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THE CRP PROMOTER POLYMORPHISM OF DOMESTIC RABBITS.  
PRELIMINARY STUDY.

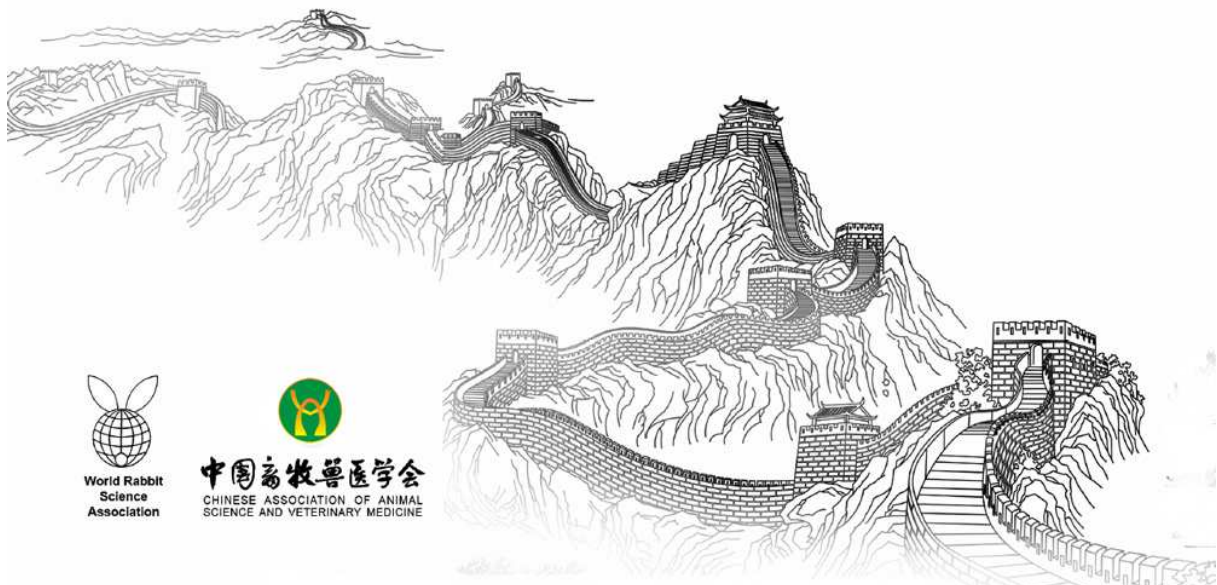
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## THE CRP PROMOTER POLYMORPHISM OF DOMESTIC RABBITS. PRELIMINARY STUDY.

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### ABSTRACT

A parental female crossbred rabbit line, based on New Zealand white rabbits, was subject to strict divergent selection. The does were mated with potential of creating population of animals with higher number of live born kits and lower variability kits per litter. Females of the parental generation were divided into two groups: 1<sup>st</sup> group, females with low variability live born kits (LV) and 2<sup>nd</sup> group, females with high variability live born kits (HV). In the so formed groups, selected reproductive variables were evaluated (average number of live kits, mortality of rabbits weaned at 42 days of age, coefficient of variation of live born kits). In addition, on the basis of ELISA technology, C-reactive protein (CRP) was analysed in the blood plasma of does. We also analysed the polymorphisms in the *CRP* gene promoter by PCR amplification and sequencing of does after strict divergent selection, and their offspring by PCR-RFLP and HRM methods. After PCR-RFLP and HRM analyzes we observed that the LV group of does was heterozygous in the part of *CRP* promoter and 5'UTR region for the nucleotide position 1419 (T/C), 1422 (T/C) and 1666 (G/T). On the other hand the HV group of does was homozygous in the *CRP* promoter nucleotide position 1419 (C), 1422 (C) and 1666 (T). Comparing LV and HV groups of does, significant differences have been observed. We found higher ( $P < 0.05$ ) average number of live-born kits/litter ( $8.2 \pm 1.21$  vs.  $6.5 \pm 3.3$ ), lower mortality ( $P < 0.01$ ) of weaned rabbits (4.06% vs. 27.47%) and lower variability of offspring at birth ( $v = 14.76\%$  vs.  $50.77\%$ , respectively). The identified SNPs of rabbit *CRP* gene promoter may be relevant in the divergent selection of appropriate parental genotypes.

**Key words:** rabbit, litter size, C-reactive protein, *CRP* promoter, single nucleotide polymorphism.

### INTRODUCTION

Selection at an early age for breeding and higher production is a prerequisite under intensive livestock production system through out the world (Niranjan et al., 2010). Studies in rabbits have revealed that both, direct and maternal influences are important for animal growth (Ferraz et al., 1992; Lukefahr et al., 1993; Lukefahr et al., 1996) and affects the phenotypic expression of the young through her genotype for maternal effects and direct additive genes for growth. On the other hand in multiparous species, such as the rabbit, maternal effect mediated by litter size and birth weight influences both growth and mortality in suckling and growing animals (Poigner et al., 2000). Researchers have demonstrated that maternal antibodies remain detectable up to 6 - 9 weeks after birth with a negligible effect afterwards (Blasco et al., 1983; Szendrő et al., 1984; Kerr, 1997). The litter size of weaned rabbits is correlated to genetic maternal predisposition, health status and overall breeding condition of the mother. A first factor, usually involved in the severity of a disease, is the immunological status of the host. One of the main profile characteristics of health, immunological and genetic predispositions is CRP (C-reactive protein). C-reactive protein (CRP) is an acute-phase plasma protein of hepatic origin that could be used for that purpose in toxicity studies with rabbits (Destexhe et al. 2013). Although a wide range of studies have been carried out to determine the usefulness of acute phase proteins in the prediction of several diseases, they are still relatively underutilised in veterinary medicine, predominantly in farm animals (Tothova et al. 2014). A specific feature of the acute phase response are the acute phase proteins (APPs), which are defined as proteins whose plasma concentration in inflammation is at least 25% increased (positive APPs) or reduced (negative APPs). CRP belongs to the positive acute phase proteins. The main characteristic feature of the biological effects of CRP is its ability to bind phosphocholine, which allows CRP to recognize foreign pathogens and damaged-cell phospholipid components. From an immunological perspective, CRP acts as an

opsonin. After binding to the foreign particles and phagocytic cells, it activates the complement system via the classical pathway and interacts with the humoral and cellular effector systems of inflammation, triggered by removal of the target cells. CRP ligand complex is directly tied into neutrophils, macrophages and other phagocytic cells and stimulates an inflammatory response and the production of cytokines. The rapid rise of the CRP-inducing stimulus suggests that CRP is a component of the innate immune response. Therefore the information about the level of CRP in plasma and polymorphisms of rabbit *CRP* gene promoter may be useful in the divergent selection of appropriate parental genotypes to increase offspring weaning viability.

## MATERIALS AND METHODS

### Animals and housing

The trial was performed on the experimental farm at the National Agricultural and Food Centre - Research Institute for Animal Production Nitra, Slovakia and was conducted on clinically healthy does crossbreed rabbit line based on New Zealand white rabbits. Females of the parental generation were divided into two groups: 1st group, females with low variability live born kits (LV), three females (assessed a total of 15 litters/123 kits), was subjected to strict divergent selection and selection criteria should have been more stringent (selected female had to have at least three litters with number of live born kits of 7-10 pieces). 2<sup>nd</sup> group, females with high variability live born kits (HV), two females (total of 14 litters/91 kits), had at least three litters and a large variation range of live born kits at birth (1-15 kits). The females from LV and HV groups were inseminated with same heterozygous male (selected after molecular analysis) in the *CRP* promoter for the nucleotide position 1419 (T/C), 1422 (T/C) and 1666 (G/T). Animals were individually housed in wire cages, arranged in flat-decks on one level. Cages were equipped with a hopper for food. The rabbits were fed with a commercial diet (pellets of 3 mm in diameter). All animals were given access to the feed *ad libitum*. Drinking water was provided with nipple drinkers *ad libitum*. A cycle of 16 hrs of light and 8 hrs of dark (minimum light intensity of 80 lux) was used throughout the trial. Temperature and humidity in the building were recorded continuously by a digital thermograph positioned at the same level as the cages. Heating and forced ventilation systems allowed the building temperature to be maintained within 17 - 21 °C throughout the trial. Relative humidity was about 60±5%.

### Blood plasma samples

Peripheral blood (1 ml) from each experimental rabbit was taken from the *vena auricularis centralis*. Within 30 minutes after collection, the blood was centrifuged for 15 minutes at 1000 x g at 4 ° C to obtain the blood plasma for the subsequent challenge testing. The levels of rabbit fragment C-reactive protein in the blood plasma were quantified with a commercial rabbit ELISA kit (SunRed Bio, Shanghai, China, catalogue No. 201-09-0003, <http://www.sunredbio.com/eindex.asp>). The detection range of fragment CRP is 50 µg/l-1000 µg/l and a microtiter spectrophotometer XS PowerWave at a wavelength of 450 nm was used. This commercial ELISA kit expresses not the whole protein, but just a fragment. We received the value of whole CRP after conversion in accordance with the instructions of manufacturer. The standard of this ELISA Kit corresponds with the value 32mg/l of the whole CRP protein, (personal communication with SunRedBio).

### Molecular analyses

The DNA from buffy coat was isolated using a Maxwell 16 Magnetic Particle Processor and Maxwell Blood DNA purification kit (Promega, USA) following the manufacturer's instructions. A 501 bp-long fragment containing the *CRP* gene promoter and 5'UTR region was amplified by PCR using rCRP F and rCRP R primers in strictly divergent selected does (Table 1 – group LV three females with 123 kits) and group HV (two does with 91 kits). PCR amplicons from all does and six males were checked by agarose-gel electrophoresis, purified, and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 310 ABI PRISM Genetic Analyzer from both sites. Sequencing primers were designed from NW\_003159286 sequence: forward (rCRP F: 5'- CTGTCAGCTTGGCTCTGTCA -3') and reverse (rCRP R: 5'- GATCAGGAAACACCACAGCA -3').

## Detection of polymorphism in rabbit offspring

PCR-RFLP method with Hinf I (1419 T/C) and Nla III (1422 T/C) has been used to detect polymorphism in indicated nucleotides. For the detection of polymorphism in 1666 nucleotide, the PCR-HRM method was used and a 87 bp fragment was amplified by primers rCRPnt1666F 5'-TGATTTTGCTTCCCTTCCTC -3' and rCRPnt1666R 5'-GGGAATCTGTGCCACTTTGT -3' in Rotor-Gene 6000 using Type-it HRM PCR Kit (Qiagen).

## Statistical analyses

Obtained data were statistically analyzed using  $\chi^2$ -test in SigmaPlot software (Systat Software Inc., Germany), expressed as the means  $\pm$  standard deviation and a commercially available statistics package SAS 9.1 (SAS Institute Inc., USA) using t-test. Statistical significance was indicated by *P* values of less than 0.05, 0.01 and 0.001.

In this animal study, institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by ethical committee.

## RESULTS AND DISCUSSION

The females of parental generation were subjected to strict divergent selection with potential of creating population of animals with higher viability of litters at weaning. The basic selection criterion for the creation of two groups of females was the variation range of live born kits at least after 3 litters. Females with 7-10 kits per litter were assigned to the LV group and females with a higher variability of kits in individual litters (1-15 kits/litter) were selected for the HV group. In the so formed groups, reproductive variables (average number of live kits, mortality range of rabbits weaned at 42 days of age, coefficient of variation of live born kits) were evaluated and CRP levels were quantified by ELISA in the blood plasma of females.

From the results shown in Table 1 it follows that directional selection for lower variability of live born kits per litter in LV group is associated with lower coefficient of variation of live born kits  $v$  (14.76%), higher average number of live born kits per litter ( $8.2 \pm 1.21$ ) and lower mortality of weaned rabbits (4.06%).

**Table 1** Key parameters of rabbits after divergent selection

Group (n)	Number of litters/total number of live born kits (n)	The average number of live born kits/litter $\pm$ sd	The average number of weaned kits at 42 days of age $\pm$ sd	Mortality of rabbits weaned at 42 days of age (%)	Coefficient of variation of live born kits $v$ (%)	Levels of fragment CRP in plasma rabbits ( $\mu\text{g.l}^{-1}$ )	Levels of whole CRP in plasma rabbits ( $\text{mg.l}^{-1}$ )
LV (3 females)	15/123	$8.2 \pm 1.21$	$7.87 \pm 1.25$	4.06	14.76	$225.95 \pm 91.94$	$4.02 \pm 1.64$
HV (2 females)	14/91	$6.5 \pm 3.3^*$	$4.7 \pm 2.43^{***}$	27.47**	50.77	$230.10 \pm 15.98$	$4.09 \pm 0.28$

\* statistical significance  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; LV = females with low variability live born kits; HV = females with high variability live born kits

Achieved results (Table 1) confirm the importance of divergent selection on key parameters of rabbits. By t-test statistics between LV and HV group of does and their offspring, significant differences have been observed. We found higher ( $P < 0.05$ ) average number of live-born kits/litter ( $8.2 \pm 1.21$  vs.  $6.5 \pm 3.3$ ), higher ( $P < 0.001$ ) average number of weaned kits ( $7.87 \pm 1.25$  vs.  $4.7 \pm 2.43$ ) and lower mortality ( $P < 0.01$ ) of weaned rabbits (4.06% vs. 27.47%, respectively). The unequal variation of the levels of fragment CRP in plasma of rabbits between female groups LV and HV it may be due to different genotypes in partial *CRP* promoter, too. Based on the research results we recommend a

.LOCUS *Proceedings 14th World Rabbit Congress, June 15-18, 2016, Qingdao, China*  
 NW\_003159286 5041 bp DNA linear CON 07-MAY-2010  
 DEFINITION *Oryctolagus cuniculus* breed Thorbecke inbred chromosome 13  
 genomic scaffold, OryCun2.0, whole genome shotgun sequence.  
 ACCESSION [NW\\_003159286](#) REGION: 13347320..13352360 GPS\_000400798  
 VERSION [NW\\_003159286.1](#) GI:283557908  
 DBLINK BioProject: [PRJNA42933](#)  
 KEYWORDS WGS; RefSeq.  
 SOURCE *Oryctolagus cuniculus* (rabbit)

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                                1395          rCRP_F          1419          1422
1381 tccatctcca taaaCTGTCA GCTTGGCTCT GTCAGacttc atgccagagc tgaagggtgtt
1441 ggagacacag ctatcgtgtg gacacagatg gccactcgtc tgtaaagatt ttccattatt
1501 ttctcaggat agatagccaa agtggaaacct gaggaagctt cttcattttt cctgtcaciaa
1561 aaagttggca attcggtagt cacaggagtt tgtaataaat aaccacatt gatttctctg

                                1666
1621 ttccagaatg attttgcttc ccttcctccc agagccctga cacttgccct aagaggcggt
1681 gttagaaaat catttaciaa gtggcacaga ttcccatct actgtggata taaatccagg
1741 caggatggag tggcgtgag gcaagggatg caggacttct agtacctgag cttcagcca
1801 gagacgtttt ctccaaagga gtggattctg agcctgctcg gtagcactgg tggcagggag
1861 tgaccATGga gaagctgctg tgggttttcc tgatc
                                ACGAC ACCACAAAGG ACTAG
                                rCRP_R          1895

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**Figure 1:** SNPs *Oryctolagus cuniculus* CRP promoter, (GenBank, NCBI)

divergent selection of females after three litters on low variability in the number of live born kits (7-10 pcs) in the litter with a coefficient of variation ( $v < 15\%$ ).

We have also amplified by PCR the part of *CRP* gene promoter and 5'UTR region in does after strict divergent selection. The amplicons were sequenced to identify SNPs (Single Nucleotide Polymorphisms).

LV group of females and 63 pcs (51.22%) from 123 of their kits were heterozygous in the CRP promoter for the nucleotide position 1419 (T/C), 1422 (T/C) and 1666 (G/T). Other kits of this group were homozygous, while the frequency of the C/C, C/C, T/T genotype was 25.2% and frequency of the T/T, T/T, T/T genotype was 23.58% in the same nucleotide position. On the other hand, HV females and 42 pcs (46.15%) from their 91 kits were homozygous. Nineteen of these rabbits (20.88%) were homozygous in the promoter nucleotide position 1419 (C), 1422 (C) and 1666 (T) and 23 rabbits (25.27%) in nucleotide position 1419 (T), 1422 (T) and 1666 (T). The frequency of the heterozygous genotype T/C, T/C, C/G in this nucleotide position was 34.07% and frequency of the T/C, T/C, G/T genotype was 19.78%.

## CONCLUSIONS

These results from preliminary study show a promising application of rabbit CRP promoter polymorphisms for molecular genotyping and classification of individuals in the early stages of ontogeny. The SNPs of rabbit CRP promoter may be useful in the divergent selection of appropriate parental genotypes – heterozygous (males and females) in the nucleotide position 1419 (T/C), 1422 (T/C) and 1666 (G/T) to decrease the offspring mortality at weaning. These results still need to be confirmed by further studies on larger numbers of animals and on other breeds of rabbits.

## ACKNOWLEDGEMENTS

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# The CRP promoter polymorphism of domestic rabbits. Preliminary study.

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## 1. The Message

- The function of C-reactive protein has related to its role in the innate immune system.
- The results suggest the potential contribution and influence of CRP promoter polymorphism on viability of rabbit.

## 3. Methods

- **Animals** - crossbreed rabbit line based on New Zealand white rabbits had at least three litters
  - 1st group - females with low variability live born kits (LV),
  - 2nd group, females with high variability live born kits (HV)
- **Blood plasma samples**
  - ELISA analyses of CRP level
- **Molecular analyses and detection of polymorphism**
  - identification of SNPs in CRP gene promoter

## 2. Introduction

In multiparous species, maternal effect mediated by litter size and birth weight influences both growth and mortality in suckling and growing animals. One of the main profile characteristics of health, immunological and genetic predispositions is CRP (C-reactive protein). Information about the level of CRP in plasma and polymorphisms of rabbit CRP gene promoter may be useful in the divergent selection of appropriate parental genotypes to increase offspring weaning viability.

## 4. Results

Table 1: Key parameters of rabbits after divergent selection

Group (n)	Number of litters/total number of live born kits (n)	The average number of live born kits/litter	Mortality of rabbits weaned at 42 days of age (%)	Level of CRP in plasma rabbits (mg.l <sup>-1</sup> )
LV	15/123	8.2±1.21	4.06	4.02±1.64
HV	14/91	6.5±3.3*	27.47**	4.09±0.28

\* statistical significance P<0.05; \*\* P<0.01; LV = females with low variability live born kits; HV = females with high variability live born kits

Table 2: Nucleotide positions and genotype frequency of partial CRP promoter *Oryctolagus cuniculus*

Group	LV				HV			
	1419	1422	1666	Frequency %	1419	1422	1666	Frequency %
Genotype	T/C	T/C	G/T	51.22	T/C	T/C	G/T	19.78
	C/C	C/C	T/T	25.20	C/C	C/C	T/T	20.88
	T/T	T/T	T/T	23.58	T/T	T/T	T/T	25.27
	-	-	-	-	T/C	T/C	C/G	34.07

LV = females with low variability live born kits; HV = females with high variability live born kits

Figure 1: NZW - crossbreed rabbit line



Figure 2: Sequence of CRP promoter with detected of SNP (G/T)

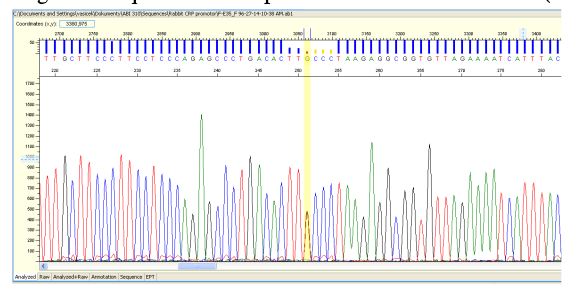
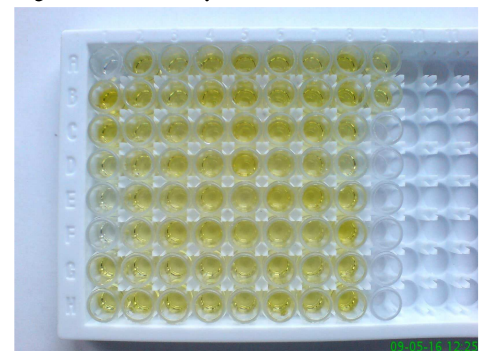


Figure 3: ELISA analyses of CRP level



## 5. Conclusions

- Application of rabbit CRP promoter polymorphisms for molecular genotyping and classification of individuals in the early stages of ontogeny.
- The parental heterozygous rabbits in the nucleotide position 1419 (T/C), 1422 (T/C) and 1666 (G/T) of the CRP promoter may be useful to decrease the offspring mortality at weaning.

## Acknowledgements

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