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Influence of glycolic, oxidative, and mixed metabolism on growth in swine skeletal muscle

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Contents

List of Tables 5

List of Figures 6

List of Annex..7

Abstract..8

Resumen 9

Introduction 10

Materials and Methods...13

Experiment Description 13

Quick DNA Miniprep Plus Kit Protocol 15

Materials 15

Reagents...15

Equipment..15

Protocol..16

TaqMan q-PCR for Mitochondrial DNA Quantification..17

Materials 17

Reagents...17

Equipment..17

Protocol..18

Hematoxylin & Eosin Staining 20

Materials 20

Reagents...21

Equipment..21

Protocol 21

Statistics Section 23

Results and Discussion 24

Mitochondrial DNA Quantification 24

Hypertrophy and Adipose Tissue Growth 27

Analysis of the Cross-Sectional Area of Muscles 28

Conclusions 31

Recommendations 32

References 33

Annexes 35

List of Tables

Table 1 Group of pigs according to age 14
Table 2 Equipment used in the DNA extraction protocol. 15
Table 3 Equipment used in the Mitochondrial DNA quantification protocol. 17
Table 4 Pig primers/ Mitochondrial DNA 18
Table 5 Eukaryote primers / Nuclear DNA 18
Table 6 Samples used for standards 19
Table 7 Standards 19
Table 8 Cycling Parameters 20
Table 9 Equipment used in H&E staining protocol. 21
Table 10 Treatments 23
Table 11 Statistical Analysis PCR 25
Table 12 Least Square Means for effect AGE 26
Table 13 Least Square Means for effect MUSCLE 27
Table 14 Statistical Analysis CSA 30

List of Figures

Figure 1 Diagram for obtaining the Histological sample 14

Figure 2 Diagram process of obtaining tissue section sample 21

Figure 3 Abundance of Mitochondrial DNA throughout the growth cycle 25

Figure 4 Abundance of Mitochondrial DNA in different muscles 27

Figure 5 Body weight 28

Figure 6 Assessment of adipose tissue growth 28

Figure 7 Assessment of muscle growth with different types of metabolism 29

List of Annex

Annex A DNA Extraction 35

Annex B Standars curve 36

Annex C Layout of the Standard 96-well plate 37

Annex D Layout of the Pigs 96-well plate 38

Annex E Assess Mitochondrial DNA as an indicator of metabolic changes 39

Annex F Least Squares Means of the analysis of the CSA of muscles 40

Abstract

The present work focuses on analyzing skeletal muscle metabolism in pigs, throughout different stages of growth and investigate how muscle fiber growth varies in three types of muscle with different metabolic profiles: *Longissimus dorsi* (glycolytic muscle), *Latissimus dorsi* (mixed muscle) and *Masseter* (oxidative muscle). The interaction between age and muscle has influence on hypertrophy muscle is also explored.The study was conducted with 25 selected castrated pig from the Kansas State University research facility. The pigs were fed according to a staggered feeding plan to meet their nutritional requirements at different stages of growth, were sampled at five slaughter time points from 20 to 180 days of age. The results revealed that there is not significant difference in Mitochondrial DNA abundance between oxidative and glycolytic muscle fibers. This contrasts with earlier research suggesting that oxidative muscle fibers tend to have a higher amount of Mitochondrial DNA. It is suggested that other factors than metabolic activity may influence this difference. In terms of the amount of Mitochondrial DNA (mtDNA), a peak was observed at 87 days of age, which could be related to the increase's energy demands of muscle at this stage and the transition from a nursery diet to finisher diet. In addition, it was found that the interaction between age and muscle does significantly affect muscle growth. While locomotive muscle, such as Longissimus dorsi and Latissimus dorsi, showed continuous growth after 53 days, the Masseter muscle, related to chewing and located in the head, experienced significant growth after 120 days.

Keywords: Fibers, hypertrophy, mtDNA, pig, nutrition.

Resumen

El presente estudio se enfocó en el analisis del metabolismo del músculo esquelético en cerdos, a través de diferentes etapas de crecimiento e investigar cómo el crecimiento de la fibra muscular varía en tres tipos de músculo con diferentes perfiles metabólicos: *Longissimus dorsi* (músculo glicolico), *Latissimus dorsi* (músculo mixto) y *Masseter* (músculo oxidativo). El estudio se llevó a cabo con 25 cerdos castrados que fueron alimentados de acuerdo con un plan de alimentación para satisfacer sus necesidades nutricionales en diferentes etapas de crecimiento, fueron muestreados en cinco puntos de tiempo de sacrificio de 20 a 180 días de edad. Los resultados revelaron que no hay diferencia significativa en la abundancia de ADN mitocondrial entre las fibras musculares oxidativas y glicolicas. Contrastando con investigaciones anteriores que sugieren que las fibras musculares oxidativas tienden a tener una mayor cantidad de ADN mitocondrial. Sugiriendo que otros factores además de la actividad metabólica pueden influir en esta diferencia. En términos de la cantidad de ADN mitocondrial (ADNmt), se observó un pico a los 87 días de edad, que podría estar relacionado con el aumento en la demanda de energía del músculo en esta etapa y la transición de una dieta de inicio a una final. Además, se encontró que la interacción entre la edad y el músculo afecta significativamente el crecimiento muscular. Los músculos locomotores, como *Longissimus dorsi* y *Latissimus dorsi*, mostraron crecimiento continuo después de 53 días, el músculo Masseter, relacionado con la masticación, experimentó un crecimiento significativo después de 120 días.

Palabras clave: ADNmt, cerdo, fibras, hipertrofia, nutrición.

Introduction

According to the Organization of Economic Co-operation and development (OECD) and the Food Agriculture Organization of the United Nations (FAO), over the next ten years, global consumption of pork will rise to 127 million tons, making up 33% of the overall growth in meat. Pork consumption has increased quickly per person in Latin America as result of competitive prices that have brought pork to the international market (Organization for Economic Co-operation and Development (OECD)/ 2021). Meat demand is increasing globally because of demographic and economic growth. The swine business is currently in a production phase in which improving productive yields is critical to ensuring the sustainability of farms and the food chain. Improved swine farm performance and efficiency are connected to good health and nutritional management (Biovet 2021).

The optimization of feed efficiency appears as a critical topic in swine husbandry, as it the significant consequences not only for production yields but also for farm economic viability. Given that feed expenses account for a significant portion of total production costs, ranging from 80 to 85%, producers must devise meticulously devised strategies to expedite attainment of optimal body weight within a shortened timeframe while simultaneously fostering increased feed efficiency (Campabadal 2009).

The composition of tissues, particularly muscle and fat, is critical to the quality of meat products of animal origin, particularly pig. This composition is influenced by various factors including as diet, age, gender, and genetic type. Among these, nutrition is a simple instrument for modulating tissue composition and adapting it to quality criteria (Benítez et al. 2012). Skeletal muscle makes up between 30 to 65% of a pig's body weight and 45% of its total protein, depending on the breed. Muscle fibers account for 75 to 90% of muscle volume, with the remainder made up of the adipose tissue, connective tissue, arteries and nerves (Graziotti et al. 2000).

Pig breeders are increasingly interested in improving meat quality features, as it is difficult to improve meat quality using conventional selection methods. The quality of meat depends on the

composition of different types of muscle fiber and intramuscular fat content. Research of muscle transcriptional profiles is particularly interesting to understand the mechanisms of muscle development and find molecular methods to improve meat quality traits in pig breeding. In addition, muscle fiber are adaptable beings that can chance their phenotypic properties in response to changing functional needs.

The early prenatal environment is critical for the development of the skeletal muscle. Several studies have revealed that intralitter variation in utero influences postnatal skeletal muscle growth in the pig (Dwyer et al. 1994). Muscle growth is recognized to be influenced by the number, size, and type of muscle fibers. In most mammalian species, the total number of the skeletal muscle fibers (hyperplasia) is fixed before birth. Skeletal muscle fibers only expand postnatally through hypertrophy (growth in size) (Karunaratne et al. 2005). According to previous studies, the increase in the number (hyperplasia) and size (hypertrophy) of myofibers contributes significantly to the postnatal muscle phenotype of pigs; However, the number and composition of muscle fiber vary significantly during prenatal development (Xu X et al. 2019).

Due to the fact that genetic background has a significant impact on animal performance, a constructive interaction between genetics and nutrition is required to improve our understanding of what constitutes optimal responses to fiber-rich diets. A high-fat diet for pigs can cause variations in muscle fiber ratios due to Mitochondrial expansion or decrease, resulting in alterations in cellular metabolism (Vincent et al. 2015). Different breeds of pigs perform differently because proteins synthesis and Mitochondrial energy consumption of muscular tissue are not comparable. Furthermore, there is a link between the gene networks that control Mitochondrial energy metabolism and muscle growth (Pérez de Nanclares et al. 2017).

Diet has a significant impact on muscle physiology and one of the best-known instances is the enzymatic activity of muscle in pigs fed a high protein diet, which results in increased glycolytic metabolism, which has ramifications for the animal’s growth. To meet the energetic requirement of

muscle, resources are used via biochemical reactions that allocate different nutrients to anabolic growth or catabolism. Substrates are oxidized during a condition of catabolism in which substrates are predominantly assigned to oxidative metabolic pathways, which creates carbon dioxide (CO_2) and represents a loss of carbon that could be employed in anabolic reactions and muscle building (Kong and Adeola 2014).

Whether nutrients are digested in muscle via glycolytic or oxidative mechanisms influences feed conversion. Understanding the cellular processes that govern food allocation to glycolytic or oxidative metabolic pathways will thus help us create novel strategies to increase swine feed efficiency (Sarri Espinosa 2018).

Therefore, the objectives of this study are:

Analyze the changes in skeletal muscle metabolism at various stages of the growth cycle of the pig depending on the type of muscle.

Understand how muscle fibers growth differs in three skeletal muscles with different metabolisms: *Longissimus dorsi* (glycolytic muscle), *Latissimus dorsi* (mixed muscle) and *Masseter* (oxidative muscle).

Investigate the metabolic changes that accompany skeletal muscle hypertrophy.

Materials and Methods

Experiment Description

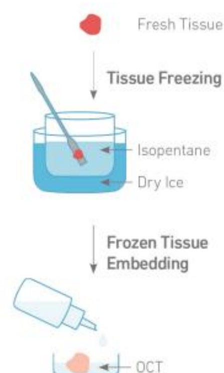
The study used 25 castrated pigs chosen among the 1,500 pigs (300 pigs per litter of the same age) housed at Kansas State University (KSU) Swine Teaching and Research Center in Manhattan, Kansas. The pigs had *Ad libitum* access to feed and water, with a single automatic drinker and a trough in each pen. Staged feeding was implemented (Nursery diet 12-80 days and Finisher diet 80-280 days), to satisfy the nutrient requirements for these growing pigs to promote optimum growth.

The pigs were brought to the KSU meat lab and slaughtered at 20 days (5.7 kg), 53 days (20.8kg), 87 days (42.2kg), 120 days (83.4kg) and 180 days of age (130.5kg) (Table 1). Within 15 minutes of exsanguination, three muscles samples were taken: *Longissimus dorsi (LD), Latissimus dorsi (LAT) and Masseter (MS).*

The samples were split and stored in liquid nitrogen for further use in histology (CSA) or Mitochondrial isolation (PCR). The liquid nitrogen-frozen samples were kept at -80°C until further analysis. Histological samples were frozen in liquid nitrogen-cooled isopentane and stored at -80 °C after being immersed in plastic molds containing appropriate cutting material (Fisher Scientific, Hampton, NH) (Figure 1). Mitochondrial were isolated immediately for metabolite tracing (The protocol for this study was authorized by Kansas State University Institutional Animal Care and Use Committee).

Figure 1

Diagram for obtaining the Histological sample



Note. Acquired from the manual Xenium In Situ for Fresh Frozen- Tissue Preparation Guide (10X Genomics, Inc. [date unknown])

Table 1

Group of pigs according to age

Age (days)	Age group	Pig #	Harvest group
20	1	P 16	4
		P 17	
		P 18	
		P 19	
		P 20	
53	2	P 11	3
		P 12	
		P 13	
		P 14	
		P 15	
87	3	P 6	2
		P 7	
		P 8	
		P 9	
		P 10	
120	4	P 1	1
		P 2	
		P 3	
		P 4	
		P 5	
180	5	P 21	5
		P 22	
		P 23	
		P 24	
		P 25	

Note. Pig #: identification given to each pig for study, P: pig and #: pig number.

Quick DNA Miniprep Plus Kit Protocol

Materials

Powder skeletal muscle samples

Microcentrifuge tubes

Zymo-Spin IIC-XLR column

Collection tube

Eon BioTek microplate

Reagents

Proteinase K storage buffer

Proteinase K

MQ water

Solid tissue buffer

Genomic Binding Buffer

DNA Pre-wash buffer

g-DNA wash buffer

DNA elution buffer

Equipment

Table 2 presents the equipment used in the Quick DNA Miniprep Plus Kit protocol, along with their respective brads.

Table 2

Equipment used in the DNA extraction protocol.

Name of the equipment	Brad
Centrifuge 5810R	Eppendorf
Barnstead MaxQ 4000 Digital Orbital Incubator Shaker	Thermo Fisher Scientific
Mini vortex	Thermo Fisher Scientific
Eon plate reader	BioTek

Protocol

Incubator was preheated to 55°C and 80 rpm and 0.1g of skeletal muscle sample was weighed and placed in a microcentrifuge tube.

In a bottle 1.040 µl of proteinase K storage buffer and 20 mg of proteinase k were mixed. The final concentration was 20 mg/ml, which was stored at -20°C after mixing.

A solution of 190 µl of MQ water, 190 µl of solid tissue buffer (blue liquid) and 20 µl of proteinase K was added to the microcentrifuge tubes with the muscle sample, then taken individually to the vortex for 10-15 seconds and were left for an hour in conditions of 55°C to 80rpm in the incubator room.

At the end of this time, each microcentrifuge tube was mixed with 800 µl of genomic binding buffer and vortex for 15 seconds. The mixture was transferred to a Zymo Spin IIC-XLR column in a collection tube, this step was repeated twice for the liquid amount, then all the mix was centrifuged at 12,000 rpm for one minute (x2), when these minutes passed the collection tube was discarded.

400 µl of DNA pre-wash buffer was added to the spin column in a new collection tube and centrifuged at 12,000 rpm for one minute and the collection tube was emptied. A 700 µl of g-DNA wash buffer was added to the spin column and centrifuged at 12,000 rpm for one minute and emptying the collection tube was emptied. 200 µl of g-DNA wash buffer was added to the spin column, centrifuged at 12,000 rpm for one minute and the collection tube with the supernatant was removed.

The spin column was transferred to a clean microcentrifuge tube (1.5-1.7 mL), and a 50 µL DNA elution buffer was added directly into the matrix, it was left to rest for five minutes at room temperature and centrifuged at 14,000 rpm for one minute to elute the DNA, this step was repeated once again (Note: The eluted DNA was immediately used for molecular based applications or stored at <20 °C for future use).

When DNA abundance was analyzed 2µL of DNA and one blank of 2µL at ultrapure water were loaded into the Eon BioTek microplate and was read using the Eon BioTek reader. Gen5 application was opened and used the nucleic acid setting.

TaqMan q-PCR for Mitochondrial DNA Quantification

Materials

MicroAmp™ Fast Optical 96-Well Reaction 4346906

MicroAmp™ Optical Adhesive Film 4311971

Pipettes and pipette tips with filter

1.6 mL autoclaved tubes

Ice bucket with ice

10 µM Primer and probe stocks

DNA samples ON ICE

Reagents

DNase Away

DEPC-treated RNase

Equipment

Table 3 presents the equipment used in the TaqMan q-PCR for Mitochondrial DNA Quantification protocol, along with their respective brads.

Table 3

Equipment used in the Mitochondrial DNA quantification protocol.

Name of the equipment	Brand
Centrifuge with adapter for 96-well plates	No specific brand
Fast Advanced Master Mix Applied Biosystems stored at -20oC freezer until 1st thaw, then at 4 °C	TaqManTM
7500 Fast Real Time PCR System in rooms	Thermo Fisher Scientific

Protocol

Prepared 96-well plate layout that ran on the instrument and cleaned up the work area and pipets using DNase Away.

To prepared PCR reaction mix: thawed TaqMan^{TM} Fast Advanced Master Mix on ice and blended gently while dipping the tube several times. Table 4 and Table 5 shows the total volumes of Reaction Mix's both Mitochondrial DNA and Nuclear DNA.

Table 4

Pig primers/ Mitochondrial DNA

Reagent	Initial (µMole/L)	Final (µMole/L)	Vol/Reaction (µL)	# of reactions (µL)	Total volume (µL)
TaqMan	2X	1X	10	54	540
H2O			6.75	54	364.5
Primer F	100	1	0.5	54	27
Primer R	100	1	0.5	54	27
Probe	10	0.5	0.25	54	13.5
DNA	up to 20ng		2		

Table 5

Eukaryote primers / Nuclear DNA

Reagent	Initial (µMole/L)	Final (µMole/L)	Vol/Reaction (µL)	# of reactions (µL)	Total volume (µL)
TaqMan	2X	1X	10	54	540
H2O			6	54	324
Primer F	100	1.5	0.75	54	40.5
Primer R	100	1.5	0.75	54	40.5
Probe	10	1	0.5	54	27
DNA	up to 20ng		2		

Note. The total number of reactions was calculated by multiplying the number of individual samples x 3 and adding 5%

Added each component to a 1.6 mL tube labeled "RM mt" (reaction mix) and "RM EUK" in the order indicated above (largest volumes first, smallest volumes last). Changed the pipet tips between each component and after the addition of each component, pipet up and down a few times to rinse the tip and mix the solution.

The reaction solution was mixed gently inverted a few times and centrifuged briefly to bring all the liquid to the bottom of the tube. Finally, a serial dilution of 1:10 was made for 8 standard curve points. Standard 1 is pooled of all the sampler (Table 6) and the other standards are dilutions of the above (Table 7).

Table 6

Samples used for standards

Number of pigs	Muscle
2	LD
29	LAT
5	MS
14	LAT
23	MS

Table 7

Standards

Standards	DNA Quantity (µL)	Water Quantity (µL)
STD 1	10 from each sample = 100	0
STD 2	5 of STD 1	45
STD 3	5 of STD 2	45
STD 4	5 of STD 3	45
STD 5	5 of STD 4	45
STD 6	5 of STD 5	45
STD 7	5 of STD 6	45
STD 8	5 of STD 7	45

The following steps were taken for the standards:

20 µl of each standard mixture (Standard and reaction mix) and the plate was covered with MicroAmpTM Optical Adhesive Film, ensuring the edges were well covered. The plate was then briefly centrifuged before being placed in the thermocycler and ran the PCR reaction plate.

The plate was placed in the thermocycler and the instrument was closed, verifying that the cycling parameters coincided with the above. Clicked "Start Run" and configured the plate under

"plate set-up" with the standards and samples in the assigned wells, when finished cleaned the work area using 10% ethanol, turned on the UV light and turned off the white light.

For the duplicate sample: added 18 µl of the master mixture to each well (as marked on the plate layout), the pipet tips between tubes/wells were changed. All DNA samples were then briefly agitated and centrifuged before use. 2 µl of DNA was added to each well according to the tube/ plate layout and added the sample directly to the master mix at the bottom of each tube/well and pipetted up and down 2-3 times to rinse and mix, changed the pipet tips between each sample.

Then the plate was centrifuged down to 1000rpm and placed on the machine running used 7500 Fast ABI; Taqman Assay. Finally, Table 8 shows the parameters used to verify the process.

Table 8

Cycling Parameters

Instrument	Step	Temperature (°C)	Duration (s)	Cycles
7500 Fast ABI	Ampli Taq^{Tm} Fast DNA Polymerase Up activation	95	20	Hold
	Denature	95	3	40
	Anneal/ extend	60	30	

Hematoxylin & Eosin Staining

Materials

Tissue sections

Microscope slides

Slides cove

Coplen jar

Kimwipes

Shandon Mounting media.

Reagents

Hematoxylin 1%

Deionized water

Eosin 50%

Ethanol (50%, 70%, 95%, 100%)

MQ water

Equipment

Table 9 presents the equipment used in the Hematoxylin & Eosin Staining protocol, along with their respective brads.

Table 9

Equipment used in H&E staining protocol.

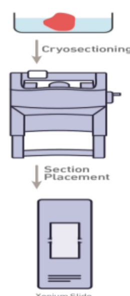
Name of the equipment	Brand
Cryostat microtome	Thermo scientific
Microscope	Nikon Eclipse Ti2

Protocol

Tissue sections of 5 µM were cut and thick on saline coated slides using a cryostat microtome, then stored the slides at -80 °C (Figure 2).

Figure 2

Diagram process of obtaining tissue section sample



Note. Acquired from the manual Xenium In Situ for Fresh Frozen- Tissue Preparation Guide (10X Genomics, Inc. [date unknown])

The cuts were removed from the freezer and allowed to thaw/dry for 15 minutes. After 15 minutes, the slide was placed into the 1% prepared hematoxylin for three minutes, then placed in a coplen jar with running deionized water for five minutes. After this time submerged in prepared eosin (diluted 1:50) for one second (one dip). Then rinsed with deionized water for one minute.

The slides were immersed in 50% ethanol for 10 seconds, followed by 70% ethanol for 10 seconds, 95% ethanol for 30 seconds and finally 100% ethanol for 30 seconds.

The slide was quickly dipped into xylene seven times, then dried with a Kimwipe, making sure not to dry the area containing the section, as this would have cleaned the fabric.

A few drops of mounting media were added to cover the entire whole slide and the slide cover was gently placed over the tissue section. The slide cover was left to dry and was ready for imaging after approximately 30 minutes.

To take pictures of the slides with the sample the following steps were followed:

The microscope cover sleeve was removed, and the different engines of the equipment were started in ascending order.

The NIS-Elements AR 5.30.02 application was opened and the Nikon Ti2 button was clicked. Then the slide was placed face down and the live view button was pressed, ensuring that Fi3-DIA functions were activated and setting the target to 10x. The image was focused, and the capture was taken, making sure to save it in ND2 format. When the imaging was finished, the different engines of the microscope were switched off in descending order.

The following steps were followed for image analysis:

The computer was turned on, the NIS-Elements AR 5.30.02 application was introduced, and the passive mode button was clicked. Opened the folder of the images made in the previous steps and accessed the toolbar, selecting the tool to draw perimeters.

Zoomed in, if necessary, to find a piece of the image that could be clearly seen, and the edge of the fibers was drawn using the tool mentioned above (20 fibers per image). At the end the data was exported to an Excel sheet and analyzed the averages of the perimeters per image taken.

Statistics Section

The experiment followed a Completely Randomized Design (CRD) with effects on the variables muscle, age, and their interaction, separated into five treatments with five repeats (Table 10), yielding a total of 25 experimental units. When Pr ≤ 0.05, data were judged significant, and peer comparisons were separated using Tukey's HSD. The data is show as Least Squares Means (LSMeans) ± Standard Error (SE). R Studio (version 4.2.1) was used to analyze all data.

Table 10

Treatments

Treatments	Description	
	Age (days)	Types of muscles
TRT 1	20	MS
		LAT
		LD
TRT 2	53	MS
		LAT
		LD
TRT 3	87	MS
		LAT
		LD
TRT 4	120	MS
		LAT
		LD
TRT 5	180	MS
		LAT
		LD

Note. TRT: Treatments, MS (Masseter), LAT (Latissimus dorsi) and LD (Longissimus dorsi).

Results and Discussion

Mitochondrial DNA Quantification

The content of Mitochondrial DNA (mtDNA) in *Longissimus dorsi (LD), Latissimus dorsi (LAT) and Masseter (MS)* muscles with glycolytic, mixed, and oxidative metabolism, respectively, studied during five stages over a period of 180 days, was determined by selecting differences sequences and then performing a qPCR analysis.

Table 11 shows the probabilities of muscle, age, and their interaction. The variance analysis (ANOVA) revealed that there were no significant differences between type of muscle (Pr= 0.936), while there were significant differences between ages (Pr=9.04e-05) allowing us to indicate that there is a difference in the quantity of mtDNA. The interaction between muscle types and age factor was not statistically significant (Pr= 0.298), indicating that the relationship between age and mtDNA abundance does not vary according to muscle type.

Figure 3 provides a visual demonstration of the significance of the age facto. In this figure, a peak of growth in mtDNA abundance is observed at 87th day for all age groups.

Table 12 shows the separation of means from the age factor. This table shows the average, minimum and maximum value of mtDNA abundance for each age group. The separation of means is important because it indicates that age 3 (87 days) is statistically different from other ages.

The higher mtDNA content observe on 87th day can be associated with two reasons: Greater energy needs required by the muscle at this age, this assumed by the analysis performed by Pesce et al. (2001) which indicates: greater muscle development will have greater energy demand. We can compare this with the contractile activity of the heart muscle and the high energy demand required for this process, which generates a higher demand of mtDNA (Xie YM. et al. 2015). On the other hand, the transition from nursery diet (12-80 days) to a finisher diet (80-280 days) can generate a higher energy demand, because when transitioning to a diet with the objective of creating greater muscle growth. According to Whittemore (1993), and Fang LHu et al. (2019) the pig presents changes in

development, blood profiles and other parameter, thus causing the pig to enter into stress and sped more energy as a survival method to gain more weight and develop muscle.

Table 11

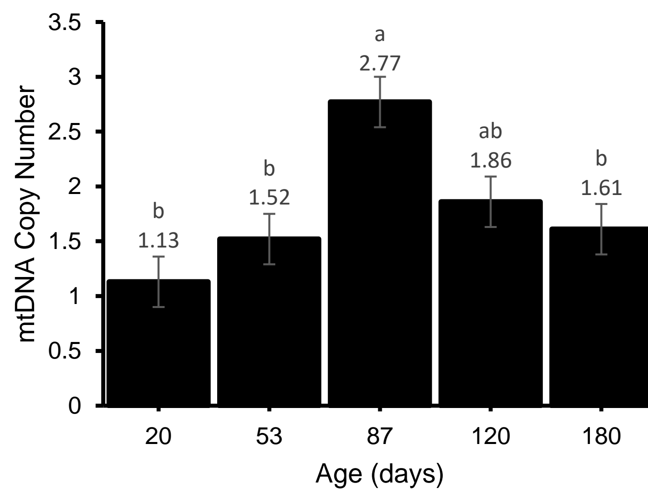
Statistical Analysis PCR

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Muscle	2	0.10	0.052	0.066	0.936
Age	4	22.61	5.653	7.139	9.04e-05
Muscle: Age	8	7.79	0.974	1.230	0.298
Residuals	60	47.51	0.792		

Note. Df: degree freedom, Sum Sq: Sum of Squares, Mean Sq: Mean of Square, Pr (>F): probability

Figure 3

Abundance of Mitochondrial DNA throughout the growth cycle



*Note.*20 days: max 1.59, min:0.67; 53 days: max 1.98, min 1.07; 87 days: max 3.23, min 2.31; 120 days: max 2.32, min 1.40; 180 days max 2.07 min 1.15.

Table 12

Least Square Means for effect AGE

Age	emmean	SE	Df	Lower.CL	Upper.CL	Group
1	1.13	0.23	60	0.67	1.59	b
2	1.52	0.23	60	1.06	1.98	b
5	1.61	0.23	60	1.15	2.07	b
4	1.86	0.23	60	1.40	2.32	ab
3	2.77	0.23	60	2.31	3.23	a

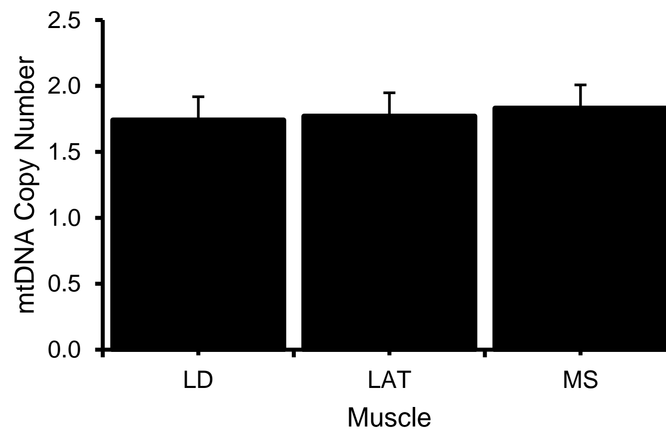
Note. SE: Standard Error, Df: degree freedom, Lower.CL: lower value, Upper CL: higher value.

Figure 4 shows that, since the probability of mtDNA abundance in muscle was not statistically significant (Pr=0.936) (Table 11), all the bards representing the three types of muscles studied are at the same level. This can be sustained with the media separation shown on Table 13.

These results differ with date obtained by Bakala et al. (2013), who reported that oxidative muscle fibers tend to have higher mtDNA copy number than glycolytic muscle fiber. It is also possible that there are other factors than metabolic activity influence mtDNA abundance un muscle cells. The date are also inconsistent with the analysis produced by: Hoeks and Schrauwen (2012) the abundance of mtDNA is an indicator of the metabolic activity if Mitochondria, which are cellular organelles responsible for energy production. Therefore, oxidative muscle fibers, which are primarily responsible for aerobic energy production, were expected to have higher mtDNA copy number than glycolytic muscle fibers, which are primarily responsible for anaerobic energy production. However, the results of our study did not support this hypothesis. It is possible that the differences in mtDNA abundance between oxidative and glycolytic muscle fibers are less marked that previously thought.

Figure 4

Abundance of Mitochondrial DNA in different muscles



Note: Longisssimus dorsi (LD), Latissimius dorsi (LAT) and Masseter (MS).

Table 13

Least Square Means for effect MUSCLE

Muscle	emmean	SE	Df	Lower.CL	Upper.CL	Group
LD	1.74	0.178	60	1.38	2.09	1
LAT	1.77	0.178	60	1.41	2.13	1
MS	1.83	0.178	60	1.47	2.18	1

Note. SE: Standard Error, Df: degree freedom, Lower.CL: lower value, Upper CL: higher value.

Hypertrophy and Adipose Tissue Growth

Assessment of muscle hypertrophy and adipose tissue in pigs was determined by measuring muscle Cross- Sectional Area (CSA) and Adipose Tissue Size (Backfat Thickness), respectively. Body weight increased gradually between the ages of 20 to 87 days, subsequently a rapid increase in body weight was observed between the ages of 87 to 180 days (Figure 5). While intensive growth of adipose tissue occurred between the ages of 120 to 180 days (Figure 6). According to the study conducted by Garrido de la Osa (2022) can indicate that the increase in body weight is due to an increase in muscle and adipose tissue. Intensive growth of adipose tissue occurs during the finishing period (finisher diet), when pigs are fed high-energy and low- fiber diets.

Figure 5

Body weight

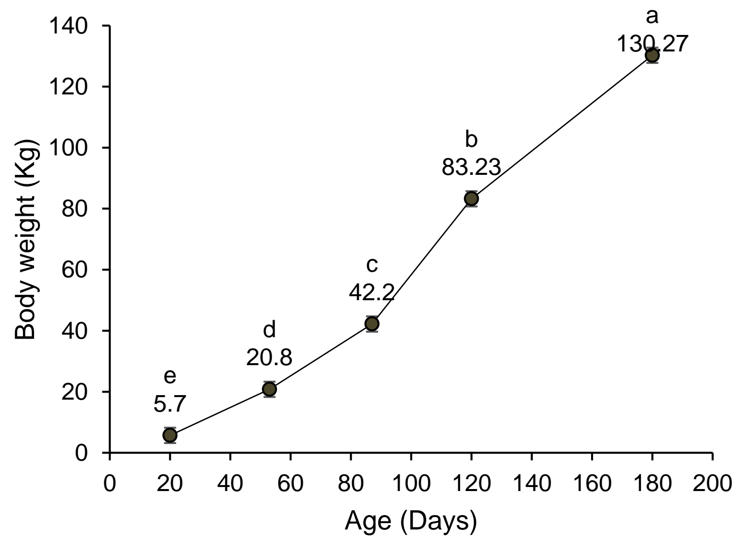
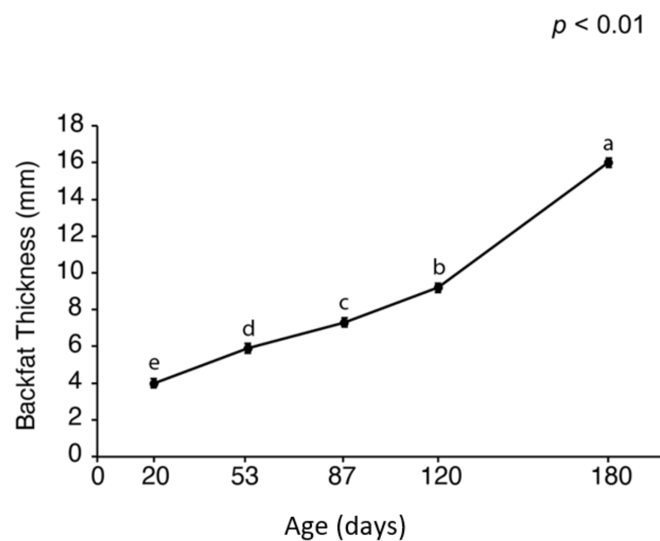


Figure 6

Assessment of adipose tissue growth



Analysis of the Cross-Sectional Area of Muscles

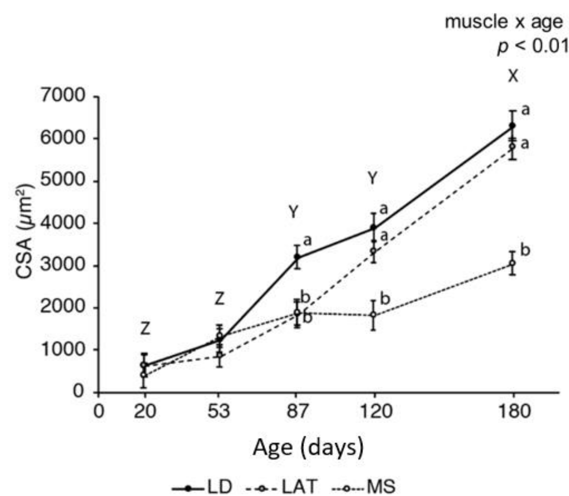
Table 14 shows the probabilities of different factors, such as muscle, age, and interaction of these, to know their relationship with the cross section are. In this case, muscle and age values are

statistically significant, indicating that the perimeter of muscle fibers varies depending on the muscle and the age in which it is found. In addition, an interaction between muscle and age was show.

Figure 7 demonstrates that after 53 days that *Longissimus dorsi (LD) and Latissimus dorsi (LAT)* muscles grew at each point. Nevertheless, after 120 days *Masseter (MS)* muscle grew. Notably, between 87 and 120 days, MS muscle size seemed to be stuck. According to Ramírez (2004) Two reasons can be given this observation: The oxidative muscles (MS) have smaller fibers than glycolytic muscle (LD) and because the LD and LAT are locomotor muscles, whereas the MS muscle is a masticatory muscle. The locomotor muscle is subjected to a greater mechanical loas than the masticatory muscle, which may explain their greater growth and the location of the MS muscle is in the head may also contribute to its slower growth, is surrounded by other muscle and tissues, limit its ability to expand.

Figure 7

Assessment of muscle growth with different types of metabolism



Note. Longisssimus dorsi (LD), Latissimius dorsi (LAT) and Masseter (MS).

Table 14

Statistical Analysis CSA

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Muscle	2	9551888	4775944	13.308	2.32e-05
Age	4	149459462	37364865	104.116	< 2e-16
Muscle:Age	8	22628365	2828546	104.116	8.65e-07
Residuals	50	17943905	358878		

Note. Df: degree freedom, Sum Sq: Sum of Squares, Mean Sq: Mean of Square, Pr (>F): probability

Conclusions

The results of the study show that changes in skeletal muscle metabolism in the pig depend on the type of muscle and age. In general, glycolytic, and mixed muscles showed greater metabolic activity than oxidative ones.

The three types of muscle studied (Longissimus dorsi, Latissimus dorsi and Masseter) have distinct growth patterns. Longissimus dorsi, a glycolytic muscle, showed steady growth over time. Latissimus dorsi, a mixed muscle, showed accelerated growth after 53 days. Masseter, an oxidative muscle, showed slower growth after 53 days.

There is a relationship between muscle hypertrophy, age, and muscle type. Depending on whether the muscle is glycolic, oxidative, or mixed will be the change in growth at different stages.

Recommendations

Consider other factors that may influence the abundance of mtDNA in muscle cells, such as diet, exercise, or stress, may also influence this abundance.

Evaluate how each of the nutrients incorporated in different diets throughout the life cycle affects muscle growth.

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Annexes

Annex A

DNA Extraction

Using the Quick-DNA Miniprep Plus Kit (Zymo Research 11-397B) following the manufacturer's protocol for tissue with an overnight digestion period of 25-50mg of ground tissue at 56 ^{o}C. We elute with 50 µl of DNA elution buffer and then pass through the eluate again.

Nanodrop and Dilute samples 1:1 as needed to get concentrations in a relatively close range to one another. They don't have to be exactly the same concentration but +/- 20 µg/µl is OK.

Annex B

Standars curve

1. Eight points are used in the standard curve

2. Two replicates are used for each point. Replicates are identical reactions, containing identical reaction components and volumes.

3. The starting quantity is dependent on the total ng/ µl of the pooled samples to make Standard 1 and the serial factor is 1:10).

Vortex between each standard

This makes 4 sets of standards:

Standard	DNA quantity	Water Quantity (Ultra pure RNase-free)
STANDARD 1	Pool 10 µl from 10 samples = 100 µl total (briefly vortex then nanodrop for quantity in ng/µl)	0 µl
STANDARD 2	5 µl from **standard 1**	45 µl
STANDARD 3	5 µl from **standard 2**	45 µl
STANDARD 4	5 µl from **standard 3**	45 µl
STANDARD 5	5 µl from **standard 4**	45 µl
STANDARD 6	5 µl from **standard 5**	45 µl
STANDARD 7	5 µl from **standard 6**	45 µl
STANDARD 8	5 µl from **standard 7**	45 µl

4. Be sure to have a standard curve for each assay (or gene) to extrapolate copies/ng for samples.

5. Combine the Reaction Mix with each individual standard (36 µl MM + 4 µl standard) in separate tubes (8-tube PCR strip) and then add 20 µl of this mixture to each assigned well of the 96-well plate.

6. Each gene (mitochondrial and eukaryote) needs its own reaction mix and its own set of standards.

Annex C

Layout of the Standard 96-well plate

96-well Plate 1 layout

	Nuclear DNA/ EUK						mtDNA/ PIG					
	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	Std 1	P1 LD	P1 LD	P1 LD	Std 1	Std 1	Std 1	P1 LD	P1 LD	P1 LD
B	Std 2	Std 2	Std 2	P1 MS	P1 MS	P1 MS	Std 2	Std 2	Std 2	P1 MS	P1 MS	P1 MS
C	Std 3	Std 3	Std 3	P1 LAT	P1 LAT	P1 LAT	Std 3	Std 3	Std 3	P1 LAT	P1 LAT	P1 LAT
D	Std 4	Std 4	Std 4	P2 LD	P2 LD	P2 LD	Std 4	Std 4	Std 4	P2 LD	P2 LD	P2 LD
E	Std 5	Std 5	Std 5	P2 MS	P2 MS	P2 MS	Std 5	Std 5	Std 5	P2 MS	P2 MS	P2 MS
F				P2 LAT	P2 LAT	P2 LAT				P2 LAT	P2 LAT	P2 LAT
G	Pos Control	Pos Control	Pos Control				Pos Control	Pos Control	Pos Control			
H	Neg Control	Neg Control	Neg Control				Neg Control	Neg Control	Neg Control			

Note. Positive control: Isolated Mitochondria DNA, Negative control: MQ water

Annex D

Layout of the Pigs 96-well plate

96-well Plate 2 layout

	Nuclear DNA/ EUK						mtDNA/ PIG					
	1	2	3	4	5	6	7	8	9	10	11	12
A	P3 LD	P3 LD	P3 LD	P5 LD	P5 LD	P5 LD	P3 LD	P3 LD	P3 LD	P5 LD	P5 LD	P5 LD
B	P3 MS	P3 MS	P3 MS	P5 MS	P5 MS	P5 MS	P3 MS	P3 MS	P3 MS	P5 MS	P5 MS	P5 MS
C	P3 LAT	P3 LAT	P3 LAT	P5 LAT	P5 LAT	P5 LAT	P3 LAT	P3 LAT	P3 LAT	P5 LAT	P5 LAT	P5 LAT
D	P4 LD	P4 LD	P4 LD	P6 LD	P6 LD	P6 LD	P4 LD	P4 LD	P4 LD	P6 LD	P6 LD	P6 LD
E	P4 MS	P4 MS	P4 MS	P6 MS	P6 MS	P6 MS	P4 MS	P4 MS	P4 MS	P6 MS	P6 MS	P6 MS
F	P4 LAT	P4 LAT	P4 LAT	P6 LAT	P6 LAT	P6 LAT	P4 LAT	P4 LAT	P4 LAT	P6 LAT	P6 LAT	P6 LAT
G	Pos Control	Pos Control	Pos Control				Pos Control	Pos Control	Pos Control			
H	Neg Control	Neg Control	Neg Control				Neg Control	Neg Control	Neg Control			

Note. Positive control: Isolated Mitochondria DNA, Negative control: MQ water

96-well Plate 3 layout

	Nuclear DNA/ EUK						mtDNA/ PIG					
	1	2	3	4	5	6	7	8	9	10	11	12
A	P7 LD	P7 LD	P7 LD	P9 LD	P9 LD	P9 LD	P7 LD	P7 LD	P7 LD	P9 LD	P9 LD	P9 LD
B	P7 MS	P7 MS	P7 MS	P9 MS	P9 MS	P9 MS	P7 MS	P7 MS	P7 MS	P9 MS	P9 MS	P9 MS
C	P7 LAT	P7 LAT	P7 LAT	P9 LAT	P9 LAT	P9 LAT	P7 LAT	P7 LAT	P7 LAT	P9 LAT	P9 LAT	P9 LAT
D	P8 LD	P8 LD	P8 LD	P10 LD	P10 LD	P10 LD	P8 LD	P8 LD	P8 LD	P10 LD	P10 LD	P10 LD
E	P8 MS	P8 MS	P8 MS	P10 MS	P10 MS	P10 MS	P8 MS	P8 MS	P8 MS	P10 MS	P10 MS	P10 MS
F	P8 LAT	P8 LAT	P8 LAT	P10 LAT	P10 LAT	P10 LAT	P8 LAT	P8 LAT	P8 LAT	P10 LAT	P10 LAT	P10 LAT
G	Pos Control	Pos Control	Pos Control				Pos Control	Pos Control	Pos Control			
H	Neg Control	Neg Control	Neg Control				Neg Control	Neg Control	Neg Control			

Note. Positive control: Isolated Mitochondria DNA, Negative control: MQ water

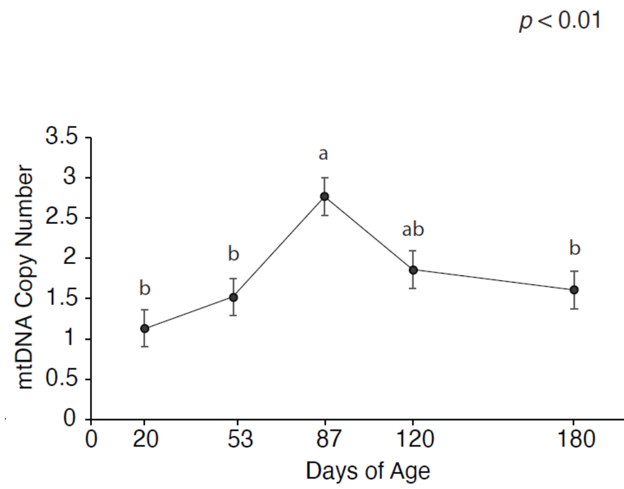
96-well Plate 4 layout

	Nuclear DNA/ EUK						mtDNA/ PIG					
	1	2	3	4	5	6	7	8	9	10	11	12
A	P11 LD	P11 LD	P11 LD	P13 LD	P13 LD	P13 LD	P11 LD	P11 LD	P11 LD	P13 LD	P13 LD	P13 LD
B	P11 MS	P11 MS	P11 MS	P13 MS	P13 MS	P13 MS	P11 MS	P11 MS	P11 MS	P13 MS	P13 MS	P13 MS
C	P11 LAT	P11 LAT	P11 LAT	P13 LAT	P13 LAT	P13 LAT	P11 LAT	P11 LAT	P11 LAT	P13 LAT	P13 LAT	P13 LAT
D	P12 LD	P12 LD	P12 LD	P14 LD	P14 LD	P14 LD	P12 LD	P12 LD	P12 LD	P14 LD	P14 LD	P14 LD
E	P12 MS	P12 MS	P12 MS	P14 MS	P14 MS	P14 MS	P12 MS	P12 MS	P12 MS	P14 MS	P14 MS	P14 MS
F	P12 LAT	P12 LAT	P12 LAT	P14 LAT	P14 LAT	P14 LAT	P12 LAT	P12 LAT	P12 LAT	P14 LAT	P14 LAT	P14 LAT
G	Pos Control	Pos Control	Pos Control				Pos Control	Pos Control	Pos Control			
H	Neg Control	Neg Control	Neg Control				Neg Control	Neg Control	Neg Control			

Note. Positive control: Isolated Mitochondria DNA, Negative control: MQ water

Annex E

Assess Mitochondrial DNA as an indicator of metabolic changes



Annex F

Least Squares Means of the analysis of the CSA of muscles

Least Squares Means for effect MUSCLE: AGE

Muscle	Age	emmean	SE	Df	Lower.CL	Upper.CL	Group
MS	1	401	300	50	-201	1003	1
LAT	1	640	268	50	102	1178	1
LD	1	649	268	50	111	1187	1
LAT	2	870	268	50	332	1408	1
LD	2	1237	268	50	699	1776	1
MS	2	1329	268	50	791	1867	1
MS	4	1815	346	50	1120	2510	123
LAT	3	1850	300	50	1248	2452	12
MS	3	1892	300	50	1290	2493	12
MS	5	3054	268	50	2516	3592	234
LD	3	3199	268	50	2661	3737	234
LAT	4	3336	268	50	2798	3874	34
LD	4	3901	346	50	3206	4595	4
LAT	5	5801	300	50	5200	6403	5
LD	5	6306	346	50	5611	7000	5

Note: Ages: 1 (20 days), 2 (53 days), 3 (87 days), 4 (120 days) and 5 (180 days). Muscles: Masseter (MS), Latissimius dorsi (LAT) ans Longisssimus dorsi (LD).

Least Squares Means for effect AGE

Age	emmean	SE	Df	Lowe.CL	Upper. CL	Group
1	563	161	50	240	887	1
2	1146	155	50	835	1456	1
3	2314	167	50	1978	2649	2
4	3017	186	50	2644	3391	2
5	5054	177	50	4699	5409	3

Note: Ages 1 (20 days), 2 (53 days), 3 (87 days), 4 (120 days) and 5 (180 days).

Least Squares Means for effect MUSCLE

Muscle	emmean	SE	Df	Lowe.CL	Upper. CL	Group
MS	1698	133	50	1431	1965	1
LAT	2500	126	50	2247	27523329	2
LD	3058	135	50	2788	2649	3

Note: Masseter (MS), Latissimius dorsi (LAT) ans Longisssimus dorsi (LD).