Association analysis of seven candidate genes with performance traits in Czech Large White pigs

F. Weisz[1,2], T. Urban[1], P. Chalupová[1], A. Knoll[1]

[1]Department of Animal Morphology, Physiology and Genetics, Faculty of Agronomy, Mendel University in Brno, Brno, Czech Republic
[2]Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Liběchov, Czech Republic

ABSTRACT: In this study association analyses were performed between genes tagged SNP (*IGF2*, *NAMPT*, *DGAT1*, *MYF4*, *MC3R*, *MC4R* and *MYOD1*), performance traits (backfat thickness, lean meat content, average daily gain from birth to the end of the test, average daily gain in test) and estimated breeding values (EBVs) in a population of Czech Large White sows (n = 101). Genotyping of all SNPs was performed by SNapShot with the exception of SNP within *NAMPT* gene for which *Hpa*II *PCR-RFLP* assay was used. The following significant associations between genes tagged SNPs and traits or EBVs were found out: *DGAT1* – lean meat content (*AG* > *AA*, $P \leq 0.05$), *MC4R* – EBV for lean meat content (*GG* > *AA*, $P \leq 0.05$; *GG* > *AG*, $P \leq 0.05$), *IGF2* – EBV for reproduction (piglets born alive in the second and subsequent parity) (*AG* > *AA*, $P \leq 0.05$) and total EBV (*AG* > *AA*, $P \leq 0.01$) and *MC3R* – EBV for average daily gain (*CT* > *TT*, $P \leq 0.05$).

Keywords: pig; genotyping; SNP; performance trait; estimated breeding value (EBV); Czech Large White

Currently, pig breeding plays a key role in meat production, and the high consumption of pork corresponds to this. On the other hand, the high efficiency of pig breeding is absolutely necessary for competitiveness under today's difficult economic conditions. So meat performance, reproduction, and product qualities are the most economically important factors for production efficiency. These quantitative traits are under the polygenic control of many genes as well as environmental factors, and each underlying gene contributes a small proportion of genetic variation (Hu et al., 2009). The knowledge of genes affecting meat performance could be used in marker assisted selection (MAS), which combines traditional selection techniques and molecular biology for genetic improvement and which can make the production of pork more efficient.

The gene set for our analysis was composed of seven previously reported polymorphic genes with an influence on meat performance (*IGF2*, *NAMPT*, *DGAT1*, *MYF4*, *MC3R*, *MC4R*, *MYOD1*).

Paternally expressed insulin-like growth factor 2 (IGF2) is one of the polypeptide growth factors which play an important role in foetal growth and development, tumour cell proliferation, and muscle growth; its causative mutation is located in intron 3 of the *IGF2* gene (*IGF2*-int3-3072G>A) and affects muscle development and backfat thickness; *A* allele is accompanied by higher muscle development and lower fat deposition (Jungerius et al., 2004; Vykoukalová et al., 2006). *NAMPT* (visfatin) is an essential enzyme in the NAD biosynthetic pathway and current research suggests a role of visfatin in glucose metabolism and the pathogen-

Supported by the Internal Grant Agency of Faculty of Agronomy, Mendel University in Brno, Czech Republic (Project No. 16/2009).

esis of type 2 diabetes, it can be associated with subcutaneous or visceral fat, and insulin resistance contributes to abnormal lipid metabolism. Previously reported SNP AM999341:g.669T>C in intron 9 of the *NAMPT* gene is just a marker that is in linkage disequilibrium with an unknown causative mutation affecting fatness and muscling and linkage phase of the SNP and causative mutation differs in different breeds, but in Large White *TT* genotype showed higher backfat thickness than other genotypes (Wang et al., 2007; Čepica et al., 2008; Zrůstová et al., 2009). Diacylglycerol acyltransferase (DGAT1) is a microsomal enzyme that catalyses the final and only committed step in the formation of triglycerides, which are the major form of stored energy in eukaryotes. The A/G SNP at position 103 of intron 2 was used according to Nonneman and Rohrer (2002). The myogenin (MYOG, MYF4) is a transcription factor specific to skeletal muscle and fulfils a key function in muscle differentiation by controlling the onset of myoblast fusion and the establishment of myofibres. Genetic variation in the *MYF4* gene may be associated with differences in myoblast and myofibre numbers (Soumillion et al., 1997; Kim et al., 2009). Soumillion et al. (1997) described *Msp*I polymorphism at 3' end of the *MYF4* gene. The melanocortin 3 receptor gene (*MC3R*) encodes a G-protein coupled receptor for melanocyte-stimulating hormone and adrenocorticotrophic hormone, and it has an important role in energy homeostasis. An association between polymorphism in the *MC3R* gene and obesity has been detected in humans (Boucher et al., 2002). Civáňová et al. (2004) found out silent SNP polymorphism in position 522 of sequence AJ744762. Of particular interest among candidate signalling molecules involved in the regulation of energy homeostasis is the melanocortin-4 receptor (*MC4R*) because of its association with food intake, body weight, and growth in pigs; the G/A substitution changes an amino acid in protein MC4R (at codon 298 aspartic acid to asparagine) and allele *G* is associated with lower backfat thickness, slower growth rate and lower feed intake, allele *A* with fatter, higher feed consuming and faster-growing animals (Kim et al., 2000). The myogenic regulatory factor gene (*MYOD1*) induces the differentiation of fibroblasts into myoblasts and its genetic variation may influence some meat production traits and meat quality (Verner et al., 2007). Knoll et al. (1997) described *Dde*I polymorphism in the intron of *MYOD1* gene.

The aim of the study was to search for an association between the above-mentioned genes and selected performance traits and estimated breeding values (EBV's) in the Czech Large White population in the Czech Republic, and for a verification of gene effects in this population.

MATERIAL AND METHODS

A total of 101 Czech Large White sows were included in this study. Blood samples of purebred sows (born between 2003 and 2008) with defined relationship were selected randomly from one commercial herd. Peripheral blood was stored with EDTA at 8°C until genomic DNA purification performed by JETQUICK® Blood & Cell culture DNA Spin Kit (Genomed, Bad Oeynhausen, Germany), according to the user manual. Purified DNA was stored at –20°C until PCR amplification.

The PCR amplification included multiplex amplification of 5 genes (*DGAT1*, *MYF4*, *MC3R*, *MC4R*, *MYOD1)* and single amplifications of the *IGF2* gene and the *NAMPT* gene in accordance with the previously described methods using GeneAmp PCR System 2400 (Applied Biosystems, Forster City, USA). The *NAMPT* gene was genotyped according to Zrůstová et al. (2009) by *Hpa*II PCR-RFLP (Figure 1). Civáňová and Knoll (2007) described the SNaPshot minisequencing system (Applied Biosystems, Forster City, USA) for a set of 7 candidate genes – we used this methodology for the genotyping of *IGF2*, *DGAT1*, *MYF4*, *MC3R*, *MC4R*, *MYOD1* with the following modifications: *FOS* gene was monomorphic in their study and for this reason the *FOS* gene was disabled and concentration of SH2 primer (sequencing primer for *MC4R* gene) was decreased from 0.4 to 0.2μM. Other parameters were identical. Products of minisequencing were analysed using an ABI PRISM 3100 Avant Genetic Analyser (Applied Biosystems, Forster City, USA), and genotypes were determined by GeneMapper software vers. 3.7 (Applied Biosystems, Forster City, USA).

The phenotypic values of average daily gain (ADGb, g), average daily gain in test (ADGt, g), backfat thickness (BFT, cm), lean meat content (LM, %) and the EBVs of average daily gain from birth (EBVadg), of lean meat content (EBVlm), of reproduction (EBVr), and total EBV (EBVt) were studied. All data were collected by the Association of Pig Breeders in Bohemia and Moravia during the field test in compliance with appropriate methodol-

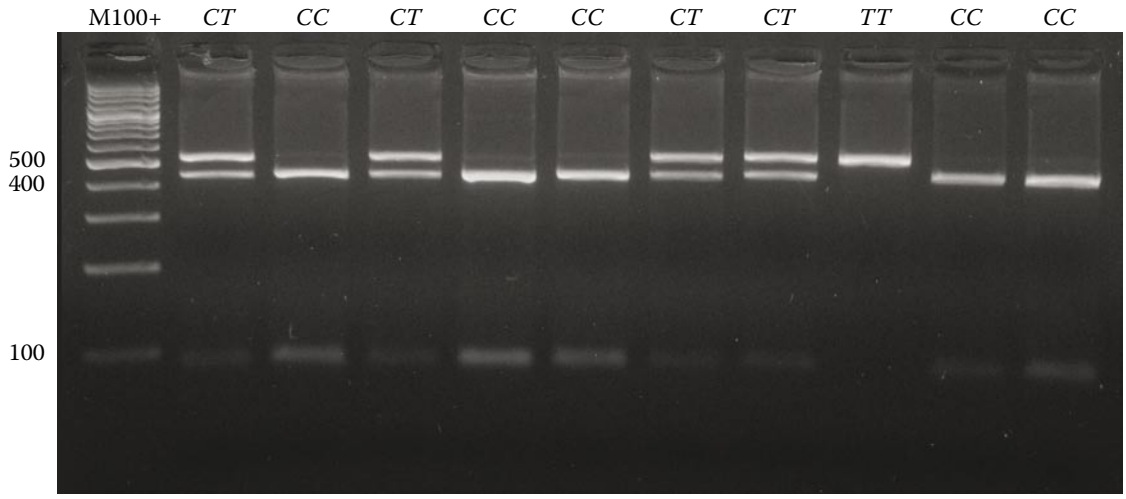

Figure 1. Agarose gel (3%) showing the polymorphism of *NAMPT* gene after *Hpa*II digestion. M100+ GeneRuler^{TM} 100bp Plus DNA Ladder (Fermentas), *C* allele was characterized by 432 bp and 92 bp product, *T* allele 524 bp

ogy between 2003 and 2008. The field test started at 12 weeks of age of animals and lasted for 57 days (± 7 days). ADGb and ADGt were calculated from birth to the end of the field test, and from the start to the end of the test, respectively. LM and BFT (measured at the end of the field test) were determined at two predetermined anatomical points by SonoMark SM-100 M: (1) backfat between the third and the fourth last lumbar vertebra 7 cm aside from the centre of the back; (2) backfat and longissimus muscle thickness was measured between the third and the fourth last rib and 7 cm from the centre of the back, in accordance with the performance recording methodology. The values of EBVadg, EBVlm, EBVr (piglets born alive in the second and subsequent parity) and EBVt (including EBVadg, EBVlm and EBVr) were estimated according to standard methodology (BLUP Animal Model) guaranteed by the Association of Pig Breeders in Bohemia and Moravia.

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by exact test (R version 2.12.1). The values of phenotypic correlations between performance traits and EBV's are shown in Table 1. Tested genotypes were subjected to association analysis performed by a mixed linear model (PROC MIXED) with the Bonferroni correction used to adjust significance levels of the test in SAS for Windows 9.1.4 using the equation:

Table 1. The values of phenotypic correlations between performance traits and estimated breeding values (EBV's)

	LM	ADGb	ADGt	EBVadg	EBVlm	EBVr	EBVt
BFT	–0.9011***	–0.2148*	–0.1759	–0.0891	–0.6100***	–0.0607	–0.13151
LM		0.3215**	0.2787*	0.16231	0.7482***	0.1223	0.2315
ADGb			0.9025***	0.6527**	0.3229**	–0.0471	0.4100***
ADGt				0.6376***	0.3202**	–0.0657	0.3858***
EBVadg					0.3753***	–0.0128	0.6661***
EBVlm						0.1874	0.4311***
EBVr							0.7361***

BFT = backfat thickness; LM = lean meat content; ADGb = average daily gain from birth; ADGt = average daily gain in test; EBVadg = breeding value for average daily gain; EBVlm = breeding value for lean meat content; EBVr = breeding value for reproduction; EBVt = total breeding value

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

$$y_{ijklmnopqrs} = \mu + IGF2_i + NAMPT_j + DGAT1_k + MYF4_l + MC3R_m + MC4R_n + MYOD1_o + M \times Y_p + f_q + m_r + e_{ijklmnopqrs}$$

where:

$y_{ijklmnopqrs}$ = phenotypic or estimated breeding value of the analysed trait

μ = population mean

$IGF2_i$ = fixed effect of the i^{th} genotype of *IGF2* gene (i = *AA* and *AG*)

$NAMPT_j$ = fixed effect of the j^{th} genotype of *NAMPT* gene (j = *CC*, *CT* and *TT*)

$DGAT1_k$ = fixed effect of the k^{th} genotype of *DGAT1* gene (k = *AA*, *AG* and *GG*)

$MYF4_l$ = fixed effect of the l^{th} genotype of *MYF4* gene (l = *AA*, *AG* and *GG*)

$MC3R_m$ = fixed effect of the m^{th} genotype of *MC3R* gene (m = *CT* and *TT*)

$MC4R_n$ = fixed effect of the n^{th} genotype of *MC4R* gene (n = *AA*, *AG* and *GG*)

$MYOD1_o$ = fixed effect of the o^{th} genotype of *MYOD1* gene (o = *AA*, *AC* and *CC*)

$M \times Y_p$ = effect of the interaction of year and month of birth

f_q = random effect of q^{th} father

m_r = random effect of r^{th} mother

$e_{ijklmnopqrs}$ = random error effect of each observation

RESULTS AND DISCUSSION

The allele frequencies were determined in 101 Czech Large White sows (Table 2). All polymorphic systems were in the Hardy-Weinberg equilibrium. The results of the association analysis are shown in Table 3.

No statistically significant differences were detected between individual genotypes of the *NAMPT* gene and the traits analysed in our study. Čepica et al. (2008) found out that the *TT* genotype of Large White was associated with higher backfat thickness and Zrůstová et al. (2009) stated that the *CC* genotype of Czech Large White was associated with lower backfat thickness in comparison with the *TT* and *CT* genotypes, and the *CC* genotype was probably associated with higher lean meat content compared to the *TT* genotype. Zrůstová et al. (2009) expected that this SNP of *NAMPT* gene in pigs is in linkage disequilibrium with an unknown causative mutation affecting the energy metabolism and linkage phase of the SNP and that the causative mutation differs in different breeds (Czech Large White compared to Landrace and Black Pied Přeštice).

Although *DGAT1* does not map within the major quantitative trait loci (QTL) an interval for back-

Table 2. Allele frequencies of the analysed genes in 101 Czech Large White sows

Gene	Allele frequency		HWE (*P*-value)
IGF2	*G* 0.16	*A* 0.84	0.0667
NAMPT	*T* 0.49	*C* 0.51	1.0000
DGAT1	*A* 0.66	*G* 0.34	0.6566
MYF4	*G* 0.77	*A* 0.23	0.0901
MC3R	*C* 0.04	*T* 0.96	1.0000
MC4R	*G* 0.19	*A* 0.81	1.0000
MYOD1	*A* 0.40	*C* 0.60	1.0000

HWE = Hardy-Weinberg equilibrium

Table 3. Associations between the genotypes of 7 candidate genes and performance traits and estimated breeding values – EBVs (least square means ± standard error are given for each genotype)

Marker	BFT	LM	ADGb	ADGt	EBVadg	EBVlm	EBVr	EBVt
NAMPT								
CC (*n* = 27)	0.66 ± 0.05	63.83 ± 0.51	634.52 ± 26.88	1054.76 ± 60.20	34.47 ± 5.51	1.50 ± 0.17	1.63 ± 0.15	1397.53 ± 83.02
CT (*n* = 50)	0.61 ± 0.05	64.30 ± 0.49	637.77 ± 25.97	1057.96 ± 57.93	34.48 ± 5.34	1.60 ± 0.16	1.51 ± 0.14	1346.66 ± 79.96
TT (*n* = 24)	0.59 ± 0.05	64.76 ± 0.47	643.10 ± 24.76	1075.20 ± 55.54	40.12 ± 5.33	1.61 ± 0.15	1.31 ± 0.14	1375.60 ± 87.77
DGAT1								
AA (*n* = 43)	0.63 ± 0.04	64.24 ± 0.44^{a}	646.36 ± 23.40	1070.24 ± 52.65	37.57 ± 4.93	1.36 ± 0.14	1.36 ± 0.13	1299.80 ± 74.14
AG (*n* = 48)	0.58 ± 0.05	64.89 ± 0.47^{a}	647.75 ± 24.66	1078.75 ± 55.48	36.07 ± 5.07	1.71 ± 0.15	1.40 ± 0.14	1289.69 ± 78.70
GG (*n* = 10)	0.64 ± 0.06	63.77 ± 0.59	621.25 ± 31.23	1038.95 ± 69.57	33.13 ± 6.27	1.46 ± 0.19	1.70 ± 0.17	1462.17 ± 96.33
MC4R								
AA (*n* = 66)	0.65 ± 0.04	63.77 ± 0.38	637.24 ± 17.72	1048.02 ± 44.33	34.09 ± 4.41	1.29 ± 0.12^{a}	1.45 ± 0.11	1292.90 ± 60.30
AG (*n* = 32)	0.66 ± 0.04	63.81 ± 0.44	645.82 ± 23.07	1068.57 ± 51.93	34.22 ± 4.87	1.40 ± 0.14^{b}	1.52 ± 0.13	1380.57 ± 72.96
GG (*n* = 3)	0.54 ± 0.08	65.31 ± 0.79	632.31 ± 42.29	1071.34 ± 93.17	38.47 ± 7.79	2.01 ± 0.26^{a,b}	1.48 ± 0.25	1378.19 ± 139.14
MYF4								
AA (*n* = 2)	0.62 ± 0.08	64.35 ± 0.77	671.62 ± 41.90	1135.86 ± 91.70	40.49 ± 7.55	1.57 ± 0.26	1.52 ± 0.26	1436.12 ± 154.20
AG (*n* = 43)	0.63 ± 0.04	64.13 ± 0.42	613.80 ± 21.75	1003.65 ± 49.08	32.82 ± 4.73	1.57 ± 0.13	1.47 ± 0.12	1314.30 ± 65.10
GG (*n* = 56)	0.60 ± 0.04	64.41 ± 0.44	629.06 ± 23.58	1048.42 ± 51.27	33.46 ± 4.93	1.57 ± 0.14	1.46 ± 0.12	1301.24 ± 65.24
MYOD1								
AA (*n* = 16)	0.60 ± 0.05	64.44 ± 0.53	620.19 ± 27.79	1021.60 ± 61.87	33.84 ± 5.62	1.47 ± 0.17	1.64 ± 0.15	1385.42 ± 83.98
AC (*n* = 49)	0.63 ± 0.05	64.17 ± 0.46	649.17 ± 24.61	1091.23 ± 55.29	37.10 ± 5.06	1.57 ± 0.15	1.38 ± 0.14	1312.99 ± 79.70
CC (*n* = 35)	0.62 ± 0.05	64.28 ± 0.47	646.01 ± 24.91	1075.11 ± 55.78	35.83 ± 5.18	1.66 ± 0.15	1.43 ± 0.14	1353.26 ± 78.94
IGF2								
AA (*n* = 68)	0.62 ± 0.04	64.10 ± 0.42	624.13 ± 28.29	1019.62 ± 62.94	36.71 ± 4.73	1.51 ± 0.14	1.35 ± 0.12^{a}	1275.83 ± 70.52^{A}
AG (*n* = 33)	0.62 ± 0.05	64.50 ± 0.53	666.92 ± 41.85	1148.20 ± 90.68	34.47 ± 5.66	1.62 ± 0.17	1.62 ± 0.15^{a}	1425.28 ± 85.64^{A}
MC3R								
CT (*n* = 8)	0.60 ± 0.06	64.53 ± 0.60	663.83 ± 32.00	1113.60 ± 71.82	42.29 ± 6.38^{a}	1.66 ± 0.20	1.39 ± 0.18	1358.83 ± 103.29
TT (*n* = 93)	0.62 ± 0.04	64.06 ± 0.43	613.09 ± 22.31	1011.68 ± 50.13	28.89 ± 4.78^{a}	1.47 ± 0.14	1.58 ± 0.12	1342.28 ± 67.27

The same superscripts in a column show significant differences: $^{A}P \leq 0.01$; $^{a,b}P \leq 0.05$

BFT = backfat thickness (cm); LM = lean meat content (%); ADGb = average daily gain from birth (g); ADGt = daily gain in test (g); EBVadg = breeding value for average daily gain; EBVlm = breeding value for lean meat content; EBVr = breeding value for reproduction; EBVt = total breeding value

fat thickness or intramuscular fat in pigs, it does map within suggestive QTL intervals for growth rate, intramuscular fat, and fatty acid composition (Nonneman and Rohrer, 2002). In this study the *AG* genotype within the *DGAT1* gene was associated with higher lean meat content compared to *AA* genotype ($P \leq 0.05$). Civáňová and Knoll (2007) did not find out any significant differences among animals of the examined population. That is probably because of the low frequency of homozygous genotype (*GG*) in the Black Pied Přeštice breed.

In the *MC4R* gene the *GG* genotype was associated with higher EBV for lean meat content compared to the *AA* genotype ($P \leq 0.05$) and to the *AG* genotype ($P \leq 0.05$). Civáňová and Knoll (2007) reported a statistically significant association of the *MC4R* gene and lean meat content in the Black Pied Přeštice pigs (*GA* > *AA*, $P \leq 0.05$), and Kim et al. (2000) revealed the *G* allele association with lower backfat thickness, slower growth, and lower daily gain and lower feed intake in four different commercial lines of PIC (international pig breeding company) pigs derived from European/American breeds. Dvořáková et al. (2011) found out an association between the *G* allele and higher values of lean meat content, muscle depth of *musculus gluteus* and *musculus longissimus lumborum et thoracis* in the population that included seven commercial crosses and one pure breed. The influence of *G* allele was similar in our study and in the studies of Civáňová and Knoll (2007), Kim et al. (2000) and Dvořáková et al. (2011) in Black Pied Přeštice, European/American PIC pigs and commercial crosses, respectively. On the other hand, the *G* allele was associated with the fattest animals in the line which was derived by crossing a Chinese (Meishan) breed with a line of Large White origin. This result could be explained by the erosion of linkage disequilibrium between the analysed mutation and causative mutation in this line of pigs, or it could also be due to epistatic or sampling effect (Kim et al., 2000).

It can be expected that structural changes in the *MYOD1* gene will have an impact on some muscle characteristics (Cieslak et al., 2000). Our results for the *MYOD1* gene *DdeI* polymorphism (510A>C), which is in the non-coding region (intron 1), revealed no association with any analysed traits. Civáňová and Knoll (2007) detected a significant association between *AC* and *CC* genotype of the *MYOD1* gene and lean meat content (*CC* genotype was associated with higher lean meat content than *AC*) in the Black Pied Přeštice breed. However, no statistically significant association with carcass and meat quality traits was found in the study of Liu et al. (2008). In the study of Cieslak et al. (2000) pigs from farm I with *AA* genotype of *MYOD1* gene displayed significantly lower values for meatiness, weight and ratio of ham or loin meat and loin eye area, but the tendency was reverse in pigs from another farm. The results of the presented studies indicate that the effect of *Dde*I polymorphism in the *MYOD1* gene may vary in different breeds or lines due to linkage disequilibrium between this SNP and the causative QTL, or due to background gene effects.

In the *IGF2* gene the *G* allele was associated with higher feed intake and lower average daily gain (Oczkowicz et al., 2009). In our study the *AG* genotype was associated with higher EBV for reproduction ($P \leq 0.05$) and higher total EBV ($P \leq 0.01$) in comparison with the *AA* genotype. Our results could be influenced by the absence of *GG* genotype, and by the low frequency of *G* allele (0.18) in the studied population of Czech Large White. The low frequency of *G* allele was observed in other studies in Large White (Vykoukalová et al., 2006; Yang et al., 2006; Oczkowicz et al., 2009). Previously reported associations of the *IGF2* gene and lean meat content and backfat thickness in the Black Pied Přeštice pig breed (Civáňová and Knoll, 2007) and in Large White (Vykoukalová et al., 2006) were not confirmed by our study, however the *IGF2* gene was associated ($P \leq 0.01$) with total EBV as the only one of the analysed genes.

In the *MC3R* gene, the *CT* genotype was associated with the EBV for average daily gain compared to *TT* ($P \leq 0.05$). Civáňová and Knoll (2007) did not show any significant differences among animals of the studied population. This is probably so because the homozygous genotype (*CC*) occurred at a low frequency (Civáňová and Knoll, 2007). In our study we did not detect any homozygous genotypes (*CC*), and only eight heterozygous *CT* genotypes were observed, which could limit the results of this association study.

No statistically significant differences were detected between individual genotypes of *MYF4* genes and the traits analysed in our study. In the study of Civáňová and Knoll (2007) the *MYF4* gene showed a tendency to regulate backfat thickness. However, Te Pas et al. (1999) reported that in Large White pigs the *AA* genotype was associated with increased birth weight, growth rate and lean meat content,

and similarly Verner et al. (2007) described a statistically significant association between *AG* and *GG* genotypes and backfat thickness in the Czech Large White (*AG* genotype was associated with higher backfat thickness than *AA*).

In conclusion, the associations between genotypes and performance traits and EBVs were detected for *DGAT1*, *IGF2*, *MC3R* and *MC4R* genes in 101 sows of Czech Large White breed. The associations suggest that these genes probably have effects on the performance traits, and could be used as a selection tool for the next generation of sows in this herd. Because of the low number of animals involved in this study and the low frequency of some alleles, a higher number of animals from this and other herds have to be investigated to get a more representative sample and to pronounce a general conclusion.

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Received: 2010–05–13
Accepted after corrections: 2011–06–17

Corresponding Author

Prof. RNDr. Aleš Knoll, Ph.D., Mendel University in Brno, Faculty of Agronomy, Zemědělská 1, 613 00 Brno, Czech Republic
Tel. +420 545 133 184, e-mail: knoll@mendelu.cz