Effect of g.2728G>A and g.3996T>C Polymorphisms at the Leptin Gene Locus on Microstructure and Physicochemical Properties of longissimus lumborum Muscle of Polish Landrace Pigs*

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The influence of HindII (g.2728G>A) and BglII (g.3996T>C) polymorphisms at the leptin gene locus on muscle fibre characteristics and meat quality of longissimus lumborum muscle was studied in 146 barrows of the Polish Landrace breed. Leptin gene polymorphism was identified by PCR-RFLP. Fibre type percentage, fibre diameter and the following technological parameters of meat were also determined: pH<sub>1h</sub>, pH<sub>24h</sub>, L<sub>a</sub>, a<sup>b</sup>, b<sup>c</sup> colour, drip loss, water holding capacity, shear force and intramuscular fat content. Polymorphism was not detected in the locus studied in the Landrace pig herd analysed with the BglII restriction enzyme (g.3996 T>C). For the HindIII enzyme (g.2728G>A), there was a high frequency of GG homozygotes (0.78) and G allele (0.89), but the AA genotype was not present. Moreover, the genotypes of leptin gene RFLP-HindIII polymorphism had no effect on intramuscular fat content and muscle fibre type percentage, but had a significant effect on muscle fibre size. Heterozygous GA fatteners had a significantly larger (P<0.05) diameter of type IIb and type I fibres compared to homozygous GG fatteners. Generally, meat quality parameters were comparable among the examined genotypes except for water holding capacity (which was the lowest for the GG genotype) and colour lightness (L<sub>a</sub>) (which was the lightest for GA genotype). Moreover, regardless of genotype, large differences were observed between each animal in the distribution of intramuscular fat.

Key words: leptin gene polymorphisms, microstructure, meat quality, pig.

Meat quality parameters in farm animals, determined by both environmental and genetic factors, are of economic importance to breeders. The advances in molecular biology over the last few decades have made it possible to identify genes regulating feed intake, growth, meat quality or body fat content. Breeders use two methods for identification of quantitative trait loci (QTL) — candidate genes and genome scanning (ROTHSCHILD 2003). Out of over 20 candidate genes associated with growth and especially with fatness, a mention should be made of leptin, which in addition to leptin, agouti and carboxipeptidase E receptors is part of the central melanocortin system, which plays a key role in maintaining the body’s energy homeostasis (BOLZE & KLINGENSPOR 2009; ŚWIOTOŃSKI et al. 2010). Leptin is a protein secreted from white adipocytes and results in a decrease in feed intake, loss of body weight, loss of fat depots and an increase in energy metabolism. In pigs, several polymorphisms were described for the leptin gene at the positions 867C>T, 1112A>G, 3469C>T, 3714G>T, A2845T, 3996T>C and 2728G>A (STRATIL et al. 1997; JIANG & GIBSON 1999; KENNES et al. 2001). The 3469T>C polymorphism was observed to have a significant effect on the fat depth and daily weight gain in Large White and Landrace pigs (JIANG & GIBSON 1999; KENNES et al. 2001), and on the lean meat content in Landrace pigs (KULIG et al., 2001). SZYDŁOWSKI et al. (2004) observed a weak association between the 3469T>C polymorphism and intramuscular fat content in Polish Large White pigs and loin weight in the synthetic line 990. The 2845A>T polymor-

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phism may be associated with feed intake in Landrace pigs (KENNES et al. 2001).

The relationship between polymorphism of the leptin gene and histological characteristics (muscle microstructure) is still not clear. It is well known that many characteristics of muscle tissue such as size, proportion of muscle fibres, collagen and intramuscular fat content directly affect meat quality (RYU & KIM 2005; LEPETIT 2008; OSHIMA et al. 2009; YANG et al. 2010). Therefore, the purpose of this paper is to evaluate the polymorphism at the leptin gene locus of Polish Landrace pigs and their relationship with histological characteristics of muscle and several other traits that affect meat quality.

Material and Methods

A total of 146 Polish Landrace barrows (only CC genotypes in analysis of mutation g.1843C>T in the ryanodine receptor 1 gene) were kept in individual pens equipped with automatic drinkers and fed ad libitum. All animals were reared under the same environmental and production regime. Animals were fattened from 50 to 105 kg body weight using a complete diet containing 13.3 MJ metabolizable energy and 161 g crude protein. This level was calculated based on diet composition, using chemical composition we used a modified combined method of NADH-tetrazolium reductase activity and immunohistochemical determination of slow myosin heavy chain on the same section. First, sections were air-dried for 1 h and incubated with monoclonal antibodies against skeletal slow myosin heavy chain was performed for 1 h at RT (DUBOVITZ et al. 1973). Next, on the same section, immunohistochemical staining with monoclonal antibodies against skeletal slow myosin heavy chain was performed for 1 h at RT (clone WB-MHCs Leica, Germany, dilution 1:80). The reaction was visualized by the Novolink™ Polymer Detection System (Leica, Germany), according to the manufacturer’s instruction. Finally, all sections were dehydrated in a graded series of ethyl alcohol, cleared in xylene and mounted in DPX mounting medium (Fluka, Buchs, Switzerland). On the same section three different fibre types were recognized, namely, type I (red, slow twitch oxidative) which were blue-brown granulated; blue granulated type IIA (intermediate, fast-twitch oxidative glycolytic); and unstained type IIB (white, fast-twitch glycolytic) fibres. A minimum of 300 fibres were counted in each section using a NIKON E600 light microscope.

Moreover, frozen sections were also stained with Oil red O (Sigma, Germany) to evaluate lipid content. Intramuscular fat content and distribution was evaluated based on 10 randomly chosen areas (1000 m²) in each preparation.

The percentage and diameter of muscle fibre types and percentage of intramuscular fat were quantified with an image analysis system using the Multi Scan v. 14.02 computer program.

The samples for meat quality assessment were taken from the right carcass side from the m. longissimus lumborum at the level of the 5th lumbar vertebra and deep within the muscle. Muscle samples were immediately cut into 1 cm³ pieces (parallel to the muscle fibres) and frozen in isopentane that was cooled using liquid nitrogen and the samples were stored at -80°C until histochemical analyses were performed. Samples were mounted on a cryostat chuck with a few drops of tissue-freezing medium (Tissue-Tek; Sakura Finetek Europe, Zoeterwoude, The Netherlands). Transverse sections (10-μm thick) were cut at -20°C in a cryostat (Slee MEV, Germany). To determine the muscle histochemical composition we used a modified combined method of NADH-tetrazolium reductase activity and immunohistochemical determination of slow myosin heavy chain on the same section. First, sections were air-dried for 1 h and incubated for 1 h at 37°C with medium for the determination of NADH-TH (DUBOVITZ et al. 1973). Next, on the same section, immunohistochemical staining with monoclonal antibodies against skeletal slow myosin heavy chain was performed for 1 h at RT (clone WB-MHCs Leica, Germany, dilution 1:80). The reaction was visualized by the Novolink™ Polymer Detection System (Leica, Germany), according to the manufacturer’s instruction. Finally, all sections were dehydrated in a graded series of ethyl alcohol, cleared in xylene and mounted in DPX mounting medium (Fluka, Buchs, Switzerland). On the same section three different fibre types were recognized, namely, type I (red, slow twitch oxidative) which were blue-brown granulated; blue granulated type IIA (intermediate, fast-twitch oxidative glycolytic); and unstained type IIB (white, fast-twitch glycolytic) fibres. A minimum of 300 fibres were counted in each section using a NIKON E600 light microscope.

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The samples for meat quality assessment were taken from the right carcass side from the longis-
**Results and Discussion**

In the present study, we identified g.2728G>A and g.3996T>C polymorphisms at the leptin gene locus of the Polish Landrace population of pigs using HindIII and Bg/II restriction enzymes. Analysis of the genetic structure of Landrace pigs using Bg/II restriction enzyme (g.3996T>C) revealed no polymorphism at the locus studied. All the pigs were TT homozygotes.

The identification of g.2728G>A polymorphism using HindIII enzyme showed a high frequency of GG homozygotes (0.78) and the G allele (0.89), but no presence of the AA genotype. An earlier study by KENNES et al. (2001), which analysed this mutation in Duroc, Landrace and Yorkshire populations showed equally high G allele frequencies of 0.91, 0.85 and 1.0, respectively.

Identification of the RFLP-HindIII polymorphism made it possible to investigate its possible influence on the composition of muscle fibres and quality parameters of the meat from the analysed fatteners.

The effect of genotypes of the leptin gene on muscle fibre traits of *m. longissimus lumborum* of Polish Landrace pigs is shown in Table 1 and Fig. 1A-B.

The data revealed that the genotypes of the leptin gene had no effect on muscle fibre type percentage, but it affected the size of muscle fibres. Both type I and type IIB fibres from pigs with the GA genotype were characterized by considerably greater diameter compared to GG fatteners. These results were not confirmed by the literature data. However, the differences found in the present study in the diameter of muscle fibres of both types indicate a higher gain in muscle tissue of *m. longissimus lumborum* in GA genotype pigs compared to GG pigs. This was also indicated by KENNES et al. (2001), who showed that LEP/HindIII polymorphism is associated with the growth rate of fattened animals. In addition, the greater muscle fibres in GA pigs found in the present study would indicate that these animals have a greater loin eye. It must be remembered, however, that loin eye size is determined not only by the size but also by the amount of muscle fibres. The increase in the diameter of muscle fibres, especially white fibres (IIB) with glycolytic metabolism, is connected with the increase in carcass meat content and lower fat content. On the other hand, it contributes to poorer meat quality, as reflected in a decreased number of capillaries and the associated muscle oxygen deficiency and increased production of lactic acid during glycolysis (RUUSUNEN & PUOLANNE 2004). This may ultimately be indicative of poorer meat quality in heterozygous (GA) animals.

The intramuscular fat content of meat is an important factor to be considered when evaluating the consumption quality of meat. DASZKIEWICZ et al. (2005) reported that a certain amount of intramuscular fat, which results in the marbling of meat and relaxation of connective tissue, is essential for the sensory traits of meat to be favourable. The results of the present analysis did not show a significant effect of the leptin gene g.2728G>A polymorphism on the intramuscular fat content (IMF) of *m. longissimus lumborum* from Polish Landrace pigs (Table 1). Meanwhile, KENNES et
al. (2001) suggest that lower frequency of the A allele (0.09) in Duroc pigs is related to the highest fatness of animal carcasses (0.66) compared to Landrace pigs, in which the frequency of this allele was 0.15 and carcass fatness level was 0.20. Different results were obtained by JIANG & GIBSON (1999), who studied C/c174T substitution at position 3469 (exon III) of the leptin gene, identified by STRATIL et al. (1997). Experiments with four different populations of pigs (Duroc, Hampshire, Landrace, Large White) showed a relationship between higher frequency of the C allele and lower fatness of Large White pigs. Meanwhile, KENNES et al. (2001), who studied the same polymorphism at the leptin gene locus obtained results suggesting that in the group of analysed animals (Duroc, Landrace, Yorkshire) the higher frequency of the C allele was responsible for greater carcass fatness. This indicates that the inconsistency of the mentioned results may be due to differences in the gene pools of the analysed populations (breeds), as evidenced by different genotype effects. It should also be recalled that these mutations are localized in the non-coding regions of the leptin gene. 3’UTR is an untranslatable region, but it is conjectured that these mutations may determine mRNA stability or translation efficiency, although this hypothesis needs to be confirmed. They may occur as molecular markers conjugated with specific regions controlling growth and feed intake (KENNES et al. 2001). These mutations may also determine the level of gene expression, as reported by TE PAS et al. (1999), who showed that point mutation in the 3’ region of the myogenin gene has a significant effect on carcass meat content. It is also important that the quantity and quality of fat raw material results from the interaction between genetic and many other factors such as the animal’s age and sex, management and feeding conditions, and location of adipose tissue. The latest research (VILLALBA et al. 2009) on g.3469C>T polymorphism confirms the association between the leptin gene polymorphism and the level of carcass fatness according to the age of pigs and location of adipose tissue. Based on the measurements of Duroc pigs at different developmental stages, it was found that the genotype effect changes during individual growth and thus animal’s age may influence the effect being estimated. In the investigated population, it was found that in the case of g.3469C>T leptin gene polymorphism, 160-day-old TT pigs had a 0.096 mg/kg higher intramuscular fat content compared to TC pigs, whereas at 220 days TT pigs had a 1.64 mg/kg lower intramuscular fat content compared to TC animals. In addition, GARDAN et al. (2006) discovered that the leptin

### Table 1

Percentage (%) and diameter (µm) of muscle fibre types and physico-chemical properties of *longissimus lumborum* muscle as related to leptin gene polymorphism

<table>
<thead>
<tr>
<th>Item</th>
<th>GG N=114</th>
<th>GA N=32</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibre type percentage (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>71.09</td>
<td>71.53</td>
<td>0.92</td>
<td>0.37</td>
</tr>
<tr>
<td>IIA</td>
<td>15.27</td>
<td>14.38</td>
<td>0.59</td>
<td>0.25</td>
</tr>
<tr>
<td>I</td>
<td>13.64</td>
<td>14.09</td>
<td>0.63</td>
<td>0.08</td>
</tr>
<tr>
<td>Fibre diameter (µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>56.68</td>
<td>65.26</td>
<td>0.79</td>
<td>0.02</td>
</tr>
<tr>
<td>IIA</td>
<td>42.49</td>
<td>44.32</td>
<td>1.12</td>
<td>0.14</td>
</tr>
<tr>
<td>I</td>
<td>47.45</td>
<td>55.93</td>
<td>0.63</td>
<td>0.03</td>
</tr>
<tr>
<td>pH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>6.68</td>
<td>6.71</td>
<td>0.02</td>
<td>0.31</td>
</tr>
<tr>
<td>pH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>5.63</td>
<td>5.65</td>
<td>0.01</td>
<td>0.47</td>
</tr>
<tr>
<td>Colour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L&lt;sup&gt;*&lt;/sup&gt;</td>
<td>53.07</td>
<td>54.26</td>
<td>0.68</td>
<td>0.04</td>
</tr>
<tr>
<td>a&lt;sup&gt;*&lt;/sup&gt;</td>
<td>14.35</td>
<td>14.18</td>
<td>1.19</td>
<td>0.69</td>
</tr>
<tr>
<td>b&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.57</td>
<td>4.08</td>
<td>0.84</td>
<td>0.12</td>
</tr>
<tr>
<td>Drip loss (%)</td>
<td>2.63</td>
<td>2.48</td>
<td>0.31</td>
<td>0.38</td>
</tr>
<tr>
<td>WHC (%)</td>
<td>37.62</td>
<td>39.78</td>
<td>0.34</td>
<td>0.02</td>
</tr>
<tr>
<td>Shear force (kg/cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>6.64</td>
<td>6.35</td>
<td>0.63</td>
<td>0.09</td>
</tr>
<tr>
<td>IMF %</td>
<td>1.72</td>
<td>1.86</td>
<td>0.27</td>
<td>0.42</td>
</tr>
<tr>
<td>IMF-H %&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.23</td>
<td>1.39</td>
<td>0.41</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<sup>1</sup> Intramuscular fat content evaluated histochemically
structure of pig muscles, that lipids are also deposited in type I and IIA fibres, i.e. in fibres with higher oxidative capacity, and to a lesser extent in white fibres (type IIB) with glycolytic metabolism.

The comparison of meat quality traits (Table 1) did not reveal differences in pH\textsubscript{L90min} and pH\textsubscript{2.8}, between the analysed genotypes. Our results are similar to those reported by BORGES & GOULART (2002), who identified g.3469T>C polymorphism in the populations of Landrace, Large White and Pietrain pigs but also did not find the effect of animal genotype on pH of meat. In addition, in the present study, no significant differences in shear force values were identified between GA and GG genotypes. In the literature there are only a few studies on the influence of polymorphism at the leptin gene locus on meat quality of pigs. An earlier study with two sheep populations (Dorset, Suffolk) revealed the association between 103A>G polymorphism at the leptin gene locus and greater tenderness of \textit{longissimus muscle} in the Suffolk breed (BOUCHER et al. 2006). On the other hand, marked differences were noted in the meat colour as affected by genotypes. \textit{L*} values were higher in GA than in GG genotypes, which suggests poorer quality of meat raw material from GA pigs. The lighter meat colour in GA pigs is probably related to the greater diameter of type IIB fibres in these animals. This is confirmed by LARZUL et al. (1997) and RYU & KIM (2005), who showed positive correlations between parameters of \textit{L*} colour and parameters of type IIB muscle fibres. Meanwhile, BORGES & GOULART (2002), who identified 3469T>C polymorphism in the leptin gene in Landrace, Large White and Pietrain pigs, showed no relationship between animal genotype (TT, TC) and meat colour. This may be due to the fact that meat colour, like other meat traits, is affected by several factors such as IMF content, pH and water binding in muscle, the interaction of which determines the variation of this parameter.

Some of the major quality traits of pork, important for technological processing, are water holding capacity and drip loss. In our study we did not find the effect of polymorphism, identified using \textit{HindIII}, on drip loss. Significantly higher water holding capacity was found, however, in \textit{m. longissimus lumborum} of pigs with the GA genotype compared to GG animals. SAFFLE & BRATZLER (1959) hold that muscles with a higher content of intramuscular fat have higher water holding capacity, which is probably due to the relaxation of muscle fibre microstructure by intramuscular fat, which allows for higher water activity in tissue (HAMM 1960). This finding is reflected in the results of the present study, in which GA pigs showed higher water holding capacity in \textit{m. longissimus lumborum} and a tendency towards greater deposition of intramuscular fat compared to GG pigs.

**Fig. 1.** Exemplary cross-sections of \textit{m. longissimus lumborum} of Polish Landrace fatteners: NADH-TH and immunohistochemical MHC\textsubscript{slow} staining: I – red fibres; IIA – intermediate fibres; IIB – white fibres; A – genotype GA; B – genotype GG; C, D – intramuscular fat between muscle bundles (*); E – inside muscle bundles (†); F – intracellular fat (▲). Scale bar 50 \(\mu\text{m}.\)
Analysis of the microstructure and technological parameters of ML in the pigs studied revealed the effect of g.2728G>A polymorphism at the leptin gene locus on muscle fibre diameter and meat colour and water holding capacity. The results obtained do not provide a basis for definite conclusions because each of the analysed traits is most often modulated by the interaction of a pool of different genes and environmental factors. It is also possible that the leptin gene may influence other muscle parameters for which no genotype effect was found.

References


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