ANDRÉ MAURIC FROSSARD RIBEIRO

GENE EXPRESSION OF MYOSIN HEAVY CHAIN ISOFORMS IN PIGS OF DIFFERENT GENETIC GROUPS AND AGES

Dissertation presented to the Breeding and Genetics Graduate Program of the Universidade Federal de Viçosa, in partial fulfillment of the requirements for degree of *Magister Scientiae.*

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BIOGRAFIA

André Mauric Frossard Ribeiro, filho de Teresa Francisca Mauric Ribeiro e Araken Frossard Ribeiro, nasceu em Santos, São Paulo, no dia 22 de março de 1986.

Em maio de 2006 ingressou no curso de Zootecnia da Universidade Federal de Viçosa, Viçosa, Minas Gerais, concluindo a graduação em julho de 2010. Ingressou no mestrado em Genética e Melhoramento da Universidade Federal de Viçosa no ano de 2012, defendendo a dissertação em 28 de abril de 2015.

SUMMARY

ABSTRACT

RIBEIRO, André Mauric Frossard, M.Sc., Universidade Federal de Viçosa, April, 2015. **Gene Expression of myosin heavy chain isoforms in pigs of different genetic groups and ages.** Adviser: Simone Eliza Facioni Guimaraes. Co-advisers: Marcio de Souza Duarte and Fabyano Fonseca e Silva.

The objective of this study was to evaluate gene expression pattern of myosin heavy chain isoforms in pigs of three different genetic groups at four ages. 48 castrated males of Piau, Commercial and Crossbred (Piau X Commercial) genetic group, slaughtered at birth, 56, 112 and 156 days. *Longissimus dorsi* muscle samples were taken for RNA extraction. The expression of MyHC I, MyHC IIA, MyHC IIX and MyHC IIB were analyzed by quantitative PCR (qPCR). Interactions between genetic groups and slaughtered ages to MyHC I, MyHCII A and MyHC IIB relative transcript abundance were observed. The expression of MyHC I was significantly different between birth and 56 days in Piau and Crossbred, mainly in Piau, which showed 81-fold decrease ($P < 0.0001$). At birth the expression of MyHC I in Piau was nearly 23 and 9.6 times greater than Commercial ($P = 0.0028$) and Crossbred ($P = 0.0258$), respectively, explaining the higher proportion of MyHC I transcripts in Piau. The expression of MyHC IIA was significantly higher at birth in comparison to 56 days for all genetic groups, especially in Commercial ($P < 0.001$, FC=16.04). The expression of MyHC IIA at 56 days in Commercial was 3.76 ($P = 0.0346$) and 5.4 times lower ($P = 0.0082$) than Crossbred and Piau. The difference in expression of MyHC I and MyHC IIA across genetic groups at birth can be explained by embryonic development .The expression of MyHC IIB relative to birth, contrary to MyHC I and MyHC IIA, had a significant increase across the ages with highest value at 156 days in Commercial ($P < 0.001$, FC = 110.22) and Crossbred ($P = 0.014$, FC=10.49) while in Piau was at 112 days ($P = 0.012$, FC = 24.34). There was a reversion of proportion of oxidative MyHC (MyHC I and MyHC IIX) to glycolytic MyHC (MyHC IIX and MyHC IIB) in all genetic groups however the rates of this reversion were different. The earlier inflexion in Piau and Crossbred may be due to the difference in partitioning of energy undergoing to protein and/or lipid synthesis, and the respective efficiencies of

synthesis. Commercial and Crossbred at 56 days shows higher proportion of oxidative isoforms than Piau (P-value=0.012, P-value=0.023) while at 112 and 156 days Piau shows higher proportion of these isoforms compared to Commercial. The difference of MyHC proportions across genetic groups are caused by difference in expression of oxidative MyHC.

RESUMO

RIBEIRO, André Mauric Frossard, M.Sc., Universidade Federal de Viçosa, abril de 2015. **Expressão Gênica das Isoformas da Cadeia Pesada da Miosina em Suínos de Diferentes Grupos Genéticos e Idades.** Orientadora: Simone Eliza Facioni Guimaraes. Coorientadores: Marcio de Souza Duarte e Fabyano Fonseca e Silva.

O objetivo deste estudo foi avaliar padrão de expressão gênica das isoformas de cadeia pesada miosina em suínos de três diferentes grupos genéticos em quatro idades. 48 machos castrados dos grupos genético Piau, Comercial e Cruzado (Comercial x Piau), foram abatidos ao nascimento, 56, 112 e 156 dias. Amostras do músculo *Longissimus dorsi* foram retiradas para extração de RNA. A expressão de MyHC I, IIA MyHC, MyHC IIX e MyHC IIB foram analisadas por PCR quantitativo em tempo real (qPCR). Foram observadas interações entre grupos genéticos e idades para MyHC I, MyHC IIA e MyHCIIB. A expressão de MyHC I foi significativamente diferente entre o nascimento e 56 dias em Piau e Cruzado, principalmente no Piau, que mostrou queda de 81 vezes (P <0,0001). No nascimento, a expressão de MyHC I em Piau foi cerca de 23 e 9,6 vezes maior do que em Comercial (P = 0,0028) e Cruzado (P = 0,0258), respectivamente, o que explica a maior proporção de transcritos de MyHC I em Piau. A expressão de MyHC IIA foi significativamente maior ao nascimento em comparação com 56 dias para todos os grupos genéticos, especialmente em Comercial (P <0,001, Fold Change = 16,04). A expressão de MyHC IIA aos 56 dias em Comercial foi de 3,76 (P = 0,0346) e 5,4 vezes menor (P = 0,0082) do que Cruzudos e Piau, respectivamente. A diferença na expressão de MyHC I e MyHC IIA entre grupos genéticos ao nascimento pode ser explicado pelo desenvolvimento embrionário. A expressão de MyHC IIB em relação ao nascimento, ao contrário do MyHC I e MyHC IIA, teve um aumento significativo entre as idades com valor mais elevado aos 156 dias em Comercial (P <0,001, Fold Change = 110,22) e Cruzado (P = 0,014, Fold Change = 10,49), enquanto em Piau foi aos 112 dias (P= 0,012, Fold Change = 24,34). Houve uma reversão da proporção de MyHC oxidativas (MyHC I e MyHC IIX) para MyHC glicolíticas

(MyHC IIX e MyHC IIB) em todos os grupos genéticos no entanto, as taxas dessa reversão foram diferentes. A inflexão precoce em Piau e Cruzado pode ser devida à diferença no particionamento de energia submetida a síntese de proteína e/ou de lipídios, e as respectivas eficiências de síntese. Comercial e Cruzado aos 56 dias demonstra maior proporção de isoformas oxidativas do que Piau (valor de P = 0,012, P = 0,023), enquanto que a 112 e 156 dias Piau mostra maior proporção destas isoformas comparado de Comercial. A diferença de proporção MyHC entre os grupos genéticos são causados pela diferença na expressão de MyHC oxidativo.

GENERAL INTRODUCTION

Until the 90s, pig breeding programs were essentially aimed to improve growth rates, feed conversion efficiency and carcass quality. The meat quality was not taken into account, with the exception of problems related to the presence of the halothane gene (Wood et al., 2004). Physiological and metabolic characteristics and the final size of muscle tissue depend mainly on the proportion of fiber types. Thus, the meat quality is directly influenced by the frequency of muscle fiber types that forms the muscle, these will determine the *post-mortem* metabolism changes of muscle to meat (Essen-Gustavsson, 1993; Karlsson., 1993; Karlsson et al., 1999; Klont et al., 1998;. Maltin et al., 1997). Studies have found high correlations between the type of fiber and organoleptic qualities of meat (Scheffler and Gerrard 2007). Thus, the understanding of muscle fibers characteristics contributes to optimization of muscle growth and meat quality, two important concerns in animal production.

Fiber Types and Myosin Isoforms

The classification of muscle fibers can be made using different methods. One of the first classification methods is based on the sensitivity to acidic or alkaline pH of the ATPase activity of the Myosin Heavy Chain isoforms (MyHC) (Picard, 2010). Myosin is a fundamental structural and functional component of all skeletal muscles, and about 1/3 of the total muscle proteins are from myosin making it the vast majority of the contractile apparatus of muscle fibers (Picard et al., 2002). Four out of eight isoforms known in mammals have been identified in

porcine muscle according to their specific expression of MyHC (Chang and Fernandes .,1997). The I and IIB fibers, also known as slow-oxidative and fast-glycolytic fibers, respectively, represent two extreme metabolic profiles. The IIA and IIX fibers are defined as intermediates with the transition that IIA fibers are more similar to I type and IIX fibers are more in relation to IIB fibers (Chang et al. 2003). Thus, the expression of a particular MyHC isoform in a fiber defines its biochemical and functional phenotype, and reflects the coordinate pattern of gene expression in that fiber (Schiaffino and Reggiani 1996)

Oxidative fibers, mainly type I, contain high level of slow contractile proteins, high volume density of mitochondria, high levels of myoglobin, high capillary densities and high oxidative enzyme capacity (Spangenburg and Booth 2003); they are responsible for posture maintenance. On the other hand, glycolytic fibers (type IIB) with low volume of mitochondria, high glycolytic enzyme and myosin ATPase activity, high level of fast contractile protein and increased rate of contraction (Spangenburg and Booth 2003) are more involved in producing movement (Table 1).

Table 1: Classification of skeletal muscle fiber types, modified from Spangenburg & Booth (2003).

	Muscle Fiber types			
Myosin heavy chain	Type I	Type IIA	Type IIX	Type IIB
Contractile speed	Slow	Fast	Fast	Fast
Metabolic	Oxidative	Oxidative	Glycolytic	Glycolytic
Color	Red	Red	White	White
Fiber CSA	Small	Medium	Large	Large
Mitochondria	Many	Many	Few	Few
Capillaries	Many	Many	Few	Few
Fatigue resistance	High	Intermediate	Low	Low
Function	Postural, endurant movement	Postural, endurant movement and fast movement		Fast movement

CSA: cross-sectional area

In the pig, gene coded for type I fiber is found on chromosome 7 in one cluster, whereas genes responsible for embryonic, IIA, IIX, IIB, neonatal and extra ocular fibers are located on chromosome 12 (Davoli et al. 1998). The identification of the genes allows the precise interspecies comparison; the homology between paralogue MHCs is much less than that between orthologue MHCs. The identification of the expressed mRNA either by in situ hybridization or by RT-PCR gives precise information on which isoform is expressed in a given muscle or fiber (Pas et al., 2004).

Factors affecting porcine muscle fiber types

In pigs, the number of muscle fibers is determined genetically before birth (Stickland and Goldspink, 1973), while muscle fibers differentiation continues through life. At birth, muscle fibers are especially oxidative but they have the capacity to be transformed from an aerobic state to an anaerobic state of

metabolism (Ruusunen and Puolanne, 2004). However, this transformation rate is still genetically and molecular unknown.

Muscle metabolism and MyHC expression can change in response to several factors some of them genetically originate from species, breed, sex, muscle and individuals, whereas others are environmental factors such as age, nutrition, temperature, exercise and some growth promoters. Fiber type transition follows a reversible fixed pattern: I ↔ IIA ↔ IIX ↔ IIB. In pigs, the factors of major influence on fiber type composition are genetic factors, in particular breed or line. In general, genetic differences across different breeds lead to the variation in mature body size and the age, at which, pigs can be slaughtered (Wimmers et al. 2008).

Oxidative fibers have smaller cross-sectional area and slower growth than glycolytic fibers (Spangenburg and Booth, 2003). Thus, intensive selection for lean muscle growth in pigs may have caused a large genetic change in fiber type composition, which resulted in a higher proportion of glycolytic fibers and an increase in the mean fiber diameter in domestic pigs compared to more unselected breeds. Studies show that wild pigs contain more oxidative and less glycolytic fiber than domesticated swine (Rahelic and Puac 1981; Ruusunen and Puolanne, 2004). Lefaucheur et al. (2004) found higher expression of MyHC IIB in Large White in comparison to Meishan (Chinese traditional breed). Dal Pai et al. (1997) observed that Large White has more glycolytic fibers than Brazilians native breed (Piau and Sorocaba).

Muscle fiber type and quality of meat

The composition of the fiber types is directly related to the final quality and technological properties of meat. The glycolytic fibers use anaerobic metabolism to produce ATP from glycogen, leading to a rapid postmortem glycolysis and lactate accumulation, which results in a rapid decline in muscle pH while muscle temperature is still high. This combination of low muscle pH and high temperature results in higher protein denaturation, and generally poorer meat quality. Muscles harboring a high glycogen and lactate content at 45 min postmortem are significantly composed by higher type IIB fiber and lower type I fiber, and also show rapid postmortem glycolysis, paler color, higher drip loss than muscles harboring a high glycogen and low lactate content in the early postmortem period (Choe et al. 2008). Ryu and Kim (2005) reported that the accelerated metabolic rate and poor quality of meat in PSE are explained by an increase in the percentage of type IIB fiber.

In addition, the percentage of type I fiber is positively correlated with intramuscular fat content in pig (Wood et al., 2004), that contributes to more to juiciness and flavor, whereas a high content of type IIB fiber tends to be associated with tougher meat.

Myosin isoforms also differ in their susceptibility to protein denaturation, (Bowker et al. 2005). Choi et al. (2006) reported that muscles with a lower MHC slow isoform content exhibited more pronounced protein denaturation at 24 h postmortem than muscles presenting a higher MHC slow isoform content.

Muscle energy metabolism and growth

A better knowledge of the developmental factors driving muscles towards a glycolytic or oxidative type is needed in order to determine the crucial developmental stage at which muscle energy metabolism can be easily reversible. But this reversion depend on the original characteristics of these muscles and the metabolic characteristics that really need to be optimized in order to improve growth rate or to enhance specific meat properties.

Within resting muscles, glucose can follow several metabolic pathways: (1) direct oxidation, (2) glycolysis to L-lactate, which is then released to circulation, (3) glycogen synthesis, which constitutes a major fate of glucose (Pethick, 1984) and (4) fatty acid synthesis, in particular in intramuscular adipose (Smith and Crouse, 1984). Thus, the type of fiber is highly influenced by the type of glucose metabolism.

Energy status can be modified by alterations in energy intake and energy expenditure, and the postnatal development of skeletal muscle is susceptible to both these factors (Dauncey and Ingham 1990). Furthermore, changes in the properties of skeletal muscle due to energy status may in turn have both immediate and long-term consequences for locomotor, postural, thermogenic function and meat quality.

Postnatal growth between birth and puberty is positively related to the glycolytic metabolism of the muscle tissue (Hocquette et al., 1996). However, in cattle beyond sexual maturity, an increase in oxidative muscle metabolism and fat content are observed (Jurie et al. 1995). This change may be explained by the

partition of metabolites of the metabolic fate of glucose between muscle fibers and intramuscular adipocytes during the period.

Therefore, the objective of this study was to evaluate gene expression pattern of myosin heavy chain isoforms in pigs of three different genetic groups at four ages.

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CHAPTER 1

INTRODUCTION

Pork industries need to add value to their products to turn their activities profitable and competitive and to engage in a high quality supply chain. Parameters of meat quality must be known to obtain *in natura* or processed products with better quality and higher value-added, which ensures consumer satisfaction and higher economic returns. In this way, the entire meat industry has taken several steps to improve meat tenderness, juiciness, and flavor, and reduce or eliminate pale, soft, exudative (**PSE**) meat condition. In the last years, pig breeders have focused on elimination of PSE pork via including meat quality traits in their selection programs (Wood et al., 2004).

The physiological and metabolic characteristics and the final size of muscle tissue depend mainly on the proportion of fiber types. For this reason, understanding the characteristics of muscle fibers contributes to optimization of muscle growth and meat quality, two important concerns in animal production. The differences between muscle fiber types are related to their myosin complement such as myosin heavy chain (MHC) isoforms (Lefaucheur et al., 2002). Four MHC isoforms known as MHC slow/I, 2A, 2X, and 2B have been observed in porcine skeletal muscle (Abreu et al. 2006; Chang et al. 2003; Kim et al. 2013; Lefaucheur et al. 2002). These MHC isoforms have been identified in ATPase based fiber types: MHC-1 in type I fiber, MHC-2a in fiber type IIA fiber, MHC-2x in type IIX fiber and MHC-2b in IIB type fiber (Pette and Staron, 2000).

Type IIB fibers have larger area and faster growth rate than other fibers (Dall Pai and Curi, 1992). Thus, selection of pigs to increase muscle deposition

leads to an indirect increase in the frequency of these fibers. However, type IIB fibers have anaerobic metabolism to produce ATP from muscle glycogen, which leads to increase rate of post-mortem lactic acid production. Thus, as higher is frequency of fibers type IIB as meat quality tends to be worst.

In pigs, the number of muscle fibers is determined genetically before birth (Stickland and Goldspink, 1973), while muscle fibers differentiation continues through life. At birth, muscle fibers are especially oxidative (da Costa et al., 2002) but they have the capacity to be transformed from an aerobic state to an anaerobic state of metabolism (Ruusunen and Puolanne 2004). However, this transformation rate is still genetically and molecular unknown. The MHC isoforms protein level is correlated with their correspondent mRNA levels. Therefore, the objective of this study was to evaluate gene expression pattern of myosin heavy chain isoforms in pigs of three different genetic groups at four ages.

MATERIAL AND METHODS

All methods involving animal breeding handling were done in accordance with regulation by the institutional animal welfare and ethics/ protection commission of the Universidade Federal de Viçosa (UFV).

Animals

A total of 48 castrated males pigs from pig breeding farm of Animal Science department of UFV were used. These animals consisted of three genetic groups: the Brazilian local breed Piau (**Piau**), a Commercial Pietrain base Line (**Com**) and Crossbred group (produced from Piau males and Commercial Line females; **CrB**). For each genetic group, 16 animals were slaughtered at four slaughter ages: at

birth, 56, 112 and 156 days. Therefore, for each combination of genetic group and slaughter ages were used 4 animals. The pigs were fed with same diet according to Rostagno et al. (2011) and free water access.

Slaughters and sampling

Animals were weighed and slaughtered via electrical stunning and jugular section. Immediately after slaughter about 500 mg of muscle from *Longissimus dorsi* (**LD**) at P2 site (between 12th rib and 13th rib) were taken, sectioned into 1-2 mm, immediately immersed in RNALater (Ambion, Austin, TX, USA) and stored at -80˚C. In order to correct the data, slaughter weight from each animal was recorded to be tested as a covariate in statistical analysis.

RNA isolation of muscle samples

Total RNA was isolated from individual LD skeletal muscle by using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. In brief, muscle samples were first grinded and homogenized with 750µl TRIzol using polytron. To ensure complete dissociation of nucleoprotein complexes, samples were allowed to stand for 5 min before adding 0.2 ml of chloroform. The mixture was shaken and left at room temperature for 5 min and centrifuged at 12,000 x g for 15 min at 4˚C. The upper aqueous phase was transferred to another fresh centrifuge tube and RNA was precipitated with 0.6 ml of isopropanol. After 10 min incubation at room temperature, samples were centrifuged at 10,560 rpm for 10 min at 4˚C to pellet RNA , which was subsequently washed by 75% (v/v) ethanol. Centrifugation was then performed and the RNA pellets were air-dried and resuspended in 40 µl of UltraPure™ DNase/RNase-Free.

In order to remove possible contaminating genomic DNA, the extracted RNA for all samples were treated with 5 µl RQ1 DNase buffer, 5 units DNase and 40 units of RNase inhibitor in a 40 µl reaction volume. Concentration of cleaned-up RNA was determined spectrophotometrically at 256 and 280 nm; the purity of RNA was estimated by the ratio A256/A280 with respect to contaminants that absorb in the UV. Additional examination of integrity was done by denaturing agarose gel electrophoresis and ethidium bromide staining (data not shown). Finally, the purified RNA was stored at -80˚C for further analysis.

Reverse transcription and cDNA synthesis

The cDNA was synthetized using the GoScript Reverse Transcription (**RT**) System (Promega, Madison, WI) following the manufacturer's instructions. Briefly, 1 µg total RNA and 1 µl oligo $(dT)_{15}$ primer were incubated at 70°C. Thereafter,1 µl random primer, 4 µl GoScript 5× reaction buffer, 1.2 µl MgCl2, 1 µl nucleotide mix, 0.5 µl recombinant RNasin ribonuclease, and 1 µl GoScript reverse transcriptase were added in the system and then incubated at 25°C for 1 h, 40°C for 1 h and 70°C for 1 h.

Primer Design

Primers design (Table 1) was obtained according to Wimmers et al. (2008), except primers for MyHCIIA and GAPDH which were designed using PrimerQuest (http://www.idtdna.com/primerquest/Home/Index). The gene GAPDH was chosen as housekeeping because its efficiency was around one and there was not variation across treatments (data not shown).

Table 1 – List of primers used to quantify myosin heavy chain (MyHC) isoforms.

Gene	Primer Sequence (5`-3`)	Acession number –NCBI (Citation)
MyHC I	Fw:AAGGGCTTGAACGAGGAGTAGA	AB053226 (Wimmers et al.,2008)
	Rev:TTATTCTGCTTCCTCCAAAGGG	
MyHC IIA	Fw: AACACCCTGACCAAAGCTAAA	AB025256
	Rev: TCCTCTTGGCTCTCTCTAAGTC	
MyHC IIX	Fw:AGAAGATCAACTGAGTGAACT	AB025262 (Wimmers et al.,2008)
	Rev:AGAGCTGAGAAACTAACGTG	
MyHC IIB	Fw:ATGAAGAGGAACCACATTA	AB025261 (Wimmers et al.,2008)
	Rev:TTATTGCCTCAGTAGCTTG	
GAPDH	Fw: CAAGTGGACATTGTCGCCATCA	NM_001206359
	Rev: GCTTCCCATTCTCAGCCTGACT	

Amplification Efficiency

Each gene amplification efficiency was calculated according to the standard curves in order to point out the best combination of cDNA (1, 10, 20 e 40 ng) and primer (100, 200 e 400 nM) concentration. The C_T values were used to calculate and plot a linear regression line by plotting the logarithm of template concentration (*X*-axis) against the corresponding threshold cycle (*Y*-axis). The slope of the line was used to determine the efficiency of target amplification (***E***) using the equation $E = (10^{-1/\text{slope}}) - 1$ (Pfaffl 2001). Finally, the coefficient of determination (r^2) shows whether a linear relation is observed.

Real-time RT-PCR

Real-time RT-PCR were carried out in an ABI Prism 7300 Detection System thermocycler (Applied Biosystems,Foster City, CA) using GoTaq qPCR Master Mix (Promega Cororation, Madison, WI).

All reactions were done in duplicate and the coefficient of variation (CV) of cycles Ct value from replicates within each sample was less than 3%, indicating acceptable accuracy and reproducibility. Each final assay consisted of cDNA as template, forwards and reverse primers and mix which includes a proprietary dsDNA-binding dye, a low level of carboxy-X-rhodamine reference dye (identical to ROX™ dye), GoTaq® Hot Start Polymerase, MgCl2, dNTPs and a proprietary reaction buffer in a total volume of 15 µl reaction. The amplification conditions of all reactions were: initial denaturation at 95°C for 10 min followed by 40 cycles of 95ºC for 15s denaturation and 56°C for 1 min annealing and extension.

The threshold cycle values (Ct) of each reaction were obtained and normalized (ΔCt) based on the Ct values obtained for GAPDH (Livak and Schmittgen, 2001)

Statistical analyses

Statistical analysis of gene expression data was carried out following the routine QPCR_MIXES:SAS [https://www.msu.edu/~steibelj/JP_files/QPCR.html], a method proposed by Steibel et al. (2009) which consists on the analysis of cycles to threshold values (Ct), for the targets and endogenous genes using a linear mixed model. The following model was used for analyzing the joint expression of the target and control genes:

$$Y_{gijkr}=TG_{gi}+P_j+B_{gik}+D_{ik}+b(W_{ik}-W)+e_{gijkr;}$$

where Y_{gijkr} corresponds to the Ct value obtained from the thermocycler software for the g^{th} gene (reference or targets) from the r^{th} well, which corresponds to the kth animal submitted to the i^{th} treatment (genetic groups and ages combination); TG_{gi} the effect of the i^{th} treatment on the expression of gene g; P_j is the systematic effects of j^{th} plate ; B_{gik}~N(0,σ_B^2) the gene-specific random effect of the k^{th} animal; D_{ik}~N(0, σ_D^2) the sample-specific random effect (common to reference and target genes); W_{ik} the covariate slaughter weight of the k^{th} animal submitted to the i^{th} treatment; W the mean of the slaughter weight; b the regression coefficient; and e_{gijkr}~N (0, σ_e^2) the residual term. Using a significance level of 5% the covariate slaughter weight (W) had no statistical effect and was removed.

To test differences and interactions between classes in the expression rate of genes of interest (ΔΔCT) normalized by the endogenous gene (GAPDH), different contrasts were performed between the appropriate estimates of TG levels. Significance of ΔΔCT estimates was determined with the t-test and the Satterthwaite method was used to obtain denominator degrees of freedom estimates due to of heterogeneity of variances. To obtain fold change values (FC) from the estimated ΔΔCT values, the following equation was applied: $FC= 2^{-\Delta\Delta CT}$. Asymmetric (95%) confidence intervals were calculated for each FC value by using the standard error (SE) of the estimated difference 95% confidence interval from $2^{-(\Delta\Delta CT+ t \times SE)}$ to $2^{-(\Delta\Delta CT- t \times SE)}$.

To study the pattern of gene expression during post-natal life were adjusted regression equations adjusted as follow: the amount of each target was normalized to endogenous reference and calculated $2^{-\Delta CT}$, where ΔCT is equal

to the difference in threshold cycles for target and reference (Ct target - Ct reference). Over the fact that E of each gene were similar (Figure 1), the relative transcript abundance of each MyHC mRNA was determined was determined by dividing the amount of a particular MyHC on the total of others MyHCs. Thus, regression equations to proportion of each MyHC mRNA for all genetic groups were adjusted using lm ( ) function of free software R (R Core Team, 2013) .

RESULTS

The standard curve gradients of all genes had similar slopes (3.36 ± 0.06) implying similar PCR amplification efficiency (Figure 1), which allows quantitative comparisons across isoforms (Costa et al., 2002).

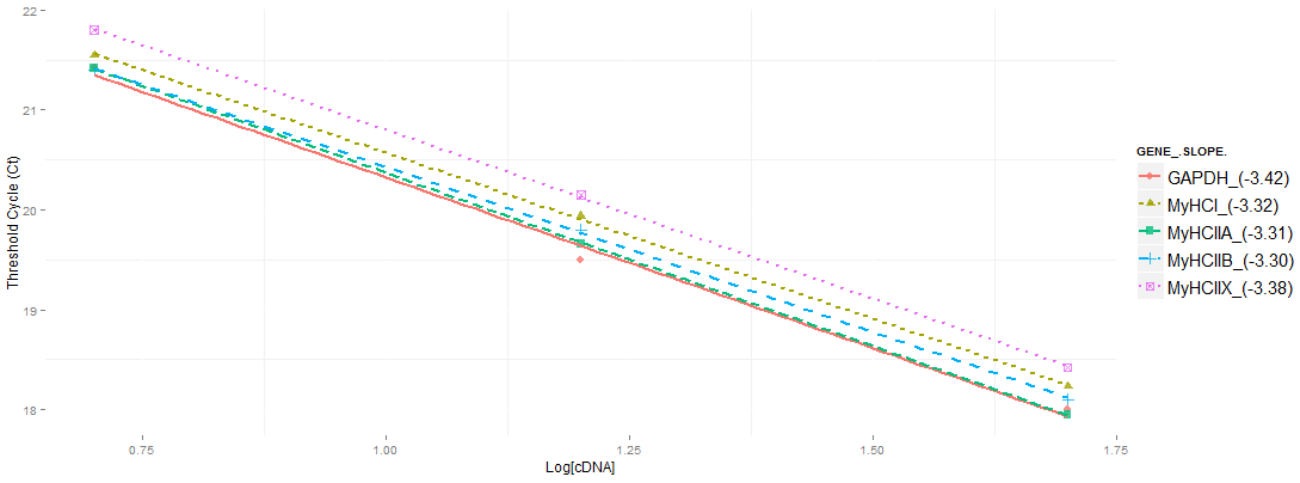

Figure 1. Relative standard curves of five genes GAPDH, MyHC I, MyHC IIa, MyHC IIx and MyHC IIb. Similar slope values suggest the similarity in amplification efficiency.

Interactions between genetic groups and slaughter ages to MyHC I, MyHC IIA and MyHC IIB relative transcript abundance were observed (Table 2).

Therefore, relative expression changes (fold change) results were interpreted by contrast comparison pairwise within each factor.

Table 2. Results of the analysis of variance - ANOVA (P values)

MyHC Isoforms	Genetic Groups	Age	GG x Age
MyHC I	0.3915	0.0001	0.0006
MyHC IIA	0.4067	<.0001	<.0001
MyHC IIX	0.4862	0.1248	0.3727
MyHC IIB	0.4067	<.0001	<.0001

The statistical analysis results for each contrast between treatments are shown in supplementary table 1 and 2 (Appendix). The expression of MyHC IIX showed no interaction between genetic groups and slaughtered ages (Table 2), thus its expression was used just to calculate the relative proportion of each MyHC isoform.

The expression of MyHC I was significantly different between birth and 56 days in Piau and CrB (Figure 2), mainly in Piau which showed 81-fold decrease ($P < 0.0001$). The higher expression in Piau and CrB at birth can explain the higher proportion of this isoform in comparison to other days (Table 3). The comparison of 56 -112 and 112-156 days showed no difference in any genetic groups (supplementary Table 2) however in Com the proportion of MyHC I decreases between 56 to 112 days ($P= 0.021$). Furthermore, at birth the expression in Piau was nearly 23 and 9.6 times greater than Com ($P = 0.0028$) and CrB ($P = 0.0258$) (Figure 3), respectively, explaining why the proportion of MyHC I transcripts in Piau at birth was the highest (Table 3). There was no difference in expression

between Com and CrB at birth (P=0.3799), assuming Com and Piau are divergent breeds and their alleles are homozygous, we can consider that the expression of Com allele is dominant over Piau allele. Absence of dominance was observed to other ages.

Table 3. Proportion of MyHC I in different genetic groups and ages quantified by real-time RT-PCR.

	MyHC I			
	0	56	112	156
Com	0.298±0.15 A a	0.410 ±0.32 A b	0.214±0.10 A a	0.307±0.43 A a
Piau	0.708±0.21 C b	0.223±0.06 A a	0.204±0.14 A a	0.175±0.10 A a
CrB	0.514±0.12 B b	0.330±0.15 A a	0.241±0.17 A a	0.137±0.05 A a

Means with the same uppercase letter in column and lowercase letter in row are not significantly different (P>0.05). Com=Commercial line, Piau= Piau, CrB= Crossbreed.

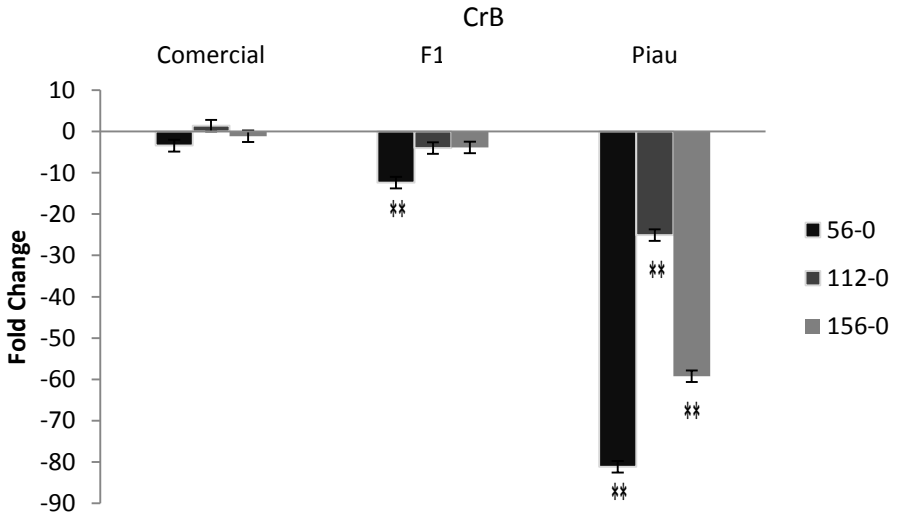

Figure 2. Fold change for MyHC I expression across slaughtered age within genetic groups. Results are presented as fold change for expression at 56,112 and 156 days relative to expression at birth (56-0, 112-0 and 156-0; respectively), such that the bars above the origin indicate lower expression at birth and bars below the origin indicate higher expression at birth. * P < 0.05; **P < 0.01.

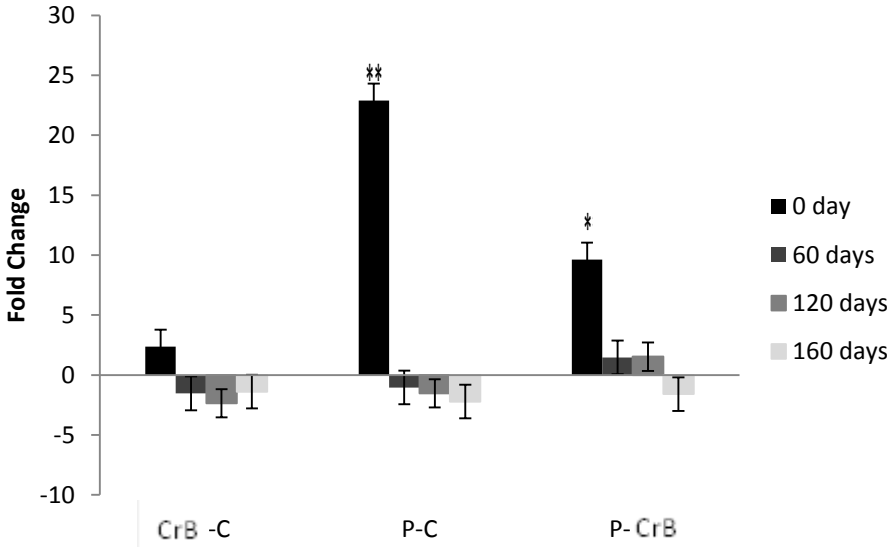

Figure 3. Fold change for MyHC I expression across slaughtered age within genetic groups (C=Commercial, CrB=Crossbread, P=Piau). Results are presented as fold change for expression across CrB-C , P-C and P-CrB, such that the bars above the origin indicate higher expression of first level of contrast, and bars below the origin indicate higher expression of second level of contrast.* P < 0.05; **P < 0.01.

The expression of MyHC IIA was significantly higher at birth in comparison to 56 days for all genetic groups (Figure 4), especially in Com ($P < 0.001$, FC=16.04). Although Com showed a 7.55 fold increase ($P = 0.002$) in expression at 112 days in comparison to 56 days (supplementary table 2), the proportion of MyHC IIA decrease while in Piau this proportion is higher at 112 days and in CrB at 156 in comparison to 56 days (Table 4) . Moreover, at 56 days the expression in Com was 3.76 ($P = 0.0346$) and 5.4 times lower ($P = 0.0082$) than CrB and Piau (Figure 5). Again, we can consider that the expression of Piau allele is dominant over Com allele, since Piau and CrB showed no difference in expression of MyHC IIA at 56 day, absence of dominance was observed to other ages. Despite to another ages there was no difference in expression of this gene across

genetic groups, the proportion in Com at 156 day was significantly lower than Piau (P>0.001) and CrB (P>0.001) (Table 5) .

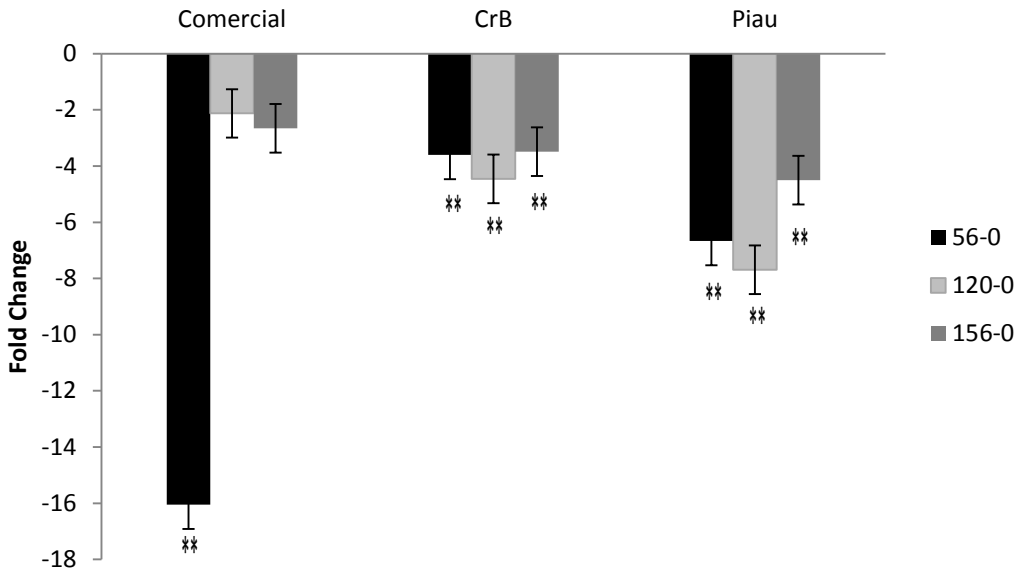

Figure 4. Fold change for MyHC IIA expression across slaughtered age within genetic groups. Results are presented as fold change for expression at 56,112 and 156 days relative to expression at birth (56-0, 112-0 and 156-0; respectively), such that the bars above the origin indicate lower expression at birth and bars below the origin indicate higher expression at birth. * P < 0.05; **P < 0.01

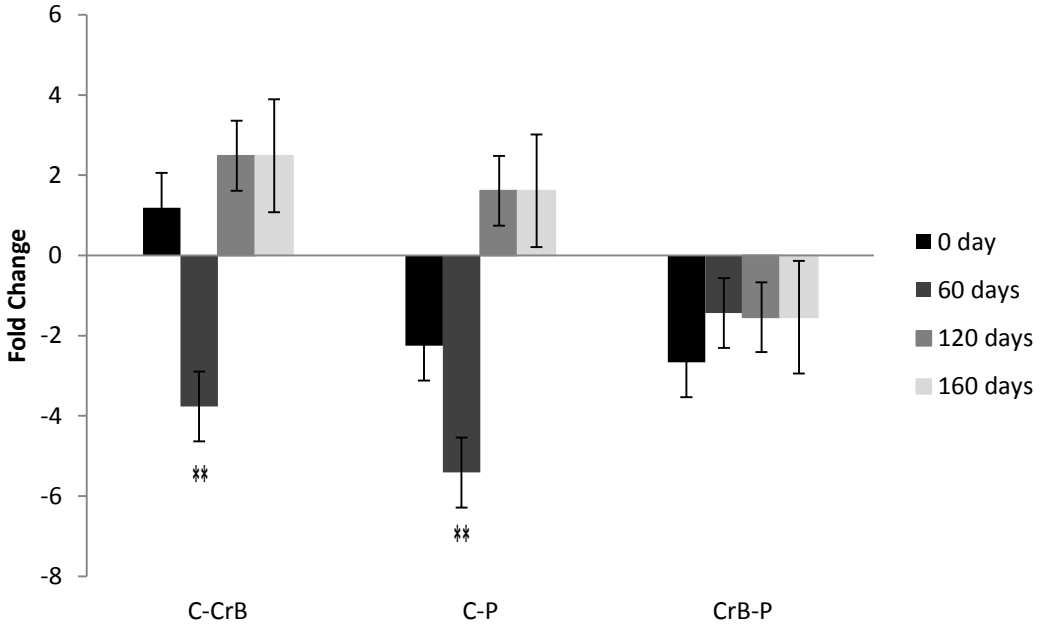

Figura 5. Fold change for MyHC IIA expression across genetic groups within slaughtered ages. (C=Commercial, CrB=Crossbread, Piau=Piau). Results are presented as fold change for expression across CrB-C, P-C and P-CrB, such that the bars above the origin indicate higher expression in first level of contrast, and bars below the origin indicate higher expression in second level of contrast. * P < 0.05; **P < 0.01.

Table 5. Proportion of MyHC IIA in different genetic groups and ages quantified by real-time RT-PCR.

	MyHC IIA			
	0	56	112	156
Com	0.520±0.06 C c	0.273±0.23 A b	0.185±0.07 A b	0.047±0.04 A a
Piau	0.174± 0.1 A a	0.171±0.08 A a	0.386±0.05 B b	0.386±0.06 B b
CrB	0.391±0.11 B b	0.206±0.05 A a	0.152±0.05 A a	0.367±0.15 B b

Means with the same uppercase letter in column and lowercase letter in row are not significantly different (P>0.05). Com=Commercial line, Piau= Piau, CrB= Crossbreed.

Although expression of MyHC IIB showed no significant difference across genetic groups within slaughtered ages (Table 2), a strong difference across slaughtered age within each genetic group was found (Figure 6). The expression of MyHC IIB relative to birth, contrary to MyHC I and MyHC IIA, had a significant increase across the ages with highest value at 156 days in Com (P < 0.001, FC = 110.22) and CrB (P = 0.014, FC=10.49) while in Piau was at 112 days (P = 0.012, FC = 24.34). The proportion of MyHC IIB transcripts in all genetic groups was lowest at birth (Table 6) and the highest proportion was found at 112 and 156 days in Com.

Table 6. Proportion of MyHC IIB in different genetic groups and ages quantified by real-time RT-PCR.

	MyHCIIB			
	0	56	112	156
Com	0.005±0.003 A a	0.199±0.21 A b	0.403±0.09 B c	0.442±0.28 B c
Piau	0.005±0.006 A a	0.357±0.21 B b	0.229±0.01 A b	0.144±0.11 A c
CrB	0.028±0.024 A a	0.256±0.1 AB b	0.312±0.1 AB b	0.209±0.20 A b

Means with the same uppercase letter in column and lowercase letter in row are not significantly different (P>0.05). Com=Commercial line, Piau= Piau, CrB= Crossbreed.

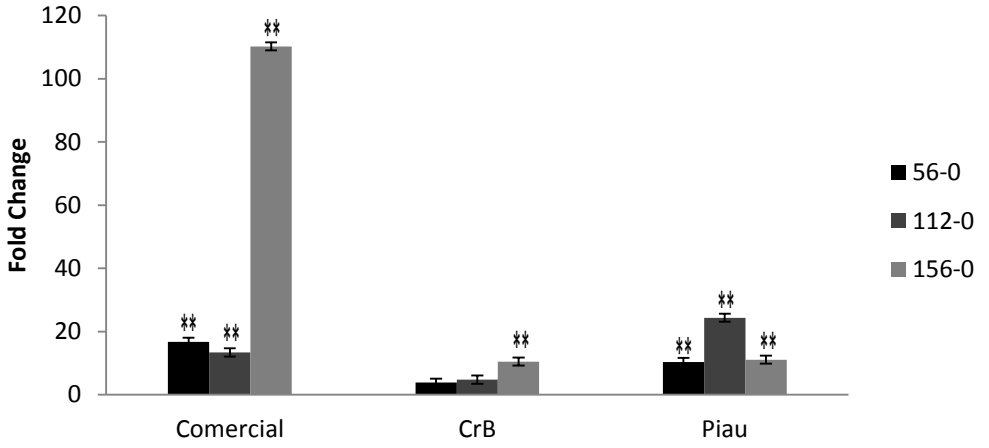

Figure 6. Fold change for MyHC IIB expression across slaughtered ages within genetic groups.). Results are presented as fold change for expression at 56,112 and 156 days relative to expression at birth (56-0, 112-0 and 156-0; respectively), such that the bars above the origin indicate lower expression at birth and bars below the origin indicate higher expression at birth. * P < 0.05; **P < 0.01.

The figure 7 was obtained from the sum of proportions of MyHC I with MyHC IIA (isoforms related to oxidative fibers) and MyHC IIB with MyHC IIX (isoforms related to glycolytic fibers) and estimate equations to study the pattern of this proportion during postnatal development. As we can see, both graphs show a linear equation to Com while in Piau and CrB a quadratic equation had a best fitting; however, the performance was symmetrically antagonistic. The vertex

(maximum and minimum points) of equations was estimate by derivation of the equations based on age. The proportion of glycolytic isoforms transcripts were observed to intensively increase up to 94 and 109 days to Piau and CrB, respectively and thereafter this proportion decrease. The reverse is true to oxidative isoforms.

Figure 7. Proportion of oxidative (A) and glycolytic MyHC (B) during postnatal development.

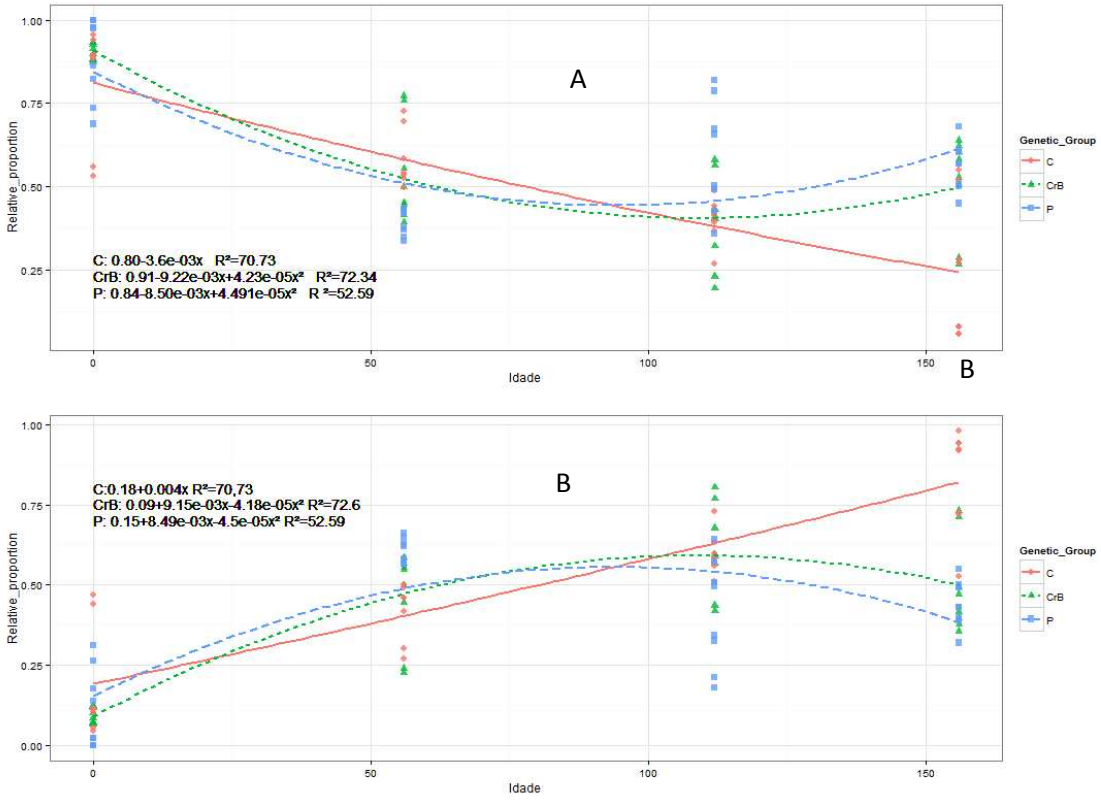

Table 7 and 8 shows that although all genetic groups demonstrate equal proportions of oxidative and glycolytic isoforms expression, respectively, at birth and this proportion change during development. At 56 days, Com and CrB shows higher proportion of oxidative isoforms than Piau (P =0.012, P =0.023; Table 7)

while at 112 and 156 days Piau shows higher proportion of these isoforms compared to Com.

Table 7. Proportion of MyHC I+MyHCIIA in different genetic groups and ages quantified by real-time RT-PCR.

	MyHCI+MyHCIIA			
	0	56	112	156
Com	0.817±0.17 A b	0.683±0.21 B b	0.4±0.06 A a	0.354±0.41 A a
Piau	0.882±0.12 A b	0.394±0.04 A a	0.59±0.17 B b	0.561±0.07 B b
CrB	0.905±0.02 A b	0.536±0.14 AB a	0.393±0.14 A a	0.505±0.14 B a

Means with the same uppercase letter in column and lowercase letter in row are not significantly different (P>0.05). Com=Commercial line, Piau= Piau, CrB= Crossbreed.

Table 8. Proportion of MyHC IIX+MyHCIIB in different genetic groups and ages quantified by real-time RT-PCR.

	MyHCIIX+MyHCIIB			
	0	56	112	156
Com	0.182±0.16 A a	0.317±0.21 A a	0.560±0.06 B b	0.646±0.41 B b
Piau	0.117±0.12 A a	0.565±0.04 B c	0.410±0.17 A b	0.439±0.07 A b
CrB	0.095±0.2 A a	0.463±0.14 AB b	0.567±0.14 B b	0.495±0.14 A b

Means with the same uppercase letter in column and lowercase letter in row are not significantly different (P>0.05). Com=Commercial line, Piau= Piau, CrB= Crossbreed.

DISCUSSION

Fiber type transition follows a reversible fixed pattern: I ↔ IIA ↔ IIX ↔ IIB. During postnatal development, there is no fiber formation and degradation is rare. Thus the properties of fibers changes by the remodeling of individual fibers (Pas et al., 2004).

The higher expression and proportion of oxidative MyHC (I and IIA) at birth are in accordance to Laffecheteur et al. (1986) who showed, by histochemical

analyses, that muscles at birth are essentially oxidative. The main heat production mechanisms in newborn pigs is shivering thermogenesis and oxidative fiber produce more heat per unit time than other fibers, when used in shivering (Herpin et al. 2002) . Thus, the differences in fiber type induced by changes in energy status reflect changes in the concentration of contractile proteins within specific muscles and hence in the functional properties of these muscles. We can observe in Figure 7 , although the proportion of oxidative MyHC decreases, this proportion still higher than glycolytic MyHC (IIX and IIB) until nearly 60 days to Piau and CrB and 83 days to Com, suggesting that proportion of oxidative fibers is higher until these dates but in decreasing rates. Laffecheteur et al. (1986) found that proportion of fibers type I increased from birth up to 2 months of age and little rate occurred thereafter.

The difference in expression of MyHC I and MyHC IIA across genetic groups at birth can be explained by embryonic development where the differentiation of fibers in first wave give originates to oxidative fibers, mainly type I, while fibers differentiated in second wave originate fibers IIA and IIX (Figure 8). Results of our scientific group, not published yet, found that Piau showed higher expression of myogenic networks in first wave, while a Com line had higher expression at the second wave. These results can explain why the expression of MyHC I in Piau was so higher than Com.

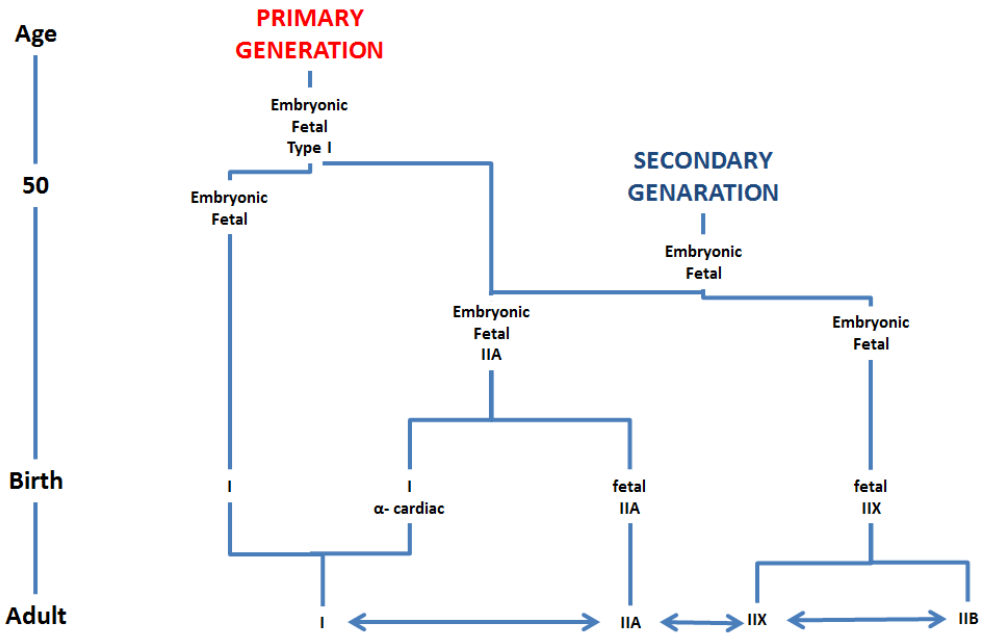

Figure 8. Schematic representation of fiber differentiation in developing skeletal muscle based on myosin heavy chain isoform transitions, adapted from Lefaucheur and Gerrard (1998)

There was a reversion of proportion of oxidative MyHC to glycolytic MyHC in all genetic groups (Figure 7), however the rate of this reversion were different. The glycolytic MyHC in Piau and CrB reached 50% of MyHC total together, however CrB kept this proportion higher than 50% for longer. In the other hand, Com reached this proportion later than Piau and CrB, but the proportion still increase linearly until 156 days, contrary to Piau and CrB which reached maximum proportion of glycolytic MyHC at 94 and 109 days , respectively. The increasing percentages of type IIb fiber, and decreasing percentages of types I and IIa fibers, are related to increases in drip loss and lightness, which are deteriorative to pork quality. Ryu and Kim (2006) mentioned that fast-glycolyzing

PSE pork contained higher proportion of IIx/IIb fiber, which may be more prone to undesirable pork because of its anaerobic nature, greater glycogen content, lower ultimate pH and higher drip loss.

The earlier inflexion in Piau and CrB may be due to the difference in partitioning of energy undergoing to protein and/or lipid synthesis, and the respective efficiencies of synthesis. Piau is considered a breed which has a low growth rate and high fat content in carcass (Faria et al., 2009). Additionally, it has been shown the precocity for intramuscular fat deposition in Piau animals (Serão et al., 2011). Thus, Piau and CrB animals had a faster increase of the proportion of glycolytic MyHC. When protein deposition rate decreases, lipid deposition becomes the major component of the weight gain and energy request to fat tissues increases (Hocquette et al. 2000). Consequently, less energy substrate to contraction will be available to muscles fibers and the energy metabolism of glycolytic fibers switch towards to oxidative energy metabolism, which is more efficient pathway to produce energy (Hocquette Ortigues-Marty, I., Pethick, D., Herpin, P., Fernandez, X. 1998). Differently than was observed in Piau and CrB animals, Com pigs no inflexion on proportion of glycolytic MyHC was observed, supporting the hypothesis that the intensive selection for lean muscle growth in modern pigs has, over time, induced a shift in muscle metabolism toward a more glycolytic and less oxidative muscle fiber type (Lefaucheur et al., 2004). Lin and Hsu (2005) compared the patterns of metabolic gene expression in the *Longissimus dorsi* muscle of adult Duroc and Taoyuan pigs, which differ in their post-natal muscle growth rate, and observed overexpression of glycolytic metabolism and glycolytic MyHC in Duroc. Benevenuto (2001) evaluated meat

quality in commercial Landrace x Large White, Piau and F2 pigs, found better meat quality traits in Piau .

In Com pigs, it was found that despite the proportion of MyHC I have increased from birth to 56 days (Table 3), relative abundance of this transcript was not changed during the postnatal development (Figure 2). Indeed, this proportion increases because relative abundance of MyHC IIA dramatically decreases (Figure 4) at the same stage. In addition, there was a significant increase in relative expression and proportion of MyHC IIB (Figure 6). This result suggests a remodeling of fibers type IIA to fibers type IIB in Com.

Since both of glycolytic MyHC relative expression showed no differences across genetic groups within each age, they do not explain the difference of MyHC proportions across genetic groups. Thus, we detected that difference of MyHC proportions across genetic groups are caused by difference in expression of oxidative MyHC.

The higher proportion of MyHC IIX and MyHC IIB in Com at 112 and 156 days can explain why growth rate in Com is faster and greater than Piau e CrB since the diameter of type II fibers increase faster than that of type I (Kim et al., 2013).

CONCLUSION

There was a reversion of proportion of oxidative MyHC to glycolytic MyHC in all genetic groups, however the rates of this reversion are different. Although all genetic groups were born with same proportion of oxidative MyHC, Com showed more MyHC IIA and Piau and CrB showed more MyHC I. Difference between proportions of each MyHC across genetic groups can be explained by difference

in expression of oxidative MyHC. The higher proportion of glycolytic MyHC in Com at 112 and 156 days can explain their faster growth rate.

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Appendix

Table 3. Fold change results for each contrast between genetic groups within each slaughtered ages.

Isoforms	Age	Comparison	FC	SE	p-value
MyHC I	0	CrB-C	2.378	1.405	0.3799
		CrB-P	-9.627	1.405	0.0258
		C-P	-22.889	1.405	0.0028
	56	CrB-C	-1.525	1.405	0.6674
		CrB-P	-1.471	1.405	0.6944
		C-P	1.037	1.405	0.9705
	112	CrB-C	-2.351	1.405	0.3861
		CrB-P	-1.533	1.405	0.6637
		C-P	1.534	1.405	0.6633
	156	CrB-C	-1.390	1.405	0.737
		CrB-P	1.593	1.405	0.6354
		C-P	2.215	1.405	0.420
MyHC IIA	0	CrB-C	-1.185	0.871	0.780
		CrB-P	-2.663	0.871	0.113
		C-P	-2.247	0.871	0.188
	56	CrB-C	3.763	0.871	0.035
		CrB-P	-1.438	0.871	0.551
		C-P	-5.411	0.871	0.008
	112	CrB-C	-2.484	0.871	0.140
		CrB-P	-1.542	0.871	0.478
		C-P	1.611	0.871	0.434
	156	CrB-C	-1.556	0.871	0.469
		CrB-P	-2.066	0.870	0.237
		C-P	-1.328	0.870	0.642
MyHC IIB	0	CrB-C	6.111	1.441	0.079
		CrB-P	2.111	1.441	0.456
		C-P	-2.895	1.441	0.295
	56	CrB-C	1.397	1.441	0.226
		CrB-P	-1.284	1.441	0.740
		C-P	-1.794	1.441	0.804
	112	CrB-C	2.182	1.441	0.440
		CrB-P	-2.420	1.441	0.383
		C-P	-5.279	1.441	0.105
	156	CrB-C	-1.720	1.441	0.591
		CrB-P	1.995	1.441	0.494
		C-P	3.431	1.441	0.226

FC: fold change; SE: standard error; CI: confidence interval. Positive values of FC mean lower expression at the first level relatively to the second level. (C=Commercial, CrB=Crossbread, Piau=Piau)

Table 4. Fold change results for each contrast between slaughtered ages within each genetic groups.

Isoform	Genetic Group	Comparison	FC	SE	p-value
MyHC I	C	0-56	3.419	1.405	0.215
		0-112	-1.399	1.405	0.732
		0-156	1.169	1.405	0.874
		56-112	-4.785	1.405	0.117
		56-156	-2.926	1.405	0.278
		112-156	1.635	1.405	0.617
	CrB	0-56	12.399	1.405	0.014
		0-112	3.994	1.405	0.164
		0-156	3.864	1.405	0.174
		56-112	-3.105	1.405	0.253
		56-156	-3.209	1.405	0.239
		112-156	-1.034	1.405	0.973
	P	0-56	81.156	1.405	<.0001
		0-112	25.088	1.405	0.002
		0-156	59.265	1.405	<.0001
		56-112	-3.235	1.405	0.236
		56-156	-1.369	1.405	0.749
		112-156	2.362	1.405	0.383
MyHC IIA	C	0-56	16.047	0.863	<.0001
		0-112	2.125	0.863	0.218
		0-156	2.658	0.863	0.113
		56-112	-7.552	0.863	0.002
		56-156	-6.036	0.863	0.005
		112-156	1.251	0.863	0.711
	Crb	0-56	3.598	0.863	0.041
		0-112	4.454	0.863	0.018
		0-156	3.490	0.863	0.046
		56-112	1.238	0.863	0.724
		56-156	-1.031	0.863	0.956
		112-156	-1.276	0.863	0.687
	P	0-56	6.662	0.863	0.004
		0-112	7.693	0.863	0.002
		0-156	4.498	0.864	0.018
		56-112	1.155	0.863	0.812
		56-156	-1.481	0.864	0.517
		112-156	-1.710	0.864	0.377

Table 4 (cont.). Fold change results for each contrast between slaughtered ages within each genetic groups.

MyHC IIB	C	0-56	-16.714	1.294	0.004
		0-112	-13.352	1.294	0.008
		0-156	-110.224	1.294	<.0001
		56-112	1.252	1.294	0.804
		56-156	-6.594	1.294	0.045
		112-156	-8.255	1.294	0.026
	CrB	0-56	-3.821	1.294	0.147
		0-112	-4.767	1.294	0.093
		0-156	-10.488	1.294	0.014
		56-112	-1.247	1.294	0.807
		56-156	-2.745	1.294	0.270
		112-156	-2.200	1.294	0.387
	P	0-56	-10.359	1.294	0.015
		0-112	-24.346	1.294	0.001
		0-156	-11.097	1.294	0.012
		56-112	-2.350	1.294	0.349
		56-156	-1.071	1.294	0.939
		112-156	2.194	1.294	0.389

FC: fold change; SE: standard error; CI: confidence interval. Positive values of FC mean lower expression at the first level relatively to the second level. (C=Commercial, CrB=Crossbread, Piau=Piau)