

Expression of *NR3C1*, *INSR* and *SLC2A4* genes in skeletal muscles and *CBG* in liver depends on age and breed of pigs

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Abstract: The genes encoding glucose transporter 4 (*SLC2A4*, *GLUT4*), insulin receptor (*INSR*) and glucocorticoid receptor (*NR3C1*) are considered regulators of energy metabolism that may influence fat content in skeletal muscle at different ages and breeds of pigs. In the study we performed analysis of expression of *NR3C1*, *INSR* and *SLC2A4* genes in two skeletal muscle tissues: *semimembranosus* and *longissimus dorsi* muscle in gilts from three breeds of pigs that differed in intramuscular fat content: Duroc (DU), Pulawska (PUL) and Polish Large White (PLW) at 60, 90, 120, 150, 180 and 210 days after weaning. We also analyzed the expression of cortisol binding globulin (*CBG*) in the liver. Expression was analyzed with real time PCR (qPCR) using a relative quantification method. In blood, the concentration of cortisol, insulin and leptin were evaluated with radioimmunoassay. The concentration of metabolites (triglycerides and glucose) related to the changes of lipids content was also measured. We observed a significant relationship between the breed and the age of pigs and the expression of analyzed genes in muscle tissues ($P < 0.01$ for all analyzed genes) as well as with two hormones (for insulin $P = 0.001$; for cortisol $P < 0.0001$). Leptin level and metabolites in serum were not significant in the model ($P > 0.05$). The expression of analyzed genes (*SLC2A4*, *INSR* and *NR3C1* in muscles and *CBG* in liver) strongly correlates with intramuscular fat content in pigs and is associated with age and breed.

Keywords: muscle fat; liver; adipogenesis; gene

Skeletal muscle is a complex tissue and has a considerable plasticity in its response to dietary intakes of protein and energy (Matsakas and Patel 2009). Fat content in skeletal muscles (e.g. intramuscular fat or marbling fat), which is the total lipid within the skeletal muscle, has a significant impact not only on meat quality, but also on health as suggested previously (Quintanilla et al. 2011). In humans and farm animals excessive accumula-

tion of fat in muscle tissues is associated with a number of pathological conditions such as insulin resistance, type 2 diabetes and obesity (Hafizi Abu Bakar et al. 2015). Accumulation of fat in skeletal muscle in pigs is controlled by a number of genes involved in various pathways e.g. adipogenesis and lipid metabolism as well as the regulation of appetite and energy intake. Therefore, understanding the factors which affect the fat accumulation of

animal muscle tissue seems important for animal and human health and metabolism.

Fat content in skeletal muscle and intramuscular fat (IMF) accumulation is breed-specific. The most common breeds in Poland are Duroc (DU), Pulawska (PUL), which is a Polish autochthonous pig breed, and Polish Large White (PLW). They are included in the national breeding programme (Tyra et al. 2011). The analyzed breeds show different fat content in skeletal muscles, with DU and PUL having the highest fat content in muscle tissue, and PLW with the lowest fat content (Tyra et al. 2011). We hypothesized that the analysis of genes involved in glucose metabolism in skeletal muscle could provide an explanation for the variability of breed-specific fat content.

Muscle development and fat formation undergoes significant changes during ontogenesis. Muscle and bone develop first, with muscle growing relatively quickly and bone relatively slowly. During the following growth stages fat formation accelerates, whereas the rates of muscle and bone deposition decline. These developmental changes in muscle and fat formation have been confirmed on modern-type pigs (Wagner et al. 1999).

The deposition of fat in the muscle tissue is influenced by energy metabolism and involves glucose signaling (Katsumata 2011). Glucose availability is determined by the amount of feed consumption, age and the rate of synthesis and is stimulated by cortisol in the liver. The latter hormone is transported in complex with cortisol binding globulin (CBG) and exerts its biological action through glucocorticoid receptor (GR). This influences insulin-stimulated glucose transport into the cell by glucose transporter 4, the main glucose transporter in muscle and fat tissues. However, their role in the regulation of lipid metabolism in muscle tissue has not been well understood so far.

The aim of this study was to determine if the expression of three genes involved in glucose metabolism (solute carrier family 2 member 4 – *SLC2A4*, also known as *GLUT4*, insulin receptor – *INSR*, also known as *HHF5* or *CD220* and glucocorticoid receptor – *NR3C1*, also known as *GR*) changes at different developmental stages between three breeds of pigs (PLW, DU and PUL) in two muscle tissues (*m. semimembranosus*, *m. longissimus dorsi*). Additionally, we also analyzed the expression of cortisol binding globulin gene (*CBG*, also known as *SERPINA6*) in liver during

ontogenesis in pigs as well as glucose, triglycerides, leptin and cortisol levels in serum.

MATERIAL AND METHODS

Animals. The study included 118 gilts representing three different breeds, i.e. Duroc (DU, $n = 39$), Polish Large White (PLW, $n = 36$) and Pulawska (PUL, Polish autochthonous breed, $n = 43$). The breeds differ in back fat tissue and intramuscular fat content. Animals were kept in the Pig Station of the National Research Institute of Animal Production in Pawłowice under the same housing conditions and standard feed *ad libitum*. All consents were obtained as required. From six to eight pigs (two pigs from litter) of each breed were slaughtered after overnight fast and dissected at the age of 60, 90, 120, 150, 180 and 210 days after weaning.

Tissue. After slaughter, blood, liver, *semimembranosus* and *longissimus dorsi* muscle were collected as described previously (Tyra et al. 2013). Tissues were frozen at -80°C for gene expression analysis. Serum was obtained from blood by centrifugation (3200 rpm, 10 min) and frozen at -80°C for analysis of hormones, glucose and triglyceride levels. To measure indicators of adiposity and lipid metabolism, we performed enzymatic assays (glucose, triglycerides) and radioimmunoassays (insulin, leptin and cortisol) in serum as described below.

Glucose and triglycerides concentration. Glucose concentration in serum was measured by the use of Glucose Hexokinase reagent according to the described protocol (Pointe Scientific, USA). Triglycerides in serum were measured using an enzymatic kit (Pointe Scientific) according to provided protocol.

Hormones assay. Cortisol concentration in serum was measured using a radioimmunoassay kit (IBL International, Germany). Insulin and leptin concentration in serum were determined by Porcine Insulin RIA kit and Multi-Species Leptin RIA kit (Merck Millipore, USA) according to the enclosed protocol. Intra- and inter-assay variations for CBG were 6.2 and 11.0%, for insulin 5.6 and 10.8%, whilst for leptin 4.7 and 9.2%, respectively.

RNA isolation and real-time PCR (RT-qPCR). The total RNA from liver and muscles were isolated by the use of TriPure isolation reagent (Roche Diagnostics, Germany) according to manufacturer's protocol for tissue. After RNA isolation, the DNase

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digestion using RQ1 RNase-Free DNase (Promega, USA) was done and 1 µg of total RNA was used for reverse transcription with the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostic, Germany). In this reaction both oligo dT and random hexaprimers were used. For all genes RT- (without reverse transcriptase) control was prepared. Obtained cDNA was amplified using real-time PCR (qPCR) for analyzed genes which included cortisol binding globulin (*CBG*), glucocorticoid receptor (*NR3C1*), glucose transporter 4 (*SLC2A4*) and insulin receptor (*INSR*). As an internal control we used TATA-box binding protein (*TBP*). All primers were designed by freely available Primer 3.0 software (<http://primer3.ut.ee/>) and are listed in Supplementary Table S1 in Supplementary Online Material (SOM). We have chosen *TBP* gene after analyzing the different internal standards (*ACTB*, *HPRT1*, *TBP*, *TOP2B*, *GAPDH*) using Best Keeper program (www.gene-quantification.de/bestkeeper.html). qPCR was performed using Roche 2.0 instrument and LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostic). Analysis was made using LightCycler software Version 4.5 based on the relative quantification method (standard curve method) with the efficiency correction. Results were presented as a ratio of studied gene expression to internal standard *TBP* (arbitrary units).

Statistical analysis. To test if gene expression and protein serum levels are influenced by age and breed of analyzed pigs, we performed two-way ANCOVA analysis with gene expression and serum levels as dependent variables and breed and age as independent predictors. This was further confirmed by regression analysis. The statistical model included breed, age and interactions among these effects as fixed factors. When required, data were transformed to logarithms or by Box-Cox transformation to obtain a normal distribution of residuals in ANOVA (one-way analysis of variance) or MANOVA (multivariate analysis of variance). Normality of distribution was tested by the Kolmogorov-Smirnov test. All tests were two-sided. *P*-value less than 0.05 was regarded significant. Statistical calculations were performed using STATISTICA software (Version 10).

RESULTS

Analysis comparing relative mRNA levels of analyzed genes in two skeletal muscle tissues in three breeds showed significant differences during de-

velopment. We found that these changes correlated with the intramuscular fat (IMF) accumulation during ontogenesis (results from analysis of the same animals (Tyra et al. 2013) and increased IMF content in *l. dorsi* and *semimembranosus* muscles were observed from day 120 up to day 180 in all analyzed pigs (Supplementary Figure S1 in SOM). The expression of analyzed genes was significantly influenced by breed and correlated with the IMF content which was the highest in DU pigs.

Gene expression analysis stratified by age and breed. The expression of *SLC2A4* in *semimembranosus* muscle showed the highest increase in days 120–180 in all breeds analyzed together (Supplementary Figure S2A in SOM). When we stratified the *SLC2A4* expression by breed, we observed similar expression pattern in all three breeds, but the expression was significantly higher in DU pigs in comparison to the two other breeds (Figure 1A). The expression of *SLC2A4* in *l. dorsi* muscle was more constant across different developmental stages with an increase in days 150–180 in all breeds analyzed together (Supplementary Figure S2D in SOM). However, analysis stratified by breed indicated that DU pigs showed a significantly increased expression in days 120–180 in comparison to the other breeds (Figure 1B).

The expression of *INSR* in *semimembranosus* muscle demonstrated gradual increase up to day 150 and then gradual decrease in all breeds (Supplementary Figure S2B in SOM). The analysis stratified by breed revealed significant differences, with the lowest expression and different expression pattern for PLW in comparison to other breeds (Figure 1A). The expression of the *INSR* in *l. dorsi* muscle showed an increased expression in days 120–180 followed by decrease at day 210 (Supplementary Figure S2E in SOM). The analysis by breed showed significantly higher expression in DU pigs in comparison to the other breeds (Figure 2B).

The *NR3C1* expression in *semimembranosus* muscle was increased gradually up to day 180 followed by a decrease in all breeds (Supplementary Figure S2C in SOM). The analysis stratified by breed showed the same expression pattern for PLW and PUL, but not for DU pigs, that showed an increased expression also at day 210 (Figure 3A). The analysis of *NR3C1* expression in *l. dorsi* muscle showed the highest level in days 150–180 followed by a decrease at day 210 (Supplementary Figure S2F in SOM). In the analysis

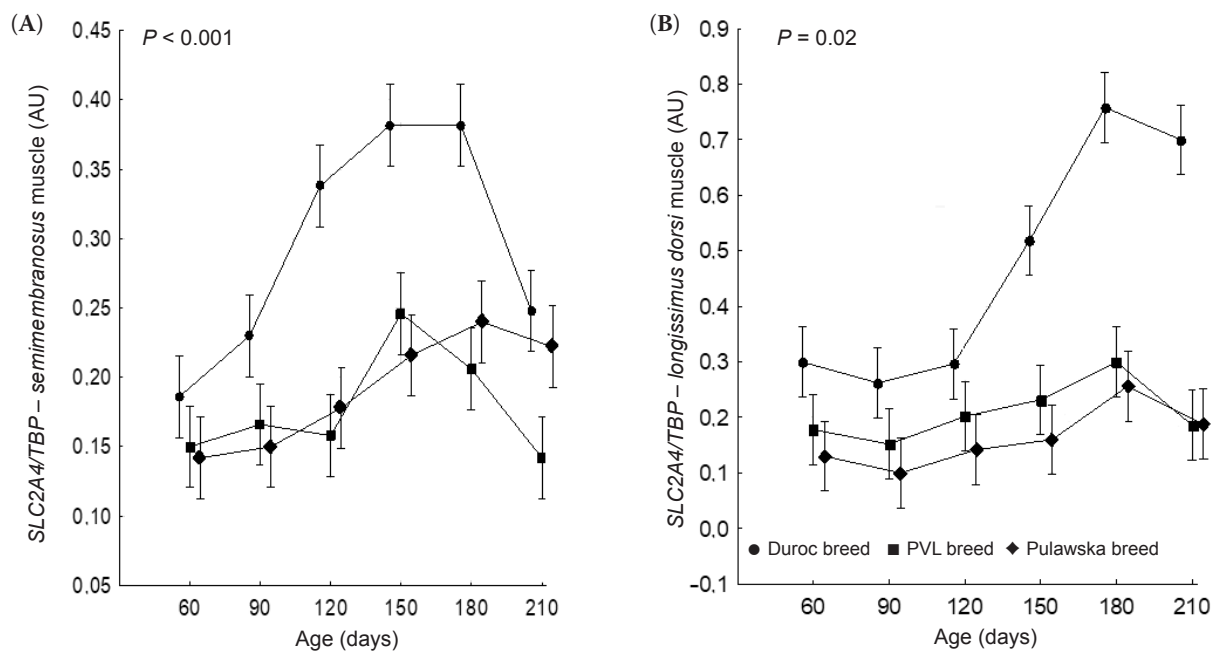


Figure 1. Interaction between breed and age significantly influences the *SLC2A4* gene expression level (arbitrary units) in (A) *semimembranosus* and (B) *longissimus dorsi* muscle (MANOVA, Least Squares Means with 95% confidence intervals)

stratified by breed we found a significantly higher *NR3C1* expression for PLW pigs in comparison to DU and PUL pigs (Figure 3B).

Analysis of age and breed interaction on gene expression. Linear regression analysis confirmed that age and breed significantly influenced the expression of *SLC2A4*, *INSR* and *NR3C1* genes in skeletal muscles when analyzed independently.

Interaction analysis of age and breed showed their significant influence on *SLC2A4* expression in both muscle tissues and on *INSR* expression in *semimembranosus* muscle, whereas the expression levels of *INSR* in *l. dorsi* muscle and the *NR3C1* expression in both muscle tissues were not affected by the interaction of breed and age of pigs (see Figures 1–3).

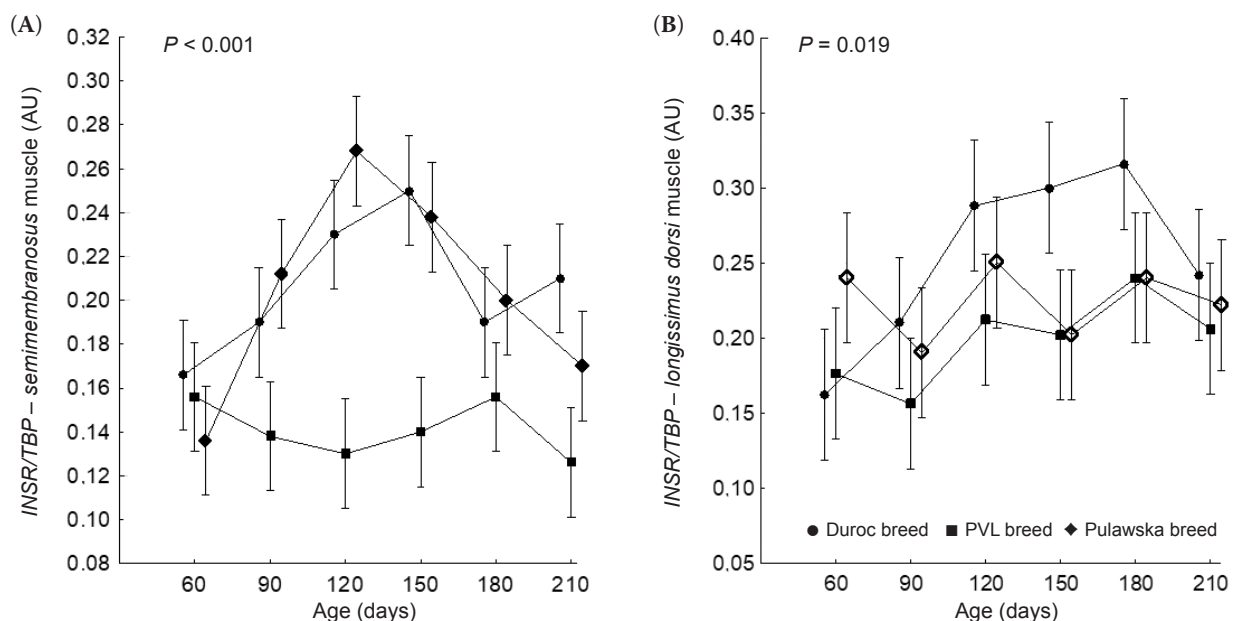


Figure 2. Interaction between breed and age significantly influences the *INSR* gene expression level (arbitrary units) in (A) *semimembranosus* and (B) *longissimus dorsi* muscle (MANOVA, Least Squares Means with 95% confidence intervals)

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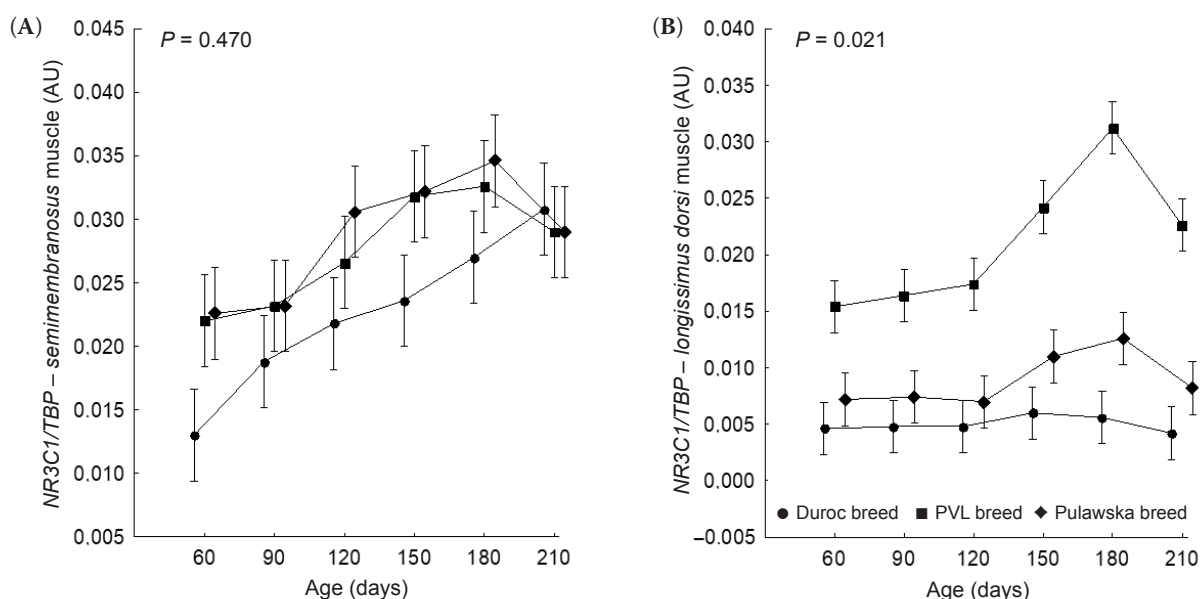


Figure 3. Interaction between breed and age significantly influences the *NR3C1* gene expression level (arbitrary units) in (A) *semimembranosus* and (B) *longissimus dorsi* muscle (MANOVA, Least Squares Means with 95% confidence intervals)

Breed and age significantly influenced also the serum level of two hormones involved in energy metabolism (insulin and cortisol) as well as *CBG* expression in the liver when analyzed separately as well as in the interaction. The exception was observed for leptin, where breed and age significantly influenced its serum level only when analyzed separately, but not in the interaction (Figure 4C–F).

The level of glucose and triglycerides increased gradually during the development (Supplementary Figure S3A, B in SOM) independent of breed (Figure 4A, B). Leptin and insulin level gradually increased during ontogenesis of analyzed pigs (Supplementary Figure S3C, D in SOM), but the analysis by breed revealed significant differences. PLW pigs showed insulin increase after day 150, whereas in PUL pigs the insulin level decreased at day 210 (Figure 4C). For DU, we observed gradual increase with age (Figure 4C). The cortisol level showed a gradual increase during the development in pigs up to day 180 (Supplementary Figure S3E in SOM), and showed significant changes between breeds: for PLW pigs we found an increase up to 150 days, followed by a decrease in the later development; for PUL pigs we observed a gradual increase up to 180 days; for DU pigs the cortisol level remained high but unaltered during development (Figure 4E). The expression of *CBG* in liver showed the same pattern as cortisol in serum, with gradual increase during ontogenesis up to

180 days. The analysis of *CBG* expression by breed revealed that DU pigs had the lowest and unaltered expression, PUL pigs demonstrated an increase up to 180 days, whereas PLW pigs showed the highest expression at days 120–180 with a significant decrease afterwards (Figure 4F).

DISCUSSION

The main finding of this study is that the expression of four genes related to glucose metabolism in the skeletal muscle tissue is significantly influenced by the age and breed of pigs.

We observed that the expression of *SLC2A4*, *INSR* and *NR3C1* genes in muscles and also the *CBG* gene in liver reflects alterations in fat accumulation in the muscle tissue during the development and in genetically different pigs.

Age effect. The developmental stage exerts a considerable influence on lipids content, composition and oxidative stability in Guizhou mini-pigs (Yang et al. 2010) in which lipid content decreased from day 90 to day 180 and increased afterwards in both adipose tissue and muscle. Other study reported a constant increase in intramuscular lipids content as pigs grew (Bosch et al. 2012). This is in accordance with the previous observation that IMF increased from day 120 to day 180 in muscle tissue (Tyra et al. 2013). In our study we observed increasing levels of glucose

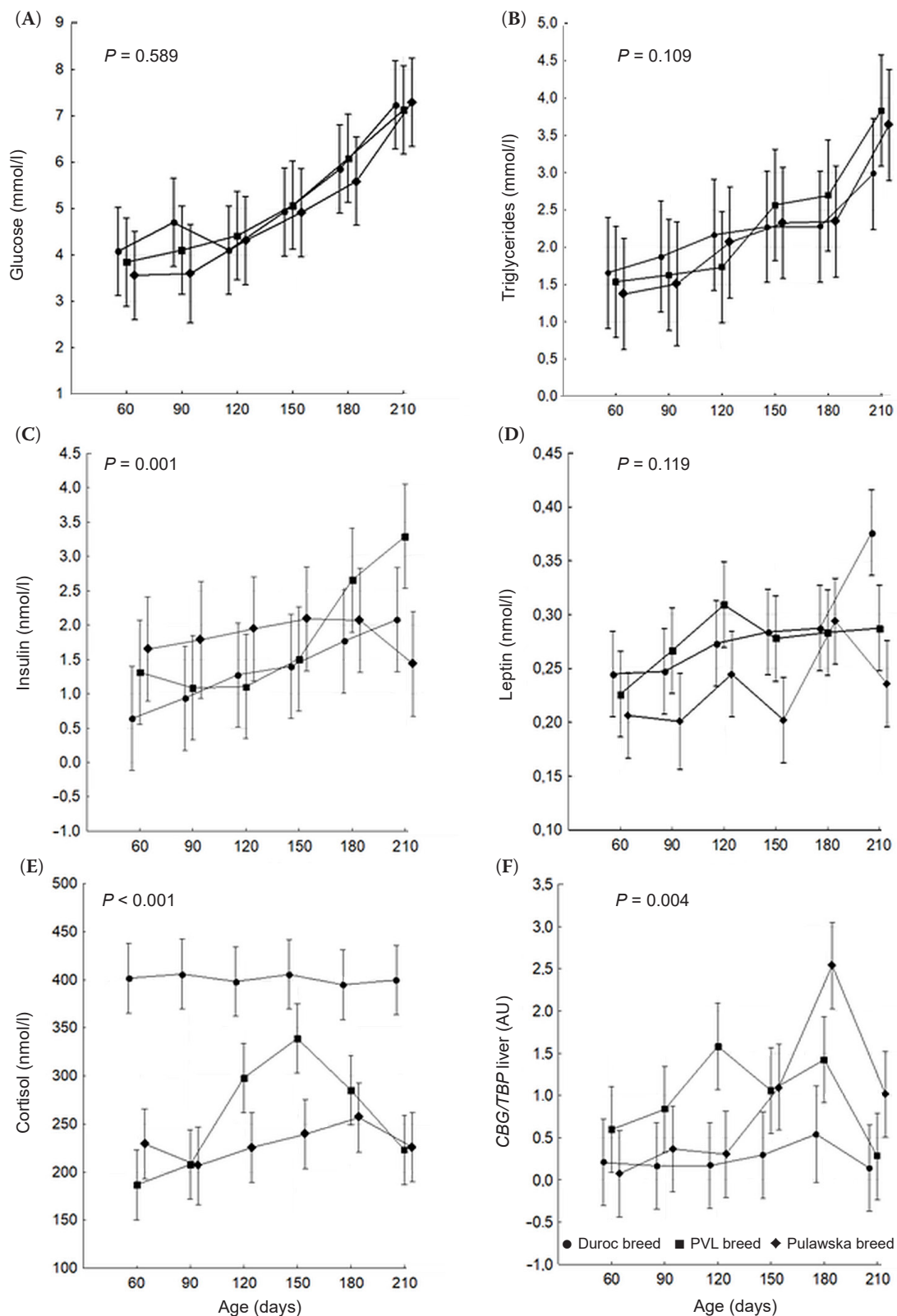


Figure 4. Effect of the interaction between breed and age on (A) glucose, (B) triglycerides, (C) insulin and (D) leptin serum levels (MANOVA, Least Squares Means with 95% confidence intervals). The effect of the interaction between breed and age significantly influences (E) cortisol serum level and the changes of (F) CBG gene expression in liver (arbitrary units) (MANOVA, Least Squares Means with 95% confidence intervals)

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and triglycerides with age, independent of breed that indicates reduction in insulin sensitivity during ontogenesis (Larsen et al. 2001). The previous reports also showed increased serum insulin and leptin levels with age and that the leptin mRNA expression in adipose tissue increased with age in breed-specific manner (Zhou et al. 2004) and this is consistent with our findings that insulin and leptin levels increased with age.

Glucose transporter 4 (GLUT-4) is the main glucose transporter expressed in skeletal muscle that plays a key role in cellular glucose uptake and is stimulated by insulin (Hou and Pessin 2007). Upon insulin stimulation, the insulin receptors (INSR) undergo tyrosine phosphorylation and regulate glucose uptake in muscle (Saltiel and Pessin 2002). Previous study (Zuo et al. 2010) reported a decrease in *SLC2A4* and *INSR* expression in porcine skeletal muscle by day 7 and a significant increase by day 30 suggesting that *SLC2A4* plays a role in glucose metabolism in muscle tissue during the pig development. Moreover, the developmental changes in the *SLC2A4* expression coincided with these observed for *INSR* expression which was also confirmed in our study, as the highest expression of these genes in muscles was observed in days 120–180 when pigs are rapidly growing and increase the amount of fat in skeletal muscle tissue. This is consistent with the previous study (Bosch et al. 2012) who reported that IMF content increased in days 160–220 of age in muscle tissue in pigs. Moreover, the most recent data showed an increased expression of *GLUT-4* and *GLUT-2* in Tibetan and Yorkshire pigs during the first 6 months of ontogenesis (Liang et al. 2015).

The glucocorticoid receptor (GR) mediates catabolic effects of glucocorticoids (GCs) thus regulating energy metabolism in many tissues including muscles. It was shown that *NR3C1* expression in skeletal muscle in diabetic patients correlated with the degree of insulin resistance with normalization of GR levels following treatment (Vestergaard et al. 2001). Dysregulated GR signaling resulting from elevated GCs activity contributes to muscle catabolism and insulin resistance in several metabolic disorders (Vegiopoulos and Herzig 2007). On one hand, GCs inhibit glucose uptake and glycogen synthesis, but on the other hand, they suppress protein synthesis and enhance protein degradation and amino acid export and these processes are regulated by the insulin/IGF 1

signaling pathway (Glass and Czikk 2005). The ability of GCs to reduce ligand-induced insulin receptor (IR) phosphorylation in skeletal muscle was reported in an animal model (Giorgino et al. 1993) after dexamethasone administration in rats. In our study, an increased expression of *NR3C1* in both muscle tissues with age implies that glucocorticoid action may be enhanced in the growing skeletal muscle thus suppressing glucose uptake.

The effect of GR expression and signaling is affected by cortisol binding protein (CBG) that influences the bioavailability as well as metabolic clearance rate of cortisol within the circulation (Bright 1995). The amount of cortisol taken up by target tissue is estimated by the non-CBG bound (free and bound with albumin) fraction of total circulating steroid. A developmental increase in CBG has been demonstrated previously in the rat (Smith and Hammond 1991), whereas the decrease in CBG with accompanying unaltered cortisol levels indicates a higher amount of biologically available free cortisol that in turn decreases fat accumulation in muscle due to reduced glucose uptake. Increased *CBG* expression between days 150 and 180 in our study may reduce free cortisol thus increasing *INSR* signaling and glucose uptake that subsequently stimulates fat accumulation in muscles at this age. This is in accordance with a previous study (Ousova et al. 2004) reporting a positive correlation between the binding capacity of CBG and fat content, and a negative correlation with muscle mass in two pig breeds in France. However, this was not confirmed in the other study (Geverink et al. 2006) where no major effect of CBG level on fat or muscle content in five European breeds of pigs was found.

Breed effect. Breed is an important factor determining the ability to store lipids in the muscle, with IMF values ranging from 1.2% in Pietrain pigs to 9% in Iberian swine (Casellas et al. 2013). This is not only due to differences in the management and feeding, but also to the specific genetic background of each breed. For instance, a comparison of Berkshire, Duroc, Tamworth and Large White pigs fed with the same standard diet showed differences in their IMF deposition rate (Berkshire > Duroc > Tamworth > Large White) (Wood et al. 2008).

The growth rate of intramuscular fat relatively to carcass weight is lower than in subcutaneous adipose tissue. The development of intramuscular

adipose tissue seems to be determined at an early stage, before 20 kg of body weight, especially in lean breeds such as Pietrain (Kouba and Sellier 2011). Analyzing other fat depots, some breeds (Pietrain, Duroc) do not show a linear gradient of increasing ratio of intermuscular to subcutaneous fat with decreasing carcass fatness. In this regard, the DU pigs exhibit much higher intermuscular fat than could be expected from their overall fatness (Wood et al. 2004) and develop large amount of intermuscular fat resulting in a high ratio of intermuscular to subcutaneous fat (Kouba and Sellier 2011).

Results in this study showed that Duroc has the highest expression of *SLC2A4* and *INSR* in muscles during development thus indicating insulin-stimulated glucose uptake. This correlated positively with higher ability to accumulate intramuscular fat in comparison to the other analyzed breeds as showed previously (Tyra et al. 2013). In addition, DU pigs showed the highest level of serum cortisol and the lowest expression of *CBG* in liver, suggesting enhanced GR signaling in this breed resulting in reduced glucose uptake by inhibiting *INSR* signaling and *SLC2A4* translocation to the cell surface (Weinstein et al. 1998). This is of particular importance as fat deposition may affect the amount of IMF and fatness parameters in pigs further influencing not only meat quality, but also animal health. An increased tendency to accumulate fat in muscles may contribute to insulin resistance, and it seems that Duroc pigs are at the highest risk in comparison to the other breeds analyzed in this study. However, this is our speculation how results can be interpreted and requires further investigation, but indicates the direction of potential future studies.

PUL pigs showed similar IMF content as DU, but had the highest subcutaneous fat level in comparison to the other breeds (Tyra et al. 2013). Interestingly, these pigs showed a decreased expression of *SLC2A4*, *INSR* and *NR3C1* genes in muscle tissue after day 180, which coincided with the decrease in insulin and leptin level at the same age and this correlation was not observed for the other breeds. This may indicate that older PUL pigs show decreased glucose uptake and lipogenesis and thus lower fat content in muscles in comparison to the other breeds. This breed also showed the highest decrease in *CBG* expression in liver after day 180 suggesting that at this age increase of free cortisol levels may be responsible for reduced glucose uptake and fat deposition in

muscles. This confirms the previous observation that the rate of IMF deposition significantly slowed down after day 180, especially in relation to the rate of muscle tissue deposition (Tyra et al. 2013).

PLW pig is characterized by lower IMF content in comparison to DU and PUL breeds (Tyra et al. 2013). This breed has also higher basal cortisol level and higher GR expression than other breeds suggesting that muscle catabolism is increased in those pigs. Our observation demonstrated a significant increase in cortisol serum level until day 150, followed by a rapid decrease with a parallel increase in *CBG* expression in liver. This age was also characterized by decreased leptin level and increased insulin level thus indicating that lipogenesis and fat accumulation in muscle tissue occurs faster in PLW than in the other analyzed breeds.

CONCLUSION

Our results indicated that the developmental stage and breed significantly influence the expression of genes involved in energy metabolism in muscle tissue. Moreover, we found that the interaction between breed and age significantly influences the *CBG* gene expression in liver. In conclusion, the analyzed genes participate in the intramuscular fat accumulation in different breeds during ontogenesis.

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