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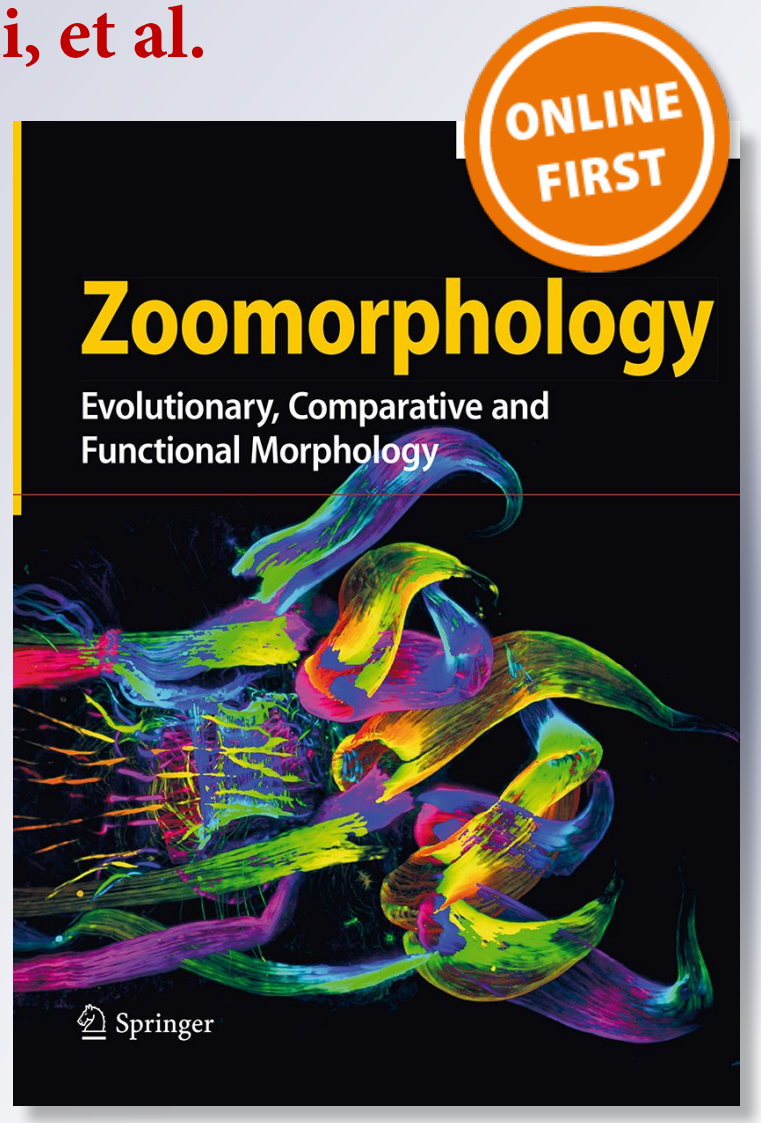

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ORIGINAL PAPER

Influence of genetic selection on the myofibre type composition of porcine biceps femoris muscle: a comparative study of a purebred (Nero di Parma) and commercial hybrid pigs (Large White × Landrace × Duroc)

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Abstract This study is aimed to evaluate histological features related to the different quality of meat cuts obtained from different pig breeds. We compared animals genetically selected to restore the original local purebred "Nero di Parma" pig, and highly selected fast-growing commercial hybrids. As weight is the main factor determining the time of slaughter, we compared equally weighing animals, sampled from slaughter for edible use, regardless of their age and sex, and immunohistochemically demonstrated the myofibre type composition of their biceps femoris muscle. In both groups we observed type I myofibres, situated as central islets, encircled by type IIA, IIX and IIB myofibres ordered in concentric rings according to the dynamic of their differentiation/maturation. However, the purebred pig muscles contained a smaller quantity of myofibres expressing the MyHC-IIb isoform, related to rapid postmortem glycolytic rate, meat toughness and poorer quality, in comparison to commercial hybrids. This proves that the latter are subjected to a more rapid transition in the expression of the different MyHC, probably as a consequence of genetic selection and breeding conditions, such as different feeding and housing reducing the possibility of continuous physical exercise. Further studies on the postnatal transitions timing of myosin heavy chain isoforms in functionally different muscles of various breeds are necessary to verify if they might be "artificially modulated", with the aim to design breeding programs allowing a good balance between growth performance, muscularity and meat quality.

Keywords Nero di Parma pig · Commercial hybrid pigs · Immunohistochemistry · Myosin heavy chain · Myofibre composition

Introduction

The European domestic pig (*Sus scrofa domesticus*) originated from a long process of domestication of the Eurasian wild boar (*Sus scrofa*) (Larson et al. 2007), enhanced with the formation of organized breeding systems, in which animals were bred for specific quality traits such as rapid growth and increased prolificacy (Darwin 1868). Once the beneficial effects of the genetic selection were recognized, a stronger selection was imposed for the improvement of the lean meat content and muscularity to satisfy the preferences of the consumers (Porter 1993). This system, particularly used in the twentieth century, led to the domination of breeding farms of commercial hybrids, such as Large White and Landrace, which necessarily reduced the breeding trend of purebred pigs, causing a decrease of their population as it has been described for the Korean Black Pig (Hur et al. 2013). The province of Parma, Italy, is known for the traditional production of Protected Designation of Origin (DOP) meat products. Originally they were obtained from local purebred Nero di Parma pig whose meat, known for its intense red color and taste, was a first-class raw material. However, with the arrival of more productive and prolific cross-bred pig breeds from northern Europe, the population of Nero di Parma, significantly declined (Rozzi 1934). Today, selection programs are being designed to reintroduce the Nero di Parma pig breeding tradition in the

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province of Parma, increasing growth performance and meat quality.

These characteristics are related to the morphological and physiological features of myofibres forming the skeletal muscles different among them by their function, structure and metabolism (Hur et al. 2013). Myosin heavy chain (MyHC) proteins are directly involved in the force–velocity features of muscle myofibres and good indicators of their functional characteristics. Four MHC isoforms exist that consequently define four types of muscle myofibres: I, IIA, IIX and IIB with different shortening velocities (Pette and Staron 2000). Each MHC isoform has a different energy demand which defines the metabolism of the myofibres. Type I myofibres are slow, oxidative myofibres, responsible for continuous contractions. On the other hand, type IIB myofibres are glycolytic and support quick and transitory contractions. Finally, IIA and IIX are intermediate (fast-twitch oxidative-glycolytic) fast myofibres (Brooke and Kaiser 1970; Schiaffino et al. 1989).

On the contrary to only two fast MyHC isoforms (MyHC-IIa and -IIx) expressed in some large mammals like humans (Smerdu et al. 1994), cattle (Tanabe et al. 1998), dogs (Strbenc et al. 2004; Smerdu et al. 2005; Maccatrozzo et al. 2007), cats (Talmadge et al. 1996), horses (Eizema et al. 2003) and bears (Smerdu et al. 2009), three fast isoforms, MyHC-IIa, -IIx and IIb, are expressed in domestic pigs (Lefaucheur et al. 1995, 2004; Toniolo et al. 2004; Fazarinc et al. 2016) like in llamas (Graziotti et al. 2001), rodents and lagomorphs (Schiaffino and Reggiani 1994; Pette and Staron 2000). Furthermore in pigs, the myofibre types are specifically spatially distributed in fascicles, forming islets of type I fibres surrounded by type IIA and IIX, furthermore encircled by type IIB fibres (Lefaucheur et al. 1995).

Several studies have shown that there is positive correlation between the percentage of type I myofibres and higher meat juiciness and flavor, determined by their elevated content of phospholipids. On the contrary, type IIB myofibres are associated with lower water holding capacity making the meat firmer and tougher (Kauffman et al. 1998; Gil et al. 2003; Ryu and Kim 2005, 2006; Choi and Kim 2009; Lee et al. 2012). It has been showed by comparative studies among pigs of different breeds that modern cross-bred pigs have high muscular-growth capacity and contain an elevated number of type IIB muscle fibres as a consequence of genetic improvement and breeding conditions. (Weiler et al. 1995; Maltinet al. 1997; Lee et al. 2012).

The goal of the present study was to verify whether biceps femoris muscle (BFM) of pure bred Nero di Parma pigs differs in the myofibre type composition from that of the same muscle of commercial hybrids and contains less fast type myofibres, as previously found in other pure breeds (Ryu et al. 2008). We chose this muscle, because it is mainly used for evaluation of pig meat quality, besides being one of the main muscles forming the cutting of ham, one of the most important DOP meat products of Parma.

Materials and methods

Animals and muscle sampling

Five Nero di Parma pigs (NP) and seven commercial hybrids Large White × Landrace × Duroc (H) were used in this study. H were raised until reaching 160–170 kg and a minimum age of 9 months, following the standards established for the production of Parma ham (Consortium for Parma Ham 1992). NP deriving from local farms were raised until the age of 18 months and weight of 170 kg. At the end of the trial all pigs were transported to abattoir. Instantaneous insensibility was induced by electrical stunning and animals were exsanguinated. Muscle samples (1 cm^3) from the medial portion of the BFM at the point of maximal circumference were taken for immunohistochemical analysis. Particular attention was payed to unify the sampling of muscles, taking the samples exactly from the same site in all the animals, to avoid variations in the characteristics of the myofibres between the functionally differentiated neuromuscular compartments, macroscopically defined by the distribution of primary branches of the gluteal caudal and sciatic nerves (Graziotti et al. 2012, 2014). The samples were promptly frozen in isopentane precooled in liquid nitrogen and stored at −80 °C until subsequent analysis. The animals exhibited no locomotor dysfunction and no pathological changes were found postmortem, which could influence the locomotion ability and muscle fibre characterization.

Immunohistochemistry and determination of myosin heavy chain-based myofibre types

In serial cross sections of biceps femoris muscle, type I, IIA, IIX and IIB fibres were immunohistochemically demonstrated by three monoclonal antibodies specific to MyHC isoforms: BA-D5 (MyHC-I), A4.74 (MyHC-IIA and -IIX) and BF-F3 (MyHC-IIB). The antibodies were supplied by the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242, USA. Details about the reactivity of the antibodies with different myofibres can be found in Table 1. The specificity of these antibodies to porcine MyHC isoforms has been previously established (Lefaucheur et al. 2002; Graziotti et al. 2011; Fazarinc et al. 2016). In our study, the antibodies were used at a concentration of 2 µg/ml.

Table 1 Reactivity of the antibody panel used for the characterization of myofibres forming biceps femoris muscle

	Myofiber type			
	I	IIA	IIX	IIB
Antibody				
BA-D5	++	−	−	−
A4.74	−	++	+	−
BF-F3	−	−	−	+

++, Strong reactivity; +, moderate reactivity; −, absence of reactivity

To evaluate the presence of the different types of myofibres, transverse serial sections of 10 µm were cut from entire blocks on a cryostat microtome (HM505, Microm GmbH, Germany) at −20 °C, directly mounted on polysine-coated microscope slides and air-dried. From each muscle sample, three serial sections were collected and stained with one of the three antibodies.

The immunoperoxidase staining protocol with the avidin–biotin complex (ABC) was used. In brief, 10-µm thick air-dried sections were treated, with 0.3% hydrogen peroxide in 0.1% sodium azide for 10–15 min for quenching of endogenous peroxidase, washed in buffer for 5 min and pre-incubated for 20 min in a blocking solution of normal goat serum. Then excess serum was blotted and the primary antibodies BA-D5, A4-74 or BF-F3 were applied overnight at 4 °C. After incubation, the sections were washed for 5 min in buffer and then incubated for 30 min with secondary antibodies (biotinylated sheep anti-mouse IgG, code n. RPN 1001 dilution 1:100, supplied by Amersham Pharmacia Biotech, Little Chalfont, UK). The sections were then washed and reacted for 30 min with VECTASTAIN®ABC Reagent (Vector Laboratoires, Inc, Burlingame, USA). After another washing, immunocomplexes were visualized by incubating the sections, until desired staining intensity development, with a chromogen agent in formulation of SIGMA*FAST*™ 3,3′-diaminobenzidine tablets (Sigma, Saint Louis, Missouri, USA).

Histomorphometric analysis

The histological sections were analyzed with a microscope Nikon Eclipse 90i (Nikon, Tokyo, Japan) equipped with a digital camera (Nikon model 5 M) and an image analysis software (NIS-Elements AR 3.1; Nikon). Histomorphometric assessment was performed on each immunohistochemistry stained section, within three regions of interest (ROIs) represented by three micrographs taken at 10× magnification and selected at 1 mm of distance from each other on a line passing through the center of the section. For the numerical estimation of the different types of myofibres, only those profiles (whole cells) which were entirely present within the counting ROIs and did not enter the "border line" of the edges of the frame were counted and measured. On the contrary, those, crossed by the "border lines", were not taken into consideration. For the histomorphometric analysis, each myofibre was encircled manually, and the cross-sectional area (CSA) was automatically calculated by the software.

For each animal, the data relative to three ROIs were summed, so that approximately 400 to 500 myofibres per sample were counted. Mean CSA of each myofibre type was calculated and myofibre type relative area (%) was considered to be the ratio between the total CSA of each myofibre type and the total area of all measured myofibres. Myofibre type relative number (%) was calculated as ratio between the number of cells counted per each myofibre type and the total number of myofibres. Myofibre type density (number/mm^2) was presented as the number of individual myofibres of a given type per 1 mm^2 of muscle CSA.

Statistical analysis

The experimental data were presented as mean and standard error (SE). Myofibre characteristics were analyzed using PAST statistical software package version 3.0 (Hammer et al. 2001, http://folk.uio.no/ohammer/past). To compare the muscles of the two groups of animals for each quantitative feature, the statistical analysis of the data was carried out by means of one-way ANOVA model followed by Tukey's post hoc test to confirm the differences between groups. Statistical significance was assigned to $p < 0.05$.

Results

Immunohistochemical staining allowed the differentiation of the four types of myofibres in both NP (Fig. 1a, c, e) and H (Fig. 1b, d, f). Observing the different reactivity of the same myofibre for each antibody in serial sections, it was also possible to identify a few myofibres that were contemporaneously strongly positive for BA-D5 and moderately positive for A4-74; therefore, classifiable as hybrid myofibre type I/IIA, (Fig. 1a, c). Moreover, it was possible to identify some myofibres that were contemporaneously strongly positive for BF-F3 and moderately positive for A4-74; therefore, classifiable as hybrid myofibre type II/XB, (Fig. 1c–f). In both H and NP, the spatial distribution of myofibres resembles rosette clusters in which MyHC isoform expression followed the rank order of I → IIa → IIx → IIb from the center to the periphery.

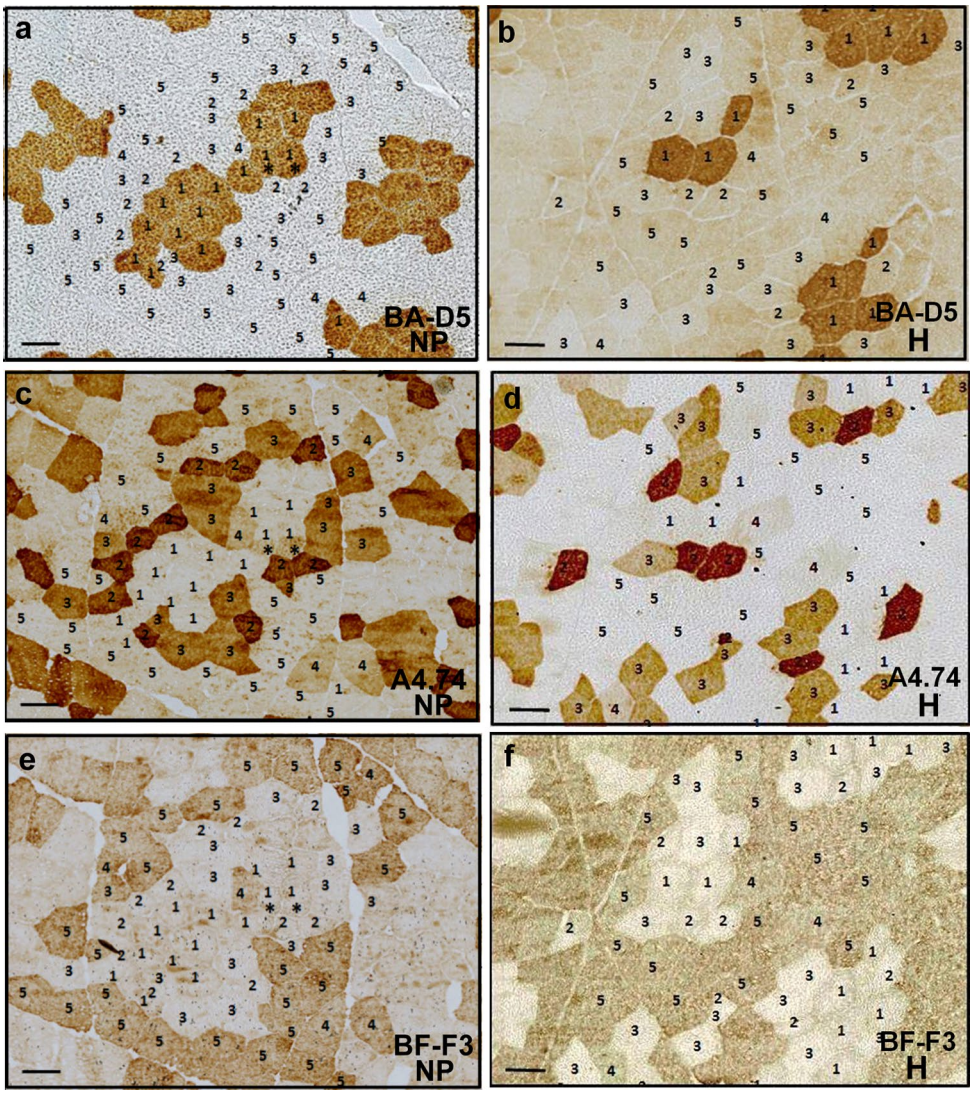

Fig. 1 Serial sections of the biceps femoris muscle of a Nero di Parma pig (NP) and a commercial hybrid pig (H) stained for monoclonal antibodies BA-D5 (**a, b**), A4.74 (**c, d**), BF-F3 (**e, f**). *1, 2, 3, 4* and *5* correspond to myofibre types I, IIA, IIX, IIX/B and IIB, respectively. *Asterisks* indicate two myofibres completely positive for BA-D5, but only partially moderately positive for A4-74; they might be hybrid myofibre type I/IIA. *Scale bar* 100 µm

Nero di Parma pigs (NP) vs. commercial hybrids (H)

The relative area occupied by myofibres stained with the same antibody in the two groups resulted significantly different ($p < 0.001$) only comparing myofibers positive for BF-F3. In fact, in NP, on average, a half of the area of the samples taken from the BFM was occupied by type IIB myofibers, while the mean relative area of type IIB myofibers in H was around 65% (Fig. 2a).

Within each group, both type I and IIA myofibers resulted smaller in size ($p < 0.001$) than type IIX and IIB myofibers (Fig. 2b). Comparing the mean CSA of the myofibres of the same type between NP and H, no statistically significant difference was found. So the significantly smaller relative area occupied by type IIB myofibres in NP may be related to their relative number that resulted smaller ($p < 0.01$) (around 50% of the total number of the myofibers composing the samples) compared to H, in which this percentage raised to approximately 61% (Fig. 2c).

In both groups, the greatest number of cells per mm^2 of muscle CSA was observed for type IIB myofibres. However, in NP type IIB myofibers were significantly ($p < 0.001$) denser only compared to type IIA myofibres. Instead, in H type IIB myofibres were significantly denser than all the other myofibre types ($p < 0.001$). Type IIX myofibres were denser than IIA both in NP ($p < 0.05$) and H ($p < 0.001$), while type IIA myofibres were less numerous per mm^2 than type I myofibers only in NP ($p < 0.05$) (Fig. 2d).

Discussion

Several studies have demonstrated a correlation between composition, number and size of myofibre types within

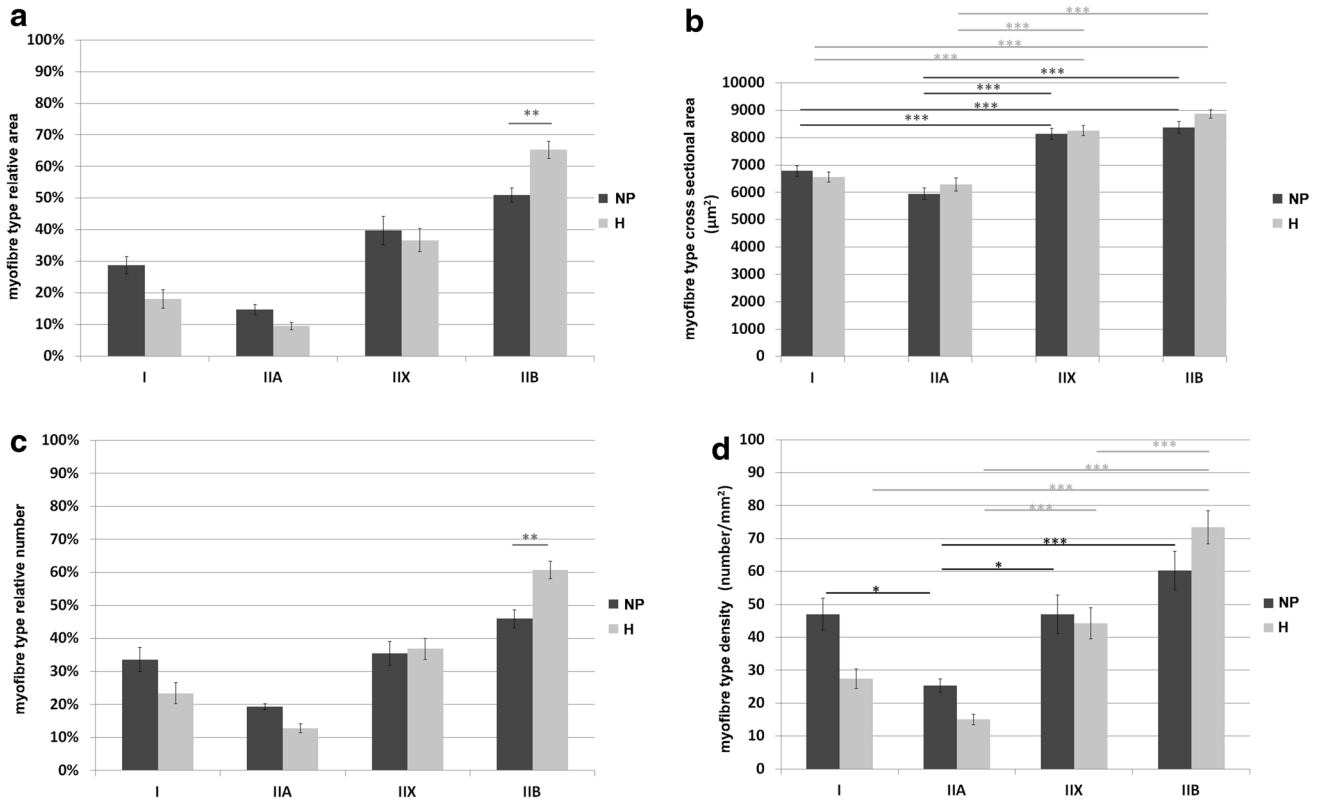

Fig. 2 Comparison of **a** myofibre type relative area, **b** myofibres type cross-sectional area, **c** myofibre type relative number and **d** myofibre type density between Nero di Parma pigs (NP) vs. commercial hybrids (H). Data are represented as means ± SE. Significant differences between myofibre types are presented with *asterisks* $*p<0.05$, $**p<0.01$, $***p<0.001$

muscles (Ryu et al. 2004), and highlighted their influence on meat quality traits in pigs (Eggert et al. 2002; Ryu and Kim 2005; Kim et al. 2014) as well as in other species (e.g., in cattle: Maltin et al. 1998; Ozawa et al. 2000).

The aim of our study was to perform a preliminary screening of the myofibre type composition of muscles at slaughtering that could give a histological basis predicting the quality of identical products obtained from NP and H. As weight is the main factor determining if an animal is ready for slaughtering, we performed an immunohistochemical analysis on samples obtained from animals of different sex and age and, therefore, in a different stage of development of their muscle fibres.

The most relevant result of the present investigation was that, in 18-month-old NP, type IIB myofibres were present in smaller numerical percentage and occupied a smaller proportion of muscle CSA in comparison to 8–9-month-old commercial hybrids. Moreover, no difference in the average myofibre type size and density was found between the two groups.

We would like to point out that in this phase of our study, we focused on the whole section, considering it as a microscopic model of the entire meat cut. Therefore, histochemical and metabolic typification of myofibres was not performed analyzing correspondent microscopic fields of serial sections of the same sample. Moreover, we used the monoclonal antibody A4.74 that recognizes both MyHC-IIa and MyHC-IIx isoforms in the pig (Fazarinc et al. 2016) but has a higher affinity for the first isoform (Graziotti et al. 2011), to simply classify the myofibre type as IIA or IIX according to their strong or moderate reaction for this antibody. We did not consider important to identify and quantify hybrid myofibre type IIA/X because, like pure IIA and IIX myofibres, they have been positively related to desirable pork (Kim et al. 2014). In the same way, we used the monoclonal antibody BF-F3, specific for the MyHC-IIb isoform, and classified the stained myofibre type as IIB. We made no distinction between myofibres strongly reactive for BF-F3 (pure IIB myofibres) and moderately reacting for both BF-F3 and A4.74 (hybrid IIX/B myofibres co-expressing MyHC-IIx and IIb), because both of them have been related to poor quality traits (Kim et al. 2014).

The method used allowed us to highlight, in both NP and H, the type grouping distribution of myofibres, characteristic for pig muscles. Type I myofibres were, in fact, situated as central islets encircled by IIA, IIX and IIB myofibres, ordered in concentric rings from the center to the periphery. According to Lefaucheur et al. (2002) and

Fazarinc et al. (2016) this arrangement follows the dynamics of myofibre differentiation/maturation with a decrease in oxidative metabolism and an increase in myofibre size.

Type I myofibres showed similar size and occupy the same area percentage in the two groups, indicating that the genetic selection programs performed to restore an ancient pig breed with genetic characteristics more similar to the wild boar apparently did not influence this type of myofibres that instead resulted larger in wild pigs (Weiler et al. 1995).

Type IIA myofibres showed similar size in the two groups, but were significantly less dense than all the other types in NP. As their number is very low, they probably have, however, scarce effect on muscle size and characteristics.

Type IIB myofibres, instead, despite having similar size in the two groups, were significantly denser than any other type only in H and occupied consequently a larger proportion of muscle CSA in H compared to NP.

This proves that the dynamics of MyHC transition scheme I→IIa→IIx→IIb are slower in NP and that, when they reach slaughter weight, their muscles contain a smaller amount of myofibres related to a worse meat quality (Ruusunen and Puolanne 2004). On the contrary, H are subjected to a more rapid transition in the expression of the different MyHC resulting in higher numerical and areal proportion of type IIB myofibres. This could probably be a consequence of the genetic selection and breeding conditions, such as different feeding and housing that reduces the possibility of continuous physical exercise resulting in a faster weight increase.

Conclusion

The main factor determining when pigs are ready for slaughtering is weight. The comparison between H and NP of the same weight shows that, at the time of slaughter, their muscles have a different myofibre type composition. The selection program, performed to restore the original pure breed NP, led to obtain slowly growing pigs. Their muscles contain a smaller quantity of myofibres expressing the isoform MyHC-IIb having clear glycolytic metabolic properties, associated to poor quality of the final products. Further studies on the postnatal transition timing of MyHC isoforms in functionally different muscles of various breeds are necessary to verify if they might be "artificially modulated", with the aim to design breeding programs allowing a good balance between growth performance, muscularity and meat quality. Moreover, to deepen the investigation on the factors related to the quality of traditional pork products obtained from purebred Nero di Parma pigs, also additional factors such as age, sex and muscle changes occurring post-mortem must be analyzed.

Compliance with ethical standards

Funding This research was supported by the Research Project Grant FIL 2014—University of Parma.

Conflict of interest The authors declare no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. However, the ethical impact of this experimentation is low due to the fact that we used animal samples from slaughter for edible use.

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