CHARACTERIZING THE PROTEOMIC AND MITOCHONDRIAL FUNCTIONAL DIFFERENCES BETWEEN NORMAL-pH AND DARK-CUTTING BEEF

By

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CHARACTERIZING THE PROTEOMIC AND

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Title of Study: CHARACTERIZING THE PROTEOMIC AND MITOCHONDRIAL FUNCTIONAL DIFFERENCES BETWEEN NORMAL-pH AND DARK-CUTTING BEEF

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Abstract: Dark-cutting beef is an example of color deviation, in which beef fails to have a characteristic bright-red color. The overall goal of the current research was to determine the biochemical basis of dark-cutting beef. Two separate experiments were conducted to determine differential protein expression and mitochondrial functional differences between normal-pH and dark-cutting beef. Protein profiles were identified using proteomic analysis and mitochondrial respiration function was evaluated using a Clark-oxygen electrode. Mass spectrometry analysis identified 1,162 proteins. Of these, 92 proteins were differentially expressed ($p < 0.05$) between normal-pH and dark-cutting beef. Out of the 92 proteins, we confidently identified 52 proteins whose expression levels changed (by $\geq$ 1.3 fold). Of these, 27 were up-regulated and 25 were down-regulated in dark-cutting beef. Upregulated proteins were involved in mitochondrial function, muscle contraction, and mitophagy while down-regulated proteins were involved in glycogen and energy reserve metabolism processes and associated with phosphorylase kinase activity, calmodulin, and polysaccharide binding molecular functions. In addition, we observed that mitochondria isolated from dark-cutting beef had greater ($P < 0.05$) mitochondrial complex II respiration, state III oxygen consumption and uncoupled respiration ($p < 0.05$), with a greater respiratory control ratio (RCR), and a lower ADP/O ratio compared to normal-pH beef mitochondria. However, no differences ($p > 0.05$) in mitochondrial complexes I and IV respiration, state IV oxygen consumption and mitochondrial membrane integrity from normal-pH and dark-cutting beef. The results from the current study suggest that a greater expression of mitochondrial electron transport chain proteins supports mitochondrial respiratory function in dark-cutting beef, which can influence color development and stability via oxygen consumption. Understanding the biochemical basis will help to elucidate the etiology of the dark-cutting phenotype.

Keywords: Dark-cutting, meat color, proteomics, mitochondria oxygen consumption, mass spectrometry, bioinformatics

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CHAPTER I

LITERATURE REVIEW

Meat color

Meat color is the most important quality attribute that determines consumers' purchasing decisions. Consumers associate a bright cherry-red color of meat with wholesomeness and freshness (Faustman & Cassens, 1990; Mancini & Hunt, 2005). Consumers do not purchase meat when meat surface discoloration reaches 30-40% metmyoglobin (Cornforth, 1994). Jeyamkondan et al. (2000) reported that meat discoloration limits shelf life and marketability of fresh red meat. Thus, retailers either trim off discolored areas or discount prices for discolored meat. As a result, the US meat industry loses approximately $1 billion due to discoloration annually (Smith et al., 2000).

The color of meat is primarily determined by a sarcoplasmic protein called myoglobin (AMSA, 2012; Bekhit & Faustman, 2005; Cornforth, 1994; Faustman & Cassens, 1990). Physiologically, myoglobin functions as an oxygen reservoir in muscle. The molecular structure of myoglobin is composed of 153 amino acids, with a porphyrin ring containing iron. The Iron atom contained within the heme protein is capable of forming up to six coordination bonds (AMSA, 2012). Four of these bonds are coordinated with the pyrrole nitrogen rings. The fifth is attached to the proximal imidazole group of histidine (histidine

residue at position 93), while the sixth is available for ligand binding (Richards, 2013). The heme prosthetic groups consist of a hydrophobic pocket and a globin polypeptide moiety. Iron moiety within the heme can exist in a ferric or ferrous state (AMSA, 2012). The valance state of iron and the nature of the ligand-bound at the sixth coordination site determines meat color (AMSA, 2012). In fresh meat, myoglobin exists in three redox states, namely oxy-, deoxy- and met-myoglobin (AMSA, 2012). Deoxymyoglobin is formed when no ligand is bound to the ferrous heme iron, producing a purple color. The bright-red color results from oxymyoglobin when oxygen is bound as a ligand. Oxidation of both deoxymyoglobin and oxymyoglobin forms brown metmyoglobin, with ferric heme iron and water as the bound ligand. The formation of metmyoglobin is a proton-mediated process (Brantley et al., 1993; Wallace et al., 1982). Low oxygen concentrations less than 7 mm Hg (about 1-2% oxygen) facilitate metmyoglobin formation (AMSA, 2012). In addition to myoglobin, other sarcoplasmic proteins such as hemoglobin and cytochrome *c* also contribute to meat color (Ahn and Maurer, 1990; Yu et al., 2017).

Other factors such as species-specificity, environment, slaughter processes, and storage conditions can influence meat color. In addition, variability in meat color between seasons is attributed to differences in physical activity, stress, and nutritional status of the animal (Kadim et al., 2004; Kim et al., 2003). The inherent biochemical profiles of different muscles also determine meat color stability (Faustman and Cassens, 1990; Mckenna et al., 2005; Salim et al., 2019). Further, differential abundance of sarcoplasmic proteins in color-labile compared with color-stable muscles influences their color stability characteristics

(Canto et al., 2015). Additionally, Abraham et al. (2017) showed that the inherent differences in metabolic profiles contribute to differences in muscle-specific redness and metmyoglobin reducing ability (MRA) in beef longissimus compared to psoas muscles under retail display conditions.

Biochemical changes during pre- and post-mortem environments influence postmortem muscle-pH and meat color. For example, depletion of muscle glycogen before slaughter limits pH decline, and is associated with a high ultimate muscle pH greater than 5.6 (Mancini & Hunt, 2005; Rosenvold et al., 2001). A greater muscle pH can result in dark-colored muscles due to decreased myoglobin oxygenation (Ashmore et al., 1971). Additionally, high pH levels allow muscle proteins to hold more water limiting the muscle's capacity to scatter light and resulting in darker muscle color (Lawrie, 1958 & MacDougall, 1982).

Dark-cutting beef

Dark-cutting beef is a meat quality defect in which meat does not display a characteristic bright-red color typical in normal-pH beef (Apple et al., 2011; Lawrie, 1958; McKeith et al., 2016; Wills et al., 2017). About $165-$170 million is lost by the USA beef industry due to dark-cutting carcasses (McKenna et al., 2002). In the 2011 National Beef Quality Audit, 3.2% of the total carcasses assessed were dark- cutters (Moore et al., 2012). However, in the 2016 National Beef Quality Audit, the incidence of dark-cutting beef declined with the highest of 0.74% in October and the lowest of 0.33% in January (Boykin et al., 2017). In Canada, dark-cutting beef is graded as B4 with a reduction in carcass price

of 50 cents per pound causing losses of up to $1.4 million annually to Canadian beef producers. The incidences of carcasses graded B4 in Canada was reported to increase by 1.3% in 2011 from 0.8% in 1998 (Holdstock et al., 2014). Brown et al. (1990) reported that about 4.1% of carcasses slaughtered in the United Kingdom were dark-cutters and the cause was associated with short and long transport distance. In South Chile, incidences of dark-cutting beef were reported at about 17- 40% per year (Gallo et al., 2003). Therefore, dark-cutting beef presents economic challenges to the beef industry worldwide. Thus, understanding the mechanism of occurrence may help mitigate this problem.

The occurrence of the dark-cutting condition is associated with a high ultimate post-mortem muscle-pH in excess of 5.8. (Mahmood et al., 2018; Ponnampalam et al., 2017; Wulf et al., 2002). The depletion of glycogen before slaughter limits muscle metabolism, preventing muscle pH decline below 6.0 (Briskey et al., 1966; Ferguson and Gerrard, 2014). Muscles with a high ultimate pH have reduced levels of glucose-6-phosphate and low rates of anaerobic glycolysis (Baker et al.,2005; Briskey et al., 1966; Jensen et al., 2011). Thus, the decline in muscle glycolysis limits the muscles' capacity to form lactate.

Lactate accumulation in the muscle initiates post-mortem pH decline. Additionally, Campbell (1993) reported that lactate formed during post-mortem glycolysis is mainly utilized in the regeneration of nicotinamide adenine dinucleotide residues (NAD^+), a co-enzyme required in glyceraldehyde phosphate dehydrogenase activity. This enzyme initiates glycogen degradation pathways, producing glucose-6-phosphate (Komoda and

Matsunaga, 2015). The formed glucose-6-phosphate is utilized in the production of lactate post-mortem. Therefore, impaired glycolytic metabolism can influence post-mortem muscle pH through reduced lactate accumulation.

Ashmore et al. (1971) noted that high ultimate muscle pH could result in sustained mitochondrial function post-mortem. Increased mitochondrial function can increase mitochondrial oxygen consumption while decreasing available oxygen for blooming. Therefore, the biochemical and physical characteristics of dark-cutting beef result from a high ultimate muscle pH which can influence muscle metabolism and color development post-mortem.

Several other factors are also associated with the occurrence of dark-cutting beef. Guilbert (1937) postulated that the causes of dark-cutting beef are connected to hereditary factors. However, Lawrie (1958) suggested that the occurrence of dark-cutting beef is associated with stress prior to slaughter. Different stressors pre-slaughter, especially during transport, reduce muscle glycogen reserves resulting in elevated pH levels post-mortem. Recently, Jerez-Timaure et al. (2019) showed that an overexpression of genes involved in stress-related signaling pathways such as growth arrest and DNA damage-inducible beta (GADD45B), protein-lysine methyl transferase (METTL21C) and amine oxidase [Flavin-containing] A (MAOA), contribute to the high muscle pH by regulating calcium signaling and oxidative metabolism.

On-farm factors such as nutrition, feeding systems, gender and climatic conditions also contribute to the occurrence of dark-cutting beef. For example, the prevalence of dark-cutting beef is higher in bulls than in steers or heifers (Martin et al., 1978). Bulls are highly susceptible to stress compared to heifers (Field, 1971). Animals finished on pastures are more likely to produce dark-colored meat compared with feedlot animals (Muir et al., 1998). This is attributed to low energy levels of pasture-based diets (Sainz et al., 1995). In addition, animals finishing on pastures have high-energy demands for digestion and locomotion while grazing, which reduces their glycogen reserves. Seasonal variations in pasture distributions and environmental weather conditions may also contribute to dark-cutting beef. Kim et al. (2003) reported that animals slaughtered in winter seasons with temperatures below 5°C are more likely to produce undesirable meat color characteristics compared to those slaughter at warmer temperatures. However, high temperatures especially very hot summers can also increase dark-cutting conditions.

Although several factors are connected with the occurrence of dark-cutting beef, the mechanistic basis is still unknown. Therefore, employment of comprehensive analysis at a molecular level using modern analytical tools such as proteomics, metabolomics, and genomics may widen our understanding of the dark-cutting problem.

Mitochondrial structure and biochemistry

Mitochondria are double membrane-bound organelles responsible for maintaining cellular energy homeostasis through the production of adenosine triphosphate (ATP). Live

cell microscopic studies revealed that mitochondria are highly dynamic cellular organelles capable of building large interconnected intracellular networks (Bereiter-Hahn, 1990), with numerous fusion and fission processes (Westermann, 2010). The outer membrane of the mitochondria appears as a continuous sheath with different physical and chemical composition from the inner membrane. The inner mitochondrial membrane is folded into numerous structures called cristae (Chappell, 1968). The surface membrane of the cristae creates a proton gradient necessary for oxygen diffusion, supporting mitochondrial respiration (Osellame et al., 2012). Various proteins including proteins involved in redox and oxidative phosphorylation pathways, ATP synthase complex, and transport proteins are embedded in the inner mitochondrial membrane (Chappell, 1968).

Large conductance pores called permeability transport pores (PTP) span the inner mitochondrial membrane and are responsible for regulating mitochondrial membrane permeability and function (Bernardi and Di Lisa, 2015; Osellame et al., 2012; Palmer et al., 2011). The opening of mitochondrial membrane pore is associated with the collapse of mitochondrial membrane potential, ATP depletion, and rapid progression to cell death (Palmer et al., 2011). Changes in mitochondrial membrane permeability result from releases of cytochrome *c*, a major electron transport protein capable of initiating apoptosis (Bernardi and Di Lisa, 2015; Kroemer et al., 2007; Nilsson et al., 2009). When released, cytochrome *c* binds to Apoptotic protease-activating factor 1 (Apaf-1) exposing card domains of Apaf-1 proteins (Osellame et al., 2012). The combination of cytochrome *c* and Apaf-1 complex recruits procaspase-9, forming an active apoptome, which in turn,

activates apoptotic caspase 3 proteins. Apoptotic caspase 3 proteins, can attack other proteins causing changes in mitochondrial cell metabolism and programmed cell death (Bernardi and Di Lisa, 2015; Osellame et al., 2012).

The proton gradient across the mitochondrial membrane is maintained by electron transport and active transfer of calcium (Ca^{2+}) ions in and out of the matrix (Chaudhuri et al., 2013). Ca^{2+} ion movement into the mitochondrial matrix is proposed to up-regulate the rate-limiting enzymes of the tricarboxylic cycle (TCA) necessary for increasing cellular energy levels. Jouaville et al. (1999) showed that NAD-isocitrate, pyruvate and α-ketoglutarate dehydrogenases contained in the mitochondrial matrix are up-regulated by excess Ca^{2+}ion uptake, which increases TCA substrate oxidation. However, an increase in mitochondrial enzymatic activity depends on constant supply of TCA substrates from complete oxidation of sugars, fats, and proteins (Osellame et al., 2012). The reduced cofactors (NADH and FADH) resulting from such processes are shunted into the mitochondrial shuttle system across a series of multi-subunit enzyme complexes (mitochondrial electron transport chain proteins) embedded in the inner mitochondrial membrane for ATP production (Wedell and Merker, 1964; Collins et al., 2012).

The mitochondrial electron transport chain is made up of four protein complexes. Electrons picked up by NADH and FADH are transferred from NADH-Coenzyme Q-reductase (Complex I) to succinate dehydrogenase (complex II), cytochrome bc1 (complex III) and finally to cytochrome *c* oxidase (Complex IV; González et al., 2005; Jung et al., 2002; Osellame et al., 2012; Palmer et al., 2011). Movement of electrons along the electron

transport chain reduces electron free energy with a decrease in the redox potential of their carriers (Osellame et al., 2012). Oxygen at complex IV with a high redox potential picks up the electrons and forms water (González et al., 2005). The structure, content, and function of the mitochondria are thus most important in determining the efficiency of muscle metabolism (Erlich et al., 2016). Therefore, changes in mitochondrial structure can reduce mitochondrial bioenergetics, influencing muscle metabolism post-mortem.

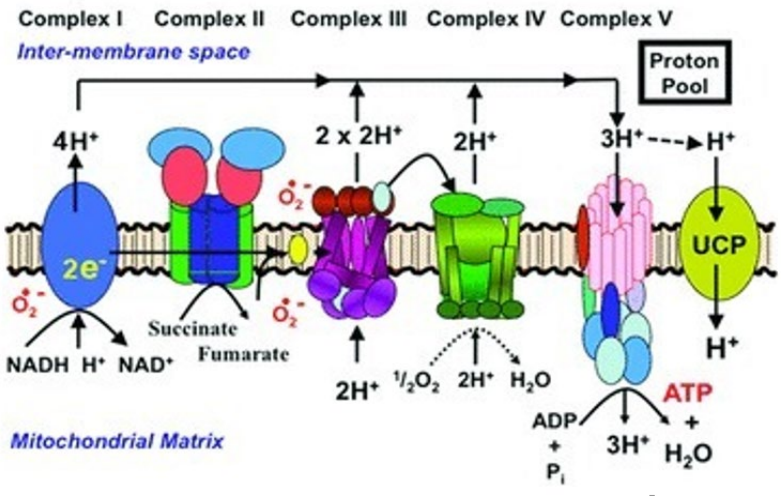

Figure 1: Schematic diagram of the mitochondria electron transport chain

Mitochondria and meat color

Mitochondria influences meat color development and stability via oxygen consumption and metmyoglobin reducing activity (Tang et al., 2005a). Oxygen consumption by mitochondria competes with myoglobin for oxygen. Thus, increased oxygen consumption can produce dark-colored muscles (McKeith et al., 2016). However, mitochondrial respiration can also provide reducing equivalents necessary for

metmyoglobin-reducing activity, promoting color stability (Mitacek et al., 2019). More specifically, electrons available between complex III and IV of the electron transport chain mediate metmyoglobin-reduction (Tang et al., 2005a). Additionally, the reduction of mitochondrial pyridines via reversal electron transport chain can produce NADH needed for metmyoglobin reductase activity (Belskie et al., 2015).

Mitochondrial oxygen consumption varies across muscles and tissues. Madhavi and Carpenter (1993) suggested that the differences in color stability between *semimembranosus* (color stable) and *psoas major* (color labile) are associated with a greater mitochondrial density and oxygen consumption. However, Lanari and Cassens (1991) reported a reverse relationship with relatively greater mitochondrial density and oxygen consumption in muscles of low color stability (*gluteus medius*) compared to color stable *longissimus dorsi*. The authors attributed that greater mitochondrial density and oxygen consumption of the low color stable muscle (*gluteus medius*) to greater enzyme activity. However, Kelly and Scarpulla (2004) suggested that mitochondrial number in cells is regulated in adaptation for changes in metabolic demand. Therefore, muscles with a high-energy demand are more likely to have greater mitochondrial content with lower color stability.

The functionality of mitochondria is important for meat color development and stability. Loss of mitochondria function affects myoglobin stability through reduced regeneration of NADH and metmyoglobin reducing ability (Ramanathan et al., 2012; Tang et al., 2005a). Biochemical changes in mitochondrial electron transport pathways from a

coupled state to an uncoupled state can produce reactive oxygen species (ROS). (Barbour and Turner, 2014; Benedetti et al., 2015; Bonekamp et al., 2015). These ROS can cause oxidation of myoglobin (Ladikos and Lougovois, 1990). Additionally, myoglobin demise by oxidation of lipids within the mitochondrial membrane forming various alkenals, aldehydes, and hydroxyl alkenals, which are highly reactive and cause cytotoxic effects to proteins (Esterbauer et al., 1991; Tang et al., 2005b). Therefore, changes in mitochondrial functionality may influence meat color development and stability by inducing changes in myoglobin structure and functionality.

Mitochondria and substrates

Mitochondrial oxidative phosphorylation is influenced by the nature of cellular substrates including fatty acids, proteins, and carbohydrates utilized by the cells. Changes in substrates levels may result in decreased mitochondrial function. (Bendall, 1979; Mancini et al., 2018). .

Mitochondria can utilize substrates via a series of catabolic pathways. For example, $NADH^{+}$ is oxidized via three sequential coupling sites including complex I, II, and IV while oxidation of $FADH_2$ involves only complex III and IV (Leverve and Fontaine, 2001). Although NADH is a key substrate for mitochondrial respiration, it is impermeable to the inner mitochondrial membrane. NADH transport into mitochondria thus occurs via malate/aspartate and glycerol-3-phosphate/dihydroxyacetone phosphate shuttle systems (Leverve and Fontaine, 2001). In meat, NADH is a key substrate in determining color stability through non-enzymatic and enzymatic metmyoglobin- reduction processes

(Echevarne et al., 1990; Kim et al., 2006). Regeneration of NADH from NAD^+ when added to ground beef, resulting in increased metmyoglobin-reduction (Watts et al. 1966).

Succinate is the main mitochondrial respiratory substrate oxidized by succinate dehydrogenase. Mitochondrial oxygen consumption in presence of succinate as a substrate is high due to the capacity of succinate to enter directly into complex II of the mitochondrial electron transport (Hederstedt, 1999). Several reports have described the role of succinate in mitochondrial function and metmyoglobin-reduction (Ramanathan and Mancini, 2010; Tang et al., 2005a). Wollenman et al. (2017) reported a lower mitochondrial oxygen consumption (20-35 nmol O_2/mg/min) when using non-succinate substrate combinations compared with succinate (100-150 nmol O_2/mg/min).

Lactate is another important energy substrate in mitochondrial cellular respiration (Pallotta et al., 2004). Mitochondria can utilize extraneous lactate as energy sources increasing cellular respiration and oxygen consumption (De Bari et al., 2004). Lactate is transported into the mitochondria by monocarboxylic transporters, where it is oxidized to pyruvate under catalysis of lactate dehydrogenase, producing NADH (Atlante et al., 2007; Brooks et al., 1999; Passarella et al., 2014). Pyruvate is another mitochondrial substrate mainly oxidized via the tricarboxylic acid cycle and electron transport chain (De Bari et al., 2004). Therefore, substrates utilized by the mitochondrial can modify mitochondrial respiration.

Relationship between mitochondria and myoglobin function

Several reports have described the relationship between mitochondria and myoglobin function (Lanari and Cassens, 1991; Tang et al., 2005b; Postnikova et al., 2009; Ramanathan and Mancini, 2018). Myoglobin is loosely bound to the lipid layer in the outer mitochondrial membrane (Criddle 1969). The association of myoglobin with the mitochondrial membrane allows transfer of molecular oxygen to mitochondrial enzymes, facilitating mitochondrial respiration.

Myoglobin functions as an oxygen reservoir and carrier in body systems (Jürgens et al., 2000; Mancini and Ramanathan, 2014). The structural arrangement of oxymyoglobin facilitates oxygen release in cells (Wittenberg and Wittenberg, 2003). The exchange of oxygen between oxymyoglobin and mitochondria occurs at the mitochondrial outer membrane when the oxygen partial pressures are between 2-5mm Hg (Postnikova et al., 2009). The increase in oxygen flux into mitochondrial cells results from a greater mitochondrial cytochrome *c* oxidase affinity for oxygen than myoglobin's affinity to bind oxygen. (Sidell 1998). Thus, increased mitochondrial competition for oxygen can limit development of a cherry bright-red colored muscle. However, mitochondria's capacity to function effectively depends on myoglobin's ability to provide molecular oxygen. Therefore, a balanced mitochondrial activity is necessary for enhanced bright and stable red color.

Proteomic approaches in meat quality research

Comparing changes in protein expression has shown promise in understanding molecular and cellular mechanisms that regulate meat characteristics such as tenderness (Bjarnadóttir et al., 2012; Zhao et al., 2014), quality (Lametsch & Bendixen, 2001), and color (Canto et al., 20s15; Joseph et al., 2012; Maheswarappa et al., 2016; Mahmood et al., 2018)). The majority of these proteomic studies have utilized both comparative and expression proteomics approaches. These are usually based on protein separation in at least two dimensions, using a chromatographic or electrophoresis method and then followed by mass spectrometry. Bouley et al. (2004) reported detection of about 500 reproducible protein spots in bovine *semitendinosus* muscles while using two gel electrophoresis (2-DE) and mass spectrometry. The coupling of 2-DE techniques with MS is for large scale identification of protein mixtures and analysis of differential protein expression between two or more samples.

As mentioned above, possible relationships between meat color and differential protein expression have been mostly characterized in 2-DE proteomic studies. Greater abundance of antioxidant proteins and chaperones proteins were observed in color-stable muscles compared to color-liable muscles (Joseph et al. 2012). In addition, Nair et al. (2016) observed a positive correlation between creatine kinase M-type and triosephosphate isomerase with metmyoglobin reducing activity; while Canto et al. (2015) noted an over-abundance of myofibrillar proteins (Myosin regulatory light chain 2 and myosin light 1/3) in color-stable compared with color-labile muscles.

While several studies have utilized 2-DE in differential proteomics, new techniques for measuring differential protein expression have been developed. These involve incorporation of non-perturbed protein extracts with different stable isotopes mostly with carbon and nitrogen (Pratt et al., 2002). Tandem mass tag labeling for example was employed by Yu et al. 2017 to quantify phospho-proteomic differences among muscles of different color stability. Utilizing a combination of proteomic methods, we may be able to understand molecular mechanisms that underlay the occurrence of dark-cutting beef.

Conclusion

The occurrence of dark-cutting phenotype in meat is associated with elevated pH levels resulting from depletion of glycogen post-mortem. The high ultimate pH influences the biochemical and physical properties of post-mortem dark-cutting beef. However, the mechanistic basis for the occurrence is still unknown. Despite increased applications of proteomics tools in understanding post-mortem metabolism, limited research has characterized the proteomic profile expressional and mitochondrial functional differences between normal-pH and dark-cutting beef (high pH beef). Therefore, the overall objective of this research was to characterize protein expression and mitochondrial functional differences between normal-pH and high-pH beef, to better understand the molecular and mechanistic basis for the occurrence of dark-cutting phenotype.

CHAPTER II

DIFFERENTIAL PROTEIN EXPRESSION OF NORMAL-PH AND DARK-CUTTING BEEF

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Abstract

Dark cutting beef is a meat quality defect in which meat does not display a marketable bright-red color due to high post-mortem muscle pH. Various proteins are involved in post-mortem muscle pH development, and the mechanistic basis for greater muscle pH is not clear. Biochemical properties and differentially expressed proteins (DEPs) of normal-pH and dark-cutting beef were analyzed using LC-MS/MS mass spectrometry-based proteomics. Mass spectrometry analysis identified 1,162 proteins. Of these, 92 proteins were significantly differentially expressed between normal-pH and dark-cutting beef samples ($P < 0.05$). We identified 52 of the 92 proteins whose expression levels changed (by ≥ 1.3 fold). Of these, 27 were up-regulated and 25 were down-regulated in dark-cutting beef. Upregulated proteins were involved in mitochondrial function, muscle contraction, and mitophagy while down-regulated proteins were involved in glycogen and energy reserve metabolism processes and associated with phosphorylase kinase activity, calmodulin, and polysaccharide binding molecular functions. The results suggest that increased abundance of mitochondrial proteins and lower expression of glycogen and energy metabolism may be responsible for greater muscle-pH and associated dark meat color.

Keywords: Dark-cutting, Proteomics, Mass spectrometry, Meat color, Bioinformatics

Introduction

Meat color is the most important quality attribute determining consumers' purchasing decisions in terms of wholeness and freshness (Faustman & Cassens, 1990; Mancini & Hunt, 2005; Smith et al., 2000). Dark-cutting beef is a meat quality defect in which meat does not display a characteristic bright-red color typical in normal-pH beef when the cut surface is exposed to oxygen (Apple et al., 2011; McKeith et al., 2016; Wills et al., 2017). The color of meat is primarily determined by myoglobin. Depending on the redox state and ligand-bound, myoglobin can exist in oxy-, deoxy- or metmyoglobin producing bright-red, purple, or brown, respectively (AMSA, 2012). Therefore, development of bright-red color in meat is highly dependent on the extent of myoglobin oxygenation.

The typical dark-red color in dark-cutting beef is associated with post-mortem muscle pH greater than 5.8 (Ashmore et al., 1971; Ponnampalam et al., 2017; Wulf et al., 2002). Various researchers noted that dark-cutting beef has lower glycogen content compared with normal-pH beef (Mahmood et al., 2018). Further, depletion of muscle glycogen ante-mortem reduces muscle metabolism and pH decline (Chauhan and England, 2018; England et al., 2015; Li et al., 2017; Shen and Du, 2005). Previous research also noted that high ultimate muscle pH could sustain mitochondrial function post-mortem, influencing myoglobin redox stability via oxygen consumption (Ashmore et al., 1971). Hence, greater mitochondrial oxygen consumption can promote myoglobin deoxygenation, producing dark-colored meat.

The evidence for the model that the occurrence of dark-cutting beef is due to depleted glycogen ante-mortem is primarily based on a high post-mortem muscle pH greater than normal. However, the mechanistic basis for the occurrence is still not clear. Several mitochondrial and glycolytic proteins are involved in muscle metabolism and pH decline (Bowker et al., 2004; Chauhan and England, 2018; Estrade et al., 1994; Newbold and Scopes, 1971). For example, a decrease in abundance of phospho-fructose kinase (PFK) and lactate

dehydrogenase B (LDH-B) is associated with a slow post-mortem pH decline (Canto et al., 2015; Jerez-Timaure et al., 2019; Nair et al., 2016). A recent study suggested that atypical dark-cutting beef (based on Canadian beef grading system) had defective glycolytic enzymes compared with normal colored beef (Mahmood et al., 2018).

Despite increased applications of proteomics tools in understanding post-mortem metabolism, limited research has evaluated the protein expression profiles of normal-pH vs dark-cutting beef. Therefore, the objective of the current research was to characterize protein profiles of normal- and high-pH beef *longissimus* muscle.

Materials and Methods

Materials and reagents

Corning Costar 96 well black flat-bottomed polystyrene plates, trypsin, guanidine-HCL, chloroacetamide (CAA), tryptophan, HEPES, tris-hydroxymethyl aminomethane hydrochloride (Tis-HCL) was obtained from Sigma-Aldrich Chemicals CO. (St. Louis, MO). All chemicals were of reagent analytical grade or greater.

Characterizing the biochemical properties of normal-pH and dark- cutting beef

Six USDA Low Choice and six dark cutting (no-roll) beef strip loins (IMPS #180, NAMP, 2002) were purchased from Tyson Fresh Beef Plant in Amarillo, TX, within 3 d of harvest. Care was taken to avoid variation between loins within each pH category, and the average pH of loins were 5.6 and 6.4 for normal-pH and dark-cutting, respectively. Loins were transported on ice to Oklahoma State University, Food and Agricultural Products Center. Following arrival, three 2.54-cm thick steaks were cut from the anterior end of each loin and randomly assigned for analyses. The first steak was assigned to proteomic analysis and mitochondrial content determination, the second steak was assigned to measure color and to

determine biochemical properties of steaks, and the third steak was used to determine pH and proximate composition.

Mitochondrial protein content was determined utilizing differential centrifugation on fresh tissue according to method of Ke et al. (2017). Fresh-cut surface of each steak was allowed to oxygenate at 4 °C for 2 h. The surface color was measured using a HunterLab MiniScan XE Plus spectrophotometer (HunterLab Associates, Reston, VA) with a 2.5 cm diameter aperture, Illuminant A, and 10° standard observer. CIE L* (indicates lightness/darkness), and a* (redness)values were used to characterize the surface color. Following surface color measurements, each steak was cut into half. The first half was used to estimate oxygen consumption and the second half was used to determine metmyoglobin reducing activity (Ramanathan et al., 2019). Reflectance approach was utilized to determine oxygen consumption and metmyoglobin reducing activity. pH was recorded using an Accumet 50 pH meter (Fisher Scientific, Fairlawn, NJ), and proximate composition was determined using a NIR-based method (English et al., 2016).

Protein extraction

Freeze-dried skeletal muscle tissue samples of 0.5 g from six (n = 6) normal-pH and six (n = 6) dark-cutting beef were extracted in 5 mL of lysis buffer (6 M guanidine hydrochloride, 100 mM HEPES, 50 mM chloroacetamide (CCA), 10 mM TRIS (2-carboxyethyl)phosphine TCEP, pH 8.0). Tissue lysates were homogenized using a small tissue teaser (Model 985370-395, Biospec product, INC) for 2 min. Subsequently, homogenized tissue sample slurry was boiled for 10 min in a water bath and cooled down to room temperature. Cooled samples were sonicated in a bioruptor diagenode at high setting with 30 s on, 30 s off cycles for 15 min. Subsequently, sonicated tissue lysates were centrifuged at 10,000 g at room temperature for 10 min. Extracted samples were kept at -80°C for further analysis.

Protein concentration

Protein concertation was determined using a tryptophan fluorescence standard assay according to Wišniewski and Gaugaz (2015) with minor modifications. Stocks of tryptophan standard were prepared in guanidine lysis buffer (guanidine hydrochloride, 100 mM HEPES, 50 mM chloroacetamide (CCA) and 10 mM TRIS- (2-carboxyethyl) phosphine TCEP, pH 8.0), and diluted before analysis. The fluorescence of the whole protein samples was measured using a Corning Costar 96 well black flat-bottomed polystyrene plate (Sigma-Aldrich, CLS3915). Protein lysates and standards were directly spiked into the 96 well plate prefilled with 200 µL of lysis buffer. Fluorescence was excited at 295 nm, and the emission was recorded at 350 nm using a microplate reader SpectraMax M_3 (Molecular Devices, LLC. San Jose, CA, USA). The microplate reader was installed with the tryptophan fluorescence block programmed to shake the plate at 25°C for 15 s and resting for 2 s. Protein concentration was calculated (assuming 0.0117 g of tryptophan per gram of total protein).

Protein digestion and peptide mass spectrometry analysis

Tryptic peptides from extracted crude protein lysates were generated using a fast aided sample preparation method (FASAP) as described previously by Wiśniewski, et al. (2009). Samples were digested with 4 µg/mL trypsin/Lys-C (Promega V5072) overnight at 37°C, with an additional 2 µg/mL of protease, and samples digestion were continued for another 8 h. Digested normal-pH and dark-cutting beef trypsinolytic peptides were acidified to 1% trifluoroacetic acid. Trypsinolytic peptides were then purified by solid-phase extraction with C18 affinity media (Agilent OMIX tips) and analyzed on an LTQ-Orbitrap mass spectrometer (Thermo Scientific) coupled to a New Objective PV-550 Nano-electrospray ion source and Eksigent NanoLC-2D chromatography with a vented trap column configuration as previously described by Voruganti et al. (2018).

Database searching

MaxQuant (V1.5.3.8) was used to perform peak analysis and searching the generated raw LC-MS/MS data files against a reference proteome database of 23,968 bovine proteome sequences downloaded from Uniprot in March of 2018. Default MaxQuant settings were used with the addition of a match between runs (Cox et al., 2009). These parameters were as follows: full trypsin specificity with no more than two missed cleavages allowed, carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine as the variable modification. The precursor ion mass tolerance of 20 ppm and a fragment ion mass tolerance of 0.3 Da was used for all MS/MS spectra obtained. The sequence of common contaminates was included in the searches.

Label-free quantification (LFQ) intensity data files were analyzed using Perseus software (V.1.6.3.3). Protein groups were filtered for potential contaminants. Fold changes of the differentially expressed proteins were filtered out on a volcano plot using a t-Test. Significant changes of protein expression were determined at cut off of $P \leq 0.05$ of the log2 expression values. A parameter power calculation analysis with a targeted statistical power of 80% (https://www.dssresearch.com/knowledgeCenter/toolkitcalculators/statisticalpowercalculator.aspx) was used to determine the magnitude of change in protein expression between dark-cutting and normal-pH beef that could be validated for 6 samples, to enable bioinformatics pipeline analysis.

Bioinformatics analysis of the differentially expressed proteins (DEPs)

The proteomic results were analyzed by multiple approaches. David version 6.8 (https://david.ncifcrf.org/) was utilized to classify gene ontology enrichment analysis of the functional categories of DEPs. In addition, the Go database was used to identify the subcellular locations of the differentially expressed proteins. The background list for go-analysis comprised of a gene list of all proteins quantified in both normal-pH and dark-cutting beef

samples to avoid sampling bias. Cytoscape (V.3.7.1; https://cytoscape.org/) was employed to query the STRING plugin database of protein-protein interaction (PPI), and the nodes of the subnetworks were used to create protein-protein interaction networks of the corresponding DEPs up-and down-regulated in dark-cutting beef relative to normal- pH beef. Key pathways involved in post-mortem metabolism (glycogen metabolism and mitochondrial electron transport) pathways were analyzed using the wiki pathway and visualized in Cytoscape (V.3.7.1; https://cytoscape.org/).

Non-specific peptide cleavage searching to differentiate in-situ versus trypsinolytic proteolysis

To determine differences in non-specific peptide cleavage/in-vivo proteolysis in normal-pH and dark-cutting beef, non-specific cleavage peptide mapping and fragmentation analyses were compared. Collected raw data files generated from the differential protein expression study were loaded into MaxQuant (V1.6.2.10), and semi-specific trypsin cleavage searching was done. Search parameters were set as follows: semi-trypsin specificity with no more than two missed cleavages allowed, carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine as a variable modification. The precursor ion mass tolerance of 20 ppm and a fragment ion mass tolerance of 0.3 Da was used for all MS/MS spectra obtained. Generated semi-specific trypsin cleavage raw data file intensities were normalized and imported into Perseus (V1.6.2.1). Peptide intensity ratios were calculated to avoid the domination of the data by only the major peptides. Calculated ratios were kept separate for all the six samples for statistical analysis. Combined median peptide intensity ratios were then determined, and a 1-sample t-test was calculated. Significant changes of peptide intensities were determined at cut off of $P \leq 0.05$ of the log2 expression values.

Statistical Analysis

A completely randomized block design was utilized to determine the effects on meat color and biochemical parameters. Each loin served as a block. The fixed effect included normal-pH and dark-cutting beef, and the random effect included loin. The data were analyzed using the Mixed Procedure of SAS. Least square means were separated using the PDIFF option and were considered significant at $P < 0.05$. For the proteomics analyses, details included in the previous section.

Results

Biochemical properties of normal-pH and dark-cutting beef

The biochemical properties and mitochondrial content in normal-pH and dark-cutting beef are summarized in Table 1. Consistent with previous findings, dark cutting beef had a greater pH ($P < 0.001$) than normal-pH beef (X = 6.3, SD 0.06). However, the increase in muscle pH can affect color (lower L* values/lightness), redness (lower a* values), which had lower values ($P < 0.001$) and oxygen consumption, and metmyoglobin-reducing activity which had greater values ($P < 0.05$) compared with normal-pH beef. In addition, dark-cutting beef had greater ($P < 0.05$) mitochondrial content than normal-pH beef.

Protein identification and differentially expressed proteins (DEPs)

To characterize the differences in protein expression between normal-pH and dark-cutting beef, proteomic expression profiles were measured. The proteomic analysis yielded 1,162 proteins quantified from in normal-pH and dark-cutting beef samples. The t-test revealed that 92 proteins (11.06 % of the total 832 proteins remaining after data filtering) showed significant changes in expression at a cutoff of $P \leq 0.05$ (Figure 2 A and 2 B). Out of the 92 DEPs, 53.3% were up-regulated, while 46.7% were down-regulated in dark-cutting beef (Figure 2 C).

To assess what magnitude of change in protein expression that could be validated for 6 samples, we utilized a power analysis calculation with a target of 80%. Results revealed validation of changes in protein expression between < 0.77 to > 1.3 (Log2 +/-0.3). Using this cutoff, 51 proteins (55.4% of the 92 DEPs proteins) showed changes in expression with a magnitude of 1.3 at P-value cut-off of 0.05 (Figure 2 D). Out of these 51 DEPs, 27 (52.9%) were up-regulated while 25 (47.1%) were down-regulated in dark-cutting beef (Figure 2D).

Gene ontology analysis of the differentially expressed proteins (DEPs)

To determine the biological process of DEPs in normal-pH compared with dark-cutting beef and to understand the pathways affected (Kyoto encyclopedia of genes and genomes; KEGG), we conducted a Go-analysis. The DEPs up-regulated in dark-cutting beef were enriched ($P < 0.001$) in oxidation-reduction, muscle contraction, mitophagy in response to mitochondrial depolarization, positive regulation of protein secretion and chaperone-mediated protein folding processes (Figure 3A). The DEPs down-regulated in dark-cutting beef were enriched ($P < 0.001$) in glycogen metabolic process, cellular glucan metabolic process, energy reserve metabolic process, glycogen, and glucan biosynthetic processes (Figure 3B). These results suggest that dark-cutting beef has a defective energy metabolism.

Similarly, Go analysis for molecular function indicated that up-regulated proteins in dark-cutting beef were enriched ($P < 0.001$) in metal ion binding, zinc ion binding, protein homodimerization, and electron carrier activities (Figure 3C). While DEPs down-regulated in dark-cutting beef were enriched ($P < 0.001$) in phosphorylase kinase activity, calmodulin-binding, calmodulin-dependent protein kinase activity, protein kinase activity, polysaccharide binding and phosphotransferase activity (alcohol accepter) molecular functions (Figure 3D). This comparison shows that the defects in energy metabolism in dark-cutting beef may be due to decreased expression of phosphorylases which are key in regulating energy metabolic pathways.

For cellular compartmentalization, DEPs upregulated in dark-cutting beef were located in the nucleus, mitochondrial membrane, sarcolemma, intercalated disc (Figure 3E) while DEPs down-regulated in dark-cutting beef were located in phosphorylase kinase complex, serine/threonine kinase complex, protein kinase complex and transferase complex-transferring phosphorous contain groups (Figure 3F). In addition, the results revealed that DEPs up-regulated in dark-cutting beef were involved in metabolic, oxidative phosphorylation, tricarboxylic acid cycle, and tight junction (Figure 3 G) while DEPs down-regulated in dark-cutting beef were mostly involved in calcium signaling, glucagon signaling, and insulin signaling KEGG pathways (Figure 3 H). On the basis of the above ontologies, results suggest an increased expression of mitochondrial and decreased expression of glycolytic metabolic proteins in dark-cutting beef.

Protein-protein interaction networks in the DEPs

In order to visualize the possible protein-protein interaction networks between the up and down-regulated DEPs in dark-cutting beef, we performed a protein-protein interaction network analysis using the STRING database and visualized the networks in Cytoscape (Figure 4). AutoAnotation of protein subnetworks revealed that the interactions among the up-regulated DEPs in dark-cutting beef were mostly related with muscle contraction (Figure 4A) and mitochondrial electron transport chain (Figure 3 C). Similarly, the interactions among the DEPs down-regulated in dark-cutting beef were mostly related with glycogen metabolism (Figure 3 B). Mitochondrial and glycolytic pathways were significantly enriched ($P < 0.05$). This thus suggests that the two pathways may play a part in the development of dark-cutting beef.

DEPs involved in glycogen metabolism and mitochondrial electron transport chain

To further determine the extent to which changes in protein expression induced by the dark-cutting condition affect muscle metabolic pathways (glycolytic and mitochondrial pathways), we performed a pathway analysis using the wiki pathway and visualized in

Cytoscape. The results showed that DEPs up-regulated in dark-cutting beef, including NADH: Ubiquinone oxidoreductase subunit A2 (Ndufa2), Succinate dehydrogenase [Ubiquinone] flavoprotein subunit, mitochondrial (Sdha), Succinate dehydrogenase complex iron-sulfur subunit B (Sdhb), cytochrome *c* oxidase subunit 6A2 (Cox6a2), and ATP synthase membrane subunit e (Atp5me), where specifically located in the mitochondrial electron transport chain pathway (Figure 5).

On the other hand, DEPs down-regulated in dark-cutting beef including amylo-alpha-1-6-glucosidase, 4-alpha-glucotransferase (Agl), glycogen phosphorylase, muscle (Pygm), phosphorylase b kinase regulatory subunit beta (Phkb), non-specific serine/threonine protein kinase (Prkaa1), phosphorylase kinase regulatory subunit gamma (Phkg1), Bis-phosphoglycerate mutase (Pgm1), and phosphorylase kinase regulatory subunit alpha (Phka1), were specifically located in the glycolytic metabolic pathway (Figure 6). These data, suggest involvement of defective glycogen metabolism and increased mitochondrial electron transport chain activity in the development of the dark-cutting phenotype.

Non-specific peptide cleavage searching to differentiate in-situ versus trypsinolytic proteolysis

To determine differennces in non-specific peptide cleavage/in-vivo proteolyis in normal-pH and dark-cutting beef, non-specific cleavage peptide mapping and fragmentation analyses were compared. We hypothesized that dark-cutting beef has more non-specific cleavage peptides compared to normal-pH beef. The intensity of all observed trypisonlytic cleavaged peptides was compared to non-trypsin N-and C-terminus cleavaged peptides between normal-pH and dark-cutting beef samples. Results showed that there were no significant differences ($P > 0.05$) in the intensity ratios of non-specific cleavaged (N-and C-terminus) peptides between normal-pH and dark- cutting beef (Figure 7). Thus, this result suggests that the level of in-vivo proteolysis/ protein degradation is similar in normal-pH and dark-cutting beef.

Discussion

Color and biochemical properties

It has been suggested that dark-cutting beef has lower L* (darker color) and a*(redness) values compared with normal-pH (Apple et al., 2011; Hunt and Hedrick, 1977). In agreement, our results showed lower L* and a* values in dark-cutting beef samples. The decrease in surface color characteristics of dark-cutting beef can be attributed to the influence of high muscle pH (Table 1). Increased muscle pH can enhance the activity of enzymes involved in oxygen consumption (Ramanathan and Mancini, 2010; Tang et al., 2005a) and metmyoglobin reducing activity. Thus, this may in part explain the observable differences in muscle biochemical properties between dark-cutting and normal-pH beef.

Differential protein profiles of normal-pH and dark-cutting beef

Our data suggest that dark-cutting beef has impaired glycolytic metabolism. Consistent with this observation, dark-cutting beef had enrichment of down-regulated proteins in glycogen metabolism. Additionally, pathway analysis showed that DEPs including; glycogen phosphorylase, phosphoglycerate mutase, phosphorylase kinase regulatory subunit alpha, beta and gamma, and non-specific serine/threonine-protein kinase were specifically located in the glycogen degradation pathway (Figure 6). However, the role played by these proteins in the occurrence of dark-cutting beef has not yet been described.

Previous studies examining post-mortem metabolism have demonstrated that the etiology of dark-cutting beef is connected to low glycogen content and glycosidic potential (Ponnampalam et al., 2017; Xing et al., 2019). While down-regulation of glycolytic proteins is not definitive proof for the occurrence of dark-cutting beef, from our data we conclude that reduced expression of glycolytic enzymes can influence muscle glycogen content. Down-regulated DEPs such as glycogen phosphorylase initiates glycogen degradation pathways by

creating phosphoroelastic cleavages in glycogen (Komoda and Matsunaga, 2015). Therefore, down-regulation of glycolytic metabolic proteins can reduce the muscle's capacity to mobilize glycogen, and the ability to accumulate lactate post-mortem. Thus, this may explain the high muscle pH typically associated with dark-cutting muscles.

In addition, our data suggest that dark-cutting beef has an increased mitochondrial metabolism supported by greater expression of mitochondrial electron transport proteins. Results showed that up-regulated proteins in dark-cutting beef were enriched in the mitochondria electron transport chain. These up-regulated mitochondrial enzymes including; NADH: Ubiquinone oxidoreductase subunit A2, succinate dehydrogenase (Ubiquinone) flavoprotein subunit, succinate dehydrogenase complex iron-sulfur subunit B, ATP synthase membrane subunit e and cytochrome *c* oxidase subunit 6A2 were specifically located in the mitochondria electron transport chain pathway (Figure 6). However, the exact mechanisms and functional links between these proteins to the occurrence of the dark-cutting phenotype are yet to be described.

The greater expression of mitochondrial transport chain proteins in dark-cutting beef may suggest increased mitochondrial respiration. The increase in mitochondrial respiration coupled with ATP production could be for supporting high-energy consuming metabolic processes including glycogen break down (Timmons, & Greenhaff, 2005), calcium homeostasis (Baughman et al., 2011; Chaudhuri et al., 2013 and Kirichok et al., 2004), biosynthesis of heme and steroids (Nilsson et al., 2009). However, the increase in mitochondrial respiration increase oxygen consumption which in turn may increase competition for oxygen with myoglobin (Ramanathan and Mancini 2018; Wittenberg and Wittenberg, 1981). Therefore, deoxygenation of myoglobin can produce a darker red color in meat (Suman & Joseph, 2013; Tang et al., 2005a). Additionally, the increase in mitochondrial functionality is associated with increased production of reactive oxygen species (ROS). These are mostly generated for signaling and

defense (Cunningham et al., 2015; Simioni et al., 2018). However, ROS can oxidize myoglobin increasing myoglobin deoxygenation and hence muscle darkening.

Determination of non-specific peptide cleavage to differentiate in-situ versus trypsinolytic proteolysis

While evaluating differences in non-specific peptide cleavage, we hypothesized that the number of non-specific cleavage peptides is greater in dark-cutting beef compared to normal-pH beef. However, results showed no differences in in-situ proteolysis between dark-cutting and normal pH beef (Figure 7). Non-specific cleaved products have been reported as byproducts of enzymatic digestions and are detected by mass spectrometry (König et al., 2001). Detection of nonspecific peptide cleavages is a reflection of protein degradation in-situ. Additionally, the increase in abundance of these peptides is a common signal for heat shock proteins (HSP) induction (Picard et al. 2018). Heat shock and chaperone proteins are important in protein repairs and correction of misfolded proteins (Xing et al., 2019). Therefore, the increase in expression of such proteins is indicative of in-situ proteolysis. Thus, in-situ proteolytic mechanism is similar between normal-pH and dark-cutting beef.

Conclusion

Dark-cutting beef had lower redness, darker color, and greater oxygen consumption compared with normal-pH beef. Greater pH can be attributed to the down-regulation of glycolytic proteins such as Epm2a, Agl, Pygm, Phkb, Prkaa1, Phkg1, Bpgm and Phka1. Further, mitochondrial proteins such as Ndufa2, Sdha, Sdhb, Mdh1, Cox6a2, Coq8a, Atp5me, and Echs1 were up-regulated in dark-cutting beef than normal-pH beef. The results suggest that greater mitochondrial activity and higher pH can be conducive for enhanced oxygen consumption and less oxygenation of myoglobin.

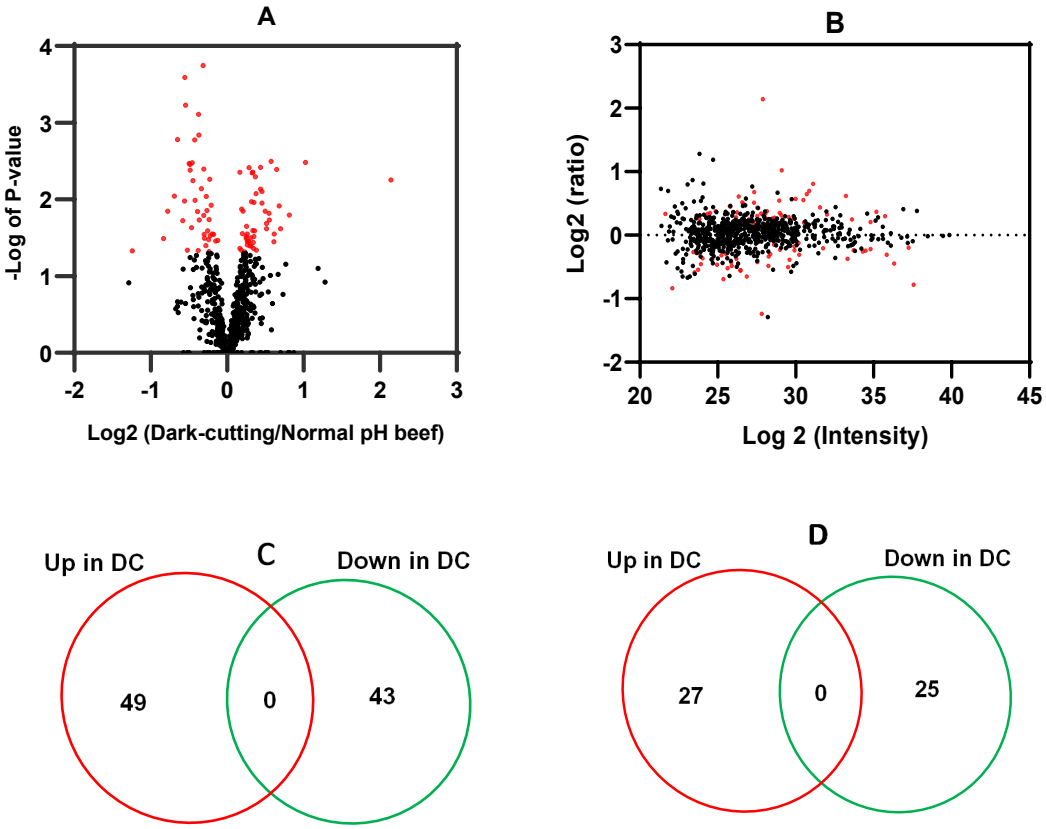
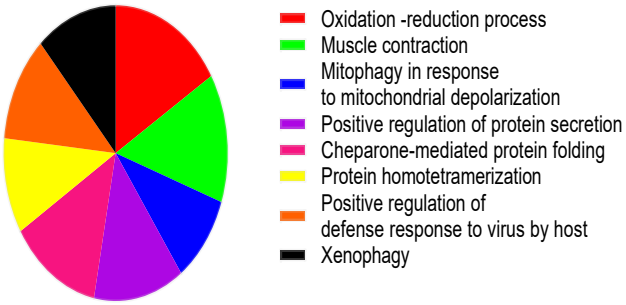


Figure 2: Identification and quantification of differentially expressed proteins in dark-cutting beef compared with normal-pH beef.

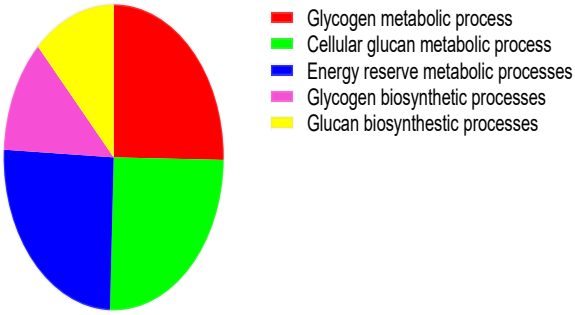
Differences in protein expression between normal-pH and dark-cutting beef were analyzed using LC-MS/MS mass spectrometry-based proteomics. Fold changes of the differentially expressed proteins were filtered out on a volcano plot using a t-Test. Significant changes in protein expression were determined at cut off of $P \leq 0.05$ of the log2 expression values. (A) Volcano plot of log2 transformed protein expression changes against a -log of the P-value from t-test of dark-cutting beef compared with normal-pH beef. Significantly changed ($P \leq 0.05$) proteins are represented by red dots and insignificantly changed proteins are represented in black. (B) Christmas tree plot showing the log2 of differentially expressed proteins (DEPs) up- and down-regulated in dark-cutting beef against a log2 expression intensity. (C) Number of up-regulated (red) and down-regulated (green) proteins in dark-cutting beef. (D) Number of up-

regulated (red) and down-regulated proteins (green) with ≥ 1.3-fold change in protein expression.

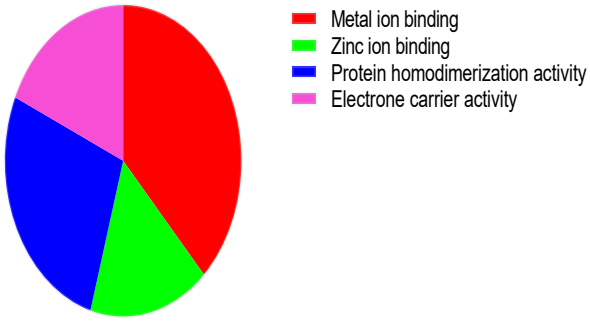
A) Biological processes of DEPs up in DC



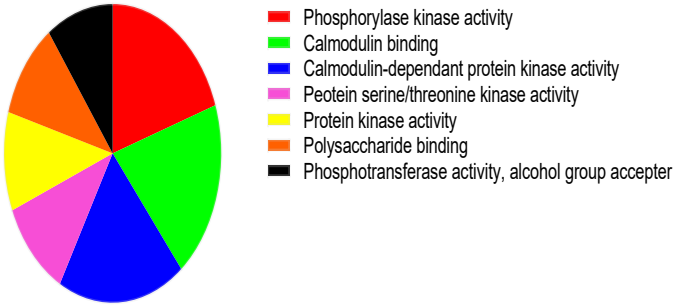
B) Biologica processes of DEPs down in DC



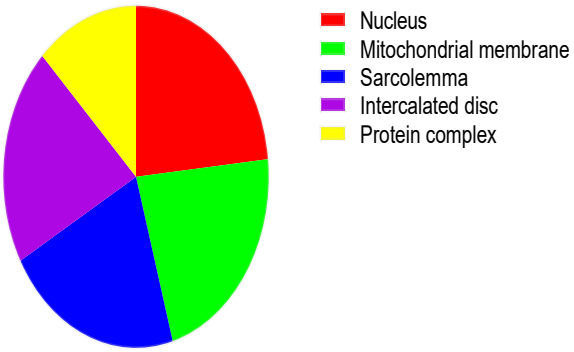
C) Molecular functions of DEPs up in DC



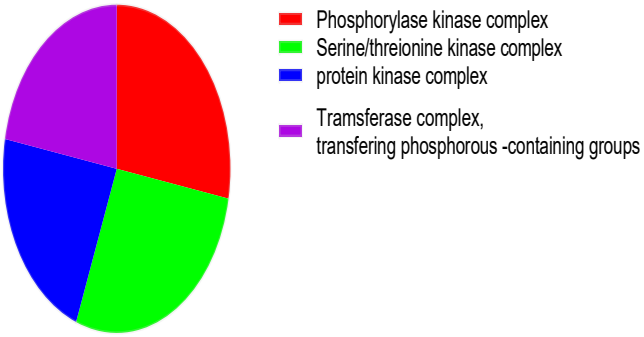
D) Molecular functions of DEPS down in DC



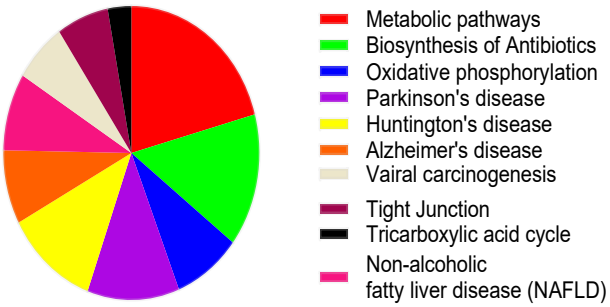
E) Cellular compartmentalization of DEPs up in DC



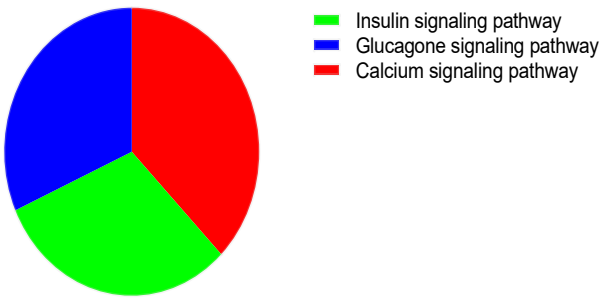
F) Cellular compartmentalization of DEPs down in DC



G) KEEG Pathways of DEPs up in DC



H) KEEG Pathways of DEPs down in DC



$P \leq 0.05$

Figure 3: Functional enrichment analysis of the differentially expressed proteins

Proteins that had their expression significantly altered by the dark-cutting condition were analyzed for functional enrichment of Go processes using David. The background list used comprised of a list of all proteins identified and quantified in dark-cutting and normal-pH beef samples. Panels (A, B) enrichment analysis of biological process, (C, D) enrichment analysis of molecular functions, (E, F) enrichment analysis of cellular compartment and (G, H) enrichment analysis of KEGG pathways ($P \leq 0.05$). The bigger the slice, the greater the P-value.

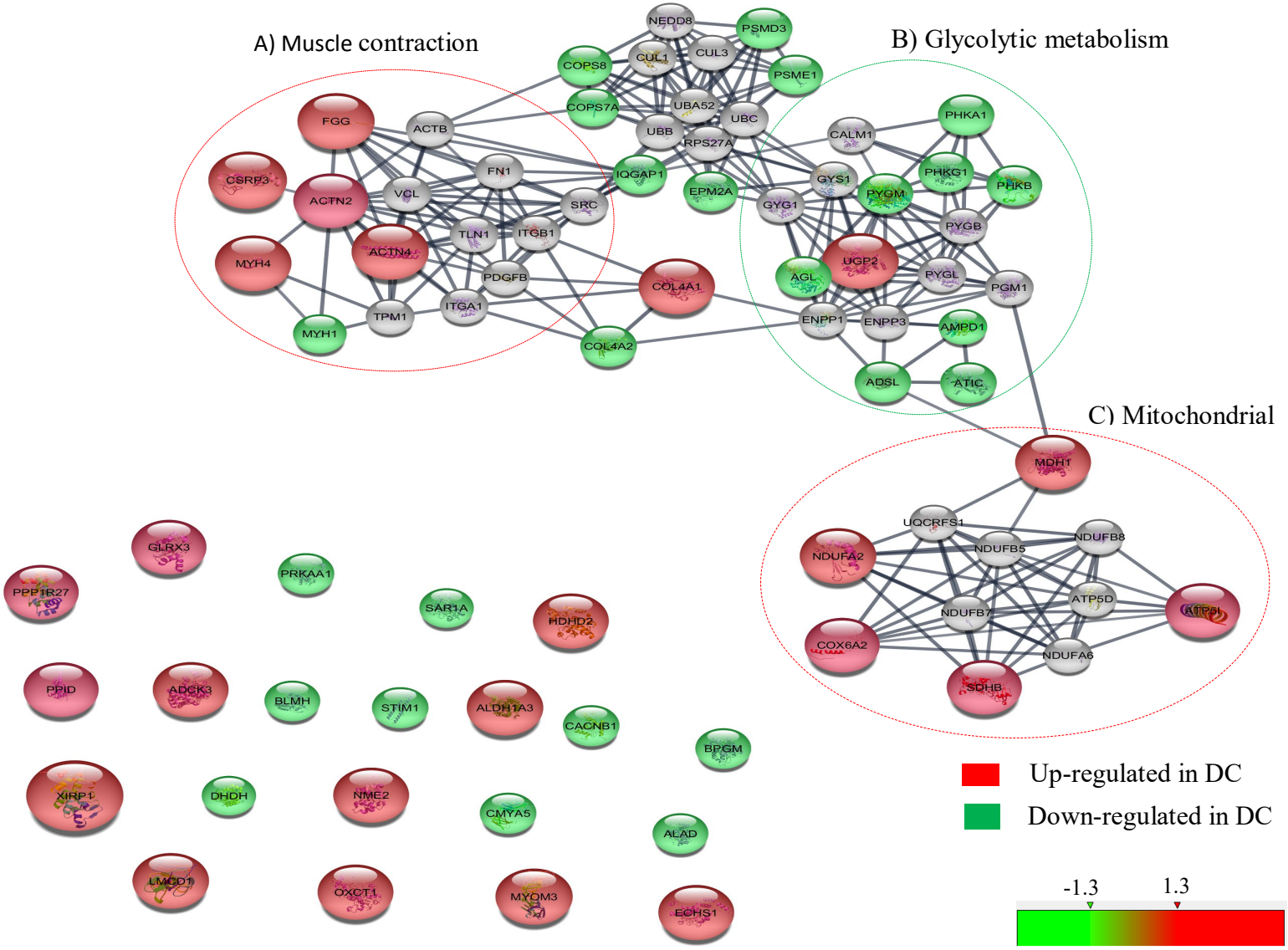

A) Muscle contraction
B) Glycolytic metabolism
C) Mitochondrial
NEDD8
PSMD3
COPS8
CUL1
CUL3
PSME1
COPS7A
UBA52
UBC
UBB
RPS27A
FGG
ACTB
CSRP3
ACTN2
FN1
VCL
IQGAP1
SRC
TLN1
ITGB1
ACTN4
MYH4
PDGFB
COL4A1
TPM1
ITGA1
MYH1
COL4A2
PHKA1
CALM1
PHKG1
PHKB
GYS1
PYGM
EPM2A
GYG1
PYGB
UGP2
AGL
PYGL
PGM1
ENPP1
ENPP3
AMPD1
ADSL
ATIC
MDH1
UQCRFS1
NDUFB8
NDUFB5
NDUFA2
ATP5D
NDUFB7
ATP5L
COX6A2
NDUFA6
SDHB
GLRX3
PRKAA1
PPP1R27
SAR1A
HDHD2
PPID
ADCK3
BLMH
STIM1
ALDH1A3
CACNB1
BPGM
XIRP1
DHDH
NME2
CMYA5
ALAD
LMOD1
OXCT1
MYOM3
ECHS1
Up-regulated in DC
Down-regulated in DC
-1.3
1.3

Figure 4: Enrichment of protein-protein interaction subnetworks of differentially expressed proteins in dark-cutting beef

Proteins that were significantly expressed in dark-cutting beef were used to query the STRING protein-protein database using Cytoscape. Protein subnetworks with more than 10 proteins were Annotated using the AutoAnnotation Plugin and visualized in Cytoscape. The changes in protein expression (up and down-regulated) in dark-cutting beef were then mapped onto the nodes. A) protein-protein interaction subnetwork of DEPS involved in muscle contraction. B) Protein-protein interaction subnetwork of DEPs involved in glycolytic metabolism. C) Protein-protein interaction subnetwork of DEPs involved in mitochondrial respiratory transport chain. The red color represents up-regulated proteins while the green color indicates down-regulated proteins. The bigger the size, the greater the fold change.

Mitochondrial Electron Transport Chain

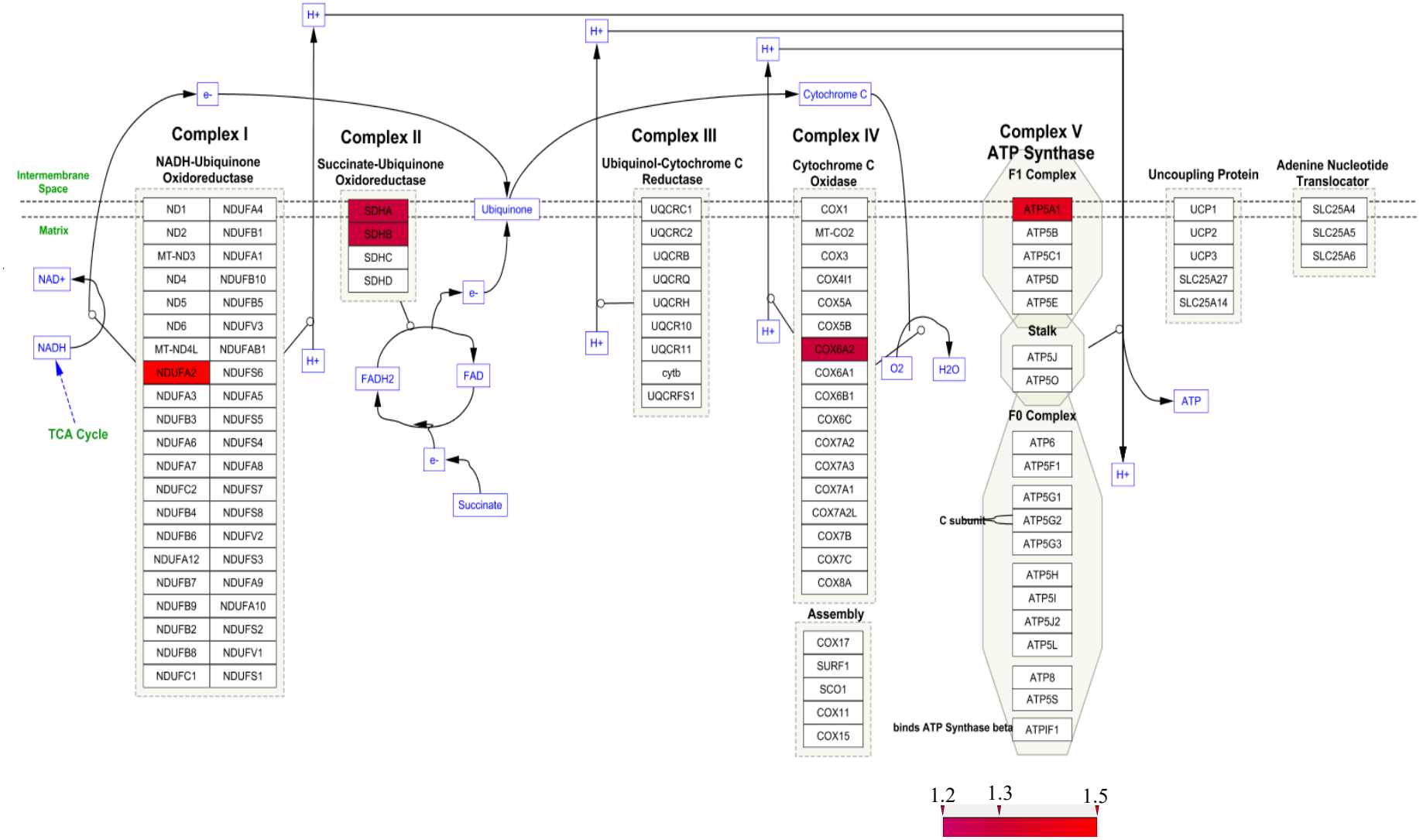


Figure 5: Representation of differentially expressed proteins (DEPs) in dark-cutting beef that are involved in the mitochondrial respiratory electron transport pathway

The DEPs in dark-cutting beef with significant change in expression were used to query the wiki mitochondrial electron transport chain pathway for enrichment and visualized in Cytoscape. The red color represents up-regulated DEPs that were enrichment in the mitochondrial electron transport chain pathway $P < 0.05$.

Glycogen Metabolism

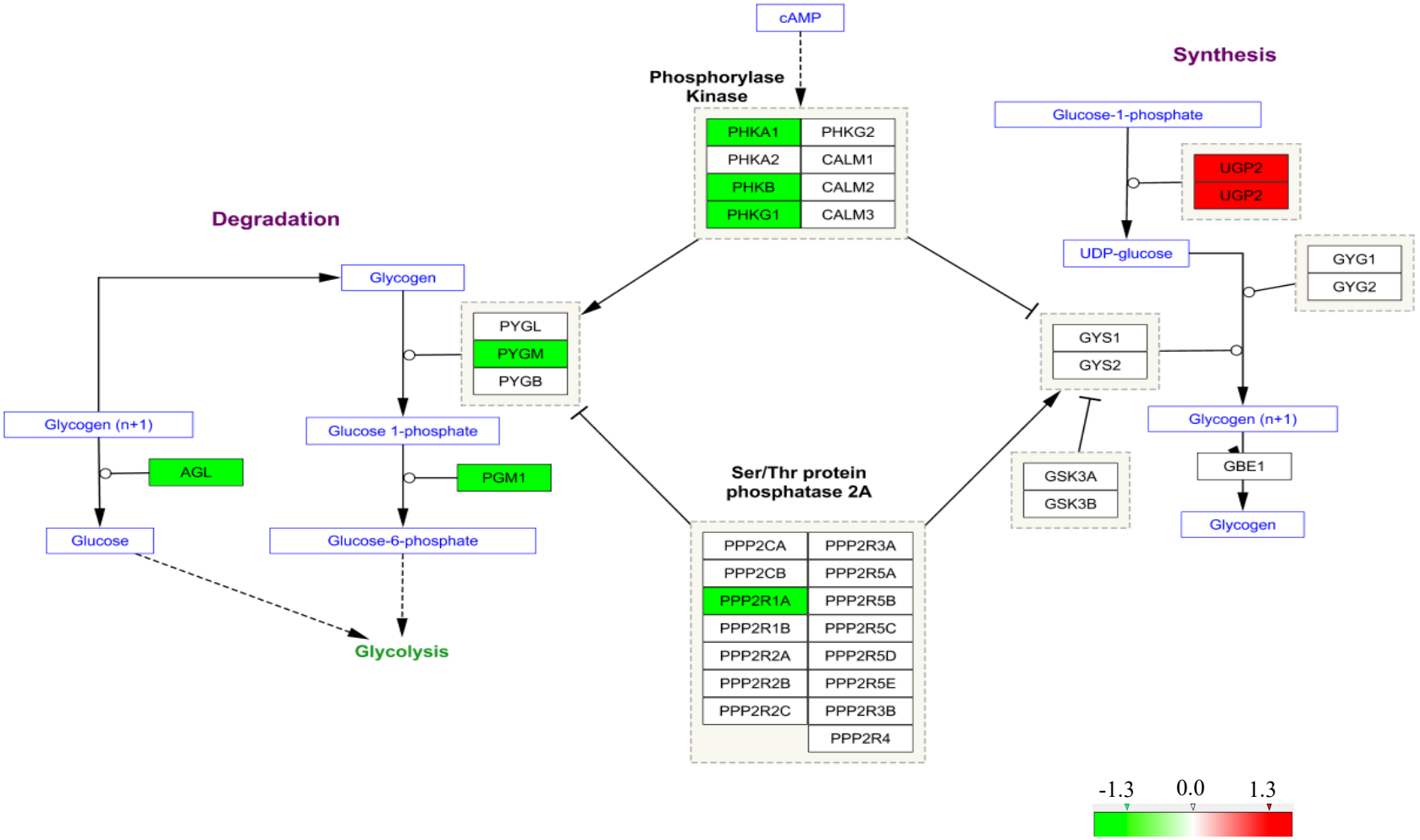


Figure 6: Representation differentially expressed proteins (DEPs) in dark-cutting beef involved in glycogen metabolism pathway

The DEPs in dark-cutting beef with significant change in expression were used to query the wiki glycogen metabolism pathway for enrichment and visualized in Cytoscape. The red color represents up-regulated DEPs while the green color represents down-regulated DEPs that were enrichment glycogen metabolism pathway.

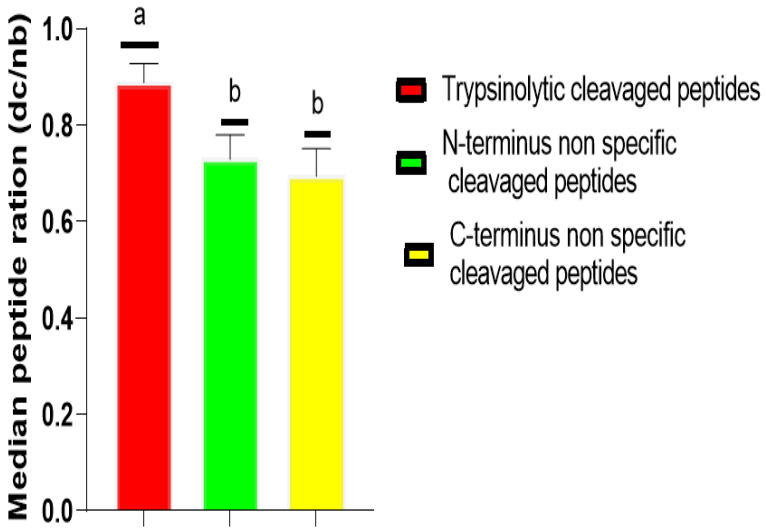


Figure 7: Non-specific peptide cleavage analysis to validate the differential abundance of active proteolytic degradation in-situ in dark-cutting beef compared with normal-pH

Digestion of proteins is usually performed using trypsin due to its high specificity at the C-terminal to Lysine and Arginine. Non-specific peptide analysis is used as an indicator of pre-existing protein fragmentation in-vivo. We utilized non-specific peptide cleavages analysis to understand

the potential differences in in-vivo proteolysis between normal-pH and dark-cutting beef samples. The ratio of non-specific cleavaged peptides was compared with trypsin- peptide

a-b, Least square means with different letters are different ($P < 0.05$).

CHAPTER III

MITOCHONDRIAL FUNCTION DIFFERENCES IN NORMAL-pH AND DARK-CUTTING BEEF

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Abstract

Mitochondria remain active post-mortem and can influence meat color. However, limited research has characterized the functionality of mitochondria isolated from normal-pH and dark-cutting beef (high pH beef). Mitochondrial complex I, II, and IV functionality, state III- and IV-oxygen consumption, mitochondrial membrane integrity, and uncoupled respiration rate in normal-pH and dark-cutting beef were determined using a Clark-oxygen electrode. Mitochondria respiratory control ratio (RCR) and number of moles of adenosine diphosphate (ADP) phosphorylated by moles of oxygen (O_2; ADP/O ratio) were also determined. Dark-cutting beef had greater ($P < 0.05$) mitochondrial complex II respiration, state III oxygen consumption, uncoupled respiration, RCR, and a lower ADP/O ratio compared with normal-pH beef. However, no differences in mitochondrial complexes I and IV respiration, state IV oxygen consumption, and membrane integrity ($P > 0.05$) were observed between normal-pH and dark-cutting beef. The results suggest that there are differences in mitochondrial functional properties between dark-cutting and normal-pH mitochondria, and this, in part, can contribute to darker meat color.

Keywords: Mitochondria, myoglobin, oxygen consumption, meat color, Clark electrode, dark-cutting beef

Introduction

A characteristic bright-red color during beef grading and at the retail display is important to maximize the economic benefits associated with the sale of beef. Any deviation from bright-red color can lead to economic losses to the meat industry (Smith et al., 2000). Predominant oxymyoglobin at the cut surface gives bright-red color, while deoxymyoglobin gives darker-meat color (AMSA, 2012).

Interaction between mitochondria and myoglobin is a key determinant of meat color (Lanari and Cassens, 1991; Tang et al., 2005b; Postnikova et al., 2009; Ramanathan and Mancini, 2018). Physiologically, myoglobin and mitochondrial functions are inter-related, and both involve cellular homeostasis. In post-mortem muscle, mitochondria remain functional influencing myoglobin redox state (Tang et al., 2005b; Ashmore et al., 1971). For example, addition of substrates such as succinate or lactate to oxymyoglobin-mitochondria mixture resulted in more deoxymyoglobin or limited myoglobin oxygenation (Giorgi-Coll et al., 2017). Therefore, actively respiring mitochondria can limit myoglobin oxygenation .

In post-mortem muscle, diffusion from the atmosphere is the main source of oxygen. Various processes such as mitochondrial oxygen consumption, microbial growth, protein- and lipid oxidation compete for the available oxygen (Ramanathan and Mancini, 2018; Tang et al., 2005b). However, on a freshly cut surface, competition between mitochondria and myoglobin determines the formation of bright-red color. Numerous factors such as pH, temperature, and substrate concentration can determine mitochondrial activity (De Bari et al., 2004; Kim et al., 2006; Ramanathan et al., 2009). Hence, any deviation in post-mortem biochemistry can influence mitochondrial activity and its impact on meat color.

Dark-cutting beef has a worldwide occurrence, and chronic stress before slaughter has been attributed to high-pH postmortem (Mahmood et al., 2018; Ponnampalam et al., 2017; Wulf et al., 2002). Dark-cutting beef has aberrant glycolytic enzyme activity, leading to greater

than normal-pH (Mahmood et al., 2018). Further, McKeith et al. (2016) reported that dark-cutting beef had less efficient mitochondria. More specifically, dark-cutting beef mitochondria generated more reactive oxygen species compared with normal-pH beef. However, mitochondria isolated from both epinephrine-injected dark-cutting (experimentally-induced) and normal-pH muscle had similar mitochondrial function properties (Ashmore et al., 1971). However, limited research is available on mitochondrial functional properties of naturally occurring dark-cutting conditions. Therefore, the objective of the current study was to evaluate mitochondrial functional differences in normal-pH and dark-cutting beef by accessing mitochondrial respiration at complex I, II, and IV, state III- and IV- oxygen consumption rate, mitochondrial membrane integrity and uncoupled respiration using a Clark oxygen type electrode.

Materials and Methods

Materials and Chemicals

Trypsin, Sucrose, HEPES, EDTA, Tris-HCl, potassium chloride, bovine serum albumin (BSA), rotenone, antimycin A, succinic acid, glutamate, malate, magnesium chloride, potassium monophosphate, adenosine diphosphate (ADP), cytochrome C, trypsin, N,N,N,N-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) and L-ascorbic acid were all obtained from Sigma-Aldrich Chemicals CO. (St. Louis, MO). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was obtained from Cayman Chemicals (Ann Arbor, MI). All chemicals were of reagent analytical grade or greater.

Mitochondrial Isolation and protein quantification

Eleven Normal-pH and 11 dark-cutting beef loins (*longissimus lumborum*; IMPS #180, NAMP, 2002) from maturity carcasses were obtained 72-h post-mortem from Creekstone Farms, Arkansas City, KS at two different occasions (five normal-pH and dark-cutting loins

during first time and six normal-pH and dark-cutting loins during the second time). Loins were transported on ice, and upon arrival, three 2.54-cm-thick steaks were cut from the anterior end of loin. The first steak was utilized for color and biochemical analysis, the second steak was utilized for mitochondrial studies, and the third steak was used to determine pH and proximate compositions.

Color characteristics and biochemical properties

Fresh-cut surface of each steak was placed on a styrofoam tray overwrapped with PVC film (oxygen-permeable polyvinyl chloride fresh meat film; 15,500 to 16,275 cm^3 O_2 m^{-2} $24h^{-1}$ at 23 °C, E-Z Wrap Crystal Clear Polyvinyl Chloride Wrapping Film, Koch Supplies, Kansas City, MO) and were allowed to oxygenate at 4 °C for 2 h. Surface color was measured using a HunterLab MiniScan XE Plus spectrophotometer (HunterLab Associates, Reston, VA) with a 2.5 cm diameter aperture, Illuminant A, and 10° standard observer. Both CIE L*, a*, and b* values and reflectance values from 400 to 700 nm were used to characterize the surface color. Following surface color measurements, each steak was cut into half. The first half was used to estimate oxygen consumption and the second half was utilized to measure pH. Reflectance approach was utilized to determine oxygen consumption. The steak half was bloomed for 1 h at 4 °C. Following blooming, steak section was vacuum packaged and incubated at 25 °C for 30 min. Surface color reading was taken after incubation to measure deoxymyoglobin level to indicate oxygen consumption. A greater deoxymyoglobin indicates greater oxygen consumption. A greater pH in dark-cutting beef can change reflectance properties. Hence a modified method was utilized to measure oxygen consumption (Ramanathan et al., 2019). Reflectance values were converted to K/S ratios at isobestic points to estimate deoxymyoglobin. Reflectance values were converted to K/S ratios using the equation: $K/S = (1 - R)^2 \div 2R$, where R represents the % reflectance expressed as a decimal. The ratio of K/S 474 ÷ K/S 525 was used to estimate deoxymyoglobin (AMSA color guide; AMSA, 2012).

The pH of each steak was recorded using an Accumet 50 pH meter (Fisher Scientific, Fairlawn, NJ). Protein, moisture, and fat contents were reported on a percent (%) basis and determined using an AOAC-approved (Official Method 2007.04; Anderson, 2007) near-infrared spectrophotometer (FOSS Food Scan 78800; Dedicated Analytical Solutions, DK-3400 Hillerod, Denmark).

Mitochondrial isolation

Mitochondria from skeletal muscle were isolated according to the method of Smith (1967) with minor modifications. Five g muscle tissue visibly devoid of fat and connective tissue from each normal-pH and dark-cutting beef loin (n = 11 each) were minced using tissue teaser. Following mincing, samples were suspended in 10 mL ice-cold suspension buffer (250 mM sucrose and 12 mM HEPES, pH 7.4), containing 5 µL of proteinase (0.25% (w/v) trypsin) for each 1 mL of suspension buffer in a 250 mL beaker. The beaker containing the tissue suspension mixture was placed in a 500 mL beaker prefilled with ice and stirred gently for 15 min using a glass stirring rod. After stirring, 40 mL of ice-cold mitochondria isolation buffer (67 mM sucrose, 10 mM EDTA, 50 mM Tris, 50 mM KCl, and 0.2% bovine serum albumin (BSA), pH 7.4) was added to the suspension mixture, and the pH was readjusted to 7.2.

The samples were homogenized two times; first using a pinpointed glass/Teflon Potter Elvehjem homogenizer and secondly with a round-bottomed glass/Teflon Potter Elvehjem homogenizer, tightly fitted on Potter-Eberbach corporation tissue grinder (Model E7000, Volt 130, St. Belleville, MI) at 160 rpm. The homogenate was then centrifuged at 800 g for 10 min at 4°C using a mini centrifuge (Hermle Z446 series high capacity centrifuge, swing-out rotor, 68*15 mL or 28 x 50 mL). The pellet was discarded, and the supernatant was decanted through a double-layered cheesecloth. The collected supernatant was then centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was discarded. The mitochondrial pellet was gently

resuspended in medium containing 75 mM sucrose, 10 mM Tris and 0.1 mM EDTA, pH 7.4. Total mitochondrial protein content was determined spectrophotometrically using a Biuret method with BSA as the protein standard.

Mitochondrial respiratory activity

Extracted mitochondria were used to assess functional and biochemical parameters of normal-pH and dark-cutting beef using a Clark Oxygen type electrode (DW 1, Hansatech, Norfork, UK, a polarizing voltage of 0.6 V and 8 mL incubation chamber). The reaction chamber was maintained at a reaction temperature of 25°C. A 10 mm Teflon covered bar was used to stir the chamber at 600 rpm. The electrode was attached to a Rank Brothers digital model 20 oxygen controller (Cambridge, England), connected to a personal computer and data logger. For all experiments, mitochondrial oxygen consumption measurements were initiated by suspending 2 mg/mL in 1 mL reaction buffer (250 mM sucrose, 5 mM potassium mono phosphate, 5 mM magnesium chloride, 0.1% BSA, 0.1 mM EDTA and 20 mM HEPES, pH 7.4) followed by the addition of test compounds.

Mitochondria from both normal-pH and dark-cutting beef were evaluated for the activity of complex I, II, and IV (Figure 8). After 1 min equilibration period in the incubation chamber, mitochondrial respiration at complex I was initiated by the addition of glutamate (5 mM) and malate (2 mM). Following steady-state respiration, complex I activity was blocked with rotenone (2 μM). Mitochondrial oxygen consumption at complex II was then initiated by adding succinate (5 mM)After steady-state respiration, activity was inhibited by the addition of Antimycin A (4 μM). Finally, complex IV oxygen consumption was determined by adding ascorbate (6 mM) and TMPD (300 μM).

Determination of RCR and ADP/O ratios

State III is the rate of oxygen consumption in the presence of ADP phosphorylation, while state IV is oxygen consumption in absence of ADP phosphorylation (Jacoby et al., 2015). To evaluate state III and IV mitochondrial oxygen consumption, mitochondria (2 mg/mL) was suspended in reaction buffer (250 mM sucrose, 5 mM potassium mono phosphate, 5 mM magnesium chloride, 0.1% BSA, 0.1 mM EDTA and 20 mM HEPES, pH 7.4). Mitochondrial complex I was first blocked using rotenone (2 µM). State IV oxygen consumption was initiated by addition of succinate (5mM), and rate of oxygen consumption was measured. State III oxygen consumption was initiated by addition of ADP at final concentration of 100 µM. Respiratory control ratio (RCR), the ratio between rate of mitochondrial oxygen consumption under state III and state IV was then determined. The number of nanomoles of ADP phosphorylated by the nanomoles of oxygen consumed per mitochondria (ADP/O) ratio was calculated according to Estabrook, (1967), using 235 nmol O_2/mL as the value of oxygen solubility at 25°C.

Mitochondrial outer membrane permeability and uncoupled oxidative phosphorylation

To characterize the integrity of the outer mitochondrial membrane, cytochrome *c* was added at a final concentration of 10 µM (Figure 10). The outer membrane permeability is characterized by release of cytochrome *c* to sarcoplasm (Gouspillou et al., 2011). When cytochrome *c* is released, it interferes with oxygen consumption and ATP synthesis. An increased response to exogenous cytochrome *c* added to the mitochondria, therefore, indicates loss of the outer membrane mitochondrial permeability. Mitochondria outer membrane permeability/integrity was evaluated by addition of substrates, rotenone (2 µM), and succinate (5 mM). State III was initiated by addition of ADP, and cytochrome *c* was added at a final concentration of 10 µM.

The degree of coupling between the electron transport chain (ETC; Figure 13) and the oxidative phosphorylation machinery (OXPHOS) was evaluated by adding mitochondrial (2 mg/mL) suspended in reaction buffer (250 mM sucrose, 5 mM potassium mono phosphate, 5 mM magnesium chloride, 0.1% BSA, 0.1 mM EDTA and 20 mM HEPES, pH 7.4). Mitochondria were started in a coupled state with substrates, rotenone (2 µM), and succinate (5 mM). State III was initiated by the addition of ADP, and FCCP-induced maximal uncoupler-stimulated respiration was initiated by the addition of FCCP at final concentration of 100 µM.

Statistical analysis

A completely randomized block design was utilized to determine muscle-specific differences in mitochondrial functionality and color attributes. The experiment was replicated 11 times (n = 11) using a completely randomized design (CRD). Eleven loins were collected during two different phases. There was no significant effect of collection day on mitochondrial functionality. Hence, the collection day was not included in the model. Each loin from normal-pH and dark-cutting beef was considered a block. Least square means and standard error of mean were analyzed using Proc GLM procedure in SAS (Version 9.1, SAS Institute Inc. Cary, NC). Least square means were separated using the pdiff option and were considered significant at $P < 0.05$.

Results

Muscle pH and mitochondrial protein content

To characterize differences in muscle pH and mitochondrial protein content between normal-pH and dark-cutting beef, we measured muscle pH and mitochondrial protein content. Results showed that dark-cutting beef had a significantly greater ultimate muscle pH and protein content compared to normal-pH beef (Figure 8, $P < 0.05$).

Mitochondrial complex I, II and IV oxygen consumption

To determine the functionality of mitochondrial complexes (Complex I, II, and IV), different substrates and complex inhibitors were added. The addition of glutamate and malate to isolated mitochondria resulted in measurable oxygen consumption in both normal-pH and dark-cutting beef (Figure 10A). However, there were no significant differences in the oxygen consumption rate between normal-pH and dark-cutting beef ($P > 0.05$). Thus, these results suggest that a close link between mitochondria complex I function in normal-pH and dark-cutting beef.

The addition of succinate to stimulate complex II oxygen consumption resulted in greater mitochondrial oxygen consumption in dark-cutting beef than normal-pH beef (Figure 10B, $P < 0.05$). The addition of TMPD and ascorbate to stimulate complex IV oxygen consumption resulted in no differences in the rate of oxygen consumption between normal-pH and dark-cutting beef mitochondria (Figure 10C, $P > 0.05$). The addition of complex I and II inhibitors (rotenone and Antimycin A), inhibited oxygen consumption in both normal-pH and dark-cutting beef mitochondria (Figure 9). Thus, the results suggest that complex II activity may play a role in the development of the dark-cutting condition.

Mitochondrial outer membrane integrity and respiratory control ratio

To determine the integrity of the outer membrane, we added cytochrome *c* to isolated mitochondria. The addition of cytochrome *c* resulted in measurable oxygen consumption in both normal-pH and dark-cutting beef (Figure 11). However, there were no significant differences in the rate of oxygen consumption between normal-pH and dark-cutting beef ($P > 0.05$). Therefore, results suggest similar mitochondrial outer membrane integrity in normal-pH vs dark-cutting beef. Additionally, we measured the mitochondrial respiratory control ratio (RCR) by dividing the rate of oxygen consumption between state III and IV. Results showed that dark-cutting beef had a greater RCR compared to normal-pH beef ($P < 0.05$). Thus, a

greater RCR in dark-cutting beef is an indicator of intact inner mitochondrial membrane and greater oxidative phosphorylation capacity.

Mitochondrial coupled and uncoupled oxidative phosphorylation

To further evaluate the oxidative phosphorylation efficiency, we measured ADP/O ratio and the rate of oxygen consumption in presence of the uncoupler FCCP. Results revealed that normal-pH beef had a greater ADP/O ratio compared to dark-cutting beef ($P < 0.05$; Figure 14 B). Addition of FCCP resulted in measurable oxygen consumption (Figure 14). Dark-cutting beef had greater uncoupled respiration compared to normal-pH beef ($P < 0.05$; Figure 15A). A greater uncoupled respiration and low ADP/O ratio in dark-cutting beef may suggest greater oxidative phosphorylation capacity.

Discussion

Biochemical properties of normal-pH and dark-cutting steaks

It has been previously shown that dark-cutting beef has a greater muscle pH compared to normal-pH beef (English et al., 2016; Mahmood et al., 2018). Consistent with these studies, our data showed that dark-cutting beef has a greater muscle pH compared to normal-pH beef (Figure 8B). The high ultimate muscle pH observed in dark-cutting beef can be attributed to the depletion of muscle glycogen ante-mortem. McKeith et al. (2016) noted that the development of dark-cutting beef occurs when glycogen concentration is less than 80 mmol/kg tissue.

Further, reduced muscle glycogen content in dark-cutting beef suggests an increased metabolic capacity. The depletion of muscle glycogen can result in reduced lactate accumulation post-mortem (Briskey et al., 1966; Ferguson & Gerrard, 2014; Rosenvold et al., 2001). A reduction in lactate concentration can lead to elevated muscle pH, which in turn influences muscle metabolism. Additionally, the high muscle pH can sustain mitochondria

activity post-mortem (Ashmore et al., 1971; Tang et al., 2005 b). Hence, in the current research, steak oxygen consumption was greater in dark-cutting steaks than normal-pH steaks. Previous research also noted greater oxygen consumption and more deoxymyoglobin content in bloomed steaks than normal-pH steaks (English et al., 2016; McKeith et al., 2016).

Mitochondrial protein content

The incidences of stress before slaughter may trigger a bioenergetics survival mechanism ensured to maintain energy homeostasis through increased mitochondrial density. Consistent with previous findings (McKeith et al., 2016), we observed greater mitochondrial protein content in dark-cutting beef compared with normal-pH beef. The increase in mitochondrial content is reflective of adaptive responses to cellular stressors (Barbour & Turner, 2014; Erlich et al., 2016; Olsen et al., 2015; Picard et al., 2018). Therefore, cellular adaptive pathways might boost mitochondrial bioenergetics and biogenesis via fission and fusion pathways (Erlich et al., 2016; Palmer et al., 2011; Westermann, 2010). Thus, the increase in mitochondrial division and multiplication processes ensures adequate energy supply. However, it is still not clear how fusion and fission mechanisms are activated and coordinated in post-mortem dark-cutting beef.

Effects on mitochondrial functionality

Mitochondrial membrane integrity

Due to practical difficulty in procuring loins immediately after harvest from a commercial packing plant, mitochondrial functionality was evaluated 72 h postmortem. Inherent pH differences between normal-pH and dark-cutting beef can influence postmortem proteolytic changes, which can influence mitochondrial function. Hence, mitochondrial membrane integrity was determined to make sure mitochondrial functional differences were not due to pH. No significant differences in mitochondrial membrane integrity were observed between normal-pH and dark-cutting mitochondria.

Additionally, extraneous cytochrome *c* additions showed no significant effect on mitochondrial respiration in normal-pH and dark-cutting beef. The response to cytochrome *c* addition indicates loss of outer membrane integrity and is associated with release of cytochrome *c*. However, when release of cytochrome *c* was accessed by addition of TMPD with ascorbate, a similar response in TMPD-induced respiration (complex IV respiration) between normal-pH and dark-cutting beef was observed. TMPD-induced respiration is cytochrome *c*-dependent, as TMPD is a membrane-permeable electron donor capable of reducing cytochrome *c* (Gottlieb, Armour, & Thompson, 2002). Thus, these results might suggest a similar out membrane integrity with no cytochrome *c*.

Oxygen consumption of different mitochondrial complexes

Our results suggest that dark-cutting beef has a greater oxidative phosphorylation capacity primarily due to mitochondrial complex II respiration. However, the mechanistic basis for greater complex II activity is not clear. Jardim-Messeder et al. (2015); Yadava and Nicholls, (2007) noted that mitochondrial complex II plays a central role in mitochondrial metabolism by linking the tricarboxylic acid cycle (TCA) with the electron transport chain (ETC), catalyzing oxidation of succinate to fumarate and reduction of ubiquinone (UQ) to ubiquinol (UQH_2). Consistent with the proteomic analysis (Chapter II), we observed greater expression of mitochondrial complex II proteins in dark-cutting beef. Thus, mitochondrial respiration at complex II may play part in the development of dark-cutting beef.

Additionally, our results suggest that dark-cutting beef has an efficient oxidative phosphorylation capacity with a well intact inner membrane compared to normal-pH beef. This might be attributed to greater RCR and state III oxygen consumption. A grater RCR can be attributed to an intact inner mitochondrial membrane integrity. While the increase in oxygen consumption under state III in dark-cutting beef might be associated with increased utilization of substrate-dependent respiration. Further, the greater RCR and State III OCR correlate with

effective ADP-coupled oxidation phosphorylation (Estabrook, 1967; Gottlieb et al., 2002). Thus, the decreased ability of the mitochondria to carry out ADP coupled respiration as observed in normal-pH beef might be attributed to a less active ATP synthase or to a decreased ability to transport ADP.

Further, the greater ADP/O ratios observed in normal-pH beef could also be associated with coupling of nucleotides and phosphate transport to electrochemical proton gradient across the mitochondrial inner membrane (Gottlieb et al., 2002). Additionally, the significantly greater response to addition of uncoupler, FCCP in dark-cutting beef might suggest that the proton gradient across the inner membrane was not dissipated, and mitochondrial respiration was thus not limited by electron transport or access to oxidizable substrate.

The increased oxidative phosphorylation capacity coupled with increased complex II respiration observed in dark-cutting beef may suggest greater mitochondrial oxygen consumption. Thus, this might inhibit myoglobin oxygenation and hence, cause muscle darkening. Egbert & Cornforth (1986) noted that inhibition of pre-rigor mitochondrial respiration with rotenone results in a bright-red color. Thus, increased mitochondrial respiration may explain, in part, the dark-color in dark-cutting beef.

Conclusion

Dark-cutting beef has a greater oxidative phosphorylation capacity primarily due to mitochondrial complex II respiration. The incidences of stress before slaughter may trigger a bioenergetics survival mechanism ensured to maintain energy homeostasis through increased mitochondrial density and respiration. This activity is supported by a high ultimate post-mortem muscle pH, increasing mitochondria's capacity to utilize oxidizable substrates and consume oxygen.

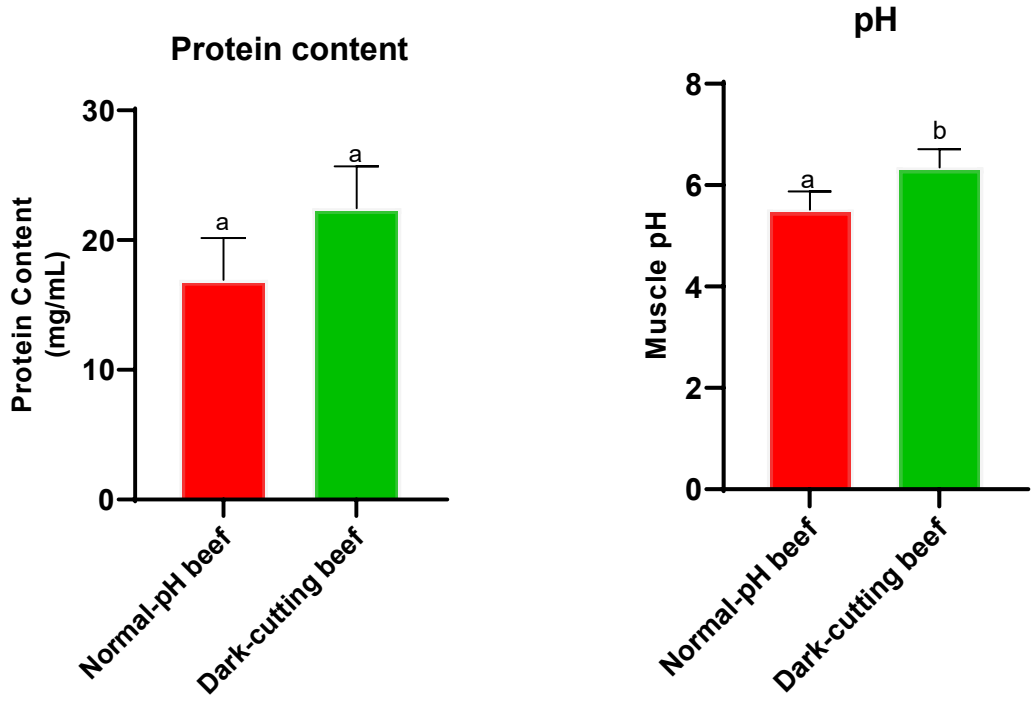

Figure 8: Differences in mitochondrial protein content (A) and Muscle pH (B) in normal-pH (green) and dark-cutting beef (red)

Mitochondria were isolated from 11 normal-pH and dark-cutting beef samples. Mitochondria protein content was determined using a BCA assay. The pH of each steak was recorded using an Accumet 50 pH meter. a-b, Least square means with different letters are different ($P < 0.05$).

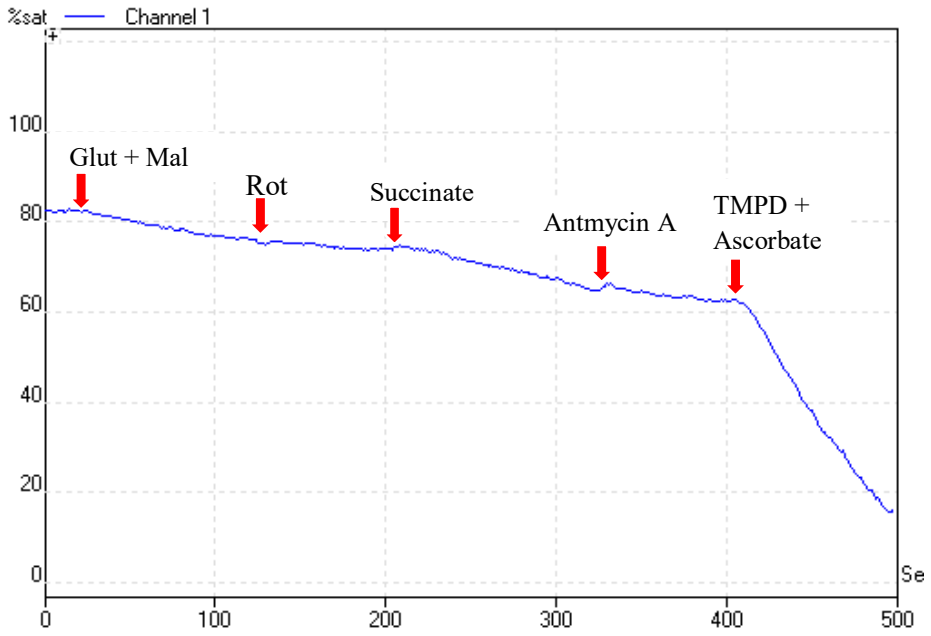

Figure 9: Evaluation of mitochondrial respiration at complex I, II, and IV

Mitochondria isolated as described in the methods was used to measure mitochondrial respiration at complex I, II and IV. Mitochondrial basal respiration was measured for 20 seconds and then complex I was initiated by the addition of glutamate and malate, and run for 80 seconds. Subsequently, respiration was blocked by addition of rotenone at 120 seconds . Succinate was added to stimulate complex II respiration and then blocked by addition of Antimycin A. Finally, complex IV respiration was measured in presence of TMPD and ascorbate as substrates for about 100 seconds.

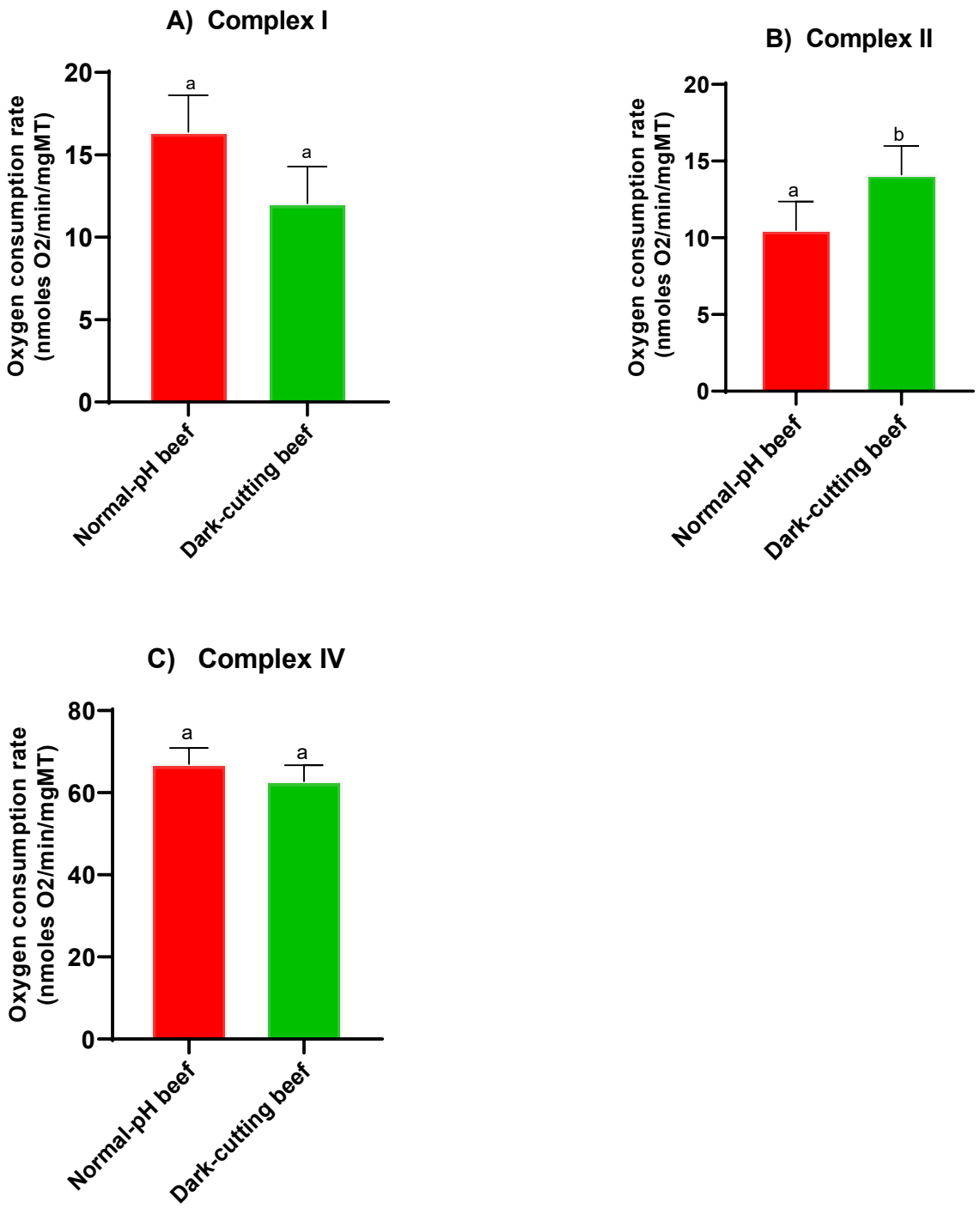

Figure 10: Differences in mitochondrial OCR measured in normal-pH beef (red) vs. dark-cutting beef (green)

Differences in mitochondria respiration at complex I, II and IV between normal-pH and dark-cutting beef was measured as described in Figure 8. A) Represents mitochondrial OCR measured at complex I after the addition of glutamate and malate. B) Represents mitochondrial

OCR measured at complex II after addition of succinate. C) Represents mitochondrial OCR measured at complex IV after addition of TMPD and ascorbate. a-b, Least square means with different letters are different ($P < 0.05$).

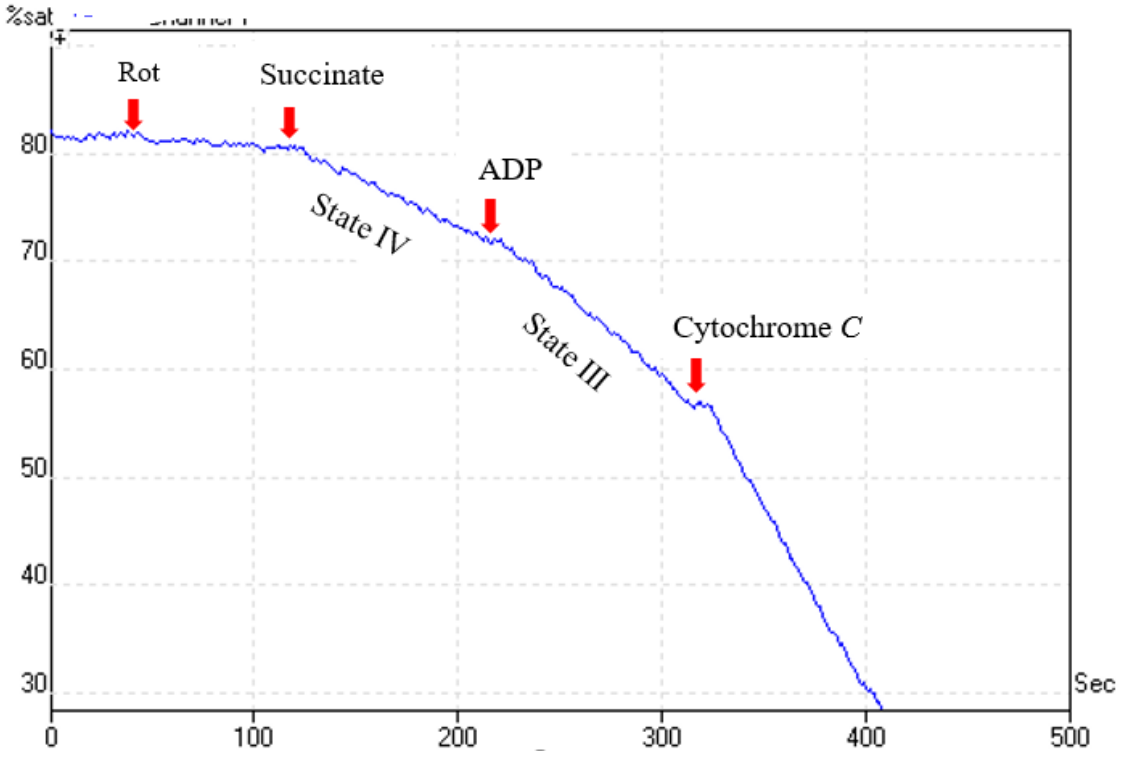

Figure 11: Evaluation of mitochondrial state III, IV and outer membrane integrity

To evaluate mitochondrial state III, IV and outer membrane integrity, mitochondria were isolated as described in the methods. Mitochondrial basal respiration was measured for 20 seconds, and then inhibited by addition of rotenone. State IV OCR was initiated by addition of succinate while state III OCR was initiated by addition of ADP. Finally, mitochondrial membrane integrity was evaluated by addition of cytochrome *c*.

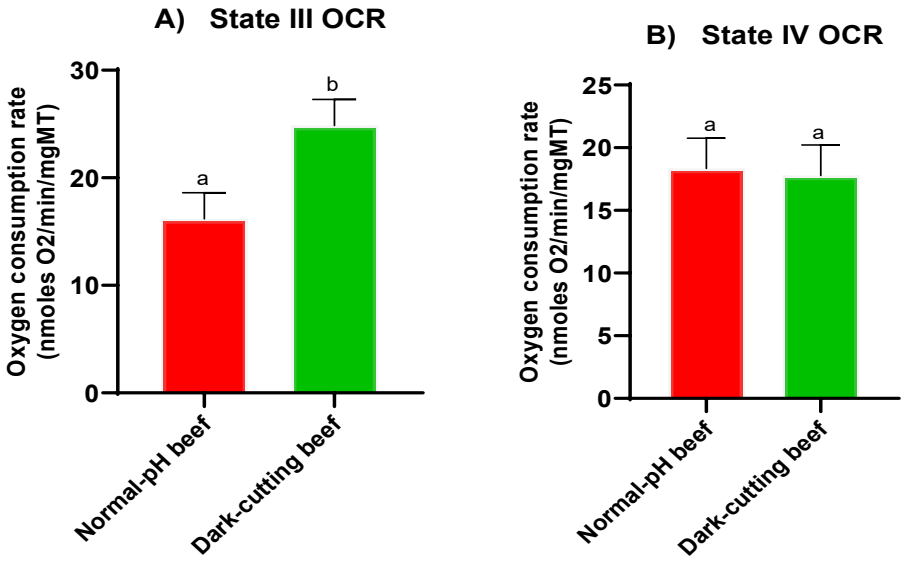

Figure 12: Differences in mitochondrial OCR under state III and state IV in normal-pH and dark-Cutting beef

Mitochondrial state III and IV between normal-pH and dark-cutting beef, was evaluated as described in Figure 10. A) Represents state III OCR determined by measuring the rate of oxygen consumption by mitochondria in presence of ADP. B) Represents state IV OCR was determined by measuring mitochondrial respiration in presence of succinate as the substrate. a-b, Least square means with different letters are different ($P < 0.05$).

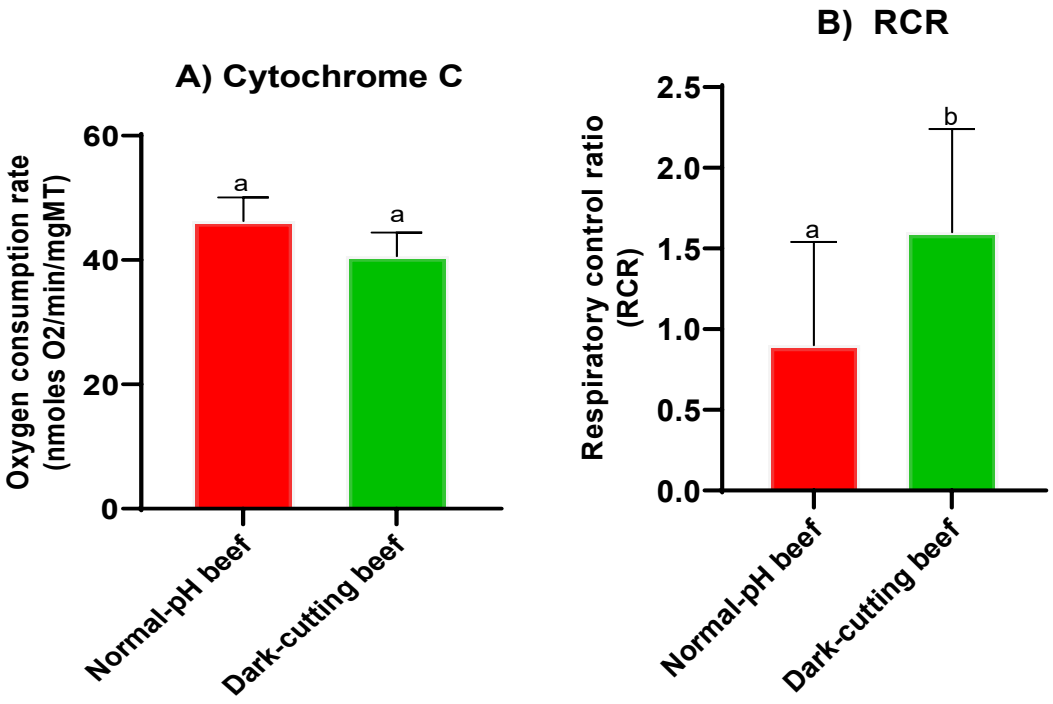

Figure 13: Differences in mitochondrial outer membrane integrity and respiratory control ratio in normal-pH (Red) and dark-cutting beef (green)

Mitochondrial outer membrane integrity in dark-cutting vs normal-pH beef, was evaluated as described in Figure 10. A) Represents mitochondrial membrane integrity measured by adding extraneous cytochrome *c*. B) Represents mitochondrial RCR calculated as a ratio between state III and state IV OCR. a-b, Least square means with different letters are different ($P < 0.05$).

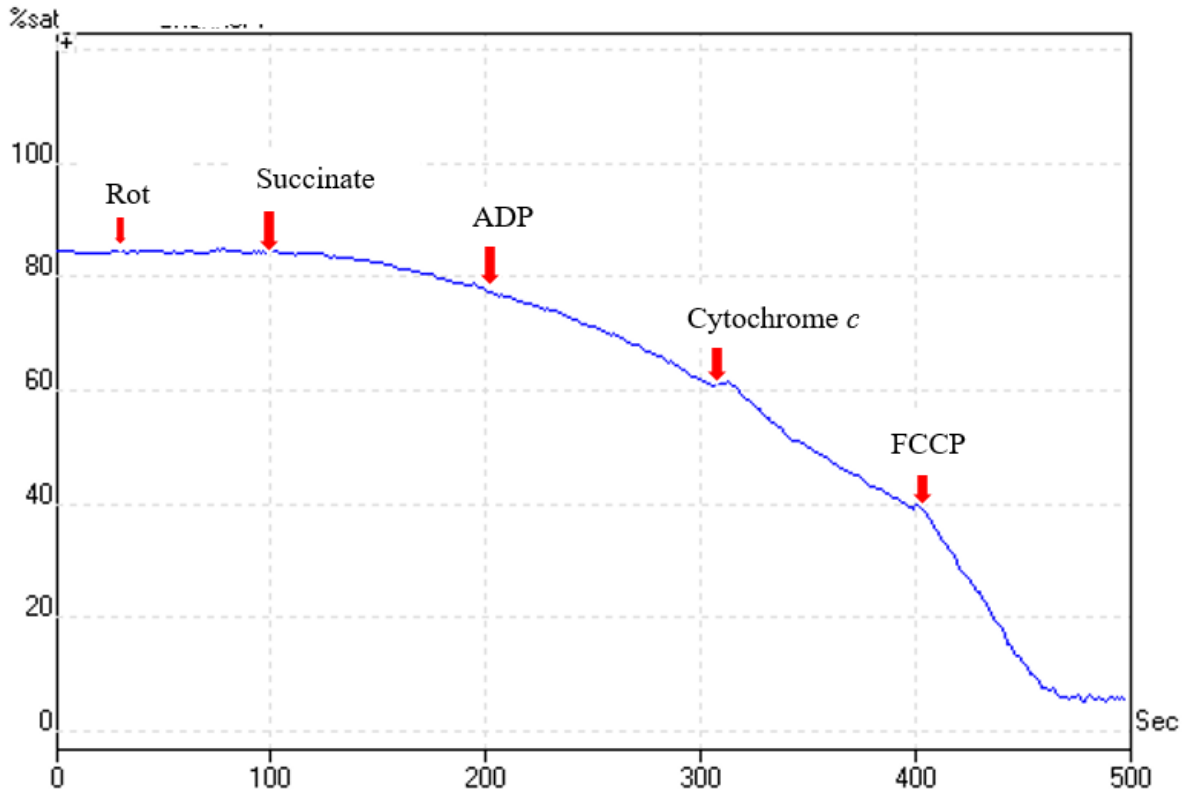

Figure 14: Evaluation of mitochondrial uncoupled respiration using FCCP uncoupler

Mitochondria isolated as described in the methods was used to evaluate mitochondrial uncoupled respiration using FCCP as the uncoupler. Mitochondrial basal respiration was measured for 20 seconds and then inhibited by the addition of rotenone. State IV OCR was initiated by addition of succinate while state III OCR was initiated by the addition of ADP. Membrane integrity was evaluated by addition of cytochrome c. FCCP was added to determine mitochondrial OCR under uncoupled state.

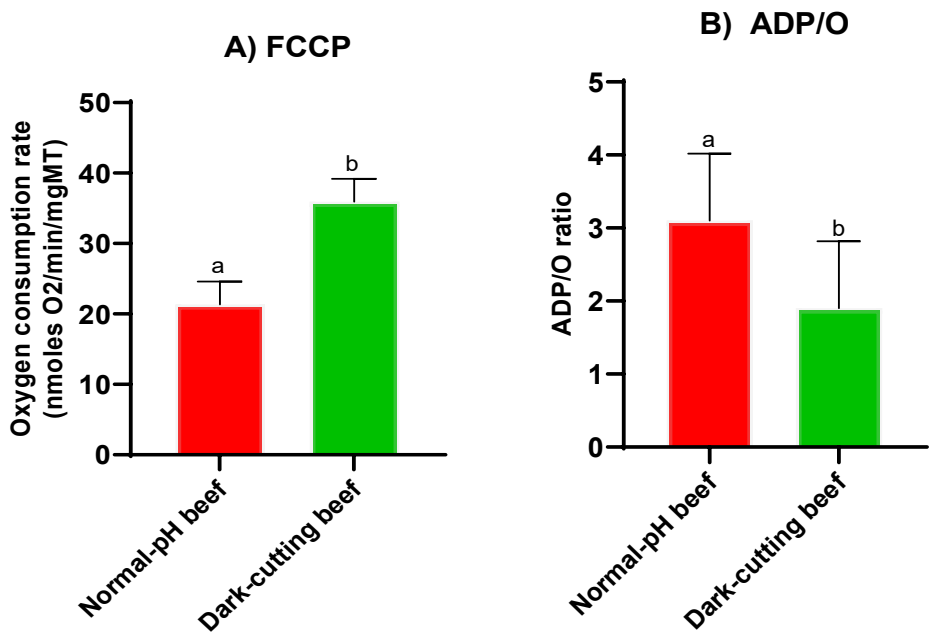

Figure 15: Differences in mitochondrial coupled and uncoupled respiration between normal-pH (red) and dark-cutting beef (green)

Mitochondria uncoupled respiration was evaluate between dark-cutting and normal-pH beef using as described in Figure 13. A) Mitochondrial uncoupled respiration was measured by the addition of uncoupler FCCP and effects on subsequent respiration was measured for 100 seconds. B) Mitochondrial coupled oxidative phosphorylation calculated as a ratio between amounts of ADP (nmoles), added under state III OCR to number of oxygen molecules consumed (nmoles).

Table 1. Comparison of mitochondrial content and biochemical properties of normal-pH and dark-cutting beef longissimus muscle.

Parameters		Normal	Dark cutting beef	SEM	P-value
a) Mitochondrial comparison[1]	Mitochondrial protein yield	1.85	2.62	0.13	0.03
b) Surface color[2]	L*	40.5	29.4	0.84	< 0.001
	a*	28.4	21.5	0.93	< 0.001
c) Biochemical properties[3]	pH	5.6	6.3	0.06	< 0.001
	Myoglobin concentration (mg/g meat)	5.7	7.9	0.27	< 0.001
	%Oxygen consumption (OC)	61.4	74.2	2.40	< 0.001
	Metmyoglobin reducing activity (MRA)	0.82	1.32	0.09	< 0.001

[1] Mitochondrial protein yield represents total mitochondria isolated from normal pH and dark cutting muscle by differential centrifugation. It represents mg of protein per gram of tissue

[2] Surface color was measured using a HunterLab Miniscan Spectrophotometer after exposing the steaks in atmospheric oxygen for 1 h at 4 °C. L* represents lightness and a lower number represents darker meat color; a* value represents redness and a lower number indicates less red color.

[3] pH was measured using a probe type pH meter; OC determined by changes in % deoxymyoglobin level after blooming and vacuum packing for 30 min at 30 °C. A greater number represents more oxygen consumption; MRA was determined as initial metmyoglobin formation of steaks immersed in nitrite solution for 20 min at 30 °C. K/S ratio at K/S572 ÷ K/S525 was used to determine metmyoglobin. A lower number represents more metmyoglobin formation and less MRA.

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APPENDICES

APPENDIX 1: SUPPLEMENTAL TABLES FOR DIFERENTIALLY EXPRESSED PROTEINS

TABLE SUPPLIMENT 1

Table 2: Proteins with higher (↑) expression ($P < 0.05$) in dark cutting beef relative to normal beef

Group	Proteins	Fold change	Expression in DC/NB	Gene name
Mitochondrial and metabolic	NADH: Ubiquinone oxidoreducatase subunit A2	1.5	↑DC	NDUFA2
	Succinate dehydrogenase [Ubiquinone] flavoprotein subunit, mitochondrial	1.3	↑DC	SHDA

Succinate dehydrogenase complex iron sulfur subunit B	1.3	↑DC	SHDB
ATP synthase membrane subunit e	1.3	↑DC	ATP5ME
Cytochrome C oxidase subunit 6A2	1.3	↑DC	
Enoyl-CoA hydratase, mitochondrial	1.4	↑DC	ECHS1
Coenzyme Q8A	1.4	↑DC	COQ8A
Aldehyde dehydrogenase1 family member A3	1.5	↑DC	ALDH1A3
3-Oxoacid CoA transferase			
UDP-glucose prophosphorylase 2	1.4	↑DC	OXCT1

		1.5	↑DC	UGP2
	Malate dehydrogenase	1.4	↑DC	MDH1
	Haloacid dehydrogenase like hydrolase domain containing 2	1.4	↑DC	HDHD2
Actin associated and muscle contraction	Xin actin binding repeat containing 1	4.4	↑DC	XIRP1
	Alpha-actin -4	1.6	↑DC	ACTN4
	Myomesin-3	1.6	↑DC	MYOM3
	Alpha-actin 2	1.3	↑DC	ACTN2
	Myosin heavy chain 4	1.5	↑DC	MYH4

Immune and inflammatory	Immunoglobulin heavy constant gamma 4	2.0	↑DC	IGHG4
Others	Cysteine and glycine-rich protein 3	1.8	↑DC	CSRP3
	Protein phosphatase 1	1.3	↑DC	PPP1R27
	Glutaredoxin 3	1.3	↑DC	GLRX3
	Fibrinogen gamma-B chain	1.6	↑DC	FGG
	Peptidylproyl isomerase D	1.3	↑DC	PPID
	Lim and cysteine rich domains protein 1	1.5	↑DC	LMCD1
	Eukaryotic translation initiation factor 1	1.4	↑DC	E1F1B
	Non-metastatic cell 2, expressed in NM23	1.4	↑DC	NME2

	PDZ and LIM domain 5	1.3	↑DC	PDLMI5

Table supplement 2.

Table 3: Proteins with lower (↓) expression ($P < 0.05$) in dark cutting beef relative to normal beef

Group	Proteins name	Fold change	Expression in DC/NB	Gene name
Glycolytic and metabolic	Glycogen phosphorylase, muscle	1.4	↓DC	PYGM
	Phosphorylase b kinase regulatory subunit beta	1.4	↓DC	PHKB
	Amylo-alpha-1-6-glucosidase,4-alpha-glucotransferase	1.3	↓DC	AGL
	Bis-phosphoglycerate mutase	1.3	↓DC	BPGM

Non-specific serine/threonine protein kinase	1.3	↓DC	PPP2R1A
AMP deaminase	2.4	↓DC	AMPD1
Adenylo-succinate lyase	1.3	↓DC	ADSL
Bifunctional purine biosynthesis protein (PURH)	1.3	↓DC	ATIC
EPM2A glucan phosphatase, Iaforin	1.5	↓DC	EPM2A
Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	1.6	↓DC	DHDH
Delta-aminolevulinic acid dehydratase	1.4	↓DC	ALAD
GTP-binding protein SAR1b	1.6	↓DC	SAR1b
Myosin-1	1.7	↓DC	MYH1

Myosin and muscle contraction	Voltage – dependent L-type calcium channel subunit beta 1	1.3	↓DC	CACNB1
Proteasome	Proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	1.5	↓DC	PSME1
	26s proteasome non ATPase regulatory subunit 3	1.4	↓DC	PSMD3
Others	Cop9 Signalosome subunit 8	1.5	↓DC	COPS8
	Collagen alpha-1 (IV) chain	1.5	↓DC	COL4A1
	Collagen alpha-2 (IV) chain	1.3	↓DC	COL4A2

Cop9 Signalosome complex subunit 7a isoform X4	1.8	↓DC	COPS7A
Cardiomyopathy associated 5	1.3	↓DC	CMYA5
Bleomycin hydrolase	1.4	↓DC	BLMH
Stromal interaction molecule 1	1.3	↓DC	STIM1

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