MAYARA MORENA DÉL CAMBRE AMARAL WELLER

EFFECTS OF THERMAL ENVIRONMENT AND DIETARY PHOSPHORUS LEVELS IN PIG GENE EXPRESSION

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento para obtenção do título de *Magister Scientiae*.

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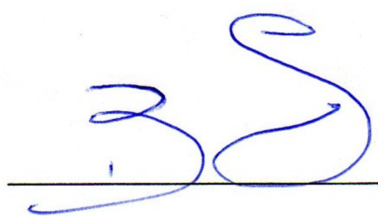

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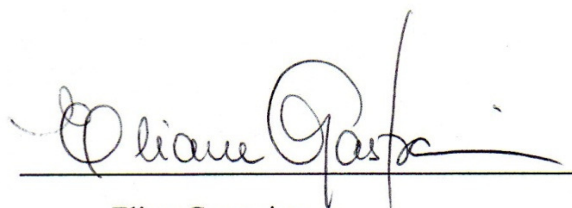

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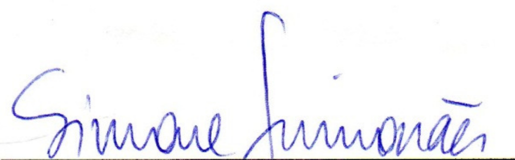

Simone Eliza Facioni Guimarães
(Orientadora)

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BIOGRAFIA

Mayara Morena Dél Cambre Amaral Weller, filha de Ailton Alves Weller e Remy Gomes do Amaral, nasceu em 4 de abril de 1987 em Cachoeiro de Itapemirim, Espirito Santo, Brasil.

No 2005, iniciou o curso de Zootecnia, pelo Departamento de Zootecnia da Universidade Federal de Viçosa, em Viçosa, MG, onde foi bolsista de iniciação científica, acompanhando e conduzindo pesquisas junto ao Programa de Melhoramento Genético de Suínos do Departamento de Zootecnia da UFV.

Em janeiro de 2010, graduou-se em Zootecnia, pela Universidade Federal de Viçosa.

Em março do mesmo ano, iniciou o Mestrado pelo Programa de Pós-Graduação em Genética e Melhoramento Animal da Universidade Federal de Viçosa, submetendo-se à defesa de dissertação para obtenção do título de Magister Scientiae em Genética e Melhoramento, dia 16 de fevereiro de 2012.

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RESUMO

WELLER, Mayara Morena Dél Cambre Amaral, M. Sc, Universidade Federal de Viçosa, fevereiro de 2012. **Efeitos da temperatura ambiente e níveis de fósforo dietético na expressão gênica de suínos.** Orientador: Simone Eliza Facioni Guimarães. Coorientadores: Juarez Lopes Donzele and Paulo Sávio Lopes.

O objetivo do estudo foi identificar genes codificantes para os complexos enzimáticos da cadeia tranpostadora de elétrons (CTE) que apresentam-se diferencialmente expressos no músculo *Longissimus dorsi* (LD) de suínos bem como seus padrões de expressões de acordo níveis de fósforo disponíveis (Pd) na dieta e ambientes térmicos em duas fases de crescimento. Dois experimentos foram realizados utilizando-se 48 suínos de alto potencial genético para deposição de carne em duas diferentes fases de crescimento: a fase 1 de 15 a 30 kg e fase 2 de 30 a 60 kg. Estas experimentos foram construidos em modelo fatorial 2 X 3 (2 diferentes temperaturas ambientais X 3 níveis de Pd na dieta) constituindo 6 tratamentos em cada fase de crescimento. Vinte e quatro suínos da fase da 1 (15 a 30 kg) com peso inicial de 15 ± 0,41 kg, foram distribuídos em delineamento inteiramente casualizado, com 6 tratamentos e 6 repetições. Estes animais foram alojados ou no ambiente termoneutro (24,5 ± 1,2 º C e umidade relativa em 76,3 ± 8,5%) ou no ambiente de calor (34,1 ± 0,8 º C e umidade relativa em 70,1 ± 8,1%) e, em seguida, distribuídos para um dos 3 níveis de Pd na dieta (0.107, 0.321 ou 0.535%). Vinte e quatro suínos da fase de 2 (30 a 60 kg) com peso inicial de 30,19 ± 0,30 kg, foram distribuídos em delineamento inteiramente casualizado com 6 tratamentos e 6 repetições. Estes animais foram alojados ou ambiente termoneutro (21,9 ± 1,4 º C e umidade relativa em 76,9 ± 5,7%) ou ambiente de calor (31,6 ± 0,7 º C e umidade relativa em 72,8 ± 5,9%) e, em seguida, distribuídos para um dos 3 níveis de Pd na dieta (0.116 , 0.306 ou 0.496%). Os perfis de expressão dos genes *ND1*, *ND2*, *SDHD*, *CYTB*, *COX1*, *COX2*, *COX3*, *ATP5J2* e *ATP6* foram analisados pelo PCR quantitativo em tempo real (qRT-PCR) a partir de amostras de LD. Os genes *ND1*, *ND2*, *CYTB*, *COX1*, *ATP5J2* e *ATP6* foram menos expressos ($P< 0.05$) nos suínos da fase 1 mantindo em estresse por calor em relação aqueles mantidos no ambiente termoneutro. Nos suíno da fase 2 de crescimento que estavam mantidos em estresse por calor apresentaram redução ($P < 0.05$) na expressão dos genes *ND2*, *SDHD*, *CYTB*, *COX1*, *COX2*, *COX3*, *ATP5J2* e *ATP6,* em relação aqueles submetido ao ambiente termoneutro. A menor expressão desses genes nos suínos estressados por

calor, prove evidências dos efeitos das altas temperaturas sob o metabolismo oxidativo a fim de reduzir a produção de calor o que refletiu na redução da exigência de consumo diário de fósforo. Parte desse efeito pode estar associado a concomitante redução do consumo vonlutário e outra parte é sugerido ser devido ao aumento da produção de espécies reativas de oxigênio (ROS) nas mitocôndrias induzido pelo estresse por calor. Quanto a mudança nos padrões de expressão desses genes entre os níveis de Pd, verificou-se que nos suínos da fase 1, os genes *ND1*, *ND2*, *CYTB*, *COX1*, *ATP5J2* e *ATP6* mostraram-se diferencialmente expressos (P<0.05) entre os níveis de Pd no ambiente termoneutro e o gene *ND2* no ambiente de calor (P<0.05). Nos suínos da fase 2, os genes *COX3* e *ND2* foram diferencialmente expressos (P<0.05), respectivamente, entre os níveis de Pd no ambiente termoneutro e ambiente de calor. Os dados apresentados revelam uma forte evidência de que o nutriente fósforo é um dos fatores chave que regulam a fosforilação oxidativa com implicação direta sobre a performance animal. Para a confirmação dessa hipótese de que suínos estressados por calor apresentam aumento na produção de ROS, foi realizado um segundo experimento que objetivou avaliar o perfil de expressão dos genes associados sistema antioxidante (manganes- superóxido dismutase, glutathiona peroxidase e catalase) em suínos de 2 diferentes fases de crescimeto submetido à ambientes de termoneutralidade e estresse por calor. Baseado em análises de qRT-PCR, estes genes foram significativamente mais expressos em suínos expostos ao estresse de calor. Em geral, os dados deste estudo são os primeiros a revelar os efeitos de diferentes níveis de fósforo disponível na expressão de genes relacionados com a fosforilação oxidativa e suas implicações sobre o desempenho de suínos. Além disso, é demonstrado que elevadas temperaturas não só induzem a redução da capacidade metabólica oxidativa, como também, estimulam a produção de ROS em mitocôndrias de músculo LD o que leva a danos oxidativos mitocondriais, tais como, repressão da transcrição e provavelmente redução na atividade dos complexos da cadeia transportadora de elétrons, o que refletiu na piora da performance animal. Tais informações irão contribuir para melhor compreensão do papel do fósforo no metabolismo energético e maior entendimento sobre as mudanças no metabolismo e funcionamento mitocondrial em resposta à altas temperaturas.

ABSTRACT

WELLER, Mayara Morena Dél Cambre Amaral, M. Sc, Universidade Federal de Viçosa, February 2012. **Effects of thermal environment and dietary phosphorus levels in pig gene expression.** Adviser: Simone Eliza Facioni Guimarães. Co-advisers: Juarez Lopes Donzele and Paulo Sávio Lopes**.**

The purpose of this study was to identify genes encoding the electron transport chain (ETC) complexes that are present differentially expressed in the *Longissimus dorsi* (LD) of pigs and their expression patterns according to levels available of phosphorus (aP) in the diet and thermal environment in the two different growth phase. Two experiments were carried out using 48 higher-lean growth pigs from two different growth phases: phase 1 from 15 to 30 kg and phase 2 from 30 to 60 kg. These experiments were designed as a 2 x 3 (2 different environment temperatures X 3 levels of dietary aP) factorial treatments comprising of 6 treatments and each growth phase. Twenty -four pigs from growth phase 1 (15 to 30 kg) with initial weight of 15 ± 0.41kg, distributed in a completely randomized design with 6 treatments and 6 replications. These pigs were allotted to either thermoneutral (24.5 ± 1.2ºC and RH at 76.3 ± 8.5% or heat stress (34.1 ± 0.8ºC and RH at 70.1 ± 8.1%) and then allotted to one of the 3 levels of dietary aP (0.107, 0.321 or 0.535%). Twenty-four pigs from growth phase 2 (30 to 60 kg) with initial weight of 30.19 ± 0.30 kg, distributed in a completely randomized design with 6 treatments and 6 replications. These pigs were allotted to either thermoneutral (21.9 ± 1.4ºC and RH at 76.9 ± 5.7%) or heat stress (31.6 ± 0.7ºC and RH at 72.8 ± 5.9%) and then allotted to one of the 3 levels of dietary aP (0.116, 0.306 or 0.496%). The expression profiles of *ND1*, *ND2*, *SDHD*, *CYTB*, *COX1*, *COX2*, *COX3*, *ATP5J2* and *ATP6* genes were analyzed by quantitative real-time PCR from samples of LD. The *ND1*, *ND2*, *CYTB*, *COX1*, *ATP5J2* and *ATP6* genes showed reduce expression ($P<0.05$) in phase 1 pigs maintained under heat stress compared to those kept in thermoneutral environment. In pigs from growth phase 2 maintained under heat stress, the *ND2*, *SDHD*, *CYTB*, *COX1*, *COX2*, *COX3*, *ATP5J2* and *ATP6* genes showed reduce expression ($P<0.05$) compared to those kept in thermoneutral environment. The lower expression of these genes in pigs exposed to high temperatures provides evidence of the effect of heat stress by decreasing oxidative metabolism (oxidative phosphorylation) in order to reduce heat production, which reflecting on reduction of requirement for daily aP

intake. Part of this effect is associated with concomitant reduction in voluntary feed intake and another part is suggested to be due to an increased production of reactive oxygen species in the mitochondria induced by heat stress resulting in downregulation of these genes. Significant different levels of expression were observed across aP levels. In pigs from growth phase 1, the *ND1*, *ND2*, *CYTB*, *COX1*, *ATP5J2* and *ATP6* genes were differentially expressed across aP levels (P<0.05) in the thermoneutral and *ND2* gene in the hot environments. In pigs from growth phase 2, *COX3* and *ND2* genes were respectively differentially expressed across aP levels (P<0.05) in the thermoneutral and hot environments. These data revealed strong evidence that phosphorus is one of the key factors that regulates oxidative phosphorylation with direct implications on animal performance. To confirm the hypothesis that heat-stressed pigs have enhanced production of ROS, we performed a second experiment focused to identify changes in expression of genes encoding antioxidant systems (manganese superoxide dismutase, glutathione peroxidase e catalase) in pigs from high lean meat deposition at 2 different growth phases (15 to 30kg and 30 to 60kg) submitted to hot and thermoneutral environments. Based on qRT-PCR analyses, these genes were significantly upregulated in heat-exposed pigs. In general, data from this study are the first to reveal the effects of dietary phosphorus levels on gene expression related to oxidative phosphorylation and its implications on animal performance. Moreover, it is shown that high temperatures not only causes reduction in oxidative metabolic capacity, but also stimulate the ROS production in LD mitochondria which leading to oxidative damages, such as, repression of transcription of ETC complexes and likely reducing their activity in the mitochondria, which resulted in worse pig performance. The results obtained with this study will contribute to better understand the role of phosphorus in energy metabolism and will bring new insights into the comprehension of the changes metabolism and mitochondrial functioning in response to high temperature.

GENERAL INTRODUCTION

Rising costs of feed, fuel, and fertilizer due to the implementation of biofuel policies and growing global demand for animal protein (Steinfeld et al., 2006) have intensified focus of pork industry on the creation and selection of leaner and more efficient pigs.

Studies have shown that animals that are less efficient in converting feed into body weight may present changes in the activity of the sodium and potassium pump, in the expression of genes of the electron transport chain, or in the concentrations of important hormones, including insulin-like growth factor I (IGF-I), growth factor (GH), thyroxin, triiodothyronine. This may influence nutrient utilization and basal metabolism, changing body energy expenditure, and consequently, caloric increment in animals (Bottje and Carstens, 2009; Johnson et al., 2003; Yunianto et al., 1997; Rosebrough and Murtry, 1993).

Since the nineties, there have been several reports on the relationship of mitochondrial function and feed conversion ratio in poultry (Bottje and Carstens, 2009; Iqbal et al., 2004), cattle (Krueger et al, 2008; Castro-Bulle et al, 2007; Archer et al., 1999), sheep (Wolanis et al., 1980) and swine (Dazpo and Wassmuth, 1983), the trials were carried out within a single breed of animals fed same diet. The results suggest that animals with worse feed conversion ratio present imperfect mitochondrial function, as the mitochondria is responsible for 90% of the cellular production of ATP which negatively influences feed conversion. However, little has been published on the effect of nutrients or their dietary levels on the expression of gene related to important metabolic pathways such as Krebs cycle and oxidative phosphorylation.

Among the nutrients supplemented in diets for pigs phosphorus (P) is of particular interest, due to its importance to several metabolic processes within the organism, such as energy metabolism, therefore, it is a critical nutrient for the synthesis of body protein. Moreover, due to the harmful effects of P excretion to the environment, its consumption by the animals must be regulated. However, little has been published on the analyses of gene expression when studying different dietary levels of available phosphorus (aP) for pigs.

In addition, it is important to investigate the effects of the thermal environment on the regulation of gene expression, especially in tropical countries, where high temperatures persist throughout the year. Several studies have demonstrated that high temperatures have negative effects on the performance of pigs during most phases of production (Christon, 1988; Quiniou *et al*., 2000; Collin et al., 2001a; Collin *et al*., 2002; Kerr *et al*., 2003), but little is known about the changes in muscular energy metabolism in heat-stressed pigs (Rinaldo and Le Dividich, 1991; Collin *et al*., 2001b) or on the interaction between dietary levels of aP and the thermal environment to which the animals are submitted.

The goal of this study was to evaluate the expression profile of genes encoding electron transport chain proteins in the *Longissimus dorsi* (LD) muscle of pigs from a commercial line at 2 different growth phases: 15 to 30 kg and 30 to 60 kg, submitted to two thermal environments and fed different dietary aP levels. We also evaluate the expression profile of superoxide dismutase 2, glutathione peroxidase 1, glutathione reductase, catalase and UCP 3 in LD muscle of pigs from high lean meat deposition at 2 different growth phases exposed to heat stress.

CHAPTER I
(Article I)

Gene expression in pigs exposed to two thermal environments and fed different dietary available phosphorus levels

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ABSTRACT

The purpose of this study was evaluate the effects of temperature and different levels available phosphorus on the expression of *ND1*, *ND2*, *SDHD*, *CYTB*, *COX1*, *COX2*, *COX3*, *ATP5J2* and *ATP6* genes encoding electron transport chain proteins in the *Longissimus dorsi* (LD) muscle of pigs. Two experiments were carried out using 48 higher-lean growth pigs from two different growth phases: phase 1 from 15 to 30 kg and phase 2 from 30 to 60 kg. These experiments were designed as a 2 x 3 (2 temperatures X 3 levels of dietary aP) factorial treatments comprising of 6 treatments and each growth phase. Twenty-four pigs from growth phase 1 (15 to 30 kg) with initial weight of 15 ± 0.41kg, distributed in a completely randomized design with 6 treatments and 6 replications. These pigs were allotted to either thermoneutral (24.5 ± 1.2ºC and RH at 76.3 ± 8.5% or heat stress (34.1 ± 0.8ºC and RH at 70.1 ± 8.1%) and then allotted to one of the 3 levels of dietary aP (0.107, 0.321 or 0.535%). Twenty-four pigs from growth phase 2 (30 to 60 kg) with initial weight of 30.19 ± 0.30 kg, distributed in a completely randomized design with 6

treatments and 6 replications. These pigs were allotted to either thermoneutral (21.9 ± 1.4ºC and RH at 76.9 ± 5.7%) or heat stress (31.6 ± 0.7ºC and RH at 72.8 ± 5.9%) and then allotted to one of the 3 levels of dietary aP (0.116, 0.306 or 0.496%). The expression profiles of 9 genes were analyzed by quantitative real-time PCR. Six genes showed reduce expression ($P<0.05$) in phase 1 pigs and 8 genes ($P<0.05$) in phase 2 pigs maintained under heat stress compared to those kept in thermoneutral environment. The lower expression of these genes under high temperatures evidences the effects of heat stress by decreasing oxidative metabolism (oxidative phosphorylation) in order to reduce heat production, which reflecting on the reduction of the daily requirements for aP. Part of this effect is associated with concomitant reduction in voluntary feed intake and another part is suggested to be due to increased production of reactive oxygen species (ROS) in the mitochondria induced by heat stress resulting in downregulation of these genes. In pigs from phase 1, the *ND1*, *ND2*, *CYTB*, *COX1*, *ATP5J2* and *ATP6* genes were respectively differentially expressed across aP levels ($P<0.05$) in the thermoneutral and *ND2* gene in the hot environments. In pigs from phase 2, *COX3* and *ND2* genes were respectively differentially expressed across aP levels ($P<0.05$) in the thermoneutral and hot environments. These data reveal strong evidence that phosphorus is one of the key factors to regulate oxidative phosphorylation with direct implications on animal performance.

Keywords: electron transport chain, oxidative phosphorylation, pig lean deposition, qRT-PCR

Implications

Data from this study are the first to reveal the effects of different available phosphorus levels on gene expression related to oxidative phosphorylation and its implications on animal performance. Moreover, this study showed the possible effects of high temperatures on the regulation of the expression of these genes and how this may alter the physiological response to the phosphorus. This information will contribute to better understand the role of phosphorus in energy metabolism and will bring new insights into the comprehension of the effects of high temperatures on nutritional requirements and mitochondrial function.

RESUMO

O objetivo do estudo foi avaliar o perfil de expressão de genes codificantes para proteínas da cadeia tranpostadora de elétrons no músculo *Longissimus dorsi* (LD) de suínos. Quatro diferentes conjuntos de dados (n=12) foram utilizadas a partir de 4 experimentos que avaliaram em duas diferentes fases de crescimento: fase 1 (15 a 30 kg) e fase 2 (30 a 60 kg), a exigência de níveis de fósforo disponível (Pd) em dietas para suínos machos castrados de alta deposição de carne magra mantidos em ambiente termoneutro e de estresse por calor. Os perfis de expressão de genes 9 foram analisados por PCR quantitativo em tempo real a partir de amostras de LD provenientes de quatro experimentos que avaliaram a exigência de níveis aP em dietas para suínos durante a fase de crescimento 1 (15 a 30 kg) mantidos a temperatura de 24.5 ± 1.2ºC e 34.1 ± 0.8ºC e durante a fase de crescimento 2 (30 a 60 kg) mantindos a temperatura de 21.9 ± 1.38ºC e 31.6 ± 0.7ºC. Os genes *ND1*, *ND2*, *CYTB*, *COX1*, *ATP5J2* e *ATP6* foram menos expressos (P< 0.05) nos suínos da fase 1 mantindo em estresse por calor em relação aqueles mantidos no ambiente termoneutro. Nos suíno da fase 2 de crescimento que estavam mantidos em estresse por calor apresentaram redução (P < 0.05) na expressão dos genes *ND2*, *SDHD*, *CYTB*, *COX1*, *COX2*, *COX3*, *ATP5J2* e *ATP6,* em relação aqueles submetido ao ambiente termoneutro. A menor expressão desses genes nos suínos estressados por calor, prove evidências dos efeitos do estresse térmico sob o metabolismo oxidativo a fim de reduzir a produção de calor o que refletiu na redução da exigência de consumo diário de fósforo. Parte desse efeito pode estar associado a concomitante redução do consumo vonlutário e outra parte é sugerido ser devido ao aumento da produção de espécies reativas de oxigênio (ROS) nas mitocôndrias induzido pelo estresse por calor. Quanto a mudança nos padrões de expressão desses genes entre os níveis de Pd, verificou-se que nos suínos da fase 1, os genes *ND1*, *ND2*, *CYTB*, *COX1*, *ATP5J2* e *ATP6* mostraram-se diferencialmente expressos (P<0.05) entre os níveis de Pd no ambiente termoneutro e o gene *ND2* no ambiente de calor (P<0.05). Nos suínos da fase 2, os genes *COX3* e *ND2* foram diferencialmente expressos (P<0.05), respectivamente, entre os níveis de Pd no ambiente termoneutro e ambiente de calor. Os dados apresentados

revelam uma forte evidência de que o fósforo é um dos fatores chaves que regulam a fosforilação oxidativa com implicação direta sobre a performance animal.
Palavras-chaves: cadeia transportadora de elétrons, fosforilação oxidativa, suínos alto potencial genético, qRT-PCR

Introduction

In order to improve the efficiency of lean meat production in modern pig lines, the requirements of important functional nutrients need to be re-estimated. Among these nutrients, phosphorus (P) is of particular interest, due to its importance to many processes within the organism, such as energy metabolism and protein synthesis. Moreover, due to the harmful effects of P excretion to the environment, its consumption by the animals must be regulated.

Several studies have indicated that P requirements in pigs selected for high lean deposition differ across the different growth phases (Hendricks and Moughan, 1993; Stahly *et al.*, 2000; Saraiva *et al.*, 2009). However, little has been published on the analyses of gene expression when studying dietary levels of available phosphorus (aP) for pigs. Only two studies have shown that the need of aP is genotype specific (Hittmeier *et al.*, 2006; Alexander *et al.*, 2008).

In addition, it is important to investigate the effects of the thermal environment on the regulation of gene expression, especially in tropical countries, where high temperatures persist throughout the year. Several studies have demonstrated that high temperatures have negative effects on the performance of pigs during most phases of production (Christon, 1988; Quiniou *et al.*, 2000; Collin *et al.*, 2001a; Collin *et al.*, 2002; Kerr *et al.*, 2003), but there is little information available about the changes in muscular energy metabolism in heat-stressed pigs (Rinaldo and Le Dividich, 1991; Collin *et al.*, 2001b) or on the interaction between dietary levels of aP and the thermal environment to which the animals are submitted.

Therefore, this study was realized to evaluate the expression of genes encoding electron transport chain proteins in the *Longissimus dorsi* (LD) muscle of pigs from high lean meat deposition at 2 different growth phases: 15 to 30 kg and 30 to 60 kg, submitted to two thermal environments and fed different aP levels.

Material and Methods

All methods involving animal handling were realized in accordance with the regulations approved by the Institutional Animal Welfare and Ethics/Protection commission from the Universidade Federal de Viçosa, Brazil.

Experimental design

Two experiments were carried out using 48 higher-lean growth pigs from two different growth phases: phase 1 from 15 to 30 kg and phase 2 from 30 to 60 kg. These experiments were designed as a 2 x 3 (2 different environment temperatures X 3 levels of dietary aP) factorial treatments comprising of 6 treatments and each growth phase.

Twenty -four pigs from growth phase 1 (15 to 30 kg) with initial weight of 15 ± 0.41kg, were assigned randomly on basis of live weigh to one of the 6 treatments. These pigs were allotted to either thermoneutral (24.5 ± 1.2ºC and RH at 76.3 ± 8.5% or heat stress (34.1 ± 0.8ºC and RH at 70.1 ± 8.1%) and then allotted to one of the 3 levels of dietary aP (0.107, 0.321 or 0.535%).

Twenty-four pigs from growth phase 2 (30 to 60 kg) with initial weight of 30.19 ± 0.30 kg were assigned randomly on basis of live weigh to one of the 6 treatments. These pigs were allotted to either thermoneutral (21.9 ± 1.4ºC and RH at 76.9 ± 5.7%) or heat stress (31.6 ± 0.7ºC and RH at 72.8 ± 5.9%) and then allotted to one of the 3 levels of dietary aP (0.116, 0.306 or 0.496%).There were used six replications of each treatment in both experiments.

Pig Management

The pigs were housed in suspended metal cages, with wired mash floor and sides, equipped with semi-automatic feeders and nipple drinkers, located in rooms with controlled air temperature.

Experimental diets (Table 1 and Table 2) were mainly composed of corn/soybean meal supplemented with minerals, vitamins and industrial amino acids to meet the requirements of castrated male pigs from high lean meat deposition for each growth phase, as recommended by Rostagno *et al*. (2005) for all the nutrients, except for aP. Dietary concentrations of dicalcium phosphate, limestone, and kaolin were adjusted to achieve the desired concentrations of aP. Pigs had free access to feed and water throughout the entire experimental period (25 days). The pigs were weighed at beginning and end of the experimental period for calculation of average daily gain and feed conversion.

RNA Extraction and cDNA Synthesis

Four samples of LD muscle were collected during slaughter and immediately immersed in tubes containing 15 ml of RNAHolder® (BioAgency, São Paulo, Brasil) and stored at -20°C for subsequent RNA extraction.

The total RNA from each LD sample was isolated using approximately 40 mg of tissue previously stored in RNAHolder®. The samples were homogenized in buffer RLT containing 1% β-mercaptoethanol (RNeasy Mini Kit) and lysed with a tissue ruptor (Qiagen, Valencia, CA) homogenizer. The total RNA from the LD muscle samples was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. Additional

treatment with DNase was performed on the columns using the RNase-free DNase Set (Qiagen, Valencia, CA), according to the manufacturer's recommendations. RNA concentrations were checked by NanoVue Plus Spectrophotometer (GE Healthcare) with an optimal 260/280 ratio between 1.8 and 2.1. Purity and integrity were determined with an Agilent RNA 6000 Nano Kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.)

The ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England BioLabs Inc., Beverly, MA) was used to produce cDNA immediately after the RNA extraction. The reactions were performed with 6 μg of total RNA (for each sample) and 2 μL of 50 μM oligo$(dT)_{23}$VN primer, following the manufacturer's recommended protocol. The cDNA concentrations from the samples were estimated on a NanoVue Plus^{TM} (GE Healthcare, Munich, Germany) spectrophotometer. Finally, the single-stranded cDNA samples were stored at –20ºC for analysis.

Quantitative Real-time PCR

From the 10 analyzed genes, 7 were chosen based on Lin et al. (1999), and their sequences were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov; Accession No. AF034253). The sequences based on Lin et al. (1999) consisted of 7 subunits from enzymatic complexes in the mitochondrial respiratory cascade: ND1, NADH dehydrogenase subunit 1, and ND2, NADH dehydrogenase subunit 2, both from the NADH ubiquinone oxidoreductase (complex I); *CYTB*, Cytochrome b, from the ubiquinone cytochrome c reductase (complex III); *COX1*, Cytochrome oxidase subunit 1; *COX2*, Cytochrome subunit 2; and *COX3*, Cytochrome

subunit 3; from Cytochrome c oxidase (complex IV); and *ATP6*, ATPase subunit 6, from the F_1F_0 ATPase complex (complex V).

The other 3 genes, *SDHD*, Succinate dehydrogenase subunit d, which is part of the succinate ubiquinone reductase complex (complex II); *ATP5J2*, ATPase subunit f, isoform 2 (complex V); and *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase, were obtained from the nuclear genome of *Sus scrofa* (Accession No. NM_001097516.1, NM_001097464.1 and AF017079.1, respectively). The primers for the 10 genes were designed using *PrimerQuest* (www.idtdna.com/Scitools/Applications/PrimerQuest), provided by Integrated DNA Technologies, Inc. (Coralville, IA). The gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as a reference gene for normalization because it had been better efficiency and less variation across treatments than β-actin (data not shown). The primer pairs are listed in Table 3.

Quantitative real time PCR (qRT-PCR) reactions were performed using the GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI) following the manufacturer's instructions in an ABI Prism 7300 Sequence Detection System thermocycler® (Applied Biosystems, Foster City, CA). The reaction consisted of an initial step at 95ºC for 10 minutes, a second step of 40 cycles with the same temperature for 15 seconds and a final extension step at 60ºC for 60 seconds. After the amplification cycles, an additional gradient step from 60ºC to 95ºC was used to obtain a melting curve. The efficiency of each reaction was assessed in order to choose the best combination of cDNA and primer concentration in the subsequent reactions. Once the slope of a linear regression for each primer concentration was obtained, the PCR efficiency (E) was calculated using the following formula: $E = 10^{(-1/slope)}$. The amplification efficiency for all tested genes varied from 0.90 to 0.97 (Table 3). All reactions

were done in duplicate and the coefficient of variation (CV) of cycle C_t value from replicates within each sample was low, less than 5%, indicating acceptable accuracy and reproducibility (not shown).

Statistical Analyses

To study the combined effects of the dietary available phosphorus levels (aP) and the thermal environment on expression gene within each growing phase, a completely randomized design was applied in a factorial design 3 (aP) X 2 (thermal environment) using four replicates per treatment. All statistical procedures were performed using SAS (v. 9.1.3). The routine QPCR_MIXED SAS® [https://www.msu.edu/~steibelj/JP files/QPCR.html] developed to generate commands in SAS PROC MIXED suitable to analyze data from qRT-PCR, assuming independent random effects for reference gene and target genes in each biological replicate, was used in the analysis (Steibel et al., 2009). This statistical method is more accurate, powerful and flexible than existing alternatives for qRT-PCR data analysis, and it is especially useful in more complex experimental designs involving more than two treatments or time points and multiple experimental factors (Steibel *et al.*, 2009). It was observed an interaction between the aP levels and the thermal environments, therefore the data was analyzed using the linear mixed model:

$$y_{gikr} = TG_{gi} + C_{gik} + D_{ik} + b(W_{ik} - \overline{W}) + e_{gikr}$$

In this equation, y_{gikr} corresponds to the C_t for the *gth* gene (control or target) from the *rth* well, which corresponds to the *kth* animal submitted to the *ith* treatment (thermal environment and aP level combination); TG_{gi} is the effect of the *ith* treatment on the expression of gene *g*; $C_{gik} \sim N(0, \sigma^2_C)$ is the gene-

specific random effect of the *kth* animal; $D_{ik} \sim N(0, \sigma^2_D)$ is the sample-specific random effect (common to both genes); W_{ik} is the covariate slaughter weight of the *kth* animal submitted to the *ith* treatment; $\overline{W}$ is the mean of the slaughter weight; b is the regression coefficient; and $e_{gikr} \sim N(0, \sigma^2_e)$ is the residual term.

For each target gene, the comparison of the fold change values between treatments was performed by CONTRAST statement of the GLM procedure (SAS software) using Student's test to the level of 5%. The "estimates" of the contrasts between treatments generated as results of statistical analysis, are the differences in C_t values between the treatments under evaluation and were used to estimate the fold change (relative expression). The fold change values were obtained by transformation of the estimates (C_t) detected after statistical analyses by calculation: fold change = $2^{-Estimate}$) .Once the efficiency (E) of the qRT-PCR reaction was close or equal to 2.0, one cycle (C_t) of difference between two levels (or samples) means 2.0 fold change of difference, or still, twice as much expression in the first level in comparison with the second level. Also, understanding that higher C_t values means lower transcriptional expression of a specific gene for a specific sample relatively to others and that lower C_t values means higher transcriptional expression, negative values of "estimates" indicate a positive fold change (relative expression) of the first level relatively to the second under investigation, after applying the transformation (Fold change= $2^{-Estimate}$).

Performance data were analyzed using the GLM procedures of SAS software. Data were submitted to ANOVA, with six treatments (3 level aP X 2 thermal environment) each growing phase and six replicates per treatment. Means were compared by the test of Tukey ($P<0.05$).

Results

Due to the observed interactions between the aP levels and the thermal environments, the relative gene expression results were interpreted by contrast comparison two-by-two within one factor, aP levels or thermal environments (hot and thermoneutral), fixed for each level of the other factor (aP levels or thermal environments) for each target gene. The statistical analysis results for each contrast between treatments are shown in the supplementary table 1 and 2 (Appendix 1).

Pigs from growth phase 1 fed 0.107% aP in the diet showed A higher expression of the *ND1* ($P<0.01$), *CYTB* ($P<0.001$), *COX3* ($P<0.001$), *ATP5J2* ($P<0.01$), and *ATP6* ($P<0.001$) genes in the thermoneutral environment compared to those in the hot environment (Figure 1). Likewise, results for qRT-PCR analysis of the *ND1* ($P<0.05$), *CTYB* ($P<0.001$), *COX3* ($P<0.05$), *ATP5J2* ($P<0.05$) and *ATP6* ($P<0.01$) genes revealed significantly higher expression in pigs fed the 0.321% aP in the thermoneutral environment. As show in figure 1, pigs fed 0.535% aP diet had higher expression of *ND2* ($P<0.01$) and *cytb* ($P<0.001$) genes compared with those in the hot environment.

Pigs from growth phase 2 fed 0.116% aP diet had higher expression of the *ND2* ($P<0.01$), *CYTB* ($P<0.01$), *COX2* ($P<0.001$), *COX3* ($P<0.001$), *ATP5J2* ($P<0.05$) and *ATP6* ($P<0.001$) genes in the thermoneutral environment compared to those in hot environment (Figure 2). There were also difference in expression of the *ND2* ($P<0.001$), *CYTB* ($P<0.001$), *COX1* ($P<0.01$), *COX2* ($P<0.01$), *COX3* ($P<0.001$) and *ATP6* ($P<0.05$) genes indicating higher expression

for these genes in pigs fed the 0.306% aP diet in the thermoneutral environment compared to those in hot environment. Pigs fed the dietary level 0.496% aP also had higher expression of the *ND2* (P<0.001), *SDHD* (P<0.05), *COX2* (P< 0.001), *COX3* (P<0.05), *CYTB* (P<0.01) and *ATP6* (P<0.001) genes in the thermoneutral environment than those in hot environment. There was no difference in *ND1* gene expression between thermal environments irrespective aP levels.

Pigs in the thermoneutral environment (24.5 ± 1.2ºC) from phase 1 had higher expression of the *ND1* (P<0.05), *ND2* (P<0.05), *COX3* (P<0.05), *CYTB* (P<0.05), *ATP5J2* (P<0.01) and *ATP6* (P<0.05) genes when fed the 0.321% aP diet compared with pigs fed the 0.535% aP diet (Figure 3). No difference in the expression of the *ND2*, *ND1*, *SDHD*, *COX1* and *COX2* genes between aP levels in thermoneutral environment. As shown in figure 3, results for *ND2* by qRT-PCR analysis revealed significantly (P<0.01) lower expression in pigs fed 0.321% aP diet compared to those fed 0.535% of aP diet in hot environment.

Results for qRT-PCR analysis in LD of pigs from growth phase 1 between 0.107 and 0.535% aP within each thermal environment are shown in Figure 4. There were differences in the expression of *COX3* (P<0.01), *CYTB* (P< 0.05), *ATP5J2* (P<0.01) and *ATP6* (P<0.05) genes which exhibited higher expression in pigs fed the 0.107% aP diet than in pigs fed 0.535% aP diet in thermoneutral environment. The *ND2* gene exhibited significantly (P < 0.001) lower expression in pigs fed 0.107% aP diet compared with those fed 0.535% aP diet (Figure 4).There were no differences in the expression of the *ND1*, *SDHD, COX2, COX3*, *CYTB*, *ATP5J2* and *ATP6* genes in pigs kept under heat stress condition, irrespective to aP levels in the diet

Results for qRT-PCR analysis in pigs from growth phase 2 between 0.116% and 0.306% of aP within each thermal environment are shown in Figure 5. Only the *COX3* gene exhibited higher expression (*P*<0.05) in pigs fed the 0.306% of aP compared to those fed the 0.116% of aP in the thermoneutral environment. As shown in Figure 6, this same gene revealed (*P* < 0.05) higher expression in pigs fed the 0.306% of aP compared with those fed 0.496% of aP in the thermoneutral environment. In the hot environment (31.6 ± 0.7ºC), it was observed that pigs from growth phase 2 fed the 0.306% of aP had 2.64-fold higher expression (*P*<0.05) of the *ND2* gene than those fed the 0.116% of aP. This gene was also expressed 3.32-fold higher (*P*<0.01) in pigs fed the 0.306% of aP than in pigs fed the 0.496% of aP (Figure 6). There were no differences in the expression of *ND1*, *SDHD*, *cytb, COX1, COX2*, *ATP5J2* and *ATP*6 genes across aP levels irrespective to thermal environments.

There was observed an interaction between the environmental temperature and dietary aP levels and on pigs performance from growth phase 1. As shown in figure 7, pigs maintained in the thermoneutral environment (24.5 ± 1.2ºC) and fed the 0.321% of aP had higher ADG (P<0.001) and better feed conversion than pigs fed the 0.107 or 0.535% of aP (P<0.001). But in the hot environment (34.1 ± 0.8ºC), pigs fed the 0.535% of aP had greater feed conversion than that pigs of fed the 0.321% or 0.107% of aP.

There was no observed an interaction between the environmental temperature and dietary aP levels and on pigs performance from growth phase 2. As shown in the figure 8, at both temperatures pigs fed the 0.306% of aP had higher ADG (P<0.05) and greater feed conversion (P<0.05) compared with pigs receiving the 0.116% or 0.496% of aP.

Discussion

Effects of the Environment on Electron Transport Chain Gene Expression

Several studies have demonstrated the negative impacts of high temperatures on pig performance in post weaning and growing pigs (Christon, 1988; Quiniou et al., 2000; Collin *et al.*, 2002), including reduced voluntary feed intake and daily weight gain (Collin et al., 2001a; Kerr et al., 2003) as well as changes in muscular metabolic activity (Rinaldo and Le Dividich, 1991; Collin et al., 2001b). In contrast, very little has been published on the effects of the environment on the regulation of genes involved in energy metabolism, which is fundamentally important for understanding the mechanisms that underlie the metabolic changes in heat-stressed animals.

Considering that the complex activity of the electron transport chain (ETC) is closely related to levels gene expression encoding subunits of each complex. It is possible to associate the decrease in ETC activity, induced by heat stress, is a result of lower expression of some protein subunits within each complex ETC. In the present study, we showed by qRT-PCR that pigs studied during phases 1 and 2 exhibited lower expressions of genes from the electron transport chain in the hot environment, when compared to those in thermoneutral environment. Our results provide evidence of the effect of high temperatures that induce the decrease in oxidative metabolism (oxidative phosphorylation) through adaptive physiological mechanisms in order to reduce metabolic heat production. This observation is consistent with results from Christon (1988) and Collin *et al.* (2002) who reported that exposure of pigs to

high temperatures resulted in hormonal changes, including a decrease in thyroid hormones, which are known to stimulate oxidative phosphorylation and ATP production. Studies by Nicol and Johnston (1981) and Rinaldo and Le Dividich (1991) revealed that the activities of enzymes involved in oxidative metabolism and glycolysis in muscles were reduced in pigs exposed to high temperatures.

Our results indicated that exposure to high temperatures (34.1 ± 0.8ºC) resulted in reduced expression of the gene *ND1* and *COX3* in growth phase 1 when fed diets containing 0.107 and 0.321% of aP and reduced *ND2* gene expression when fed 0.535% of aP. Similarly, exposure of pigs in growth phase 2 to high temperatures (31.6 ± 0.7ºC) reduced the expression levels of the *ND2, COX2* and *COX3* genes irrespective to aP levels and reduced expression of the *COX1* gene in pigs fed 0.306% of aP. ND1 and ND2 are part of the P module of the NADH:ubiquinone oxidoreductase complex (complex I), which is responsible for pumping protons through the inner mitochondrial membrane (Brandt, 2006).Therefore changes in these genes may be critical for proper functioning electron transport chain and mitochondrial efficiency. COX1, COX2 and COX3 are essential proteins of the Cytochrome c oxidase complex (complex IV) and are responsible for electron transfer and proton pumping. They are extremely important for mitochondrial efficiency and thus for production of ATP (Scheffler, 1999).The *cytb* gene demonstrated the largest reduction in gene expression in response to high temperatures in growth phase 1 and 2 irrespective to dietary aP levels. CYTB is an essential subunit of the functional center of the ubiquinol-cytochrome-c reductase complex (complex III) and catalyzes the transfer of electrons from ubiquinol to cytochrome c (Yu *et al.*, 1999). Therefore, it has been widely used to evaluate mitochondrial activity

(Carper *et al*., 1999; Iqbal *et al*., 2004). In addition, we also observed reduced expression of the ATP5J2 gene in growth phase 1 when pigs were fed 0.107 and 0.321% of aP and in phase 2 when pigs were fed 0.116% of aP, and also a reduction in ATP6 gene expression in phase 1 independent of dietary aP levels. ATP5J2 and ATP6 are proteins in the F_0 domain of the F_1F_0 ATPase complex (complex V). ATP6 is a key component of the proton channel (Nagley, 1988), and ATP5J2 is essential for the function of the F_0 domain.

According to Drose and Brandt (2008) and Stowe and Camara (2009), mitochondria are the primary source of ROS (reactive oxygen species), such as superoxide (O_2^-), which are produced mainly by electron transport chain complexes I and III. Mitochondria have been found to be especially sensitive to oxidative damage. In the presence of ROS, mitochondria undergo morphologic and biochemical changes, including loss of electron transport capacity (Zhang *et al*., 1990), decreased transcription and levels of mitochondrial gene products (Kemp *et al.,* 1994; Kristal *et al*., 1994; Crawford *et al*., 1997; Carper *et al*., 1999), protein oxidation (Marcillat *et al*.,1988; Grune *et al*., 1995) and lipid peroxidation (Bindoli, 1988; Uchida, 2003). Thus, another factor that could be associated with decreased transcription of these genes would be due to heat stress which induced oxidative stress (imbalance of ROS production and mitochondrial ROS removal systems). This would result in decreased activity of the enzymatic complexes due to oxidative damage and would lead to down-regulation of electron transport chain genes.

Consistently with our findings, Mujahid *et al*. (2007) reported that exposure of chickens to high temperatures stimulates mitochondrial superoxide production, which resulted oxidative damage to mitochondrial lipids and

proteins. However, the actual mechanisms responsible for the induction of ROS production by heat stress are still not clear.

Effects of dietary P levels on Electron Transport Chain Gene Expression

Few studies have been done to elucidate the effects of dietary P levels on gene expression (Hittmeier *et al*., 2006; Alexander *et al*., 2008). The results obtained in this study revealed that different aP levels in the diet altered the expression of electron transport chain genes in both thermal environments.

Phosphate (P_i) has been demonstrated to directly influence dehydrogenases and other enzymes involved in oxidative phosphorylation (Rodrigues-Zavala *et al*., 2000). In addition, Bose *et al*. (2003) studied the effects of P_i on various steps of oxidative phosphorylation in pig cardiac mitochondria and showed that P_i regulated oxidative phosphorylation at several levels, including the generation of NADH, the formation of the proton electrochemical gradient and directly as a substrate for ATP formation by the F_1F_0-ATPase. However, it has become evident that the cellular regulation of oxidative phosphorylation is a complex network with various rate-limiting steps that are affected by a variety of signaling molecules, including P_i, Ca^{+2}, creatine and Mg^{+2} (Ernest *et al*., 1966; Brand and Kesseler, 1995), but the effects of each of these factors remain unclear.

During Phase 1, the expression of *ND1*, *ND2*, *cytb*, *COX3, ATP5J2* and *ATP6* genes were higher in pigs fed 0.321% of aP compared to those that received 0.535% of aP in the thermoneutral environment (Figure 3). It is possible to conclude that the lower gene expression observed in pigs receiving

0.535% of aP. This is in agreement with the performance results of pigs fed different of aP levels kept under thermoneutral environment, reported by Alebrante *et al*. (2011b) who verified that aP levels above 0.461%, which corresponded to a daily aP intake of 5.45 g, affected negatively the performance of the pigs. Thus, the consumption of P above the required level (0.535% aP) may compromise the expression of genes of the electron transport chain. Furthermore, this is consistent with the performance results of pigs fed 0.321% of aP had better feed conversion than pigs of fed 0.535% or 0.321% of aP. Thereby, it was evidenced that pigs are susceptible to inappropriate levels of phosphorus in the diet.

Interestingly, we observed higher expression of *CYTB*, *ATP6*, *ATP5J2* and *COX3* in pigs fed the 0.107% of aP compared with those fed the 0.535% of aP in the thermoneutral environment indicating that feeding sub-optimal aP levels may be less harmful to the expression of the electron transport chain genes in comparison with the excess. This is consistent with Fernández (1995) who evaluated the implications of increased aP intake on growing pigs and found that increased aP consumption did not result in higher P utilization for metabolism. On the contrary, pigs reduced bone resorption and increased urinary P excretion in response to increased intake of this nutrient.

In the hot environment, our results indicated a higher expression of the *ND2* gene for the pigs fed 0.535% of aP, compared with pigs fed 0.107 and 0.321% of aP (Figure 3). These results evidenced the effects of heat stress which decreased voluntary feed intake, therefore, the pigs that received 0.535% of aP in the diet consumed 5.12 g/day aP (in contrast to the animals under thermoneutral condition, with daily aP intake of 6.55 g), which is closer to the requirements (0.475% of aP) determined in the performance study conducted

by Alebrante *et al*. (2011b). This is confirmed by performance results of pigs fed 0.535% of aP had better feed conversion than pigs of fed 0.107% and 0.321% of aP.

During phase 2, we found a higher expression of COX3 gene in the pigs fed the 0.306% of aP compared to pigs fed the diets with 0.116 and 0.496% of aP in the thermoneutral environment. This is agreement with the performance results of pigs fed 0.306 % of aP had better feed conversion and great ADG than pigs fed 0.116% and 0.496% of aP in the thermoneutral environmental. Similarly, the diet with 0.306% aP led to increased *ND2* gene expression compared with the other diets in the hot environment. These results provide evidence that 0.306% aP led to better respiratory chain function by providing P closer to the pigs requirement verified in the performance study conducted by P. H. R. F. Campos (unpublished results) in which the diets containing 0.331% aP, corresponding to a daily aP intake of 7.32 g in the thermoneutral environment, and 0.351% aP, corresponding to a daily aP intake of 6.36 g in hot environment, resulted in greatest performance of pigs.

Interaction between dietary levels of aP and the thermal environment

An interaction between aP levels and thermal environment in the results of qRT-PCR in phase 1 was observed, as the gene expression profile of the electron transport chain was different between thermal environments. These genes had higher expression levels at 0.321% of aP in the thermoneutral environment while in the hot environment it was higher at the 0.535% aP diet. As it is evident from these data, the high ambient temperatures can cause physiological changes, such as decreased activity of enzymatic complexes of the electron transport chain due to reduced expression of these genes,

reflecting on different nutritional requirements (expressed in grams/day of feed) as demonstrated in this study by the reduction of requirement for daily aP intake.

The actual mechanisms responsible for the distinct patterns of the reduction of genes studied cannot be determined from this present study, but this may be reflected that electron transport chain genes are differentially affected to different dietary aP levels. However, we can state that in general, in pigs from phase1 the level of 0.321% aP diet under thermoneutral conditions and the level of 0.535% aP for pigs kept under high temperature conditions led to higher expression of electron transport chain genes and in pigs from growth phase 2, the level 0.306% of aP under both thermal environments led to an increased expression of electron transport chain genes. These results support that higher gene expression is associated with better respiratory chain function and likely better ATP production efficiency. The association between oxidative phosphorylation and animal performance suggested in our findings is consistent with studies that compared the performances of beef cattle (Archer *et al.*, 1999; Castro-Bulle *et al.*, 2007) and poultry (Bottje *et al.* 2002; Iqbal *et al.*, 2004; Bottje and Carstens, 2009) with mitochondrial function. Bottje *et al.* 2002 suggested that the differences in performance of genetically similar animals might be partially due to mitochondrial inefficiency, as the mitochondria is responsible for 90% of the cellular production of ATP.

In conclusion, our study revealed that exposing pigs to high temperatures results in down-regulation of genes involved in the electron transport chain. These findings indicate that physiological and biochemical adaptations to reduce metabolic heat production, which may be linked to the heat-induced increase in ROS. To confirm this hypothesis, further studies are being

conducted by our team to assign expression profiles of genes that encode the mitochondrial antioxidant systems that neutralize ROS, such as glutathione peroxidase and superoxide dismutase. We also showed that different amounts of aP in the feed alter electron transport chain gene expression. These data reveal strong evidence that the nutrient phosphorus, as well as environmental temperature, are part of the key factors regulating oxidative phosphorylation with direct implications on animal performance.

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Table 1. Diet composition from growth phase 1.

	Dietary available P(aP), %		
Item	0.107	0.324	0.535
Ingredient (%)			
Corn	62.011	62.011	62.011
Soybean meal, 45%	32.00	32.00	32.00
Soybean oil	1.810	1.810	1.810
Dicalcium phosphate	-	1.156	2.312
Limestone	1.837	1.098	0.360
Kaolin	1.200	0.783	0.365
Salt	0.456	0.456	0.456
Vitamin premix[1]	0.100	0.100	0.100
Mineral premix[2]	0.050	0.050	0.050
Growth promoter[3]	0.075	0.075	0.075
Growth promoter[3]	0.030	0.030	0.030
L-lisine HCL	0.282	0.282	0.282
DL- methionine	0.073	0.073	0.073
L- threonine	0.066	0.066	0.066
BHT (butylhydroxytoluene)	0.010	0.010	0.010
Calculated composition			
ME (kcal/kg)	3250	3250	3250
Crude protein (%)[4]	19.891	19.891	19.891
Digestible lysine (%)[4]	1.146	1.146	1.146
Calcium (%)	0.800	0.800	0.800
Sodium (%)	0.200	0.200	0.200
Total P analyzed (%)	0.344	0.546	0.782
Available P (%)	0.107	0.321	0.535

[1] Provided per kg: A vitamin - 8.000.000 IU; D3 vitamin - 2.000.000 IU; E vitamin - 10.000 mg; K3 vitamin - 1.500 mg; B12 vitamin - 20.000 mg; B2 vitamin - 5.000 mg; biotin - 50 mg; calcium pantothenate - 12.000 mg; niacin - 25.000 mg; antioxidant - 30.000 mg; B1 vitamin - 1.500 mg; B6 vitamin - 2.000 mg; folic acid - 800 mg; selenium - 320 mg; vehicle q.s.q - 1.000 g.
[2] Provide per kg of product: iron - 100.000 mg; copper - 30.000 mg; manganese - 70.000 mg; zinc - 160.000 mg; iodine - 1900 mg; and vehicle q.s.p. - 1000 g.
[3] Provided per kg of product: colistin - 80.000 mg; and tylosin- 400.000mg.
[4] Values estimated based on the digestibility of amino acids of ingredients, according to Rostagno et al. (2005).

Table 2. Diet composition from growth phase 2.

	Dietary available P(aP), %		
Item	0.116	0.306	0.496
Ingredient (%)			
Corn	55.930	55.930	55.930
Soybean meal (45% CP)	39.485	39.485	39.485
Soybean oil	1.483	1.483	1.483
Dicalcium phosphate	-	1.029	2.056
Limestone	1.353	0.696	0.041
Salt	0.415	0.415	0.415
Vitamin premix[1]	0.100	0.100	0.100
Mineral premix[2]	0.050	0.050	0.050
Growth promoter[3]	0.050	0.050	0.050
Growth promoter[3]	0.013	0.013	0.013
L-lisine HCL	0.282	0.282	0.282
DL- methionine	0.025	0.025	0.025
BHT (butylhydroxytoluene)	0.010	0.010	0.010
Calculated composition			
ME (kcal/kg)	3238	3238	3238
Crude protein (%)[4]	22.5	22.5	22.5
Digestible lysine (%)[4]	1.105	1.105	1.105
Calcium (%)	0.631	0.631	0.631
Sodium (%)	0.183	0.183	0.183
Total P analyzed (%)	0.344	0.534	0.724
Available P (%)	0.116	0.306	0.496

[1] Provided per kg: A vitamin - 8.000.000 IU; D3 vitamin - 2.000.000 IU; E vitamin - 10.000 mg; K3 vitamin - 1.500 mg; B12 vitamin - 20.000 mg; B2 vitamin - 5.000 mg; biotin - 50 mg; calcium pantothenate - 12.000 mg; niacin - 25.000 mg; antioxidant - 30.000 mg; B1 vitamin - 1.500 mg; B6 vitamin - 2.000 mg; folic acid - 800 mg; selenium - 320 mg; vehicle q.s.q - 1.000 g.

[2] Provide per kg of product: iron - 100.000 mg; copper - 30.000 mg; manganese - 70.000 mg; zinc - 160.000 mg; iodine - 1900 mg; and vehicle q.s.p. - 1000 g.

[3] Provided per kg of product: colistin - 54.000 mg; and tylosin- 174.000mg.

[4] Values estimated based on the digestibility coefficients of amino acids of ingredients, according to Rostagno et al. (2005).

Table 3. Gene names, primer pair sequences, annealing temperatures and amplification efficiencies of each target.

Gene Name	Primer sequence (5'-3')[a]	Annealing temperature , ºC	Efficiency
ND1	F- TCAACCCTAGCAGAAACCAACGA R- AAGAATATGGCGAAAGGTCGGCT	60.5	0.90
ND2	F- TTTCCTAACACAAGCCACAGCCTC R- ATGCCTTGGGTTACTTCTGGGACT	60	0.90
SDHD	F- TACAGCACATTCACCTGTCACCCA R- AGTCCATCGCAGAGCAAGGATTA	60.3	0.95
CYTB	F- TCACACGATTCTTCGCCTTCCACT R- TAGGGTTGTTGGATCCGGTTTCGT	60.4	0.93
COX1	F- ATGGGCTCACCACATATTCACCGT R- TTAATATTGCCGCCGTGCAGGGTA	60.5	0.90
COX2	F-TGGCTTACCCTTTCCAACTAGGCT R- TTGGGCATCCATTGTGCTAGTGTG	60.1	0.92
COX3	F- ACACCCGAATTAGGAGGTTGCTGA R- TACGCCTAGTGCAATGGTGATGGA	60.2	0.90
ATP5J2	F- GGCATTGCCGGAGCATTTCAAAGA R- GTATTTGCGCAGCCGCTCATGTTT	60.6	0.95
ATP6	F- TACCACACTCATTCACACCCACCA R- TGTTCCTTGTGGTAGAAAGTGGGC	59.9	0.92
GAPDH	F- CAAAGTGGACATTGTCGCCATCA R- AGCTTCCCATTCTCAGCCTTGACT	60	0.97

[a]F = forward; R = reverse.

ND1- NADH dehydrogenase subunit 1; *ND2*- NADH dehydrogenase subunit 2; *SDHD*- Succinate dehydrogenase subunit d; *CYTB* - Cytochrome b; *COX1* - Cytochrome oxidase subunit 1; *COX2* - Cytochrome subunit 2; *COX3* - Cytochrome subunit 3; *ATP5J2* - ATPase subunit f, isoform 2; *ATP6* - ATPase subunit 6 and *GAPDH* -Glyceraldehyde 3-phosphate dehydrogenase.

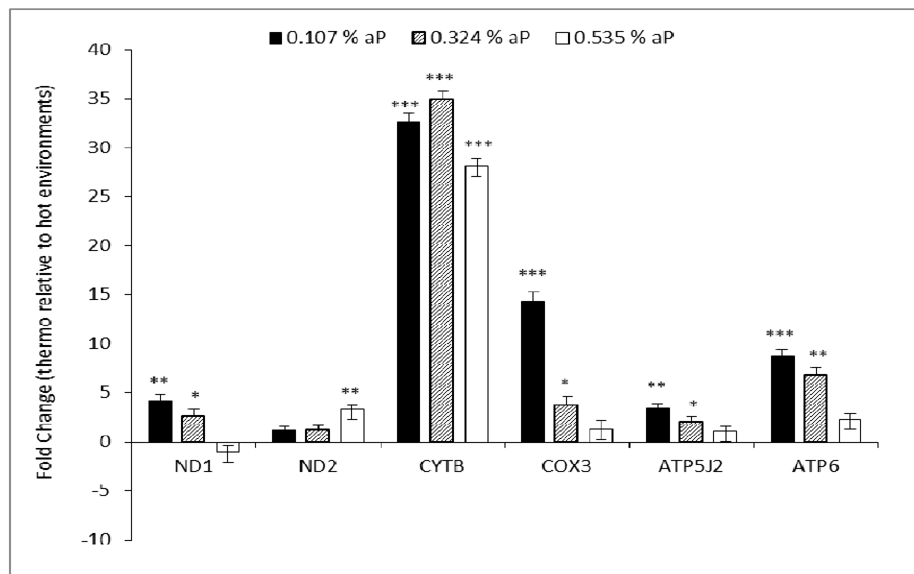


Figure 1. Quantitative RT-PCR results for six genes that exhibited differential expression on LD muscle of pigs from growth phase 1 between the thermoneutral and hot environments within each level of available phosphorus (aP). Results are presented as fold change for expression at thermoneutral environment relative to expression at hot environment, such as bars above the origin indicate higher expression at thermoneutral environment and bars below the origin indicate lower expression at thermoneutral environment for each level of aP evaluated. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

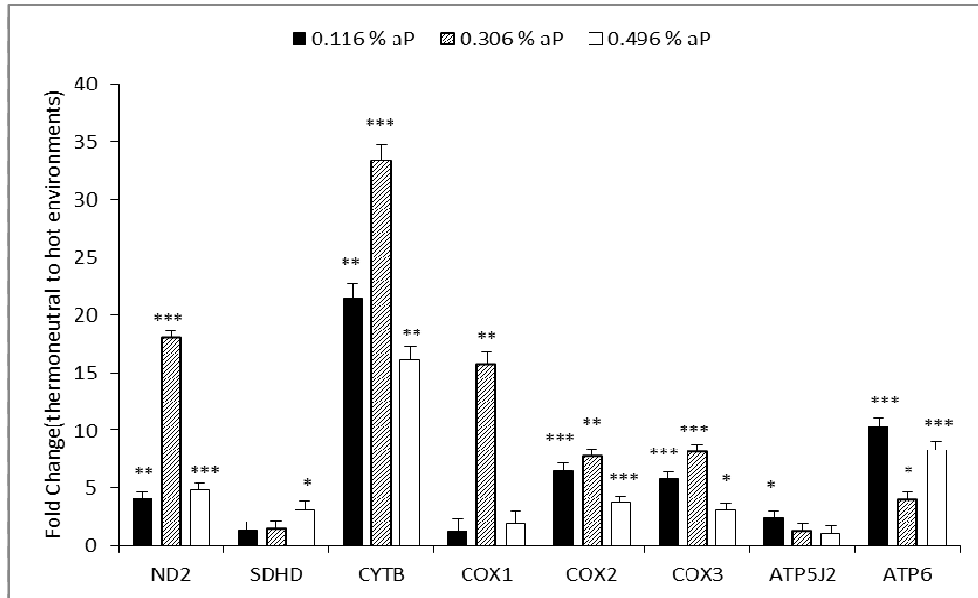


Figure 2. Quantitative RT-PCR results for eight genes that exhibited differential expression on LD muscle of pigs from growth phase 2 between the thermoneutral and hot environments within each level of available phosphorus (aP). Results are presented as fold change for expression at thermoneutral environment relative to expression at hot environment, such as bars above the origin indicate higher expression at thermoneutral environment and bars below the origin indicate lower expression at thermoneutral environment for each level of aP evaluated. *P < 0.05; **P < 0.01;***P < 0.001.

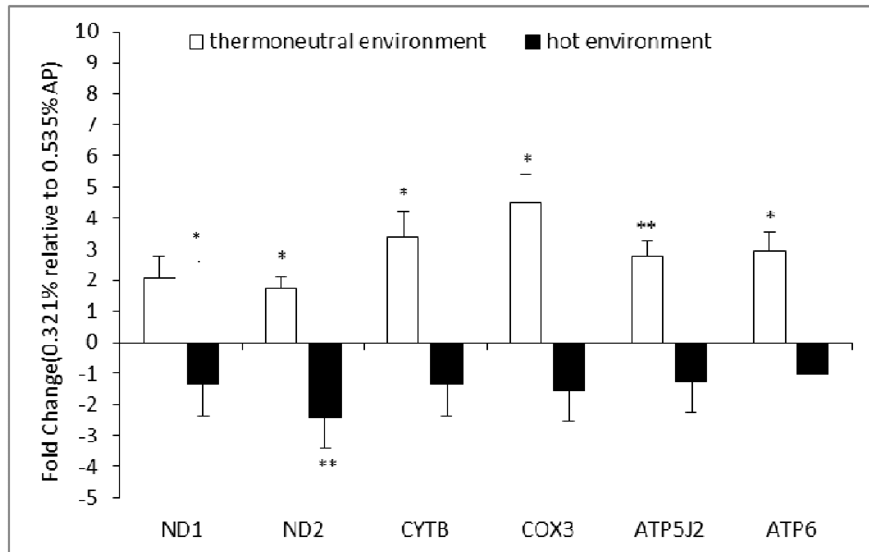


Figure 3. Quantitative RT-PCR results for six genes that exhibited differential expression on LD of pigs from growth phase 1 between 0.321 and 0.535 % aP within each thermal environment. Results are presented as fold change for expression at level 0.321% aP relative to expression at 0.535 % aP, such that the bars above the origin indicate higher expression at level 0.321% aP and bars below the origin indicate lower expression at level 0.321% aP. **P* < 0.05; ***P* < 0.01.

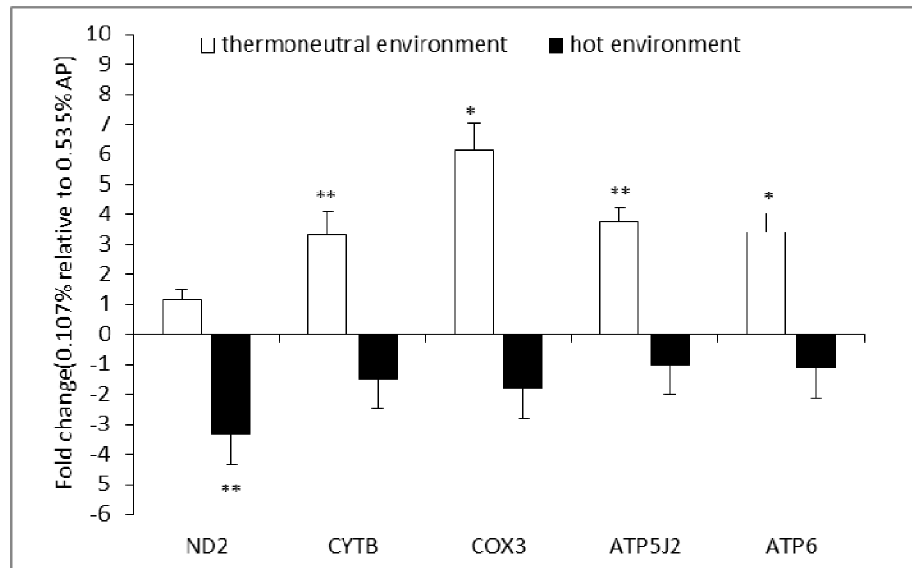


Figure 4. Quantitative RT-PCR results for five genes that exhibited differential expression on LD of pigs from growth phase 1 between 0.107 and 0.535% aP within each thermal environment. Results are presented as fold change for expression at level 0.107% aP relative to expression at 0.535% aP, such that the bars above the origin indicate higher expression at level 0.107% aP and bars below the origin indicate lower expression at level 0.107% aP. *$P < 0.05$; **$P < 0.01$.

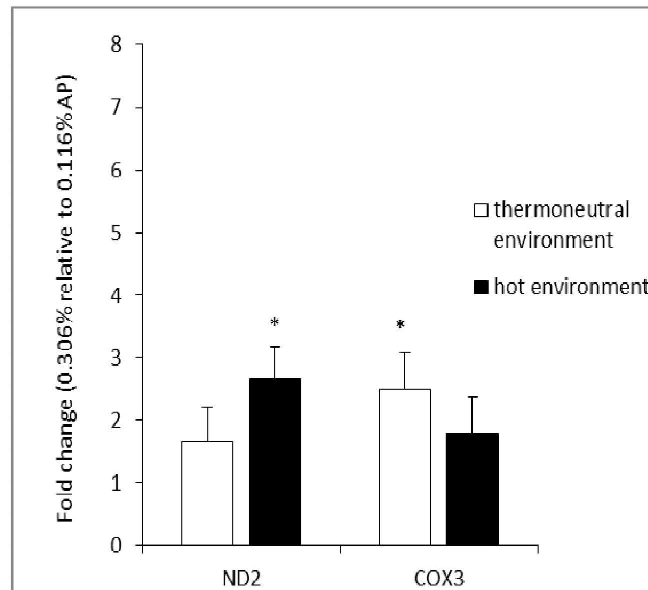


Figure 5. Quantitative RT-PCR results for two genes that exhibited differential expression on LD of pigs from growth phase 2 between 0.116 and 0.306% aP within each thermal environment. Results are presented as fold change for expression at level 0.306% aP relative to expression at 0.116% aP, such that the bars above the origin indicate higher expression at level 0.306% aP and bars below the origin indicate lower expression at level 0.306 % aP. *P < 0.05; **P < 0.01.

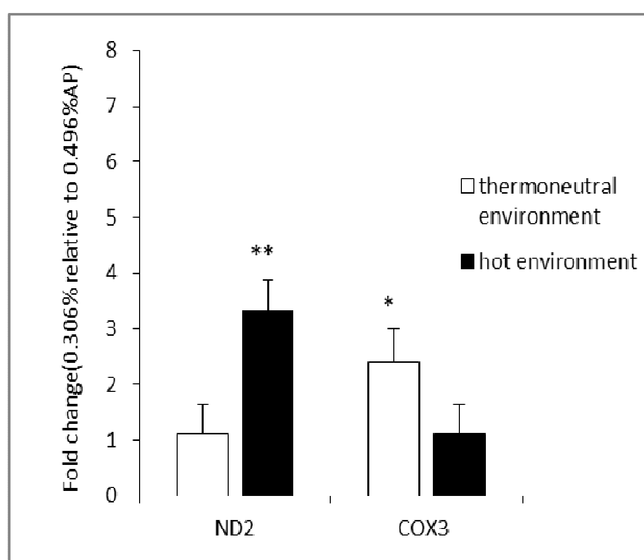
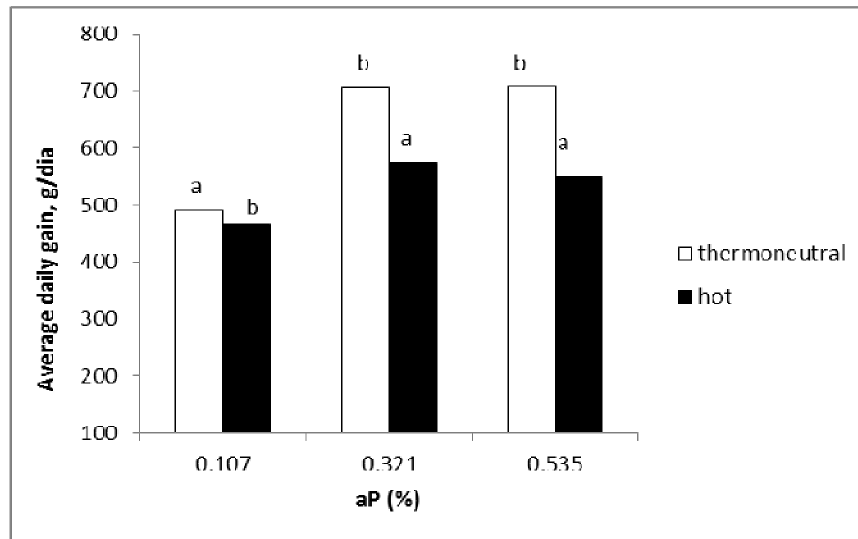
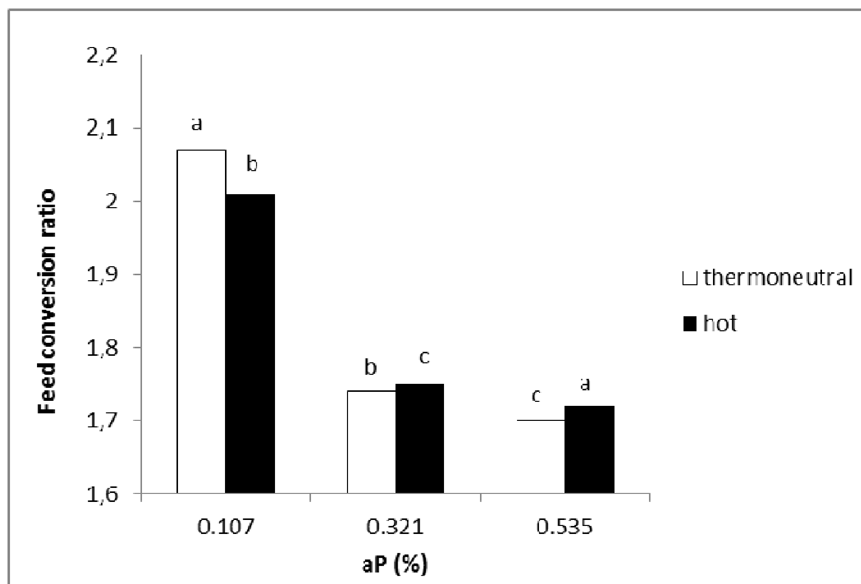


Figure 6. Quantitative RT-PCR results for two genes that exhibited differential expression on LD of pigs from growth phase 2 between 0.306 and 0.496% aP within each thermal environment. Results are presented as fold change for expression at level 0.306% aP relative to expression at 0.496% aP, such that the bars above the origin indicate higher expression at level 0.306% aP and bars below the origin indicate lower expression at level 0.306 % aP. *$P < 0.05$; **$P < 0.01$.

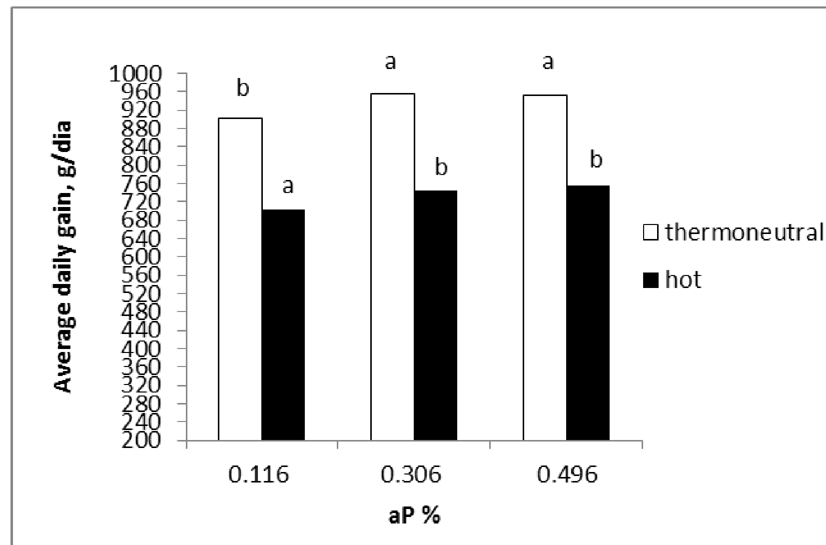


A

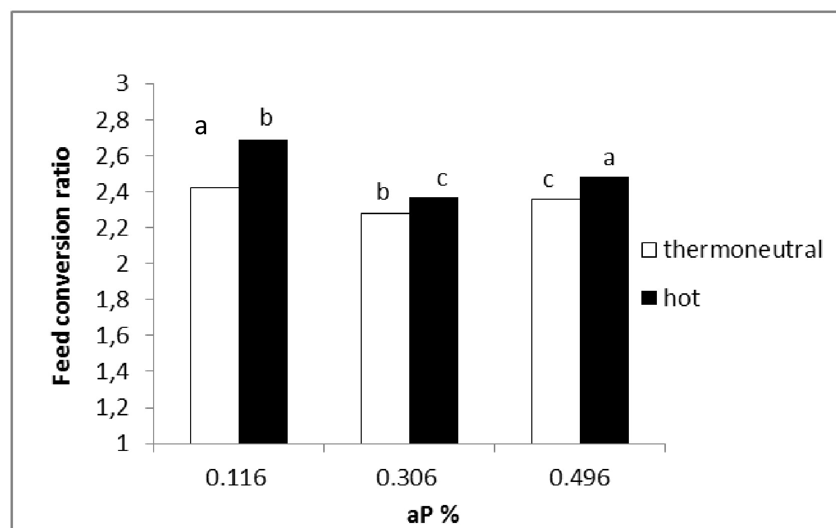


B

Figure 7. Average daily gain (A), and feed conversion ratio (B) of pigs from growing phase 1 submitted to treatments (3 different levels of aP levels within each thermal environment). Letters above the bars represent the comparison among treatment means. Different letters indicate statistical difference (P<0.05) by the test of Tukey.



A



B

Figure 8. Average daily gain (A), and feed conversion ratio (B) of pigs from growing phase 2 submitted to treatments (3 different levels of aP levels within each thermal environment). Letters above the bars represent the comparison among treatment means. Different letters indicate statistical difference (P<0.05) by the test of Tukey.

CHAPTER II
(Article II)

Heat stress stimulates mitochondrial production of reactive oxygen species in pig skeletal muscle

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ABSTRACT

Heat stress is an environmental factor that could be responsible for stimulating reactive oxygen species (ROS) production. Thus, this study was performed to evaluate the expression profile of genes encoding antioxidant systems: superoxide dismutase 2 (*SOD2*), glutathione peroxidase-1 (*GPx1*) and catalase (*CAT*) in the *Longissimus dorsi (LD)* muscle of pigs from a commercial line at 2 different growth phases: 15 to 30 kg and 30 to 60 kg submitted to heat stress. Eight pigs from growth phase 1 of similar BW, were assigned randomly to one of the two treatments groups: one group (n=4) was kept in a climate-controlled room maintained at 24.5 ± 1.2ºC and the RH (relative humidity) at 76.3 ± 8.5%, whereas, the second group (n=4) was kept in a climate-controlled room maintained at 34.1 ± 0.8ºC and RH at 70.1 ± 8.1%. Likewise, 8 pigs from growth phase 2 of similar BW, were assigned randomly to one of the two treatments groups: one group (n=4) was kept in a climate-controlled room maintained at 21.9 ± 1.4ºC and RH at 76.9 ± 5.7%, whereas, second group (n=4) was kept in a climate-controlled room maintained at 31.6 ± 0.7ºC and RH at 72.8 ± 5.9%. The qRT-PCR analyses showed that the *GPx1*, *SOD2* and *CAT* were upregulated ($P<0.05$) in heat-exposed pigs. These

results provide evidences that heat stress stimulates mitochondrial ROS production on LD muscle of pigs.
Keywords: antioxidants enzymes, pig production, qRT-PCR

RESUMO

Estresse por calor é um fator ambiental que poderia ser responsável por estimular a produção de espécies reativas de oxigênio (ROS). Assim, este estudo foi realizado para avaliar o perfil de expressão dos genes superóxido dismutase 2 (*SOD2*), glutatinona peroxidase 1 (*GPx1*) e catalase (*CAT*) no músculo *Longissimus dorsi* (LD) de suínos de 2 diferentes fases de crescimento: 15 a 30 kg e 30 a 60 kg, submetidas ao estresse por calor. Oito suínos da fase 1 de crescimento de pesos similares, foram designados aleatoriamente para um dos dois grupos de tratamentos: O grupo 1 (n=4) foi mantido em uma sala climatizada mantida em 24.5 ± 1.2ºC e 76.3 ± 8.5% UR, enquanto que, o grupo 2 foi mantido em uma sala climatizada à 34.1 ± 0.8ºC e 70.1 ± 8.1% UR. Da mesma forma, oito porcos da fase 2 de pesos similares, foram designados aleatoriamente para um dos dois grupos de tratamentos: O grupo 1 (n=4) foi mantido em uma sala climatizada à 21.9 ± 1.4ºC e 76.9 ± 5.7% UR, enquanto que, o grupo 2 (n=4) foi mantido em uma sala climatizada à 31.6 ± 0.7 ºC e 72.8 ± 5.9% UR. As análises de qRT-PCR mostraram que os genes *GPx1*, *SOD2* e *CAT* foram ($P<0.05$) mais expressos nos suínos expostos à altas temperaturas. Esses resultados provêm evidências de que estresse por calor estimula a produção mitocondrial de ROS no musculo LD de suinos.
Palavras-chaves: enzimas antioxidantes, produção de suínos, qRT-PCR

Introduction

Heat stress is of major concern for pig industry, especially in tropical countries because of the resulting deleterious effects on fertility in sows (Omtvedt et al., 1971), negative impacts on pig performance in post weaning, growing pigs (Christon, 1988; Quiniou et al., 2000; Collin *et al.*, 2002) including reduced voluntary feed intake and daily weight gain (Collin et al., 2001a; Kerr et al., 2003) as well as changes in muscle metabolism (Rinaldo and Le Dividich, 1991; Collin et al., 2001b). From the viewpoint of environmental health, the harmful effects of heat stress on organism have become matter of concern.

Heat stress was proposed to be responsible for stimulating reactive oxygen species (ROS) production because of similarities in gene expression patterns observed following heat stress compared with that following exposure to oxidative stress (Schiaffonati et al., 1990; Salo et al., 1991; Flanagan et al., 1998; Wang et al., 2000; Zuo et al., 2000). Heat-induced ROS formation may be the factor that causes molecular changes in DNA, proteins and lipids (Bruskov et al., 2002) which may be result at mitochondrial level in bioenergetics dysfunctions. Given that the mitochondria serve as the principal source of ROS in cells (Chance et al., 1979), it is likely that in heat-stressed animals ROS are mainly produced in skeletal muscle mitochondria. More recently Mujahid et al. (2005) reported the mitochondrial ROS generation in skeletal muscle of heat-exposed broilers. However, there is still no evidence of the increased ROS production in heat-stressed pigs.

The goal of this study was evaluate the expression profile of genes encoding antioxidants enzymes: superoxide dismutase 2, glutathione peroxidase 1 and catalase in the *Longissimus dorsi (LD)* muscle of pigs from a commercial line at 2 different growth phases, submitted to hot and thermoneutral environments.

Materials and Methods

All methods involving animal handling was done in accordance with regulations approved by the Institutional Animal Welfare and Ethics/Protection commission from the Universidade Federal de Viçosa, Brazil.

Data

Sixteen pigs (male castrated) from a commercial lines of two different growth phases: phase 1 (15 to 30 kg) and phase 2 (from 30 to 60 kg) were used in this trial. Eight pigs from growth phase 1 of similar BW, were assigned randomly to one of the two treatment groups: one group (n=4) were reared in suspended metal cages and kept in a climate-controlled room maintained at 24.5 ± 1.2ºC and the RH (relative humidity) at 76.3 ± 8.5%, whereas the other 4 phase 1 pigs were kept in a climate-controlled room maintained at 34.1 ± 0.8ºC and RH at 70.1 ± 8.1% considered as heat stress environment for this animal category (Coffey et al., 2000).

Likewise, 8 pigs from growth phase 2 of similar BW, were assigned randomly to one of the two treatment groups: one group (n=4) was reared in suspended metal cages and kept in a climate-controlled room maintained at 21.9 ± 1.4ºC and relative humidity of 76.9 ± 5.7% considered as thermoneutral environment for this animal category (Coffey et al., 2000) whereas others 4 pigs of growth phase 2 were kept in a climate-controlled room maintained at 31.6 ± 0.7ºC and RH at 72.8 ± 5.9%, considered as heat stress for this animal category (Coffey et al., 2000).

The experimental diet for the growth phase 1 in both thermal environments (Table 1) provided 3.25 Mcal of digestible energy/kg of diet and 19.9% of CP. The experimental diet for growth phase 2 in both thermal environments (Table 2) provided 3.23 Mcal of digestible energy/kg of diet and 22.53% CP. Feed and water were provided *ad libitum* throughout the experimental period (25days).

RNA Extraction and cDNA Synthesis

The samples of *Longissimus dorsi* (LD) were collected during slaughter and immediately immersed in tubes containing 15 ml of RNAHolder® (BioAgency, São Paulo, Brasil) and stored at -20°C for subsequent RNA extraction.

The total RNA from each LD sample was isolated using approximately 40 mg of tissue previously stored in RNAHolder®. The samples were homogenized in buffer RLT containing *1% β-mercaptoethanol (*RNeasy Mini Kit*)* and lysed with a tissue ruptor (Qiagen, Valencia, CA*)* homogenizer. The total RNA from the LD muscle samples was extracted with the RNeasy Mini *Kit (*Qiagen, Valencia, CA) following the manufacturer's recommendations. Additional treatment with DNase was performed on the columns using the RNase-free DNase Set (Qiagen, Valencia, CA), according to the manufacturer's recommendations. RNA concentrations were checked by NanoVue Plus Spectrophotometer (GE Healthcare) with an optimal 260/280 ratio between 1.8 and 2.1. Purity and integrity were determined with an Agilent RNA 6000 Nano Kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.)

The ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England BioLabs Inc., Beverly, MA) was used to produce cDNA immediately after the RNA extraction. The reactions were performed with 6 µg of total RNA (for each sample) and 2 µL of 50 µM oligo$(dT)_{23}$VN primer, following the manufacturer's recommended protocol. The cDNA concentrations from the samples were estimated on a NanoVue Plus^{TM} (GE Healthcare, Munich, Germany) spectrophotometer. Finally, the single-stranded cDNA samples were stored at –20ºC for analysis.

Quantitative Real-time PCR

The genes *GPx1*, glutathione peroxidase 1; *SOD2*- superoxide dismutase 2 mitochondrial; *CAT* – catalase and *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase, were obtained from the nuclear genome of *Sus scrofa* (Accession No. NM_ 214201.1, NM_214127.2, NM_214301.1 and AF017079.1, respectively). The primers for the 4 genes were designed using *PrimerQuest* (www.idtdna.com/Scitools/Applications/PrimerQuest), provided by Integrated DNA Technologies, Inc. (Coralville, IA). The gene *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) was used as a reference gene for normalization as it showed better efficiency and less variation across treatments than β-actin (data not shown). The primer pairs are listed in Table 3.

Quantitative real time PCR (qRT-PCR) reactions were performed using the GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI) following the manufacturer's instructions in an ABI Prism 7300 Sequence Detection System thermocycler® (Applied Biosystems, Foster City, CA). The reaction consisted of an initial step at 95ºC for 10 minutes, a second step of 40 cycles with the same temperature for 15 seconds and a final extension step at 60ºC for 60 seconds. After the amplification cycles, an additional gradient step from 60ºC to 95ºC was used to obtain a melting curve. The efficiency of each reaction was assessed in order to choose the best combination of cDNA and primer concentration in the subsequent reactions. Once the slope of a linear regression for each primer concentration was obtained, the PCR efficiency (E) was calculated using the following formula: $E = 10^{(-1/slope)}$. The amplification efficiency for all tested genes varied from 0.90 to 0.96 (table 1). All reactions were done in duplicate and the coefficient of variation (CV) of cycle C_t value

from replicates within each sample was low, less than 5%, indicating acceptable accuracy and reproducibility (not shown).

Statistical Analyses

To study the effects of the thermal environment at each growing phase, it was used a completely randomized design with four replicates per thermal environment. All statistical procedures were performed using SAS (v. 9.1.3). The routine QPCR_MIXED SAS® [https://www.msu.edu/~steibelj/JP files/QPCR.html] developed to generate commands in SAS PROC MIXED suitable to analyze data from qRT-PCR, assuming independent random effects for reference gene and target genes in each biological replicate, was used in the analysis (Steibel et al., 2009). Data was analyzed using the linear mixed model:

$$y_{gikr} = TG_{gi} + C_{gik} + D_{ik} + e_{gikr}$$

In this equation, y_{gikr} corresponds to the C_t for the gth gene (control or target) from the rth well, which corresponds to the kth animal submitted to the ith thermal environment; TG_{gi} is the effect of thermal environment on the expression of gene g; $C_{gik} \sim N(0, \sigma_C^2)$ is the gene-specific random effect of the kth animal; $D_{ik} \sim N(0, \sigma_D^2)$ is the sample-specific random effect (common to both genes); and $e_{gikr} \sim N(0, \sigma_e^2)$ is the residual term.

For each target gene, the comparison of the fold change values between treatments was performed by CONTRAST statement of the GLM procedure (SAS software) using Student's test to the level of 5%. The "estimates" of the contrasts between treatments generated as results of statistical analysis, are the differences in C_t

values between the treatments under evaluation and were used to estimate the fold change (relative expression).

The fold change values were obtained by transformation of the estimates (C_t) detected after statistical analyses by calculation: fold change = $2^{-Estimate}$. Once the efficiency (E) of the qRT-PCR reaction was close or equal to 2.0, one cycle (C_t) of difference between two levels (or samples) means 2.0 fold change of difference, or still, twice as much expression in the first level in comparison with the second level. Also, understanding that higher C_t values means lower transcriptional expression of a specific gene for a specific sample relatively to others and that lower C_t values means higher transcriptional expression, negative values of "estimates" indicate a positive fold change (relative expression) of the first level relatively to the second under investigation, after applying the transformation (Fold change = $2^{-Estimate}$).

Results and Discussion

Our previous study (chapter I) revealed that exposing pigs to high temperatures resulted in decreased transcription of mitochondrial electron transport chain (ETC) genes which may be linked to the heat-induced ROS production. It has been shown that heat-induced ROS formation may be the factor that causes molecular damages in DNA, proteins, lipids and others biological molecules (Bruskov et al., 2002). Ando et al. (1997) reported that heat stress has potential to cause increases of ROS, possibly by the disruption of electron transport assemblies of the membrane.

Heat stress was proposed to be an environment factor responsible for stimulating ROS production because of similarities in gene expression patterns observed following heat stress compared with that following exposure to oxidative stress (Schiaffonati et al., 1990; Salo et al., 1991). Indeed, Flanagan et al. (1998) demonstrated an increased

flux of ROS in rat intestinal epithelial cell monolayers following exposure to a temperature of 45ºC. Zuo et al. (2000) also showed heat-stress stimulated intracellular and extracellular ROS production, particularly superoxide formation, in mouse diaphragm muscle.

Oxidative stress is an expression used to describe various deleterious processes resulting from an imbalance between the excessive formation of ROS and limited antioxidants defenses. The triggering factors for the oxidative stress may be diverse, ranging from hereditary or acquired genetic defects (mutations) or environmental factors, such as radiation, toxins (Andreyev et al., 2005), beyond the suggested heat stress. More recently Mujahid et al. (2005) revealed overproduction of ROS in the skeletal muscle mitochondria of heat-exposed broilers. However, there is still no evidence of the increased ROS production in pigs exposed to heat stress.

Mitochondria are the primary source of ROS (Cadenas and Davies, 2000; Lenaz, 2001), with superoxide, which is produced at complexes I (Takeshige and Minakani, 1979) and III (Trumpower, 1990; Barja, 1999) of ETC, quantitatively the most important species. In addition, mitochondria are sensitive to oxidative stress. Enhanced ROS production in mitochondria leads to morphologic and biochemical changes, including loss of electron transport capacity, decreased transcription of gene products (Zhang et al.,1990; Kristal et al., 1994; Crawford et al.,1997), protein oxidation and lipid peroxidation (Mujahid et al., 2007). At the same time, defense mechanisms are being induced (antioxidants systems) that repair and protect the cell against additional damage (Crawford and Davies, 1994; Grune et al., 1995; Weise et al., 1995). Based on this, the present study was performed to evaluate the expression profile of superoxide dismutase 2 *(SOD2*), glutathione peroxidase 1(*GPx1*) and catalase (*CAT*) genes on LD muscle of pigs from 2 different growth phases: 15 to 30 kg and 30 to 60 kg submitted to hot environment.

Results for qRT-PCR analyses of three genes showed that expression was ($P<0.05$) higher in heat-stressed pigs from growth phase 1 (Figure 1) and in heat-stressed pigs growth phase 2 (Figure 2). The statistical results for each contrast between treatments within each growth phase can be checked in the supplementary table 1 and 2 (Appendix 2).

Cells are equipped with many enzymes to detoxify cytoplasmic and mitochondrial ROS (Andreyev et al., 2005; Koehler et al., 2006). Up to a limit, mitochondrial and cytoplasmic antioxidant systems are capable of neutralizing excess of ROS. Superoxide dismutases (SODs) are the major ROS detoxifiers of the cell (Fridovich, 1989). Three types of SOD, encoded by different genes, exist in the cell (Fridovich, 1989). Superoxide dismutase 2 (SOD2), also known as manganese-superoxide dismutase (Mn-SOD), is expressed exclusively within the mitochondrial matrix (Weisiger and Fridovich, 1973; Okado-Matsumodo and Fridovich, 2001) and thus is an essential defender against mitochondrial superoxide radicals among antioxidative enzymes. It catalyzes the dismutation of superoxide radicals to hydrogen peroxide (H_2O_2) and molecular oxygen (Fridovich, 1995). In this study, *SOD2* gene was 12.82-fold higher ($P<0.05$) in heat-stressed pigs from phase 1(figure 1) and 15.46-fold higher ($P<0.01$) in heat-stressed pigs from phase 2 (Figure 2). Similarly, Yamashita et al. (1997) reported significant increase in levels of Mn-SOD mRNA, in rat myocytes after exposure to heat stress. Wang et al.(2000), also demonstrated heat stress stimulated overproduction of ROS in rat myocytes which led to increased activity of superoxide dismutases (Mn-SOD, Zn-SOD).

Hydrogen peroxide (H_2O_2) is also ROS, which is decomposed in water and molecular oxygen by many antioxidant enzymes, such as, glutathione peroxidase-1(GPx1). Glutathione peroxidase-1 is one of five other known glutathione peroxidase enzymes which is ubiquitously expressed in almost all tissues (Cheng et al., 1997), in

various cellular compartments including the mitochondrial matrix (Asayama et al., 1994; Esworthy et al., 1997) and intermembrane space (Panfili et al., 1991).

GPx1 has long been viewed as one of the most important parts of cellular and mitochondrial ROS-defense network, and apparently it is involved in protecting tissues and mitochondria against acute oxidative stress rather than providing a major defense against low-level endogenous mitochondrial ROS production (Andreyev et al., 2005). In this study, *GPx1* gene was 8.42-fold higher ($P<0.05$) in heat-stressed pigs from phase 1 (figure 1) and 9.84-fold higher ($P<0.05$) in heat-stressed pigs from phase 2 (Figure 2). These results are consistent with the study of Kemp et al. (2003) who reported overexpression of genes associated with detoxification and removal of ROS (superoxide dismutase, glutathione peroxidase) in rat myocytes exposed oxidative stress induced by different concentrations of H_2O_2.

Catalase is another important H_2O_2 detoxifying enzyme (Chelikani et al., 2004; Zamocky et al., 2008), which was 2.94 fold higher ($P<0.05$) in heat-stressed pigs from phase 1 (figure 1) and 3.11-fold higher ($P<0.01$) in heat-stressed pigs from phase 2 (Figure 2). There is some controversy with respect to mitochondrial catalase, this enzyme has been found in the nucleus, peroxisomes, sarcoplasm, but not mitochondria (Zhou and Kang, 2000). However, other labs have reported mitochondrial localization of catalase (Salvi et al., 2007), and the presence of catalase within the matrix of rat heart mitochondria (Radi et al., 1991). The higher expression of *CAT* gene in heat-stressed pigs indicates importance of this enzymes for neutralize increased fluxes of H_2O_2 that may diffuse from mitochondria to cytosol during oxidative stress phenomena.

This study revealed that heat stress is an environment factor responsible for stimulating production of mitochondrial ROS in skeletal muscle of pigs. This in turn was associated with induction of antioxidant enzymes expression that is directly involved to protect cell against oxidative damages. Moreover, our findings could

support that an increase of ROS in mitochondria may lead to oxidative damages, such as, nonspecific modification of lipids, proteins and nucleic acid, which then result perturbations in the physiological function of mitochondria inevitably disturb metabolism, leading to reduction in growth performance. This approach is consistent with previous study that shown downregulation of mitochondrial electron transport chain genes, and consequently, worst performance pigs resulting from heat stress. Thus, it is clear that the increase of mitochondrial ROS production by heat stress affects directly mitochondrial function and also interfere normal growth of animals.

This study reported, for the first time, the high temperatures not only cause reduction in oxidative metabolic capacity, but also stimulate the ROS production in skeletal muscle mitochondria which leading reduction in mitochondrial activity of electron transport chain complexes, repression of their transcriptions with direct implications on pigs performance.

These findings will help to elucidate the physiological mechanism response of pigs to heat stress that still is partially understood.

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Table 1. Composition and nutritional values of experimental diets from growth phase 1.

Ingredientes	Amount (%)
Corn	62.011
Soybean meal 45%	32.000
Soybean oil	1.810
Dicalcium phosphate	1.156
Limestone	1.098
Kaolin	1.200
Salt	0.456
Vitaminico Premix[1]	0.100
Mineral Premix[2]	0.050
Growth promoter[3]	0.075
Growth promoter[3]	0.030
L-Lysine HCl	0.282
DL-Methionine	0.073
L-Threonine	0.066
Antioxidant[4]	0.010
Calculated composition	
ME (kcal/kg)	3250
Crude Protein (%)[5]	19.98
Digestible lysine (%)[5]	1.146
Digestible met + cist (%)[5]	0.641
Digestible threonina (%)[5]	0.721
Sodium (%)	0.200
Calcium (%)	0.800
Available P (%)	0.321

[1] Provided per kg: A vitamin - 8.000.000 IU; D3 vitamin - 2.000.000 IU; E vitamin - 10.000 mg; K3 vitamin - 1.500 mg; B12 vitamin - 20.000 mg; B2 vitamin - 5.000 mg; biotin - 50 mg; calcium pantothenate - 12.000 mg; niacin - 25.000 mg; antioxidant - 30.000 mg; B1 vitamin - 1.500 mg; B6 vitamin - 2.000 mg; folic acid - 800 mg; selenium - 320 mg; vehicle q.s.q - 1.000 g.
[2] Provide per kg of product: iron - 100.000 mg; copper - 30.000 mg; manganese -70.000 mg; zinc - 160.000 mg; iodine - 1900 mg; and vehicle q.s.p. - 1000 g.
[3] Provided per kg of product: colistin - 80.000 mg; and tylosin- 400.000mg.
[4] Butylhydroxiluene.
[5] Values estimated based on the digestibility coefficients of amino acids of ingredients, according to Rostagno et al., 2005.

Table 2. Composition and nutritional values of experimental diets from growth phase 2.

Ingredientes	Amounts (%)
Corn	55.930
Soybean meal (45%)	39.485
Soybean oil	1.483
Dicalcium phosphate	1.029
Calcário	0.696
Inerte	0.709
Sal	0.415
Vitamin premix[1]	0.100
Mineral premix[2]	0.050
Growth promoter[3]	0.050
Growth promoter[3]	0.013
DL-Methionine	0.025
Antioxidant[4]	0.010
Calculated composition	
ME (kgcal/kg)	3.238
Crude protein (%)[5]	22.5
Digestible lysine (%)[5]	1.105
Digestible met.+cist (%)[5]	0.661
Treonina digestível (%)[5]	0.757
Sodium (%)	0.183
Calcium (%)	0.631
Availible P (%)	0.306

[1] Provided per kg: A vitamin - 8.000.000 IU; vitamin D3 - 2.000.000 IU; E vitamin - 10.000 mg; K3 vitamin - 1.500 mg; B12 vitamin - 20.000 mg; B2 vitamin - 5.000 mg; biotin - 50 mg; calcium pantothenate - 12.000 mg; niacin - 25.000 mg; antioxidant - 30.000 mg; B1 vitamin - 1.500 mg; B6 vitamin - 2.000 mg; folic acid - 800 mg; selenium - 320 mg; vehicle q.s.q - 1.000 g.
[2] Provide per kg of product: iron - 100.000 mg; copper - 30.000 mg; manganese -70.000 mg; zinc - 160.000 mg; iodine - 1900 mg; and vehicle q.s.p. - 1000 g.
[3] Provided per kg of product: colistin - 54.000 mg; and tylosin- 174.000mg.
[4] Butylhydroxiluene.
[5] Values estimated based on the digestibility coefficients of amino acids of ingredients, according to Rostagno et al., 2005.

Table 3. Gene names, primer pair sequences, annealing temperatures and amplification efficiencies of each target.

Gene Name	Primer sequence (5'-3')[1]	Annealing temperature, ºC	Efficiency
GPx1	F-AATTGCCTCAAGTACGTCCGACCA R-CATTTGCGCCATTCACCTCACACT	60	0.89
SOD2	F-ACGGTGGAGGCCACATCAATCATA R-AAAGTCGCGTTTGATGGCTTCCAG	59.9	0.88
CAT	F-ACAGCTTTAGTGCTCCCGAACAGA R-TCCGCACCTGGGTGACATTATCTT	60	0.90
GAPDH	F-CAAAGTGGACATTGTCGCCATCA R-AGCTTCCCATTCTCAGCCTTGACT	60	0.95

[1]F= forward; R= reverse.

GPx1-glutathione peroxidase 1; *SOD2*- superoxide dismutase 2; *CAT* - catalase

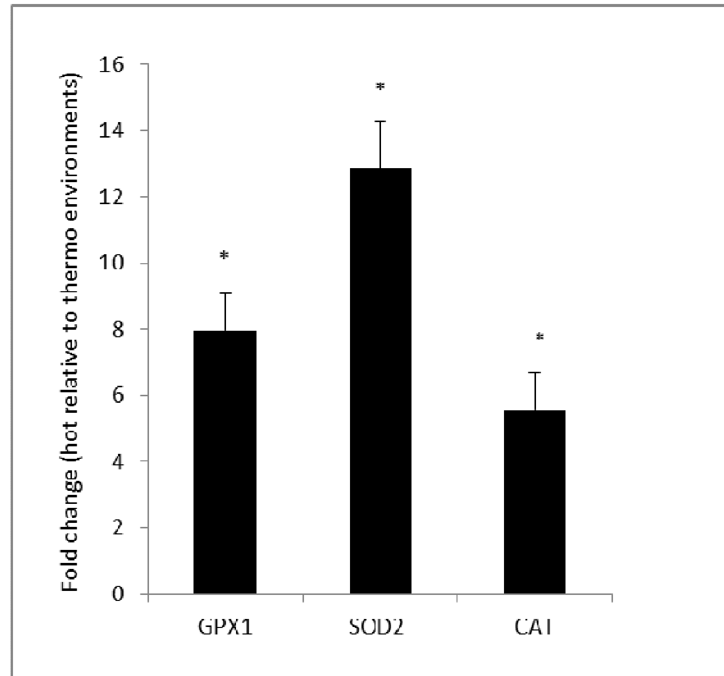

Figure 1. Quantitative RT-PCR results of genes that exhibited differential expression on LD muscle of pigs from growth phase 1 between the thermoneutral and hot environments. Results are presented as fold change for expression at hot environment relative to expression at thermoneutral environment. Bars above the origin indicate higher expression at hot environment. *$P < 0.05$.

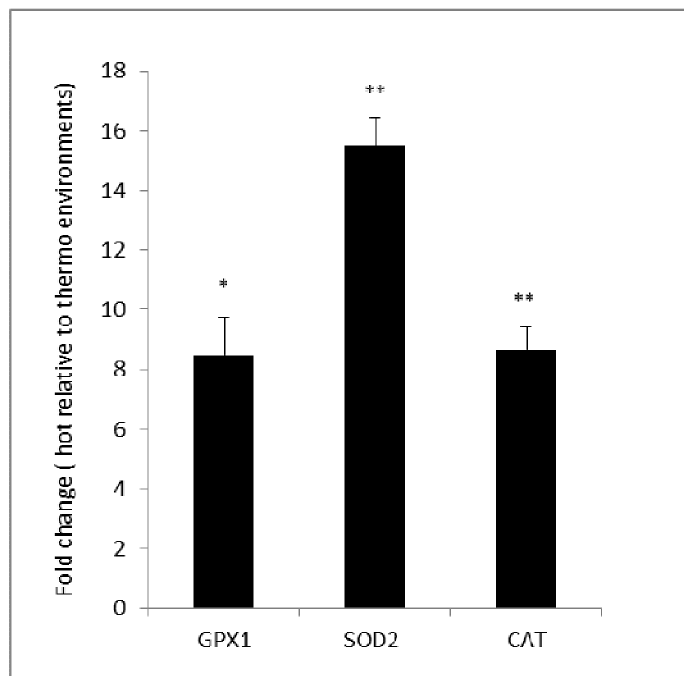

Figure 2. Quantitative RT-PCR results of genes that exhibited differential expression on LD muscle of pigs from growth phase 2 between the thermoneutral and hot environments. Results are presented as fold change for expression at hot environment relative to expression at thermoneutral environment. Bars above the origin indicate higher expression at hot environment. *$P < 0.05$; **$P < 0.01$.

GENERAL CONCLUSION

The present study is the first to reveal the effects of dietary phosphorus levels on gene expression related to oxidative phosphorylation and its implications on animal performance. Thus, was shown that phosphorus is one of the key factors regulating oxidative phosphorylation.

We have also identified that heat stress has a significant effect on mitochondrial functioning, not only causes reduction in the oxidative metabolic capacity, but also stimulate the production of mitochondrial ROS in skeletal muscle of pigs, since it was observed higher expression of antioxidant enzymes in response to heat stress. The mechanisms underlying these changes in response to heat stress is fairly understood.

Our findings could support the hypothesis that an increase of ROS in mitochondria, decreases expression of mitochondrial electron transport chain (ETC) genes, interferes with the cell growth. Thus, it is likely that heat stress inhibits normal growth of pigs, which is confirmed by worse performance under high temperatures.

This information will contribute to better understand the role of phosphorus in energy metabolism and will bring new insights into understanding the changes metabolism and mitochondrial function in response to high temperature.

APPENDIX 1

Supplementary Table 1. Fold change results for each contrast between treatments, including the comparison two-by-two within one factor, available phosphorus levels (aP) or thermal environments (hot and thermoneutral), fixed for each level of the other factor (aP levels or thermo environment) for each target gene in *Longissimus dorsi* of pigs at 30 kg slaughter weight.

Comparison	Estimate	Fold change	Std Err	t-value	p-value
aP:1-2| Environment = thermo| gene: ND1	0.2077	-1.1548	0.7017	0.30	0.7706
aP:1-3| Environment = thermo| gene: ND1	-1.2632	2.4003	0.7017	-1.80	0.0886
aP:2-3| Environment = thermo| gene: ND1	-1.0555	2.0784	0.7017	-1.50	0.0499
aP:1-2| Environment = hot| gene: ND1	0.4654	-1.3807	0.7017	0.66	0.5156
aP:1-3| Environment = hot| gene: ND1	0.8916	-1.8552	0.7017	1.27	0.2200
aP:2-3| Environment = hot| gene: ND1	0.4263	-1.3437	0.7017	0.61	0.5512
thermo-hot| aP = 1| gene: ND1	-2.0579	4.1638	0.7017	-2.93	0.0089
thermo-hot| aP = 2| gene: ND1	-1.3848	2.6114	0.7017	-1.97	0.0420
thermo-hot| aP = 3| gene: ND1	0.0970	-1.0648	0.7017	0.14	0.8916
aP:1-2| Environment = thermo| gene: ND2	0.5898	-1.5050	0.3728	1.58	0.1311
aP:1-3| Environment = thermo| gene: ND2	-0.1990	1.1479	0.3728	-0.53	0.6000
aP:2-3| Environment = thermo| gene: ND2	-0.7880	1.7276	0.3728	-2.12	0.0485
aP:1-2| Environment = hot| gene: ND2	0.4762	-1.3911	0.3728	1.28	0.2177
aP:1-3| Environment = hot| gene: ND2	1.7330	-3.3242	0.3728	4.65	0.0002
aP:2-3| Environment = hot| gene: ND2	1.2567	-2.3895	0.3728	3.37	0.0034
thermo-hot| aP = 1| gene: ND2	-0.2131	1.1592	0.3728	-0.57	0.5746
thermo-hot| aP = 2| gene: ND2	-0.3266	1.2541	0.3728	-0.88	0.3925
thermo-hot| aP = 3| gene: ND2	-1.7189	3.2919	0.3728	-4.61	0.0002
aP:1-2| Environment = thermo| gene: COX1	1.4825	-2.7943	0.799	1.85	0.0802
aP:1-3| Environment = thermo| gene: COX1	0.8775	-1.8372	0.799	1.10	0.2870
aP:2-3| Environment = thermo| gene: COX1	-0.6050	-1.5210	0.799	-0.76	0.4591
aP:1-2| Environment = hot| gene: COX1	1.7687	-3.4075	0.799	2.21	0.0512
aP:1-3| Environment = hot| gene: COX1	0.7313	-1.6601	0.799	0.91	0.3726
aP:2-3| Environment = hot| gene: COX1	1.0375	-2.0527	0.799	1.30	0.2109
thermo-hot| aP= 1| gene: COX1	-1.4287	2.6920	0.799	-1.79	0.0909
thermo-hot| aP= 2| gene: COX1	-1.1425	2.2076	0.799	-1.43	0.1702
thermo-hot| aP= 3| gene: COX1	-1.5750	2.9794	0.799	-1.97	0.0645
aP:1-2| Environment = thermo| gene: COX2	0.9290	-1.9138	1.6658	0.56	0.5839
aP:1-3|Environment = thermo| gene: COX2	-1.6800	3.2042	1.6658	-1.01	0.3266
aP:2-3| Environment = thermo| gene: COX2	-2.6090	6.100	1.6658	-1.57	0.1347
aP:1-2|Environment = hot| gene: COX2	1.2425	-2.3661	1.6658	0.75	0.4654
aP:1-3|Environment = hot| gene: COX2	0.9081	-1.8765	1.6658	0.55	0.5923
aP:2-3| Environment = hot| gene: COX2	0.3344	-1.2608	1.6658	0.20	0.8432

thermo-hot| aP = 1| gene: COX2	-2.3625	5.1426	1.6658	-1.42	0.1732
thermo-hot| aP = 2| gene: COX2	-2.0490	4.1381	1.6658	-1.23	0.2345
thermo-hot| aP = 3| gene: COX2	-0.2256	1.1692	1.6658	-0.14	0.8938
aP:1-2| Environment = thermo| gene: COX3	0.4469	-1.3631	0.9017	0.50	0.6262
aP:1-3| Environment = thermo| gene: COX3	-2.6169	6.1343	0.9017	-2.90	0.0095
aP:2-3| Environment = thermo| gene: COX3	-2.1700	4.5002	0.9017	-2.41	0.0271
aP:1-2| Environment = hot| gene: COX3	1.5089	-2.8459	0.9017	1.67	0.1115
aP:1-3| Environment = hot| gene: COX3	0.8675	-1.8245	0.9017	0.96	0.3488
aP:2-3| Environment = hot| gene: COX3	0.6414	-1.5598	0.9017	0.71	0.4860
thermo-hot| aP = 1| gene: COX3	-3.8444	14.3641	0.9017	-4.26	0.0005
thermo-hot| aP = 2| gene: COX3	-1.8886	3.7028	0.9017	-2.09	0.0430
thermo- hot| aP = 3| gene: COX3	-0.3600	1.2834	0.9017	-0.40	0.6944
aP:1-2| Environment = thermo| gene: CYTB	0.0500	-1.0353	0.8084	0.06	0.9513
aP:1-3| Environment = thermo| gene: CYTB	-1.7192	3.2925	0.8084	-2.13	0.0475
aP:2-3| Environment = thermo| gene: CYTB	-1.7693	3.4089	0.8084	-2.19	0.0420
aP:1-2| Environment = hot| gene: CYTB	0.1337	-1.9070	0.8084	0.17	0.8704
aP:1-3| Environment = hot| gene: CYTB	0.5888	-1.5003	0.8084	0.73	0.4758
aP:2-3| Environment = hot| gene: CYTB	0.4550	-1.3707	0.8084	0.56	0.5805
thermo-hot| aP = 1| gene: CYTB	-5.0300	32.6724	0.8084	-8.81	<0.0001
thermo-hot| aP = 2| gene: CYTB	-5.1263	34.9277	0.8084	-8.70	<0.0001
thermo-hot| aP = 3| gene: CYTB	-4.8120	28.0903	0.8084	-5.95	<0.0001
aP:1-2| Environment =thermo| gene: SDHD	0.2212	-1.1657	1.4266	0.16	0.8785
aP:1-3| Environment =thermo| gene: SDHD	1.5220	-2,8719	1.4266	1.07	0.3001
aP:2-3| Environment =thermo| gene: SDHD	-1.7433	3.3692	1.4266	-1.22	0.2375
aP:1-2| Environment = hot| gene: SDHD	0.6287	-1.5461	1.4266	0.44	0.6647
aP:1-3| Environment = hot| gene: SDHD	1.1950	-2.2890	1.4266	0.84	0.4132
aP:2-3| Environment = hot| gene: SDHD	0.5662	-1.4806	1.4266	0.40	0.6961
thermo-hot| aP = 1| gene: SDHD	-1.7412	3.3431	1.4266	-1.22	0.2380
thermo-hot| aP = 2| gene: SDHD	-0.8913	1.8548	1.4266	-0.62	0.5400
thermo-hot| aP = 3| gene: SDHD	-2.0683	4.1939	1.4266	-1.45	0.1643
aP:1-2| Environment = thermo| gene: ATP5J2	0.4334	-1.3504	0.5004	0.87	0.3979
aP:1-3| Environment =thermo| gene: ATP5J2	-1.9006	3.7337	0.5004	-3.80	0.0013
aP:2-3| Environment = thermo| gene: ATP5J2	-1.4672	2.7648	0.5004	-2.93	0.0089
aP:1-2| Environment = hot| gene: ATP5J2	0.3125	-1.2419	0.5004	0.62	0.5401
aP:1-3| Environment = hot| gene: ATP5J2	0.0012	-1.0008	0.5004	0.01	0.9980
aP:2-3| Environment = hot| gene: ATP5J2	0.3113	-1.2408	0.5004	0.62	0.5418
thermo-hot| aP = 1| gene: ATP5J2	-1.7800	3.4334	0.5004	-3.56	0.0023
thermo-hot| aP = 2| gene: ATP5J2	-1.0341	2.0478	0.5004	-2.01	0.0460
thermo-hot| aP = 3| gene: ATP5J2	-0.1219	1.0881	0.5004	-0.24	0.8103
aP:1-2| Environment = thermo| gene: ATP6	0.2145	-1.1603	0.6218	0.34	0.7341
aP:1-3| Environment = thermo| gene: ATP6	-1.7600	3.3870	0.6218	-2.83	0.0110
aP:2-3| Environment = thermo| gene: ATP6	-1.5455	2.9191	0.6218	-2.49	0.0230
aP:1-2| Environment = hot | gene: ATP6	0.1375	-1.1000	0.6218	0.22	0.8275
aP:1-3| Environment = hot| gene: ATP6	0.1822	-1.1346	0.6218	0.29	0.7728
aP:2-3| Environment = hot | gene: ATP6	0.0445	-1.0315	0.6218	0.07	0.9434
thermo-hot| aP = 1| gene: ATP6	-3.1324	8.7689	0.6218	-5.04	<0.0001
Thermo-hot| aP = 2| gene: ATP6	-2.7804	6.8704	0.6218	-4.47	0.0003

thermo-hot| aP = 3| gene: ATP6	-1.1901	2.2817	0.6218	-1.91	0.0717

thermo= thermoneutral ; hot= hot environment

Negative values of “estimates” indicate a positive fold change (relative expression) which means high expression at the first level relatively to the second level of the comparisons two-by-two within one factor (aP levels or thermal environments) fixed for each level of the other factor (aP levels or thermo environment). Positive values of “estimates” indicate a negative fold change (relative expression) which means lower expression at the first level relatively to the second level of the comparisons two-by-two within one factor (aP levels or thermo environment) fixed for each level of the other factor (aP levels or thermo environment).

Supplementary Table 2. Fold change results for each contrast between treatments, including the comparison two-by-two within one factor, available phosphorus levels (aP) or thermal environments (hot and thermoneutral), fixed for each level of the other factor (aP levels or thermo environment) for each target gene in *Longissimus dorsi* of pigs at 60 kg slaughter weight.

Comparison	Estimate	Fold change	Std Err	t-value	p-value
aP:1-2| Environment = thermo| gene: ND1	0.8286	-1.7760	0.8607	0.96	0.3484
aP:1-3| Environment = thermo| gene: ND1	-0.5319	1.4458	0.8607	-0.62	0.5443
aP:2-3| Environment = thermo| gene: ND1	-0.2968	1.2284	0.8607	-0.34	0.7342
aP:1-2| Environment = hot| gene: ND1	-0.2402	1.1812	0.8607	-0.28	0.7833
aP:1-3| Environment = hot| gene: ND1	0.1574	-1.1153	0.8607	0.18	0.8570
aP:2-3| Environment = hot| gene: ND1	-0.3976	-1.3173	0.8607	-0.46	0.6496
thermo-hot| aP = 1| gene: ND1	-0.5591	1.4733	0.8607	-0.65	0.5241
thermo-hot| aP = 2| gene: ND1	0.0293	-1.0205	0.8607	0.03	0.9733
thermo-hot| aP = 3| gene: ND1	0.1301	-1.0944	0.8607	0.15	0.8815
aP:1-2| Environment = thermo| gene: ND2	0.7254	-1.6533	0.5397	1.34	0.1956
aP:1-3| Environment = thermo| gene: ND2	0.5831	-1.4981	0.5397	1.08	0.2942
aP:2-3| Environment = thermo gene: ND2	-0.1422	1.1036	0.5397	-0.26	0.7951
aP:1-2| Environment = hot| gene: ND2	1.4015	-2.6418	0.5397	2.60	0.0182
aP:1-3| Environment = hot| gene: ND2	0.3333	-1.2599	0.5397	0.62	0.5446
aP:2-3| Environment = hot| gene: ND2	-1.7347	3.3281	0.5397	-3.21	0.0048
thermo-hot| aP = 1| gene: ND2	-2.0421	4.1184	0.5397	-3.78	0.0014
thermo-hot |aP = 2| gene: ND2	-4.1690	17.988	0.5397	-7.73	<0.0001
thermo-hot |aP = 3| gene: ND2	-2.2920	4.8939	0.5397	-4.25	0.0005
aP:1-2| Environment = thermo| gene: COX1	2.2575	-4.7816	1.1396	1.98	0.0631
aP:1-3| Environment = thermo| gene: COX1	1.2250	-2.3376	1.1396	1.07	0.2966
aP:2-3| Environment = thermo| gene: COX1	-1.0325	2.0456	1.1396	-0.91	0.3769
aP:1-2| Environment = hot| gene: COX1	1.4087	-2.6550	1.1396	1.24	0.2323
aP:1-3| Environment = hot| gene: COX1	0.6050	-1.5210	1.1396	0.53	0.6020
aP:2-3| Environment = hot| gene: COX1	-2.0137	4.0382	1.1396	-1.77	0.0942
thermo-hot| aP = 1| gene: COX1	-0.3063	1.236	1.1396	-0.27	0.7912
thermo-hot| aP = 2| gene: COX1	-3.9725	15.697	1.1396	-3.49	0.0026
thermo-hot| aP = 3| gene: COX1	-0.9063	1.8742	1.1396	-0.81	0.4269
aP:1-2| Environment = thermo| gene: COX2	0.8513	-1.8041	0.6310	1.35	0.1941
aP:1-3| Environment = thermo| gene: COX2	-0.3075	1.2376	0.6310	-0.49	0.6319
aP:2-3| Environment = thermo| gene: COX2	-1.1588	2.2327	0.6310	-1.84	0.0829
aP:1-2| Environment = hot| gene: COX2	0.5912	-1.5065	0.6310	0.94	0.3612
aP:1-3| Environment = hot| gene: COX2	0.5037	-1.4178	0.6310	0.80	0.4351
aP:2-3| Environment = hot| gene: COX2	-0.0875	-1.0625	0.6310	-0.14	0.8913
thermo-hot| aP = 1| gene: COX2	-2.7012	6.5034	0.6310	-4.28	0.0002
thermo-hot| aP = 2| gene: COX2	-2.9613	7.7883	0.6310	-4.69	0.0078
thermo-hot| aP = 3| gene: COX2	-1.8900	3.7064	0.6310	-3.00	0.0005
aP:1-2| Environment = thermo| gene: COX3	1.3175	-2.4923	0.5959	2.21	0.0402
aP:1-3| Environment = thermo| gene: COX3	0.0525	-1.0372	0.5959	0.09	0.9308

aP:2-3| Environment = thermo| gene: COX3	-1.2650	2.4033	0.5959	-2.12	0.0479
aP:1-2| Environment = hot| gene: COX3	0.8345	-1.7832	0.5959	1.40	0.1784
aP:1-3| Environment = hot| gene: COX3	0.9807	-1.9734	0.5959	1.65	0.1171
aP:2-3| Environment = hot| gene: COX3	-0.1463	1.1067	0.5959	-0.25	0.8089
thermo-hot| aP = 1| gene: COX3	2.5382	5.8086	0.5959	-4.26	0.0005
thermo-hot| aP = 2| gene: COX3	-3.0213	8.1189	0.5959	-5.07	<0.0001
thermo-hot| aP = 3| gene: COX3	-1.6100	3.0525	0.5959	-2.70	0.0146
aP:1-2| Environment = thermo| gene: CYTB	2.0312	-4.0875	1.2215	1.66	0.1136
aP:1-3| Environment = thermo| gene: CYTB	1.4725	-2.7750	1.2215	1.21	0.2436
aP:2-3| Environment = thermo| gene: CYTB	-0.5587	1.4729	1.2215	-0.46	0.6528
aP:1-2| Environment = hot| gene: CYTB	1.3887	-2.6184	1.2215	1.14	0.2705
aP:1-3| Environment = hot| gene: CYTB	1.8888	-3.7033	1.2215	1.55	0.1394
aP:2-3| Environment = hot| gene: CYTB	-0.5000	1.4142	1.2215	-0.41	0.6871
thermo-hot| aP = 1| gene: CYTB	-4.4212	21.4247	1.2215	-3.62	0.0020
thermo-hot| aP = 2| gene: CYTB	-5.0637	33.4446	1.2215	-4.15	0.0006
thermo-hot| aP = 3| gene: CYTB	-4.0050	16.0555	1.2215	3.28	0.0042
aP:1-2| Environment =thermo| gene: SDHD	-0.4562	1.3719	0.6863	-0.66	0.5146
aP:1-3| Environment =thermo| gene: SDHD	-0.3663	1.2890	0.6863	-0.53	0.6001
aP:2-3| Environment =thermo| gene: SDHD	0.0900	-1.0644	0.6863	0.13	0.8971
aP:1-2| Environment =hot| gene: SDHD	0.5625	-1.4768	0.6863	0.82	0.4231
aP:1-3| Environment = hot| gene: SDHD	1.7238	-3.3031	0.6863	2.51	0.0518
aP:2-3| Environment = hot| gene: SDHD	-1.1613	2.366	0.6863	-1.69	0.1079
thermo-hot| aP = 1| gene: SDHD	-0.4475	1.3637	0.6863	-0.65	0.5226
thermo-hot| aP = 2| gene: SDHD	-0.5713	1.4859	0.6863	-0.83	0.4161
thermo- hot| aP = 3| gene: SDHD	-1.6425	3.1221	0.6863	-2.39	0.0278
aP:1-2| Environment = thermo gene: ATP5J2	1.1018	-2.1462	0.5982	1.84	0.0820
aP:1-3| Environment = thermo| gene: ATP5J2	-0.6176	1.5343	0.5982	-1.03	0.3155
aP:2-3| Environment = thermo| gene: ATP5J2	-0.4841	1.3987	0.5982	-0.81	0.4289
aP:1-2| Environment = hot| gene: ATP5J2	0.5040	-1.4181	0.5982	0.84	0.4105
aP:1-3| Environment = hot| gene: ATP5J2	0.7250	-1.6529	0.5982	1.21	0.2412
aP:2-3| Environment = hot| gene: ATP5J2	-0.2210	1.1655	0.5982	0.37	0.7161
thermo-hot| aP = 1| gene: ATP5J2	-1.2625	2.3991	0.5982	-2.11	0.0491
thermo-hot| aP = 2| gene: ATP5J2	-0.3432	1.2686	0.5982	-0.57	0.5732
thermo-hot| aP = 3| gene: ATP5J2	-0.08012	1.0571	0.5982	-0.13	0.8949
aP:1-2| Environment = thermo| gene:ATP6	0.6514	-1.5707	0.7479	0.87	0.3953
aP:1-3| Environment = thermo| gene: ATP6	-0.2105	1.1571	0.7479	-0.28	0.7816
aP:2-3| Environment = thermo| gene: ATP6	-0.4409	1.3575	0.7479	-0.59	0.5629
aP:1-2| Environment = hot| gene: ATP6	0.7330	-1.6621	0.7479	0.98	0.3401
aP:1-3| Environment = hot| gene: ATP6	0.0973	-1.0697	0.7479	0.13	0.8980
aP:2-3| Environment = hot| gene: ATP6	-0.6358	1.5538	0.7479	-0.85	0.4065
thermo-hot| aP = 1| gene: ATP6	-3.3613	10.2767	0.7479	-4.49	0.0003
thermo-hot| aP = 2| gene: ATP6	-1.9769	3.9365	0.7479	-2.64	0.0165
thermo-hot| aP = 3| gene: ATP6	-3.0535	8.3022	0.7479	-4.08	0.0007

thermo= thermoneutral ; hot= hot environment

Negative values of “estimates” indicate a positive fold change (relative expression) which means high expression at the first level relatively to the second level of the

comparisons two-by-two within one factor (aP levels or thermal environments) fixed for each level of the other factor (aP levels or thermo environment). Positive values of "estimates" indicate a negative fold change (relative expression) which means lower expression at the first level relatively to the second level of the comparisons two-by-two within one factor (aP levels or thermo environment) fixed for each level of the other factor (aP levels or thermo environment).

APPENDIX 2

Supplementary Table 1. Fold change results for each contrast between thermal environment for each target gene in *Longissimus dorsi* of pigs at 30 kg slaughter weight.

Comparison	Estimate	Fold change	Std Err	t-value	*P*-value
Environment: thermo- hotl gene: GPX1	2.991	-7.9508	1.110	3.66	0.003
Environment: thermo- hotl gene: SOD2	3.683	-12.856	1.328	3.56	0.002
Environment: thermo- hotl gene: CAT	2.472	-5.5492	1.132	2.34	0.042

Supplementary Table 2. Fold change results for each contrast between thermal environment for each target gene in *Longissimus dorsi* of pigs at 60 kg slaughter weight.

Comparison	Estimate	Fold change	Std Err	t-value	*P*-value
Environment: thermo- hotl gene: GPX1	3.087	-8.4984	1.25	3.49	0.049
Environment: thermo- hotl gene: SOD2	3.950	-15.4941	0.957	4.83	0.005
Environment: thermo- hotl gene: CAT	3.112	-8.6464	0.764	4.16	0.004