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Role of Mitochondria in Beef Color: A Review

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Abstract: In postmortem muscle, mitochondria remain active and can influence beef color by oxygen consumption and metmyoglobin reduction. Enzymes involved in glycolysis and the tricarboxylic acid cycle can generate reducing equivalents such as succinate or NADH. Mitochondrial activity is critical to maintain steaks that are bright cherry-red and improve color stability. This review seeks to characterize the role of mitochondria in beef color; more specifically to understand the effects of mitochondrial function on myoglobin redox stability.

Keywords: beef color, mitochondria, MRA, myoglobin, oxygen consumption
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Introduction

Visual perception plays an important role in the acceptability of food products. This is particularly relevant to beef purchasing decisions because consumers associate bright-red lean with freshness and wholesomeness. Discoloration of meat has resulted in an annual loss of $1 billion to the U.S. meat industry (Smith et al., 2000). Although discoloration is inevitable, understanding the biochemical pathways that influence myoglobin redox chemistry can help processors to develop strategies that improve color stability. Several reviews (Giddings, 1977; Faustman and Cassens, 1990; Cornforth, 1994; Bekhit and Faustman, 2005; Mancini and Hunt, 2005) have discussed in detail the factors affecting discoloration, however, no published reviews have focused specifically on the role of mitochondria in meat color. Hence, the objective of this review was to provide an overview of the interrelationship amongst mitochondria, myoglobin, and beef color.

Myoglobin chemistry

Myoglobin is the water-soluble sarcoplasmic protein responsible for meat color. The globular structure of myoglobin contains 153 amino acids composed of 8 α-helices and a heme prosthetic group in the protein's center. The apo-protein portion of myoglobin is colorless and therefore, the heme prosthetic group imparts color (Giddings, 1977). The heme moiety is located within myoglobin's hydrophobic pocket and contains a central iron atom that can form 6 bonds. Four of these bonds are coordinated with pyrrole nitrogens, the 5th coordinates with the proximal histidine of myoglobin, and the 6th position is available to bind ligands. Both the proximal and the distal histidine play a significant role in stabilizing the heme and the ligand bound to myoglobin. More specifically, the proximal histidine coordinates the heme ring and the distal histidine stabilizes the heme-oxygen complex (American Meat Science Association, 2012).

The valence state and the ligand present at the 6th position determine meat color. In fresh beef, myoglobin can exist in three forms: deoxymyoglobin, oxymyoglobin, and metmyoglobin. Deoxymyoglobin is formed when no ligand occupies the 6th position and iron is in the ferrous state. This results in purplish color and occurs mainly in vacuum-packaged meat. An oxygen tension of less than 1.4 mm Hg is required to maintain deoxymyoglobin (Ledward, 1970). Oxymyoglobin

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produces a bright cherry-red color and is formed when oxygen binds to the 6th position of myoglobin's centrally located iron. Oxidation of oxymyoglobin results in brown metmyoglobin, which has ferric iron and water occupies the 6th position. Accumulation of metmyoglobin results in surface discoloration.

The introduction of case-ready packaging has allowed beef purveyors to modify the gas compositions within packages. This has generated interest in the use of carbon monoxide at a level of 0.4% in low-oxygen packaging (FDA, 2004). Carboxymyoglobin can form a stable red color due to strong binding of carbon monoxide to the 6th position of iron. More specifically, binding of oxygen to iron results in a bent configuration with the distal histidine while carbon monoxide forms a stable linear configuration with iron and the distal histidine (Makinen et al., 1979).

The processes that regulate the interconversion among myoglobin redox states determine beef color. Oxygen consumption and metmyoglobin reducing activity (MRA) are 2 important biochemical properties that influence myoglobin redox state and are influenced by mitochondrial function (Figure 1).

Mitochondria and postmortem oxygen consumption

Mitochondria are double membrane-bound organelles ranging from 1 to 10 micrometers and are responsible for cellular respiration (Stevens, 1981). The outer membrane is a phospholipid bilayer completely surrounding the organelle and is readily permeable to ions and small molecules. The electron transport complex, ATP synthase complex, and transport proteins are present in the inner membrane. Between the inner and outer membranes is the inter-membrane space. The inner membrane is highly convoluted, permeable to oxygen and water, and folded to form cristae that increase surface area (Frey and Mannellab, 2000). The mitochondrial matrix contains enzymes for the tricarboxylic acid cycle and contains about 2/3 of the total protein present in mitochondria.

The inner membrane contains complexes and proteins involved in the electron transport chain, including various fat-soluble electron carrier proteins such as ubiquinone, cytochromes, and iron-sulfur proteins. There are 4 enzyme complexes within the electron transport chain, each of which transfer electrons from reducing equivalents such as NADH and $FADH_2$ to NADH-Coenzyme Q reductase (complex I), succinate dehydrogenase (complex II), and to cytochrome bc_1 (complex III) and cytochrome *c* oxidase (complex IV; Scheffler, 2007). Oxygen is consumed at complex IV to form water.

Mitochondria continue to metabolize oxygen in postmortem muscle, although oxygen consumption by meat tends to decrease as time increases. For example, mitochondria isolated from ox muscle retained normal configuration after 5 days of postmortem storage at 4 °C (Cheah and Cheah, 1971). In the presence of succinate, mitochondria isolated from bovine cardiac muscle after 60 days of postmortem storage in vacuum package at 4 °C can consume 75.4 and 22.4 nanomoles of oxygen per min per mg of mitochondria at pH 7.4 and 5.6, respectively, (Tang et al., 2005a). These authors also demonstrated that mitochondrial morphology changes with storage time, indicating swelling and membrane breakdown.

Other researchers have investigated the effects of glycolytic- and tricarboxylic substrates on postmortem bovine mitochondrial function. For example, the addition of lactate, pyruvate, malate, and lactate-LDH-NAD to isolated beef heart mitochondria increased oxygen consumption (Ramanathan et al., 2009). Pyruvate can enter mitochondria via pyruvate translocase and is converted to acetyl-CoA (Alam et al., 2015), which can take part in the tricarboxylic acid cycle and ultimately produce NADH. Similarly, NADH is produced when lactate is converted to pyruvate by lactate dehydrogenase activity. Mohan et al. (2010) reported that malate-NAD-malate dehydrogenase increased NADH formation in beef. In support, pyruvate and malate increased mitochondrial oxygen consumption in vitro (Ramanathan and Mancini, 2010). Thus, metabolites that can generate either NADH or succinate have the potential to increase mitochondrial oxygen consumption.

The US beef industry has started retailing individual muscles. Hence, it is important to understand muscle-specific differences in color stability. Mckenna et al. (2005) classified beef muscles based on color stability into 3 groups, including color labile, intermediate, and color stable. Mitochondria is one of several factors that can influence this classification. Mitochondrial concentration in muscle depends on muscle fiber type. For example, red fibers have more mitochondria and myoglobin than white fibers. The *psoas major* has more red fibers than the longissimus (Hunt and Hedrick, 1977). This suggests that mitochondrial content and metabolite utilization within these two muscle types may be different because the psoas is a color labile muscle whereas the longissimus is a color stable muscle. A greater mitochondrial content can lead to more oxidative stress; hence, more discoloration in the psoas than the longissimus. Differences in color stability between

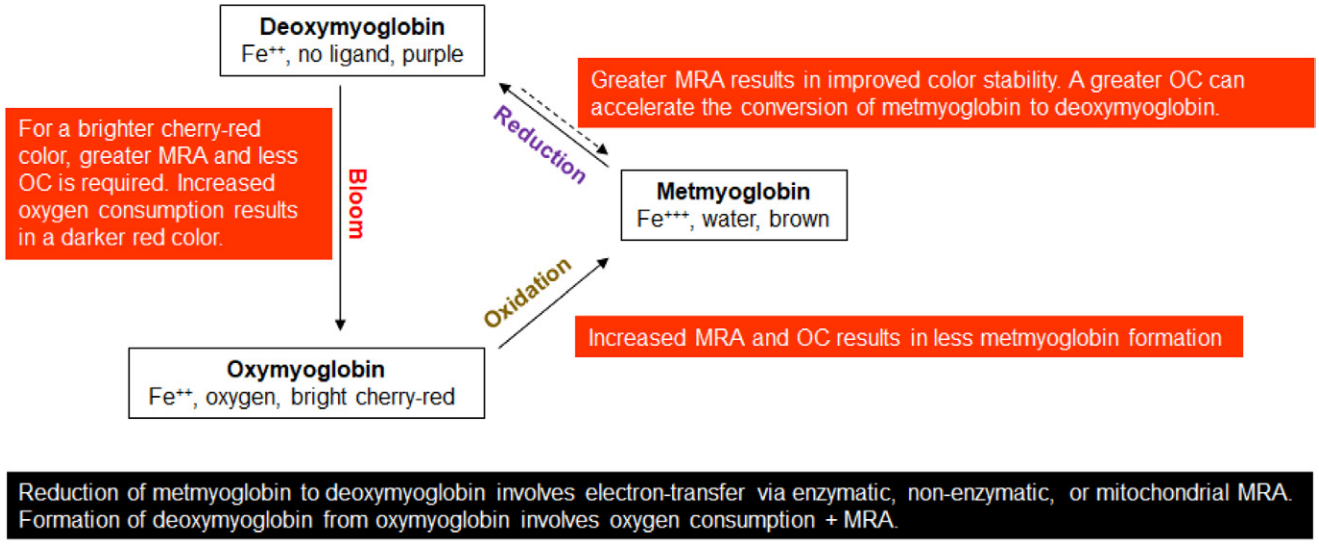

Figure 1. Effects of oxygen consumption and metmyoglobin reducing activity on redox form of myoglobin in fresh beef. OC = oxygen consumption; MRA = metmyoglobin reduction activity.

the psoas and the longissimus also can be attributed to variation in antioxidant enzymes and MRA (Mohan et al., 2010; Ke et al., 2017).

Lanari and Cassens (1991) reported that the oxygen consumption rate of mitochondria isolated from Holstein longissimus and gluteus muscles decrease during storage. Cheah and Cheah (1971) concluded that increased storage time resulted in a significant loss of cytochrome *c* activity and a decline in state III respiration. O'Keeffe and Hood (1982) reported that the oxygen consumption rate (micromole of oxygen consumed/g fresh tissue) of longissimus and psoas muscles decreased from 221.7 (d 0) to 32.7 (d 10) and 249.7 (d 0) to (d 10) 52.9, respectively, as measured using a Warburg constant volume respirometer. The oxygen penetration depth beneath the surface of steaks was deeper for the longissimus muscle compared with the psoas muscle (Joseph et al., 2012). More specifically, the depth of oxygen penetration and thickness of the oxymyoglobin layer depend on the rate of oxygen diffusion and oxygen consumed by mitochondria (O'Keeffe and Hood, 1982).

Several other factors that influence mitochondrial function are summarized in Table 1. A better understanding of the parameters involved in mitochondrial function can offer insights related to mitochondria-mediated effects on color.

Oxidative stress and mitochondrial degeneration

Immediately after slaughter, a lack of continuous oxygen and blood supply shifts energy production from aerobic to anaerobic metabolism. Although postmortem muscle attempts to maintain homeostasis, antioxidant defense mechanisms become less efficient and mitochondrial degeneration occurs (Crimi and Esposti, 2011). As a result, mitochondria are the first organelle affected by tissue anoxia (Ouali et al., 2013). More specifically, mitochondrial cardiolipin and Bcl-2 protein can interact with the outer membrane and increase mitochondrial outer membrane permeabilization. In addition, the close proximity of mitochondrial membranes to free radicals increases susceptibility to oxidative damage.

Increased mitochondrial damage can have a detrimental effect on color. The presence of cytochrome *c* (electron carrier between complex III and IV within electron-transport chain) in the cytosol is used as an indicator of mitochondrial degeneration. Recent studies have reported that the decreased color stability of psoas muscle can be attributed to greater mitochondrial damage (Ke et al., 2017; Mancini et al., 2018). In support, cytochrome *c* content was greater in psoas sarcoplasm than longissimus sarcoplasm during a 6 d display. Other research indicates that incubation of oxymyoglobin with mitochondrial lipids promoted myoglobin oxidation (Tang et al., 2005c). Conversely, supplementing lamb diets with α-tocopherol (vitamin E) decreased oxymyoglobin oxidation and increased mitochondrial function due to the accumulation of α-tocopherol in the mitochondrial membrane (Tang et al., 2005c).

Interrelation between mitochondria and myoglobin function

Lipid and protein oxidation, microorganisms, mitochondria, and myoglobin compete for oxygen in

Table 1. Factors affecting mitochondrial oxygen consumption

	Factor	Effect	System	OCR methodology	Reference
1	pH	Increased pH enhances mitochondria activity, while decreased pH limits activity	Isolated beef mitochondria	Clark oxygen electrode	Ashmore et al. 1972
			Isolated beef mitochondria	Clark oxygen electrode	Tang et al. 2005a
			Permeabilized pork tissue	High-resolution respirometer	Phung et al., 2011
			Intact pork	Measured as a change in %OxyMb using reflectance measurement	Zhu and Brewer, 1998
			Intact beef	Measured as a change in %OxyMb using reflectance measurement	English et al.,2016b
2	Temperature	Increased temperature enhances mitochondria activity, while cold temperature decrease activity	Isolated beef mitochondria	Clark oxygen electrode	Tang et al. 2005a
			Intact and minced	Manometrically in a Warburg apparatus	Bendall and Taylor, 1972
			Permeabilized pork tissue	High-resolution respirometer	Phung et al., 2011
3	Species	Lamb > pork > beef	Minced tissue	Manometrically in a Warburg apparatus	Atkinson and Folett, 1973
4	Breed	Cross bred > Holstein	Isolated beef mitochondria	Clark oxygen electrode	Lanari and Cassens, 1991
5	Muscle effect	Diaphragm > PM > Tensor fasciae late	Ground tissue	Clark oxygen electrode	Renerre and Labas, 1987
		PM = LD = SM (day 0) PM = LD > SM (day 5)	Intact muscle	As a change in carbondioxide level	Mckenna et al., 2005
		PM > LL	Intact muscle	Changes in oxymyoglobin level	Seyfert et al., 2007; Abraham et al., 2017
		PM > LL	Isolated mitochondria	Clark oxygen electrode	Ke et al., 2017
6	Muscle location	Inside semimembranosus has greater oxygen consumption than outside semimembranosus	Intact beef	Measured change in oxygen concentration in flask using Oxygen analyzer	Sammel et al., 2002
		Outside semimembranosus has greater oxygen consumption than inside semimembranosus	Isolated mitochondria (day 0)	Clark oxygen electrode	Nair et al., 2017
7	Postmortem age	Increased storage time decreased oxygen consumption	Intact beef	Measured as a change in % OxyMb using reflectance measurement	Madhavi and Carpenter, 1993; King et al., 2011
		Increased storage time decreased mitochondrial activity and electron-transport mediated MRA	Isolated mitochondria	Clark oxygen electrode	Mancini and Ramanathan, 2014
8	Substrates	Succinate, lactate, pyruvate increased oxygen consumption	Isolated beef mitochondria	Clark oxygen electrode	Tang et al. 2005a; Ramanathan et al., 2009.
		NADH increased oxygen consumption	Minced lamb	Manometrically in a Warburg apparatus	Atkinson et al., 1969
9	Packaging	PVC > High oxygen	Intact beef	Measured change in % OxyMb using reflectance measurement	Seyfert et al., 2007
10	Lipid oxidation	a) Decreased oxygen consumption	Intact beef	Measured change in % OxyMb using reflectance measurement	Seyfert et al., 2007
		b) Decreased oxygen consumption	Isolated beef mitochondria	Clark oxygen electrode	Ramanathan et al., 2012
10	Processing	a) Fabrication type: ground > saw > knife	Intact beef	Measured as a change in % OxyMb using reflectance measurement	Madhavi and Carpenter, 1993
			Minced and intact beef	Manometrically in a Warburg apparatus	Bendall and Taylor, 1972
		b) Ground v/s intact: mincing increase oxygen consumption	Isolated beef mitochondria	Clark oxygen electrode	Tang et al., 2006
		c) Sonication decrease mitochondrial activity	Isolated beef mitochondria	Clark oxygen electrode	Tang et al., 2006
		d) Freezing thaw cycle increase oxygen consumption, but decrease functional integrity	Isolated beef mitochondria	Clark oxygen electrode	Tang et al., 2006

postmortem muscle (Faustman and Cassens, 1990). Myoglobin serves as an oxygen reservoir and oxygen transporter for mitochondria. Approximately 80% of oxygen in the cell is used by mitochondria (Wittenberg, 1970). Therefore, competition for oxygen between mitochondria and myoglobin is the limiting factor responsible for the development of bright cherry-red surface color. More specifically, mitochondria can affect myoglobin redox state by (1) decreasing oxygen partial pressure via respiration, and (2) reducing metmyoglobin (Tang et al., 2005a, 2005b). Thus, mitochondria can influence both color development (oxygenation/bloom) and color stability (oxidation).

Oxygen consumption and muscle darkening

A characteristic bright-red color is required for consumer acceptance (Smith et al., 2000). Although bloomed steaks from both dark-cutting beef and lactate-enhanced meat will have a dark red color, the mechanism(s) responsible for this darkening is different. In dark-cutting beef, a greater than normal pH will prevent the decrease in mitochondrial activity that typically occurs as muscle pH declines postmortem. As a result, an increase in mitochondrial activity promotes oxygen consumption and darkens muscle due to decreased oxygen partial pressure. This encourages the maintenance of myoglobin in a deoxygenated state (Ashmore et al., 1972; Cornforth and Egbert, 1985; Egbert and Cornforth, 1986; Renerre and Labas, 1987). In this situation, meat fails to bloom (oxygenate) and does not form the characteristic bright cherry-red color associated with fresh beef. This is supported by decreased myoglobin oxygenation of bloomed dark-cutting beef compared with normal-pH beef (English et al., 2016a).

The mechanism(s) of lactate-induced darkening in beef suggests that the addition of lactate results in NADH formation by lactate dehydrogenase activity (Kim et al., 2006). This NADH can be used as a complex I substrate, resulting in increased mitochondrial oxygen consumption and muscle darkening. This was supported in vitro by the addition of lactate, NAD, and LDH to bovine longissimus mitochondria and oxymyoglobin (Ramanathan et al., 2013). Incubating mitochondria-oxymyoglobin mixture with substrates increased deoxymyoglobin by 6 h of incubation, and maintained deoxymyoglobin for 24 h. A greater amount of deoxymyoglobin results in a darker beef color. Previous research demonstrated lactate-enhanced steaks had less myoglobin oxygenation than control steaks (Ramanathan et al., 2012). The role of mitochondria in lactate-mediated darkening also has been supported by research utilizing the addition of mitochondrial inhibitor (rotenone; complex I inhibitor) to lactate-cardiac muscle homogenates, which reversed lactate-induced darkening. Both in dark-cutting and lactate-enhanced beef, physical mechanism(s) also contribute to muscle darkening. More specifically, a greater pH in dark-cutting beef can increase the ability of muscle fibers to hold more water (Swatland, 2008). However, in lactate-enhanced beef, presence of lactate can influence refractive index of sarcoplasm (Ramanathan et al., 2010). Both conditions can decrease light reflectance properties, resulting in darker meat color.

The effect of mitochondrial oxygen consumption on myoglobin redox state has been demonstrated in vitro. Mitochondrial respiration will result in the conversion of oxy- to deoxymyoglobin following the addition of mitochondrial substrate such as succinate (Tang et al., 2005a). This conversion is promoted by greater mitochondrial density and pH. Further, anaerobic conditions favor a decrease in oxygen partial pressure and deoxymyoglobin formation from oxymyoglobin. Cornforth and Egbert (1985) reported that inhibition of pre-rigor mitochondrial respiration by rotenone will result in a bright-red color.

Mitochondrial oxygen consumption and metmyoglobin reduction

In addition to the role of mitochondria in oxygen consumption (color development), mitochondrial activity also can affect myoglobin redox stability (color stability). Metmyoglobin reducing activity is an important intrinsic property that prolongs the color stability of meat during storage (Ledward, 1985). More specifically, MRA relies on the production of either NADH (by dehydrogenase enzymes) or electrons (by mitochondria), both of which can be used to reduce metmyoglobin (Giddings, 1977; Faustman et al., 1988; Bekhit and Faustman, 2005). Mitochondria-mediated MRA can occur via four different pathways:

1. *Electron-transport mediated MRA*: Cytochrome *c* is an electron carrier that transports available electrons from the electron transport chain to metmyoglobin (from the inner membrane to outer membrane). Addition of succinate to metmyoglobin-mitochondria mixtures increased ferrous myoglobin by promoting the transfer of available electrons within the electron transport chain to metmyoglobin between complexes III and IV via cytochrome *c* (Tang et al., 2005b). Other studies have reported that the addition of pyruvate and lac-

tate increased electron-transport mediated reduction (Ramanathan and Mancini, 2010; Ramanathan et al., 2010). This is likely due to the formation of NADH or succinate via utilization of pyruvate or lactate by the tricarboxylic acid cycle. On the other hand, pre-incubation of mitochondria with 4-hydroxy-2-nonenal (HNE; a secondary lipid oxidation product) decreased electron-transport mediated metmyoglobin reduction at both pH 5.6 and 7.4. The addition of antimycin A (complex III inhibitor) with succinate inhibited electron-transported mediated metmyoglobin reduction. Electron-transport mediated reduction depends on pH, temperature, mitochondrial-, and substrate-concentration.

2. *Anaerobic conditions*: Mitochondrial oxygen consumption is critical for creating anaerobic conditions that favor metmyoglobin reduction (Watts et al., 1966). The conversion of oxymyoglobin to deoxymyoglobin is a two-step process, where oxymyoglobin is first converted to metmyoglobin and then to deoxymyoglobin. Therefore, limited mitochondrial activity will produce conditions that are not ideal for reducing activity. Furthermore, when mitochondria cannot consume sufficient oxygen and the oxygen partial pressure reaches 3 to 7 mm Hg, myoglobin is prone to oxidation. Lanier et al. (1978) reported that metmyoglobin reduction occurs more efficiently in anaerobic conditions than in aerobic atmospheres.

3. *NADH-dependent reductase activity*: Similar to NADH-dependent hemoglobin reductase, muscle has NADH-dependent myoglobin reductase to convert metmyoglobin to deoxy-/oxymyoglobin. NADH-dependent metmyoglobin reductase and diaphorase are present in the outer mitochondria membrane and promote metmyoglobin reduction (Giddings, 1977; Arihara et al., 1995). These enzymes can convert metmyoglobin to ferrous myoglobin in the presence of NADH. This conversion is physiologically important because the ferric form of myoglobin cannot carry oxygen. There are muscle-dependent differences in NADH-dependent enzyme activity (Lanari and Cassens, 1991). For example, g*luteus medius* had greater enzyme activity than the *longissimus dorsi* muscle both in Holstein and crossbred cattle. The addition of lactate-LDH-NAD to metmyoglobin-mitochondria mixtures increased metmyoglobin reduction via NADH-dependent reductase activity (Ramanathan et al., 2010). However, lipid oxidation products can limit mitochondria-mediated NADH-dependent reductase activity. Pre-incubation of mitochondria with HNE decreased enzymatic metmyoglobin reduction resulting from the mitochondrial fraction.

4. *Non-enzymatic MRA*: In non-enzymatic metmyoglobin reduction, compounds such as quinone, cytochrome *c*, or methylene blue can transfer electrons from NADH to metmyoglobin (Elroy et al., 2015). Recently, Belskie et al. (2015) reported that the addition of NAD and succinate to isolated beef mitochondria increased NADH formation and metmyoglobin reduction by reverse electron transfer. More specifically, reverse electron flow occurs when substrates are available and forward electron flow is inhibited or when oxygen is absent. The NADH formed as a result can be used for either enzymatic or non-enzymatic MRA. The role of mitochondria in non-enzymatic metmyoglobin may be limited due to other MRA pathways present in mitochondria. Nevertheless, NADH formed can be used by multiple metmyoglobin reduction pathways.

Effects of aging on mitochondria function

Wet aging is commonly used to increase beef palatability. However, aging time also can influence beef color. For example, initial color development (oxymyoglobin formation) of *longissimus thoracis* steaks increased with time postmortem due to a decrease in oxygen consumption (Lee et al., 2008). Although decreased oxygen consumption might be responsible for increased in initial red color intensity, MRA is also required to maintain bloom or red color intensity. Therefore, increased aging time can decrease color stability of steaks during display (Lindahl, 2011; English et al., 2016b); in part, due to decreased mitochondrial function. Increased aging time decreased oxygen consumption and electron transport-mediated metmyoglobin reduction with the addition of succinate and lactate-LDH-NAD (Mancini and Ramanathan, 2014). Wet aging of beef beyond 14 d is detrimental to beef longissimus color. For example, Seyfert et al. (2007) reported a 3 unit decrease in beef longissimus steak redness (a^* values) when aged for 11 d and during 7 d display in PVC packaging. However, as aging time increased to 21 d, changes in redness (a^* values) during 6 d display was 16.1 units (English et al., 2016b). In addition to the role of mitochondria in decreased color stability, increased aging period also can decrease metabolites required to regenerate of NADH. The effects of aging on beef color is summarized in Figure 2.

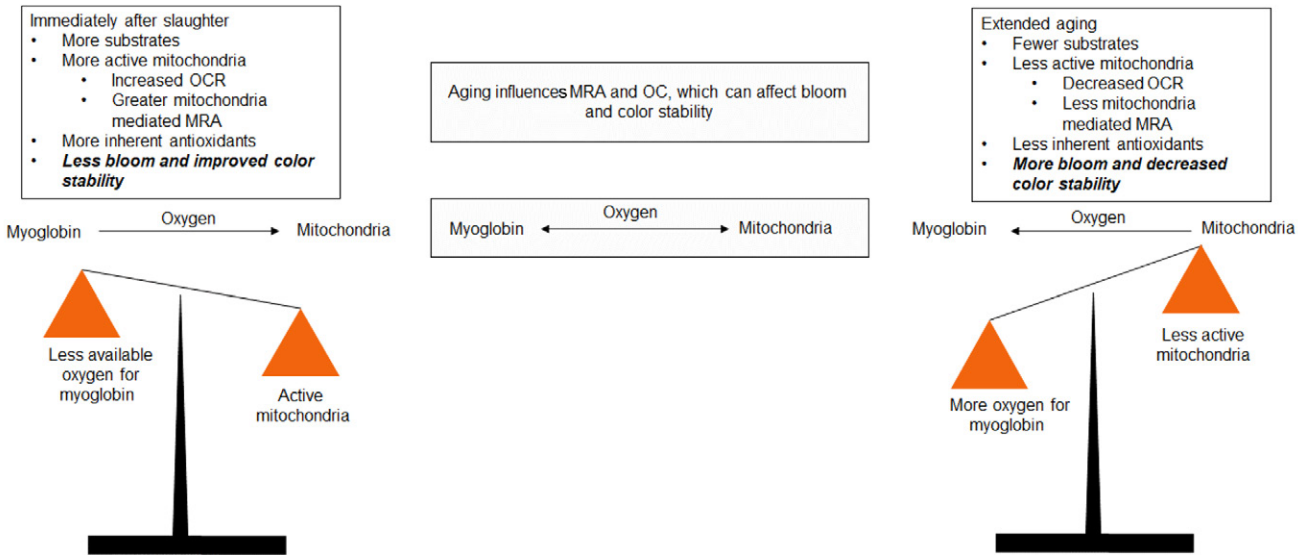

Figure 2. Effects of aging on beef color.

Mitochondria and myoglobin interaction

Myoglobin serves as an oxygen carrier and reservoir by reversibly binding oxygen. For example, in diving mammals and birds, oxygen stored in myoglobin provides the basis for aerobic cellular activity during extended periods of apnea. Myoglobin content is 10-30 times greater in muscles of diving animals compared with animals not experiencing extended periods of apnea (Noren and Williams, 2000). Furthermore, myoglobin concentration is correlated positively with dive duration (Kooyman and Ponganis, 1998).

Studies have shown that a direct interaction between mitochondria and myoglobin exists and their functions are interrelated (Wittenberg and Wittenberg, 1987; Tang et al., 2005a; Postnikova et al., 2009). Myoglobin provides oxygen to mitochondria via interaction between oxymyoglobin and the outer membrane (Wittenberg and Wittenberg, 1987). Charged residues within the C-D inter-helical region of myoglobin (C and D represents the names of alpha helix) can bind transiently to proteins present on the surface of mitochondria during oxygen delivery (Romero-Herrera et al., 1978). Oxygen can be transported from oxymyoglobin to mitochondria by facilitated diffusion (Wittenberg and Wittenberg, 2007). Postnikova et al. (2009) noted that facilitated diffusion is effective only when the oxygen partial pressure is 2 to 5 mm Hg and oxymyoglobin concentration is greater.

A decrease in oxymyoglobin and oxygen partial pressure were reported when rat mitochondria and sperm whale oxymyoglobin were incubated with succinate (Postnikova et al., 2009). More specifically, when actively respiring mitochondria were separated by a semipermeable membrane, no deoxygenation of myoglobin was observed. Therefore, these authors concluded that a mechanism other than facilitated diffusion might be present in tissue to provide effective oxygen transfer to mitochondria. More specifically, direct interaction between myoglobin and mitochondria may be necessary for oxygen transfer. However, limited research has assessed the interaction between myoglobin and mitochondria in postmortem muscle.

Mitochondria and substrates

In general, enzymes involved in glycolysis and the tricarboxylic acid cycle remain active in postmortem muscle, however, the substrates are depleted. Among the substrates and cofactors involved in color stability, NADH is a key component required for both enzymatic and non-enzymatic metmyoglobin reduction (Renerre and Labas, 1987; Echevarne et al., 1990). Although processes capable of producing NADH are continually depleted postmortem, NADH can be regenerated by dehydrogenase enzymes present in postmortem muscle, either cytoplasmic or mitochondrial (Stewart et al., 1965; Watts et al., 1966; Giddings, 1977). For example, lactate enhancement increased NADH concentration in beef longissimus by lactate dehydrogenase activity and this NADH was used for non-enzymatic metmyoglobin reduction (Kim et al., 2006). Addition of glycolytic and tricarboxylic acid (TCA) cycle intermediates to ground beef increased metmyoglobin reduction (Saleh and Watts, 1968). These authors suggested that NADH in conjunction with sarcoplasmic diaphorase enzymes facilitates metmyoglobin reduction. Atkinson et al. (1969) demonstrated a significant increase in oxygen uptake after

addition of NADH to lamb *semimembranosus* muscle. They noted that oxygen uptake was increased 3-fold with the addition of 1 to 4 micromoles NADH/ml of Ringers solution in a Warburg flask in 2 h at 15 °C. Conversely, inhibition of postmortem glycolysis decreased color stability by limiting NADH content (Jerez et al., 2003).

Enhancing beef with succinate and pyruvate increased color stability in PVC and HiOx-MAP packaging (Ramanathan et al., 2011), whereas the addition of pyruvate to steaks packaged in vacuum package increased discoloration. This suggests that the role of substrates in beef color is packaging-dependent. Enhancing beef with succinate and lactate increased MRA due to the regeneration of reducing equivalents such as NADH for MRA. Although the mechanism of pyruvate-mediated discoloration is not clear, the authors speculated that pyruvate oxidized NADH in sarcoplam via conversion of pyruvate to lactate with lactate dehydrogenase. Bjelanovic et al. (2016) also reported that pyruvate increased metmyoglobin formation in ground beef packaged in vacuum.

Mohan et al. (2010) reported muscle-specific effects of metabolites in a model system. In *semitendinosus* muscle, 2% lactate was effective in limiting discoloration, while malate was effective in longissimus and psoas muscles. Other researchers have reported that incorporating TCA substrates either individually or in combination can affect myoglobin redox stability of beef packaged in aerobic and anaerobic packaging. For example, a combination of glutamate-malate-citrate retained oxymyoglobin content in MAP packaging (70% oxygen and 30% carbon dioxide) when stored for 6 d (Bjelanovic et al. 2016). Hence, characterizing the role of metabolites will improve our understanding of beef color (Abraham et al., 2017).

Mitochondria and lipid oxidation

Polyunsaturated fatty acids, such as linoleic acid, linolenic acid, and arachidonic acid are abundant in phospholipids of cell membranes to maintain fluidity. Approximately 50% of the fatty acids present in mitochondria are unsaturated (Lass and Sohal, 1998). These fatty acids contain double bonds that are prone to oxidation. As a result, lipid oxidation within the mitochondria can form various aldehydes, alkenals, and hydroxyalkenals, all of which are highly reactive and cytotoxic to nucleic acids and proteins (Esterbauer et al., 1991). For example, oxidation of ω-6 polyunsaturated fatty acids produces HNE, which can inactivate enzymes and alter protein structure by alkylating histidine, cysteine, and lysine residues. In addition, HNE can bind covalently to histidine residues of equine (Faustman et al., 1999), bovine (Alderton et al., 2003), porcine (Suman et al., 2007), yellowfin tuna (Lee et al., 2003), chicken and turkey (Naveena et al., 2010), and ovine (Yin et al., 2011) myoglobins. The HNE can decrease color stability by covalent binding of histidine residues. The HNE also can decrease color stability by limiting functionality of enzymes involved in NADH production. For example, incubation of bovine heart LDH with HNE decreased NADH formation (Ramanathan et al., 2014). Mass spectrometric investigation indicated that HNE can covalently bind to cysteine and histidine residues, limiting LDH functionality (Ramanathan et al., 2014). Other studies have shown that HNE can bind covalently to enzymes, including glucose-6-phosphate dehydrogenase (Szweda et al., 1993), pyruvate dehydrogenase (Patel and Korotchkina, 2002), glutathione reductase (Jagt et al., 1997), and glutathione transferase (Drake et al., 2004.

In addition to the effects of HNE on myoglobin and enzymes in color stability, studies have shown that HNE can limit mitochondrial function. The HNE can bind to cytochrome *c*, which transports electrons between ubiquinol cytochrome *c* oxidoreductase (Complex III) and cytochrome *c* oxidase (Complex IV; Villani and Attardi, 2000; Isom et al., 2004). Further, HNE can covalently bind to cytochrome *c* oxidase, an enzyme that catalyzes electron transfer from cytochrome *c* to oxygen (Chen et al., 2001).

Incubation of bovine heart mitochondria with HNE decreased state III and state IV oxygen consumption with the addition of complex I and II substrates at pH 5.6 and 7.4 (Ramanathan et al., 2012). Research utilizing mitochondria from laboratory animals also noted inhibitory effects of HNE on mitochondrial function. Pre-incubation of isolated rat heart mitochondria with HNE inhibited complex I of the electron transport chain (Humphries et al., 1998). Similarly, Picklo et al. (1999) reported that the addition of HNE to mitochondria isolated from rat brain decreased oxygen consumption by complexes 1 and 3.

Electron microscopy revealed that HNE-treated mitochondria had a pH-dependent effect on morphology. Mitochondria were swollen and had increased membrane permeability at pH 7.4. Conversely, mitochondria incubated with HNE at pH 5.6 had decreased volume and permeability (Ramanathan et al., 2012). The HNE can induce swelling via stimulation of permeability transition pores present in the mitochondrial membrane (Kristal et al., 1996). Fluorescence studies indicated that HNE binds to the membrane of mitochondria isolated from bovine cardiac muscle. The interaction between HNE

and rat hepatic mitochondria results in restricted membrane mobility and fluidity, which can influence both mitochondrial morphology and function (Humphries et al., 1998). Chen and Yu (1994) reported that HNE binds to rat mitochondrial membranes, oxidizes phospholipids and protein thiols, and alters membrane fluidity.

Conclusions

Postmortem mitochondrial function, specifically oxygen consumption and metmyoglobin reduction are essential in beef color development and stability. Greater oxygen consumption and MRA can result in improved color stability. This is likely due to mitochondrial production of NADH and other reducing equivalents involved in maintaining myoglobin in ferrous form. Therefore, characterizing the factors that influence the interrelation between mitochondria and myoglobin will increase our fundamental knowledge related to beef color chemistry.

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