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PRLR, MC4R AND LEP POLYMORPHISMS, AND ADIPOQ, A-FABP AND LEP EXPRESSION IN CROSSBRED MANGALICA PIGS

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ABSTRACT

Mangalica×Duroc crossbred pigs were genotyped for the *G*1789*A* prolactin receptor (PRLR), *G*1426*A* melanocortin-4 receptor (MC4R), and *T*3469*C* leptin (LEP) single nucleotide polymorphisms (SNPs) by means of the PCR-RFLP method; genotype-trait associations were also analysed. The PRLR genotype did not influence (P>0.05) any production traits monitored, whereas the MC4R genotype significantly (P<0.05) affected backfat thickness and average daily gain during the fattening period. The LEP genotype was also significantly (P<0.05) associated with average daily gain. In crossbred gilts the expression of the adiponectin (ADIPOQ), adipocyte fatty acid-binding protein (A-FABP), and LEP genes was analysed in adipose and muscle tissues using qRT-PCR. ADIPOQ was predominantly expressed in backfat (P<0.05); however, mRNA was also detected in muscle samples indicating high intramuscular fat content. The A-FABP and LEP genes were more active in fat tissue with moderately lower levels also in muscle, contributing to intramuscular fat accumulation. MC4R and LEP polymorphisms are promising markers for production traits in the crossbred animals, whereas ADIPOQ expression is suggested as a potential indicator of elevated intramuscular fat content.

Key words: gene expression; polymorphism; backfat thickness, Mangalica pig.

INTRODUCTION

The Mangalica swine – comprising the Blond, Red and Swallow-belly breeds (Zsolnai *et al*., 2006) – was developed for fat production in the 19th century by crossing native Hungarian breeds with the Serbian Sumadia. In the 1970s the Mangalica came close to extinction due to the changing expectations of customers and the appearance of modern meat-type breeds (Egerszegi *et al*., 2003). Recently, producers have rediscovered the importance of this native breed, and Mangalica is getting much attention due to its outstanding meat quality and unique characteristics. As one of the fattest pig breeds in the world, Mangalica provides possibilities for the comparison of fat metabolism-related gene functions and expressions with commercial modern breeds. Scientific endeavours aiming to unravel the genetic background of obesity and fat metabolism-related traits are of great importance in humans due to public health concerns, whereas the findings are also applicable to livestock production, where genetic improvement in fattening characteristics offers substantial financial benefits. In a common commercial breeding scheme Duroc boars are mated to Mangalica sows, thus the production of the crossbred progenies can be increased while maintaining superior meat quality and marbled pork. In a crossbred population, prolactin receptor (PRLR), melanocortin-4 receptor (MC4R), and leptin (LEP) polymorphisms were determined to analyse genotype effects on important production traits such as backfat thickness, loin width and average daily gain.

Since the prolactin hormone acts through binding its receptor, PRLRs play major roles in several reproductive processes, mammary gland development, lactation, and regulation of maternal behaviour (Farmer *et al*., 2000). In purebred Mangalica, the *G*1789*A* single nucleotide polymorphism (SNP) in PRLR was associated (P<0.05) with the total number of piglets born and the number of piglets born alive (Tempfli *et al*., 2011). The results may not be applied to improve reproduction in the purebred population, since maintenance programs were established particularly to preserve the original characteristics of the breed; nevertheless, genetic data can be used in the selection of purebred Mangalica sows for commercial crossings with Duroc boars. Crossbred animals were genotyped in this study in order to uncover possible pleiotropic effects of the analysed PRLR polymorphism on production traits.

The melanocortin-4 receptor and the adipocyte-secreted hormone leptin play pivotal roles in controlling feed intake and energy homeostasis of pigs through hypothalamic areas associated with the regulation of appetite (Barb *et al*., 2001). External leptin administration led to a decrease in food intake indicating a hypophagic (appetite-suppressing) effect; nevertheless, plasma leptin concentration increased with the extending adipose tissue in diet-induced obesity in humans and mice (Coll *et al*., 2007). Melanocortin-4 receptors in the paraventricular nucleus of the hypothalamus are responsible for the

anorexigenic response (loss of appetite) to increasing leptin levels; however, other pathways have also been implicated since MC4R knockout mouse models showed only moderate obesity (Robinson *et al*. 2000; Lee, 2009).

Adiponectin, a protein hormone secreted by adipocytes is involved in the regulation and inhibition of lipogenesis, and the stimulation of fatty acid oxidation. Different adiponectin (ADIPOQ) genotypes were associated with elevated risks of type 2 diabetes, obesity and insulin resistance, whereas ADIPOQ plasma levels are inversely correlated with body fat levels in humans and mice (Wang *et al*. 2006; Daniele *et al*. 2008).

Adipocyte fatty acid-binding proteins (A-FABP) regulate fatty acid uptake of cells and also serve functions in the intercellular transportation of fatty acids. A-FABP plasma levels have been associated with the number of adipocytes and intramuscular fat content (IMF) in pigs (Damon *et al*. 2006).

The results of fat metabolism-related gene expression analyses presented in this study help to improve our understanding of prominent differences in the phenotype of different pig breeds.

MATERIALS AND METHODS

DNA isolation and SNP genotyping: Production data and hair follicle samples of 121 Blond Mangalica (♀) × Duroc (♂) F_1 crossbred animals (80 gilts and 41 barrows) were collected at the abattoir. Backfat thickness and loin width measurements were taken by means of an UltraFom 300 ultrasound probe between the third and fourth ribs, 6 cm off the dorsal midline. The animals involved in the experiment were fed the same diet and were kept under identical housing conditions. DNA was isolated from hair follicles using the Promega Wizard Genomic DNA Purification kit following the manufacturer's instructions. The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method was applied to determine the genotypes at the analysed loci. The following primers synthesized by IDT (Integrated DNA Technology) were used in the experiments. For PRLR (Linville *et al*., 2001), forward: 5'-CGG CCG CAG AAT CCT GCT GC-3', reverse: 5'-ACC CCA CCT TGT AAC CCA TCA TCC-3'; for MC4R (Kim *et al*., 2000), forward: 5'-TAC CCT GAC CAT CTT GAT TG-3', reverse: 5'-ATA GCA ACA GAT GAT CTC TTT G-3';and for LEP (Peixoto *et al*., 2006), forward: 5'-AAC AGA GGG TCA CCG GTT TG-3', reverse: 5'-TTT GGA AGA GCA GCT TAG CG-3'. PCR mixtures for the amplification of the three loci were similarly prepared with 12.5 µl Promega 2× PCR Master Mix (containing *Taq DNA polymerase*, dNTPs, 1.5 mM $MgCl_2$ and the required buffer), 1-1 µl forward and reverse primers (0.4 µM each), 1 µl DNA template (100 ng), adjusted with nuclease-free water up to 25 µl. The following PCR program was set in a ThermoHybaid Px2 (ThermoScientific) thermal cycler: 1 cycle initial denaturation (95°C for 3 min), 32 cycles of denaturation (95°C for 1 min), annealing (62°C for PRLR, 56°C for MC4R, and 59°C for LEP for 1 min) and extension (72°C for 1 min), finished by 1 cycle of final extension at 72°C for 5 min. The PCR products of PRLR were digested by *Alu*I restriction enzyme at 65°C, the MC4R products were digested by *Taq*I, whereas the LEP products were digested by *Hinf*I at 37°C for at least 3 hours or overnight. The digested products were loaded onto 2% agarose gels stained with Ethidium Bromide. The DNA fragments were separated by electrophoresis and made visible under UV illumination.

The non-synonymous (Gly/Ser) *G*1789*A* SNP in PRLR is located on the 16th chromosome (Vincent *et al*., 1997). The MC4R *G*1426*A* (NCBI reference sequence: NM214173.1) is a non-synonymous SNP located in the exonic region of the gene on chromosome 1, and results in an asparagin (Asn, in case of allele *A*) to aspartic acid (Asp, in case of allele *G*) change in the amino acid sequence. Asp (allele *G*) is required for normal G protein coupling (Kim *et al.*, 2004). The LEP *T*3469*C* (GenBank ID U66254.1) is a synonymous SNP located in the third exon of the gene on chromosome 18 (Villalba *et al*. 2009).

RNA extraction and preparation for gene expression analysis: Backfat and muscle (*m. levator scapulae*) samples were collected from seven crossbred gilts (live weight: 129.2±10.8 kg) at the abattoir and immediately immersed in liquid nitrogen. Tissue samples were transferred and stored in liquid nitrogen containers until further processing to avoid RNase exposure and RNA degradation.

With slight modifications, total RNA was purified from 150-250 mg backfat and muscle tissues by the guanidinium thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi (1987), using TRI Reagent Solution (Life Technologies) and 1-bromo-3-chloropropane (VWR International), and rehydrated in DEPC-treated water. Visible and intact rRNA bands and RNA integrity were verified by agarose gel electrophoresis. Isolated and rehydrated samples were quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Samples with higher than 1.8 absorbance ratios (both at 260/280 and 260/230 nm) were subjected to further procedures. RNA yield was typically higher from muscle (500-1500 ng/µl) compared to that from adipose tissue (80-200 ng/µl). To avert possible DNA contamination the samples were treated with RQ1 RNase-free DNase (Promega) according to the manufacturer's protocol. After DNase treatment, RNA was reverse-transcribed by means of the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) containing a blend of oligo (dT) and random hexamer primers. Quantitative PCR was carried out in a CFX96

Real-Time PCR Detection System (Bio-Rad Laboratories). Reactions were prepared in a total of 20 µl, containing 10 µl SsoFast EvaGreen supermix (Bio-Rad Laboratories), 1 µl cDNA, 1-1 µl primers, and nuclease-free water. As an internal reference for cDNA normalisation the housekeeping gene β-actin was used. Primer sequences are presented in Table 1.

Table 1. Sequence and annealing temperature of the primers, and the length of the expected PCR products

Gene	Sequence (5'–3')	Annealing (°C)	Length (bp)
β-actin[1]	F: CCAGGTCATCACCATCGG R: CCGTGTTGGCGTAGAGGT	-	158
ADIPOQ[2]	F: CGAGAAGGGTGAGAAAGGAG R: TAGGCGCTTTCTCCAGGTTC	60	123
LEP[2]	F: TGACACCAAAACCCTCATCA R: ATGAAGTCCAAACCGGTGAC	56	102
A-FABP[3]	F: CAGGAAAGTCAAGAGCACCA R: TCGGGACAATACATCCAACA	58	227

[1] Luo *et al*., 2009 [2]Cirera *et al*., 2013 [3] Zhao *et al*., 2009

The reactions were performed in triplicates for each sample. Gene expression levels were analysed by the $2^{-\Delta\Delta Ct}$ comparative threshold cycle method and normalised to β-actin levels.

Statistical analyses: To analyse PRLR, MC4R, and LEP genotype-trait associations the following general linear model was used (SPSS Version 16.0; SPSS, Inc.): $Y = \mu + G + S + e$, where *Y* is the phenotypic record of the observed trait, μ is the overall population mean, *G* is the effect of the PRLR genotype (*AA*, *AB*, *BB*), the MC4R genotype (*AA*, *AG*, *GG*), or the LEP genotype (*TT*, *TC*), *S* is the fixed effect of sex, and *e* is the residual error.

Additive effects (Add.) were determined as half of the difference between the estimated marginal means (EMM) of homozygotes: Add.=(AA–BB)/2; whereas dominance effects (Dom.) were calculated as the difference between the EMM of heterozygote and the mean EMM of the homozygotes: Dom.=AB–(AA+BB)/2. Gene expression differences between tissues were evaluated by Tukey's tests, and were considered significant at $P<0.05$ level.

RESULTS AND DISCUSSION

Genotype-trait associations: Three different genotypes were identified for the PRLR and the MC4R loci, whereas only two genotypes were found at the LEP locus (Table 2), which also resulted in reduced polymorphic information content (PIC) for this SNP.

The effect of the PRLR genotype on production was analysed because prolactin is a potential modulator of body composition and lipolysis in fat tissues, as its structure is highly similar to growth hormone; and prolactin receptors are commonly found on adipocytes and can contribute to the regulation of lipid metabolism (Lawrence *et al*. 2012). In crossbred animals, higher PRLR *A* allele frequency was observed compared to purebred Mangalica (29%), whereas *A* allele frequency was lower than that found in purebred Duroc (83%; Tempfli *et al*. 2011; Drogemüller *et al*. 2001). No significant associations were detected between the PRLR genotype and any of the analysed traits (Table 3); however, the *BB* genotype was characterized as having the largest backfat thickness and the lowest average daily gain.

Table 2. PRLR, MC4R, LEP allele and genotype frequency, and polymorphism information content (PIC) in the analysed population.

Gene	Allele frequency	Genotype frequency	PIC
PRLR	A = 0.52 B = 0.48	AA = 0.18 AB = 0.67 BB = 0.15	0.4455
MC4R	A = 0.28 G = 0.72	AA = 0.09 AG = 0.37 GG = 0.54	0.4766
LEP	C = 0.10 T = 0.90	TC = 0.19 TT = 0.81	0.2604

Separated MC4R genotypes were *AA* (226 bp), *AG* (226, 156 and 70 bp) and *GG* (156 and 70 bp), respectively. The crossing of purebred Mangalica with Duroc boars increased the *A* allele frequency in the crossbred population, which can be attributed to the typically high *A* allele frequency in Duroc as have been described in other studies (Ciobanu *et al*. 2001; Davoli *et al*. 2012; Kim *et al*. 2004).

In the F_1 animals the MC4R genotype was significantly ($P<0.05$) associated with backfat thickness, and the *A* allele contributed to the fattier and heavier phenotype (Table 3), which is remarkably consistent with many studies in several other breeds (Davoli *et al*. 2012;

Ovilo *et al*. 2006; Kim *et al*. 2000). Other findings suggest a breed- or line-specific MC4R effect, where the *G* allele can be associated with thicker backfat (Chao *et al*. 2012). The homozygous *A* and the heterozygous animals achieved greater average daily gain compared to homozygous *G* animals, and the differences were significant (P<0.05).

Table 3. Association of PRLR, MC4R, and LEP genotypes and production traits (estimated marginal mean±standard error).

Genotype (n)	BF (mm)	ADG (g)	LD (mm)	LW (kg)	Age (days)
PRLR					
AA (22)	37.7±1.6	700±13.8	45.4±1.9	136.7±1.9	236.1±2.8
AB (81)	38.1±0.8	691±7.2	46.3±1.1	137.1±1.7	232.1±1.5
BB (18)	41.1±1.8	687±15.2	46.3±1.8	134.8±2.1	230.0±3.1
Additive effect	3.4	12.3	0.9	1.9	-
Dominance effect	1.3	1.3	0.5	1.4	-
MC4R					
AA (11)	44.0±2.1[a]	726±19.1[a]	43.8±2.8	140.9±2.8	232.6±4.1
AG (45)	40.7±1.1[a]	697±9.5[ab]	45.1±1.4	137.8±1.4	231.3±2.3
GG (65)	35.9±0.9[b]	683±7.9[b]	47.3±1.1	135.2±1.1	233.3±1.7
Additive effect	8.1*	42.6*	3.5	5.7	-
Dominance effect	0.8	7.2	0.5	0.3	-
LEP					
TC (23)	39.2±1.6	730±12.9[a]	44.3±1.9	140.5±2.3	234.8±2.9
TT (98)	38.3±0.8	683±6.2[b]	46.6±0.9	135.8±0.9	232.0±1.4
Dominance effect	0.9	47.0*	2.3	4.7	-

[a,b] Values with different superscripts differ significantly (P<0.05)

* Additive or dominance effect is significant (P<0.05)

BF: backfat thickness; ADG: average daily gain in the fattening period; LD: loin diameter; LW: live weight

For the analysed LEP locus, only *TT* (397 and 89 bp) and *TC* (397, 347, 89 and 50 bp) genotypes were detected, which demonstrates the low prevalence of the *C* allele. The typically lower frequency of *C* allele has been previously described in the Polish Landrace breed as well, in a Duroc×Pietrain cross (Kulig *et al*. 2001), and in an experimental population composed of Brazilian Piau, Landrace, Large White and Pietrain (Peixoto *et al*. 2006). In the crossbred population the *TC* genotype was associated with increased average daily gain (Table 3). Similar effect of the *C* allele has been observed in Duroc (Urban *et al*. 2002), Polish Landrace (Kulig *et al*. 2001) and in a Large White, Landrace and Pietrain based population (Krenkova *et al*. 1999). Although the *T*3469*C* is a silent mutation, synonymous polymorphisms also can affect production traits by the modification of mRNA transcript stability and translational efficiency, or can be closely linked to causative non-synonymous mutations.

Gene expression results: ADIPOQ, A-FABP, and LEP mRNA levels were detectable in both fat and muscle tissues of the crossbred animals (Figure 1).

ADIPOQ was predominantly expressed in backfat, and was also detectable in muscle samples; however, at significantly (P<0.05) lower levels. In the background of muscle ADIPOQ expression, the role of IMF cells needs to be emphasized. Pig breeds and crosses characterized by different IMF levels can be separated by muscle ADIPOQ expression: detectable mRNA was present in pigs with IMF content above 2.5%, whereas no gene transcripts could be detected in animals with less than 1.6% IMF (Ding *et al*. 2004). In the Mangalica×Duroc crossbred animals the abundant IMF contributed to ADIPOQ production in the analysed muscle samples. IMF content is of great importance regarding meat quality and savouriness. ADIPOQ level has been found to be typically higher in adipose tissues of lean pig breeds when compared to fat type pigs (Cho *et al*. 2011; Daniele *et al*. 2008). ADIPOQ expression has also been shown to differ between piglets with high (higher ADIPOQ level) and low birth weight (animals with lower ADIPOQ levels); however, such differences were not found at later (60, 80, and 110 kg) body weight stages (Cho *et al*. 2011). Significant differences have been detected in ADIPOQ levels of Yorkshire- and Duroc-based production pigs and fat type Gottingen minipigs (Cirera *et al*. 2013).

A-FABP and LEP showed similar distribution: both genes were more active in backfat tissue, and were observed at moderately lower levels (P>0.05) in muscle as well. High A-FABP expression in muscle potentially contributes to IMF accumulation in the crossbred animals. Similar expression tendencies have been described in several other breeds, where mRNA and

protein levels of the gene were related to IMF content. Yorkshire pigs were characterized by lower A-FABP expression compared to Berkshire individuals with elevated IMF content. Since A-FABP is involved in fatty acid transport processes, A-FABP levels were shown to be associated with marbling score, body composition, backfat thickness and growth in pigs (Cho *et al*. 2011).

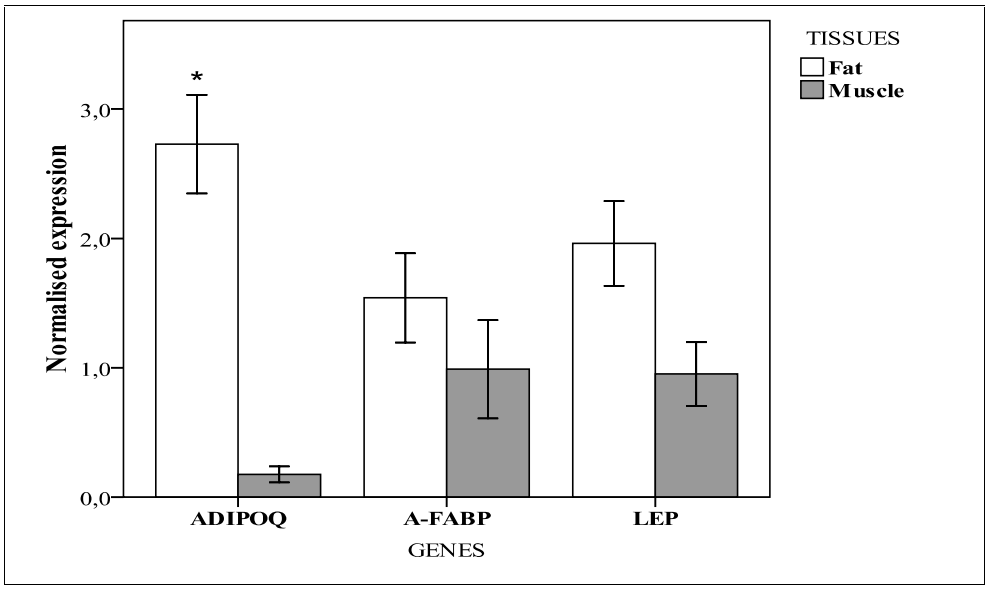

Figure 1. Normalised expression (mean±standard error) of ADIPOQ, A-FABP, and LEP genes in fat and muscle tissues of Mangalica×Duroc animals

The highest LEP expression was detected in adipose tissue, while moderate levels were also found in muscle, which is in close accordance with the results from analyses carried out in other breeds (Ramsay and Richards, 2005; Cirera *et al*. 2013). LEP levels correlated with accumulated fat content in production pigs and fat-type minipigs (Cirera *et al*. 2013), indicating the significant roles of LEP in the regulation of body composition. The data presented here are the first published ADIPOQ, A-FABP, and LEP expression results for Mangalica and Duroc crossbred pigs.

Conclusions: No pleiotropic effects of the PRLR genotype occurred on production traits in the crossbred population, thus selection of purebred Mangalica sows with the advantageous *AA* genotype may be recommended.

Since the MC4R and LEP genotypes significantly ($P<0.05$) correlated with important production traits (such as average daily gain and backfat thickness) the studied SNPs can potentially be involved in the selection of parents for commercial crossings of Mangalica and Duroc breeds.

In addition, by comparing the current results to available literature data from other breeds we concluded that different ADIPOQ, A-FABP, and LEP expression levels largely contribute to breed type differences and lipid metabolism in pigs. Our data also indicate that ADIPOQ expression in muscle can be used as an indicator of IMF content. In further studies gene expression pattern of crossbred animals should be compared to that of purebred Mangalica in order to identify crossing-related changes in gene activities.

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