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Expression of contractile protein-encoding genes *TPM2* and *TNNT3* during ontogenesis in pigs raised in Poland*

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Troponin is a regulatory proteins complex composed of subunits TnC, TnI, and TnT encoded by separate genes – *TNNT1*, *TNNT2* and *TNNT3*. It is a component of thin filaments (along with actin and tropomyosin), to which calcium binds to accomplish this regulation. The *TPM2* gene encodes beta-tropomyosin. An *imprinted* QTL for muscle mass deposition has been detected near the *TNNT3* gene and several significant linkages between *TPM2* and QTLs on chromosome 1 have been identified. However, no significant correlation was found between polymorphism in both genes and economically important pig traits. The study aimed at analysing the level of expression of *TNNT3* and *TPM2* in the developing muscle (day 60-210 of life) and determining the expression differences among Polish Large White, Polish Landrace, Pulawska, Duroc and Pietrain pigs. Within the mentioned period the expression level of both genes in question did not change significantly. No developmental pattern characteristic for all breeds was revealed. In PL gilts a highly significant correlation was found among the level of expression in both white muscles (*longissimus dorsi* and *semimembranosus*) and the animals' maternal origin. The above results suggest that an unknown polymorphism, probably located in regulatory part of the gene has an effect on the level of *TNNT3* expression. Expression of the *TPM2* did not change during ontogenesis and was not correlated with maternal origin. However, significant differences among gilts of different breeds were identified.

KEY WORDS: gene expression / muscle development / TNNT3 / TPM2 / pigs

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Muscle fibre is composed of contractile proteins packed into thin and thick filaments. The thin filaments are composed of actin, troponin and tropomyosin molecules, with one troponin complex and one tropomyosin molecule for every seven actin molecules (A7TmTn) – Gong *et al.* [2005] Troponin molecules, each of which has three subunits (C, I and T) of different properties are attached to tropomyosin. Troponin C binds calcium ions, troponin I is responsible for interaction with actin, and troponin T anchors the troponin complex to tropomyosin and actin. Tropomyosin and the troponin complex constitute about one third of the mass of the thin filament [Stryer 1997].

Tropomyosin is a contractile protein that blocks the interaction of actin and myosin in the muscle contraction process. It is composed of two similar subunits (α and β) that form a helical structure. Tropomyosin 2(β) is encoded by the *TPM2* gene located in pigs in chromosome 1 (q23-q27 region) – Sherwood *et al.* [2002]. To date no significant correlation between *TPM2* polimorphism and pig productive traits was observed, although Kim *et al.* [2000] found QTLs on chromosome 1 near *TPM2 locus*.

Troponin T is composed of a single polypeptide chain (250-300 bp). In vertebrates, it occurs in three tissue-specific forms: fast skeletal TnT, slow skeletal TnT and cardiac TnT [Perry 1998, Chaudhuri *et al.* 2005]. The slow skeletal TnT isoform is characterized by higher Ca^{2+} sensitivity than does the fast skeletal TnT [Jin *et al*. 2003]. Each isoform is encoded by a separate gene (in humans *TNNT3*, *TNNT1* and *TNNT2*, respectively) – Mao *et al.* [1996]. Variation of fast TnT sequences in species that have been analysed to date (rat, chicken and rabbit) occurs within the N-end of the molecule and in a short 14-amino acid sequence near the C-end. Multiple isoforms are generated by alternative splicing of the *TNNT3* gene [Perry 1998, Stefancsik *et al.* 2003, Chaudhuri *et al.* 2005]. Troponin T is degraded rapidly by chymotrypsin and other proteolytic enzymes, resulting in two fragments [Grabarek 2001]. This trait affects meat quality in fattening animals [Christensen *et al*. 2003**]**

The human gene encoding fast skeletal TnT was mapped to chromosome 11 (11p.15.5) – Mao *et al.* [1996], at a distance of 100 kbp from the paternally imprinted H19 gene [Yuan *et al.* 1996]. Moreover, the *TNNT3* promoter contains binding sites for *MyoD* and *MEF-2* transcription factors [Firulli *et al*. 1997]. The *human* fast skeletal troponin T (*TNNT3*) gene shows high homology with that of other species [Wu *et al.* 1994]. It may be supposed, therefore, that structure of the gene in pig is similar.

Data on the *TNNT3* gene in pigs are scarce. It maps to the short arm of chromosome 2 at 14-17 (2p14-17), but its full nucleotide sequence has not been adequately studied. To date, a fragment of about 250 bp containing part of exons 14 and 15 and intron 14 has been sequenced with several mutation changes: insertions of three-nucleotide sequence CCG and three single nucleotide polymorphisms (SNPs) – Davoli *et al.* [2002]. No significant correlations were revealed between these changes and productive traits in pigs [Brym *et al.* 2006]. However, Davoli *et al.* [2003] when working on the PigMap project identified an *imprinted* quantitaitve trait *locus* (QTL) for muscle mass deposition near the *TNNT3* gene.

In muscles of 5-6-month old Landrace × Large White × Duroc crossbreds as many as eight isoforms of the *TNNT3* gene encoding fast skeletal TnT were expressed differing in the presence or absence of exons 4, 5, 6, 7, 16 and 17 [Kitamura *et al.* 2005]. In 2007 the LongSAGE method was used to show expression differences in *TNNT3* and *TPM2* genes in the muscle (*biceps femoris*) between 90-day old Landrace and Tongcheng fetuses (90 days *post coitus*) – Tang *et al.* [2007]. A higher transcript level for both genes in question was found in the Tongcheng pigs. Because the expression of *TNNT3* and *TPM2* genes has never been studied in postnatal pigs, it seemed interesting to consider this issue.

Material and methods

A total of 180 Polish Large White (PLW), Polish Landrace (PL), Puławska, Duroc and Pietrain gilts were raised at the Pig Progeny Testing Station, Pawłowice under the same environmental and feeding conditions. Pigs were assigned to 6 groups (6 gilts of each breed per group) according to the age at slaughter: 60, 90, 120, 150, 180 and 210 days. Within each breed group the gilts shared the same sire (except Puławska pigs, which had three sires), and their mothers were sisters. All the animals were resistant to stress (*RYR1* CC) except Pietrain pigs, which were of the CC and CT genotype.

The level of *TNNT3* and *TPM2* gene expression was determined in *longissimus dorsi* and *semimembranosus* muscles. Muscle samples were collected 20 min *post mortem* and frozen in liquid nitrogen.

RNA isolation was performed using TRI reagent (SIGMA ALDRICH) according to the method of Chomczyński [1993]. Samples were homogenized using a Silent Crusher S homogenizer (HEIDOPH). Quantitative and qualitative evaluation of the isolated ribonucleic acid was performed with a BioPhotometer spectrophotometer (EPPENDORF) and in 2% agarose gel.

1 μg of RNA was transcribed into cDNA at 37°C using a High Capacity cDNA Reverse Transcription Kit (APPLIED BIOSYSTEMS) according to the protocol given below.

Table 1. Sequences of primers and probes used for expression analysis of *TNNT3* and *TPM2* genes

Gene	Primer	Probe
TNNT3	F – gag gac gac ctg aag aaa aag aag R – ttg gcc agg tag ctg ctg ta	FAM – cgc tgt cct cca tgg gcg c – TAMRA
TPM2	F – gga aaa gta ttc cga atc agt gaa g R – tga atg cgg cgg ttc ag	FAM – ctg aaa gaa ggc cac cga cgc c – TAMRA

Primers and probes for *TNNT3* and *TPM2* (Tab. 1) were designed using PRIMER EXPRESS software and synthesized by APPLIED BIOSYSTEMS. The *GAPDH* gene was used as the endogenous control [Van Laere *et al.* 2003].

Relative quantification (RQ) of the transcript level of the genes studied was performed with 7500 Real-Time PCR System using TaqMan® TAMRA probes labelled with FAM and TaqMan® Universal Master Mix. Reactions for each sample were carried out in two replicates in 50 µl volume according to the following protocol: incubation for 2 min at 50°C and 10 min at 95°C (activation of AmpliTaq polymerase), 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/ elongation). The results were analysed using Sequence Detection System software v. 2.0 (APPLIED BIOSYSTEMS). Statistical verification was performed using the General Linear Model (SAS) with origin (dam) and breed as factors. The RQ of *TNNT3* and *TPM2* of white muscle was estimated as mean of RQ of *longissimus dorsi* and *semimembranosus* muscles.

Results and discussion

The study involved gilts of five breeds – Polish Large White (PLW), Polish Landrace (PL), Pulawska, Duroc and Pietrain, differing in muscularity. PLW and PL are the most popular breeds in Poland. They have similar muscling and are used as maternal breeds, which is why the principal selection pressure was placed on number of piglets per litter and rearing traits [Milewska 2006]. Duroc and Pietrain pigs, used by Polish breeders as a father component, were selected for high lean meat content. Pietrain pigs are characterized by excellent muscling but have low daily gain and are vulnerable to stress that leads to low quality PSE meat. The Durocs are opposite to Pietrains and are characterized by higher fatness, high daily gain and resistance to stress [Liu 2005]. Puławska pigs is a conservation breed. They show the highest fatness of all the breeds considered in this study and produce good quality meat [Szyndler-Nędza *et al.* 2008].

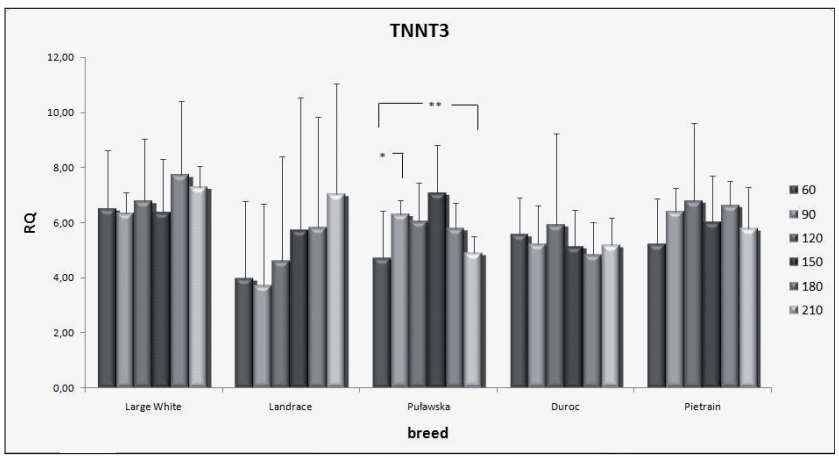

Fig. 1. Relative quantification (RQ) of *TNNT3* transcript level in porcine white skeletal muscle from day 60 to 210 of fattening. *P<0.05, **P<0.01.

In PLW, PL, Pietrain and Duroc gilts the level of *TNNT3* gene expression was not found related to the age of animals. Only in the Puławska gilts there was initially a significant increase in the transcription level, which peaked at 150 days of age and started to decline thereafter (Fig. 1). In Puławska gilts, expression of the *TNNT3* gene did not depend on the maternal origin of the pigs studied (Fig. 2).

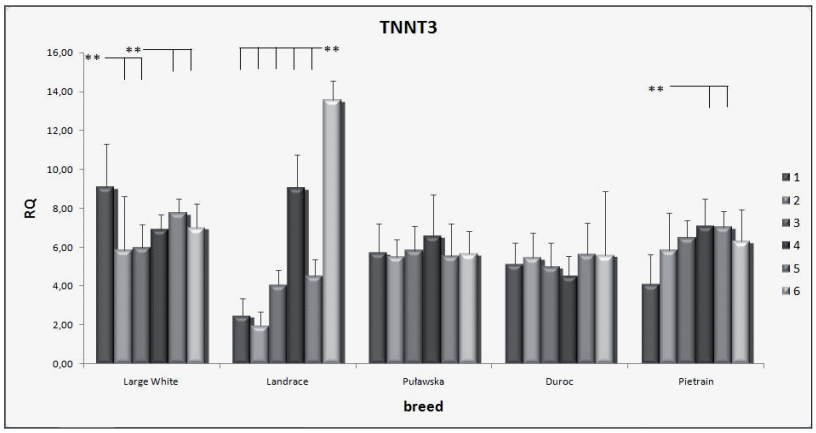

Fig. 2. Relative quantification (RQ) of *TNNT3* transcript level in porcine white skeletal muscle as related to animal origin (1-6 – consecutive pairs of parents). *P<0.05, **P<0.01.

Comparison of different breeds at the same age revealed significantly higher level of *TNNT3* transcript in the youngest 60-days old PLW than in youngest PL (P<0.01), Puławska and Pietrain gilts (P<0.05). Similarly, at the age of 180 and 210 days PLW pigs displayed the highest level of *TNNT3* transcript. In 90-days old gilts - the lowest level of *TNNT3* gene expression was observed in PL compared to gilts of other breeds (P<0.01 for Puławska and Duroc, P<0.05 for PLW) – Figure 3.

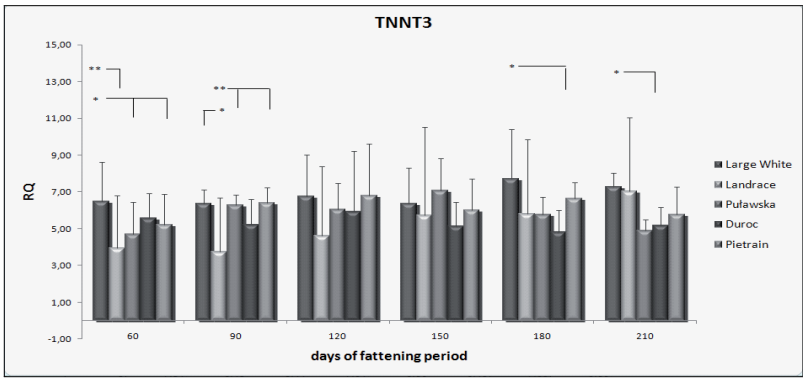

Fig.3. Relative quantification (RQ) of *TNNT3* transcript level in porcine white skeletal muscle in five breeds. *P<0.05, **P< 0.01.

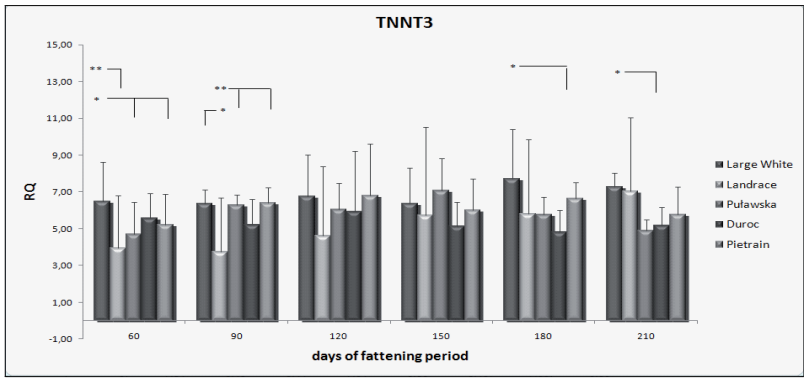

Fig. 4. Relative quantification (RQ) of the mean *TNNT3* transcript level in five pig breeds in white skeletal muscle, with no reference to day of fattening. *P< 0.05, **P< 0.01.

Comparison of mean expression of the *TNNT3* between breeds revealed significantly higher level of *TNNT3* mRNA in PLW than in PL and Duroc (P<0.01) and in Pulawska (P<0.05) gilts – Figure 4. Although these differences were statistically confirmed their biological meaning is not clear and needs further studies.

In PL gilts, a highly significant correlation was found between the level of expression and the animal's maternal origin (all PL gilts shared the same sire). Regardless of age, animals born to a second dam were characterized by the lowest transcription level, which was 6 times lower than that of animals born to dam sixth. A similar pattern was observed for PLW and Pietrain, but the differences were much smaller compared to PL gilts (Fig.1). Classification of gilts based on maternal origin showed the same expression profiles in both white muscles *(longissimus dorsi* and*. semimembranosus*) studied. The above results suggest the presence of unknown polymorphism affecting expression of *TNNT3* which is probable located in the regulatory part of the gene. In humans the expression of the troponin T gene is regulated by MyoD and MEF-2 transcription factors [Firulli *et al*. 1997]. In pigs, the regulatory parts of the *TNNT3* gene have not been adequately studied and the promoter of this gene perhaps contains binding sites for one of the transcription factors.

In the two white muscles (*longissimus dorsi* and *semimembranosus*) the highest level of *TPM2* expression in PLW, Puławska and Duroc gilts was found on day 60 of age, with no downward trend during the subsequent days of ontogenesis (Fig. 5). However, these results were rather consequence of high standard deviation in 60-days old PLW, and 180- and 60-days old Duroc gilts than the general trend during development.

No relationship was observed between the *TPM2* gene transcript level and the maternal origin of the animals (Fig. 6).

In Puławska gilts on 210 day was observed the lowest level of *TPM2* transcript (P<0,05) (Fig. 7).

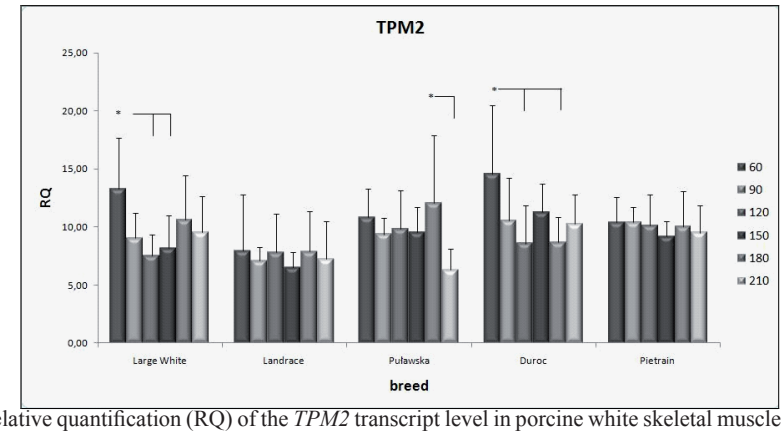

Fig. 5. Relative quantification (RQ) of the *TPM2* transcript level in porcine white skeletal muscle from day 60 to 210 of fattening. *P<0.05, **P<0.01.

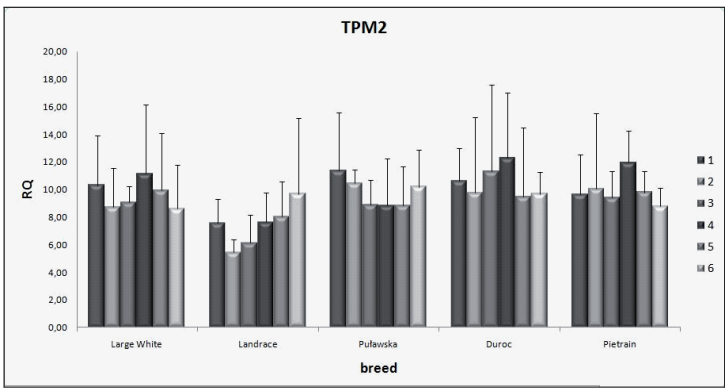

Fig. 6. Relative quantification (RQ) of the *TPM2* transcript level in porcine white skeletal muscle as related to animal origin (1-6 – consecutive pairs of parents). *P<0.05, **P< 0.01.

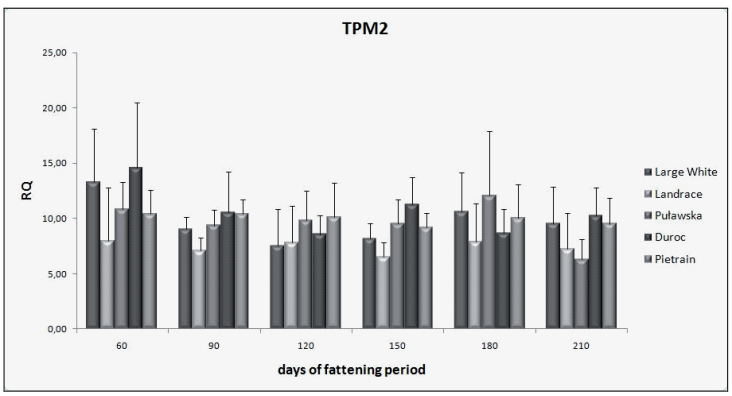

Fig.7. Relative quantification (RQ) of *TPM2* transcript level in white skeletal muscle in five pig breeds. *P<0.05, **P<0.01.

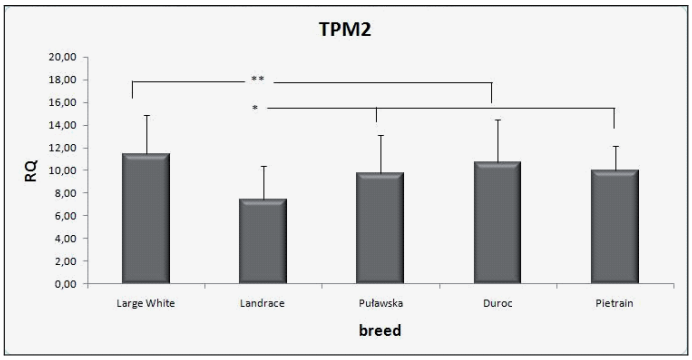

Fig. 8. Relative quantification (RQ) of the mean *TPM2* transcript level in white skeletal muscle in five pig breeds, with no reference to day of fattening. *P<0.05, **P<0.01.

On the other hand, mean expression of the *TPM2* gene was lowest in the PL, and highest in PLW and Duroc gilts, which showed comparable values (Fig. 8). In Pietrain and Puławska gilts the level of *TPM2* transcript was similar. Earlier Tang *et al*. [2007] reported the *TPM2* prenatal expression level to be higher in the muscles of animals characterized by greater fatness. The present study did not confirm the relationship between muscle fatness and *TPM2* postnatal expression.

In conclusion, the study described here has confirmed that the expression level of *TNNT3* and *TPM2* genes is not related to the age of the animals examined. However, a significant effect of the animals' maternal origin on the transcript level for the *TNNT3* gene was found indicating that an unknown polymorphism affects the transcript level of this gene. The objective of further research will be to correlate the expression level with the production traits of pigs raised in Poland.

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