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GENETIC BASIS OF PHYSIOLOGICAL STRESS RESPONSE TO SLAUGHTER IN AVILEÑA-NEGRA IBÉRICA SPANISH BREED

Jaqueline Oliveira Rosa
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DADOS CURRICULARES DO AUTOR

JAQUELINE OLIVEIRA ROSA – nascida em 09 de julho de 1989, na cidade de Ribeirão Preto, São Paulo, filha de Ricardo Ribeiro Rosa e Eliana de Souza Oliveira Rosa. Iniciou o curso de Ciências Biológicas na Faculdade de Ciência Agrárias e Veterinárias, UNESP, Campus de Jaboticabal em março de 2009. Durante o ano de 2012 desenvolveu trabalho de Iniciação Científica com bolsa de iniciação científica PIBIC na mesma instituição de ensino, sob orientação do Prof. Dr. Danísio Prado Munari. Em 2012 obteve o título de Bacharel em Ciências Biológicas. Em março de 2013, ingressou no curso de mestrado pelo Programa de Pós-Graduação em Genética e Melhoramento Animal na Faculdade de Ciência Agrárias e Veterinárias, UNESP, Campus de Jaboticabal sob orientação da Profa. Dra. Vera Fernanda Martins Hossepian de Lima e coorientação do Prof. Dr. Danísio Prado Munari, Dra. Mônica Corrêa Ledur e Dra. Valdecy Aparecida Rocha da Cruz. Foi bolsista CAPES de mestrado pelo período de março de 2013 a fevereiro de 2015. Em março de 2015, ingressou no curso de doutorado pelo Programa de Pós-Graduação em Genética e Melhoramento Animal na Faculdade de Ciência Agrárias e Veterinárias, UNESP, Campus de Jaboticabal sob orientação do Prof. Dr. Danísio Prado Munari e coorientação da Dra. Clara Díaz Martin. Realizou estágio sanduíche com bolsa estágio do programa de doutorado sanduíche no exterior (PDSE) no Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria no período de março de 2017 a fevereiro de 2018 sob supervisão da Dra. Clara Díaz Martin. Foi bolsista CAPES de março de 2015 a fevereiro de 2019.

Inteligência é a habilidade
de se adaptar às mudanças.
(Stephen Hawking)

Dedico

Aos meus pais Ricardo e Eliana,

Ao meu irmão Alisson e avós,

Sinônimos de amor, respeito, união e dedicação

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GENETIC BASIS OF PHYSIOLOGICAL STRESS RESPONSE IN AVILEÑA-NEGRA IBÉRICA SPANISH BREED

ABSTRACT- In beef production, animals are exposed to slaughter conditions that induce different responses to stress and these differences may be are genetics. Therefore, this study aimed: (1) to characterize the pattern of response of heat shock protein genes (HSP) between *Psoas major* (PM) and *Flexor digitorum superficialis* (FD) in response to stress at slaughter in two different seasons (summary and winter). This was so to determine if response of HSP to the stress of slaughter was conditioned on muscle type and thermal stress; (2) (a) to characterize the physiological response to stress generating by handling the animals at two different periods in time, feedlot (F) and slaughter (S) with a set of biomarkers; (b) to identify a subset of the biomarkers of stress that best discriminate between these two stress periods and to evaluate if the early response in F could be used to anticipate response at S, and (c) find evidences of a genetic component in the physiological response to stress in beef cattle. Estimate expression differences between FD and PM for eight heat shock protein genes were used in HSPs study. Results showed that both muscles seem to have similar patterns in the expression of HSPs at the thermal stimuli except the HSPB6 and HSPB8 that seemed to show a larger level of expression in PM muscles of animals slaughtered at summer than those that were sampled in the winter slaughter. The susceptibility and, therefore, the response to thermal stress seems to be differentially mediated by specific families of chaperones as a function of the role of the specific tissue in the restoration of protein homeostasis. For stress biomarkers study blood samples from eighty Avileña-Negra Iberica male calves were collected in the feedlot and at the slaughterhouse. Biomarkers, albumin, cortisol, creatine phosphokinase (CK), glucose, lactate, lactate dehydrogenase (LDH) and globulin were determined in both periods. The stress response in feedlot appeared to be associated with mechanisms that generate an organic response at the hepatic level. Lactate and cortisol indicated that the response to stress at slaughter occur at muscle level. Lactate, glucose and albumin best discriminate the two different stress periods. Therefore, there are other four biomarkers (cortisol, CK, LDH and globulin) that could be used as global set of physiological response to stress regardless the source of stress. The same set of markers to some extent also discriminates between farms of origin. The miss-matching occurred mostly between herds with common genetic background.

Keyowords: Biomarker, heat stress, RNA-Seq, skeletal muscle, stress response, welfare

BASE GENÉTICA DA RESPOSTA FISIOLÓGICA AO ESTRESSE NA RAÇA ESPANHOLA AVILEÑA-NEGRA IBÉRICA

RESUMO- Na produção de carne bovina, os animais são expostos a condições de abate que induzem diferentes respostas ao estresse e essas diferenças podem ser genéticas. Os objetivos deste estudo foram: (1) caracterizar o padrão de resposta de genes de proteínas de choque térmico (HSP) entre *Psoas major* (PM) e *Flexor digitorum superficialis* (FD) em resposta ao estresse ao abate em duas estações diferentes (verão e inverno). Isso foi feito para determinar se a resposta das HSPs ao estresse ao abate estava condicionada ao tipo muscular e ao estresse térmico; (2) (a) caracterizar a resposta fisiológica ao estresse gerado pelo manejo dos animais em dois períodos distintos no tempo, manejo em confinamento (F) e manejo ao abate (S) com um conjunto de biomarcadores; (b) identificar um subgrupo dos biomarcadores de estresse que melhor discriminam entre os dois períodos e avaliar se a resposta inicial em F poderia ser usada para antecipar a resposta em S, e (c) encontrar evidências de um componente genético na resposta fisiológica ao estresse em bovinos de corte. Estimativas das diferenças de expressão entre FD e PM para oito genes de proteínas de choque térmico foram usadas neste estudo. Os resultados mostraram que ambos os músculos parecem ter padrões semelhantes na expressão de HSPs sob estímulos térmicos, exceto HSPB6 e HSPB8, que mostraram maior nível de expressão no músculo PM de animais abatidos no verão em relação aos mesmos abatidos no inverno. A suscetibilidade e, portanto, a resposta ao estresse térmico parece ser diferencialmente mediada por famílias específicas de chaperonas em função do papel específico do tecido na restauração da homeostase proteica. No estudo de biomarcadores de estresse, foram colhidas amostras de sangue de oitenta bezerros machos da raça Avileña-Negra Iberica, no confinamento e no abatedouro. Biomarcadores, albumina, cortisol, creatinafosfoquinase (CK), glicose, lactato, lactato desidrogenase (LDH) e globulina foram determinados em ambos os períodos. A resposta ao estresse em confinamento parece estar associada a mecanismos que geram uma resposta orgânica a nível hepático. Lactato e cortisol indicaram que a resposta ao estresse ao abate ocorre a nível muscular. Lactato, glicose e albumina foram os biomarcadores que melhor discriminaram entre os dois diferentes períodos de estresse. Entretanto, existem outros quatro biomarcadores (cortisol, CK, LDH e globulina) que podem ser usados como um conjunto global de respostas fisiológicas ao estresse, independentemente da fonte estressora. O mesmo conjunto de biomarcadores também discriminou entre as fazendas de origem dos animais e os indivíduos que não foram corretamente discriminados às fazendas pelos biomarcadores eram de rebanhos com ancestrais comuns.

Palavras-chave: Bem-estar, biomarcadores, estresse térmico, músculo esquelético, RNA-Seq, resposta ao estresse,

LIST OF ABBREVIATIONS

PM: *Psoas major*

FD: *Flexor digitorum superficialis*

DE: Differential expression.

DEGs: Differentially expressed genes

FDR: False discovery rate

TPR: True positive rate

ROC: Receiver operator characteristic

GO: Gene Ontology

F: Feedlot handling

S: Slaughter handling

ANI: Avileña-Negra Iberica

HPA: Hipothalamic-pituitary-adrenocortical

CRH: Corticotrophin releasing hormone

CNS: Central nervous system

AVP: Arginine vasopressin

ACTH: Adrenocorticotropic hormone

CPK: Creatine phosphokinase

LDH: Lactate dehydrogenase

HSP: Heat shock proteins

TGF-β2: Transforming growth factor β2

DFD: Dark cutting

PSE: Pale soft exudative

Cp: Crossing point

PCs: Principal components

PCA: Principal Component Analysis

LDA: Linear Discriminant Analysis

Dim: Dimension values

ctr: Contribution

TCTR: Total contribution

CHAPTER 1 – General Considerations

1. INTRODUCTION

The global commercialization of beef, and its products and an increasing demand for animal products (meat, milk, and eggs) have been resulted in a considerable increase of the number of the animals raised, transported and slaughtered around the world, which has sharpened welfare problems at various points in the beef supply chain (Blokhuis et al., 2008; Rostagno, 2009). One of the main objectives of livestock industry is to ensure meat safety and quality at the consumer level (Miranda De La Lama, 2013). Stress and the psychological and physiological responses to traditional husbandry procedures have become a central issue in the discussion between consumers and producers on animal welfare (Lyles et al., 2014). All concerns comprise food safety, animal health, management practices and the sustainability of cattle production.

Along the beef production chain, animals are exposed to a series of factors and procedures such as changes in weather, presence of humans, transport conditions, handling practices, the pre-slaughter waiting period, the stunning procedure, and the slaughter method that may result in physiological and behavioral stress responses (Grandin, 1997; Apple et al., 2005; Terlouw, 2005). The magnitude of any negative effect is a function of the type of stressor, duration and intensity of the individual stressors, beef production system and susceptibility of each animal to stress (Ferguson and Warner, 2008; Terlouw et al., 2008). Such susceptibility depends to a great extent on the animal's genetic background, therefore genetic selection may be used as a tool to improve animal´s resilience to stress (Haskel et al., 2014)

In general, it is known that the most reactive animals are more predisposed to stress, making it difficult to handle and predisposing to increased aggressiveness (Macedo et al., 2004). The high reactivity affects negatively almost all traits of economic importance to the beef production system. Animals that express higher reactivity have lower weight gains (Voisinet et al., 1997a; Petherick et al., 2002; Del Campo et al., 2010; Sebastian et al., 2011), worse reproductive performance (Cooke,

2011; Cooke et al., 2017), increase the incidence of pathologies (Friedrich et al., 2016) and lower carcass yields and meat quality, with greater occurrence of dark cuts (Voisinet et al., 1997b; Paranhos Da Costa, 2000; King et al., 2006; Behrends et al., 2009; Del Campo et al., 2010; Cafe et al., 2011; Hall et al., 2011).

Stress causes important changes in the metabolism of the animals, affecting the biochemical processes of transformation of muscle in meat and, consequently, the final quality of the product (Ferguson and Warner, 2008). Moreno-Sanchez et al. (2008) studied two different muscles, *Flexor digitorum superficialis*, *membri thoraci* (FD) and *Psoas major* (PM), known as fore shank and filet mignon, respectively, in samples of Avileña Negra-Ibérica calves after slaughter. These two muscles were chosen because they were the anatomical basis of two meat cuts with a different meat quality and market values. PM muscle have more intramuscular fat content and are more tender than FD (Diaz et al., 2006). The same muscles also have different anatomical locations and potentially distinctive metabolism. FD is a limb muscle while PM is a trunk muscle.

According to Moreno-Sanchez et al. (2008), FD have a larger composition of oxidative fibers (type I) and lacked pure glycolytic (type IIX) and a pure oxidative metabolism whereas PM have a large proportion of glycolytic fibers and a high metabolic profile. Moreno-Sanchez et al. (2010, 2012) also studied with microarrays of cDNA the differential expression (DE) of genes between these two muscles sampling at slaughter Avileña Negra-Ibérica calves that were fattened under similar conditions. DE genes were accordingly muscle characteristics.

Moreno-Sanchez et al. (2012) results suggested a differential ability of muscles type to react to the same stressing factor such as slaughter as indicated by the DE observed in a number of heat-shock proteins as well as ribosomal DNA that had been described as co-chaperones factors in humans. However, in that study the level of physiological stress suffered by animals at slaughter was not taken into account in the experimental design and the number of contings of chaperones present in the non-commercial cDNA microarray was unknown. Therefore, the aims of this study were to:

1. Characterize the pattern of response of heat shock protein genes in PM and FD in response to stress at slaughter in two different seasons (summer and winter).

In order to determine if response of HSP to the stress of slaughter was conditioned on muscle type and thermal stress

2. (a) Characterize the physiological response to stress generated by handling the animals at two different periods in time, feedlot (F) and slaughter (S) with a set of biomarkers; (b) to identify a subset of the biomarkers of stress that best discriminate between these two stress periods and to evaluate if the early response in F could be used to anticipate response at S, and (c) find evidences of a genetic component in the physiological response to stress in beef cattle.

2. LITERATURE REVIEW

2.1. Avileña Negra-Ibérica spanish breed (ANI)

Avileña Negra-Ibérica breed is one of the major local breeds in Spain. They produce a meat very well-known by the Spanish consumers with a brand named “Carne de Avila”. The cow-calf production systems are under extensive conditions until weaning when male calves which are not selected to become sires of the next generations, will be taken to the feedlot. ANI farmers are organized in an integrated system where farmers own common facilities, to produce and commercialize the meat product straight to consumers.

The animals of that breed have uniform black color throughout the animal. The ANI animals coping behavior is sometimes no easy to handle. Coping behavior is a response to aversive situations. Though farmers have traditionally assumed this “temperament” as a trait of the breed. There is an increasing the concern among farmers of what is the impact of having very reactive animals on the productivity of their production systems as well as the difficulties of handling such type of animals. Therefore, animals of this breed are a good model to accomplish a research topic on the genetic background of response to stress.

2.2. Definition of stress and stressors in animals

Cannon (1914, 1932) and Selye (1956, 1974) are considered as the pioneers of stress research. Selye expanded the understanding of the stress response by indentifying the role of the hipothalamic-pituitary-adrenocortical (HPA) axis, and the secretion of cortisol. According to Selye (1974), the term stress is the organism´s response to a stressor that included physiological and cognitive components. Cannon focused on the sympathetic nervous system and the role of the hormones, epinephrine and noradrenaline, and described the “fight or flight” response that creates a mobilization of energy (increased heart rate, blood pressure, blood sugar) which potentiates physical action.

The term stress is characterized by abnormal or extreme adjustment in the physiology of the animal to cope with adverse changes in its environment and management (Fraser et al., 1975). A stressor can be defined as any internal or external *stimuli* or threat that disrupts homeostasis of the body and elicits a coordinated physiological response in an attempt to reestablish homeostasis (Asres and Amha, 2014).

In general, stressors can be grouped into three categories: psychological stressors, based on a learned response to the threat of an impending adverse condition (e.g., fear, anxiety, and exposure to a novel environment); stressors that consist of a physical *stimulus* and have a strong psychological component (e.g., pain, shock, and immobilization); and stressors that challenge cardiovascular homeostasis (e.g., haemorrhage, exercise, and heat exposure). Whether a stressor can be considered harmful depends on how an organism is able to cope with a threatening situation as it maintains a state of homeostasis (Van De Kar and Blair, 1999).

2.3. Biological response to stress

The response to stress has two major axis; hypothalamic-pituitary-adrenal (HPA) axis initiates the endocrine response to stress, mediated by the release of glucocorticoids from the adrenal cortex. It is initiated at the level of the hypothalamus which releases corticotrophin releasing hormone (CRH) and vasopressin. The second axis of this response is sympathetic-adrenal-medullary (SAM) axis, which is very rapid

and culminates in the release of catecholamines from the adrenal medulla (Chen et al., 2015).

A comprehensive biological model (Figure 1) outlining the general stages involved in the stress response in animals is divided into three general stages (Moberg, 1996; Moberg, 2000): recognition (perception) of a stressor, biological defense against the stressor, and, consequence of the stress response. Recognition of a stressor occurs within the central nervous system (CNS), which organizes an adaptive defense encompassing behavioral, physiological (autonomic, metabolic, neuroendocrine) and immunological responses used by the animal in its attempts to cope with the stressor. The final stage of this response determines whether an individual is suffering from poor welfare or merely experiencing a brief episode that will have no impact on its welfare. Failure to regain homeostasis results in altered biological function that can lead to a pre-pathological state, such as immunosuppression or metabolic disorder or pathology and/or death (Moberg, 2000).

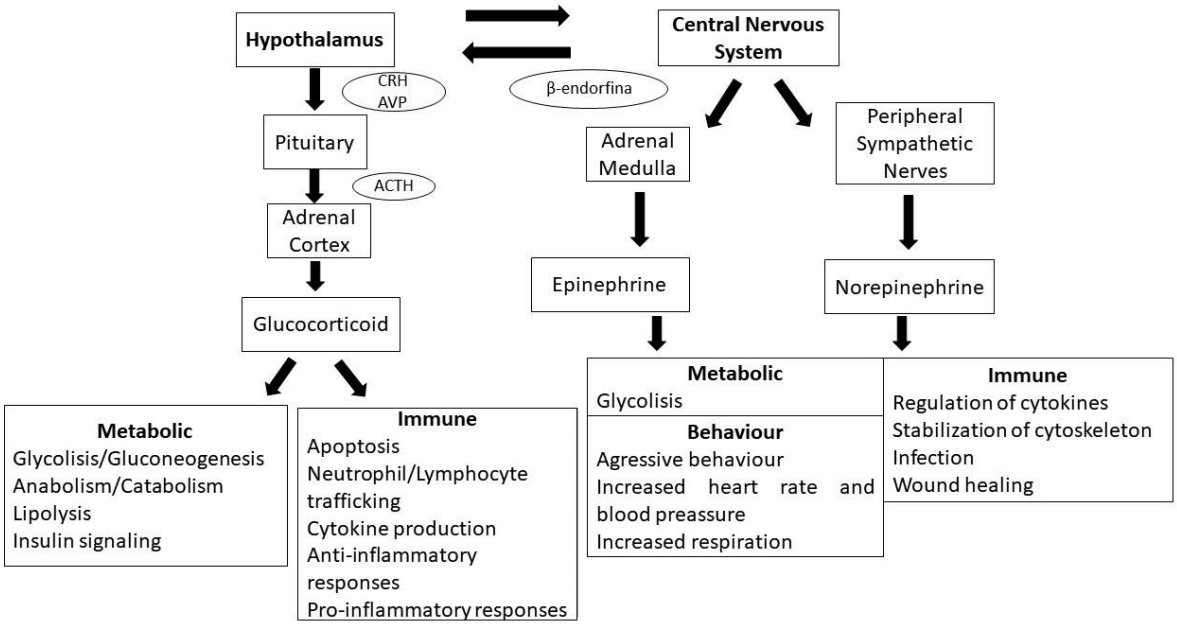


Figure 1. Biological functions regulated by the two stress axes (hipothalamic-pituitary-adrenocortical and sympathetic-adrenal-medullary) adapted from Chen et al. (2015). The hypothalamus-pituitary-adrenal (HPA) axis is activated when the body perceives a physical or psychological stressor. Corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) release from the hypothalamus results in adrenocorticotropic hormone (ACTH) secretion by the pituitary. ACTH then stimulates release of glucocorticoids from the adrenal cortex.

2.4. Stress and temperament

In animals, temperament is defined as the reactivity, or fear response to humans or novel environments (Fordyce et al., 1988). Temperament comprises a number of behavioral traits including shyness-boldness, exploration avoidance, reactivity, sociability and aggressiveness. It is an important aspect of behavioral genetics linked to fitness and to several other traits of importance to ecology and animal evolution (Réale et al., 2007).

The possible genetic background of temperament and its potential impact on cattle welfare and production traits have created the opportunity of using genetic selection to drive temperament to optimize production needs (Friederich et al., 2015). Temperament differs among beef cattle gender, breeds, strains and age (Gauly et al., 2001; Voisinet et al., 1997a; Voisinet et al., 1997b; Lanier et al., 2001; Hoppe et al., 2010). The heritability estimate of temperament has been obtained in several breeds and results ranging across breeds (Morris et al., 1994; Le Neindre et al., 1995; Burrow and Dillon, 1997; Burrow and Corbet, 2000; Gauly et al., 2001; Hoppe et al., 2010; Haskel et al., 2014). In Holstein cows, estimates for milking temperament ranged from 0.11 to 0.17 (Lawstuen et al., 1988; Visscher and Goddard, 1995; Rupp and Boichard, 1999; Schrooten et al., 2000). In Canadian Holstein cattle, heritability reached values of 0.13 and 0.25 for milking temperament and milking speed (Sewalem et al., 2011).

Individuals with a calm temperament may adapt more easily and become less stressed with repeated handling treatments, however individuals with a very excitable temperament may become increasingly stressed (Grandin, 1997), thus they are more difficult to handle, have carcasses with greater incidence of injuries in their carcass (Mcnally and Warriss, 1996), inferior meat quality traits (Voisinet et al., 1997a; Ferguson et al., 2006; Vann, 2006), and a reduction in growth rates, reproduction, and immune functions reduced (Voisinet et al., 1997a; Cooke et al., 2009; Burdick et al., 2011). Excitable temperament is observed more frequently in *B. taurus indicus* compared with *B. taurus taurus* cattle (Hearnshaw and Morris, 1984; Fordyce et al., 1988). Docility resulted in up to 0.19 kg higher average daily weight gains in *B.*

taurus steers (Voisinet et al., 1997a). Calm animals were observed to have higher postmortem pH values (King et al., 2006) and more tender meat (Hall et al., 2011).

There are several methods being developed and used to assess beef cattle temperament, both subjective and objective in nature, with varying advantages and disadvantages (Jones, 2013). Burrow et al. (2000), suggested that, instead of subjective measures, flight speed should be used as the test of choice to assess temperament because it is quantifiable and objective. Prayaga (2003) used chute exit speed and showed that there were significant differences in the exit speed of different breeds. To eliminate the subjective factor in rating temperament, Stookey et al. (1994) developed a movement-measuring device (MMD) to objectively quantify movement.

There is a complexity of behavioral traits and there is no single objective measurement that is able to capture all characteristics of temperament (Boissy et al., 2005) several studies have also included physiological and endocrinological measurements to evaluate, for example, the activity of the hypothalamic-pituitary-adrenal axis (eg. cortisol) and sympatho-adrenal medullary system (eg. epinephrine, heart rate) which are well known systems in the stress response. According to Burdick et al. (2011), the concomitant response of cortisol, epinephrine, and associated increase in heart rate, body temperature, and metabolic processes could provide biomarkers of animal's temperament. The most important prerequisite to identify genetic loci affecting temperament is the development of distinct informative and reproducible phenotypes characterizing different temperament types (Friedrich et al., 2015).

The genetic impact on behaviour results from a complex response network of neurophysiological and structural factors, like hormones and proteins, themselves products of indirect genetic effects (Johnston and Edwards, 2002). A frequently investigated physiological pathway with a high inter-individual variability that can modulate behavioural characteristics is the stress response mediated through the HPA axis. HPA axis activity and aggressive behaviour were recently reported to be associated with two single nucleotide poly-morphisms (SNPs) in pigs (Muráni et al., 2010). A detailed investigation of the genetic correlation between behaviour and HPA axis parameters could be a valuable approach to identify relevant pathways and physiological responses resulting from the genetic predisposition of temperament.

2.5. Animal welfare

Animal welfare is a complex and multidimensional concept (Mason and Mendl, 1993; Fraser, 1995). In 1979, the UK Farm Animal Welfare Council (FAWC) first presented the widely accepted “five freedoms” for animals: (1) freedom from hunger and thirst, (2) freedom from discomfort, (3) freedom from pain, injury, or disease, (4) freedom to express normal behavior, and (5) freedom from fear and distress (http://webarchive.nationalarchives.gov.uk/20121010012427/http://www.fawc.org.uk/freedoms.htm).

Fraser (2008) unified the different animal welfare concepts in a three-pillar comprising from an ethical and a scientific viewpoint: animal health, emotions (affective states), and behavior (natural living). The public interest in animal welfare, sustainable, high quality and safe food have been increasing in most countries of the world (Broom, 2011; Chulayo and Muchenje, 2015; Grunert et al., 2018). Factors affecting the status of welfare of slaughter animals begin at the farm and occur during transportation and at the slaughterhouse (Chulayo and Muchenje, 2015).

The activities that animals pass through before slaughter not only affect the welfare of the animals, but also has an impact on the quality of meat produced from animals of different species (Ferguson and Warner, 2008; Muchenje et al., 2009a; Muchenje et al., 2009b; Hemsworth et al., 2011; Cetin et al., 2012). According to Grandin (1995), animal welfare is an issue that the meat industry must take very seriously because a good welfare reduces damage to carcasses by reducing bruises and injuries; helps preserve meat quality by reducing dark cutting (DFD) and pale soft exudative (PSE) meat; improve employee safety by reducing accidents during stunning and handling; reduce labor requirements because the animals will move through the pens, races and restrainer more easily; reduces costly line stoppages which are caused by delays during stunning or handling and improve the public’s perception of the meat industry and serve as a marketing tool.

2.6. Biomarkers of stress

Biomarkers are molecular components of biological processes regulating phenotypes of animals and derived from improved knowledge of the biological

mechanism underlying economically interest and complex phenotypic traits (Te Pas et al., 2017). Amtmann et al. (2006) report that some indicators such as cortisol, globulin, total protein, plasma albumin, activity of creatine phosphokinase (CK), haptoglobin, fibrinogen, and the leukocytes are used as biomarker of stress, especially when comparing previous values and after a certain management that induces stress.

Cortisol is the primary glucocorticoid released from the adrenal cortex in cattle and distributed via the circulatory system to various target tissues, organs or systems in the body (Burdick et al., 2011). Abnormal cortisol concentrations may result from *stimuli* such as fear, hipoglycemia, fever, trauma, shock, and despair. Plasma measures, such as creatine phosphokinase (CK), lactate dehydrogenase (LDH) and cortisol, are reported to change in response to stressors (Helmreich et al., 2006, Jarvis et al., 2006). Physiological functions of cortisol include control of carbohydrate metabolism, electrolyte homeostasis, water circulation, anti-inflammatory and immunosuppressant processes.

Cortisol is associated with various others physiological and immunologic activities (Keevil, 2016), and vary widely between individual animals (Ray et al., 1972). Assessment of blood cortisol concentration is used to monitor impacts of farming practices on animal health and welfare (Raff and Singh, 2012). Besides that, cortisol concentration in hair from the tail switch has been identified as biomarker of chronic stress in cattle (Burnett et al., 2014; Marti et al., 2015; Moya et al., 2015).

Albumin is the most commonly found protein in the body, comprising 35% to 50% of total serum protein in animals (Kaneko, 1997). This protein plays an important role in maintaining homeostasis, in transport of substances, and acts as a free-radical scavenger (Hankins, 2006). Serum albumin is the major negative acute phase protein and it is a major source of amino acids that can be utilized by the animal's body when necessary (Tóthová et al., 2013). In cases of stress, the serum concentration of this protein decrease, this is the reason why albumin is known to be a negative acute phase protein (Eckersall and Bell, 2010).

Globulins are a heterogeneous group of proteins which includes antibodies and other inflammatory molecules (Alberghina et al., 2010). Physiological and pathological states can result in variation in albumin and globulin concentrations of blood. Therefore, measurement of their concentrations could be a useful tool for evaluating

physiological states that affect animal welfare (Boboo et al., 2017). Concentration of total serum globulin has been suggested as indicator of the animal's immune response (Chorfi et al., 2004).

Creatine phosphokinase (CK) is a muscle specific enzyme, found in skeletal muscle, cardiac muscle, the brain, bladder, stomach, and colon, which plays an important role in the muscle energy metabolism by the activation of the phosphocreatine system which promotes a resynthesis of ATP (Tietze, 2012; Ferreira, 2014; Darras et al., 2015). This enzyme is currently used as an indicator of pre-slaughter physical stress/muscle damage, which are directly associated with animal welfare and meat quality (Fàbrega et al., 2002; Tuomola et al.,2002). The measurement of blood CK levels have been employed in the assessment of physical stress, or stress susceptibility, in poultry (Sandercock et al., 2001), goats (Kannan et al., 2002), pigs (Fàbrega et al., 2002), red deer (Pollard et al., 2002) and calves (Van De Water et al., 2003).

Lactate dehydrogenase (LDH) is an enzyme that catalyzes the conversion of lactate to pyruvate (Valvona, et al., 2016) being found in a variety of tissues, including skeletal muscle. CK and LDH are both released when an animal is experiencing stress in its environment. The activity of this enzyme changes in response to factors associated with stress and muscle fatigue or weariness (Nakyinsige et al., 2013). In muscle damage such as bruising or in vigorous exercise, LDH and CK are released into the blood affecting the quality of meat (Wickham et al., 2012).

Glucose is the primary source of metabolic energy for the central nervous, it is required for the turnover and synthesis of fat, and it is a precursor of muscle glycogen (Mcdowell, 1983). After short and long–term transport stress, glucose level increases indicating metabolic depletion associated with mobilization of energy reserves (Miranda De La Lama et al., 2011).

Lactate is the final product of anaerobic glycolysis in many organisms (Gladden, 2004; Kristensen et al., 2005) and has been proposed as an active metabolite, playing important roles in muscle glycogen production (Cori and Cori, 1993) and on causing muscle fatigue (Fabiato and Fabiato, 1978; Kristensen et al., 2005). The higher plasma lactate levels at slaughter in cattle are indicative of sympathetic-adrenal stress response (Shaw and Tume, 1992) with negative consequences in meat quality.

2.7. Skeletal muscle and fiber types

Skeletal muscle is the most abundant tissue, constituting approximately 40% of the body of mammals (Yan et al., 2001; Xu et al., 2012) and traditionally, can be distinguished as red (type I and IIa) and white (type IIb) fibers. Red skeletal muscles, such as the *Psoas major*, popularly known as filet mignon, in cattle, have a higher percentage of capillaries, myoglobin, lipids and mitochondria than white skeletal muscles such as the *Longissimus dorsi* (Kim et al., 2004). After the animal's death, muscle is converted into meat by a complex biological process, which biochemistry, physiology, nutrition, health, technology, combine to affect the structure and integrity of the muscle (Paredi et al., 2012).

Skeletal muscle is an extremely heterogeneous tissue and has the capacity to adapt when stimulated by several factors, such as variations in contractile activity, supply of energy substrate and changes due to environmental factors and exercises (Flyck and Hoppeler, 2003). The proteins myosin, actin, protein C, protein M, tropomyosin, α- and β-actin form the thick and thin filaments constituting the myofibrils, tubular organelles that almost completely fill the cytoplasm of muscle cells and are responsible for muscle contraction function (Sgarbieri, 1996).

From a functional point of view, a simple classification of these muscle fibers is based on their speed of contraction and metabolic properties. Muscle contraction requires energy from ATP, whose requirements differ widely among the muscle fiber types (Picard et al., 1998). The contractile properties primarily depend on myosin heavy-chain isoforms (MyHCs) present within the thick filaments. Four types of MyHC are expressed in most mature mammalian skeletal striated muscles: I, IIA, IIX, and IIb. The ATPase activity of these MyHCs is related to the speed of contraction: slow (type I) and fast (types IIA, IIX, and IIb) (Listrat et al., 2016). Type I fibers are capable of contracting repeatedly with moderate force, they preferably use fatty acids as energy source and are very resistant to fatigue. The contraction of type II fibers are capable of producing much more force. However, type II fibers have low resistance to fatigue, and are rich in glycogen (Pette and Staron, 2001). Studies in beef cattle of the same breed of this study demonstrated that the *Flexor digitorum* muscle is composed mainly of slow type I and IIA contractile fibers, whereas *Psoas major* is a mixed muscle

composed of large amounts of I, IIA and mainly IIX fibers and high metabolic profile. Compared with other mammalian species, these muscles present a small proportion of hybrid fibers (Moreno-Sánchez et al., 2008).

2.8. Heat shock proteins (HSP) as main factors of response to stress

Heat shock proteins are highly conserved proteins which get activated by heat and other stressors and act as molecular chaperons conferring thermotolerance and the ability of the cell to survive injury and oxidative stress (Yang et al., 2006; Lindiquist and Craig, 1988). During stress conditions in the cell, HSPs interact with denatured proteins and inhibit the formation of cytotoxic protein aggregates, thereby maintaining the protein homeostasis of a cell (Mayer and Bukau, 2006).

Based on the molecular weight and biological functions, HSPs are classified as Hsp110, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, Hsp10, and small HSP families, of which thermo-tolerance development is mainly correlated with Hsp70 and Hsp90 of rice (Hue et al., 2013).The 70 kDa family (Hsp70) is the most highly conserved family of these proteins, which is controlled by 13 genes in human and four genes in bovine (Grosz et al., 1992; Gallagher et al., 1993; Kampinga et al., 2009). Cytoprotective function of Hsp70 has been established in many organs such as intestine, kidney and embryo of cattle (Bhat et al., 2016).

Increased expression of many HSPs including HSP32, HSP40, HSP60, HSP70, HSP90, HSP110 and many others are also observed in goat during hyperthermic stress (Gade et al., 2010; Gupta et al., 2013; Sharma et al., 2013). The HSP25, HSP90AA1, HSPA2 were found in overexpression in heat-stressed chickens' testes (Wang et al., 2015). The HSPA1A was up-regulated following heat shock in blood Sahiwal heifer (Mehla et al., 2014). The stress and chronic exercise have been consistently shown to induce increases in HSP content in the skeletal muscle of several animal species (Morton et al., 2009).

The most abundant Hsps in skeletal muscle are the small Hsps, Hsp70 and Hsp60 or Hsp90 (Liu and Steinacker, 2001), which has been associated with several meat quality traits including color, flavor and juiciness (Lomiwes et al., 2014). In farm animals, elevation in HSP70 and HSP90 expression was observed in blood of sheep

(Romero et al., 2013), in blood mononuclear cells of Murrah buffaloes (Kishore et al., 2014), in blood mononuclear cells of cattle (Kishore et al., 2014), blood of goats (Shaji et al., 2015) and heart of broilers (Yu et al., 2008), during heat stress. Some members of the Hsp families are strictly inducible by stress, whereas others are constitutively expressed at normal condition and are only slightly increase its expression in cases of stress (Neuer et al., 1999). Hsp90 as well as Hsp70 have two isoforms. The isoforms of Hsp90 are HSP90AA1 (inducible form) and HSP90AB1 (constitutive form), while the isoforms of the Hsp70 family are HSPA1A (inducible) and the HSPA8 (constitutive) while others are extrictly stress inducible (Whitley et al., 2009). Moreover, the specificity of HSP for fibers types have been also described in response to stress (Cumming et al., 2014; Cassar-Malek and Picard, 2016).

There are several studies comparing the differential expression of genes between muscles (Moreno-Sanchez et al., 2010; Moreno-Sanchez et al., 2012; Del Pino et al., 2017). Moreno-Sanchez et al. (2010) studied the differential gene expression by cDNA microarrays between *Psoas major* (PM) and *Flexor digitorum superficialis* (FD), in male calves of Avileña-Negra Ibérica beef cattle breed fattened with the same diet during a finishing period, slaughtered with the same age, and noted that approximately 54% of genes differentially expressed (DE) encoded metabolic enzymes and structural-contractile proteins. Genes up-regulated in PM, were associated with metabolic and contractile functions, and mainly encoding fast-glycolytic fiber structural components and glycolytic enzymes. Genes related to muscle development and regeneration were three times more represented in FD than in PM. In FD, the genes were related to cell support. The DE genes coding for contractile and constituent proteins of muscle, as well as those related to the different mechanisms involved in gene expression and protein modification, presented similar percentages in both muscles. According to these authors, the results confirmed the existence of a muscle dependent transcription.

Studies with the same muscles to illustrate muscle-specific transcription were performed by Moreno-Sanchez et al. (2012). Genes involved in repairing mechanisms such as ribosomal and heat shock proteins were DE between muscles, suggesting a differential ability of muscles to react to a similar stressing factor. Heat shock proteins were upregulated in the FD, the muscle aimed to movement and exercise. Different

transcription regulators were DE (ANKRD1, MEF2C, ATF4 overexpressed in PM; ACTN2, CREG1, NFE2L1, UBA52 overexpressed in FD).

2.9. Stress and meat quality

There is a large individual variation in meat quality both within and between animals of the same breed, sex and environment (Lawrie, 1985). This variation is likely to be caused by differences in various known and unknown intrinsic (genetic) and extrinsic (environmental) factors, which interact and determine the outcome of metabolic processes in the peri- and post-mortem period. It has been long known that pre-slaughter conditions are an extrinsic factor that does not only affect animal welfare, but also have an impact on meat quality attributes, such as colour, pH and texture (Lahucky et al., 1998; Ferguson and Warner, 2008; Muchenje, 2009a; Muchenje, 2009b), resulting in economic losses.

If stressful conditions occur immediately prior to slaughter, the presence of high lactic acid concentration reduces muscle pH, while carcass temperature is still high. The combination of low pH and high temperature in the meat causes the denaturation of some muscle proteins leading to reduction in their water holding capacity and to changes of the meat colour (Offer, 1991). When animals are exposed to chronic or long-term stress before slaughter, glycogen is depleted, less lactic acid is formed, and the meat does not acidify normally which consequently results in high pH in meat (i.e., dark, firm and dry) (Schaefer et al., 1997). Warner et al., (1998), related dark cutting in beef with the time spent in lairage pre-slaughter. Gajana et al. (2013) reported that longer transportation time and higher stocking density affected pH and thus reduced meat quality.

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CHAPTER 2- Heat shock protein genes show a differential response to stress type according to muscles in Spanish Avileña-Negra Ibérica beef cattle

ABSTRACT - The differential gene expression (DE) of two phenotypically distinct muscles, *Flexor digitorum superficialis* (FD) and *Psoas major* (PM), in Avileña-Negra Ibérica (ANI) beef cattle breed, was studied with the aim to characterize the pattern of response of heat shock protein (HSP) genes between PM and FD in response to stress at slaughter in two different seasons (summer and winter). In order to determine if response of HSP to the stress of slaughter was conditioned on muscle type and thermal stress. The expression differences between FD and PM muscles were estimated for eight heat shock protein genes (HSPB1, HSPB6, HSPB8, HSP90AA1, HSP90AB1, HSPA1A, HSPA8 and HSPA14) in three sets of biological replicates using qRT-PCR. From the models tested, the best model was adjusted with the least square estimates of each level of the triple interaction. Two sets of contrast to test gene by muscle interaction and gene by season interaction were compared by linear contrast. HSPs were significantly ($P<0.001$) up-regulated in FD as compared to PM muscle except for the HSPA8 (constitutive) and HSPA1A (inducible) genes members of the Hsp70 family. HSPB6 transcript was the most expressed in the oxidative muscle. When we compared the expression of these HSP genes in muscles of animals at slaughter under heat and cold stress, HSPs were upregulated during the summer. Both muscles seem to have similar patterns in the expression of HSPs at the thermal *stimuli* except the HSPB6 and HSPB8 that seemed to show a larger level of expression in PM muscles of animals slaughtered at summer than those that were sampled in the winter slaughter. The inducible Hsp70 (HSPA1A) presented the same level (1.84) of response for both muscles. Small heat shock family of proteins showed a larger level of expression in PM. Not all heat shock protein genes were expressed differentially between FD and PM. However, those that demonstrated difference were strong differential pattern maybe as a response to stress associated to events occurring during the pre-slaughter period. Hsp70 family seems to be more susceptible to thermal stress. Differences in thermal status influence the expression profile of specific HSPs depending on muscle type. The susceptibility and, therefore, the response to thermal stress seems to be differentially mediated by specific families of chaperones as a function of the role of the specific tissue in the restoration of protein homeostasis.

Key words: Bovine, Chaperones, Heat Stress, Mixed Model, Skeletal Muscle

1. INTRODUCTION

The importance of heat shock proteins (HSPs) in skeletal muscle has drawn attention, due to its induction in response to several physiological and pathophysiological *stimuli*, and its speculated role in the muscle adaptations to these *stimuli*. HSPs, also known as chaperones or stress-proteins, are critical for protein folding and stability, and cellular localization of newly synthesized proteins. These functions imply the key roles of these proteins in apoptosis, cell differentiation, and regulation of the embryo cell cycle (Luft and Dix 1999; Lanneau et al., 2007). Some studies have interrogated the role of this specialized class of proteins in muscle biology (Liu and Steinacher 2001; Pulford et al., 2008; Etard et al., 2015) and the role of some of HSPs in the protection of isolated contracting muscles of stressor agents (Locke and Celotti, 2014).

A major challenge to the beef meat industry is to produce high value table cuts with a good and consistent meat quality. The effect of pre-and slaughter conditions is of great interest for the industry because its impact on meat quality (Ferguson and Warner, 2008). Positive correlations have been found between expression of muscle-specific genes (including genes relative to oxidoreduction, and oxidative phosphorylation) and tenderness in the stressed animals (Cassar-Malek and Picard, 2016). Thus, establishing well-defined animal models of stress may help to a better understanding of the biological responses to individual or combined stressors and the signaling pathways that mediate these responses at a cellular level. The selection of animal models that best reflect human molecular responses would provide increased confidence in the selection or testing of therapeutics (Chen et al., 2015).

Recognizing the fundamental importance of muscle proteins to meat quality attributes, there has been a growing interest on how muscle proteins and their genes regulating their coding relate to meat quality (Lomiwes et al., 2014). The transcriptomic evaluation showed a muscle-specific response to stress (Cassar-Malek and Picard, 2016). Several of these studies have consistently reported the differential expression of HSPs in muscle (Lametsch and Bendixen, 2001; Bernard et al., 2007; Kim et al., 2008; Pulford et al; 2008, 2009, Moreno-Sanchez et al., 2012).

Skeletal muscle adapts to the stress at slaughter activating changes in the expression of HSP genes (Moreno-Sánchez et al., 2012) among others. Mammalian Hsps have been classified into five families according to their molecular size: Hsp100, Hsp90, Hsp70, Hsp60 and the small Hsps. The 70 kDa family (Hsp70) is the most highly conserved family of these proteins, which is controlled by 13 genes in human and four genes in bovine (Grosz et al., 1992; Gallagher et al., 1993; Kampinga et al., 2009). The most abundant Hsps in skeletal muscle are the small Hsps, Hsp70 and Hsp60 or Hsp90 (Liu and Steinacker; 2001), which has been associated with several meat quality traits including color, flavor and juiciness (Lomiwes et al., 2014). Some members of the Hsps families are strictly inducible by stress, whereas others are constitutively expressed at normal condition and are only slightly increase its expression in stress conditions (Neuer et al., 1999). Hsp90 as well as Hsp70 have two isoforms. The isoforms of Hsp90 are HSP90AA1 (inducible form) and HSP90AB1 (constitutive form), while the isoforms of the Hsp70 family are HSPA1A (inducible) and the HSPA8 (constitutive) while others are extrictly stress inducible (Whitley et al., 1999).

In the response to stress, cells accelerate the expression rate of heat shock protein genes (Morimoto et al., 1990; Morimoto, 1993) that play crucial roles in environmental stress tolerance and adaptation (Sorensen et al., 2003). The stress has been consistently shown to induce increases in HSP content in the skeletal muscle of several animal species (Morton et al., 2009) and this induction in skeletal muscle seems to be muscle fiber-type specific (Liu and Steinacker, 2001, Cumming et al., 2014; Cassar-Malek and Picard, 2016).

Therefore, we studied the differential gene expression in two phenotypically distinct muscles, *Flexor digitorum superficialis* (FD) and *Psoas major* (PM), in Avileña-Negra Ibérica beef cattle breed. The aim was to characterize the pattern of response of heat shock protein genes between PM and FD in response to stress at slaughter in two different seasons (hot and cold). In order to determine if response of HSP to the stress of slaughter was conditioned on muscle type and thermal stress.

2. MATERIALS AND METHODS

2.1. Ethics statements

Animal Care and Use Committee approval was not obtained for this study because the samples were collected from carcasses at slaughter.

2.2. Animal material and nucleic acid isolation

Samples of nineteen males of Avileña-Negra Ibérica (ANI) beef cattle breed were used for the experiment. The animals were fattened in a common feedlot utility own by farmers of the Avileña-Negra Ibérica Breed Association located in Riocabado (Avila, Spain). All animals were fattened under the same diet and location conditions, and slaughtered when they were fit for commercial requirements, at weight around 500 kg, normally achieved at around 450 days of age. At the slaughterhouse, samples of approximately 1 × 1 × 0.5 cm^3 were removed from portion of the belly of the PM and FD of each animal just after slaughter. We defined two seasons at slaughter, the “hot” season for animals slaughtered during June and July, and “cold” season for animals slaughtered between November and February in the following year. Tissue samples of *Flexor digitorum superficialis* (FD) and *Psoas major* (PM) skeletal muscles were collected at slaughter, immediately frozen in liquid nitrogen, and stored at -80ºC until RNA isolation. These two muscles were chosen because of their physiological and metabolic differences, FD is a purely oxidative muscle and PM has a strong glycolytic profile (Moreno-Sanchez et al., 2008).

Total RNA was isolated from frozen muscle tissues using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany) following manufacturer’s instructions. RNA quality and quantity were verified by 28S:18S rRNA ratio in 1% agarose gel and by NanoDrop ND-1000UV/Vis spectrophotometer (NanoDrop Technologies, Inc., DE, USA), respectively. Furthermore, the RNA integrity and concentration were assessed by RNA Nano Labchips in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). To ensure the maintenance of the purity, all RNA samples were treated with RQ1 RNase-Free DNase (Promega Corporation, WI, USA) following manufactures

guidelines. RNA integrity number (RIN) average to FD was 7.33 (minimum 5.3 and maximum 8.6) and 7.24 (minimum 5.1 and maximum 8.1) to PM.

2.3. Real-time quantitative PCR (qRT-PCR)

Differential expression of eight target genes (Table 1) from the heat shock protein family were studied: HSPB1 (heat shock protein family B (small) member 1; inducible), HSPB6 (heat shock protein family B (small) member 6; constitutive), HSPB8 (heat shock protein family B (small) member 8; inducible), HSP90AA1 (heat shock protein 90 alpha family class A member 1; inducible), HSP90AB1 (heat shock protein 90 alpha family class B member 1; constitutive), HSPA1A (heat shock 70kDa protein 1A; inducible), HSPA8 (heat shock protein family A (Hsp70) member 8;constitutive) and HSPA14 (heat shock protein family A (Hsp70) member 14; inducible). In addition, UBB (ubiquitin B) gene was chosen as internal reference (endogenous) gene because, according to our previous results in a cDNA microarray experiment (Diaz et al., 2009) it did not show DE between muscles and proved to be very stable as indicated by the magnitude of the standard deviation of the estimate that was negligible.

Table 1. Gene symbol, name, location and aliases.

Gene symbol	Gene ID[a]	Name	Location	Aliases
HSPA1A	282254	Heat shock 70kDa protein 1A	BTA 23, AC_000180.1	*HSP70 HSP70-2, HSP70-1, HSPA1B, HSPA2 Hsp701A/B*
HSPA14	51182	Heat shock 70kDa protein 14	BTA 13, AC_000170.1	*HSP70L1, HSP70-4*
HSPA8	281831	Heat shock 70kDa protein 8	BTA 15, AC_000172.1	*HSPA10, Hsc70 HSC70 HSC71 HSP73*
HSP90AA1	281832	Heat shock protein 90kDa alpha (cytosolic), class A member 1	BTA 21, AC_000178.1	*HSPCA HSP90A*
HSP90AB1	767874	Heat shock protein 90kDa alpha (cytosolic), class B member 1	BTA 23, AC_000180.1	*HSPCB*
HSPB1	516099	Heat shock 27kDa protein 1	BTA 25, AC_000182.1	*Hs.7606, HSP27, Hsp25, HSP28, CMT2F*
HSPB6	534551	Heat shock protein, alpha-crystallin-related, B6	BTA 18, AC_000175.1	*HSP20 PPP1R91, Hsp20, FLJ32389*
HSPB8	539524	Heat shock 22kDa protein 8	BTA 17, AC_000174.1	*CMT2L, H11, HSP22, E2IG1, HspB8*
Reference gene				
UBB		Ubiquitin B	BTA 19, AC_000176.1	*FLJ25987, MGC8385*

The sequences of primers and hydrolysis probes were designed with the GenScript Real-time PCR (TaqMan) Primer Design software (GenScript, Piscataway, NJ, USA) (Table 2). All primers/probes sequences were chosen according to two criteria: to be located in the invariable region of each gene and to avoid amplification of possible DNA contamination the primers/probes crossing exons junction were picked. Each single probe contained two labels, a 3' dark quencher dye (BBQ) for all hydrolysis probes, a fluorescent 5' reporter for all target genes probes (6FAM) and a fluorescent 5' reporter for reference gene probe (YAK).

Table 2. The sequences of primers, hydrolysis probes and amplicon sizes.

Name	Taq Man Probes and Primers sequences	Amplicon sizes (bp)
HSPB1-Fw	5' GGA CGT CAA CCA CTT CGC 3'	
HSPB1-Probe	5' **6FAM**-TTG ACC GTC AGC TCC TCG GG--**BBQ** 3'	77
HSPB1-Rv	5' CGT GCT TGC CAG TGA TCT C 3'	
HSPB6-Fw	5' TAC CGC CCA GGT ATC GAC 3'	
HSPB6-Probe	5' **6FAM**-CCC GGG CAT TTC TCG GTG TT--**BBQ** 3'	75
HSPB6-Rv	5' ATT TCC TCG GGT GAG AAG TG 3'	
HSPB8-Fw	5' TCG TCT CCA AGA ACT TCA CAA 3'	
HSPB8-Probe	5' **6FAM**-TCC ACC TCT GCA GGA AGC TGG A--**BBQ** 3'	75
HSPB8-Rv	5' AAA GGG AGG CAA ACA CTG TC 3'	
HSP90AA1-Fw	5' ATG AGC AGT ATG CCT GGG AG 3'	
HSP90AA1-Probe	5' **6FAM**-CCT TAC TGT GAA GGA CCC TCC TGC A--**BBQ** 3'	72
HSP90AA1-Rv	5' CCA TTG GTT CTC CTG TGT CA 3'	
HSP90AB1-Fw	5' TAC ATC ACT GGT AAG AGC AAA GA 3'	
HSP90AB1-Probe	5'-**6FAM**-CAC GCG CTC CAC AAA TGC AG--**BBQ** 3'	83
HSP90AB1-Rv	5' TAC ACC ACC TCA AAG CCC CG 3'	
HSPA1A-Fw	5' CGG AGA AGG ACG AGT TTG AG 3'	
HSPA1A-Probe	5'-**6FAM**-CAC ACC TGC TCC AGC TCC TTC CT--**BBQ** 3'	75
HSPA1A-Rv	5' GGT ACA GTC TGC TGA TGA GG 3'	
HSPA8-Fw	5' ACA AAG ATG AAG GAA ATC GCA 3'	
HSPA8-Probe	5'-**6FAM**-TGG GAA GAC GGT TAC CAA CGC TG--**BBQ** 3'	79
HSPA8-Rv	5' TAA AAT AGG CAG GTA CTG TGA CG 3'	
HSPA14-F	5' GAG TTT CAG AGA TCC TTC AGA CAT 3'	
HSPA14-Probe	5'-**6FAM**-CAT GGC GCG GGC ATT TCC--**BBQ** 3'	71
HSPA14-Rv	5' GCA CCG TTC ATC AGC TTC AT 3'	
UBB-Fw	5' CAA GAC CAT CAC CCT GGA AG 3'	
UBB- Probe	5' **YAK**-GGA GCC CAG TGA CAC CAT CGA --**BBQ** 3'	73
UBB-Rv	5' CTC CTT ATC CTG GAT CTT GGC 3'	

The reaction mix for one-step qRT-PCR were set by LightCycler 480® RNA Master Hydrolysis Probes following the protocol for use with LightCycler® 480 multiwell Plate 96 (Roche, Switzerland). qRT-PCR amplification reactions (20 µl) contained 100 ng total RNA, 1X Master Mix without enhancer, 3.25 mM of activator, 400 nM each of the forward and reverse primers for internal reference gene, 400 nM each of the forward and reverse primers for the tested gene, 250 nM reference hydrolysis probe and 200 nM each of the tested hydrolysis probe. Reactions were run in triplicate on a LightCycler® 480 II (Roche, Switzerland) following manufacturer's cycling parameters. The corresponding mRNA levels were measured and analyzed by their crossing point (Cp) what represents the cycle number at which the sample fluoresce signal passes a

fixed threshold above the baseline. The threshold cycle is inversely proportional to the original relative expression level of the gene of interest. Thus, the larger the Cp values the lower the amount of mRNA and therefore the lower level of expression.

2.4. Statistical data analysis and identification of differentially expressed genes

The qRT-PCR results were analyzed for statistical significance using mixed models (PROC MIXED procedure) in SAS (SAS v9.1, SAS Institute Inc., Cary NC, USA) as proposed by Steibel et al. (2009). A total of 3136 records were contained in the data file. The design was connected having replicates of genes in different wells. Table 3 shows the equations of the mixed models tested. To indicate the best model we used the follow criterias : Akaike´s Information Criterion (AIC = -2l+2d, smaller is better), and Schwarz´s Bayesian Criterion (BIC = -2l+dlogn, smaller is better), where l is the maximum value of the log likelihood, d is the dimension of the model and N is the number of effective observations.

Table 3. Description of the models used for the analysis.

M	Model	Variance structure
1	$y_{gmaw} = MG_{mg} + w_w + a_a + e_{gm}$	$w_w \sim N(0, \sigma^2_{I_w});\ a \sim N(0, \sigma^2_a); e \sim N(0, \sigma^2_e)$
2	$y_{gsaw} = GS_{gs} + w_w + a_a + e_{gm}$	$w_w \sim N(0, \sigma^2_{I_w});\ a \sim N(0, \sigma^2_a); e \sim N(0, \sigma^2_e)$
3	$y_{gmsaw} = MG_{gm} + GS_{gs} + w_w + a_a + e_{gm}$	$w_w \sim N(0, \sigma^2_{I_w});\ a \sim N(0, \sigma^2_a); e \sim N(0, \sigma^2_e)$
4	$y_{gmsaw} = MGS_{gms} + w_w + a_a + e_{gm}$	$w_w \sim N(0, \sigma^2_{I_w});\ a \sim N(0, \sigma^2_a); e \sim N(0, \sigma^2_e)$

In the model column the random effects were *a*=animal (19 levels) and *w*=well (18 levels); the fixed effects were interactions, y_{gmsaw} is Cp obtained from the thermocycler software for the *g*th gene from the *m*th muscle in the *s*th season, corresponding to the *a*th animal in the wth well, MG_{gm} is the interaction muscle x gene, GS_{gs} is interaction gene x season, MGS_{gik} is the interaction muscle x gene x season, w_w is a random effect of *w*th well and a is a random effect of *a*th animal, and e is a residual term. M = model identification.

Differentially expressed (DE) genes were identified after setting the appropriate linear contrasts from the least square solutions of the interaction between gene, muscle and season, obtained from the best model. T test was used to determine differential expression with level of significance P <0.001. The relative quantification between muscles was expressed as $2^{-\Delta\Delta Cp_g}$ assuming that the efficiencies were 2 (Livak and Schmittgen, 2001). Thus,

$$\Delta\Delta Cp_g = \left(TG_{gPM} - RG_{PM}\right) - \left(TG_{gFD} - RG_{FD}\right)$$

where, TG refers to the target gene g for each specific muscle (FD and PM) and RG refers to the $Cp_g$of reference genes in FD and PM respectively. Instead of raw Cp_g we used the corresponding least square estimates of the effects from the chosen model.

2.5. Protein interactions

String software (http://string-db.org) was used to predict protein interactomes. This computational approach is a software to analyze protein interaction using outsource information (Franceschini et al., 2013).

3. RESULTS AND DISCUSSION

3.1. Model selection

Table 4 shows each of the tested model together with the AIC (Akaike's Information Criterion) and BIC (Schwarz's Bayesian Criterion). According to the criterions the model that better fit the data (lower values of AIC and BIC) was the model that included the third level interaction between gene, muscle and season. Therefore, all results will be referred to this model.

Table 4. Model selection criteria for the tested models.

M	AIC	BIC
1	3223.3	3242.1
2	4062.4	4081.3
3	3014.2	3033.1
4	2922.0	2940.9

M=models tested, AIC= Akaike's Information Criterion, BIC= Schwarz's Bayesian Criterion

3.2. Differential gene expression

The gene expression differences between FD and PM muscles were estimated for eight heat shock protein genes in three sets of biological replicates using qRT-PCR. The best model resulted was the M4 (Table 4). Thus, with the least square means of each level of the triple interaction we created two sets of contrasts to test whether the gene by muscle interaction (Table 5) and gene by season interaction (Table 6) were statistically significant ($P<0.001$).

Table 5. Differentially expressed genes between muscles *Psoas major* and *Flexor digitorum superficialis* muscles under stress at slaughter.

Gene symbol	Gene ID[a]	Estimate	Standard Error	P-value	Fold-change[b]
HSPA1A	282254	-0.17	0.10	0.113	1.21
HSPA14	51182	-0.67	0.07	<0.0001	1.59
HSPA8	281831	0.06	0.06	0.316	0.96
HSP90AA1	281832	-1.72	0.09	<0.001	3.33
HSP90AB1	767874	-1.41	0.09	<0.001	2.66
HSPB1	516099	-0.96	0.09	<0.001	1.94
HSPB6	534551	-2.14	0.14	<0.001	4.42
HSPB8	539524	-1.11	0.13	<0.001	2.18

[a]NCBI gene ID, [b]The fold-change ($2^{-\Delta\Delta Cp_g}$) estimates (relative expression) refer to the FD muscle.

HSPs were significantly (P<0.001) up-regulated in FD as compared to PM muscle (Table 5) except for the HSPA8 (constitutive) and HSPA1A (inducible) genes members of the Hsp70 family. Fold changes ranged from 0.96 to 4.42 for HSPA8 and HSPB6, respectively. Samples in these animals were taken after slaughter what implies that animals have gone through an uncontrolled stress generated by social changes during the transport and the effect of transport from the feedlot to slaughterhouse, waiting and stunning (Ferguson and Warner, 2008). These events may be associated to a process chronic stress and this is well known that induce increases in HSPs gene expression in the skeletal muscles of several species (Samelman, 2000; Naito et al., 2001; Morton et al., 2009). Skeletal muscle is a high plastic tissue that may be modified by use or disuse and is a very complex heterogeneous system since it comprises different muscle fiber types (Wada et al., 1995). Muscle fiber types differ in functional and biochemical properties, as does their expression and response to HSPs. Different muscles adapt to the stress by activating changes in the gene expression of HSPs genes (Moreno-Sanchez et al., 2012). In fact, all genes were upregulated in the FD, the muscle mainly used to movement, exercise and maintaining posture (Moreno-Sanchez et al., 2008).

Taking into consideration the relative expression under the stress at slaughter between muscles (Table 5), HSPB6 transcript was the most expressed in the oxidative muscle, the FD (4.42 fold change). HSPB6 may play several roles in muscle, from the contractile processes associated with troponin complexes to metabolic responses due to muscle activity or inactivity (Dreiza et al., 2010). This fact justifies a greater expression of this transcripts, once the animal was submitted to a stress situation, activating energy metabolic responses. Investigations demonstrated that muscle fiber type, aging, denervation, or disease can may alter HSPB6 expression levels and function in skeletal muscle and associated tissues (Sakuma et al., 1998; Ungvari and Koller, 2001; Huey et al., 2004). According to Li et al. (2017) cellular stress or damage increases the expression of HSPB6. When we compared the expression of these heat shock proteins genes in muscles of animals slaughtered between under thermal stress (summer *vs* winter seasons), the pattern of differences of expression was smaller (Table 6) in relation to animals under stress of slaughter (Table 5).

Table 6. Estimates of differential expression between cold and hot season at slaughter for a set of eight heat shock protein genes.

Gene symbol	Gene ID[a]	Estimate	Standard Error	P-value	Fold-change[b]
HSPA1A	282254	-0.88	0.10	<0.0001	1.84
HSPA14	51182	-0.02	0.07	0.764	1.01
HSPA8	281831	-0.17	0.06	<0.009	1.13
HSP90AA1	281832	-0.80	0.09	<0.0001	1.74
HSP90AB1	767874	-0.36	0.09	<0.0002	1.28
HSPB1	516099	-0.81	0.09	<0.0001	1.75
HSPB6	534551	-0.67	0.14	<0.0001	1.59
HSPB8	539524	-0.82	0.13	<0.0001	1.76

[a]NCBI gene ID. [b]The fold-change ($2^{-\Delta\Delta Cp_g}$) estimates (relative expression) refer to the hot season.

Range of fold-change varied between 1.01 for HSPA14 and 1.84 for the inducible Hsp70, the HSPA1A. HSPs were significantly (P<0.001) upregulated during the hot season as it indicates the negative value of the estimate, except HSPA14 and HSPA8. Changes in gene expression are an integral part of the cellular response to

thermal stress. In this study, the magnitude of DE between muscles at slaughter of HSPs seems to be more relevant than the one due to heat stress for some specific HSPs such as HSPA14 gene, Hsp90 family (HSP90AA1 and HSP90AB1) and heat shock protein family B (HSPB1, HSPB6 and HSPB8), but not for the Hsp70 family (HSPA1A and HSPA8). Thus, the Hsp70 family appears to have sensitive to thermal stress than slaughter stress.

Banerjee et al. (2014) showed that HSPA8 and HSPA1A were temperature sensitive and seasonal variate in goats, differently this study. HSPA1A was the most expressed, which can be explained by the fact that Hsp70 act as a molecular chaperone to stabilize and re-fold thermally denaturing proteins (Table 6). There is considerable evidence that the synthesis of Hsp70 is temperature dependent (Zulkifi et al., 2003) and thus Hsp70 responses could be considered as cellular thermometer. We explored if the differences between seasons were more relevant in the pattern of HSP gene expression for specific muscles (Table 7).

Table 7. Fold changes between summer and winter seasons for all HSP in each muscle.

		Flexor digitorum superficialis			*Psoas major*		
Gene symbol	Gene ID[a]	Estimate (SE)	P-value	Fold change	Estimate (SE)	P-value	Fold-change[b]
HSPA1A	282254	-0.88 (0.16)	<0.0001	1.84	-0.88 (0.16)	<0.0001	1.84
HSPA14	51182	-0.04 (0.09)	0.6437	1.03	-0.00 (0.09)	0.9871	1.00
HSPA8	281831	-0.20 (0.09)	0.0339	1.14	-0.15 (0.09)	0.1165	1.11
HSP90AA1	281832	-0.91 (0.11)	<0.0001	1.89	-0.69 (0.11)	<0.0001	1.61
HSP90AB1	767874	-0.48 (0.12)	0.0001	1.39	-0.23 (0.12)	0.0994	1.18
HSPB1	516099	-0.71 (0.11)	<0.0001	1.64	-0.90 (0.11)	<0.0001	1.87
HSPB6	534551	-0.29 (0.18)	0.1010	1.22	-1.05 (0.18)	<0.0001	2.08
HSPB8	539524	-0.55 (0.16)	0.0010	1.46	-1.09 (0.16)	0.0010	2.13

SE=Standard error, [a]NCBI gene ID, [b]The fold-change estimates (relative expression) refer to the FD muscle and hot season. Fold-change $=2^{-\Delta\Delta Cp_g}$

The activation of HSPs genes during heat challenge has been extensively studied in mammals and birds (Xie et al., 2014). Heat stress is regulated in two stages: acute (short term) and chronic (long term) (Garret et al., 2009). The acute phase includes the response at the cellular level and the chronic phase results in acclimation to the stressor and involves to the reprogramming of gene expression, including HSP expression, and metabolism activation (Horowitz, 2002; Collier et al., 2006). Therefore,

variations in expression pattern of HSP genes during different seasons may be centrally important mechanism for better adaptability in cattle.

In our study both muscles have similar patterns in the expression of HSPs at the thermal *stimuli* except the HSPB6 and HSPB8 that seemed to show a larger level of expression in PM muscles of animals slaughtered at summer than those that were sampled in the winter slaughter according to the contrasts. However, the interaction between the response to slaughter plus thermal stress ranged in magnitude according to the muscle (Table 7). On the other hand, the inducible Hsp70 (HSPA1A) presented the same level of response for both muscles, and finally, small heat shock family of proteins showed a larger level of expression in PM (HSPB1, HSPB6 and HSPB8).

Studies on Hsp70 expression and muscle fiber specificity have shown that the inducible form of Hsp70 is expressed in rat muscles comprised of type I muscle fibers, but not in those comprised of type IIb fibers. In muscles of mixed fiber type, HSP70 content is roughly proportional to the percentage of type I fibers. These results suggest a specific expression of HSP70 in type I muscle fiber (Locke et al., 1991). FD has a larger composition of oxidative fibers (type I) and lacks pure glycolytic (type IIX) and a pure oxidative metabolism whereas PM had on average a balanced proportion among the three mains pure fiber types (I, IIA and IIX) fibers (Moreno-Sanchez et al., 2008). Sanders et al. (2009), suggest that the oxidative muscle is more susceptible to heat stress mediated changes redox balance than glycolytic muscle during chronic heat stress. However, as Locke et al (2014) suggested, the effect of heat stress on the oxidative muscle and its effect on the loss of its contractile properties is achieved at higher temperatures than in the glycolytic muscle. Furthermore, it could also be that difference in the amount of type I fibers has not been sufficient to cause a difference in the expression of HSPA1A transcript between muscles or it could also be that the muscle response is mediated through other inherent factors to the muscle.

Calves of Avileña Negra-Ibérica breed are more sensitive to heat than to cold stress (Usala et al., 2018). Therefore, patterns of expression according to thermal stress seems to be consistent with those findings. The results (Table 7) suggest that differences in thermal status influence the expression profile of specific type of muscles. Heat or cold stress at slaughter should be considered thermal chronic stress because animals' exposure to the same type of thermal stress started at feedlot.

The known functional associations of proteins encoded by HSPs genes involved in this study (Figure 2) are important to identify the extensive understanding of intricate biological mechanisms and pathways. Except HSPB8, there seems to exist interaction between all proteins products of these HSPs gene. HSPs proteins showed co-expression (black line) patterns, except between HSPA8 (Hsp70 constitutive) and HSPA1A (Hsp70 inducible) what does not to occur in the Hsp90 family. The different patterns showed by HSPs as well as the difference in the interaction of encoded proteins generated or not by co-expression, may only highlight the complexity of muscles responding to the various types of stressor factors that may affect their normal physiology along the production life of farm animals.

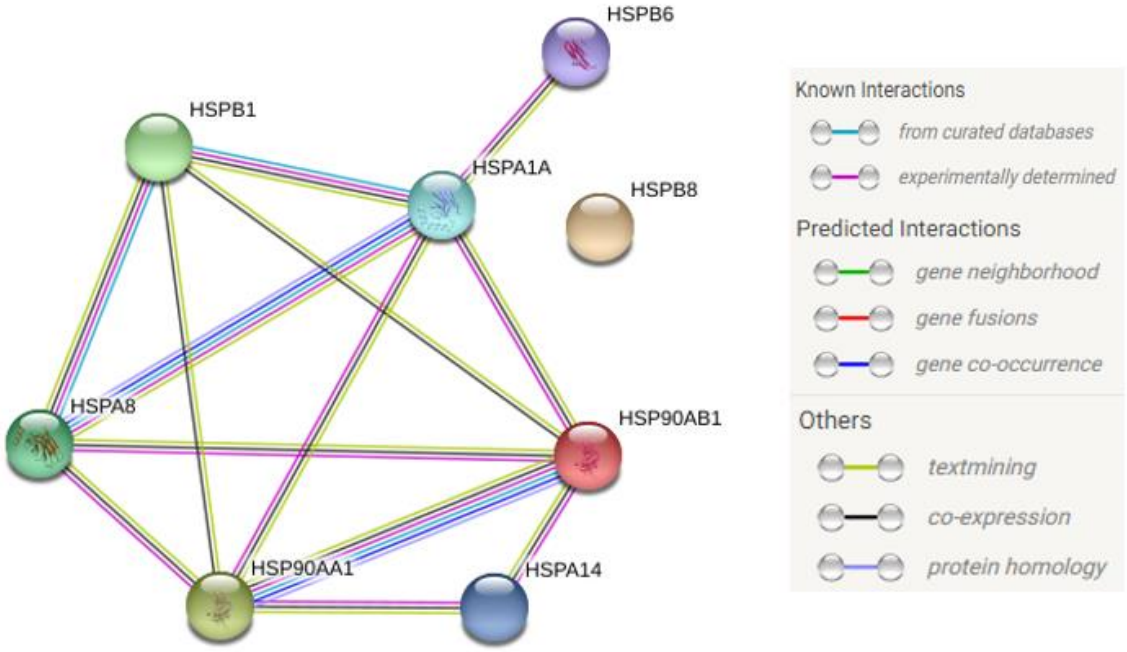


Figure 2. Interaction network of the proteins encoded by the eight HSP genes involved in the study. The network nodes (circles) represent proteins, the edges represent protein-protein functional association and line color indicates the type of interaction.

4. CONCLUSION

Not all heat shock protein genes were expressed differentially between FD and PM. However, those that demonstrated difference were strong differential pattern maybe as a response to stress associated to events occurring during the pre-slaughter period. Hsp70 family seems to be more susceptible to thermal stress. Differences in

thermal status influence the expression profile of specific HSPs depending on muscle type. The susceptibility and, therefore, the response to thermal stress seems to be differentially mediated by specific families of chaperones as a function of the role of the specific tissue in the restoration of protein homeostasis.

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CHAPTER 3 - The physiological response to stress of Spain Avileña-Negra Iberica cattle differs between two management conditions

ABSTRACT - In livestock production, animals are exposed to management conditions that induce different stress responses. The aims of the present study was to characterize the physiological response to stress generated by handling the animals at two different periods in time, feedlot (F) and slaughter (S) with a set of biomarkers; (b) to identify a subset of the biomarkers of stress that best discriminate between these two stress periods and to evaluate if the early response in F could be used to anticipate response at S, and (c) find evidences of a genetic component in the physiological response to stress in beef cattle. Blood samples of eighty Avileña-Negra Iberica male calves, were collected at the end of the fattening period in the feedlot and from 4 to 7 days later at the slaughterhouse during exsanguination. Seven biomarkers, albumin, cortisol, creatine phosphokinase (CK), glucose, lactate, lactate dehydrogenase (LDH) and globulin were determined in both periods. Logistic regression and Multinomial logistic regression models were tested. Animals seem to experience more stress at S than at F. Linear Discriminant Analysis (LDA) based on the combination of biomarkers chosen by the best logistic regression model (lactate, glucose and albumin) showed that 92.5% of animals were correctly assigned to group F and 87.5% to group S demonstrating that in order to discriminate between F and S only three biomarkers albumin, lactate and glucose are needed. Searching for evidences of a genetic component in the physiological responses, LDA among farms showed 59% and 57% of the animals were correctly assign to the origin farm in F and S groups, respectively, suggesting that the miss-matching among farms could be also attributed to an admixture genetic background between these herds. The stress response in feedlot appeared to be associated with mechanisms that generate an organic response at the hepatic level. Lactate and cortisol indicated that the response to stress at slaughter occur at muscle level. Lactate, glucose and albumin best discriminate the two different stress periods. Therefore, there are other four biomarkers (CK, cortisol, LDH and globulin) that could be used as global set of physiological response to stress regardless the source of stress. The same set of markers to some extent also discriminates between farms of origin. The miss-matching occurred mostly between herds with common genetic background.

Keywords: Biomarker, Bovine, Linear Discriminant Analysis, Principal Component Analysis, Physiological Stress, Temperament

1. INTRODUCTION

The concept of stress embraces numerous factors, which can be divided into physical and psychological. According to Moberg (1980) stress is defined as a complex multidimensional phenomenon promoted by several *stimuli* that causes a physiological response aimed to maintain or to recover the body homeostasis. In livestock production, animals are exposed to a series of factors and procedures (changes in weather, transport conditions, handling practices, pre-slaughter waiting periods, stunning procedure, and the slaughter methods) at different environments (farm, feedlots, transportation vehicle and slaughterhouse) which expose them to new challenges and experiences that induce different types of stress (Grandin, 1997; Apple et al., 2005; Terlouw, 2005).

Several biochemical parameters known as biomarkers of stress are used to study the effect of a stressor based on the physiology of the animal (Buckham et al., 2008) and, therefore, in evaluating animal welfare. According to Amtmann et al. (2006), these variables are applied as indicators of physiological stress, especially when comparing pre and post values to a certain management that is believed to induce stress, between animals of similar general groups (age, breed and breeding system). Studies have been using some these biomarkers as indicators of temperament, for example, the lactate (Boles et al., 2015; Miller et al., 2016), cortisol (Williams et al., 2015) and glucose (Miller et al., 2016).

The stress response includes several changes that may have negative effects on the performance of farm animals. Stress response depends on the stressor source, duration, intensity, and the susceptibility of the individual (Ferguson et al., 2001). According to Grandin (1981), animals do not react similarly to the stress and the response of each individual is unique, depending on its genetic background, prior experience, the types and the duration of the stressors, age, gender, physiological status, emotional state, cognitive function and season (Von Holst, 1998; Cockram, 2004; Veissier and Miele; 2014). Within this variation, an animal can show a consistent type of behavioral and physiological response in different challenge situations and on different occasions when exposed to a single type of challenge. When the behavioral

response is consistent across time and across situations it is described as a behavioral type or temperament (Sih et al., 2004; Mackay and Haskell, 2015).

In animals, temperament is defined as the reactivity, or fear response, to humans or novel environments (Fordyce et al.,1988). This is a key factor to consider when stress affects animal performance. Individuals with a calm temperament may adapt more easily and become less stressed with repeated handling treatments than individuals with a very excitable temperament that may become increasingly stressed (Grandin, 1997). Thus, the animals are more difficult to handle, have carcasses with greater incidence of injuries (Mcnally and Warriss, 1996), inferior meat quality traits (Voisinet et al., 1997; Ferguson et al., 2006), have growth rates, reproduction, and reduced immune functions (Voisinet et al., 1997; Cooke et al., 2009, 2012; Burdick et al., 2011).

Temperament has a genetic component (Burdick et al., 2011; Haskel et al., 2014) therefore it could be modulated via selection (Haskel et al., 2014). There are also studies highlighting the association between biomarkers and temperament (Burdick et al., 2011; Cooke et al., 2017) that could help to understand the complexity of the response to stress as well as its effects on animal's health, reproduction and production, in general, the animal welfare. Thus, the aim of the present study was (a) to characterize the physiological response to stress generated by handling the animals at two different periods in time, feedlot (F) and slaughter (S) with a set of biomarkers; (b) to identify a subset of the biomarkers of stress that best discriminate between these two stress periods and to evaluate if the early response in F could be used to anticipate response at S, and (c) find evidences of a genetic component in the physiological response to stress in beef cattle.

2. MATERIAL AND METHODS

2.1. Ethics statements

Animal Care and Use Committee approval was not obtained for this study because the samples were collected at feedlot commercial conditions and from carcasses at slaughter.

2.2. Handling procedures

Feedlot handling of the calves involves changes in social interactions, the passage through the chute and contact with the veterinarian for blood extraction. The handling of the animals to go to slaughter involves pre-slaughtering conditions such as load in truck, transport, unloading in slaughterhouse, short wait in pens and conduction aisle to the stunning box and finally the stunning.

2.3. Samples collection and measurements

Blood samples of eighty Avileña-Negra Iberica male calves, belonging to 14 farms, born between 2013 and 2015, were collected at two different periods. Animals were sampled at the end of the fattening period, between 4 and 7 days before finishing this period. At sampling, animals had stayed an average of 250 days in the commercial feedlot manage by the breed association. Blood samples were taken from the caudal vein using a needle connected to the vaccutainer tube and without anticoagulant when animals were restrained in the chute. The same animals were sampled at the slaughterhouse during exsanguination. Average age of animals at slaughter was 422 days.

Immediately after taking the sample, blood was transferred to two tubes of 1.5 mL one with ethylenediamine tetraacetic acid (EDTA) as anticoagulant and another with sodium fluoride (FNa) to delay the consumption of glucose by blood cells. The blood samples were centrifuged for 5 min at 6,000 rpm, to obtain plasma and serum respectively, which were transferred to 1.5 ml vials without anticoagulant, and frozen in liquid nitrogen, kept in a freezer at -80ºC until they were taken to the laboratory for analysis. Seven biomarkers, albumin, cortisol, creatine phosphokinase (CK), glucose, lactate, lactate dehydrogenase (LDH) and globulin were determined in the samples obtained at both periods.

2.4. Statistical data analysis

Statistical analysis was performed with the R Statistical Computing software (R Development Core Team, 2006). Paired T-test, Principal Component Analysis (PCA), Linear Discriminant Analysis (LDA), Logistic Regression and Multinomial Logistic Regression were used to analyse the phenotypic data of stress biomarkers, by means of R package FactomineR (Husson et al., 2011), 'lda' function of the MASS package glm function, and multinom function of nnet package (Venables and Ripley, 2002), respectively.

PCA analysis was used to characterize the physiological stress. PCA is a mathematical technique that reduces the dimensionality of variables by creating a new set of variables called principal components (PCs), which retain most of the variation in the data set. The dimension values (Dim) and contribution (ctr) were applied on the exploration of the data. Dim values refer to the coordinate of each variable on each principal component and ctr (%) provides the contribution of that coordinate to calculate that dimension of the variables. From the PCA output we calculated the percentage of the total contribution (TCTR) of each biomarker to the total variability represented by three first components to characterize the physiological components of stress response. The TCTR (%) was obtained by a weighted sum: for the first component, the ctr was multiplied by the percentage of variance that this component explained. The same was performed for components 2 and 3. Then, results were summed for two (TCTR2) and for three (TCTR3) components.

Fisher Linear Discriminant Analysis, also called Linear Discriminant Analysis (LDA) and with logistic regression were utilized to achieve our second objective. Presence of differences between F and S was our initial assumption therefore, we aimed to determine if we needed all markers to discriminate between F and S or if there were a subset of them with a common variation that we could use as proxies of stress status regardless the differences of the stressor source or stressful situation. Thus, this set of biomarkers could be a candidate of biomarkers to be used for the selection of less reactive animals at early stages.

We hypothesized that if biomarkers permit to discriminate between the status F and S of animals, markers would have somehow specificity and therefore, will not be useful as proxies of stress regardless the stressor factor. LDAs are methods used in statistics to find a linear combination of features (in our case biomarkers) that,

characterizes or separates two or more classes of objects, groups or events, in our case (management conditions, F and S). The effectiveness of LDA in classifying the groups was evaluated comparing the assignments made through matrices scatter in which the diagonal results correspond to the correct assignment and off-diagonal to the failure predictions or miss-matchings.

A logistic regression model was used to find the best fitting model to describe the relationship between the dichotomous trait of interest (feedlot handling and slaughterhouse handling) and a set of independent (biomarkers of stress) variables as well as to determine the relationship of the biomarker with the discriminated status. The robustness was judged by 5-fold cross validation and the final model was chosen taking into account the lowest value of k-fold error using the cv.glm function of the R-package boot. The best model was:

$$log\left(\frac{p(X)}{1-p(X)}\right) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots \beta_n X_n + e,$$

in which $y = log\left(\frac{p(X)}{1-p(X)}\right)$ called the log-odds ratio; β_o is the intercept; β_1, β_2, β_n are the regression coefficients associated with $X_{i=1,n}$, for n=7 which are predictors related to each of the biomarkers; e is the error of the model.

In order to find evidences of the effect of previous experience (management in the farms of origin of calves) or evidences of a genetic component in the physiological responses to stress, we used LDA one more time. In this case, we were assuming that previous experience of calves to management were characteristic of the farm in which calves were raise previously to their arrival to feedlot where they could have developed a copying behavior (Réale et al., 2007). Thus, we evaluated the discrimination ability of biomarkers between origin farms of animals. A multinomial logistic regression model was used to find the best fitting model to describe the relationship between the multinomial dependent variables (14 origin farms of animals) and a set of independent (7 biomarkers of stress) variables. The multinomial logit model was:

$$y_{ij} = \frac{\exp(x'_i \beta_j)}{1+\sum_{k=2}^{m} \exp(x'_k \beta_j)} \text{ for } j=2, \ldots, m$$

Here, y_{ij} is the probability that the response variable is in its *jth* of *m* levels for observation *i*; x'_i is the *i*th row of the model matrix X; and β_j is a vector of p regression coefficients pertaining to level *j* of the response. The first level of the response is treated specially to ensure that $\sum_{j=1}^{m} y_{ij} = 1$. The interpretation of the regression coefficients is to base in its log-odds:

$$log \frac{y_{ij}}{yi1} x'_i \beta_j \text{ for } j=2, \ldots, m$$

3. RESULTS AND DISCUSSION

3.1. Characterization of physiological stress

Table 1 shows descriptive statistics of biomarkers determined in blood serum at feedlot (F) handling and at the slaughterhouse (S) handling. Animals seem to experience more stress at S than at F as glucose, lactate, globulin and CK increased significantly ($p<0.05$) from F status to S. In addition, albumin that is known to be a negative indicator of stress. However, there was no significant difference between F and S for albumin level. Pre-slaughter stress implies a cascade of factors (social mixed, restraint, transport, novelty stunning) that generates a larger exposed to a stressor sources and might break the animal's homeostasis (Ferguson and Warner, 2008; Chen et al., 2015).

Table 1. Number of animals (N = 80), means, standard deviations (SD), minimum (Min) and maximum (Max) values of the biomarkers in feedlot and slaughterhouse.

Biomarker	**Feedlot**				**Slaughterhouse**			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Albumin (g/100 mL)	3.23	0.40	2.38	4.03	3.19	0.37	2.51	4.25
Cortisol (µg/dL)	8.06	4.20	1.05	15.60	8.62	3.91	2.57	16.14
CK (U/L)	206.93[b]	104.91	75.00	463.00	327.24[a]	108.50	126.00	569.00
Glucose (mg/100mL)	106.03[b]	16.99	72.00	146.00	215.22[a]	77.83	104.00	416.00
Lactate(mmol/L)	2.69[b]	1.69	0.85	9.66	7.27[a]	2.07	3.01	11.81
LDH (U/L)	1456.13	208.78	1006.00	1915.00	1467.00	252.35	996.00	2059.00
Globulin (g/100 mL)	2.49[b]	0.59	1.15	3.86	2.70[a]	0.55	1.73	3.84

Different letters indicate significant differences ($p<0.05$) by paired T-test. CK=creatine phosphokinase, LDH=lactate dehydrogenase.

After short- and long-term transport stress, glucose level in blood increases indicating metabolic depletion associated with mobilization of energy reserves (Miranda–de la Lama et al., 2011), as is the case in this study. Therefore, glucose is imperative during a stress response since the animal will expend more energy to maintain homeostasis. Plasma lactate is an indicator of acute stress related to handling conditions, especially physical exercise, agitation and muscular damage (Hambrecht et al., 2005). Acute stress results in the secretion of adrenaline which accelerates pre-slaughter muscle glycogen turnover (Gardner et al., 2006) through its activation of glycogen phosphorylase (Franch et al., 1999) and inhibition of glycogen synthase (Roach, 1990), resulting in elevated plasma lactate concentration, as we have found in this study.

Globulins are a heterogeneous group of proteins which includes antibodies and other inflammatory molecules (Alberghina et al, 2010) and has been suggested as indicator of the animal's immune response (Chorfi et al., 2004). Therefore, the increase in mean globulin indicates altered biological function and a failure in o regain homeostasis. CK is a muscle specific enzyme that plays an important role in the energy metabolism. Several studies use increased levels of CK as indicator of muscular damage, physical activity, and muscular fatigue, as reported in pigs (Hambrecht et al.,

2005), cattle (Early et al., 2012), lambs (Miranda–De La Lama et al., 2011), and goats (Ekiz et al., 2012). The highest CK average in slaughterhouse could have been related to cattle handling during truck loading or transportation. According to Romero et al., (2013) these activities may have increased membrane permeability or harm muscle cells due to the efforts of calves to maintain balance and avoid being thrown against the truck walls during the trip.

In feedlot handling, LDH and glucose were the variables that best explained the variability of the data set, because are positively correlated with PC1 (Table 2). Lactate and CK were the variables with the highest contribution value to PC2 and PC3, respectively. In slaughterhouse handling, the variables that most contribute to PC1 (Dim 1) and explaining the variability of the data were lactate, cortisol and albumin, respectively. LDH was the variable with the highest contribution value to PC2 and CK to PC3 (Table 2). The correlation circles of the variables between the first and the second principal component, the first and the third, and the second and third shown the relationships between the variables for F and S (Figure 1). In feedlot, the first two dimensions explain 49,37% of the variance, while in slaughterhouse 54,10%. The first PC (Dim 1) in F (Figure 1a), which explained 27.20 % of the variation, was negatively correlated to globulin and cortisol, and positively correlated to albumin, lactate, CK, glucose and LDH, and these variables were all correlated with each other. PC1 (Dim 1) in S, which explained 30.80 % of the variation (Figure 1d), was linked with LDH on the negative side and other markers on the positive side. LDH was negatively correlated with cortisol and globulin.

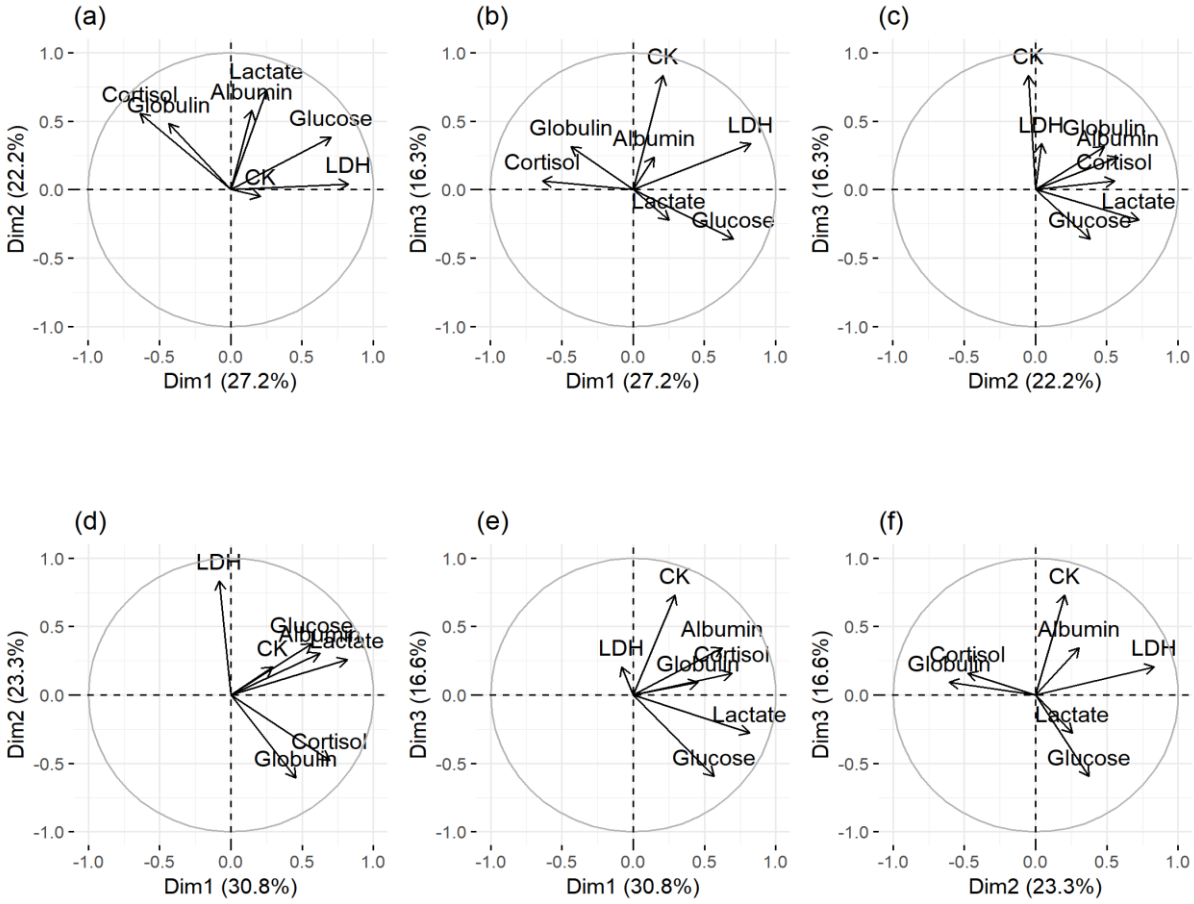


Figure 1. Correlation circle of biomarkers of stress used for the PCA, projected on the first two components (a), on the first and third components (b) and on the second and third components (c) in feedlot handling. Correlation circle of biomarkers of stress used for the PCA, projected on the first two components (d), on the first and third components (e) and on the second and third components (f) in slaughterhouse handling. CK= creatine phosphokinase, LDH=lactate dehydrogenase. Values between parentheses indicate how much the component explains the variance of the characteristics expressed as a percentage.

Table 2. Dimension (Dim) and contribution (ctr %) values of the biomarkers on principal component 1, 2 and 3 in feedlot and slaughterhouse.

	Feedlot						Slaughterhouse					
Biomarker	Dim.1	Ctr	Dim.2	ctr	Dim.3	ctr	Dim.1	ctr	Dim.2	ctr	Dim.3	ctr
LDH	0.83	35.95	0.04	0.12	0.34	9.85	-0.08	0.30	0.83	42.68	0.21	3.65
Glucose	0.70	26.05	0.38	9.44	-0.36	11.26	0.57	14.89	0.37	8.57	-0.59	30.47
Cortisol	-0.64	21.24	0.56	19.89	0.07	0.37	0.69	22.29	-0.48	13.91	0.16	2.17
Globulin	-0.44	9.96	0.48	14.96	0.31	8.63	0.46	9.62	-0.61	22.48	0.09	0.73
Lactate	0.25	3.36	0.73	33.92	-0.22	4.30	0.82	30.94	0.26	4.09	-0.28	6.67
CK	0.21	2.29	-0.05	0.15	0.83	60.67	0.29	3.96	0.20	2.53	0.73	46.05
Albumin	0.15	1.15	0.58	21.53	0.24	4.94	0.62	18.00	0.31	5.74	0.35	10.26

CK=creatine phosphokinase, LDH=lactate dehydrogenase

In feedlot, LDH, glucose and cortisol were the most important biomarkers, that is, with greater ctr in the characterization of the physiological response to stress, while in the slaughterhouse were lactate, cortisol and albumin (Table 2). The stress response in feedlot appeared to be associated with mechanisms that generate an organic response at the hepatic level, because under stressful conditions, cortisol provides the body with glucose by tapping into protein stores via gluconeogenesis in the liver (Wilson, 2013), and LDH is a liver enzyme whose activity changes in response to stress factors and muscle weariness (Brien, 1980; Klieber et al., 2007; Burdick et al., 2011; Nakyinsige et al., 2013).

Lactate and cortisol indicated that the response to stress at slaughter occur at muscle level, with possible negative impact on meat quality, because lactate is an indicator of energy use in muscle (Blache et al., 2011). We observed that the components of stress in feedlot handling and slaughterhouse handling are distinct, that is, the physiological response of the animals varied according to the environment. It is important to consider that the stress response depends not only on the environment, but also on other factors such as genetic and temperament. According to Grandin (1997), the animal's reactions will be governed by a complex interaction of genetic factors and previous experience.

The total contribution to the total variation of each biomarker using the two or three first components showed that at least two biomarkers were common among the three that most contributed in the physiological response to F and S stress (Table 3).

Glucose, LDH and cortisol were the biomarkers with greater contribution in F and globulin, cortisol and LDH in S when the sum of the first two components was considered. The biomarkers that most contributed to the characterization of the physiological response to stress were LDH, glucose and CK in F and glucose, lactate and LDH in S, when the total contribution of the biomarkers was evaluated using the first three components.

Table 3. Contribution of each biomarker in feedlot and slaughterhouse to the total variation.

	Feedlot		**Slaughterhouse**	
Biomarkers	TCTR2	TCTR3	TCTR2	TCTR3
CK	0.66	10.57	1.81	9.43
Albumin	5.09	5.90	6.88	8.59
Globulin	6.03	7.44	10.48	8.33
Lactate	8.45	9.15	8.20	11.59
Glucose	9.17	11.00	6.58	11.63
LDH	9.79	11.40	10.04	10.65
Cortisol	10.19	10.25	10.11	10.57

TCTR2=total contribution for two components; TCTR3=total contribution for three components.

3.2. Identification of a subset of markers

In our study, we have used seven biomarkers that have been previously described in association to several sources of stress (Buckham et al., 2008; Romero et al., 2014). Based on the combination of all biomarkers we observed that 91.25% and 86.25% of the animals were correctly assigned to F and to S, respectively (Table 4).

Table 4. Mismatching percentage (off-diagonal) between feedlot and slaughterhouse of linear discriminant analysis.

	All biomarkers		Predictors of the best logistic regression model used in LDA	
	F	S	F	S
F	**91.25**	8.75	**92.50**	7.50
S	13.75	**86.25**	12.50	**87.50**

F=Feedlot,S=Slaughterhouse

Lactate was the variable that most discriminated between the two situations in relation to the other biomarkers, with around 15% of incorrect assignments from S to F and 7% of incorrect assigning from F to S, respectively. CK had the lowest power of

discrimination, assigning 76% of the animals to S when they belonged to F. All markers except CK and albumin tended to incorrectly assign more animals from S to F than in the opposite direction (Appendix A). Somehow this is an expected result because pre-slaughter stress conditions represent many sources of stress. The result for albumin is within expected range, since it is a negative acute phase protein. However, CK was the worst F source of discrimination.

LDA based on the combination of biomarkers chosen by the best logistic regression model showed that 92.5% were correctly assigned to group F and 87.5% to group S demonstrating that in order to discriminate between F and S only three biomarkers albumin, lactate and glucose are needed as predictors. Thus, cortisol, LDH, CK and globulin may be candidates to be used as indicators of global response to stress (Appendix A).

3.3. Searching for some evidences of a genetic component in the physiological responses

The complexity of physiological or behavioral response to stress comes from the complexity of the mechanisms mediating such responses (Réale et al, 2007; Burdik et al, 2011; Chen et al., 2015) what involves the hipothalamic-pituitary-adrenocortical axis and the sympathetic nervous system (Burdik et al, 2011; Chen et al., 2015). As part of the development of the response individuals developed a coping style to deal with environmental challenges as it could be the handling routines in the farms. Response to stress is known to be influenced by previous experience (Réale et al., 2007) therefore, we could expect coping style to be reproduced at feedlot and slaughterhouse and therefore being discriminated by our set of biomarkers. The final model selected by the multinomial logistic regression was the one with the set of 7 biomarkers of stress with the best Akaike Information Criterion (AIC) and McFadden's R squared.

When we put all the data together (F and S) and tried to discriminate among origin farms we observed that only 49% of the animals were correctly assign to the farm where they came from (Table 5). However, when we separated the data in the two groups, F and S and applied the same model, we observed an increase in the hint-

ratio of discrimination up to 59% and 57%, respectively. In the feedlot the range of success went from 0 in farm 10 to 100% in farms 4 and 11; at S, these values varied between 17% to 100% for farm 6 and farm 4, 11 and 13, respectively. These results suggest to some extent that coping styles within farms exit allowing us to some extent predict correctly the stressor factors that are generating the animal's response. However, when we study more in detail the miss-matching patterns across farms, we have observed that those are mostly produced between farms that exchanged sires, what is to say they have a common genetic background. So these biomarkers are not enough to discriminate between these farms (Appendix B and C).

Several studies of the genetic structure of this population have been done (Vasallo et al., 1989; Gutierrez et al., 2003; Cañas-Álvarez et al., 2014) pointing out that the development of this breed has been based on the recurrent use of sires coming from a reduced number of herds (3-5). In addition, a study performed by Collado et al. (2013) using 17 microsatellites concluded that the Avileña-Negra Ibérica consists of three sub-populations and a loose pool of individuals that constitute an admixture of origins. All these suggest that the miss-matching among farms could also be attributed to an admixture genetic background between these herds what could be understood as an evidence of the genetic background of the response to stress at F and S.

Table 5. Hit-ratio of discrimination between origin farms and hit-ratio of discrimination between origin farms divided in two groups (feedlot handling and slaughterhouse handling).

Origin farms		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Hit-ratio		0.75	0.42	0.50	1.00	0.00	0.42	0.70	0.67	0.56	0.17	1.00	0.00	0.67	0.44
Hit-ratio global	0.49														
Hit-ratio	F	0.50	0.33	0.43	1.00	0.66	0.50	0.80	0.67	0.69	0.00	1.00	0.33	0.67	0.78
	S	0.50	0.50	0.71	1.00	0.33	0.17	0.80	0.67	0.65	0.33	1.00	0.33	1.00	0.44
Hit-ratio global	F	S													
	0.59	0.57													

F=feedlot handling, S=slaughterhouse handling

4. CONCLUSION

Based on blood parameters and PCA analysis, the physiological response to stress was different in feedlot handling and slaughter handling, therefore, the stress response is modulated by different mechanisms depending on the stress period. In feedlot, LDH, glucose and cortisol had the most important in the characterization of physiological stress response whereas at the slaughterhouse lactate, cortisol and albumin were the main characteristics of the response. Lactate, glucose and albumin best discriminate between the two different stress periods. Therefore, there are other four biomarkers (CK, cortisol, LDH and globulin), that could be used as global set of physiological response to stress regardless the source of stress.

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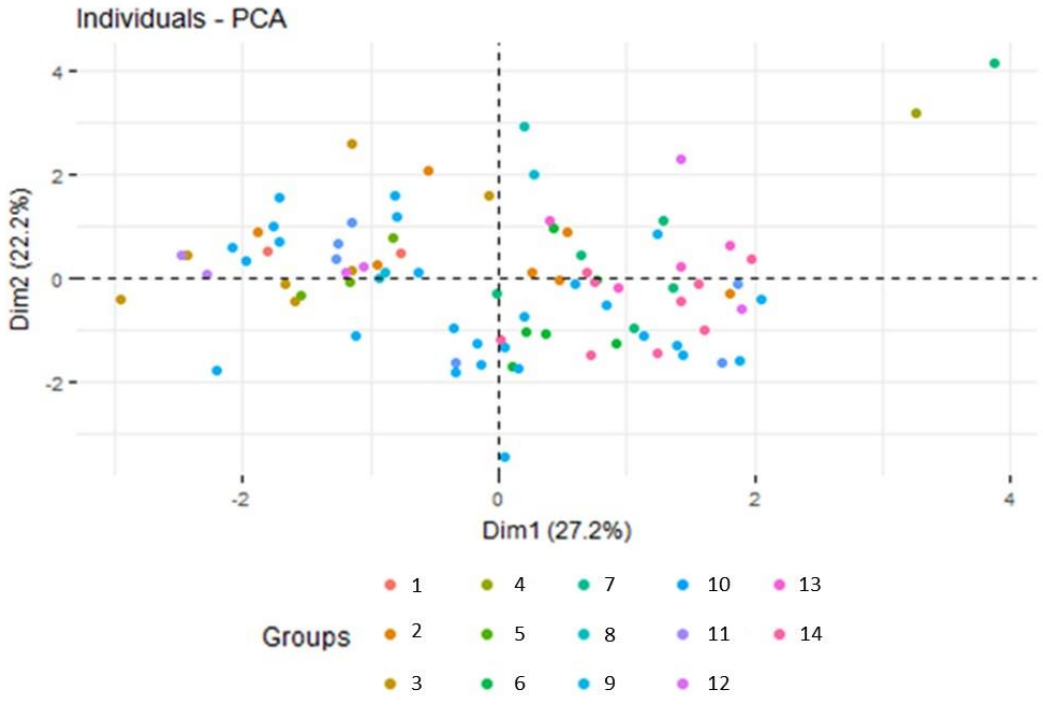
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APPENDIX

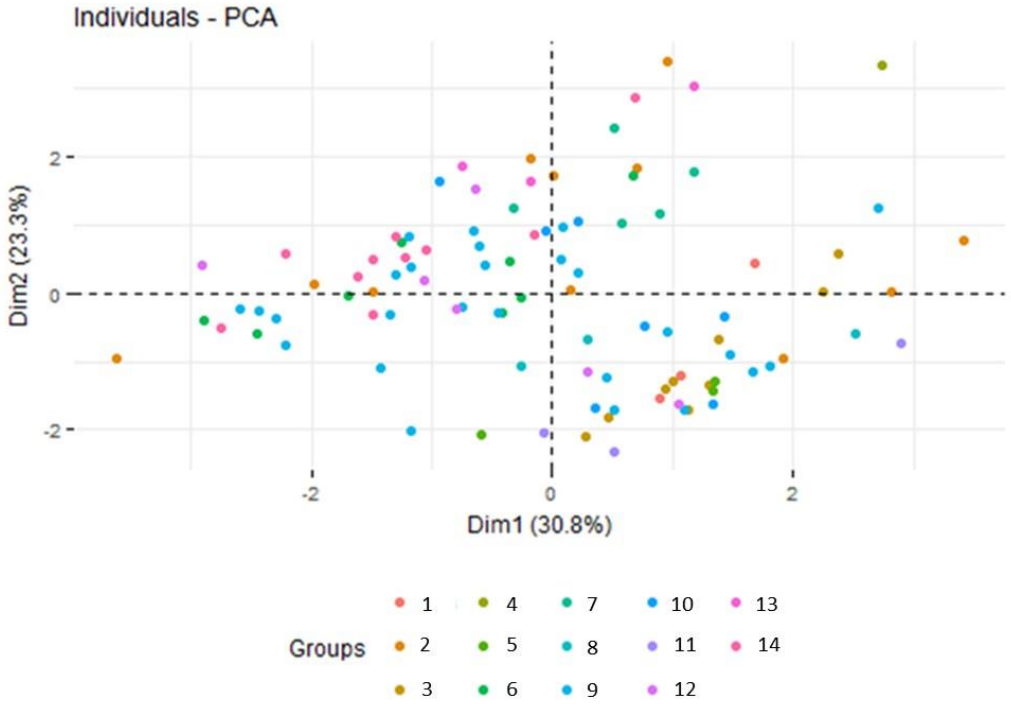
Appendix A. Miss-matching percentage (off-diagonal) between feedlot and slaughterhouse.

	Albumin			Cortisol			CK			Glucose			Lactate			LDH			Globulin	
	F	S		F	S		F	S		F	S		F	S		F	S		F	S
F	53.80	46.25	F	56.30	43.75	F	23.75	76.25	F	56.25	43.75	F	92.50	7.50	F	56.25	45.00	F	57.50	42.50
S	43.80	56.30	S	52.50	47.50	S	22.50	77.50	S	52.50	47.50	S	15.00	85.00	S	53.75	46.25	S	48.75	51.25

F=Feedlot,S=Slaughterhouse



Appendix B. Principal component analysis (PCA) of individuals in feedlot divided by groups based on farm of origin.



Appendix C. Principal component analysis (PCA) of individuals in slaughterhouse divided by groups based on farm of origin.