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EFFECT OF RESTRICTED FEEDING UNDER REARING ON REPRODUCTION, BODY CONDITION AND BLOOD METABOLITES OF RABBIT DOES SELECTED FOR GROWTH RATE

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Abstract: Young rabbit females selected for growth rate can have nutritional needs which may not be met by the common practice of feed restriction during rearing in commercial rabbit production. The aim of this study was to analyse the effect of two different feeding programmes: restricted and *ad libitum* feeding, applied in young rabbit females for one month at the end of rearing, on reproductive performance, body condition and circulating metabolic hormones and metabolites in a rabbit line selected by growth rate in 3 consecutive reproductive cycles. Thus, twenty-four 16-week-old does were randomly assigned to a group in which the daily recommended nutrient intakes were satisfied (fed restricted: 130 g/day, n=13) or a group fed to satiety (*ad libitum*: 235.5 g/day, n=11) during one month. Then, all does were inseminated in 3 consecutive cycles using a 42-day reproductive cycle. Measurements of does' body weight, perirenal fat thickness and plasma leptin, non-esterified-fatty-acids (NEFA), beta-hydroxybutyrate (BOHB) and fructosamine were performed at artificial insemination (AI), parturition and weaning time in 3 consecutive cycles. Reproductive performance of does was evaluated based on fertility, litter size at parturition, prolificacy and productivity. Differences in body weight were found only in the 1st cycle, *ad libitum* fed females being heavier than restricted ones. Nevertheless, body weight variances disappeared in later cycles. No differences were found in perirenal fat thickness. Finally, in *ad libitum* fed females slight differences were found in plasma levels of NEFAs (452 *vs.* 258 µekv/L and 527 *vs.* 306 µekv/L for 1st and 2nd cycles) and BOHB (0.26 *vs.* 0.03 mM for 2nd cycle), but disappeared in the 3rd reproductive cycle. Fertility, prolificacy and productivity was not significantly affected by the feeding programme. Nevertheless, total litter size showed to be higher in *ad libitum* fed females at second parturition (8.7 *vs.* 5.9 kits). Therefore, the evaluated feeding programmes until first AI in females selected by growth rate had no effect on their reproductive outcomes, as the global reproductive performance was not affected.

Key Words: feeding regimen, metabolic status, reproductive performance, rabbit doe, growth rate.

INTRODUCTION

Paternal rabbit lines are selected for post-weaning daily weight gain (Estany *et al.,* 1992). These lines are essential in rabbit production as terminal sires in the three-way cross scheme in order to obtain crossbred animals with both reproductive and growth traits to produce more rabbits with fast growth and high feed efficiency (Baselga, 2004). Despite the relevance of growth rate, the efficiency and profitability of rabbit meat production depends on reproductive success, defined by fertility and litter size (Piles *et al.,* 2005). Unfortunately, long-term selection for traits affecting growth rate can result in physiological, immunological and/or reproductive problems (Rauw *et al.,* 1998). Piles and Tusell (2011) founded that the genetic correlation between average daily weight and reproductive traits in female is negative but low in magnitude: selection for growth traits could have a slightly negative effect on fertility of paternal lines. However, other studies have shown that ovulation rate and litter size increase with the body weight of the doe in different breeds of rabbits ranging from 1.2 to 6 kg (Bünger *et al.,* 2005), which suggests a positive relationship between body weight and litter size (Mgheni and Christensen, 1985; Camacho and Baselga, 1990). Therefore,

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genetic correlations between growth and reproductive traits in rabbits are contradictory, perhaps due to the genetic line and the reproductive trait evaluated (Camacho and Baselga, 1990; Rochambeau *et al.,* 1994; Gómez *et al.,* 1998; Garreau *et al.,* 2000; García and Baselga, 2002).

Line R is a rabbit synthetic line created in 1990 selected by individual selection criterion of 28 to 63-day daily weight gain from weaning to slaughter (Estany *et al.,* 1992). The main objective was to improve the feed efficiency, because of its negative and important genetic correlation between growth rate and conversion index (Blasco, 1989). After 36 generations, line R has shown severe reproductive problems such as ovulation failure, implantation embryo failure and lower litter size, related with higher implantation, gestational and foetal losses (Vicente *et al.,* 2012; Naturil-Alfonso *et al.,* 2015).

The reproductive performance of animals could be affected by their feeding regimen and consequently, their nutritional balance (Wolfenson and Blum, 1988; Fortun-Lamothe, 2006). Several reports have established that an altered maternal nutritional regimen prior to mating and during pre-implantation development can influence follicular/oocyte characteristics and embryo development (Edwards and McMillen, 2002; MacLaughlin *et al.,* 2005; Watkins *et al.,* 2008; Picone *et al.,* 2011; Daoud *et al.,* 2012). In this sense, a restrictive diet during rearing is used to control body condition and prevent the negative effects of over-fattening on fertility and foetal losses (Rommers *et al.,* 2004). However, an *ad libitum* diet affords does better sexual development, in terms of receptivity, ovulation rate, blastocyst size and implantation rate, even containing more fat (Ashworth *et al.,* 1999a,b; Rommers *et al.,* 2004). Thus, our hypothesis was that the nutritional regimen is not enough to cope with the needs during reproduction of these females, causing a long-term nutrient deficiency which leads to the subsequent reproductive problems. Needs recommended for the maintenance diet of adult rabbits were established from the equation proposed by Xiccato and Trocino (2010): 340 kJ d^{-1} kg^{-1} live $weight^{0.75}$. With an average body weight of 5 kg, the nutritional requirements were established as 130 g/d with a commercial rabbit diet on dry matter (DM) basis: 17.5% crude protein (CP), 3.5% ether extract (EE), 16.7% crude fibre (CF), and 2938 kcal digesible energy (DE)/kg. Our hypothesis was that these young females with 36 generations of selection for growth rate, when subjected to a restrictive regime to avoid over-thickening, could not cope with their needs at first conception, causing long-term nutrient deficiency which leads to the subsequent reproductive problems characteristics of this synthetic line.

The aim of this study was to evaluate the influence of two feeding programmes (restricted and *ad libitum*) applied in young rabbit females for one month at the end of rearing on reproductive performance, body condition and circulating metabolic hormones and metabolites in a rabbit line selected by growth rate.

MATERIAL AND METHODS

Ethical statement

The experiment was performed in accordance with the principles of animal care published by Spanish Royal Decree 53/2013 (BOE 2013). The animal studies were approved by the Ethics and Animal Welfare Committee of the Universidad Politécnica de Valencia (procedure 2015/vsc/00061). Researchers involved in the work with the animals held an animal experimentation licence issued by the Spanish authorities.

Animals

The animals used came from a synthetic rabbit line selected for growth rate between days 28-63, for 36 generations at the experimental farm from the Universitat Politècnica de València (Estany *et al.,* 1992). Twenty-four females were housed individually at 12 wk of age, with free access to water, under a 16-h light/8-h dark photoperiod unless stated below.

Feeding

Rabbits were fed with a commercial rabbit diet (on DM basis: 17.5% CP, 3.5% EE, 16.7% CF, and 2938 kcal DE/kg). Specifically, at 16 wk of age, females were divided into 2 experimental groups: feeding restricted (n=13; provided daily with 130 g/d to comply with energy requirements for maintenance: 340 kJ d^{-1} kg^{-1} live $weight^{0.75}$; Xiccato and

Trocino 2010) or *ad libitum* (n=11; an average feed intake of 235.5 g/d). After 1 mo under these experimental conditions, females were inseminated for the first time at 20 wk of age. The feed intake of *ad libitum* does was determined from 16 to 20 wk of age by weighing the feeder at the beginning and end of each week, and all feed supplies given within a week were recorded. Additionally, when a restricted doe was tested as pregnant, the diet was established as an *ad libitum* regime.

Breeding and reproductive traits

Reproductive performance of does was evaluated for 3 consecutive cycles including the last weaning (about 7 mo after the first artificial insemination [AI]). Only receptive females were inseminated. Receptivity of does was determined observing the vulvar colour and turgescence, considering receptive those with red/purple and swollen vulva. AI was performed with 0.5 mL of fresh heterospermic pool of males from the same line selected with motility criteria and diluted 1:5 with tris-citric-glucose diluent (Viudes-de-Castro and Vicente, 1997). Immediately after insemination, ovulation was induced by an intramuscular injection of 1 µg of Buserelin Acetate (Suprefact, Hoescht Marion Roussel, S.A., Madrid, Spain).

Pregnancy was determined by abdominal palpation on day 14 post-insemination. Five days before parturition, each doe had free access to a nestbox that was put in front of the cage. At parturition, litter size was recorded. Nestboxes were removed 3 wk later and kits were placed in the cage with the doe to stimulate solid-feed intake. Weaning was performed 30 d post-partum. Second and third AI were performed 12 d post-partum (semi-intensive breeding rhythm). Non-receptive and non-pregnant does were inseminated 21 d after the previous one. Non-pregnant females after 2 successive AI were eliminated from the study (3 *ad libitum* and 2 restricted females were discarded).

At the end of the 3 reproductive cycles, the following factors were determined: kindling rate (number of does giving birth/number of inseminations as percentage), prolificacy (total kits born/number of inseminations) and productivity (total kits born per doe/total number of inseminations per doe). Figure 1 represents an schematic diagram of the experimental design.

Body condition

Body weight of rabbit does was determined at AI, parturition and weaning over 3 reproductive cycles. The perirenal fat thickness (PFT) of does was measured by ultrasound to evaluate body condition, as described by Pascual *et al.,*

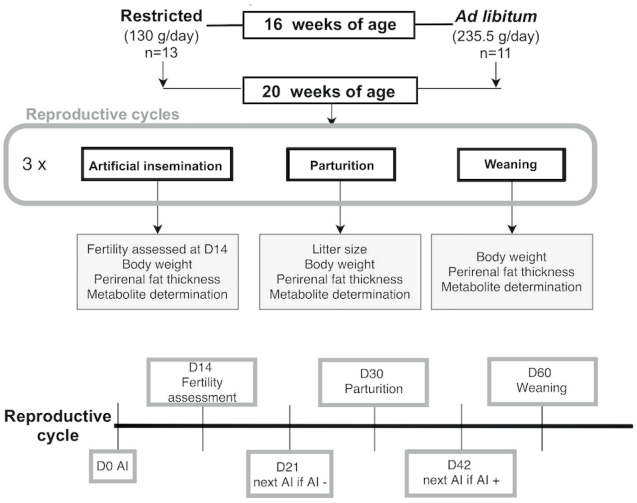

Figure 1: Schematic diagram of the experimental design comparing 2 different feeding programmes (*ad libitum* and restricted) in females during rearing. AI –: pregnancy failure. AI +: pregnancy success. D: day.

(2000a). Briefly, images were obtained with the portable colour Doppler ultrasound device stated above. PFT measures were indirectly obtained using the ultrasound unit software. When measuring the PFT, does were also weighed.

Collection of blood samples

Blood samples were taken at AI, parturition and weaning to determine the profile of non esterified fatty acids (NEFA), leptin, beta-hydroxybutyrate (BOHB: ketone body) and fructosamine concentration. They were obtained from the central ear artery into ethylenediaminetetraacetic acid (EDTA)-coated tubes and immediately centrifuged at 3000× *g* for 10 min, then stored at –20°C until analysis.

Metabolites assay

Leptin was analysed by Multispecies Leptin assay (RIA, XL-85K) (Milipore Corporation, Billerica, MA, USA), according to the manufacturer's guidelines. Intra- and inter-assay coefficient of variation (CV) were 9.1% and 9.3%, respectively. NEFAs were determined using the NEFA C ACS-ACOD assay method (Wako Chemicals GmbH, Neuss, Germany). Serum levels of fructosamine were assessed by a colorimetric assay (Roche Diagnostics). BOHB was determined as an increase in absorbance at 340 nm owing to the production of nicotinamide-adenine dinucleotide (NADH), at slightly alkaline pH in the presence of BOHB dehydrogenase. Sample blanks were included and the oxamic acid was included in the media to inhibit lactate dehydrogenase, as proposed by Harano *et al.,* (1985). Analyses of NEFA and BOHB were performed using an auto-analyser, ADVIA 1650® Chemistry System Siemens Medical Solutions, Tarrytown, NY, USA); in all instances, the intra- and inter-assay CV was below 2% and 4%, for NEFA and BOHB respectively.

All chemicals used in this study were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain) unless stated otherwise.

Statistical analysis

To compare fertility between groups, a general linear model was performed including the type of feeding with 2 levels (*ad libitum* or restricted) as a fixed factor. The error was designated as having a binomial distribution using the probit link function. Binomial data for fertility were assigned a value of one if AI was positive. Failure to inseminate resulted in a score of zero. Prolificacy and productivity differences between groups were analysed with a generalised linear model including the type of feeding with 2 levels (*ad libitum* and restricted) as a fixed effect. These analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002).

The model used to analyse litter size and metabolic data at AI, parturition and weaning in 3 consecutive reproduction cycles was a mixed model (PROC MIXED by Statistical Analysis System, SAS, 2002), in a repeated measure design that took 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 feeding programmes (*ad libitum* or restricted), the moment (AI, parturition or weaning) and their interaction as fixed effects. Random terms in the model included a permanent effect of each animal (p) and the error term (e), both assumed to have an average of zero and variance σ2p and σ2e, respectively. Different contrasts were computed to test the significance of the differences between feeding regimens.

Differences of $P<0.05$ were considered significant. Data are shown as means±standard error means (SEM).

RESULTS

Feed intake and reproductive performance

Females fed *ad libitum* showed significantly increased ingestion of 55% compared to the restricted group (235.5±6.87 g/d *vs.* 130.0 g/d) from 16 to 20 wk of age.

No significant differences were found in kindling rate, prolificacy and productivity between restricted and *ad libitum* females at the end of the 3 reproductive periods (Table 1).

Table 1: Effect of 2 different feeding programmes (*ad libitum* and restricted) in females during rearing (from 16 to 20 wk of age) on kindling rate prolificacy and productivity in 3 consecutives reproductive cycles.

Type	N	Kindling rate (%)	Prolificacy	Productivity
Ad libitum	13	56.0±8.5	7.7±0.54	6.6±1.00
Restricted	11	43.0±7.3	7.2±0.54	5.0±0.94

N: number of females. Kindling rate: number of does giving birth/number of inseminations as percentage. Prolificacy: total kits born/number of inseminations. Productivity: total kits born per doe/total number of inseminations per doe. The absence of different letters on means in the same row means no significant differences between female groups (p >0.05).

Figure 2 shows the effect of the feeding programmes on total litter size of different parturitions. Differences were only detected at second kindling, where *ad libitum* females showed higher litter size than restricted ones (8.6±0.90 *vs.* 6.0±1.14, respectively).

Body condition

Ad libitum females showed to be heavier during almost the entire reproductive cycle at the different 1st, 2nd and 3rd cycles (Figure 3). At the moment of artificial insemination, *ad libitum* does were heavier in the first AI. However, no differences were found either between *ad libitum* and restricted females in 2nd AI or in 3rd AI. Nevertheless, in restricted females, differences were observed from 2nd and 3rd reproductive cycle. In the 2nd AI, restricted females were lighter than restricted females in the 3rd reproductive cycle, which did not occur in the case of *ad libitum* females. In contrast, at third AI body weight was similar between groups. In fact, the differences found in the first reproductive cycle in body weight diminished during the second reproductive cycle until they had already disappeared by the 3rd cycle (Figure 3).

However, those *ad libitum* females with higher body weight at 1st AI were those which did not get pregnant (Table 2).

In contrast, no significant differences were observed for perirenal fat thickness between *ad libitum* and restricted females (Figure 4). When PFT was analysed for *ad libitum* and restricted females at different reproductive cycles and moments (AI, kindling and weaning), significant differences were only found at first AI (9.36±0.29 mm *vs.* 8.7±0.27 mm, for *ad libitum* and restricted females, respectively). Additionally, the reduction in PFT at first partum is higher in *ad libitum* females (–0.68) than in restricted ones (–0.44) (Figure 4). Moreover, differences in PFT fluctuations are also observed in the second cycle, with high differences between AI, parturition and weaning in restricted females, while PFT of *ad libitum* females remained more constant.

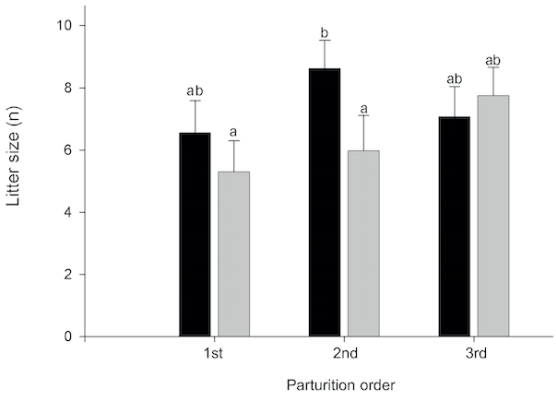

Figure 2: Average litter size at parturition in females fed *ad libitum* (n=11) or restricted (n=13) in 3 consecutives reproductive cycles. Bars show the mean value±standard error between groups. Bars with different superscripts are significantly different ($P<0.05$). ■ *Ad libitum*; ■ Restricted.

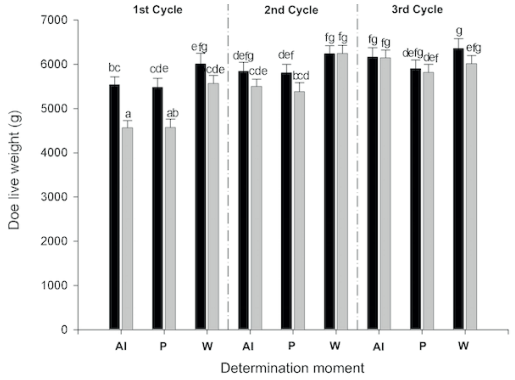

Figure 3: Live weight in females fed *ad libitum* (n=11) or restricted (n=13) in 3 consecutives reproductive cycles determined at successful artificial insemination (AI), parturition (P) and weaning (W). Bars with different superscripts are significantly different ($P<0.05$). ■ *Ad libitum*; ■ Restricted.

Blood metabolites parameters

The plasma profile of leptin, NEFAs, fructosamine and BOHB are shown in Figure 5. When metabolites were analysed for restricted and *ad libitum* females, small differences were found. Circulating leptin levels at 3rd reproductive cycle were shown to be reduced at AI and parturition in restricted females compared with levels from *ad libitum* females (–0.24 ng/mL and –0.31 ng/mL at AI and partum, respectively, Figure 5A).

NEFAs plasma concentrations showed to be different between females from different feeding regimens only at 1st and 2nd parturition. *Ad libitum* females showed higher levels of NEFAs than restricted ones (Figure 5B).

Fructosamine plasma levels are shown in Figure 5C. No differences are found at any time between *ad libitum* and restricted females except at 3rd AI and partum. At 3rd AI *ad libitum* females showed lower levels of plasma fructosamine (269.6±21.0 *vs.* 330.6±17.4 µM, for *ad libitum* and restricted females, respectively). However, at 3rd kindling fructosamine plasma concentration was higher in *ad libitum* females than in restricted ones (241.4±17.9 *vs.* 194.9±18.2 µM). On the other hand, the highest levels of plasma fructosamine for both *ad libitum* and restricted females were detected at 1st AI, while the lowest levels were detected at partum (Figure 5C).

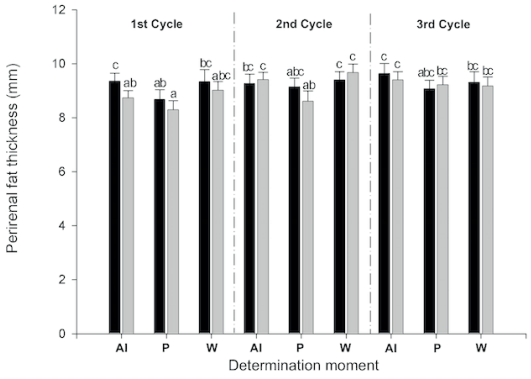

Figure 4: Perirenal fat thickness in females fed *ad libitum* (n=11) or restricted (n=13) in 3 consecutives reproductive cycles determined at successful artificial insemination (AI), parturition (P) and weaning (W). Bars with different superscripts are significantly different ($P<0.05$). ■ *Ad libitum*; ■ Restricted.

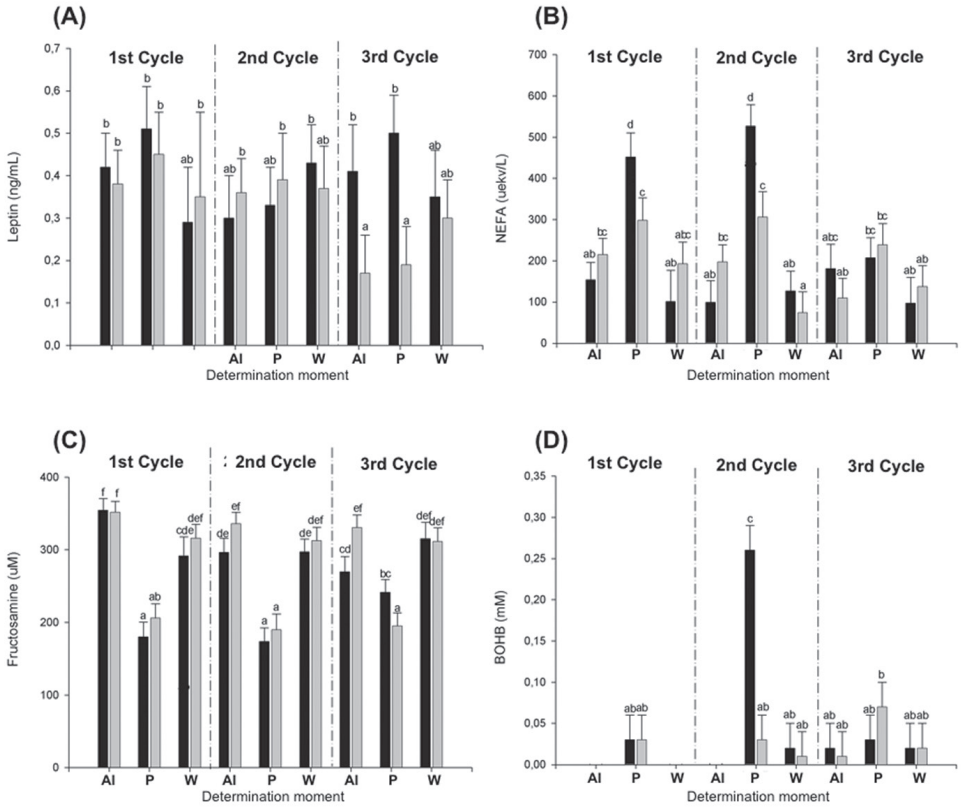

Figure 5: Evolution of blood plasma (A) leptin, (B) non-esterified fatty acids (NEFA), (C) fructosamine and (D) BOHB concentrations in females fed *ad libitum* (n=11) or restricted (n=13) in 3 consecutives reproductive cycles determined at successful artificial insemination (AI), parturition (P) and weaning (W). Bars with different superscripts are significantly different ($P<0.05$). ■ *Ad libitum*; ■ Restricted.

Levels obtained for plasma BOHB concentrations are generally too low for detection. Only at kindling were levels high enough to allow measurements. Specifically, differences were only found at 2nd partum (Figure 5D). At this point, *ad libitum* females showed higher levels of BOHB plasma concentration than restricted does (0.26±0.03 *vs.* 0.03±0.03 mM, respectively).

DISCUSSION

The relationship between nutrition and reproduction has been observed for several decades (Fortun-Lamothe, 2006). Previous studies have demonstrated that the nutritional regime before and after mating influences the profile of circulating concentrations of metabolites and reproductive hormones, such as glucose, NEFA, leptin, insulin and IGF-I, which could determine reproductive performance (Ferguson *et al.,* 2003; Brecchia *et al.,* 2006; García-García *et al.,* 2011). Our results confirm this effect of nutritional regimen on metabolic alterations in different ways, but did not show any effect on reproductive efficiency of R paternal line, except the 2nd kindling.

Our results revealed that *ad libitum* females were heavier throughout the reproductive period studied, as a consequence of the higher feed intake. The higher body weight at first AI remained during the reproductive period, but as Rommers *et al.* (2004) appreciated, the difference was compensated between groups until it finally disappeared

in the 3rd reproductive cycle. Several works have shown that selection for growth traits results in heavier animals with negative responses in reproduction performance (Mgheni and Christensen, 1985; Arias-Álvarez *et al.,* 2009; Gómez *et al.,* 1999; Ragab *et al.,* 2010). Maybe this fact meant that *ad libitum* does with excess body weight at first AI were those which did not get pregnant. However, another possibility is that the selection process influences the maturation process of the generated genotypes, so these animals could present a lower degree of physiological maturation, which would explain at least some of the reproductive failures.

Interestingly, the higher weight of *ad libitum* females is not indicative of overfat females, as no differences in PFT were detected between females fed with different rearing programmes across consecutive reproductive cycles. The only differences in PFT between experimental groups were observed at 1st AI. As previously reported, at 1st AI *ad libitum* females presented higher PFT, probably as a consequence of higher feed intake, but these differences disappeared at 1st kindling (Martínez-Paredes *et al*., 2012). Nevertheless, PFT levels decrease at the 2nd partum in restricted females, indicative of higher pre-partum fat mobilisation, which coincided with a decrease in litter size. This appreciation is contrary to other results in rabbit paternal lines which did not find effects of feed restriction on litter size when analysing until 4th parturition (Verdelhan *et al.,* 2005). Thus, metabolite analysis would help understand whether reduction in litter size at second partum was a direct effect of different feeding regimes or just a chance effect.

The differences in body weight, PFT and litter size in the 2nd reproductive cycle between *ad libitum* and restricted females were also manifested in plasma metabolite concentrations, specifically in NEFAs and BOHB levels. NEFA concentrations at kindling are the highest, indicating high reserve mobilisations due to the body fat transfer to the foetuses during the last week of pregnancy, causing a NEB (negative energy balance) in the mother (Rommers *et al.,* 2004). At 2nd kindling, NEFA plasma concentrations were higher in *ad libitum* females than in restricted ones, which may be related with the higher litter size in the former females (+3.3). The higher number of kits would increase the needs of milk production and consequently present higher body reserve mobilisations (Pascual *et al.,* 2000b; Pascual *et al.,* 2002), which was reflected in higher blood concentrations of NEFAs (Savietto *et al.,* 2014).

Similar to the NEFA results, plasma BOHB concentration were higher in *ad libitum* females than in restricted ones at 2nd partum. These higher levels of BOHB might be indicative of the energy effort of the females to cope with the high energetic needs due to the litter size in this parturition (8.6, when the average of line R females was 6.8). On the other hand, BOHB levels could only be detected at parturitions, when the highest reserve mobilisations take place (Pascual *et al.,* 2013).

In this work, we chose fructosamine as a parameter to evaluate glucose variations, as it is a more stable indicator of short-term glycaemic status, which reflects the average glucose levels for the previous 10-14 d (True, 2009). Although body weight results seemed to indicate a severe restriction in restricted females, results did not reveal any difference in plasma fructosamine concentrations between females from different feeding regimens in the 1st and 2nd reproductive cycles. These results indicated that the restriction was not too severe and restricted females had sufficient energy. However, at 3rd kindling *ad libitum* females had higher levels of blood fructosamine. The higher number of kits in the 2nd kindling might induce a higher feed intake post-weaning due to the higher suckling needs (Pascual *et al.,* 2002), which would in turn be reflected in the increase in blood fructosamine concentration.

The 3rd reproductive cycle is also when differences between groups in plasma leptin concentrations are found. The lower leptin concentration in restricted females might be indicating that restricted females reach 3rd reproductive cycle with lower body energy reserves, which may reflect female depletion. However, no differences were detected either in body weight or in PFT between females from both groups. Thus, lower leptin concentration in 3rd cycle may be a consequence of higher overlapping between gestations and weaning. Moreover, the lower litter size at 2nd partum in restricted females may lead to a lower feed intake due to lower milk production needs, with a consequent drop in leptin levels.

Therefore, few differences were found at metabolic levels between females fed *ad libitum* or restricted from rearing to reproduction, with no significant effects on reproductive performance. Differences were mainly detected in the 2nd reproductive cycle, which seemed to be compensated at later parturitions (3rd).

In our previous study, the feeding regimen showed a significant effect on embryonic quality, increasing the number of kits born when embryos were recovered from an *ad libitum* fed donor female (Naturil-Alfonso *et al.,* 2016). However,

after 36 generations of selection, the nutritional needs of these females for reproduction could have changed. Nevertheless, the different feeding programmes (restricted and *ad libitum*) during the rearing period of young rabbit females selected for growth rate did not induce changes in their productive life performance. On the other hand, the degree of physiological maturation or genotypes might have been modified through the selection process.

In the light of these results, other different strategies should be carried out. Generally, heavy animals with lower productivity need a low metabolic effort. In these animals, metabolic effort is determinant at first gestation, but mainly at second parturition, as in other rabbit lines, when lactation and gestation are overlapped, as can be appreciated in the fluctuations detected in this study (NEFAs and BOHB). Therefore, further studies testing other nutritional and rearing strategies, such as a feeding strategy at these particular times, should be carried out.

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