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Polymorphism and association of progesterone receptor gene with milk production and reproductive traits of rabbits

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Abstract: Using the PCR RFLP method polymorphism and three different genotypes (AA, AG and GG) were detected in the progesterone receptor gene (*PGR)* promoter in a local Slovak crossbred rabbit line. We have noted a slightly majority frequency of allele A (0.53) over allele G (0.47). Comparing the observed and expected genotype frequencies with the χ^2 test the results were statistically significant, which means the tested rabbit population was non-equilibrium. The best results and significantly highest milk production (P < 0.001) were recorded in the does of GG genotype compared to AA genotype. Other association studies aimed at the effect of genotypes on litter size showed the highest litter size and number of weaned rabbits per litter in GG genotype. A significantly higher (P < 0.05) average number of stillborn kits per litter was in the group of does with AA genotype (0.62) compared with GG genotype (0.34).

Keywords: PGR; milk yield; genotype; rabbit

Eukaryotic gene expression and cellular proliferation are regulated by steroid hormones and their receptors. Progesterone has a central role in reproduction, being involved in ovulation, implantation, and pregnancy (Al-Asmakh 2007). Physiological effects of progesterone are mediated by interaction of the hormone with specific intracellular progesterone receptors (*PR*s) that are expressed as two protein isoforms, *PR-A* and *PR-B* (Conneely et al. 2002). *PR-A* and *PR-B* are functionally distinct mediators of progesterone action *in vivo* and should provide suitable targets for the generation of tissue-selective progestins (Mulac-Jericevic et al. 2000). *PR-A* is necessary and sufficient to elicit the progesterone-dependent reproductive responses essential for female fertility, while *PR-B* is required to elicit normal proliferative responses of the mammary gland to progesterone (Conneely et al. 2002).

The secretion of progesterone and oestrogen takes place mainly in the ovaries, but also in the placenta

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of pregnant females. They significantly contribute to the maintenance of pregnancy and the development of the mammary glands during the period of physical adolescence of young females (Erb 1977; Schams et al. 1984; Tucker 1985; Wall and McFadden 2012). The mammary gland is another site of action for *PGR*. The development of the mammary gland occurs mainly postnatally and is controlled by the complex of endocrine hormones oestrogen, progesterone and prolactin under the influence of growth factors (Anderson 2002). It involves two phases that are initiated at the beginning of puberty and later during pregnancy. Even though mouse mammary adipose and connective tissues have high affinity binding sites for oestrogens and progesterone, the largest amount of progesterone receptors is present in the epithelial component (Haslam and Shyamala 1981). However, the lactating mammary tissue has no detectable progesterone receptors (Haslam and Shyamala 1979; Terada et al. 1988), and in fact, progesterone is unable to inhibit established lactation. Seagroves et al. (2000) found that *PGR* is formed exclusively in the mammary epithelium and its development from the immature stage to the mature mammary gland is associated with a change in *PGR* expression. They also found that *PGR*-expressing mammary cells differ from normal proliferating cells. Rabbit milk production is a very important maternal trait, because the litter growth, mainly in the first period of life, and vitality of young rabbits are dependent on the maternal ability to provide optimal conditions for kits (Lebas 1969). Several studies describe the importance of milk as the essential and only source of nutrition for kits a few days (18–19) after birth (Fortun-Lamothe and Gidenne 2000; Maertens et al. 2006) and therefore the milk production in the first 21 days of lactation has a significant impact on the growth and health of the kits and is one of the limiting factors for successful rearing during the pre-weaning period. According to the study of Bonachera et al. (2017) the total amount of milk intake seems to be the main factor affecting the performance of kits during early lactation. Peiro et al. (2007) studied early embryo survival and development in 2 lines divergently selected for high and low uterine capacity throughout 10 generations. While they found no difference in the embryonic stage of development at 25 h, but at 48 and 62 h of gestation, the high line, compared with the low line, had a greater percentage of early and compacted morulae. In another study Peiro et al. (2008) found a single nucleotide polymorphism in the rabbit *PGR* promoter and the association between different litter size, implanted embryos, and early embryo survival. The aim of this study was to verify the effect of *PGR* gene polymorphism in relation to 21 days rabbit milk production, total number of live-born kits, stillborn kits and pre-weaning mortality percentages at 35 days of age.

MATERIAL AND METHODS

Experimental design, animals and management

All experiments were performed in accordance with relevant institutional and national guidelines for the care and use of animals, and all experimental procedures involving animals were approved by an ethical committee.

The trial was performed on the experimental rabbit farm at the National Agricultural and Food Centre, Nitra, Slovak Republic. A total number of 239 clinically healthy adult animals (214 does and 25 bucks) of the local crossbred line of New Zealand White × Californian × Rabbit of Nitra were used. The experimental females in all genotype groups were at the age between 11 and 15 months and their average live weight was 5 072.86 ± 467.40 g in genotype *AA*, 5 069.64 ± 396.83 in *AG* and 5 058.21 ± 444.85 g in *GG.*

The does with kits were housed in cages made of spot-welded wire mesh and of 560 × 760 mm in size (width × length, without feeder and nest) and with a raised area for resting (560 × 310 mm) arranged in flat decks on one level. The nest (560 × 260 mm) was lined with sterile wood shavings. The nest area and cage were separated by a sheet metal wall with door.

The rabbits were fed a commercial diet (pellets of 3.5 mm in diameter). The ingredients and chemical composition of this diet are presented in Table 1.

Chemical analyses were conducted according to AOAC (1995) with the considerations mentioned by Gidenne et al. (2001) for dry matter (DM), crude protein (CP), crude fibre (CF), crude fat, nitrogen free extract, ash and organic matter. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed sequentially (Van Soest et al. 1991) with

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Table 1. Composition and nutrient content of rabbit diet

Ingredients	%	Chemical analysis	Original matter (g/kg)
Lucerne meal	36	crude protein (N × 6.25)	177.25
Extracted sunflower meal	5.5	crude fibre	168.28
Extracted rapeseed meal	5.5	fat	34.21
Wheat bran	9	ash	87.10
Oats	13	starch	125.13
Malt sprouts	15	organic matter	847.53
DDGS	5	ADF	185.21
Sodium chloride	0.3	NDF	316.19
Mineral and vitamin mixture*	1.7	calcium	7.63
Barley grains	8	phosphorus	5.93
Limestone	1	ME (MJ/kg)	11.08

ADF = acid detergent fibre; DDGS = dried distiller grains with solubles; ME = metabolizable energy; NDF = neutral detergent fibre

*Premix contains per kg: calcium 6.73 g; phosphorus 4.13 g; magnesium 1.90 g; sodium 1.36 g; potassium 11.21 g; iron 0.36 g; zinc 0.13 g; copper 0.03 g; selenium 0.2 mg; Vitamin mixture provided per kg of diet: Vitamin A 1 500 000 IU; Vitamin D3 125 000 IU; Vitamin E 5 000 mg; Vitamin B1 100 mg; Vitamin B2 500 mg; Vitamin B6 200 mg; Vitamin B12 0.01 mg; Vitamin K3 0.5 mg; biotin 10 mg; folic acid 25 mg; nicotinic acid 4 000 mg; choline chloride 100 000 mg

a thermostable amylase pre-treatment and starch according to the alpha-amyloglucosidase method.

All animals had *ad libitum* access to feed. Drinking water was provided with nipple drinkers *ad libitum*. A cycle of 16 h of light and 8 h of dark was used throughout the trial. Temperature and humidity in the building were recorded continuously with a digital thermograph. Heating and forced ventilation systems allowed the building temperature to be maintained within 18 ± 4 °C throughout the trial. Relative humidity was in the interval of 70 ± 5%.

Evaluated reproductive traits

All females were multiparous at least after the third kindling and between 8 a.m. and 9 a.m. they were artificially inseminated (A.I.) by fresh heterosperm semen doses (0.5 ml per one female), after 48 h from the application of pregnant mare serum gonadotropin (PMSG, 25 IU, Sergon; Bioveta, Ivanovice na Hané, Czech Republic). Each female was administered intramuscularly 2.5 μg of synthetic GnRH-Lecirelinum (Supergestran; BIOPHARM a.s., Jílové u Prahy, Czech Republic) immediately after A.I. Rabbit does were inseminated at 18 weeks of age for the first time, and thereafter 18 days after each parturition. Litters were standardised at birth to 8–9 kits (no cross-fostering was used). The number of live-born kits, stillborn kits per litter and pre-weaning mortality percentages were observed in 35-day-old kits.

There is a high correlation between the weight gain of rabbit kits in the first period of life and milk production. There are currently several methods to determine milk production in female rabbits. One of them is the indirect method of predicting milk production using different formulas based on a high correlation between milk production and litter live weight or live weight gain at 21 days. Maertens et al. (2006) claimed that the litter weight gain at 21 days is a better predictor of the doe milk yield than litter weight at 21 days and therefore we used the following formula recommended by them for highly productive hybrid does:

$$\text{Milk yield } 0\text{–}21\text{ d (g)} = 1.69 \times \text{weight gain of the litter } 0\text{–}21\text{ d} + 362\text{ (g)} \quad (1)$$

Molecular analyses

For the molecular analyses we have used a non-invasive sample collection method. The buccal swabs for DNA isolation were collected from all analysed rabbits. MagNA Pure LC 2.0 Instrument and MagNA Pure LC DNA Isolation Kit II (Tissue) – External Lysis Purification protocol – DNA II Tissue external proteinase K (Roche Diagnostics GmbH, Mannheim,

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Germany) were used for automated isolation and purification of DNA, after external lysis (56 °C/overnight), following the manufacturer's instructions. Total DNA concentrations were measured by a UV/VIS spectrophotometer NanoPhotometer (Implen GmbH, Munich, Germany).

For the amplification of PGR promoter fragment (558 bp) and detection of polymorphisms in this segment specific primers were designed and synthesized according to Peiro et al. (2008), using the gene sequence (GenBank, X06623):
PGR F: 5'-GAAGCAGGTCATGTCGATTGGAG-3';
PGR R: 5'-CGCCTCTGGTGCCAAGTCTC-3'.

The PCR conditions (PTC-200, BIO-RAD) were 94 °C for 2 min, 94 °C for 30 s, 66 °C for 30 s, 72 °C for 30 s, 35 cycles, with the last extension at 72 °C for 10 minutes. The reaction volume (25 µl) contained 10 mM Tris-HCl (pH 8.6 at 25 °C, 50 mM KCl, 1.5 mM $MgCl_2$, 25 units/ml *Taq* DNA polymerase, 0.2 mM dNTPs each, 5% glycerol, 0.08% IGEPAL® CA-630, 0.05% Tween-20) (New England Biolabs, Hitchin, United Kingdom), 10 pmol/µl each primer.

Genotyping

The PCR-RFLP method with restriction enzyme *Eco*31I was used for *PGR* genotyping.

PGR amplicon was digested by 5 IU of *Eco*31I (Fermentas) at 37 °C/16 hours. The restriction fragments of three different *PGR* genotypes (*AA* = 558 bp, *GG* = 416 + 142 bp and *AG* = 558 + 416 + 142 bp) obtained in digestion reactions were electrophoretically separated on 2% agarose gels containing ethidium bromide at 80 mA in 10 mM lithium borate buffer, pH 8.0 for 60 minutes. The products were visualized under UV light and photographed using a MiniBis Pro system (Bio-Imaging Systems, Neve Yamin, Israel).

Statistical analysis

Statistical analysis of the obtained parameters and allele frequency were statistically evaluated by chi-squared test, one-way analysis of variance (ANOVA) with Scheffe's test and *t*-test with the level of significance set at *P* values of less than 0.05, 0.01 and 0.001. The results are quoted as means ± standard deviation.

Allele frequencies and genotypic equilibrium were evaluated by the χ^2-test when the Hardy-Weinberg equilibrium holds, and genetic diversity was calculated using the following parameters: expected heterozygosity (H_{exp}), observed heterozygosity (H_{obs}), effective allele number (A_E) and polymorphic information content (PIC) according to the following formulas:

$$pA = \frac{2AA + AB}{2N} \;;\; qB = \frac{2BB + AB}{2N} \quad (2)$$

where:
pA, qB – allele frequencies;
N – total number in the population.

$$\chi^2_{(n-1)} = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}} \quad (3)$$

where:
n – number of genotype classes.

$$H_{obs} = \frac{N_H}{N} \quad \text{(Nei 1978)} \quad (4)$$

where:
H_{obs} – observed heterozygosity;
N_H – number of observed heterozygotes;
N – total number in the population.

$$H_{exp} = 1 - \sum (p^2 + q^2) \quad \text{(Nei 1978)} \quad (5)$$

where:
H_{exp} – expected heterozygosity;
p – frequency of allele A;
q – frequency of allele G.

$$A_E = \frac{1}{p^2 + q^2} \quad \text{(Crow and Kimura 1970)} \quad (6)$$

where:
A_E – effective allele number;
p – frequency of allele A;
q – frequency of allele G.

$$PIC = 1 - \sum (p^2 + q^2) - \left(\sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2 \right) \quad \text{(Botstein et al. 1980)} \quad (7)$$

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where:

PIC – polymorphic information content;
p – frequency of allele A;
q – frequency of allele G.

RESULTS AND DISCUSSION

The polymorphism and three different genotypes (*AA*, *AG* and *GG*) in *PGR* gene promoter were detected by the used PCR RFLP method (Figure 1). The highest frequency was reached by genotype *AG* (0.41), followed by genotype *AA* (0.33) and the lowest frequency was in genotype *GG* (0.26). The higher frequency of the allele *A* (0.53) vs allele *G* (0.47) was found by the analysis of the specific region of *PGR* receptor gene (558 bp) (Table 2). The observed heterozygosity (0.42) was lower than the expected heterozygosity (0.50), which can be attributed to inbreeding in hybrid rabbit populations.

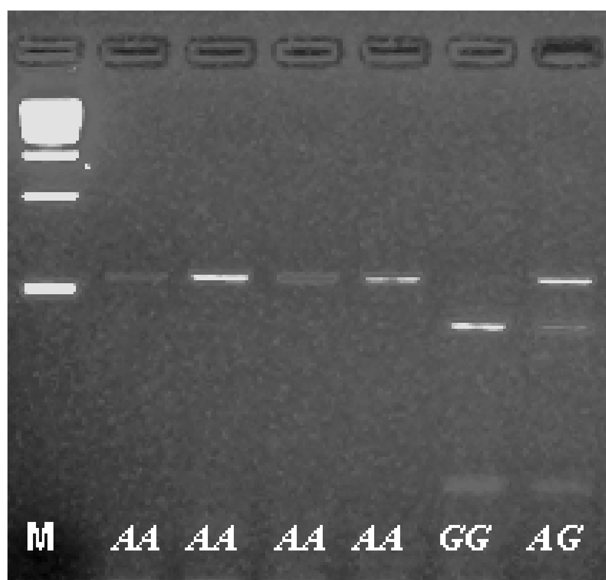

Figure 1. PCR-RFLP electrophoresis results for *PGR* gene. M = 1 kbp DNA Ladder; Genotypes: *AA* = 558 bp; *GG* = 416 + 142 bp; *AG* = 558 + 416 + 142 bp

The polymorphic information content (PIC) is an ideal parameter for the genetic diversity evaluation. Botstein et al. (1980) suggested using the PIC to evaluate the level of gene variation, where the PIC value > 0.5 corresponds to high diversity; PIC < 0.25 is low diversity and intermediate diversity is when the PIC value is in the interval between 0.25 and 0.5. In our analysed population the PIC value was 0.38, which means that it did not reach the threshold (0.5) for high diversity.

The effective number of alleles (AE) was 1.99 close to the cut-off value (Table 3), which is typical of two-allele systems where both alleles effectively participate in genotyping. When comparing the observed and expected genotype frequencies with the χ^2 test, the results were statistically significant (P = 0.019 53) and corresponded to the non-equilibrium tested rabbit population (Table 2).

The females of *GG* genotype had the significantly highest ($P < 0.001$) milk production (4 864.16 ± 450.04 g/doe) vs genotype *AG* and *AA,* during the first 21 days. The lower milk production was recorded in *AG* genotype (4 289.49 ± 397.06 g/doe) and the lowest in *AA* genotype (4 117.00 ± 546.48 g/doe) (Table 4). In the group of does with *GG* genotype we observed a significantly higher ($P < 0.05$) number of live-born kits (9.98 ± 1.53) compared to *AG* genotype (9.43 ± 2.19). The groups of *AA* and *AG* genotypes had a significantly ($P < 0.05$) higher number of stillborn kits per litter (0.62 ± 1.09 and 0.5 ± 0.9, respectively) compared with *GG* genotype (0.34 ± 0.69) (Table 4).

Table 3. Efficiency of the *PGR* alleles in the rabbit population

Locus	H_{exp}	H_{obs}	A_E	PIC
PGR	0.50	0.42	1.99	0.38

A_E = effective number of alleles; H_{exp} = expected heterozygosity; H_{obs} = observed heterozygosity; PIC = polymorphic information content

Table 2. Genetic structure of the monitored population

Locus	Genotype	Number of rabbits	Alleles	Allele frequency	$\chi 2$	*P*-value
PGR	*GG*	56	*G*	0.47	7.87	0.019 53*
	AG	88				
	AA	70	*A*	0.53		
	Σ	214				

*$P < 0.05$

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Table 4. Productive and reproductive traits in different rabbit *PGR* genotypes

Parameter	Female genotype					
	AA (*n* = 70)		*AG* (*n* = 88)		*GG* (*n* = 56)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Milk production (g)	4 117^{a}	546.48	4 289.49^{b}	397.06	4 864.16^{c}	450.04
Liveborn kits/litter (*n*)	9.88^{ab}	2.36	9.43^{a}	2.19	9.98^{b}	1.53
Stillborn kits/litter (*n*)	0.62^{a}	1.09	0.5^{a}	0.90	0.34^{b}	0.69
Weaned rabbits (*n*)	6.92^{a}	1.43	7.01^{a}	1.08	7.03^{a}	0.9
Pre-weaning mortality (%)	13.78	–	12.28	–	12.07	–

Means ± SD with different superscripts in the same row significantly differ at $P < 0.05$ (^{a} vs ^{b}); $P < 0.001$ (^{a} vs ^{c}; ^{b} vs ^{c})

Bonachera et al. (2017) evaluated the impact of variation in the milk intake of kits on performance before and after weaning while the milk intake affected the survival rate only during 0–17 days of age.

However, during the later growth period the effect of milk intake on rabbit mortality was not significant. In our study, we did not record any significant pre-weaning mortality (35 days old kits) compared to different genotypes with significantly different maternal milk yields.

Progesterone and progesterone receptor are known to be important factors influencing animal reproduction and therefore they have been used for association studies in different livestock species. Argente et al. (2000) performed an experiment to select animals for uterine size, monitoring the litter size of animals with large and small uterine capacity. They found a high correlation between litter size and uterine capacity.

Anzaldua et al. (2007) characterized the expression pattern of progesterone receptor in two regions of the oviduct and the uterus of the rabbit during early pregnancy and they observed a significant increase in the expression of PR in the uterus on the first two days of pregnancy. These differences are related to different functions of PR in the reproductive tract during early pregnancy, thereby affecting embryo implantation, survival and development.

Peiro et al. (2008) observed 589 females selected for the uterine size for ten generations. They monitored *PGR* gene as a possible candidate gene affecting litter differences and related factors (embryo number and survivability, developmental stage). In their work they focused on SNP analysis at 2 464 $G \rightarrow A$. Allele *G* was identified in 75% of animals with large uterine capacity and only in 25% of animals with smaller uterine capacity. At the same time, the *GG* genotype animals were 0.5 progeny more litter than the *AA* genotype animals and obtained the same results when observing the number of implanted embryos. Within 48 h of pregnancy they found negligible differences in the number and degree of embryo development, however at 72 h the *GG* genotype had by 0.36 embryos more than the *AA* genotype.

Also our results confirm better reproductive properties of *GG* genotype (9.98 ± 1.53 live-born kits/litter) compared with *AA* and *AG*. The *AG* genotype was not described and evaluated by Peiro et al. (2008).

CONCLUSION

We found a higher frequency of heterozygous *AG* genotype compared to *AA* and *GG*. The smallest proportion was revealed in the *GG* genotype. The frequency of *G* allele was slightly lower than that of *A* allele. The highest milk production was recorded in the females of *GG* genotype. The association studies focused on the effect of *AA*, *GG* and *AG* genotype on live-born kits per litter confirmed significant differences. The females of *GG* genotype reached the highest number of live-born kits per litter, on the other hand this genotype also had a significantly ($P < 0.05$) lower number of stillborn kits per litter compared with *AA* females. This association between the single nucleotide polymorphism in rabbit *PGR* gene and different milk production in the first 21 days of lactation can be useful for selection programmes of rabbit maternal lines, other reproduction studies as well as evalua-

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tion of diversity in populations of national breeds and rabbit lines.

The results show that selection aimed at increasing the frequency of *G* allele in the monitored rabbit population can achieve an increase in milk production. The *G* allele of *PGR* gene is a potential DNA marker for this parameter.

Conflict of interest

The authors declare no conflict of interest.

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