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POLYMORPHISMS IN OBESITY-RELATED LEPTIN GENE AND ITS ASSOCIATION WITH REPRODUCTIVE TRAITS OF SOWS

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Abstract

Getmantseva, L., A. Kolosov, M. Leonova, S. Bakoev, A. Klimenko, A. Usatov, A. Radyuk, V. Vaselenko, M. Makarenko and N. Bakoev, 2017. Polymorphisms in obesity-related leptin gene and its association with reproductive traits of sows. *Bulg. J. Agric. Sci.*, 23 (5): 843–850

Leptin and other hormones secreted by fatty tissue increasingly draw attention as mammalian reproduction markers. In this paper, we have studied the effect of c.3469 T <C SNP of the *LEP* gene on Large White sows reproductive traits according to all litters and differentially according to each litters (1st, 2nd and 3rd litter). Research was conducted on purebred pigs of Large White (♀n = 354) bread in Russia. The productivity of pigs was evaluated according to the following traits: Total Number of piglets Born (TNB), Number of piglets Born Alive (NBA), Weight of Litter (WL). The c.3469 T<C SNP of *LEP* was determined by PCR-RFLP method. Analysis of the *LEP* gene polymorphism effect on the reproductive traits was carried out using mixed linear models. The data obtained for all litters show significant differences in the reproductive traits of pigs specified by c.3469 T<C SNP of *LEP*. The best reproductive traits are associated with the genotype CC. Sows with genotype CC exceeded their analogues with genotype TT by TNB 1.3 ± 0.5 (p = 0.04), NBA 1.33 ± 0.5 (p = 0.03) and WL 3.2 ± 1.01 (p = 0.001). The effect of polymorphism of the *LEP* on TNB and NBA may depend on the litter's number. In our studies, with a differentiated estimate for the 1st, 2nd, and 3rd litter, the statistically significance effect by c.3469 T<C SNP of *LEP* on the TNB and NBA was determined only for the third litter. Further studies are needed to clarify the role of *LEP* polymorphism and other adipose tissue hormones and their receptors on the reproductive traits of pigs and opportunities to use them as SNP markers in breeding programs.

Key words: SNP; leptin; pig; reproductive traits; marker

Introduction

Adipose tissue is an active endocrine organ generating regulatory proteins (leptin, resistin, adipophilin, adipsin, agouti- protein, etc.). Leptin as one of the main adipose tissue hormones regulates energy homeostasis and thus signaling to the brain about organism's fat stores (Schwartz et al., 2000). The level of leptin is maintained due to the mechanism of negative feedback that can be performed through pituitary cells (short feedback) or neurosecretory hypothalamus cells (long feedback). Interacting with specific receptors in the hypothalamus (paraventricular, lateral, ventromedial, dorsomedial nuclei) leads to suppression of oryxigenic peptides synthesis and stimulation of anorexigenic factors (Robertson et al., 2008). As a rule, the content of leptin in the circulating blood is correlated with the body mass. Nevertheless, people and animals with an elevated level of adipose tissue rarely display leptin synthesis and leptin secretion (Yang et al.,

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2015). The decrease in the hormone's effectiveness may be associated with a malfunction of drawing hormonal signal or ability of leptin to penetrate the blood-brain barrier resulting in the leptin resistance effect (Myers et al., 2010).

Tempfli et al. (2016) studied the differences in leptin gene expression, related to pigs' breed. The experiment was carried out on pig breeds with significant phenotypic differences: fat-type Mangalica, Mangalica x Duroc, lean-type Hungarian Large White and Pietrain Duroc. The highest rate of backfat expression is found in Mangalica fat-type, and the least was observed in the Hungarian Large White lean-type. It should be noted that Mangalica pigs are very different in phenotypic characteristics from western breeds (Landrace, Large White, Duroc). According to Tempfli et al.(2016) Mangalica pigs have backfat thickness of 50.7 mm (± 5.5), an average daily gain of 592 g (± 67.0) and according to Egerszegi et al. (2003) the number of piglets per litter ranges 5-8 ones.

Leptin is mainly secreted by white adipose cells, but the presence of leptin is also found in the gastric mucosa, mammary epithelium, muscle tissue, placenta, ovaries, testicles, hair follicles (Bado et al., 1998; Wahab et al., 2015). Perez-Montarelo et al. (2013) analyzed the level of LEP expression in various tissues (backfat, liver, hypothalamus, longissimus dorsi and diaphragm) in cross pigs (Iberian x Landrace). As expected, the largest leptin expression was found in the backfat. Nevertheless, the expression is also found in other tissues that point to a potential peripheral role of the hormone. Leptin and other hormones secreted by fatty tissue increasingly draw attention as mammalian reproduction markers. Affecting through the brain, these hormones can serve as links between the adipose tissue and the reproductive system to provide and regulate energy needs for normal reproduction and pregnancy (Budak et al., 2006). Animals with congenital insufficiency of leptin exhibit a delay in sexual development and infertility together with obesity. Introduction of leptin leads to the increased mass of uterus and ovaries in females and to restoration of fertility (Chehab et al., 1996).

The data presented in the review of Priyadarshini et al. (2015) evidence that leptin can affect the axis of the hypothalamic-pituitary-gonadal region and participate in the regulation of reproductive function of pigs. Leptin plays an important role in the regulation of folliculogenesis stimulating the secretion of gonadotropic releasing hormone, follicle stimulating hormone and luteinizing hormone. Ovarian granulose and thecal cells have high affinity receptors for leptin. Porcine ovary has one of the highest levels of leptin receptor mRNA compared to other organs (Lin et al., 2000). Leptin is a modulator of oocyte maturation and follicular development in swine (Moreira et al., 2013).

LEP gene (Gene ID: 396832) in pigs is located in the 18 chromosome and studying its polymorphism is generally aimed at finding associations with the backfat thickness, growth and meat traits of pigs (Bauer et al., 2006; Chen et al., 2004; Jiang et al., 1999; Korwin-Kossakowska et al., 2002; Kulig et al., 2001). The role of leptin in the formation and regulation of reproductive functions makes one think that there are close links between *LEP* polymorphism and reproductive parameters of sows and show the relevance of studying effect of SNPs of the *LEP* gene with pig reproductive traits. In this paper, we have studied the effect of c.3469 T<C SNP of the *LEP* gene on Large White sows reproductive traits.

In this paper we studied the relations of c.3469 T<C SNP of the *LEP* gene with reproductive traits of Large White sows according to three litters and differentially according to each litters (1st, 2nd and 3rd litter). As a part of this work, the task was to assess the possibility of using c.3469 T<C SNP of the *LEP* gene as a genetic marker in breeding programs aimed at increasing reproductive characteristics of sows of the livestock under study.

Materials and Methods

Experiment material

Research was conducted on purebred pigs of Large White (♀n = 354) bread in Russia. The productivity of pigs was evaluated according to the following traits: Total Number of piglets Born (TNB), Number of piglets Born Alive (NBA), Weight of Litter (WL). Pigs of one year of birth were selected. All pigs were kept under identical and standardized conditions.

PCR- RFLP analysis

Genomic DNA was isolated from small pieces (1–10 mm^2) of ear tissue using a kit Diatom DNA Prep100 according to the manufacturer's instructions (Isogene Lab. Ltd.Russia). For the PCR we used oligonucleotide primers: F5′ – ACGTTGAAGCCGTGCCCATCTGG – 3′ and R5′ – AAGGTCCCGGAGGTTCTCCAGG – 3′(Chen, Chang, Su 2004).

The PCR reaction solution was composed of 1X PCR buffer, 100 μM dNTPs, 0.5 μM primer pair, 50-ng genomic DNA and ddH_2O. PCR amplification was performed by initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of annealing at 94°C for 30 c, 58°C for 30 c and 72°C for 45 c, and an elongation step at 72°C for 5 min. Amplified PCR products were digested by *HinfI* (Sibenzim. Russia). The genotypes (designated 'T' and 'C') were identified using 2.0% agarose gel electrophoresis.

Statistical analysis

The allelic and genotypic frequencies, observed (Ho) and expected (He), and the Hardy–Weinberg equilibrium test were calculated using PopGene 3.1 software. Analysis of the *LEP* gene polymorphism effect on the reproductive traits of Large White pigs was carried out using mixed linear models. The effect of c.3469 T <C SNP on TNB, NBA and WL was studied. The analysis was carried out using the programming language tools for statistical data processing R (3.3.3) in the R-studio system (Version 1.0.136) ("lme4" package, *lmer* function).

The fixed factors of the model were the litter number and genotypes of *LEP*. As a randomized effect, the sow effect and the mating partner effect were initially determined. In the process of preliminary analysis of the model factors, however, no impact of partner factor on the traits under study has been found. The litter number in the primary data was specified by alphabetic designation to ensure interpretation of this parameter as a factor trait. Thus, analysis models for the reproductive qualities of Large White pigs are generally represented as follows (using R-notation):

$$\begin{aligned} &TNB \sim LitterIndex + LEP\_Genotype + (1 \mid AnimalID) \\ &NBA \sim LitterIndex + LEP\_Genotype + (1 \mid AnimalID) \\ &WL \sim LitterIndex + LEP\_Genotype + (1 \mid AnimalID) \end{aligned} \quad (1)$$

where *LitterIndex* – litter number (fixed factor); *LEP _Genotype* – genotype of *LEP* (TT, TC, CC) (fixed factor); *AnimalID* – sow effect (random factor).

In analyzing mixed models by the *lmer* function the fixed and random effects are estimated in different ways. At estimating fixed effects the base point is determined: for each of these factors one of the levels is selected (the first one at sorting in alphabetical order), and the value corresponding to a combination of these fixed effect levels is defined. The values for other fixed effect levels are defined as deviations from this base point. The evaluation of randomized effects is the share of total variability specified by their influence expressed by the value of standard deviation (Winter, 2013).

To determine the statistical significance of the genotype effect on multiple pregnancies by *LEP* gene we used the likelihood ratio test (LR). The matter of this manipulation is that the model including the factors studied (in our case the genotypes according to the *LEP* gene) is conventionally designated as a "long model", being compared with a "short model", which differs from the long one by the lack of these factors. A supposition of no difference between the models (the factor effect on the dependent variable being insignificant) is the null hypothesis. The onward estimation of the likelihood ratio function distribution is done:

$$LR = 2(l_L - l_S) = 2ln\frac{L_L}{L_S} \quad (2),$$

where and are the values of the logarithmic function of the long and short models. If the models in question differ insignificantly, the distribution of χ^2 with q degrees of freedom occur. If the design value of χ^2 for the distribution under consideration exceeds the threshold value for the given level of significance and the number of degrees of freedom (in our case 0.95 and 2 respectively), then the null hypothesis is rejected. The number of degrees of freedom is defined as the difference in the number of factors of the both long and short models.

Taking into account the above, we have identified 2 models (for each of the three investigated features): LEP.model is a target model including genotype factors for the *LEP* gene (1), LEP.null is a control model including none of these factors (3). In terms of the R language, the control model:

$$\begin{aligned} &TNB \sim LitterIndex + (1 \mid AnimalID) \\ &NBA \sim LitterIndex + (1 \mid AnimalID) \\ &WL \sim LitterIndex + (1 \mid AnimalID) \end{aligned} \quad (3)$$

where *LitterIndex* – litter number (fixed factor); *AnimalID* – sow effect (random factor).

Analysis of the genotype effects by *LEP* gene differentially for each litter was carried out by using the following models:

$$\begin{aligned} &TNB \sim LEP\_Genotype + (1 \mid AnimalID) \\ &NBA \sim LEP\_Genotype + (1 \mid AnimalID) \\ &WL \sim LEP\_Genotype + (1 \mid AnimalID) \end{aligned} \quad (4)$$

where *LEP_Genotype* – genotype of *LEP* (TT, TC, CC) (fixed factor); *AnimalID* – sow effect (random factor).

To apply the likelihood ratio test to the specified models the ANOVA function (LEP.null, LEP.model) was used.

Results

The analysis data revealed the polymorphism of *LEP* in the livestock under study. The frequencies of alleles T and C were 0.7 and 0.3, respectively (Table 1). The frequencies of the genotypes TT, TC and CC were 0.50; 0.38 and 0.12 respectively. The analysis based on the Hardy-Weinberg law resulted in establishing the fact that genetic equilibrium is maintained in the studied population ($P > 0.05$).

The character of distribution reproductive traits in sows of different genotypes *LEP* in the studied selection is shown in Figure 1.The model analysis result using the *lmer*

Table 1
Allele and genotype frequencies of *LEP* at Large White pigs

Allele		Genotype				χ^2
			TT	TC	CC	
0.69	0.31	n	177	135	42	0.01
		Ho	0.50	0.38	0.12	
		He	0.48	0.43	0.10	

Note: n- number of pigs, Ho – observed frequencies, He – expected frequencies, χ^2 – test value indicates that the different genotypes are in Hardy–Weinberg equilibrium: χ^2 0.05 = 3.84, χ^2 0.01 = 6.63

Table 2
Differences in the reproductive traits of sows specified by c.3469 T <C SNP of *LEP* in all litters

Trait	Intercept	Genotypes of LEP		df	χ^2	p
		TC	TT			
TNB	12.43 ± 0.49	-1.00 ± 0.51	-1.30 ± 0.50	2	6.345	0.040
NBA	11.32 ± 0.49	-1.20 ± 0.51	-1.33 ± 0.50	2	6.930	0.030
WL	15.30 ± 0.97	-1.28 ±1.03	-3.20 ±1.01	2	13.748	0.001

Note: TNB – Total Number of piglets Born; NBA – Number of piglets Born Alive; WL – Weight of Litter; Intercept – value for the genotype CC

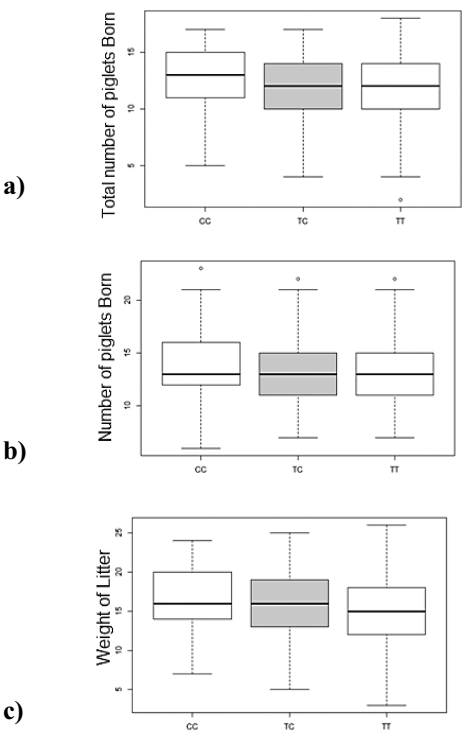

Fig. 1. Reproduction traits in sows of different genotypes of the *LEP* gene
Box-plot illustrating variation reproduction traits (A – total number of piglets born: B – number of piglet born alive; C – weight of litter) in sows of difference genotypes of LEP: horizontal lines indicate the median value; whiskers extend to a maximum of 1.5 x 3 interquartile region; outliers indicated by a point

function is presented in Table 2. In all models, the value for the CC genotype was adopted as the base point (intercept). Estimates of the remaining grades of fixed factors are presented as deviations from the intercept. The statistical significance of these differences was estimated by means of the likelihood ratio test between the models (1) and (3). The data obtained show significant differences in the reproductive traits of pigs specified by c.3469 T<C SNP of *LEP*. The best reproductive traits are associated with the genotype CC. Sows with genotype CC exceeded their analogues with genotype TT by TNB 1.3 ± 0.5 (χ^2 = 6.3447, p = 0.04), NBA 1.33 ± 0.5 (χ^2 = 6.93, p = 0.03) and WL 3.2 ± 1.01 (χ^2 = 13.748, p = 0.001).

The additional task of this work was also to evaluate the effect of genotypes of the *LEP* gene differentially for each litter (1st, 2nd and 3rd litters). The results showed that significant differences by TNB between sows of CC and TT genotypes were manifested only by the third litter (Table 3). By assessing NBA it can be noted that the sows of the CC genotype tended to the best indicators relative to the TT genotype for all analyzed litters. However, significantly differences by NBA between sows of CC and TT genotypes are established only in the third litter. The statistical significance of differences by WL between sows of CC and TT genotypes were established only by all litters.

Discussion

Lipid metabolism is a complex set of interlacing reactions influenced by various regulatory factors. The key role of leptin in the regulation of lipid metabolism provoke of

Table 3
Differences in the reproductive traits of sows specified by c.3469 T <C SNP of *LEP* in first, second and thirds litters

Number of litter	CC	TC	TT	P
Total Number of piglets Born				
First	10.22 ± 0.78	1.77 ± 0.92	0.15 ± 0.92	0.86
Second	12.43 ± 0.96	0.45 ± 1.14	0.7 ± 1.14	0.55
Third	16.78 ± 0.98	-2.57 ± 1.03	-2.32 ± 1.03	0.03
Number of piglets Born Alive				
First	9.10 ± 1.12	1.47 ± 1.20	-1.07 ± 1.20	0.38
Second	13,00 ± 0.82	-0.59 ± 0.88	-0.37 ± 0.87	0.4
Third	14.89 ± 0.75	-1.60 ± 0.89	-1.80 ± 0.89	0.04
Weight of Litter				
First	11.88 ± 1.44	3.96 ± 1.66	0.43 ± 1.67	0.79
Second	18.33 ± 1.10	-0.62 ± 1.32	-1.40 ±1.30	0.28
Third	18.89 ± 1.25	-0.93 ± 1.50	-1.75 ± 1.50	0.23

the search for associations between gene polymorphism and lipid metabolism parameters related to fat accumulation and growth and meat qualities of pigs, and later with reproductive functions. The first studies of *LEP* polymorphisms were carried out in 1997 by Stratil et al., who annotated c.3469 T>C SNP in pigs of various breeds. The further studies represented in the review of Van der Lende et al (2005) showed a relationship between c.3469 T>C SNP and average daily growth in Landrace (Kennes et el., 2001), Polish Landrace (Kulig et al., 2001), Duroc (Urban et al., 2002), backfat thickness in Large White (Jiang et al.,1999) and Duroc (Urban et al., 2002), lean meat content in Polish Landrace (Kulig et al., 2001), intramuscular fat in Polish Large White (Szydlowski et al., 2004). The findings of Nyisalovits et al. (2013) about the effect of c.3469 T>C SNP in crossbred F1 for growth and meat traits (backfat thickness, lean meat percent, average daily gain) showed a relationship only with average daily gain. Similar data were obtained by Bauer et al. (2006) at studying the association of c.3469 T>C SNP with growth and meat traits of White Improved breed pigs. The calculations proved the existence of a connection only with average daily gain and no significant effect for backfat thickness and lean meat.

Currently more than 100 SNPs in various areas of the *LEP* gene have been annotated in the results of large-scale studies and their relationship to productive indices of pigs has been studied. Kennes et al. (2001) have found a connection of c.2845 T>A SNP with total age intake and 100 kg body weight in Landrace. In addition, possible associations of SNPs at positions c.2728 G>A, c.3996 C>T (Kennes et al., 2001) and g.1112 A>G (Jiang et al., 1999) have been studied with average daily gain, backfat thickness and feed intake, but these findings showed no positive results. Perez-Montarelo et al. (2013) carried out sequencing of the *LEP* gene of Iberian × Landrace cross pigs. 39 SNPs were identified, with 8 new ones. In the intron range three SNPs were identified: g.1382C>T, g.1387C> T and g.1723A> G. Analysis of SNP g.1387 C> T, completely coincided with SNP g.1382 C> T, with additive effect on the on live and carcass weights and dominant effects on the backfat thickness being revealed. In addition, new effects of polymorphisms on the composition of fatty acids in subcutaneous fat have been found, which may be associated with the fat content of Iberian pigs. Park et al. (2015) examined the influence of *LEP / HindIII* polymorphism (Chr18: 21,204,710G>A / *HindIII*) on the growth and meat traits of pigs. The results showed a very low polymorphism in all breeds under study (Duroc, Landrace and Large White), but the influence of the minor allele on the average daily gain in Duroc was determined.

Investigations of *LEP* polymorphism were not only limited to coding fields. In Stachowiak's et al. (2007) study four polymorphisms were found in a fragment of 245 bp in the *LEP* promoter, but the authors found no evidence to associate the *LEP* promoter genotype with fatness traits. The 5' flanking region was studied in the paper of Crisà et al. (2011) and polymorphisms was examined, which may be associated with different *LEP* gene transcriptional activity. As a result, 17 polymorphic variants were identified in the 5' flanking of the gene region and differences in the activity of the promoter were determined. Liu et al. (2011) identified SNP c.2863G>A in the distal promoter region of the porcine leptin gene of pigs. Testing of 780 pigs (Duroc, Yorkshire, Laiwu, Lulai Black and Landras x Yorkshire breeds) was done by PCR-SSCP method. As a result, the effect on the backfat thickness was determined and it was suggested that SNP c.2863G>A plays a significant role in the regulation of leptin transcription.

Mankowska et al. (2015) examined SNPs in 3' UTR *LEP* and their relation to the production characteristics of Polish Landrace, Polish Large White, Duroc, Pietrain and Line 990 pigs. 8 SNPs and 1 indel were found in Polish Landrace and Polish Large White pigs. 9 SNPs were found in Duroc pigs. One SNP (g.168 C>T), observed only in the Duroc breed, was in the target site for microRNA (miR-9). Pietrain and Line 990 pigs were monomorphic. As a result, only one c.846 C>T SNP was associated with abdominal fat weight in Polish Landrace pigs. On the whole it was concluded that the contribution of polymorphisms in 3' UTR to the phenotypic variability of pig productivity is marginal.

It should be noted that investigations of *LEP* polymorphism are mainly aimed at growth and meat parameters of pigs. However, lately the adipose tissue hormones are increasingly attracting attention as potential regulators of energy homeostasis and reproductive function as well (Wahab et al., 2015). Leptin and ghrelin hormones, functional antagonists in the regulation of metabolism and energy homeostasis, probably play opposite roles in the regulation of puberty onset and gonadal function (Tena-Sempere, 2013). The existence of leptin receptors in the endometrium confirms the participation of this hormone in the preparing and providing implantation of a fertilized ovum (Brannian et al., 2002). During pregnancy, the level of leptin in the blood rises together with the gestation period. It is suggested that leptin plays a significant role during pregnancy as a placental hormone (Kerr et al., 2014). After parturition the level of leptin in the blood drops sharply, this can reflect the lactation process energy losses.

The performed works aimed at studying the association of *LEP* gene polymorphism with reproductive traits of pigs found the availability of reliable effects. The study results of Chen et al. (2004) found relation of SNP at position c.846 C>T with reproductive traits and backfat thickness in Duroc. Investigation of purebred Landrace and Yorkshire sows in the *Thai commercial* swine *population* showed the effect of c.3469 T>C SNP on litter size, mummified piglet and weaning estrus interval (Suwanasopee et al., 2011). The obtained results of studying Duroc-Pietrain crossbred boars, showed the effect of c.3469 T>C SNP on ejaculate volume, ejaculate sperm concentration and percentage of live sperm (Kmiec et al., 2003). The study of Korwin-Kossakowska et al. (2002) presented data on the significant effect of c.3469 T>C SNP on TNB and NBA of L990. In the same study the influence of c.3469 T>C SNP on the number of piglets surviving to day 21; number of piglets weaned; litter weight on day 21 and litter weight at weaning was also investigated, but reliable effects were not found. In studying three pig breeds in Hungary (Large White, Duroc, Pietren) Hunyadi-Bagi et al. (2016) determined that the homozygous CC genotype was observed only in Large White pigs, but its significant effect on TNB and NBA was not defined. In their turn, Hu et al. (2017) studied the effect of *LEP* polymorphism on reproductive qualities of British Landrace pigs and found that CC genotype in sows is reliably associated with better TNB and NBA compared to genotype TT at 1.32 and 1.68, respectively.

Our studies presented in this work, demonstrated the availability of a significant effect of c.3469 T>C SNP on the reproductive traits of Large White sows. The effect of polymorphism of the *LEP* on TNB and NBA may depend on the litter's number. In our studies significant differences by TNB and NBA between CC and TT genotypes were manifested only in the third litter. Similar results were obtained by Wierzchowska et al. (2012) where significant differences between CC and TT genotypes were established only by the second litter. These results may reflect specific features of leptin functioning associated with the age of the animals. So, in their study Kikuchi et al. (2001) assessed the effect of leptin on the growth of follicles in immature and mature female mice. The results showed that the mechanisms of leptin impact in these cases are significantly different.

In assessing the effect of c.3469 T>C SNP it should be noted that despite the results indicating a positive correlation of the CC genotype with the reproductive traits of sows all works noted a low frequency of the CC genotype. Perhaps, this can be explained partly by the fact that the selection of sows to the main herd is carried out after the first litter, but the c.3469 T>C SNP effect of the CC genotype is not manifested at the first litter. It can also be assumed that there are some negative relations of the CC genotype which prevent increasing the CC genotype homozygous frequency in pigs. Wang et al. (2017) investigated the association between c.3469 T>C SNP of the LEP gene and the emergence of follicular cysts in sows. The data obtained by these studies haven't shown such a connection.

All these results show the potential significance of gene polymorphism in forming reproductive traits of sows. General mechanisms controlling the energy homeostasis and reproductive function are still poorly investigated, but it is undeniable that puberty and reproductive functions are sensitive to the metabolic and energetic state of an organism. Further studies are needed to clarify the role of *LEP* polymorphism and other adipose tissue hormones and their receptors on the reproductive traits of pigs and opportunities to use them as SNP markers in breeding programs designed to increase the reproductive.

In conclusion, the c.3469 T>C SNP of *LEP* gene provided significant effect on reproductive traits of Large White pigs.

Therefore, these SNP can be applied in selection program to improve some reproductive traits for this studied population.

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