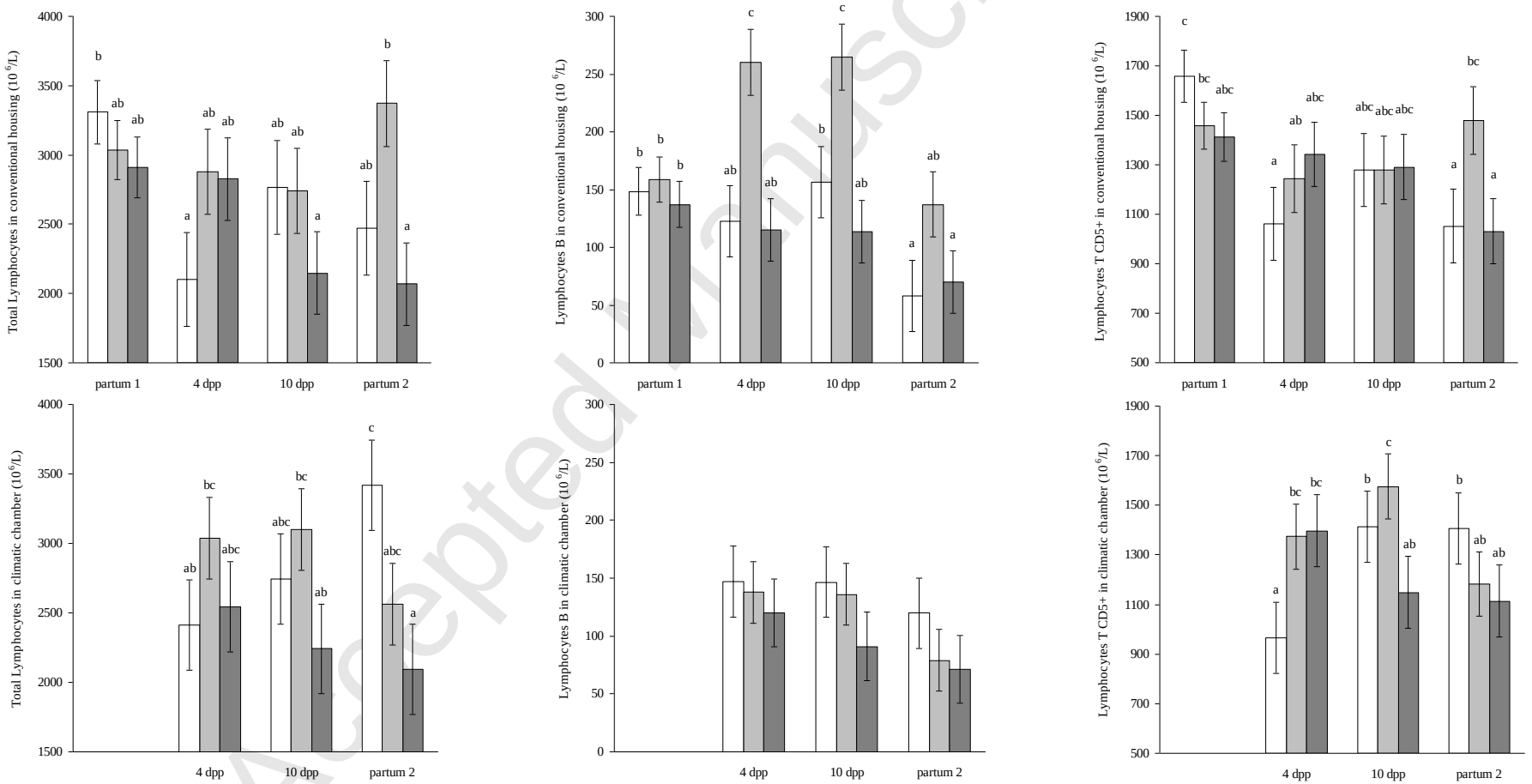

(a) (b) (c)

Figure 1. Effect of animal type (LP □, V16 ■ and V36 ■) on the evolution of (a) Total lymphocytes, (b) Lymphocytes B and (c) Lymphocytes T CD5$^+$ ($\times 10^6$/L) in the peripheral blood of rabbit does when housed in a conventional environment [upper figures] or in a climatic chamber under heat stress conditions [lower figures]. LP, the line constituted by hyperlongevity and reproductive criteria selection; V16 and V36, the populations selected for litter size at weaning for 16 and 36 generations. ^{a,b,c} The means for each environment, which do not share a superscript in the same figure were significantly different (P<0.05). Error bars correspond to the standard error for each least square mean.

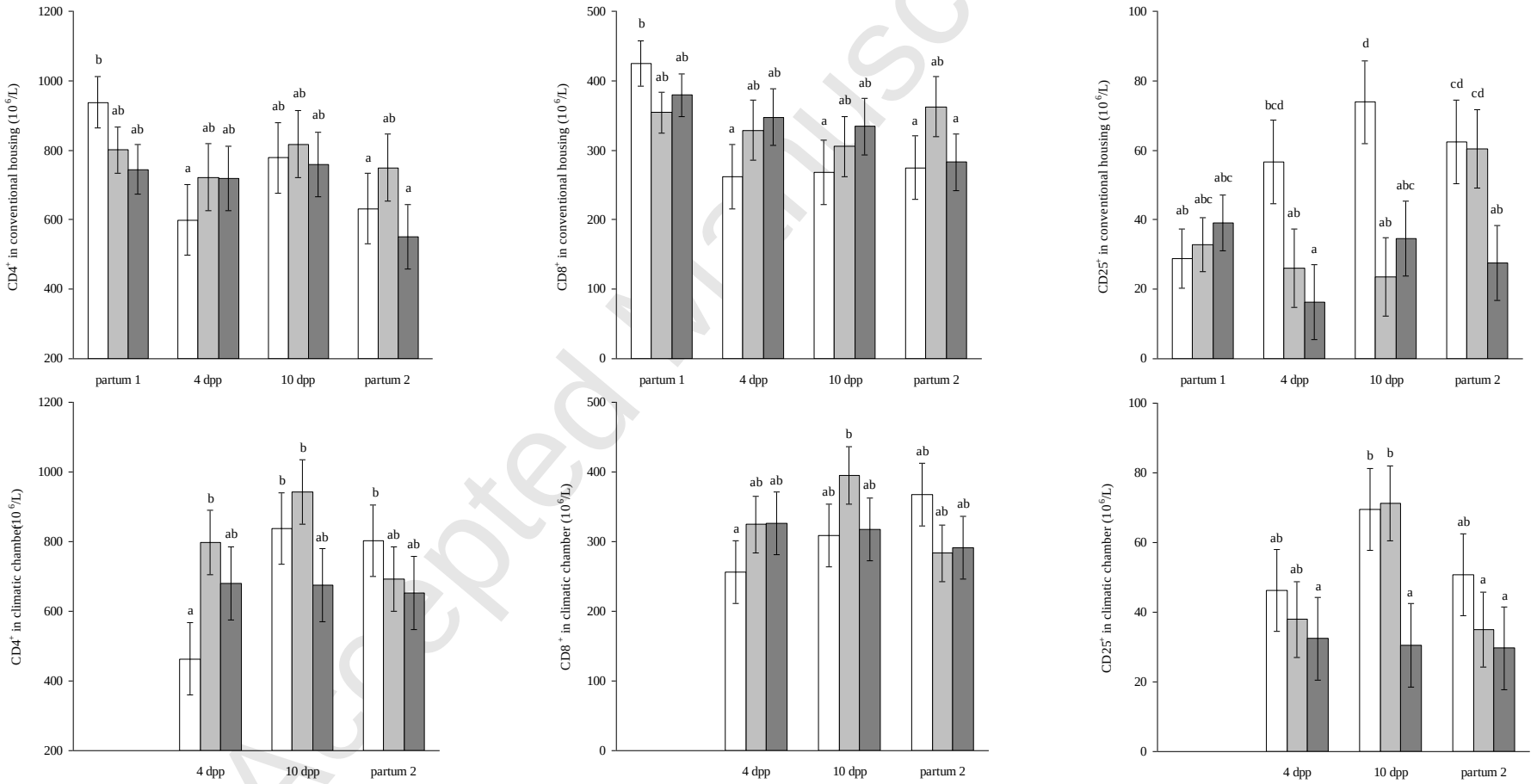

Figure 2. Effect of animal type (LP □, V16 ■ and V36 ■) on the evolution of lymphocytes (a) $CD4^+$, (b) $CD8^+$ and (c) $CD25^+$ ($\times 10^6$/L) in the peripheral blood of rabbit does when housed in a conventional environment [upper figures] or in climatic chamber under heat stress conditions [lower figures]. LP, the line constituted by hyperlongevity and reproductive criteria selection; V16 and V36, the populations selected for litter size at weaning for 16 and 36 generations. ^{a,b,c} The means for each environment, which do not share a superscript in the same figure were significantly different ($P<0.05$). Error bars correspond to the standard error for each least square mean.

Table 1. The monoclonal antibodies used in this study.

Monoclonal antibodies	Isotype	Specificity	Cell labeling	Clone	References	Company
Mouse anti-rabbit T lymphocytes: FITC[a]	IgG1	CD5	T cell	KEN-5	Kotani et al., 1993	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., 1993	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., 1993	Abd Serotec
Mouse anti-human CD14: FITC	IgG2a	CD14	Monocytes and granulocytes	TÜK4	Jacobsen et al., 1993	Abd Serotec
Mouse anti-rabbit α- CD45	IgM	CD45	All leukocytes	ISC76A	Davis and Hamilton, 2008	VMRD, Inc.

[a] 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 datasheet).

Table 2. Effect of animal type and housing of rabbit does on the lymphocyte populations (10^6/L) in peripheral blood from the first to the second parturition.

	Genetic type (G)[1]			Housing (H)[2]		P-value						
	LP	V16	V36	CC	CH	G	H	D[3]	G×H	G×D	H×D	G×H×D
Total Lymphocytes ($CD45^+$ $CD14^-$)	2816 ± 133[b]	2969 ± 119[b]	2467 ± 125[a]	2754 ± 102	2748 ± 106	0.3555	0.9701	0.0240	0.9242	0.0240	0.7180	0.0388
B Lymphocytes	130.8 ± 10.9[ab]	166.5 ± 10.4[b]	106.9 ± 10.6[a]	117.7 ± 9.2	151.7 ± 9.2	0.0536	0.0157	0.0002	0.0222	0.4269	0.2581	0.0212
$CD5^+$ T Lymphocytes	1312 ± 67	1381 ± 57	1268 ± 61	1334 ± 49	1306 ± 49	0.6851	0.6824	0.0001	0.8511	0.0074	0.7905	0.1058
$CD4^+$	748.5 ± 49.2	790.1 ± 41.7	690.5 ± 46.7	751.9 ± 37.4	734.2 ± 35.6	0.8860	0.7244	0.0001	0.8349	0.0271	0.8050	0.5792
$CD8^+$	323.4 ± 18.5	338.5 ± 16.9	332.3 ± 16.8	334.3 ± 14.0	328.6 ± 14.3	0.2685	0.7751	0.0034	0.8106	0.1566	0.7129	0.3335
$CD25^+$	52.15 ± 4.24[b]	40.02 ± 3.76[ab]	31.17 ± 3.96[a]	43.46 ± 3.29	38.77 ± 3.25	0.2962	0.3179	0.0377	0.3105	0.0859	0.2293	0.2647

[1]Animal type: LP, line constituted by selection for hyperlongevity and reproductive criteria; V16 and V36, populations selected for litter size at weaning for 16 and 36 generations.
[2]Housing: CC, climatic chamber; CH, conventional housing.
[3]D: control day (4 and 10 days post first partum and second partum).
[a,b,c] Means in a same row not sharing superscript were significant different (P<0.05) for animal type.