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Bone and Meat Discolouration of Broiler Chicken Thighs

by

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Dedicated to my
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ABSTRACT

Consumer acceptance of meat depends on its colour and there is a negative bias in North America for broiler dark meat. Freezing and cooking leads to changes in meat characteristics and colour of broiler chicken thighs. The first experiment focused on the effect of freezing and cooking on broiler thighs and comparisons with fresh uncooked thighs. Freezing and cooking resulted in pigment leakage from bones and meat heme pigment oxidation and denaturation. The meat colour was not dark under the applied conditions. The second experiment dealt with the effect of diet (vitamin D, 25-hydroxyvitamin D_3, dilute) fed to broilers (Male Ross 308) on the meat characteristics and colour differences in broiler thighs. The study revealed that bones from birds fed 25-hydroxyvitamin D_3 diet may have a better bone mineralization indicated by certain meat parameters. However the color of meat was not affected by the dietary treatments.

Keywords: Colour, broilers, thigh, discolouration, darkening, bone, pigments, vitamin D, 25-hydroxyvitamin D_3

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance

Fe^{2+}: Ferrous

Hb: Hemoglobin

Hg: Mercury

H_2O_2: Hydrogen peroxide

kDa: Kilodalton

L*: Lightness

MAP: Modified atmospheric packaging

Mb: Deoxymyoglobin

MbO_2: Oxymyoglobin

MetMb: Metmyoglobin

NADH: Reduced nicotinamide-adenine dinucleotide

O_2: Oxygen

P: Phosphorus

R.B.C: Red blood cell

TCA: Trichloroacetic acid

UV: ultraviolet

α: Alpha

β: Beta

1, $25(OH)_2D_3$: 1,25-dihydroxyvitamin D_3

$25\text{-}OHD_3$: 25-hydroxvyitamin D_3

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Consumer Market Trends - Poultry Preference

Food quality depends on its sensory qualities, safety, nutritional value and convenience (Molnar, 1995). The eating habits of consumers in the US clearly indicate a correlation between health and diet (Duewer et al., 1993). A rise in certain markets like Brazil and Thailand and more focus of consumers in western countries on high-protein but low carbohydrate diets resulted in a 158% growth in global chicken production between 1985 and 2005 (Agriculture and Agri-Food Canada, 2006). In 2005, Canada ranked 13th in global chicken production. Per capita chicken consumption in Canada (eviscerated weight basis) increased from 16.88 kg to 31.34 kg (1980-2009) while that of beef and pork (carcass weight basis) decreased from 38.81 kg to 28.60 kg and 32.16 kg to 23.36 kg respectively, during the same period. From 2006-2015 global chicken meat production is predicted to rise by 2.31%, out of which 1.79% will be from North America. Industrialization, growing populations and urbanization will also increase the demand for chicken in the future globally (Agriculture and Agri-Food Canada, 2009). Another reason for an increase in chicken consumption is that chicken is considered a leaner and healthier choice over other meat types, especially red meat like beef. Chicken is relatively cheaper, and unlike other meats, there are few religious restrictions associated with chicken (Magdelaine et al., 2008). The relative convenience of cooking chicken compared to red meat has made chicken more popular in recent years among US consumers, especially women (Haley, 2001).

1.2 Quality of Poultry Meat

For selection of any meat product at the retail level consumer satisfaction is of prime importance as it defines product acceptability whether raw or cooked. In poultry meat, appearance and texture are the two important attributes responsible for initial consumer meat evaluation as well as final product acceptance (Fletcher, 2002).

Appearance of meat includes skin and meat colour. Skin colour is a major factor in whole birds while meat colour is the main component for cut up parts or cooked further processed foods. Numerous factors like age, sex, strain, cooking temperature and freezing affect meat colour (Mugler and Cunningham, 1972). The major contribution to meat colour is because of the muscle pigment myoglobin and to some extent hemoglobin. Pigment deposition in birds depends on genetics, diet and health of the birds as well as its handling at processing (Fletcher, 2002). Variations in the raw meat colour affect the final colour of a cooked product (Fletcher et al., 2000). Stress prior to slaughter can change meat colour due to variations in the glycogen stores in muscles affecting final pH of the meat (Mellor et al., 1958). Various stunning methods before slaughter like electrical or gaseous stunning also influence meat colour (Veerkamp, 1987; Raj et al., 1990). Pinking of cooked meat is an undesirable colour defect found in poultry (Maga, I994). It occurs when nitric oxide and carbon monoxide in oven reacts with the heme pigments in meat while roasting (Pool, 1956).

Another problem is the darkening of bone-in frozen and cooked chicken products. Bruises or hemorrhages leading to blood accumulation in meat tissue and cuts at processing leading to loss of pigment from bones and are also seen as colour defects in poultry (Fletcher, 2002).

Texture is a sensory property affecting final meat quality. Earlier it was thought to be associated with breed, sex and age of bird but now it is well established that processing of birds is mainly responsible for the texture of meat (Fletcher, 2002). The texture depends on the maturity of connective tissue where the cross linkages between collagen increases with age and makes meat less tender. The other factor is myofibrillar protein contraction which depends on time and rate of *rigor mortis* development after bird culling (Fletcher, 2002). Increasing pressure for more deboned or cut ups for fresh poultry in less time resulted in separation of meat portions especially breast from bird skeleton quite early. These procedures do not allow sufficient time for rigor development and result in muscle shortening of the final meat product and losses to industry (Koonz et al., 1954).

1.3 Problems with Bone-in Chicken Meat

In North America, bone-in broiler meat like broiler thighs is considered dark as compared to broiler breasts (Haley, 2001). The high concentration of the heme pigments mainly hemoglobin (Hb) and myoglobin (Mb) and a higher proportion of blood vessels per unit muscle mass make the initial colour of broiler thighs or drumsticks redder than breasts (Lyon et al., 1976). Moreover the

pigment hemoglobin inside bone marrow of thigh bones after its leakage onto surface leads to surface discoloration of bone and meat near to bone. As a result there are consumer complaints regarding bone darkening although chances of rejection increase only when it is noticeably red (Smith and Northcutt, 2003).

Muscles have two types of fibers-red and white. The difference between the two fibers depends on their oxygen carrying capacity (Elminowska-Wenda, 2007). Red fibers have high oxidative enzyme activity as compared to the white fibers and hence have high concentration of pigments particularly myoglobin as it is an oxygen carrier for muscles (Lawrie, 1952; Ogata, 1958; Beecher et al., 1965).

1.4 Bone Darkening or Discolouration

Commercially, bone darkening is explained as a dark reddish brown or black discolouration on the surface of bone and muscle adjacent to the bone after cooking. The darkening is due to bone marrow passing from inside the bone onto the bone surface and adjoining tissue usually after freezing the meat (Lyon et al., 1976; Smith and Northcutt, 2004) and more importantly after cooking the frozen meat (Koonz and Ramsbottom, 1947). This pigment migration from femur to tissue causes darkening and is more prevalent in younger birds as their bones are less calcified, more porous and have more red marrow than yellow marrow (no red pigment) as compared to older birds. The epiphysis of long bones in older birds is more calcified than young birds so it is more difficult for pigment to escape from the bone onto surrounding tissue. The bone darkening only affects

the appearance and not the organoleptic properties of the meat product (Koonz and Ramsbottom, 1947). Cooking chicken breast samples containing bone marrow from femurs significantly increased darkness in broiler meat (Smith and Northcutt, 2004). Furthermore, there was a reduction in meat redness when the femur was removed before cooking, as there was a relationship between bone marrow and bone darkening when the broiler thighs were intact (Lyon et al., 1976).

The commercial further processing industry has reported that redness is usually accompanied by blood in bone-in chicken carcasses. The fully cooked bone-in chicken with this bloody appearance may be rejected by consumers as they think that the product is undercooked and unsafe for consumption. Evidence from commercial meat plants showed that during processing bone marrow leached onto the surface of adjacent meat through cuts in the bone (Smith and Northcutt, 2003). Surface discolouration in 15% of retail beef caused price discounts resulting in $1 billion annual revenue losses (Smith et al., 2000).

Leg meat supply has decreased as compared to white meat in recent years as it is seen as dark meat (Betti and Fletcher, 2005). Smith and Northcutt (2003) studied discolouration in commercially fully cooked breasts, thighs, and drumsticks from various market sources. They reported that severe discolouration was observed in 0.4% of fully cooked chicken while 10.6% was discoloured extensively, and hence speculated that about 11% of product could face consumer complaints or rejection. Vaillancourt (1993) reported that sometimes dark coloured broiler chicken carcasses are linked with septicemia or cyanosis in North

America and this has led to condemnation of about 0.24% percent of broiler chickens in Canada in 1995.

1.5 Important Muscle Meat Parameters in Poultry

1.5.1 Heme Pigments - Properties and Functions

Hemoglobin (Hb) and myoglobin (Mb) are the main heme pigments in poultry, while myoglobin is the main pigment determining meat colour (Lawrie, 1991) (Figure 1.1). The heme pigments supply oxygen to various tissues; hence they are quite susceptible to reaction with oxygen which gives a red colour. The heme proteins contain a heme (iron containing) part and a globin (protein) part. The heme part is a prosthetic group of iron-containing porphyrins which can bind to oxygen and provide colour to meat (Kranen et al., 1999). They are protein pigments and can coagulate and denature during cooking. There is a colour change while cooking from red to brown or grey due to the formation of hemochromogen and other breakdown products (Koonz and Ramsbottom, 1947).

The heme pigments regulate oxygen binding and their structures maintain iron in its reduced (ferrous state). The reaction of heme pigments with ligand (oxygen) leads to the formation of ferrous and then ferric Hb or Mb and the process of this ferrous to ferric conversion is known as autoxidation. Due to this oxidation the globin component of heme pigments cannot bind to its heme group which is the first indication of pigment protein denaturation (Brantley et al., 1993). The extent of pigment protein denaturation depends upon the oxygen

bound to heme iron, the redox state of iron and the stability of the globin chain (Dickerson and Geis, 1983; Slinde, 1987).

Heme pigment exudation from muscle tissue and leaks from the circulatory system affect meat quality. Bruises, hemorrhages, and *post mortem* blood are all considered to be defects in poultry meat (Griffiths and Nairn, 1984).

1.5.1.1 Hemoglobin Structure and Properties

Hemoglobin is a conjugated protein consisting of a heme (iron) part and a globin (protein) part. It is a tetrameric blood protein with two alpha (α) and two beta (β) subunits (Figure 1.2). Each subunit has one heme group per molecule (Richards et al., 2005). Thus, hemoglobin contains 4 subunits each having a core heme group inside a globular protein (Mathews and van Holde, 1990).

Red blood cells are present inside the red marrow of bones which is a hemopoietic (blood forming) tissue and contains hemoglobin (Koonz and Ramsbottom, 1947). Hemoglobin is the likely source of red pigmentation from bony marrow (Brant and Stewart, 1950). and its oxidation leads to formation of methemoglobin (Koonz and Ramsbottom, 1947).

In a well-bled bird carcass, about 20-30% of hemoglobin is still left and so it may also contribute to meat colour (Froning, 1995). Sometimes *post mortem* birds are diagnosed as not being properly bled and in a bird with an abnormal cardiac condition there is retention of hemoglobin due to its failure in bleeding properly (Herenda and Franco, 1996).

1.5.1.2 Myoglobin

Muscles have different fiber types and metabolic functions varying in oxygen (O_2) consumption rates, pigment reduction, mitochondrial respiration, and total reducing activities (Seyfert et al., 2006). Myoglobin is the main pigment determining meat colour and the colour of fresh uncooked muscle is due to myoglobin (Koonz and Ramsbottom, 1947). It is a heme protein present in red muscle fibers and its concentration remains constant in muscle even after death (Pegg et al., 1997; Liu and Chen, 2001). The concentration further depends on the species, breed, sex and age of animal, nature of nutrition, muscular activity, oxygen availability, blood circulation and muscle type as well as the way the meat is treated (Giddings and Hultin, 1974; Livingston and Brown, 1981; Postnikova et al., 1999). The oxidation state of the iron in myoglobin and its chemical interaction with small molecules like oxygen provides an ability to absorb different wavelengths of light and hence determine the chromatic shades of the whole muscle (Liu and Chen, 2001).

Myoglobin is an intracellular protein in muscle cells with one heme group bound to globin (Richards et al., 2005). Joseph et al. (2011) were the first to sequence the primary structure of amino acids in myoglobin from turkey. They reported that turkey myoglobin has 153 amino acids and it is 100% similar to chicken myoglobin. The molecular mass of turkey and chicken myoglobin was reported to be 17291 Dalton (Maheswarappa et al., 2009). Both hemoglobin and myoglobin have a tertiary structure but myoglobin is monomeric (one subunit). The subunits of hemoglobin differ from each other and from myoglobin with

respect to their amino acid sequence (primary structure) (Mathews and van Holde, 1990).

1.5.1.3 Stages of Myoglobin

Myoglobin is a major component determining the colour in muscle and exists in three reversible forms: deoxymyoglobin (ferrous, iron II and purple in colour), oxymyoglobin (ferrous, iron II but bound to oxygen released from blood and bright red in colour) and finally metmyoglobin (ferric, iron III due to further reaction with oxygen and dark brown in colour) (Cornforth, 1994). The reaction of iron containing pigments metmyoglobin and methemoglobin with hydrogen peroxide (H_2O_2) activates iron to a ferryl species. This formation attacks other heme edge molecules and produces a porphyrin radical destabilizing the whole molecule (Harel et al., 1988). As a result, heme pigments are actively involved in lipid oxidation processes (Johns et al., 1989).

1.5.1.4 Deoxymyoglobin (Mb), Oxymyoglobin (MbO_2) and Metmyoglobin (MetMb)

Deoxymyoglobin is a reduced stage of myoglobin with low oxygen tension (Mancini and Hunt, 2005) and the heme iron is in the ferrous (Fe^{2+}) state with no binding to the oxygen. This gives the purple-red colour found in vacuum packaged meat or muscle immediately after cutting. Oxygen tension of < 1.4 mm mercury (Hg) is required to keep myoglobin in this form (Wallace et al., 1982). Exposure of myoglobin to an oxygen tension excess leads to oxymyoglobin

formation and development of cherry red colour but still the valence of iron is in the ferrous (Fe^{2+}) state (Livingston and Brown, 1981; Wallace et al., 1982). Metmyoglobin is the further oxidized form of myoglobin. This occurs due to oxidation of the heme iron present inside the pigments from a ferrous to a ferric state which results in meat discolouration (Figure 1.3). The oxygen loss from oxymyoglobin and formation of metmyoglobin changes the absorption properties of the molecule and causes colour changes in meat from bright red to dark red and finally to brown (Kanner, 1994).

Exposure of heme iron to certain ligands like water and the high oxygen environment in meat itself may destabilize or denature the pigment proteins due to alteration in their tertiary structure forming metmyoglobin (Baron and Andersen, 2002). Formation of metmyoglobin also depends on the exposure of myoglobin for longer periods to light, heat, microbial growth, or freezing (Mancini and Hunt, 2005). The metmyoglobin formed can act as a catalyst and is positively correlated with lipid oxidation (Lynch and Faustman, 2000).

To maintain good colour in meat, metmyoglobin reduction is essential. The reductive enzymes in a living body keep metmyoglobin content to only 2-3% of total myoglobin content in the meat (Halliwell and Gutteridge, 1989). After death the enzyme activity ceases and the oxidation of myoglobin leads to more metmyoglobin formation resulting in darkening of the tissue (Guidi et al., 2006). Studies in human beings have also supported that the rate of formation of methemoglobin in human erythrocytes is around 3% per day (Jaffé and Neumann,

1964) which is reduced to about 1% per day due to the activity of reductive enzyme systems (Bodansky, 1951).

The reductive enzyme systems present inside muscle and red cells keep pigment proteins in their reduced form (Bunn and Forget, 1986). These enzyme systems in the presence of reduced nicotinamide-adenine dinucleotide (NADH) start decreasing with increasing time *post mortem* (Wallace et al., 1982). The enzyme metmyoglobin reductase in the presence of NADH can help in reduction of metmyoglobin to its ferrous state (Osborn et al., 2003).

John et al. (2004) reported that high oxygen modified atmospheric packaging of raw beef meat resulted in a premature meat browning while cooking. Seyfert et al. (2004) also reported a premature browning of ground beef patties in high oxygen modified atmospheric packaging (MAP). This was due to more myoglobin oxidation from increased oxygen environment causing premature browning while cooking. However, vacuum packaged product had low oxygen and hence high deoxymyoglobin resulting in a pink colour while cooking even at 71.1^{o}C. This indicated higher thermal stability of deoxymyoglobin over metmyoglobin. Moreover, exposure of deoxymyoglobin to the atmosphere resulted in bloom (redness) showing that colour development depends on the stage of myoglobin.

1.5.2 Iron Status

Meat acts as the main source of heme and non-heme iron in human diets (Carpenter and Mahoney, 1992) and bioavailability of these forms of iron is

different in different food stuffs (Monsen et al., 1978). Moreover, different muscles have varied iron content depending on extent of bleeding during slaughtering, breed type and nutritional status (Han et al., 1993). Cooking decreases the bioavailability of iron in food, so knowledge of the relative concentration of heme and non-heme iron in food after cooking is essential (Monsen et al., 1978).

1.5.2.1 Heme Iron

Heme iron is found in dietary meat sources like fish and poultry. It has a much higher bioavailability (15 to 35%) than non-heme iron and it is unaffected by other dietary constituents (Monsen and Balintfy, 1982). Different heat treatments (Igene et al., 1979) and different processing methods (Schricker and Miller, 1983) convert the more available heme iron into less available non-heme iron to varying degrees. The process involves oxidation of the porphyrin ring followed by its cleavage resulting in the release of iron due to formation of an unstable complex (Elder, 1980). Once free from the porphyrin ring the heme iron contributes to the non-heme iron pool (Garcia et al., 1996).

1.5.2.2 Non-heme Iron

Dietary non-heme iron is present in both plant and animal sources but it has a limited bioavailability varying from 2 to 20% (Monsen and Balintfy, 1982). The ratio of non-heme to heme iron increases due to heating, possibly due to pigment breakdown (Igene et al., 1979; Tichivangana and Morrissey, 1985). The

increase in non-heme iron occurs more at slow heating. This may be due to possible hydrogen peroxide (H_2O_2) formation that activates and destroys porphyrin rings causing iron release (Rhee et al., 1987; Harel et al., 1988).

1.5.3 Colour

Commercial success of meat depends on many factors such as price, presentation, and organoleptic characteristics (i.e., odor, colour, and tenderness) (Faustman and Cassens, 1990; Liu et al., 1995). The most important characteristic by which consumers judge the quality and freshness of raw meat is colour and an unacceptable colour is detected much earlier than the flavour of cooked meat (Jacobson et al., 1959). Colour is an important attribute for estimation of the economic value of meat. The factors influencing meat colour include kind of muscle, environmental factors and processing and packaging techniques (Mugler and Cunningham, 1972). Pre-harvest factors (diet, breed, housing, pre-harvest handling and glycolytic potential), postharvest factors (chilling rate, antioxidant availability, antimicrobial compounds, package atmospheres) and cooking affect the colour (Mancini and Hunt, 2005). Age of the bird also affects meat colour with meat getting darker and redder as age progresses in both uncooked and cooked meat (Froning and Hartung, 1967).

The colour of meat is mainly influenced by the heme pigment myoglobin (Lawrie, 1991). The change of meat colour from cherry red (oxymyoglobin) to reddish brown (metmyoglobin) in red meat cuts has negative impact on consumers (Giddings and Hultin, 1974; Lanari and Cassens, 1991). There is a

strong relationship between colour preference and purchase by consumers as they can refuse to buy fresh beef meat when metmyoglobin levels go beyond 40% (Greene et al., 1971). Freezing also affects meat colour and the surface meat colour formation is dependent on the duration and temperature of freezing. Longer storage of beef for about 1 year at 10^{o}F significantly resulted in a dark colour as compared to meat stored at -30^{o}F due to accelerated oxidation of hemoglobin to methemoglobin (Ramsbottom and Koonz, 1941). The meat color in fact became less dark when temperature of cooking increased from 60^{o}C to 82^{o}C (Helmke and Froning 1971).

1.5.4 pH

Muscle pH is related to various meat quality parameters like tenderness, water holding capacity, cooking loss, juiciness, and microbial stability (shelf life) (Fletcher, 2002). There is a complex relationship between meat colour and pH. The pH of muscle affects the physical meat structure and its light reflecting properties through influencing the water-binding nature of the proteins (Bkiskey, 1964). Meat with a high pH has more water-binding capacity resulting in darker meat (Cornforth, 1994). pH also affects oxygen reactivity with heme proteins by altering enzymatic activity of the mitochondria1 system (Ashmore et al., 1972; Cornforth and Egbert, 1985). Heme species have high prooxidative activity at acidic pH (Baron and Andersen, 2002). Yang and Chen (1993) observed a significant increase in pH of ground chicken meat with increased storage time. The pink or red colour of meat can be maintained during and after cooking by

higher pH as it may protect myoglobin from denaturation by heat (Hunt et al., 1999).

1.6 Bone Growth in Birds

Commercial broilers, turkeys, and ducks have undergone a significant change in growth and development over time. There has been a disproportionate increase in breast muscle growth rate compared to the overall body development for increased yields (Lilburn, 1994). But this increase has led to various metabolic and skeletal disorders (Ferket and Sell, 1989; Sullivan, 1994). Bone quality is related to bone growth rate in chickens and fast growing birds have higher incidence of bone deformities than slow growing birds (Reiland et al., 1978; Hulan et al., 1980; Duff and Thorp, 1985). This is due to low mineral content in the bones of fast growing birds as compared to slow growing meat type chickens (Leterrier and Nys, 1992). Thorp and Waddington (1997) reported that deficiency of Ca, P, vitamin D or imbalance in Ca/ P in the bones was responsible for bone deformities like rickets and tibial dyschondroplasias in lame 35 day old broilers. As ossification of the sternal tip is not complete in broilers even at 14 weeks and laying hens of 5 months of age they are still considered young poultry (Breugelmans et al., 2007). The cortical bones were less mineralized and more porous in fast growing strains of birds as compared to the slow growing bird strains and this might be either due to inadequate supply of minerals (Ca, P) in their diet or their inability to utilize minerals quickly because of their growth rates or genetic effects (Williams et al., 2000).

There is a mineral homeostasis in bones of birds where there is constant resorption (bone loss) and remodeling (bone formation) around the Haversian canals of individual osteons. All the fast growing birds showed more bone porosity than slow growing birds (Williams et al., 2000) due to an imbalance in this mineral homeostasis mechanism (Parfitt et al., 1983; Frost, 1986). Slow growing birds had a higher bone mineral content (Leterrier and Nys, 1992) and ash content (Williams et al., 2000) in comparison to fast growing birds.

1.7 Vitamin D and Diet Relationship

Vitamin D is a steroid hormone synthesized in the body when skin is exposed to ultraviolet (UV) radiation or is available from dietary intake (Holick et al., 1980; Norman, 1998). Vitamin D can either be utilized as ergosterol (D_2) from plant sources or as cholecalciferol (D_3) from animal sources (Holick et al., 1980). However performance of bones was better when birds were fed vitamin D_3 as compared to vitamin D_2 fed birds (Mattila et al., 2004). UV radiations convert 7-dehydrocholesterol (provitamin D_3) present in skin to previtamin D_3 (Holick et al., 1980). Non-enzymatic isomerisation of previtamin D_3 converts it into cholecalciferol or vitamin D_3 or vitamin D (Segart, 2008; Lehmann and Meurer, 2010). Bile salts in the intestine help its absorption (Greaves and Schmidt, 1933). After absorption vitamin D is first hydroxylated and metabolized in the liver to 25-hydroxycholecalciferol or 25-hydroxvyitamin D_3 (25-OHD_3) or calcidiol (Holick, 2007). Further, 25-OHD_3 is hydroxylated in the kidneys to 1,25-

dihydroxycholecalciferol or 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), or calcitriol which is the active circulating metabolite (Gandini et al., 2009). (Figure 1.4). Non-renal tissues like macrophages can also produce calcitriol (Liu et al., 2006).

Various feed components like plants, fish meal, and mineral supplements affect the bone development as they vary in vitamin D supplementation to the body. Further, Calcium (Ca) and phosphorus (P) mineral metabolism in the body is governed and affected by vitamin D in the diet (Rath et al., 2000). Bone mineralization depends on amount of Ca and P mineral deposition in the bone (Leterrier and Nys, 1992). Fast growth rate of modern broiler chickens has resulted in less minerals and hence high porosity in their bones making them more susceptible to injuries (Williams et al., 2000; Kwiecien, 2003). This disturbance in metabolism of Ca and P is due to problems with hydroxylation and production of active metabolites like 25-hydroxycholecalciferol ($25\text{-}OHD_3$) and the active form 1,25-dihydroxycholecalciferol ($1,25\text{-}(OH)_2D_3$) (Reichel et al., 1989; Soares et al., 1995; Whitehead et al., 2004). So feeding using these metabolites directly has proved to be beneficial to the overall health, particularly with respect to body weight, egg shell thickness and decreased risk for bone disorders (Rennie and Whitehead, 1996; Fritts and Waldroup, 2003). Janocha et al. (2009) reported that feeding broiler chickens with 25-hydroxycholecalciferol significantly increased body weight, and resulted in better feed and nutrient utilization as compared to birds fed a diet without 25-hydroxycholecalciferol. It also increased crude ash, Ca and P contents in the thigh and tibia bones of these broiler chickens. The enzyme

system of birds which is required for proper hydroxylation of vitamin D in liver is not fully functional in the starter period. Hence, supplementing 25-OHD_3 directly may contribute a beneficial effect (Soares et al., 1995).

1.8 Cooking Effect on Bone and Muscle Darkening

Cooking influences meat quality and red discolouration of a fully cooked bone-in chicken product is seen as undercooked and unsafe for human consumption (Smith and Northcutt, 2003). Brant and Stewart (1950) found that cooking method and end point temperature did not affect bone darkening while Lyon and Lyon (1986) reported that cooking method had a significant effect on bone darkening. Microwave cooked broiler meat samples were lighter than deep fried samples (Hatch and Stadelman, 1972). Further microwave heating of chicken legs for 2-4 min before freezing resulted in reduced bone darkening (Essary, 1959). High temperature leads to autooxidation of oxymyoglobin and oxyhemoglobin (Brown and Mebine, 1969; Wallace et al., 1982) resulting in formation of H_2O_2. This activates metmyoglobin and initiates lipid peroxidation (Harel and Kanner, 1985).

Cooking causes non-heme iron release from heme pigments (Tichivangana and Morrissey, 1985). Cooking also leads to conformational changes in myoglobin after its denaturation and exposes its active heme group to surrounding molecules (Eriksson et al., 1971). There was a decrease in heme/non-heme iron ratios after cooking chicken and turkey legs with losses ranging from 22 to 43% (Lombardi-Boccia et al., 2002). This is due to oxidative cleavage of porphyrin

rings leading to opening of the heme complex and causing release of iron (Schricker and Stouffer, 1982). During cooking the heme proteins still have most of the heme intact with globin, which becomes disassociated when temperature reached beyond 85^{o}C (Han et al., 1993) and the rate of denaturation increases substantially only after 55^{o}C (Kristensen and Purslow, 2001). Lyon and Lyon (1986) described the variations in bone discolouration due to different preparation methods (precook, freeze, reheat) and found that freezing before cooking increased the severity of discolouration more than cooking followed by freezing and reheating.

1.9 Freezing Effect on Muscle and Bone Darkening

Freezing is important for maintenance of product quality but storage for longer periods can result in negative attributes of colour, flavour and texture (Addis, 1986). Freezing results in metmyoglobin formation causing browning of colour (Van Laack, 1999; Coggins and Chamul, 2004). Metmyoglobin formation is a slow process in both unfrozen and frozen states but after defrosting the frozen samples, bone colour darkens due to quick methemoglobin formation. Redness of raw meat increases after freezing the chicken (Lyon et al., 1976).

Freezing results in hemolysis (breakdown) of the red blood cells present inside bone marrow of long bones. Freezing also increases bone porosity and so hemoglobin from the breakdown of red blood cells is released from these porous bones. (Koonz and Ramsbottom, 1947). This pigment release from the bones results in darkening (Brant and Stewart, 1950). The discolouration in frozen

broilers is greater when thawing time is increased between freezing and cooking. Reducing the thawing time of broilers and heating them quickly after freezing reduced discolouration as most of the bone discolouration was observed during thawing (Hatch and Stadelman, 1972). Blast freezing increased darkening in both raw as well as cooked bone-in broiler thighs, while freezing the thighs without femurs decreased darkness (Lyon et al., 1976).

Slow freezing causes cell disruption and blood migration in broiler breasts leading to discolouration of both raw and cooked tissue (Lyon and Lyon, 2002). Darkening was observed in frozen broilers but not in unfrozen controls during slow freezing (Spencer et al., 1961). Slow freezing leads to formation of larger ice crystals between muscle fibers resulting in higher cell wall destruction (Kuprianoff, 1952) while quick freezing results in small ice crystal formation inside muscle cells (Nord, 1936). During ice crystal formation in meat, the salts in muscle juice become concentrated and at higher concentrations these salts denature cellular proteins (Kuprianoff, 1952). Quick freezing of meat results in fast ice crystal formation and does not allow salts to be concentrated enough for cell wall destruction (Deatherage and Hamm, 1960). Freezing causes myofibril shrinkage due to formation of ice crystals. This damages muscle cells leading to protein denaturation and decreasing their water holding capacity (Lee et al., 2008). The size of ice crystals formed in beef increased with freezing time when the temperature was kept constant (Martino and Zaritzky, 1988). Size and distribution of ice crystals in the intracellular or extracellular spaces of frozen meat vary with the rate of freezing, while the temperature achieved during

freezing decides the amount of ice formed. So rate of freezing and storage temperature influences the structure of frozen meat and its sensory qualities (Barbut and Mittal, 1990).

Frozen storage for extended periods also causes changes in meat proteins which can be attributed to some enzymes which can remain active even at low temperatures (Smorodintsev and Bysstrov, 1937; Sinitsov, 1956). Li et al. (1969) found improved colour and appearance of chicken thighs after 45 days of storage at -17.8^{o}C, pre-treated with a liquid nitrogen process in a chamber with -101.1^{o}C for 5 minutes as compared to sharp freezing at -28.9^{o}C. However, it was reported that frozen storage of broilers (-18^{o}C) at different times (Spencer et al., 1961) and four different freezing methods did not affect the severity of bone darkening (Streeter and Spencer, 1969).

1.10 Study Objectives and Importance

There is a widespread sporadic occurrence of bone-in broiler chicken discolouration but sufficient data regarding the incidence or severity of the problem is lacking (Smith and Northcutt, 2004). Little research has been done on discolouration defects in poultry meat (Smith and Northcutt, 2003). The present situation of modern broiler chickens is different compared to broilers reared in earlier years. The growth rate of modern broilers has increased tremendously over the ones in 1950 (Schmidt et al., 2009). Bone discolouration in modern broilers can be related to more porosity in leg bones, causing leakage of marrow from the marrow cavity (Whitehead and Fleming, 2009).

Knowledge of the factors responsible for discolouration can help in development of control strategies which include modifications in processing, storage and preparation techniques to reduce the incidence. Smith and Northcutt (2003) suggested that the bone discolouration occurs periodically and consumer complaints subside in between and so the causes, incidence and severity of this problem are not addressed. Cuts in bones during processing of broilers lead to more pigment leakage from bone marrow onto the bone and meat surface. But due to inconsistency in the consumer complaints about this problem no remedial measures are taken and the incidence remains the same. This research will help to understand the possible causes of the problem. Addressing these problems can put more pressure at processing plants and retailers for increased scrutiny of bone-in products which may help to reduce the incidence. So with due reference to the above facts the study was designed with the following objectives:

- To quantify the factors responsible for meat and bone discolouration or darkening in bone-in broiler chicken thighs.
- To study the effect of cooking and freezing on broiler chicken bone-in thighs.
- To study the relationship of diet to bone growth and colour of bone-in broiler chicken thighs.

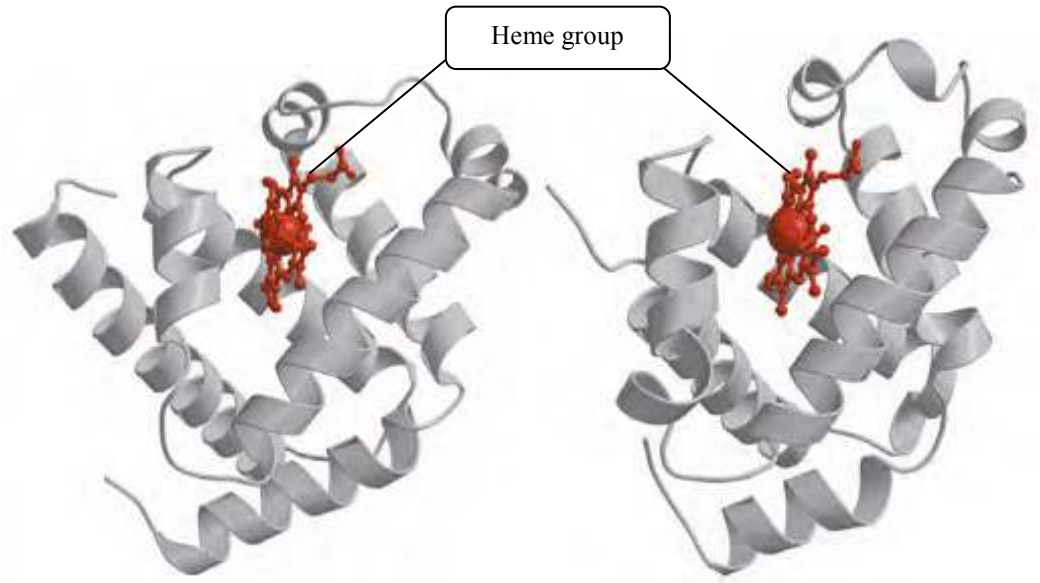

Figure 1.1. Myoglobin and a subunit of hemoglobin with a central heme (iron containing) group. Reprinted from PRINCIPLES OF BIOCHEMISTRY (4th Ed), CHAPTER 5, FIGURE 5-6. EBOOK by Lehninger. Copyright 2004 by W. H. Freeman and Co. Reproduced with permission of W. H. Freeman and Co. in the format of Dissertation via Copyright Clearance Centre.

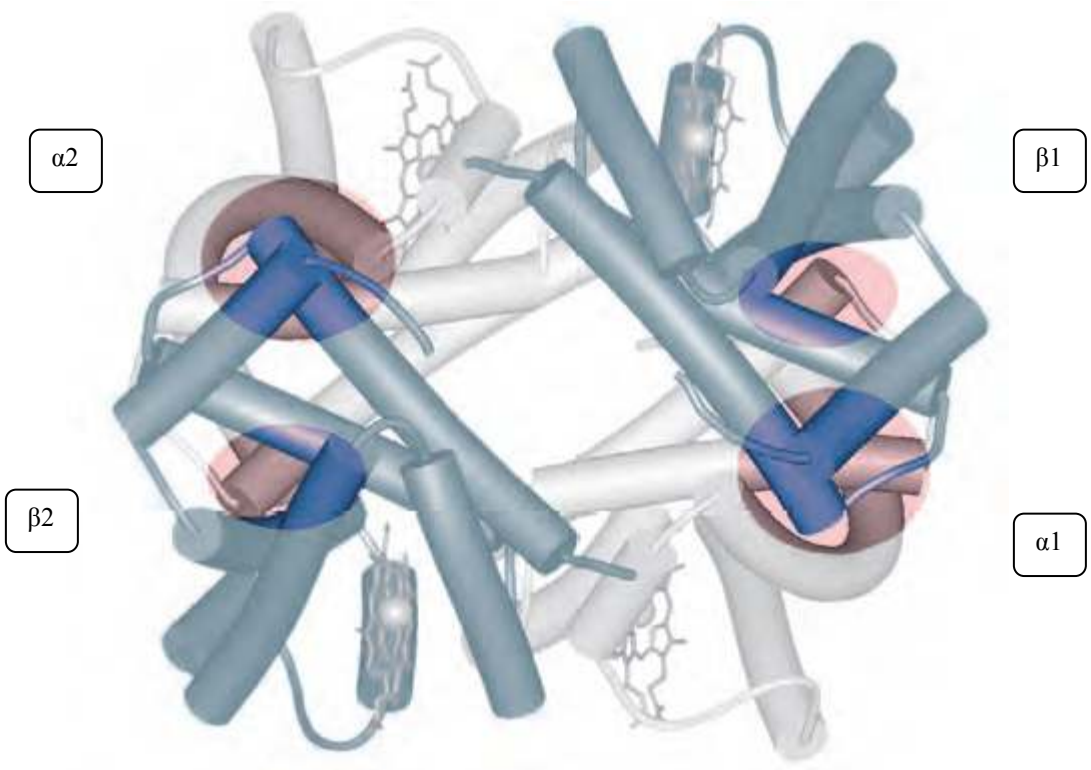

Figure 1.2. Quarternary structure of hemoglobin with its subunits joined to each other mainly by hydrophobic interactions. Reprinted from PRINCIPLES OF BIOCHEMISTRY (4th Ed), CHAPTER 5, FIGURE 5-8. EBOOK by Lehninger. Copyright 2004 by W. H. Freeman and Co. Reproduced with permission of W. H. Freeman and Co. in the format of Dissertation via Copyright Clearance Centre.

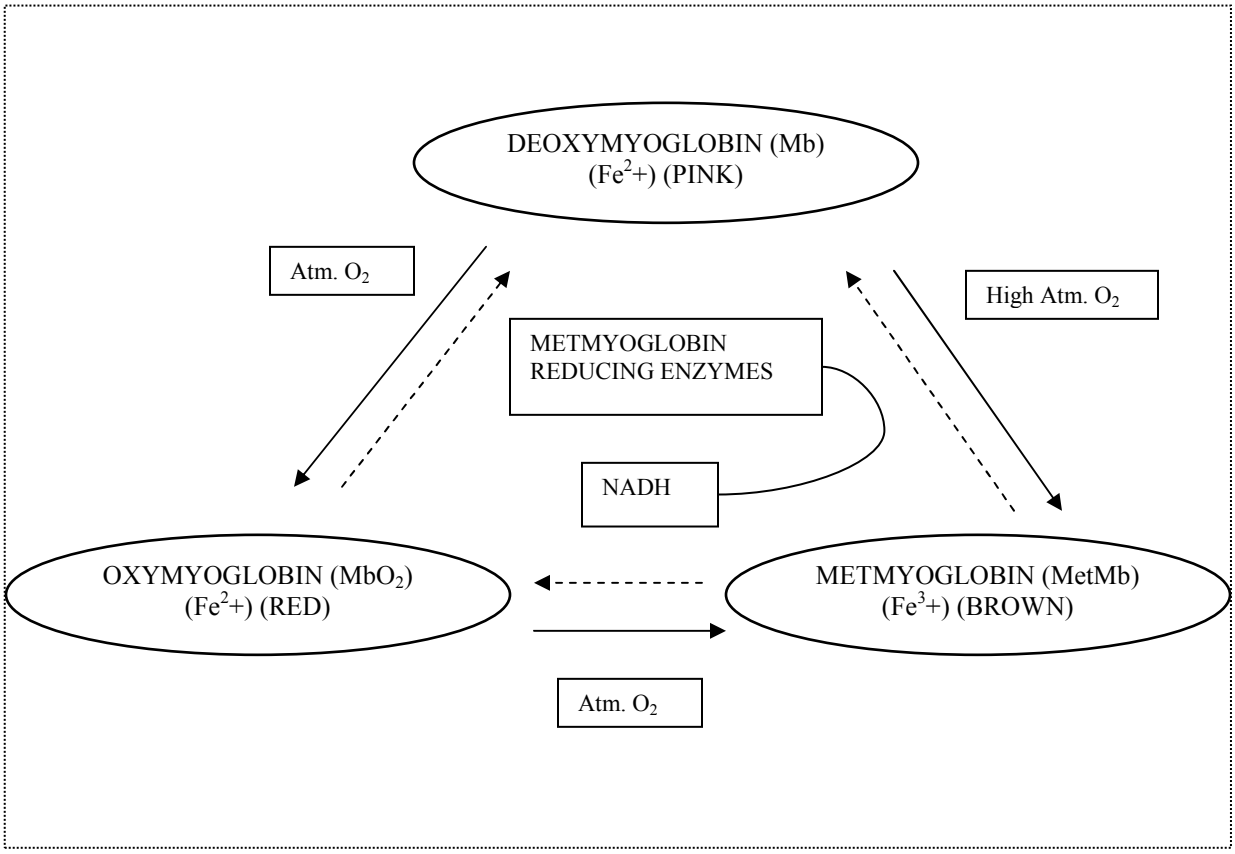

Figure 1.3. The interrelationships of different myoglobin forms after oxidation and reduction.

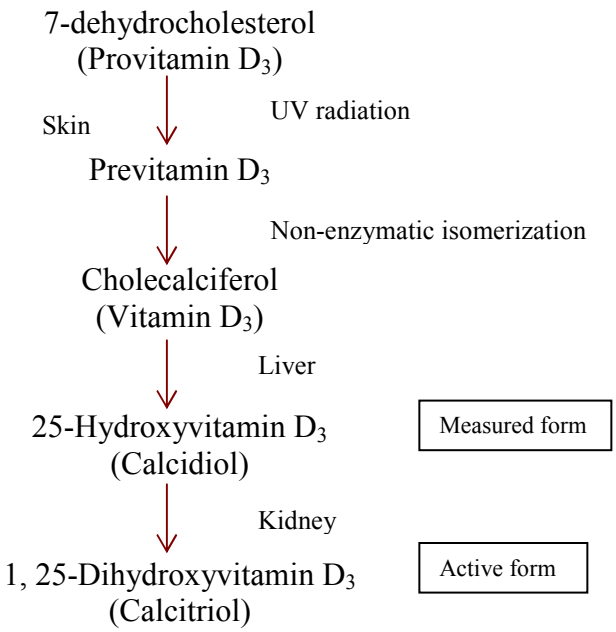

Figure. 1.4. Flow diagram for the synthesis of vitamin D in the body.

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CHAPTER 2. EFFECT OF FREEZING AND COOKING ON MEAT CHARACTERISTICS AND COLOUR OF BONE-IN BROILER CHICKEN THIGHS

ABSTRACT

The colour of broiler thigh and drum meat is dark as compared to breast meat due to increased vascular supply and high concentration of heme pigments. This can lead to consumer rejection and financial losses for the industry. Freezing or cooking the broiler thighs results in significant chemical changes in the meat. Hemoglobin leaked from bone and oxidized muscle pigment (myoglobin) undergoes structural changes resulting in their denaturation during freezing or cooking. These changes in the meat affect its colour. Research related to the effects of freezing and cooking on meat parameters and colour is limited and hence the study was conducted to understand the effect of freezing and cooking on bone-in broiler thighs. Bone-in thighs (n=40) were obtained from 20 whole-carcass broiler chickens bought from a supermarket. Effects of cooking (180^{o}C oven temperature, 80^{o}C core meat temperature), freezing (-20^{o}C for 4 weeks) and their interactions were studied. Heme iron, non-heme iron, total pigment, myoglobin, colour and pH were estimated in fresh raw, fresh cooked, frozen raw and frozen cooked thighs. Data were analyzed using two-way ANOVA using the mixed procedure of SAS. Cooking x freezing interactions were significant only for heme ($P < 0.01$) and non-heme iron ($P < 0.01$) among all the above parameters. In general, heme iron increased ($P < 0.01$) after freezing and non-

heme iron increased after both freezing ($P < 0.01$) and cooking ($P < 0.01$). Total pigment ($P < 0.01$) and myoglobin ($P < 0.01$) decreased after cooking while freezing had no significant effect ($P > 0.05$). Meat was lighter in colour as lightness (L*) increased ($P < 0.01$) and redness (a*) decreased ($P < 0.01$) after cooking while freezing had no effect ($P > 0.05$). The pH increased after freezing ($P = 0.01$) or cooking ($P < 0.01$) the samples. The study revealed that even though there was a release of pigments, the current conditions used for cooking and freezing did not lead to discolouration at a detectable level and hence had no influence on black bone discolouration.

Key words: hemoglobin, myoglobin, broilers, freezing, cooking

2.1 INTRODUCTION

The dark appearance of broiler thighs makes them an undervalued or low priced product especially in North America (Betti and Fletcher, 2005). Thigh muscles have more red fibers than breast muscles due to the presence of more myoglobin and increased vascular supply in them (Schreurs, 2000). Freezing of poultry meat is a safe and efficient means to maintain product quality, but storage for extended periods may result in altered texture, colour, and flavour of meat (Addis, 1986). Freezing results in myofibrillar shrinkage due to ice crystal formation and hence causes protein denaturation by damaging muscle cells (Lee et al., 2008). Freezing increases soluble salt concentration inside muscle cells which also results in protein denaturation (Van Laack, 1994). Freezing can also result in discolouration of meat due to brown colour formation as a result of

metmyoglobin production (Van Laack, 1994; Coggins and Chamul, 2004). Alternative freezing and thawing of broiler thighs can hemolyse red blood cells which releases hemoglobin from inside the bone onto bone surface (Dukes, 1955). Hamre (1966) reported that colour of frozen light and dark chicken meat was affected by freezing rate; faster freezing rate resulting in lighter meat. Contrary to this it was reported that different freezing methods and freezing for different times at constant temperature did not significantly affect the degree of bone darkening in broiler chickens (Spencer et al., 1961; Streeter and Spencer, 1969).

Cooking of meat results in denaturation of heme pigments hemoglobin and myoglobin. The denaturation results in release of heme iron present inside these pigments to the free form non-heme iron (Kongkachuichai et al., 2002). Cooking the broiler thigh meat without the bone resulted in lighter meat colour as compared to broiler thigh meat with the bone (Omana et al., 2010). Different authors have reported variable effects of cooking on the meat. Preheating chicken legs in a microwave for 2-4 min (Essary, 1959) or blanching in hot oil to a temperature of 88°C (Woodroof and Shelor, 1948) before freezing might reduce or even eliminate bone darkening. This may be due to heat coagulation of the bone marrow before freezing (Ellis and Woodroof, 1959). However, Lyon and Lyon (1986) reported that different preparation methods (precook, freeze, and reheat) have an effect on bone discolouration. They also mentioned that cooking and then freezing and reheating resulted in lower discolouration as compared to freezing before cooking.

The heme pigments mainly hemoglobin and myoglobin provide colour to the meat. They are iron containing pigments and are affected by the oxidation state of iron (ferrous or ferric) (Lawrie, 1991). Hemoglobin is a blood protein and is present inside the bone marrow of long bones (Koonz and Ramsbottom, 1947). Myoglobin is the main muscle pigment protein whose concentration remains constant even after bleeding the bird (Pegg et al., 1997; Liu and Chen, 2001). Myoglobin exists in three forms deoxymyoglobin (Mb), oxymyoglobin (MbO_2) and metmyoglobin (MetMb) depending on the oxidation state of iron. Myoglobin oxidation after contact with atmospheric oxygen can lead to meat darkening (Cornforth, 1994). The leakage and then oxidation of hemoglobin from inside of bone-in broiler thighs onto bone surface and adjoining meat can affect meat colour. Both hemoglobin and myoglobin are pigment proteins and undergo denaturation after heating resulting in colour change of meat (Koonz and Ramsbottom, 1947).

Little recent research has been done on the effects of freezing and cooking on meat parameters and colour of broiler bone-in thighs. Hence the present investigation is useful in understanding freezing and cooking effects on raw broiler bone-in chicken thighs. The study also increases understanding of the possible factors associated with darkening of bone and adjoining muscle. It can help to educate consumers as there is a consumer bias against using dark (broiler thigh) meat in North America (Haley, 2001). Thus the objectives of the study were to study the effects of freezing and cooking on meat parameters of broiler

thighs and to study the relationship of freezing and cooking to the colour of thigh bones and the meat adjoining it.

2.2 MATERIALS AND METHODS

2.2.1 Materials

20 whole carcass broiler chickens were purchased from a supermarket in Edmonton, Canada (same source to maintain uniformity). The chemicals used were of reagent grade and obtained either from Fisher Scientific (Waltham, MA) or Sigma-Aldrich Co. (St. Louis, MO).

2.2.2 Sample Preparation

Immediately after purchase the whole broiler chicken carcasses were brought to the lab for analysis. From the 20 whole carcasses a total of 40 broiler bone-in thighs were recovered manually after dissecting the birds. Then they were randomly divided into 4 groups with 10 thighs in each group. The groups divided were**:** fresh raw, fresh cooked, frozen raw and frozen cooked.

Immediately after manual dissection, 10 fresh raw thighs obtained were measured for both pH and colour at 2 points (meat adjoining the thigh (femur) bone and at the meat surface away from the bone) and grouped as 'fresh raw'. Another 10 fresh thighs were cooked in a hot air oven at 180^{o}C. Cooking was done until the meat core temperature reached 80^{o}C as measured with a digital thermometer. After cooking, pH and colour were measured as in the case of raw thighs; and grouped as 'fresh cooked'. The remaining half of the thighs (20) were

frozen and were stored individually at -20^{o}C for 4 weeks until analysis. After 4 weeks of frozen storage, all the thighs were thawed overnight at refrigeration temperature (4^{o}C) and were grouped into 'frozen raw' and 'frozen cooked' and the whole procedure was repeated similarly to the fresh raw and fresh cooked samples. The identity of each thigh was maintained throughout the entire analysis.

After measuring pH and colour of bone-in thighs, bones were removed from each thigh in all the treatments. The meat thus obtained was frozen stored individually at -20^{0}C for further analysis.

2.2.3 Heme Iron

Heme iron measurement was done by the acetone acid extraction method of Turhan et al. (2004). To 10 g of meat sample 20 mL of acid-acetone mixture (acetone, water and concentrated hydrochloric acid added in ratio of 16 mL: 3.6 mL: 0.4 mL respectively) was added and homogenized at 15,000 rpm for 30 sec with a homogenizer (Fisher Scientific, Power Gen 1000S1). After homogenization another 20 mL of acid-acetone mixture was added. The samples were thoroughly mixed and placed in the dark for 1 hour. Then the mixture was centrifuged using an Avanti J-E refrigerated centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) at 2200 g for 10 min at room temperature (22^{o}C). The supernatant after centrifugation was filtered using Whatman no. 1 filter paper and the absorbance was taken against a blank at 640 nm using a spectrophotometer (V-530, Jasco Corporation, Tokyo, Japan). Heme iron was calculated and expressed as μg Fe per gram meat.

2.2.4 Non-heme Iron

Non-heme iron estimation was done by the method described by Min and Ahn (2009) with slight modifications. 5 g of meat sample was mixed with 3 volumes (15 mL) of deionized water. The mixture was homogenized at 15,000 rpm for 30 sec with a homogenizer (Fisher Scientific, Power Gen 1000S1). 1.5 mL of homogenate was mixed with 0.5 mL ascorbic acid (1 % in 0.2 N HCl, w/v) and 1 mL trichloroacetic acid (TCA) solution (11.3% w/v). The mixture was thoroughly vortexed (Fisher, Scientific, ON, CA) and kept for 5 min at room temperature followed by centrifugation using an Avanti J-E refrigerated centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) at 5000 g for 15 min at 20^{o}C. After centrifugation 2 mL of the supernatant was mixed with 0.8 mL of ammonium acetate (10% w/v) and 0.2 mL of the ferrozine colour reagent (6.4 mM ferrozine and 14.4 mM neurocuproine in 0.14 N HCl). The solution was mixed thoroughly and after 10 min of colour development at room temperature, absorbance was taken at 562 nm against a reagent blank using a spectrophotometer (V-530, Jasco Corporation, Tokyo, Japan). A standard curve using an iron standard (Fluka Analytical, Sigma-Aldrich, Inc. St. Louis, USA) was made and non heme iron content was calculated from the standard curve as µg Fe per gram meat.

2.2.5 Total Heme Pigment Determination

Total pigment determination was done by the alkaline hematin method of Karlsson and Lundström (1991). 5 g of broiler thigh meat sample was weighed individually and put into an 80 mL plastic container with a lid. To each of the containers 50 mL of chilled (4^{o}C) 130 mM phosphate extraction buffer (pH 7.4) was added. Then the samples were homogenized at 10000 rpm for 30 seconds using a homogenizer (Fisher Scientific, Power Gen 1000S1) and the homogenate was stored overnight at 4^{o}C. The temperature of the samples was maintained at 4^{o}C throughout the experiment. The samples were then stirred and subjected to centrifugation using an Avanti J-E refrigerated centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) at 5000 g for 20 min at 4^{o}C. After centrifugation, samples were filtered at room temperature using Whatman No. 42 filter paper. After 30 min of filtration, 4 mL of the filtrate was mixed with 400 µL of 10% triton X-100 detergent solution. Finally 250 µL of 5.0 M sodium hydroxide was added. After mixing thoroughly the absorbance were taken after 5 min at 575 nm and 700 nm against a reagent blank using a spectrophotometer (V-530, Jasco Corporation, Tokyo, Japan). Total pigment concentration was calculated and expressed as µg per gram meat.

2.2.6 Total Myoglobin Determination

Total myoglobin and the three forms of myoglobin; deoxymyoglobin (Mb), oxymyoglobin (MbO_2) and metmyoglobin (MetMb) were determined according to the method described by Krzywicki (1982). To a 5 g of meat sample 25 mL of conc. phosphate buffer (pH 6.8 ionic strength of 0.04M) was added.

Temperature was maintained at 4^{o}C throughout the experiment. The samples were further homogenized at 15000 rpm for 30 seconds with a homogenizer (Fisher Scientific, Power Gen 1000S1). After 1 hr, the homogenates were centrifuged using an Avanti J-E refrigerated centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) at 5000 g for 30 min at 4^{0}C. After centrifugation, the supernatant obtained was further filtered through Whatman No. 1 filter paper. Absorbances were taken at wavelengths of 525 nm, 545 nm, 565 nm, 572nm, 700 nm, 730 nm against a reagent blank and total myoglobin content was calculated and expressed as mg myoglobin per gram meat. The relative content of three myoglobin classes was also calculated.

2.2.7 Colour Measurement

Colour was measured by Minolta CR-400 colourimeter (Konica Minolta Sensing Americas, Inc., Ramsey, NJ, USA) which was calibrated using a standard white ceramic tile. Readings were taken using illuminant D65 as the light of source (CIE L*a* b) where L* refers to lightness, a* refers to redness and b* refers to yellowness. Colour was measured at two points (meat adjoining the thigh (femur) bone and at the meat surface away from the bone).

2.2.8 pH Measurement

pH was also measured at two points (meat adjoining the thigh (femur) bone and at the meat surface away from the bone using a pH meter (UB-10, Ultra Basic pH meter, Denver Instrument, Bohemia, NY, USA).

2.2.9 Statistical Analysis

Data were analyzed with a two-way ANOVA statistical model using the mixed procedure of SAS (SAS version 9.2, SAS Institute, Cary, NC, USA, 2006). The model tested the main effects for storage (fresh and frozen), cooking (raw and cooked) as well as their interaction using residual errors. Differences between group means were determined using HSD Tukey differences and were reported as significant at the $P < 0.05$ level. For each thigh two samples were taken and for each parameter 20 observations were taken.

2.3 RESULTS AND DISCUSSION

2.3.1 Heme Iron

Heme iron content of meat and meat containing foods is of great significance as storage stability of food products is dependent on iron content. Iron also acts as a major catalyst of lipid oxidation after cooking and subsequent release from heme pigments (Love and Pearson, 1974; Igene et al., 1979). Heme iron content of fresh thigh was found to be 5.14 µg/g of meat (Table 2.1). These values were in the range as reported by Leonhardt et al. (1997). They reported heme iron content in raw chicken thighs ranging from 4.0 - 7.0 µg/g of meat (wet weight). Earlier researchers also found that heme iron content in broiler thighs varied from 4.9 - 5.1 µg/g of meat (Carpenter and Clark, 1995; Clark et al., 1997). They also mentioned that heme iron variations in legs were more than breast meat due to more blood vessels and higher heme pigments. However, Lombardi-Boccia

et al. (2002) reported a heme iron concentration of 2 μg/g of meat (fresh weight) in raw chicken thighs.

There was a significant interaction of freezing and cooking ($P < 0.0001$) (Table 2.1) showing that the heme iron content increased from fresh raw to fresh cooked, highly increased from fresh raw to frozen raw but decreased significantly from frozen raw to frozen cooked samples. The initial heme iron increase in fresh samples after cooking may have been due to leakage of pigment from bone marrow to the bone surface. However when we froze the fresh samples without cooking the increase was almost 3 times which may be related to the high hemolysing effect of freezing on RBC's inside bone marrow of femur bones releasing heme pigment onto bone surface. Since there was a lot of heme pigment available after its leakage while freezing as compared to fresh samples there was a high denaturation effect of cooking on frozen samples resulting in a decrease in final heme iron content in frozen cooked samples.

Lombardi-Boccia et al. (2002) reported that the increase of heme iron in fresh samples after cooking can relatively be due to moisture loss that occurred during cooking. But the ratio of heme/non-heme iron (Table 2.1) in fresh raw, fresh cooked, frozen raw, and frozen cooked samples showed a decreasing trend indicating a decrease in heme iron and a corresponding increase in non-heme iron after freezing and cooking. This indicates that the pigment denaturation occurs during both freezing and cooking. Lombardi-Boccia et al. (2002) also reported a decrease in the ratio of heme/non-heme iron in legs of both chicken and turkey during cooking.

Red blood cells (RBC) are present inside bone marrow of long bones (Koonz and Ramsbottom, 1947). Freezing may result in breakdown of these RBC inside bone marrow releasing hemoglobin to the bone surface and finally increasing the heme iron content. After cooking, the heme moiety of protein pigments breaks down leading to formation of ionic iron and porphyrin and resulting in reduced heme iron content (Buchowski et al., 1988; Ahn et al., 1993). This decrease is due to breakdown of the heme present inside heme pigments (myoglobin and hemoglobin) which are proteins and capable of coagulation and denaturation by heat (Koonz and Ramsbottom, 1947). The denaturation occurs due to the oxidative cleavage of the porphyrin rings of hemoproteins releasing the iron from heme complexes (Schricker and Stouffer, 1982). At 55°C the rate of pigment denaturation is less (Geileskey et al., 1998) but as the temperatures go above 55°C the rate of denaturation increased correspondingly (Kristensen and Purslow, 2001). Han et al. (1993) reported that the heat denaturation of hemoproteins is temperature dependant and occurs from 85 to 100^{o}C. This causes dissociation of heme from globin resulting in iron release. The core temperature used for cooking in this study was 80^{o}C which caused extended protein denaturation. Lombardi-Boccia et al. (2002) found that cooking chicken and turkey in an oven significantly reduced heme iron with losses ranging between 22 and 43% while pan frying of meat cuts showed a range between 1 to 24% indicating lower heme loss. This further indicates that the cooking method influences final heme iron content and milder processing conditions cause less heme iron reduction. In general cooking resulted in a 27% decrease in heme iron

content in the present study. Carpenter and Clark (1995) also reported a 28% decrease in heme iron content after cooking of raw chicken thighs. A mean loss of about 20% heme iron content was observed during cooking of thighs from different poultry birds (Lombardi-Boccia et al., 2002).

2.3.2 Non-heme Iron

Both plant and animal sources provide non-heme iron but it has little bioavailability (2 to 20%) (Monsen and Balintfy, 1982). Non-heme iron content of raw broiler thighs was 1.33 ug/g of meat (Table 2.1). Leonhardt et al. (1997) also reported a non-heme iron content of 1.0 ug/g fresh weight in chicken thighs.

The interaction of freezing and cooking ($P < 0.0082$) was significant indicating that freezing the samples before cooking certainly had an effect on final non-heme iron content as compared to samples cooked without freezing. After cooking the fresh samples there was a significant increase in non-heme iron content but when we froze the fresh samples before cooking the increase in non-heme iron content quadrupled. This increase was consistent with a huge increase in heme iron content after freezing the broiler thigh samples. However after cooking of frozen samples there was no further significant increase in non-heme content. But the heme/non-heme ratio (Table 2.1) decreased constantly from fresh raw, fresh cooked, frozen raw to frozen cooked samples suggesting a change of iron state from heme iron to non-heme iron after freezing and then cooking those frozen samples. The increased non-heme iron in this study showed that there was a heme pigment protein denaturation of hemoglobin and myoglobin resulting in

the release of heme iron (ferrous) inside the heme moiety of heme pigments to its free ionic non-heme iron form (ferric) after both freezing and cooking. Also the increased availability of heme iron resulted in a corresponding huge increase in non-heme iron content due to denaturation of heme pigment proteins after freezing the samples.

Denaturation of heme pigment proteins hemoglobin and myoglobin leads to opening of porphyrin structures causing release of bound iron (heme iron) as free iron or what we call non-heme iron (Igene et al., 1979; Schricker and Miller, 1983; Tichivangana and Morrissey, 1985). The freezing temperature and rate of freezing affects the structure of frozen meat and its sensory qualities (Barbut and Mittal, 1990). Slow-freezing may cause some destruction of protein structure by myofibrillar shrinkage due to formation of large ice crystal between cells (Lee et al., 2008). This freeze denaturation of pigment proteins causes formation of non-heme iron (Tichivangana and Morrissey, 1985).

Cooking converts heme iron to non-heme iron to variable extents (Igene et al., 1979; Schricker and Stouffer, 1982) and degree of heme degradation depends on different cooking methods (Igene et al., 1979; Schricker and Miller, 1983). Various cooking methods caused an increase in non-heme iron from 0.50 to 1.04 mg/g in chicken thighs (Lombardi-Boccia et al., 2002). Cooking leads to denaturation of pigment proteins. This denaturation happens due to alteration in the tertiary structure of these pigment proteins (Baron and Andersen, 2002). Heat disrupts muscle cell structure (Harel and Kanner, 1985) and the pigment protein

denaturation releases bound iron (heme iron) as non-heme iron (Tichivangana and Morrissey, 1985).

2.3.3 Total Heme Pigments

Total heme pigments hemoglobin and myoglobin are iron containing pigments and function primarily as oxygen carriers (Lawrie, 1991). Myoglobin and hemoglobin structure maintains the heme complex in the reduced Fe (II) state. It is important as the oxidized Fe (III) form cannot bind to oxygen and is inactive physiologically (Bunn and Forget, 1986).

There was no significant ($P > 0.05$) effect of freezing on total heme pigment content. However, values showed a decreasing trend indicating the freeze denaturation of pigment proteins. Total heme pigment content significantly decreased ($P < 0.0001$) after cooking the broiler thighs (Table 2.1). Cooking results in conformational changes in heme pigment proteins exposing its active heme group to surrounding molecules (Eriksson et al., 1971). These conformational changes lead to denaturation of proteins (Smith and Northcutt, 2004). After the protein denaturation the solubility of pigments decreases (Tornberg, 2005) and hence leads to their reduced extractability (Ayala et al., 2005). All these changes result in a decrease in total heme pigment content of thigh meat after cooking the samples. The freezing x cooking interaction was not significant ($P < 0.05$) for total heme pigment content indicating that cooking the broiler thigh meat samples previously frozen or unfrozen did not affect the total heme pigment content.

2.3.4 Total Myoglobin

Myoglobin is the main pigment determining meat colour (Lawrie, 1991) and its concentration remains constant in the muscle after death (Pegg et al., 1997; Liu and Chen, 2001). The total myoglobin content of fresh thighs in this study was 3.35 mg/g of meat. Min and Ahn (2009) found myoglobin content of 1.16 mg/g of meat in a fresh chicken breast sample. However thighs contain more pigment than breast due to higher pigments and more vascular supply (Schreurs, 2000). Guidi et al. (2006) reported a myoglobin content of 1.96 mg/g of meat in fresh chicken thighs. Higher myoglobin concentrations may be due to overestimation as a result of turbidity of the extracts (Kranen et al., 1999). The myoglobin content also varies depending on species, breed, sex and age of animal, blood circulation and muscle type (Giddings and Hultin, 1974; Livingston and Brown, 1981; Postnikova et al., 1999). The broiler thighs used for analysis in this study were from male birds only.

Freezing had no significant effect ($P > 0.05$) on total myoglobin content. In spite of an increase in non-heme iron content during freezing, myoglobin content remained unchanged. Similar observations were also noticed by Min and Ahn (2009) during refrigerated storage of chicken breast meat. The myoglobin content significantly ($P < 0.0001$) decreased after cooking broiler thighs, which is related to decrease of total pigment content as myoglobin is the major component of total pigments. The active heme group of myoglobin is accessed by the surrounding molecules due to change in its conformation (Eriksson et al., 1971).

The resulting denaturation may lead to decreased solubility of these pigments and hence result in reduced myoglobin content in cooked samples.

Freezing x cooking interaction had no significant effect ($P > 0.05$) on myoglobin concentration. This indicates that cooking of thigh meat samples previously frozen did not affect the total myoglobin content.

2.3.5 Deoxymyoglobin (Mb), Oxymyoglobin (MbO_2) and Metmyoglobin (MetMb)

Myoglobin is the main pigment in fresh meat and exists in three forms deoxymyoglobin (Mb), oxymyoglobin (MbO_2) and metmyoglobin (MetMb) (Cornforth, 1994). Freezing significantly decreased the levels of both Mb ($P = 0.0047$) and MbO_2 ($P = 0.0344$) while there was no significant change in MetMb ($P > 0.05$) (Table 2.1). Min and Ahn (2009) also reported that the MetMb percentage did not change during refrigerated storage of chicken breast meat. The decrease in both Mb and MbO_2 forms of myoglobin indicates a tendency of change in its oxidation status. Freezing may result in quick changes in oxidation of myoglobin wherein the deoxy and oxy forms are short lived and tend to decrease favouring MetMb formation. Mancini and Hunt (2005) reported that exposure of myoglobin for longer periods to light, heat, microbial growth, or freezing results in MetMb formation.

Cooking significantly increased Mb ($P < 0.0344$) while there was a trend for a cooking effect on MbO_2 ($P = 0.0536$) where the levels decreased after

cooking similar to the freezing effect. However the reason for an increase in Mb after cooking is not clear.

There was no significant effect of freezing x cooking interaction on Mb, MbO_2 or MetMb percentage ($P > 0.05$) (Table 2.1). This suggests that freezing the samples before cooking did not affect the oxidative changes occurring in heme pigments while cooking the thigh samples.

The percentages of Mb, MbO_2 and MetMb (fresh thighs) in the present study were 25%, 8% and 68% respectively (Table 2.1). However, Guidi et al. (2006) reported a MetMb content of 67% only in darkened tissues of turkey thighs. In the present study the ratio of Mb: MbO_2: MetMb (0.25: 0.08: 0.68) indicates that fresh raw meat already had a high content of MetMb (~70%). MetMb is an oxidized product which indicates that oxidation occurs very fast during *post mortem* (Guidi et al., 2006). A lower percentage of MbO_2 (8%) suggests that it is short lived during the oxidation process of myoglobin. After the tissue death there is fast decrease in the enzyme activity responsible for MetMb reduction. This results in MetMb accumulation leading to protein denaturation (Guidi et al., 2006). Similar trends were observed in frozen as well as cooked samples where MetMb represented highest proportion of myoglobin (70%) while Mb and MbO_2 represented proportions similar to the fresh samples (Table 2.1). MetMb is also positively related to lipid oxidation as aldehydes (products of lipid oxidation) prevent reduction of MetMb (Lynch and Faustman, 2000).

2.3.6 Colour

Meat colour is dependent on the kind of muscle, storage temperature, and gas composition of modified atmosphere packaging (MAP) (Guidi et al., 2006). The translucency of muscle also affects meat colour (Swatland, 2008). The colour of meat is further dependent on myoglobin concentration and leg muscles appear darker due to more myoglobin.

Colour data has been presented in (Table 2.2). The colour values of lightness (L*), redness (a*) and yellowness (b*) in this study were similar to the values reported in breast muscle by Galobart and Moran (2004). Freezing had no significant ($P > 0.05$) effect on L* and a* values at the meat adjoining the thigh (femur) bone and at the meat surface away from the bone. This suggests that frozen storage at -20^{o}C for 4 weeks did not influence a change in colour development. There was no significant effect of freezing rate as well as frozen storage time on meat colour darkening (Brant and Stewart, 1950). However it may be possible that with increasing time in frozen storage a* value increases and L* and b* values decrease (Davis, 1995). However, our results showed that freezing significantly increased b* value at the meat surface away from the bone ($P < 0.0001$) while it had no effect on the meat adjoining the thigh (femur) bone ($P > 0.05$). This increase in b* value at the surface may be due to increased exposure of surface meat to freezing environment and resulting oxidation as compared to the meat interior. This may lead to more protein denaturation resulting in an increase in b* value at the surface than at the meat interior. But as the L* value did not increase significantly along with b* value the exact reason for this increase is not clearly defined.

Cooking significantly increased L* value ($P < 0.0001$) at the meat adjoining the thigh (femur) bone and at the meat surface away from the bone ($P < 0.0001$) (Table 2.2). Young and West (2001) also reported an increase in L* value of raw dark muscle after cooking the meat samples. Helmke and Froning (1971) reported an increase in L* value and decrease in a* value as the end point temperatures increased. This increase in L* value was due to increased light scattering by denatured proteins due to enhanced light reflection. Also the magnitude of change in L* value was more than in a* or b* values indicating that it is the main cause of meat whiteness. Fraqueza et al. (2006) reported correlationship of L*, a* and b* values where increase in L* value resulted in decrease in a* value and an increase in b* value. The increase in L* value at the meat adjoining the thigh (femur) bone and at the meat surface away from the bone in the current study indicates that protein denaturation and the resulting increased light reflection is occurring similarly at both points, negating the effect of bone presence in the vicinity of muscle.

A significant reduction of a* value ($P < 0.0001$) was observed upon cooking at meat adjoining the thigh (femur) bone while there was no significant change ($P > 0.05$) in a* value at the meat surface away from the bone during cooking (Table 2.2). This difference in behavior of a* values is not clear. In general, both hemoglobin and myoglobin impart red colour to meat and cooking leads to pigment denaturation resulting in decreased a* value. Heme binding to globin proteins is inhibited resulting in less a* value of meat (Young and West,

2001; Choi and Park, 2002). The significant correlation of total pigment to a* values shows that they influence meat colour (Fraqueza et al., 2006).

Cooking increased b* values at meat adjoining the thigh (femur) bone ($P < 0.0001$) and at the meat surface away from the bone ($P < 0.0001$) (Table 2.2). The increase in b* values after cooking can be due to greater denaturation and oxidation of heme proteins (Kristinsson et al., 2005). Similar to L* values the increase in b* values was not dependent on the fact of whether the meat was in close proximity to the bone or away from it. However the magnitude of the increase in both L* as well as b* values was more at the meat surface than near to the bone. Thus the extent of denaturation of heme proteins may be greater at the meat surface than the meat interior due to more exposure of surface meat to heat leading to more oxidation.

The colour data (Table 2.2) shows that the L* value was lower while the a* value was much higher at meat adjoining the thigh (femur) bone as compared to the meat surface away from the bone after freezing or cooking the samples. This may be due to the presence of more pigment at the meat near to the bone as a result of pigment leakage from inside the bone marrow of bone to the bone surface and adjoining meat resulting in increased a* value. This may also be due to increased exposure of surface meat to the freezing and cooking environments causing more pigment breakdown resulting in decrease in a* value. The increase in pigment breakdown may result in more light scattering and hence increase in L* value at the meat surface as compared to the meat adjoining the thigh (femur) bone.

Freezing x cooking interaction ($P > 0.05$) had no significant effect on L*, b*, or a* values of broiler thighs at the meat adjoining the thigh (femur) bone or at the meat surface away from the bone. This indicates that whether the broiler thigh meat samples were frozen or fresh before cooking did not in any way altered the colour outcome of the final cooked product. Contrary to this study, Lyon et al. (1976) reported that freezing of broiler thighs and then cooking them after freezing resulted in darkening. Furthermore, Brant and Stewart (1950) reported a comparatively darker colour of leg, thigh bones of 12 week old birds after freezing as compared to unfrozen birds and also found that removal of bone marrow before freezing was effective in reducing bone darkening.

2.3.7 pH

Freezing caused a significant ($P = 0.0109$) increase in pH at the meat adjoining the thigh (femur) bone while there was no pH change at the meat surface away from the bone ($P > 0.05$) (Table 2.2). There was an increase in pH with increasing storage duration in lamb meat (Abdullah and Qudsieh, 2009; Muela et al., 2010). Devine et al. (1995) reported that during freezing the enzymes and microorganisms present in meat degrade the meat proteins resulting in production of ammonia and amines resulting in increase in pH.

Cooking significantly increased pH at the meat surface away from the bone ($P = 0.0013$) while at meat adjoining the thigh (femur) bone there was no significant effect of cooking ($P > 0.05$). Fletcher et al. (2000) also reported a significant increase in pH after cooking the broiler breast meat. Huang et al.

(2011) reported that after cooking there was a change in acid-base groups at the surface of sarcoplasmic proteins and their denaturation probably leads to loss of acid groups increasing the pH.

At both meat adjoining the thigh (femur) bone and at the meat surface away from the bone the freezing x cooking interaction effect was non-significant ($P > 0.05$). So cooking the samples whether previously frozen or unfrozen did not affect thigh meat pH.

2.4 SUMMARY AND CONCLUSIONS

The effect of cooking on meat characteristics and colour varies depending on different cooking temperatures and heating times. We observed a reddish brown or black discolouration at the tip of bones and area adjoining it in most of the thighs immediately after cooking in a hot air oven (Figure 2.1). This discolouration at tips may be linked to pigment leakage from the bone interior to the bone surface. However, pigment leakage onto the bone did not darken the meat adjoining it. In general, it was observed that cooked samples had lower total pigment content and were lighter in colour (from colourimetric data). However, in commercial settings or homes, cooking is done with addition of other ingredients (salts, marinating, etc.). How these additives change the final colour individually or in combination/reaction with heme pigments needs to be investigated.

The present study has also shown that freezing causes pigment leakage and oxidation, but discolouration or darkening in the surrounding muscle tissue was not visible by the naked eye. Previous studies reported that darkening of bone

or the muscle adjoining it can be influenced by different freezing rates (fast or slow) after processing and by different storage times. Processing, meat preparation-chilling type and rate, freezing, reheating, and cooking methods all influence meat discolouration (Lyon et al., 1976). In this study the conditions mentioned above for freezing and cooking did not lead to bone and muscle darkening. More research is required for conclusive evidence of bone darkening or discolouration of broiler bone-in thighs with respect to different cooking or freezing temperatures and time, different cooking methods, addition of meat ingredients and meat packaging conditions.

Table 2.1. Iron and total heme pigment content of broiler bone-in chicken thighs after freezing and cooking.

	Heme iron µg/g meat	Non-heme Iron µg/g meat	Total heme pigments µg/g meat	Total myoglobin mg/g meat	[1]Mb	[2]MbO_2	[3]MetMb
Storage (S)							
Fresh	5.14[b]	1.33[b]	40.0	3.35	0.25[a]	0.08[a]	0.68
Frozen	9.53[a]	7.96[a]	33.53	3.39	0.24[b]	0.05[b]	0.70
SEM	0.36	0.12	2.50	0.19	0.001	0.008	0.01
Cooking (C)							
Raw	8.48[a]	4.23[b]	46.50[a]	4.38[a]	0.24[b]	0.08	0.68
Cooked	6.19[b]	5.06[a]	27.03[b]	2.36[b]	0.25[a]	0.06	0.70
SEM	0.36	0.12	2.50	0.19	0.001	0.008	0.01
Interaction (S x C)							
Fresh raw	4.10[c]	0.67[c]	52.12	4.35	0.25	0.09	0.67
Fresh cooked	6.19[b]	2.00[b]	27.87	2.34	0.25	0.08[a]	0.68
Frozen raw	12.87[a]	7.78[a]	40.87	4.41	0.24	0.07	0.69
Frozen cooked	6.19[b]	8.13[a]	26.19	2.38	0.24	0.04	0.71
SEM	0.51	0.17	3.53	0.25	0.001	0.01	0.01
Source of variation			*P*-value				
S	<0.0001	<0.0001	0.0771	0.8702	0.0047	0.0213	0.1803
C	0.0001	<0.0001	<0.0001	<0.0001	0.0344	0.0536	0.4296
S x C	<0.0001	0.0082	0.1860	0.9810	0.9011	0.5903	0.9833

[a, b, c] Means within column (storage, cooking, storage x cooking interaction) with no common superscript are significantly different ($P < 0.05$).
[1]Mb (deoxymyoglobin), [2]MbO_2 (oxymyoglobin), [3]MetMb (metmyoglobin) represent the relative content of the three forms of myoglobin.
n=40

Table 2.2. Colour and pH characteristics of broiler bone-in chicken thighs after freezing and cooking.

	Colour						pH	
	L*	a*	b*	L*	a*	b*		
Storage (S)		[1]Meat			[2]Meat near bone		[1]Meat	[2]Meat near bone
Fresh	63.68	5.40	9.42[b]	60.17	11.03	14.11	6.29	6.45[b]
Frozen	62.08	5.18	12.81[a]	58.82	9.99	15.48	6.32	6.59[a]
SEM	0.95	0.36	0.45	0.86	0.49	0.55	0.03	0.03
Cooking (C)								
Raw	54.91[b]	5.16	4.91[b]	56.59[b]	14.25[a]	11.79[b]	6.21[b]	6.51
Cooked	70.85[a]	5.43	17.32[a]	62.40[a]	6.77[b]	17.80[a]	6.39[a]	6.54
SEM	0.95	0.36	0.45	0.86	0.49	0.55	0.03	0.03
Interaction (S x C)								
Fresh raw	54.37	5.54	3.78	56.72	14.99	10.82	6.15	6.39
Fresh cooked	72.99	5.27	15.06	63.63	7.07	17.39	6.42	6.51
Frozen raw	55.44	4.78	6.05	56.47	13.50	12.76	6.28	6.62
Frozen cooked	68.71	5.59	19.57	61.17	6.48	18.21	6.36	6.57
SEM	1.35	0.51	0.64	1.22	0.69	0.77	0.04	0.05
Source of variation					*P*-value			
S	0.2460	0.6688	<0.0001	0.2777	0.1452	0.0876	0.4846	0.0109
C	<0.0001	0.6103	<0.0001	<0.0001	<0.0001	<0.0001	0.0013	0.5517
S x C	0.0571	0.3019	0.0915	0.3739	0.5190	0.4773	0.0666	0.1105

[a, b, c] Means within column (storage, cooking, storage x cooking interaction) with no common superscript are significantly different ($P < 0.05$).
L* a* b* values are colour characteristics referring to lightness, redness and yellowness of meat respectively.
[1]Meat refers to meat surface away from bone and [2]Meat near bone refers to meat adjoining thigh (femur) bone in bone-in broiler thighs.
n=40

Figure 2.1. The effect of cooking (-180^{o}C hot air oven, core temp. 80^{o}C) on the bone in broiler chicken thighs.

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CHAPTER 3. INTERRELATIONSHIPS AMONG DIET, BONE GROWTH AND DISCOLOURATION OF BROILER CHICKEN THIGHS.

ABSTRACT

The rapid growth rate of modern broilers for higher muscle yield has affected their bone performance as the bone mineralization is not proper within this short time period. The poor mineralization may result in pigment leakage from weak bones during freezing and may cause darkening of the bone and adjoining meat during cooking, leading to consumer rejection. Vitamin D helps in bone mineralization by enhancing calcium and phosphorus uptake after their absorption from dietary intake. The present study was conducted to study the effects of diet on meat characteristics and colour of bone-in broiler chicken thighs. Male Ross 308 broilers (n=320) were reared to 40 d of age and fed either a standard, nutritionally-complete diet with only vitamin D_3 as the supplemental vitamin D source (2,760 IU/ Kg feed; Control), a diet with equivalent vitamin D activity (69 μg/kg feed) as 25-hydroxyvitamin D_3 (25-OHD_3) only or a reduced nutrient density diet (Reduced; Control diet diluted with 25% wheat bran). Heme iron, non-heme iron, total heme pigments, total myoglobin, colour and pH were measured in fresh raw, fresh cooked, frozen raw and frozen cooked bone-in broiler thighs (n=4 per diet). Cooking (180^{o}C oven temperature, 80^{o}C core meat temperature), freezing (-20^{o}C for 4 wk), diet and their interactions were studied. Data were analyzed using ANOVA; differences were considered significant at $P < 0.05$. The freezing x diet ($P < 0.01$) interaction for heme iron and cooking x

freezing x diet ($P < 0.01$) interaction for non-heme iron showed that their concentrations were lowest in fresh uncooked samples from birds fed with 25-OHD_3 diet as compared to control and diluted diet. Increased bone mineralization might lead to less pigment leakage in 25-OHD_3 birds. However after freezing or cooking, significant differences were not observed among the various dietary treatments. The diet x cooking x freezing interactions for total pigment ($P < 0.01$) and myoglobin ($P < 0.01$) indicated a decrease in their concentrations after freezing or cooking the fresh thigh meat irrespective of the dietary treatment. Freezing favoured myoglobin oxidation indicated by decreased oxymyoglobin and increased metmyoglobin ($P < 0.01$) without any dietary effect. The study revealed that dietary 25-OHD_3 may increase bone quality but diet did not significantly affect bone discolouration under the applied conditions.

Key words: broiler, growth rate, vitamin D_3, 25-hydroxyvitamin D_3, darkening

3.1 INTRODUCTION

Colour of meat is an important characteristic that influences buying preferences of consumers as it is an indicator of freshness (Jacobson et al., 1959; Rentfrow et al., 2004). In North America bone-in broiler thighs are considered as dark meat and are undervalued. This makes them less saleable as compared to other meat parts like chicken breasts (Betti and Fletcher, 2005). Chicken thighs have greater concentration of heme pigments and blood supply than chicken breasts (Schreurs, 2000). Another problem associated with chicken thigh is the presence of pigment hemoglobin inside the bone marrow of thigh bones. Leakage

of this pigment from inside the bone to its surface leads to the unpleasant appearance in fresh thighs as well as after cooking (Koonz and Ramsbottom, 1947). The pigment leak occurs on bone surface and the meat adjacent to the bone (Smith and Northcutt, 2003). Myoglobin is another iron-containing pigment protein of muscles and affects the meat colour due to its oxidation and change in the valence of iron in it (MacDougall, 1982; Lawrie, 1991; Liu and Chen, 2001).

The growth of bones is slow compared to the overall body growth. In recent times the growth rate of broiler chickens has increased substantially, making broilers reach market weight sooner for better profits. The breast muscle yield has also been increased as a result of this change (Lilburn, 1994). However the quality of bone has suffered a lot due to this selection (Sullivan, 1994). Birds require proper mineralization of bones for better performance. The main components for maintaining proper bone mineral density are calcium (Ca) and phosphorus (P). The faster growing birds have less bone mineralization as compared to the slow growing birds (Leterrier and Nys, 1992). As a result of faster growth the commercial broiler flocks are faced with bone problems like tibial dyschondroplasia (Mitchell et al., 1997). The optimum bone density and breaking strength is formed only at 35 wk of age in broilers (Rath et al., 2000). As a result of fast growth rates, they are unable to form a tight, compact bone matrix which results in porous bone (Thorp and Waddington, 1997; Williams et al., 2000). This affects broiler performance as improper mineralization due to rapid growth rates leads to various skeletal deformities like lameness and falls facing condemnation at processing (Julian, 1998). The porous bones also allow more

pigment leakage from inside to the bone surface and adjoining meat changing the final colour of cooked products (Koonz and Ramsbottom, 1947).

Modern broiler chickens today are reared for improved feed conversion rate and rapid growth rate (Bao and Choct, 2010). Micronutrients play an important part in the feed of birds and vitamin D is an important component of bird diet (Norman and Hurwitz, 1993). The intestinal absorption of Ca and P, bone formation and Ca and P resorption by the kidney are governed by vitamin D (Combs, 1998; Atencio et al., 2006). Vitamin D maintains normal blood calcium levels by stimulating the intestinal absorption of dietary calcium (Soares et al., 1995; Van Leeuwen et al., 2001). In chickens about 70% of the Ca uptake in gut is vitamin D dependent (Hurwitz et al., 1983). The uptake of Ca and P in bones is enhanced by vitamin D and hence it helps in proper mineralization of bones (DeLuca, 2004). Vitamin D is formed in skin with reaction of ultraviolet radiation or is taken in through the diet (Soares et al., 1995). From either of the sources, vitamin D enters circulation and after its hydroxylation forms metabolites 25-hydoxyvitamin D_3 in liver and 1, 25-dihydroxyvitamin D_3 in kidneys (Norman and Hurwitz, 1993; Soares et al., 1995). The hydroxylation of vitamin D_3 in liver may be impaired due to certain factors like heat stress, infection or toxins in feed (Sahin et al., 2006; Waldenstedt, 2006). Impaired hydroxylation of vitamin D and formation of its metabolites leads to disturbances in Ca and P metabolism (Reichel et al., 1989; Soares et al., 1995; Whitehead et al., 2004). 25-hydroxyvitamin D_3 resulted in an increased body weight and ash content as well

as a lower risk for bone disorders than vitamin D in broiler chickens (Rennie and Whitehead, 1996; Fritts and Waldroup, 2003).

There are a few studies focused on the effects of feeding 25-hydoxyvitamin D_3 in broiler diets and its relation to bone mineralization (Soares et al., 1995; Sahin et al., 2009). However studies showing the effect of 25-hydoxyvitamin D_3 on meat characteristics and bone discolouration are scarce. Hence the objective of the present study was to find the effect of diet (Vitamin D_3, 25-hydroxyvitamin D_3, diluted) on the various meat characteristics and colour of broiler thighs and meat adjoining it. The study also focussed on the interrelationships of diet, freezing, cooking and their comparisons with fresh uncooked thighs.

3.2 MATERIAL AND METHODS

3.2.1 Materials and Sample Preparation

Male broiler chickens (Ross 308) were bought at day of age and raised for 40 days at the Poultry Research Centre (PRC) facility of University of Alberta. Birds were divided into three groups based on diet fed: Control (standard, nutritionally-complete broiler feeds with vitamin D_3), 25-hydroxyvitaminD_3 (25-OHD_3) supplemented (control + 25-OHD_3 at the manufacturer's recommended level of 69 μg/kg feed) and a reduced nutrient density diet (control diet diluted with 25% wheat bran), during the feeding period.

Birds were slaughtered on 39 and 40 days of age and after processing and air chilling, a total of 120 left broiler thighs were obtained and immediately taken

to University of Alberta poultry meat laboratory for meat analysis. In the laboratory thighs were divided into 3 groups of 40 thighs each (total 120 thighs) based on the diet fed to broilers (vitamin D_3, 25-OHD_3, Dilute). Each one of the 3 groups (40 thighs) was further divided into four sub-groups: fresh raw, fresh cooked, frozen raw and frozen cooked. Then each of the 40 thighs in a sub-group were analysed similarly to chapter 2 (sample preparation). The chemicals used for analysis were of reagent grade and obtained either from Fisher Scientific (Waltham, MA) or Sigma-Aldrich Co. (St. Louis, MO).

All the methods mentioned below have been explained in detail in Chapter 2 (Materials and Methods).

3.2.2 Heme Iron

Heme iron was determined by acidified acetone extraction method of Turhan et al. (2004).

3.2.3 Non-Heme Iron

Non-heme iron estimation was done by method as described by Min and Ahn (2009).

3.2.4 Total Heme Pigment Determination

Total heme pigment determination was done by alkaline hematin method of Karlsson and Lundström (1991).

3.2.5 Total Myoglobin Determination

Total myoglobin determination was estimated in accordance with the method described by Krzywicki (1982). The three forms of myoglobin, deoxymyoglobin (Mb), oxymyoglobin (MbO_2) and metmyoglobin (MetMb) were also determined.

3.2.6 Colour Measurement

Colour was measured at two points: meat adjoining the thigh (femur) bone and at the meat surface away from the bone by a Minolta CR-400 colorimeter using illuminant D65 as the light of source (CIE L*a* b*); L*-lightness, a*-redness, b*-yellowness.

3.2.7 pH Measurement

pH was also measured at two points: meat adjoining the thigh (femur) bone and at the meat surface away from the bone using a pH meter (UB-10, Ultra Basic pH meter, Denver Instrument, Bohemia, NY, USA).

3.2.8 Statistical Analysis

Data analysis was using a 3 x 2 x 2 ANOVA statistical model using the mixed procedure of SAS. The main effects were diet (Vitamin D, 25-OHD_3 and Dilute) storage (fresh and frozen), and cooking (raw and cooked). Their interactions were also studied using residual errors. HSD Tukey differences were

used to determine differences between group means and results were significant at the $P < 0.05$ level. Two samples per thigh were taken and 20 observations were taken for each meat parameter for each sub-group.

3.3 RESULTS AND DISCUSSION

3.3.1 Heme Iron

Heme iron is the available iron from heme pigment proteins, hemoglobin and myoglobin. It is found in animal sources and has high bioavailability (15-35%) (Carpenter and Mahoney, 1992; Hunt, 2002). There was a significant effect of diet x storage interaction ($P = 0.0001$) on heme iron content of broiler thigh samples in this study (Table 3.1). The fresh raw samples had the lowest heme iron content in birds fed with 25-OHD_3 (3.51 µg/g of meat) as compared to vitamin D (5.49 µg/g of meat) or dilute (4.39 µg/g of meat) diet.

Heme iron variations in these three groups might be due to differences in bone mineralization of the birds. Due to less bone mineralization, the pigment contained inside bone marrow leaks to the outside and discolours the bone surface and meat adjoining it (Koonz and Ramsbottom, 1947). In this study 25-OHD_3 fed birds may have a better bone mineralization as compared to the birds fed with the vitamin D or the dilute diet. The better bone mineral density and increased bone strength as a result may result in less bone porosity. This may further lead to less leakage of pigment from bones resulting in lower heme iron content. However, the heme iron content increased after freezing the bone-in thighs in all the diet groups. This indicates that the beneficial effect of 25-OHD_3 may not be there after

freezing the thigh samples. The meat shelf life depends on variations in species, freezing rate, storage temperature and oxidation protection (Careche et al., 1999). Bone marrow contains hemoglobin and the increase in heme iron content after freezing indicates that there may be pigment leakage after red blood cell lysis from inside the bone onto the bone surface and adjoining meat (Koonz and Ramsbottom, 1947).

Beneficial effects of feeding 25-OHD_3 in comparison to vitamin D have also been reported in the previous studies. Feeding of 25-OHD_3 was more effective in reducing the incidence of bone disorders in broilers as compared to vitamin D. Feeding 25-OHD_3 with lower vitamin D levels may be more useful under stress conditions as it is metabolically more potent (Fritts and Waldroup, 2003). The intestinal absorption of 25-OHD_3 is greater than vitamin D (Bar et al., 1980). Moreover, Yarger et al. (1995) reported that feeding 25-OHD_3 to broilers resulted in increased feed conversion efficiency as well as higher breast muscle yield. They also mentioned that feeding of either vitamin D or 25-OHD_3 resulted in higher circulating plasma levels of 25-OHD_3. Sahin et al. (2009) found that feeding of 25-OHD_3 and soy isoflavones together in Japanese Quails resulted in an increased concentration of serum vitamin D as well as increased bone mineral density and tibial ash content. They also found increased deposition of Ca and P in bones with increase in the 25-OHD_3 in feed. There was a significant increase in body weight as well as tibia ash content indicating better bone mineralization in chicks with increasing levels of 25-OHD_3 in the diet (Atencio et al., 2005).

In the present study feeding of the low energy dilute diet to restrict the growth of birds for increased bone mineralization may have been effective, as heme iron levels were still lower than with the vitamin D diet; however beneficial effects of feeding 25-OHD_3 still seems higher. Leterrier et al. (1998) reported that reducing the growth rate of broilers by feeding a low energy diet did not improve their bone quality. The mineral content of tibiotarsi bones did not increase by feeding either a protein or energy restricted diet in turkeys (Turner and Lilburn, 1992; Kirn and Firman, 1993). Contrary to this, it was reported that the incidence of leg problems in birds can be reduced by reducing their growth rate (Classen, 1992; Thorp, 1994). However, Wise (1970) mentioned that genetics rather than diet affects the bone composition.

The storage x cooking interaction was also significant ($P = 0.0438$) where the heme iron content increased significantly especially after cooking of frozen samples and there was an increasing trend after cooking or freezing of fresh samples (Table 3.1). The heme iron increase after freezing can occur due to the hemolyzing effect of freezing temperatures on the red blood cells inside bone marrow releasing hemoglobin. The increase in heme iron content while cooking the samples can occur due to moisture loss (Lombardi-Boccia et al., 2002). The increase can also be due to some pigment leakage while cooking the samples as discussed earlier. However the huge increase in heme iron content after cooking of frozen samples indicates that freezing of broiler thighs resulted in changes in the bone which resulted in a higher heme pigment leakage from bones after cooking. The presence of this leaked pigment after freezing and cooking of broiler

thighs especially at meat near bone and bone itself may not be desirable for consumers.

3.3.2 Non-Heme Iron

Both plant and animal sources contain non-heme iron and its bioavailability is less than heme iron (2-20%) (Monsen and Balintfy, 1982). There was a significant effect of storage x cooking x diet interaction ($P < 0.0001$) on non-heme iron content of broiler thighs (Table 3.1). 25-OHD_3 diet fed birds had less non-heme iron (0.61 μg/g of meat) than vitamin D (1.44 μg/g of meat) or dilute (1.24 μg/g of meat) diets in fresh raw samples. However after freezing or cooking the samples, concentration of non-heme iron increased to a similar extent in all the 3 diets and differences were not seen as for fresh raw samples. Thus the difference in diet fed or the positive effect of 25-OHD_3 was seen only in fresh uncooked samples but was negligible after freezing or cooking the samples. The non-heme iron content in the present study was also consistent with the heme iron content observed in fresh raw samples. The less available heme iron content in fresh raw samples lead to the corresponding lower non-heme iron content in fresh raw samples from birds fed 25-OHD_3 diet. However, after freezing and cooking of samples the increase in heme iron content irrespective of the type of diet fed resulted in an increase in non-heme iron content also.

Freezing leads to ice crystal formation inside the meat and the size of crystal formation depends on the rate of freezing (Smith et al., 1968; Berry, 1990; Uttaro and Aalhus, 2007). This crystal formation may lead to denaturation of

proteins including heme pigment proteins. Myofibrillar protein denaturation due to ice crystal formation was reported by Lee et al. (2008). Slow freezing of meat forms large ice crystals and causes more structural damage after thawing as compared to the fast freezing (Devine, et al., 1995; Zhu et al., 2004)**.** Due to some residual enzyme activity in meat the frozen meat is also prone to deterioration and lipid oxidation as well as protein degradation (Devine et al., 1995; Akköse and Aktas, 2008). The protein denaturation opens up the porphyrin structure of heme pigments and releases the bound heme iron as free non-heme iron (Igene et al., 1979; Schricker and Miller, 1983; Tichivangana and Morrissey, 1985).

Cooking causes red pigment protein denaturation (Smith and Northcutt, 2004). The pigment denaturation results in conformational changes in myoglobin wherein the heme group inside the molecule gets activated and exposed to surrounding molecules (Eriksson et al., 1971). Cooking converts heme iron to non-heme iron due to pigment breakdown (Igene et al., 1979; Tichivangana and Morrissey, 1985). Purchas et al. (2003) reported that cooking leads to loss of soluble heme iron after pigment denaturation as insoluble heme iron inside the meat, soluble or insoluble non-heme iron after its release from the heme ring inside the meat or as soluble heme or non-heme iron in cooking juices.

3.3.3 Total Heme Pigments

Hemoglobin and myoglobin are the major components of total heme pigments (Romans et al., 1965). The study revealed a significant ($P < 0.0001$) interaction effect between diet x storage x cooking on total heme pigment content

and hence the results are described in the context of this interaction (Table 3.1). There was a significant decrease in total heme pigment content after freezing or cooking the thighs from birds fed with vitamin D and 25-OHD_3. The hydrophilic and hydrophobic balance of proteins reflects their solubility which further indicates their extent of denaturation (Van Laack et al., 2000; Omana et al., 2010). In the present study the increased denaturation of pigments may result in exposure of hydrophobic groups from inside the pigments to their surface resulting in decreased solubility. This decreased solubility may result in less extractability and hence lower total heme pigment content after freezing or cooking.

Heme pigments are sarcoplasmic proteins and are highly water soluble (Hultin et al., 1995; Papadopoulos et al., 2000). Freezing and frozen storage causes loss of water during thawing. This red aqueous solution of proteins is called drip and contains water soluble sarcoplasmic proteins (glycolytic enzymes and myoglobin) (Howard et al., 1960; Savage et al., 1990). After freezing the sarcoplasmic protein solubility decreases (Farouk and Swan, 1998). The decreased solubility leads to low extractability of proteins which may be due to modifications in the molecular structure or interaction with certain insoluble components of the muscle (Hultin et al., 1995). The water loss after thawing of frozen samples in this study as drip might also have resulted in a decreased total heme pigment content after freezing.

Cooking leads to changes in muscle structure wherein the hydrophobic groups in the interior of the proteins come to the surface and this leads to unfolding of globin chains (Dickerson and Geis, 1969). These conformational

changes in proteins are called denaturation and lead to protein-protein interaction and aggregation resulting in decreased protein solubility (Tornberg, 2005). In the present study the decrease in total heme pigment content after cooking might be due to the lower extractability of proteins because of denaturation and resulting decreased solubility. Heating leads to protein coagulation and muscle shrinkage resulting in loss of meat juices as cook loss. The cook loss during cooking also depends on time, temperature and method of cooking along with the meat type (Ziauddin et al., 1994). The loss of pigments along with the cook loss may also lead to decrease in total heme pigments.

The denaturation of sarcoplasmic proteins occurred in the temperature range of 50-80^{o}C (Okayama et al., 1991). In general, the proteins aggregate at 50-60^{o}C (Hamm, 1977) while in beef the protein aggregation occurred at the higher temperature of 90^{o}C (Davey and Gilbert, 1974). Cooking the meat results in formation of hematin and denatured proteins (MacDougall, 1982). The thermal coagulation of proteins resulted in structural changes in muscle as well as its water content. The dense aggregates of proteins were found during cooking which might be due to denaturation of sarcoplasmic proteins (Ayala et al., 2005). Since heme pigments are sarcoplasmic proteins and the oven temperatures used for cooking the thigh meat samples was 80^{o}C this resulted in heme pigment protein denaturation after cooking the samples in the present study.

In the present study freezing resulted in a greater reduction of total heme content and cooking those frozen samples resulted in a further decrease in total heme content. However when fresh samples were cooked, a highly significant

decrease was seen in total heme content. This indicates that when fresh samples were cooked most of the protein denaturation occurred during cooking. However while freezing the samples before cooking, most of the denaturation occurred while freezing.

3.3.4 Total Myoglobin

Total myoglobin forms most of the total heme pigment content and hence the results are also consistent with the total heme pigment content. The diet x storage x cooking interaction was significant (P = 0.0035) where freezing the samples resulted in a decrease in total myoglobin content among all the diet groups (Table 3.2). Cooking of frozen samples resulted in a further decrease in total myoglobin content in all the diets although the extent of decrease was not that high. However, cooking the fresh samples resulted in a huge decrease in total pigment content among all the diet groups. This suggests that although the diet effect was significant in the interaction, mainly freezing or cooking rather than diet were responsible for the change in total myoglobin content. This also indicates that denaturation of pigment proteins starts while freezing and continues on further cooking the samples.

Myoglobin is the major component of total heme pigments and hence after freezing or cooking its denaturation may have resulted in its reduced extractability after its decreased solubility. Studies in bovines showed that the myoglobin amounts remained unchanged till 60^{o}C then started decreasing gradually after further increase in temperatures (Laakkonen et al., 1970). At 65^{o}C or below the

denaturation of myoglobin may be due to some enzymatic processes while at 80^{o}C the denaturation is mainly thermal (Bernofsky et al., 1959). The decrease in total myoglobin in this study might also be due to drip or cook loss as mentioned earlier for the total heme pigment content. Purchas et al. (2003) also found drip losses after freezing / thawing and cook losses after cooking of meat.

3.3.5 Deoxymyoglobin (Mb), Oxymyoglobin (MbO_2) and Metmyoglobin (MetMb)

Fresh meat immediately on exposure to oxygen changes its colour from the purple deoxy state of myoglobin to bright cherry red oxy state but soon forms the brown metmyogobin. This is called autoxidation as it is oxygen dependent and is non-enzymatic in nature (Livingston and Brown, 1981). There was a significant effect of diet x cooking interaction (P = 0.0008) with an increase in the Mb content of 25-OHD_3 and dilute diet but not vitamin D after cooking the samples (Table 3.2). This difference between various dietary treatments was not clearly understood but in general it can be speculated that the loss of oxygen from the meat samples may lead to increase in Mb content while cooking.

The rate of oxygen consumption and its diffusion inside the muscle decides the formation of MbO_2 induced red colour in meat (Hall et al., 1944; Lawrie, 1958; Bendall and Taylor, 1972). The diet x storage x cooking interaction for MbO_2 was significant (P = 0.0008). There was a decrease in MbO_2 content after cooking fresh samples, after freezing fresh samples and after cooking the frozen samples in all the diet groups (Table 3.2). This suggests that although diet

effect was significant in the interaction the oxidation of pigments occurred after freezing and cooking which was not affected by differences in diets given to birds (vitamin D_3, 25-OHD_3 or dilute diet). Freezing and cooking promotes oxidation which can further deteriorate colour of meat (Guidi et al., 2006). Freezing and thawing of fish muscle resulted in more dark meat probably as a result of unfolding and autoxidation of myoglobin (Chow et al., 1989).

The storage x cooking ($P = 0.0002$) interaction was significant for MetMb content. After cooking the fresh samples there was a huge increase in MetMb content. In frozen samples, increase in MetMb content was mostly influenced by freezing which further increased during cooking of those frozen samples; however the increase was not as high as that of fresh samples. MetMb is formed after oxidation of myoglobin and is indicative of protein denaturation (Guidi et al., 2006). Rate of diffusion of oxygen is greater at low temperatures (Urbin and Wilson, 1958) and it may be possible that while freezing at -20^{o}C in the present study, more oxygen promoted oxidation resulting in MetMb formation. In living systems the metmyoglobin is reduced back to its oxy form with the help of reducing enzymes (Halliwell and Gutteridge, 1989). The MetMb reducing enzymes lose their activity with increased time in frozen storage which was seen even after one month of storage (Farouk and Swan, 1998). So after death of birds there is more accumulation of MetMb and darkening of meat colour (Guidi et al., 2006). Contrary to this, Osborn et al. (2003) reported that MetMb can be reversibly reduced back to MbO_2 by NADH–metmyoglobin reducing system while cooking. Cornforth et al. (1991) reported another colour problem pinking of

cooked pork under refrigeration storage and linked it back to the heme iron reduction from ferric to ferrous form. This suggests that some reduction mechanisms may still be working in meat after culling of the birds.

The significant diet x cooking interaction ($P < 0.0001$) shows that MetMb content increased after cooking the samples from birds fed with vitamin D and dilute diets with no change in samples from birds fed 25-OHD_3 diet. Oxidation of myoglobin leads to MetMb formation. However the protective action of 25-OHD_3 on myoglobin oxidation cannot be established as the MbO_2 content also decreased after cooking in 25-OHD_3 diet group.

The initial state of myoglobin (Mb, MbO_2 or MetMb) decides final cooked meat colour rather than cooking rate (Ryan et al., 2006). Both MbO_2 and MetMb are less stable to heat than Mb and denature quickly on cooking leading to premature browning (Hunt et al., 1999).

3.3.6 Colour

3.3.6.1 L* value (Lightness)

Meat colour depends on its light scattering properties (MacDougall, 1982) which are a result of gaps occurring between myofibrils (Offer et al., 1989). It also depends on rate and temperature of freezing, light intensity during display and packaging method (Jakobsson and Tsson, 1973). The leakage of blood proteins from blood vessels at the meat surface and from bones due to cuts while processing influences meat colour and quality. The denaturation of these proteins

during freezing or cooking also affects its colour (Koonz and Ramsbottom, 1947; Lyon and Lyon, 1986).

Freezing resulted in a significant decrease in L* value ($P = 0.0181$) at the meat surface away from the bone while at the meat adjoining the thigh bone there was no significant ($P > 0.05$) change in L* value (Table 3.3). The rate of freezing of meat affects its lightness value. Differences in lightness values during freezing are due to different rates of ice crystal formation. Small ice crystals from fast freezing scatter more light and produce lighter meat whereas large crystals from slow freezing scatter less light and produce dark meat (Voyle, 1974). The decrease in lightness in the present study may also be due to higher oxidation of pigments forming metmyoglobin and dark colour while freezing. However as a* values did not significantly change after freezing so more evidence is required for such conclusions.

There was a significant increase in L* value ($P < 0.0001$) at both the meat adjoining the thigh bone and at the meat surface away from the bone after cooking the samples. Cooking causes pigment protein denaturation which results in more light scattering from the wet meat surface resulting in higher L* values (Scopes, 1964; MacDougall, 1982). The increase in L* value after cooking was also reported by Young and West (2001) in raw dark meat samples. Helmke and Froning (1971) concluded that the increase in L* value occurs due to more scattering of light as a result of protein denaturation and enhanced light reflection. However, Cunningham (1974) concluded that cooking of bone-in broiler thighs to internal temperature of 85^{o}C after slow freezing (8^{o}C to -20^{o}C) resulted in a dark

bone colour while cooking after fast freezing (-197^{o}C) did not produce a dark colour.

There was no significant effect ($P > 0.05$) of diet on L* values in thigh meat samples at meat adjoining the thigh bone or at the meat surface away from the bone. This suggests that type of diet fed to birds did not affect the lightness of meat from broiler thighs.

3.3.6.2 a* value (Redness)

Freezing and cooking effects (Table 3.3) showed that a* value was higher in fresh samples at meat adjoining the thigh bone as compared to the meat surface away from the bone. Values were higher even after freezing or cooking the samples. This suggests that near the bone, pigment content may be higher as compared to the meat away from the bone. This seems relevant as pigment leakage from bones onto their surface and the surrounding meat may result in increase in redness.

There was a significant decrease in a* value ($P < 0.0001$) at meat adjoining the thigh bone but no significant change in a* value ($P > 0.05$) at the meat surface away from the bone after cooking the thigh samples (Table 3.3). The pigments provide red colour to meat and their denaturation results in a decrease in redness of meat (Young and West, 2001; Choi and Park, 2002). Probably there was a higher pigment content at meat adjoining the thigh bone also indicated by a higher a* value in raw samples. Hence the resulting oxidation and denaturation of

heme pigment was also greater while cooking for meat near the bone as compared to the meat surface resulting in a decrease in a* value.

There was no significant effect of freezing ($P > 0.05$) or diet ($P > 0.05$) on a* values either at the meat adjoining the thigh bone or at the meat surface away from the bone in the samples.

3.3.6.3 b* value (Yellowness)

There was a significant diet x storage x cooking ($P = 0.0194$) interaction at the meat surface away from the bone. Although the diet effect was significant in this interaction it did not contribute much to the increase in b* values. Freezing caused an increase in b* values but cooking frozen/unfrozen samples resulted in a huge increase in b* values indicating greater influence of cooking on colour. Cooking results in a greater extent of protein denaturation of heme pigments, thereby increasing b* values (Kristinsson et al., 2005). Decrease in pigment concentration is associated with increased light scattering and increased b* values (MacDougall, 1982).

Freezing caused a significant increase in b* values at the meat adjoining the thigh bone ($P = 0.0345$) (Table 3.3). Cooking also caused significant increase in b* values at the meat adjoining the thigh bone ($P < 0.0001$). The increase in b* values was much higher after cooking as compared to freezing the samples. There was no significant ($P > 0.05$) effect of diet on b* values in thigh meat samples at the meat adjoining the thigh bone.

3.3.7 pH

The storage x cooking interaction was significant at both meat adjoining the thigh bone ($P < 0.0001$) and at the meat surface away from the bone ($P < 0.0001$). At the meat surface away from the bone cooking the fresh samples resulted in a significant increase in pH, freezing the fresh samples did not significantly changed the pH while cooking the frozen samples again resulted in a significant increase in pH. At the meat adjoining the thigh bone cooking the fresh samples did not result in a significant increase in pH, freezing the fresh samples in fact significantly decreased the pH while cooking the frozen samples resulted in a significant increase in pH. The increase in pH during frozen storage was also reported in lamb meat (Abdullah and Qudsieh, 2009; Muela et al., 2010). During freezing the meat proteins are degraded due to the presence of enzymes and microorganisms resulting in production of ammonia and amines causing increase in pH values (Devine et al., 1995).

A significant increase in pH after cooking was also found in broiler breast meat (Fletcher et al., 2000) and in beef, pork, and chicken meat (Kwon et al., 2008). Vasanthi et al. (2007) also reported an increase in pH after cooking of raw meat at 80^{o}C, 90^{o}C and 100^{o}C for different times in a water bath. Cooking results in denaturation of sarcoplasmic proteins. This may cause disturbances in acid-base groups at the surface of proteins probably due to loss of acidic groups causing increase in pH (Lawrie, 1979; Huang et al., 2011).

There was a significant diet x storage interaction at the meat adjoining the thigh bone ($P = 0.011$) and significant diet x cooking interaction at both the meat

adjoining the thigh bone ($P = 0.0012$) or at the meat surface away from the bone ($P = 0.0006$). However, diet alone had no significant effect on pH in thigh meat samples at meat adjoining the thigh bone ($P > 0.05$) or at the meat surface away from the bone ($P > 0.05$). Thus the significant effects were produced by freezing or cooking in the interactions and not by the type of diet fed as there was an increase in pH in all the diet groups after freezing or cooking.

3.3 SUMMARY AND CONCLUSIONS

Type of diet fed to broilers may have an important effect on their performance with regard to bone mineralization. This study showed that birds fed with 25-OHD_3 supplemented diets might have an increased bone performance as heme iron content was lowest in this diet group indicating less heme pigment leakage. The 25-OHD_3 supplemented diet may help to increase bone mineralization by better Ca and P uptake and ultimately more deposition of these minerals in the bone. This may lead to less porous bones with increased bone density. We did not find any change in colour of thigh meat samples by feeding different diets to broilers. However, we found increased redness at the meat near to the bone as compared to the meat surface away from the bone in fresh, frozen and cooked samples in all the diet groups which may indicate pigment leakage from bones. This leakage of pigments at processing plants due to cuts in bones or after freezing or cooking may not be liked by consumers. The better bone mineralization may result in less porous bones resulting in less leakage of pigments which may affect the final appearance of a frozen or cooked product. It

may also help to reduce leg problems thus reducing the culls or trims at processing, increasing the meat yield. Since the genetic selection of a bird also affects its growth rate and feed conversion efficiency (Hulan et al., 1980). Hence more trials with different dietary vitamin D combinations should be tested in the varied broiler breeds for conclusive evidence of the effect of diet and its relationship to bone density and ultimately to colour change or darkening.

Table 3.1. Effect of storage, cooking and diet on heme iron, non-heme iron and total heme pigment content of broiler chicken thighs.

Fixed effect	Heme iron µg/g of meat	Non-heme iron µg/g of meat	Total heme pigments µg/g of meat
Storage (S)			
Fresh	4.46[b]	1.45	36.72[a]
Frozen	5.26[a]	1.52	32.64[b]
SEM	0.15	0.04	1.28
Cooking (C)			
Raw	4.58[b]	1.21[b]	34.98
Cooked	5.14[a]	1.75[a]	34.37
SEM	0.15	0.04	1.28
Diet (D)			
vitamin D_3	5.37[a]	1.67[a]	33.54[ab]
25-OHD_3	4.56[b]	1.46[b]	32.55[b]
Dilute	4.64[b]	1.32[b]	37.95[a]
SEM	0.18	0.05	1.57
Interaction (S x C)			
Fresh raw	4.40[b]	1.10[b]	43.76[a]
Fresh cooked	4.52[b]	1.80[a]	29.68[b]
Frozen raw	4.76[b]	1.33[b]	26.21[b]
Frozen cooked	5.75[a]	1.70[a]	39.07[a]
SEM	0.21	0.06	1.81
Interaction (S x D)			
Fresh D_3	5.49[a]	1.68[a]	42.74[a]
Fresh 25-OHD_3	3.51[c]	1.22[b]	40.57[a]
Fresh Dilute	4.39[bc]	1.45[ab]	26.85[b]
Frozen D_3	5.26[ab]	1.66[a]	24.34[b]
Frozen 25-OHD_3	5.61[a]	1.71[a]	24.53[b]
Frozen Dilute	4.89[ab]	1.18[b]	49.05[a]
SEM	0.26	0.08	2.22
Interaction (C x D)			
Raw D_3	5.02	1.48	38.83[ab]
Raw 25-OHD_3	4.23	1.14	37.63[bc]
Raw Dilute	4.49	1.02	28.49[cd]
Cooked D_3	5.72	1.86	28.26[d]
Cooked 25-OHD_3	4.90	1.78	27.46[d]
Cooked Dilute	4.79	1.61	47.40[a]
SEM	0.26	0.08	2.22

(table 3.1 cont.)

Interaction (S x C x D)			
Fresh Raw D_3	5.31	1.44^{ab}	50.15^{b}
Fresh Raw 25-OHD_3	3.66	0.61^{d}	51.20^{b}
Fresh Raw Dilute	4.23	1.24^{bc}	29.93^{c}
Fresh Cooked D_3	5.66	1.92^{a}	35.34^{c}
Fresh Cooked 25-OHD_3	3.36	1.83^{a}	29.93^{c}
Fresh Cooked Dilute	4.54	1.67^{ab}	23.76^{c}
Frozen Raw D_3	4.74	1.51^{ab}	27.50^{c}
Frozen Raw 25-OHD_3	4.79	1.68^{ab}	24.06^{c}
Frozen Raw Dilute	4.75	0.81^{cd}	27.05^{c}
Frozen Cooked D_3	5.78	1.80^{a}	21.17^{c}
Frozen Cooked 25-OHD_3	6.44	1.74^{ab}	25.00^{c}
Frozen Cooked Dilute	5.04	1.56^{ab}	71.04^{a}
SEM	0.37	0.11	3.14
Source of variation		*P*-value	
S	0.0003	0.3299	0.0268
C	0.0109	<0.0001	0.7373
D	0.0041	0.0002	0.0415
S x C	0.0438	0.0126	<0.0001
S x D	0.0001	<0.0001	<0.0001
C x D	0.6894	0.2742	<0.0001
S x C x D	0.1706	<0.0001	<0.0001

[a, b, c, d] Means within a column or within storage, cooking, diet or their interactions with no common superscript are significantly different ($P < 0.05$).
n=120

Table 3.2. Effect of storage, cooking and diet on total myoglobin content and its various forms in broiler chicken thighs.

Fixed effect	Total myoglobin mg/g of meat	Myoglobin content		
		[1]Mb	[2]MbO_2	[3]MetMb
Storage (S)				
Fresh	3.45[a]	0.24	0.08[a]	0.68[b]
Frozen	2.70[b]	0.23	0.06[b]	0.72[a]
SEM	0.06	0.001	0.002	0.002
Cooking (C)				
Raw	3.49[a]	0.23[b]	0.10[a]	0.69[b]
Cooked	2.66[b]	0.25[a]	0.05[b]	0.72[a]
SEM	0.06	0.001	0.002	0.002
Diet (D)				
D_3	2.57[c]	0.23[b]	0.07	0.70
25-OHD_3	3.52[a]	0.24[a]	0.07	0.70
Dilute	3.13[b]	0.24[a]	0.07	0.70
SEM	0.07	0.002	0.003	0.003
Interaction (S x C)				
Fresh raw	3.98[a]	0.23	0.12[a]	0.66[c]
Fresh cooked	2.91[b]	0.25	0.05[c]	0.71[b]
Frozen raw	2.99[b]	0.22	0.07[b]	0.71[b]
Frozen cooked	2.41[c]	0.25	0.04[c]	0.73[a]
SEM	0.09	0.002	0.003	0.003
Interaction (S x D)				
Fresh D_3	2.77[cd]	0.23	0.09	0.68[b]
Fresh 25-OHD_3	4.16[a]	0.24	0.09	0.68[b]
Fresh Dilute	3.42[b]	0.24	0.08	0.69[b]
Frozen D_3	2.38[d]	0.23	0.06	0.72[a]
Frozen 25-OHD_3	2.88[c]	0.23	0.06	0.72[a]
Frozen Dilute	2.85[c]	0.24	0.06	0.71[a]
SEM	0.11	0.003	0.004	0.004
Interaction (C x D)				
Raw D_3	3.07	0.23[b]	0.10[a]	0.68[b]
Raw 25-OHD_3	3.91	0.23[b]	0.08[a]	0.70[b]
Raw Dilute	3.47	0.23[b]	0.10[a]	0.68[b]
Cooked D_3	2.07	0.23[b]	0.05[b]	0.73[a]
Cooked 25-OHD_3	3.12	0.25[a]	0.06[b]	0.70[b]
Cooked Dilute	2.79	0.26[a]	0.03[c]	0.72[a]
SEM	0.11	0.003	0.004	0.004

(table 3.2 cont.)

Interaction (S x C x D)				
Fresh Raw D_3	3.36^{bcd}	0.23	0.13^{a}	0.65
Fresh Raw 25-OHD_3	4.50^{a}	0.23	0.11^{ab}	0.67
Fresh Raw Dilute	4.09^{ab}	0.23	0.11^{ab}	0.67
Fresh Cooked D_3	2.18^{ef}	0.23	0.05^{d}	0.71
Fresh Cooked 25-OHD_3	3.81^{abc}	0.26	0.06^{d}	0.69
Fresh Cooked Dilute	2.74^{de}	0.26	0.04^{de}	0.71
Frozen Raw D_3	2.79^{de}	0.23	0.07^{cd}	0.71
Frozen Raw 25-OHD_3	3.32^{cd}	0.22	0.06^{d}	0.73
Frozen Raw Dilute	2.86^{de}	0.22	0.09^{bc}	0.69
Frozen Cooked D_3	1.97^{f}	0.23	0.05^{de}	0.74
Frozen Cooked 25-OHD_3	2.43^{ef}	0.24	0.06^{d}	0.71
Frozen Cooked Dilute	2.84^{de}	0.26	0.02^{e}	0.73
SEM	0.15	0.004	0.006	0.006
Source of variation		*P*-value		
S	<0.0001	0.0667	<0.0001	<0.0001
C	<0.0001	<0.0001	<0.0001	<0.0001
D	<0.0001	0.0005	0.2473	0.6142
S x C	0.0084	0.4319	<0.0001	0.0002
S x D	0.0003	0.1105	0.3982	0.0581
C x D	0.3649	0.0008	<0.0001	<0.0001
S x C x D	0.0035	0.2576	0.0008	0.0969

[a, b, c, d, e, f] Means within a column or within storage, cooking, diet or their interactions with no common superscript are significantly different ($P < 0.05$).
[1]Mb (Deoxymyoglobin), [2]MbO_2 (Oxymyoglobin), [3]MetMb (Metmyoglobin) represent the relative content of the three forms of myoglobin.
n=120

Table 3.3. Effect of storage, cooking and diet on colour and pH characteristics of broiler chicken thighs at meat and meat near bone.

	Colour						pH	
	L*M	a*M	b*M	L*MNB	a*MNB	b*MNB	pHM	pHMNB
Storage (S)	L*	a*	b*	L*	a*	b*		
Fresh	60.58[a]	5.85	13.06[b]	59.44	12.08	13.96[b]	6.31[b]	6.57[b]
Frozen	59.39[b]	6.29	13.62[a]	58.57	11.98	14.80[a]	6.38[a]	6.65[a]
SEM	0.35	0.20	0.19	0.59	0.38	0.27	0.01	0.01
Cooking (C)								
Raw	51.53[b]	6.12	6.57[b]	54.60[b]	15.41[a]	12.79[b]	6.17[b]	6.54[b]
Cooked	68.64[a]	6.03	20.12[a]	63.41[a]	8.66[b]	15.98[a]	6.51[a]	6.68[a]
SEM	0.35	0.20	0.19	0.59	0.38	0.27	0.01	0.01
Diet (D)								
D_3	59.50	6.19	14.21[a]	59.10	11.81	14.44	6.34	6.60
25-OHD_3	60.32	5.94	13.28[b]	58.54	12.18	14.48	6.32	6.63
Dilute	60.13	6.10	12.54[b]	59.39	12.11	14.23	6.37	6.60
SEM	0.43	0.24	0.23	0.72	0.47	0.34	0.01	0.01
Interaction (S x C)								
Fresh raw	51.53	5.94	5.98[c]	54.45	15.65	12.33	6.18[c]	6.57[b]
Fresh cooked	69.64	5.77	20.14[a]	64.43	8.80	15.60	6.43[b]	6.58[b]
Frozen raw	51.13	6.30	7.15[b]	54.76	15.17	13.25	6.16[c]	6.52[c]
Frozen cooked	67.64	6.29	20.10[a]	62.39	8.80	16.35	6.60[a]	6.78[a]
SEM	0.49	0.28	0.27	0.83	0.55	0.39	0.01	0.01
Interaction (S x D)								
Fresh D_3	60.18	5.71	13.50[b]	59.89	12.46	13.64	6.31	6.56[b]
Fresh 25-OHD_3	60.79	5.65	12.93[b]	60.23	11.57	14.54	6.27	6.56[b]
Fresh Dilute	60.79	6.20	12.75[b]	59.20	12.22	13.71	6.34	6.59[b]
Frozen D_3	58.83	6.67	14.91[a]	59.30	11.16	15.24	6.37	6.64[ab]
Frozen 25-OHD_3	59.84	6.22	13.62[ab]	56.84	12.80	14.42	6.37	6.69[a]
Frozen Dilute	59.48	5.99	12.33[b]	59.57	12.00	14.75	6.40	6.61[b]
SEM	0.61	0.35	0.33	1.02	0.66	0.47	0.02	0.01
Interaction (C x D)								
Raw D3	51.12	6.51[a]	6.86[c]	53.85	15.88	12.58	6.20[b]	6.56[c]
Raw 25-OHD_3	51.70	5.49[a]	6.75[c]	55.37	14.82	13.30	6.10[c]	6.52[c]
Raw Dilute	51.18	6.37[a]	6.09[c]	54.60	15.53	12.48	6.21[b]	6.55[c]
Cooked D_3	67.90	5.87[a]	21.56[a]	64.34	7.74	16.29	6.47[a]	6.65[b]
Cooked 25-OHD_3	68.93	6.39[a]	19.81[b]	61.71	9.55	15.66	6.54[a]	6.74[a]
Cooked Dilute	69.08	5.82[a]	19.00[b]	64.18	8.69	15.97	6.53[a]	6.65[b]
SEM	0.61	0.35	0.33	1.02	0.66	0.47	0.02	0.01

table 3.3 cont.

Interaction (S x C x D)								
Fresh Raw D_3	51.59	6.13	5.71^{e}	52.60	17.10	11.50	6.23	6.59
Fresh Raw 25-OHD_3	51.80	5.03	6.63^{de}	56.02	14.41	13.37	6.08	6.49
Fresh Raw Dilute	51.60	6.67	5.61^{e}	54.73	15.44	12.11	6.24	6.62
Fresh Cooked D_3	69.16	5.29	21.30^{ab}	65.17	7.83	15.77	6.39	6.54
Fresh Cooked 25-OHD_3	69.78	6.28	19.24^{bc}	64.44	8.74	15.72	6.46	6.64
Fresh Cooked Dilute	69.97	5.73	19.90^{abc}	63.68	9.00	15.30	6.45	6.56
Frozen Raw D_3	51.03	6.90	8.00^{d}	55.09	14.66	13.66	6.18	6.53
Frozen Raw 25-OHD_3	51.60	5.95	6.87^{de}	54.71	15.22	13.23	6.12	6.55
Frozen Raw Dilute	50.76	6.06	6.57^{de}	54.48	15.63	12.85	6.18	6.47
Frozen Cooked D_3	66.64	6.44	21.82^{a}	63.52	7.65	16.81	6.56	6.75
Frozen Cooked 25-OHD_3	68.08	6.49	20.38^{ab}	58.97	10.37	15.60	6.62	6.84
Frozen Cooked Dilute	68.20	5.92	18.10^{c}	64.67	8.38	16.64	6.61	6.74
SEM	0.86	0.49	0.47	1.44	0.94	0.67	0.03	0.02

Source of variation	*P* –value							
	L*M	a*M	b*M	L*MNB	a*MNB	b*MNB	pHM	pHMNB
S	0.018	0.1235	0.0416	0.3027	0.8548	0.0345	0.0001	<0.0001
C	<0.0001	0.7347	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
D	0.3878	0.7681	<0.0001	0.691	0.8403	0.8528	0.0717	0.2427
S x C	0.1128	0.7793	0.0281	0.1615	0.489	0.8294	<0.0001	<0.0001
S x D	0.937	0.2311	0.0257	0.1007	0.1707	0.1851	0.4718	0.011
C x D	0.6638	0.0518	0.014	0.1035	0.103	0.3181	0.0006	0.0012
S x C x D	0.8442	0.5354	0.0194	0.3166	0.5206	0.6623	0.4634	0.0589

[a, b, c, d, e] Means within a column or within storage, cooking, diet or their interactions with no common superscript are significantly different ($P < 0.05$).
L*M, a*M, b*M, pHM refers to the lightness, redness, yellowness and pH of thigh meat respectively at the meat surface away from the bone.
L*MNB, a*MNB, b*MNB, pHM refers to the lightness, redness, yellowness and pH of thigh meat respectively at the meat adjoining the thigh bone.
n=120

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CHAPTER 5. PROJECT SUMMARY AND IMPLICATIONS

The success of any meat product depends on consumer acceptance. The eating habits of consumers change continuously with the change in their living standards. Globally the demand for broiler meat has increased as compared to other meat types like beef and pork (Agriculture and Agri-food Canada, 2009). Different factors contribute to this rise like convenience to cook, lower fat content and low price relative to other meat products (Haley, 2001; Magdelaine, 2008). Various parameters affect meat quality, but colour remains one of the most important deciding factors for consumer acceptance of meat products (Mugler and Cunningham, 1972). In North America, consumers prefer white meat like broiler breasts as compared to leg (thigh) meat which is considered as dark (Haley, 2001). The presence of more pigments and increased supply of blood vessels makes it darker in colour (Lyon et al., 1976). Moreover, the presence of bones makes it susceptible to changes in meat colour. The bones contain pigment and its leakage from inside the bones to surface and further spillage onto surrounding meat may not be desirable for visual perception. Further freezing and cooking of meat cause denaturation of these pigments, which are protein in nature (Koonz and Ramsbottom, 1947, Smith and Northcott, 2004). It changes to various forms after its denaturation and can lead to formation of darker colour. Myoglobin is the major pigment protein in the muscles (Lawrie, 1991). After the death of birds the pigment starts denaturing as a result of its oxidation, changing its forms and hence the colour of meat (Brantley et al., 1993). This oxidation or denaturation may be enhanced by freezing or cooking (Van Laack, 1999; Smith and Northcott, 2004).

The broiler industry has undergone a huge change with regard to the growth rate of the birds. Broilers are reared today much faster as compared to the birds from 1960's. This has been done for more profits within a shorter time. The breast muscle yield has also increased as a result of this selection (Lilburn, 1994). However the bones have suffered a lot due to this as they become weak as bone growth is slow and cannot keep up with the increasing growth rate of birds (Reiland et al., 1978; Hulan et al., 1980). Calcium (Ca) and Phosphorus (P) are the two main minerals required for proper bone density. The balance of these minerals is governed by vitamin D, which is present in the body itself or given mainly as a feed ingredient in the diet of birds (Holick et al., 1980; Norman, 1998; Rath et al., 2000). After entering the body it forms various metabolites and one of them is 25-hydroxyvitamin D_3 (25-OHD_3) (Holick, 2007).

The first experiment was designed to study the effect of freezing and cooking on meat parameters and colour of bone-in broiler chicken thighs. Comparisons were also drawn with the fresh uncooked thighs. Cooking was done in a hot air oven at 180^{o}C until the core meat temperature reached 80^{o}C and freezing was done at -20^{o}C for 4 weeks. pH, colour, heme iron, non-heme iron, total heme pigments, total myoglobin, deoxymyoglobin (Mb), oxymyoglobin (MbO_2) and metmyoglobin (MetMb) were determined in all the individual thighs. The study revealed that there was a pigment leakage from the bone marrow onto the bone surface indicated by high heme iron content after freezing or cooking. The study also showed that there was heme pigment denaturation indicated by an increase in non-heme iron content of thighs. The decrease in total heme pigments

and total myoglobin after freezing or cooking also confirmed the denaturation of proteins as their extractability was less. The high MetMb content than the other two forms in the broiler thighs indicated the increased oxidation of pigments after their exposure to oxygen and resulting denaturation *post mortem*. The pH of thighs was higher after freezing or cooking the samples. The study concluded that although there was a leakage of pigments as well as pigment oxidation and denaturation, the colour of thighs did not become dark under the applied conditions of freezing or cooking. However, the meat colour may be affected by different freezing and cooking methods. Hence effect of different freezing and cooking methods need to be studied to understand the meat colour characteristics.

The second experiment was designed to study the effect of diet on bone growth, meat characteristics and colour of broiler thighs. The freezing and cooking effects were also studied along with the diet effect. Comparisons were also made with the fresh uncooked thighs. Male Ross 308 broiler chickens (n=320) were reared for 40 days. Birds were fed either a standard, nutritionally-complete diet with vitamin D_3 as the supplemental vitamin D source (Control), a diet containing an equivalent amount of vitamin D activity (69 μg/kg feed) as 25-hydroxyvitamin D_3 (25-OHD_3) or a reduced nutrient density diet (Reduced; Control diet diluted with 25% wheat bran). pH, colour, heme iron, non-heme iron, total myoglobin and total heme pigments were measured in fresh raw, fresh cooked, frozen raw and frozen cooked bone-in broiler thighs (n=4 per diet). Cooking (180^{o}C oven temperature, 80^{o}C core meat temperature), freezing (-20^{o}C for 4 weeks), diet and their interactions were studied. Heme iron and non-heme

iron contents were lowest in fresh uncooked samples from birds fed with 25-OHD_3 diet as compared to control and diluted diet. This may be due to less porosity leading to less pigment leakage in birds supplemented with 25-OHD_3. However, after freezing or cooking significant differences were not seen. Both total pigment and total myoglobin decreased after freezing or cooking the meat in all diets. This is due to pigment protein breakdown during the treatments. Freezing favored myoglobin oxidation indicated by a decrease in MbO_2 and an increase in MetMb content with no diet effect. The study revealed that 25-OHD_3 supplementation may be useful in improving bone quality but the dietary treatments did not have any significant darkening effect on colour of meat near the bone under the applied conditions of freezing and cooking.

The study is useful for better understanding of factors related to colour of meat and bones in bone-in broiler thighs. Freezing and cooking of meat has an effect on its quality and appearance. The colour also depends on the addition of various ingredients in the meat due to their reaction with meat pigments. The handling of meat at processing plants can also be manipulated to avoid cuts in meat and bones as much as possible for less pigment leakage as blood is sometimes not desirable from consumer point of view. Packaging techniques and materials can be modified like providing the appropriate oxygen environment to maintain the desirable meat colour as oxidation of meat pigments changes the meat colour. Different dietary modifications can be made with regard to vitamin D in the feed as it is directly responsible for bone mineralization which affects the overall performance in birds. Diets with 25-OHD_3 supplementation can be made

more in practice as earlier studies with this metabolite and the current study indicates that it may be more effective in increasing the bone mineralization of birds then its parent compound vitamin D.

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