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STUDY OF FAILURES IN A RABBIT LINE SELECTED FOR GROWTH RATE

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Abstract: Selection for growth rate is negatively related with reproductive fitness. The aim of this work was to analyse the causes of fertility failure in rabbit does selected for growth rate and characterised for reproductive deficiencies (line R). In the experiment, 82 does were divided into 2 groups: naturally mated (NM) and artificially inseminated (AI), to relate luteinizing hormone (LH) concentration with ovulation induction and pregnancy rate by laparoscopic determination. Additionally, in 38 of these females ovulation rate and metabolites determination (leptin, NEFA, BOHB and glucose) were analysed and perirenal fat thickness measurement and live body weight (LBW) determined. The results showed that all ovulated does (both NM and AI) presented higher concentrations of LH than non-ovulated females. In addition, non-ovulated females showed high levels of leptin and BOHB, as well as LBW. Females from line R have an inherit reduced fertility due to ovulation failure as a consequence of a reduction in LH release, which could be explained by a heavier body weight and higher leptin concentrations.

Key Words: ovulation failure, growth rate, LH, mating.

INTRODUCTION

In rabbit, as in other species, selection for growth traits shows negative correlated responses in reproductive performance (Mgheni *et al.,* 1985; Gómez *et al.,* 1999; Ragab and Baselga, 2010). Nevertheless, estimates of genetic correlation in rabbits between the litter size and growth traits are low and contradictory, as the magnitude and sign of the correlations could depend on the correlated reproductive trait, as well as the population or strain under study. In previous studies comparing paternal and maternal lines, differences have been reported in ovulation frequency, ovulation rate, gestational losses and consequently in litter size (Vicente *et al.,* 2003, 2012, 2013). Recent estimations of the effects of inbreeding on litter size exclude it as the factor explaining the differences observed between the paternal and maternal lines (Gadsby *et al.,* 1983). The selection process for the rabbit paternal line showed a response per generation of 0.5 g/d in daily gain from weaning to slaughter and 118% overweight at end of fattening period, specifically 139% in adult females compared to the maternal line (Gómez *et al.,* 1999). Reproductive troubles described in these rabbit parental lines have been reported in many obesity models (Pasquali and Casimirri, 1993; Tortoriello *et al.,* 2004). Body fat excess has shown a negative effect on pulsatile luteinizing hormone (LH) release from the pituitary (Cano *et al.,* 2008; Uenoyama *et al.,* 2008), early embryonic gene expression (Picone *et al.,* 2011), disturbances in pre-implantation development (Sjoblom *et al.,* 2005), reduction in implantation and foetal development (Hendrickse *et al.,* 1985; Haggarty, 2002) and reduced birth weights of the kits (Montoudis *et al.,* 1999; Cordier *et al.,* 2013).

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Rabbit is a reflexively ovulating species in which sensory and neuroendocrine stimuli act together to induce a LH preovulatory surge and determine ovulatory response (Dufy-Barbe *et al.,* 1973; Fisher *et al.,* 2012). Genital somatosensory stimulation is necessary to activate GnRH neurons and generate a preovulatory LH surge from the pituitary gland (Jones *et al.,* 1976; Dal Bosco *et al.,* 2011). Currently, lines selected for growth rate are characterised by a high growth rate and adult live weight. Nevertheless, a reduced reproductive performance (mice, Brien *et al.,* 1984; Eisen *et al.,* 1973; pig, Chen *et al.,* 2003; Kuhlers and Jungst, 1992 and rabbits, Estany *et al.,* 1992; Gómez *et al.,* 1998; Vicente *et al.,* 2012), and also elevated disease incidence, despite having a higher body condition score (Sánchez *et al.,* 2012), have been described.

In this study, we evaluated whether the ovulation induction failures previously observed in a rabbit line selected for growth rate could be due to an inadequate neuroendocrine reflex in the hypothalamic-pituitary system.

MATERIALS AND METHODS

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

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 Committee of Ethics and animal Welfare of the Universitat Politècnica de València. Researchers involved in the work with animals held an animal experimentation licence issued by the Spanish authorities.

Animals

A total of 82 females from a synthetic rabbit line selected for growth rate between weaning and slaughter time (9th wk of life) for 35 generations (line R, Instituto de Ciencia y Tecnología Animal, Spain, Estany *et al.,* 1992) were housed individually, at 12 weeks of age, with free access to water, under a 16 hours light/8 hours dark photoperiod unless stated below. Animals were fed a commercial rabbit diet (on dry matter (DM) basis: 17.5% crude protein, 3.5% ether extract, 16.7% crude fibre, 2938 kcal of digestible energy (DE) per kg of DM).

Determination of LH concentration and ovulation relationship

At the age of 5 mo, receptivity of does was determined observing the vulvar colour and turgescence, considering those with red/purple and swollen vulva receptive. Receptive does were divided into 2 groups: naturally mated (NM, n=50) or artificially inseminated (AI, n=32). All NM females mated successfully. Artificial insemination was performed with 0.5 mL of fresh heterospermic pool selected for motility criteria (more than 70% of motility rate and less than 15% of abnormal sperm) 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, Hoechst Marion Roussel, S.A., Madrid, Spain).

According to the literature, LH determinations were performed taking as average point 90 min after AI/NM (based on Meunier *et al.,* 1983; Muelas *et al.,* 2008; Quintela *et al.,* 2004). Blood samples were taken from the marginal ear vein, placed in EDTA-coated tubes and immediately centrifuged at 2500 rpm for 10 min and stored at –20°C until analysis.

Ovulation and pregnancy were confirmed by laparoscopy after 12 d for both groups. Females were anaesthetised by an intramuscular injection of 16 mg xylazine (Rompun; Bayern AG, Leverkusen, Germany), followed by an intravenous injection of ketamine hydrochloride at the rate of 25 mg/kg body weight (Imalgene 1000; Merial S.A, Lyon, France) to keep does under anaesthesia during laparoscopy. The absence of *corpora lutea* was indicative that the ovulation process did not occur (ovulation failure).

Additionally, in 38 of these does (NM=28 and AI=10) at laparoscopy, the number of *corpora lutea* was recorded per female (assumed as ovulation rate, OR). For the NM group (15 ovulated and 13 non-ovulated), serum blood samples

were taken just after NM or AI to analyse for leptin, non-esterified fat acids (NEFA), beta-hydroxybutyrate (BOHB) and glucose, and the body condition was determined.

Hormone and metabolite analysis

Plasma LH concentrations were determined by a homologous ELISA method validated for rabbits and previously described (Rebollar *et al.,* 2012; Dall'Aglio *et al.,* 2013). Briefly, the RbLH antigen (provided by Dr A.F. Parlow; Pituitary Hormones and Antisera Center, National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, Harbor-University of California Los Angeles Medical Center, CA, USA) was biotinylated with EZ-Link® Biotinylation Kit (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's instructions. EIA microtitre plates (Corning Inc, Corning, NY, USA) were coated with secondary antibody anti-guinea pig IgG (Southern Biotech, Birmingham, AL, USA). Then, the anti RbLH were added into wells. Duplicates of 50 µL of appropriate standards (range 0.781 to 400 ng/mL), buffer (zero standard), plasma samples and assay controls were pipetted into respective wells. Biotinylated-RbLH (EZ-Link® Biotinylation Kit), Streptavidin-peroxidase (Sigma Chemical, St Louis, MO, USA) and the substrate (3, 3', 5, 5'–tetramethylbenzidine, 1-Step Ultra TMB-ELISA; Pierce Biotechnology, Rockford, IL, USA) were added across the entire plate. The lowest concentration of RbLH that could be distinguished from zero concentration was 0.78 ng/mL. The intra-assay coefficient of variation of the analysis was 5.2%. Inter-assay precision calculated by the 9 replicate measurements of coefficient of variation for pools of high and low concentrations was 3.1 and 6.84, respectively. The accuracy of the EIA, determined by measuring the recovery rates of known amounts of RbLH (5, 25, and 125 ng/mL) added to different plasma samples, was 90.0, 96.0, and 88.6% for low, medium and high values, respectively.

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 CV were 9.1 and 9.3%, respectively. Non esterified fatty acids (NEFA) were determined using the NEFA C ACS-ACOD assay method (Wako Chemicals GmbH, Neuss, Germany). Beta-hydroxybutyrate (BOHB) was determined as an increase in absorbance at 340 nm owing to the production of NADH, at 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). Glucose was determined according to standard procedures (Siemens Diagnostics® Clinical Methods for ADVIA 1650). Analyses of NEFA, BOHB and glucose were performed using an auto-analyser, ADVIA 1650® Chemistry System 8Siemens Medical Solutions, Tarrytown, NY, USA); in all instances, the intra- and inter-assay CV was below 2 and 4%, respectively.

Body condition

The perirenal fat thickness (PFT) of does was measured by ultrasound to assess body condition at the moment of NM or AI, as described by Pascual *et al.* (2000). Briefly, images were obtained with an ultrasound unit (Justvision 200 "SAS-320A" real-time sound machine, Toshiba), equipped with an electronic micro-convex transducer of multi-frequency (5.0, 6.0 and 7.0 –MHz; PVG-681S). PFT measures were indirectly obtained using the software of the ultrasound unit. At the moment of PFT measurement, does were also weighed.

Statistical analysis

To compare gestation rate between groups including the fecundation type with 2 levels (AI or NM) as a fixed factor, a general linear model (GLM) was performed, using the probit link function. Binomial data for fertility were assigned a value of 1 if pregnancy was positive. Failure to AI/NM resulted in a score of 0.

The GLM procedure was performed to determine differences in LH concentration related to ovulation and pregnancy, including as fixed effects the type of fecundation (NM or AI), ovulation or pregnancy (positive or negative) and their interaction. Ovulation rate was analysed with a GLM including the type of fecundation (NM or AI) as fixed factors.

All metabolites, body weight and perirenal fat thickness were analysed by a GLM including ovulated or non-ovulated naturally mated females as fixed factors. Correlations between these traits were analysed.

Differences of $P<0.05$ were considered significant. Data are shown as means±standard error means (S.E.M). All analyses were performed with Statgraphics Plus 5.1.

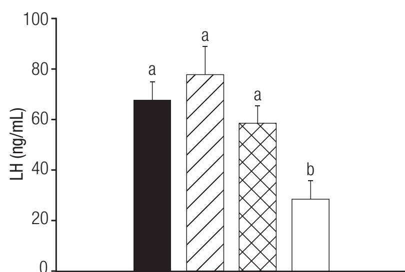

Figure 1: Mean LH concentration 90 min after natural mating or artificial insemination in females, related to ovulation and gestation diagnosis. Bars show the mean value±standard error of mean of the females involved in each group. Bars with different letters are significantly different (P<0.05). ■ Artificial insemination: ovulation & gestation. ▨ Artificial insemination: ovulation & non-gestation. ⊠ Natural mating: ovulation & gestation. □ Natural mating: non-ovulation & non-gestation.

RESULTS

Some 34.3% of the NM females were pregnant, while for AI females the pregnancy rate was 66.7%.

All the AI does ovulated successfully. Four groups were determined: (i) AI: ovulation & gestation; (ii) AI: ovulation & non-gestation; (iii) NM: ovulation & gestation; (iv) NM: non-ovulation & non-gestation; No NM female showed ovulation & non-gestation. The average plasma LH levels in ovulated and non-ovulated females are shown in Figure 1.

The relation between LH concentration and ovulation showed that LH is higher in ovulated females (NM or AI) than in non-ovulated females. Additionally, non-significant differences in LH concentration between NM and AI pregnant females were observed (58.34±7.11 and 67.39±7.57, respectively). The number of CL for ovulated females (OR) was significantly decreased in the NM group compared to the AI group (12.50±0.64 *vs.* 14.90±0.83, respectively; P<0.05).

Table 1 summarises metabolite results between non-ovulated and ovulated NM females. Serum leptin levels in non-ovulated NM females were significantly high compared to ovulated does (0.60±0.09 *vs.* 0.40±0.04 ng/mL, respectively; P<0.05). Similarly, plasma BOHB levels in non-ovulated NM females were significantly higher than in ovulated does (0.10±0.01 *vs.* 0.01±0.01 mM, respectively; P<0.05). In contrast, the presence/absence of ovulation did not affect plasma glucose and NEFA (9.00±0.72 *vs.* 8.20±0.34 mM for glucose and 192.00±43.80 *vs.* 131.00±20.90 µekv/L, for NEFA, respectively). Finally non-ovulated females were heavier than ovulated females (6230±232 g *vs.* 5389±111 g, respectively; P<0.05). Although a positive correlation (0.34) was found between body weight and BOHB levels, no other correlations between body weight and metabolites were found. Nevertheless, no significant differences were observed for perirenal fat thickness (9.70±0.32 *vs.* 9.10±0.15 mm, for non-ovulated and ovulated females, respectively).

DISCUSSION

The female line used in this study (paternal line or Line R) has previously been extensively characterised for reproductive deficiencies (Vicente *et al.,* 2012). Specifically, our previous findings indicate that these females showed a lower ovulation rate when compared with a control line (70 *vs.* 86% for line R and control line, respectively), with higher failures in ovulation induction (30%) (Vicente *et al.,* 2012). These results of ovulation failure seem to be similar to those observed in this work, with a 32% reduction in fertility when females are naturally mated.

Our results showed that LH concentration seems to be related with the ovulation success, as all ovulated females presented higher levels of LH concentration, but additionally NM females (with lower release of LH, although not significant) presented an ovulation rate lower than AI (with higher release of LH). When line R does are induced to

Table 1: Metabolite concentration and body conditions of naturally mated females if ovulated or non-ovulated after mating.

Females		Metabolite				Body condition	
Type	No	Glucose (mM)	BOHB (mM)	NEFAs (µekv/L)	Leptins (ng/mL)	Body weight (g)	PFT (mm)
Ovulated	15	8.20±0.34	0.01±0.01	131.00±20.90	0.40±0.04	5389±111	9.10±0.15
Non-ovulated	13	9.00±0.72	0.10±0.01	192.00±43.80	0.60±0.09	6230±232	9.70±0.32

BD: Body weight; PFT: Perirrenal Fat Thickness.

ovulate with GnRH analogues, no significant failures are observed in ovulation frequency: between 95-100% of AI females treated with a GnRH analogue ovulated (Viudes-de-Castro *et al.,* 1995; Mehaisen *et al.,* 2004; Vicente *et al.,* 2003, 2012). Additionally, the GnRH analogue injected after AI could increase systemic LH blood concentrations and could promote more LH receptors in the follicles than GnRH naturally secreted after natural mating (Pieper *et al.,* 1981). Therefore, these results suggest that problems due to LH secretion or to LH follicular receptors can be discarded. Moreover, Balasubramanian *et al.* (2012) showed that lower LH levels impair ovulation process, causing a lower number of fresh *corpora lutea*, as we detected in this work.

In natural mating, a nervous stimulus is needed for LH release and triggering ovulation in rabbits. The coitus provokes the release of norepinephrine in the hypothalamus, followed by the delivery of GnRH into the portal vessels, which in turn triggers the release of pituitary LH into the peripheral blood stream (Bakker and Baum, 2000). The absence of any of these events decreases LH concentration and consequently affects the ovulation process in rabbit (Rebollar *et al.,* 2012). Hence, ovulation deficiencies seem to be due to reduced LH concentrations, because a failure in the nervous stimuli to the hypothalamus or an inadequate neuroendocrine reflex in the hypothalamic-pituitary system to trigger the adequate GnRH response caused the decrease in mean LH concentration and the lower ovulation and, consequently, the lower global pregnancy rates observed in this work.

GnRH neurons are modulated by several hypothalamic neuropeptides related to energy metabolism and steroid hormones, metabolic hormones and metabolites, such as leptin, insulin, glucose, etc. The results from this study provide evidences that female rabbits that are selected for growth rate have significant changes in their neuroendocrine system, observing lower levels of LH and higher levels of leptin and BOHB in these females. This may indicate that leptin could be interfering with ovulation. When we analysed metabolites and hormones related to lipid metabolism, we observed an increase in leptin and BOHB in non-ovulated females. On the one hand, leptin effects directly act on kisspeptin neurons, a peptide which directly signals GnRH neurons to control pulsatile GnRH release, and thus stimulates LH secretion (Quennel *et al.,* 2011). On the other hand, it has been suggested that an increase in serum leptin concentration may have a role in the steroidogenesis of pre- and post-ovulatory follicles: leptin suppresses estradiol production and interferes with the development of dominant follicles and oocyte maturation (Moschas *et al.,* 2002). Thus, the absence of ovulation observed in non-ovulated mated females may be related to leptin concentrations, in accordance with other authors that showed how an elevation in leptin levels is likely to cause a reduction in secretion by decreasing LH production by the pituitary, also preventing the release of the oocytes from the preovulatory follicles (Duggal *et al.,* 2000; Brecchia *et al.,* 2006; Balasubramanian *et al.,* 2012). Our results also showed an increase in BOHB in non-ovulated females. Increased concentrations of BOHB and NEFA have been related with negative effects on reproductive performance (Opsina *et al.,* 2010; Kiani, 2012).

Additionally, high weight and diet could suppress reproductive functions by acting directly on the pituitary or the ovary (Balasubramanian *et al.,* 2012). Excessive lipid storage in mice was shown to induce ovarian dysfunction disorders with advanced follicular atresia, apoptosis and defective steroidogenesis (Seke *et al.,* 2012). In this study, non-ovulated females showed to be heavier than ovulated females. Cardinali *et al.* (2008) showed that rabbit does with optimal body condition reach higher fertility rates and show better LH secretion than rabbit does with either excess or deficit of fat depots.

CONCLUSIONS

Rabbits selected for growth rate showed a decrease in fertility when naturally mated, which seemed to be due to an ovulation failure. This absence of ovulation comes as a consequence of a reduction in LH concentrations, which could be due to the heavier body weight of these females, which may involve alterations in hormones and metabolites, such as leptin and BOHB. Further studies in reproductive effectiveness and its relation with overweight should be carried out to determine these factors' relationship with metabolism.

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