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Characterization of fresh muscle pigments in channel catfish and their influence by chilling stress

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CHARACTERIZATION OF FRESH MUSCLE PIGMENTS IN CHANNEL CATFISH AND THEIR INFLUENCE BY CHILLING STRESS

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Color and color components are important quality attributes for fresh channel catfish muscle. The objective of the present research was to obtain inside understanding regarding color distribution, color component stability and the pre-slaughter conditions influence on color and color components and other quality attributes. Catfish fillets were rendered to be paler and redder on iced storage; and paler, less red and less yellow by ice-water chilling process. Visual classified "reddish" catfish fillets had higher heme-protein content than "white" fillets and these pigments were derived mainly from hemoglobin. Pre-slaughter live fish chilling in ice water and bleeding at slaughter also slightly affected muscle color and total heme-protein content, but they were not the main factors for the "reddish" color on catfish muscle. Catfish hemoglobin autoxidation rate was similar at room and at refrigeration temperature. Myoglobin gene and hemoglobin beta gene were cloned and expressed in *Eschericia coli* cells in insoluble form. The myoglobin gene had an open reading frame of 444 nucleotides and translated into 147 amino acids with

molecular weight of 16,909 and pI of 9.43. The myoglobin gene in vivo expression in white muscle was not affected by pre-slaughter chilling. Pre-slaughter chilling is a stressor to channel catfish and shortens the pre-rigor time postmortem; thus, it is not recommended in catfish processing.

Key words: catfish, color, chilling

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TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS ii

LIST OF TABLES vi

LIST OF FIGURES vii

CHAPTER

I. INTRODUCTION 1

II. LITERATURE REVIEW 8

1. Fresh fish color 8
2. Myoglobin and hemoglobin in fish 10
3. Myoglobin gene and its expression in muscle 14
4. Myoglobin and hemoglobin concentrations in fish 16
5. Stress in fish 18
6. Pre-slaughter temperature 24
7. Fish muscle pH 25
8. Rigor mortis in fish 27
9. Stress, plasma cortisol and fish quality 28

III. EXPERIMENT I: EFFECTS OF POSTMORTEM CHILLING, STORAGE TIME ON CHANNEL CATFISH FRESH FILLET COLOR AND THE RELATIONSHIP OF COLOR AND TOTAL HEME-PROTEIN CONTENT 35

1. Introduction 35
2. Materials and Methods 37
   (1). Establishment of color category of fresh fillets by visual rating and instrumental measurement 37
   (2). Postmortem chilling effect on color of fillets on the bone-side 38
   (3). Total heme-protein content in “white” and “reddish” fillets 39
   (4). Iced storage effect on color of fillets on the bone-side 40
   (5). Experimental design and statistical analysis 40

3. Results and Discussion 41
(1). Establishment of color category of fillets by visual rating and instrumental measurement.....41
(2). Postmortem chilling effect on color of fillets on the bone-side 43
(3). Total heme-protein content in "white" and "reddish" fillets.....47
(4). Iced storage effect on color of fillets on the bone-side 48
4. Conclusions.....50

IV. EXPERIMENT II: CHARACTERIZATION OF CHANNEL CATFISH HEMOGLOBIN AND MYOGLOBIN.....66

1. Introduction.....66
2. Materials and Methods.....69
(1). Hemoglobin preparation and its autoxidation 69
(2). Hemoglobin beta-subunit gene and myoglobin gene cloning from cDNA library.....70
(3). SDS-PAGE confirmation and purification of expressed hemoglobin beta-subunit and myoglobin.....72
(4). Sequencing of myoglobin cDNA 73
(5). Antibody production 74
(6). Extraction of myoglobin and hemoglobin from "white" and "reddish" fillets and separation by HPLC.....74
(7). Native- and SDS-polyacrylamide electrophoresis 75
(8). Immunoblotting of myoglobin and hemoglobin 76
3. Results and Discussion 76
(1). Hemoglobin autoxidation.....76
(2). Cloning and expression of hemoglobin beta-subunit gene and myoglobin gene in *E. coli* BL21 (DE3) strain 78
(3). Myoglobin gene sequence.....80
(4). Myoglobin and hemoglobin separation from "white" and "reddish" fillets by HPLC and confirmed by SDS-PAGE and immunoblotting.....81
4. Conclusions.....82

V. EXPERIMENT III: EFFECT OF PRE-SLAUGHTER CHILLING AND BLEEDING ON PLASMA CORTISOL LEVEL, WHITE MUSCLE MYOGLOBIN GENE EXPRESSION, RIGOR DEVELOPMENT AND MUSCLE COLOR OF CHANNEL CATFISH 93

1. Introduction.....93
2. Materials and Methods.....96
(1). Live fish pre-slaughter chilling stress and bleeding protocol 96
(2). Rigor index measurement.....97
(3). pH measurement.....98
(4). Color measurement and incident of "reddish" fillet determination 98

(5). Total heme-protein determination 99
(6). Plasma cortisol determination 99
(7). Total RNA extraction and conversion to first-strand cDNA 99
(8). Quantitative RT-PCR for expression of myoglobin gene in white muscle 100
(9). Experimental design and statistical analysis 101
3. Results and Discussion 101
(1). Pre-slaughter chilling effect on blood plasma cortisol level 101
(2). Pre-slaughter chilling effect on expression of myoglobin gene in white muscle 104
(3). Pre-slaughter chilling effect on rigor development of iced stored catfish 106
(4). Pre-slaughter chilling effect on muscle total heme-protein content 109
(5). Pre-slaughter chilling effect on muscle pH 110
(6). Pre-slaughter chilling effect on color of fillets on the bone-side 111
(7). Bleeding effect on muscle total heme-protein content 114
(8). Bleeding effect on muscle pH and color of fillets on the bone-side 115
4. Conclusions 115

VI. SUMMARY AND CONCLUSIONS 125

REFERENCES 127

LIST OF TABLES

TABLE Page

3.1. Visual color rating scale and Hunter color values of fresh catfish fillets on the bone-side 51

3.2. Visual color rating scale and CIE L*, a*, b* color values of fresh catfish fillets on the bone-side 52

3.3. The CIE L*, a*, b* values of fresh catfish fillets before and after postmortem chilling process....53

3.4. CIE hue*, SI*, WI* values of fresh catfish fillets before and after postmortem chilling process 54

3.5. Distribution of CIE L*, a*, b* values of fresh catfish fillets before and after postmortem chilling process....55

3.6. Hunter color values and total heme-protein content in "white" and "reddish" catfish fillets....56

LIST OF FIGURES

FIGURE Page

3.1 Linear correlation between visual rating scores and Hunter a values of fresh catfish fillets on the bone-side 57

3.2 Linear correlation between visual rating scores and Hunter L values of fresh catfish fillets on the bone-side 58

3.3 Linear correlation between visual rating scores and Hunter hue angle values of fresh catfish fillets on the bone-side 59

3.4 Linear correlation between visual rating scores and whiteness values of fresh catfish fillets on the bone-side 60

3.5 Hunter L values of fresh catfish fillets on the bone-side changed during iced storage 61

3.6 Hunter a values of fresh catfish fillets on the bone-side changed during iced storage 62

3.7 Hunter b values of fresh catfish fillets on the bone-side changed during iced storage 63

3.8 Hunter hue angle values of fresh catfish fillets on the bone-side changed during iced storage 64

3.9 Hunter SI values of fresh catfish fillets on the bone-side changed during iced storage 65

4.1 Autoxidation of hemoglobin at room temperature (25^{o}C) and refrigeration temperature (4^{o}C) at pH 6.3 84

4.2 1.5% Agarose gel electrophoresis of PCR product of channel catfish myoglobin cDNA (with six-histidine tag sequence) from *Taq* amplified PCR product of head kidney cDNA library using *Pfu* polymerase 85

4.3 1.5% Agarose gel electrophoresis of PCR product of channel catfish hemoglobin beta cDNA (with six-histidine tag sequence) from head kidney cDNA library using Taq polymerase.....86

4.4 1.5% Agarose gel electrophoresis of *Ned*I and *Hind*III digested recombinant DNA of pET-29a (+) with either myoglobin or hemoglobin beta cDNA insert isolated from transformed *E. coli* DHα5, for successful cloning confirmation 87

4.5 The expressions of channel catfish hemoglobin beta and myoglobin in *E. coli* BL21 (DE3) with either hemoglobin beta gene or myoglobin gene insert were confirmed by SDS-PAGE 88

4.6 SDS-PAGE (15%) of expressed channel catfish hemoglobin beta-subunit before and after zinc ion chelated affinity chromatography with 250 mM imidazole elution.....89

4.7 cDNA sequence and deduced amino acid sequence of channel catfish myoglobin.....90

4.8 Channel catfish myoglobin and hemoglobin in SDS-PAGE from "white" and "reddish" fillets extracts by silver staining.....91

4.9 Immumoblotting of myoglobin and hemoglobin in "white" and "reddish" fillet extracts by polyclonal antibodies against short peptides selected from hemoglobin beta and myoglobin.....92

5.1 Pre-slaughter chilling effect on rigor development of ice-stored catfish 117

5.2 Pre-slaughter chilling effect on catfish muscle total heme-protein content 118

5.3 Picture of pre-slaughter chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter 119

5.4 Hunter L values of pre-slaughter chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter 120

5.5 Hunter a values of pre-slaughter chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter 121

5.6 Hunter b values of pre-slaughter live-chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter 122

5.7 Hunter hue values of pre-slaughter chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter 123

5.8 Hunter SI values of pre-slaughter live-chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter 124

CHAPTER I

INTRODUCTION

Channel catfish (*Ictalurus punctatus*) is a fresh water fish in the Ictaluridae family. Its natural habitat is comprised in the central United States of America, extending from the north to the south (Jackson 2004). Fast growth rate, high feed turnover efficiency, mild flavor, combined with other social-economic factors contributed to this being the most predominate aquaculture species in the USA (Hargreaves and Tucker 2004). The production and processing of channel catfish are concentrated in two main areas in the southeast United States with similar climate conditions: the Mississippi river alluvial valley, centralizing from the Mississippi Delta extending to the surrounding areas of Mississippi, Arkansas, and Louisiana; and a west-central Alabama, and east-central Mississippi area (Hargreaves and Tucker 2004). The duration of temperatures in these areas is above 20^{o}C for 200 days and above 25^{o}C for 150 days annually. Channel catfish is considered to be a warm-water fish. Processed channel catfish in the USA reached 225,000 metric tons in 2007 (USDA NASS 2008).

Despite the success of channel catfish industry, it is currently facing many challenges including market competition from basa (*Pangasius bocourti*) that is imported from Vietnam and channel catfish that is imported from China (USDA FAS 2008). Maintenance or even expansion of the markets is the predominate concern of the catfish

industry in the USA. Maintaining a long-term competition requires producing a highly acceptable product. Genetics and nutrition are the most important factors that influence product quality (Pottinger 2000). In addition, cultural environment, handling practices of production and processing also play very important roles in catfish quality.

Appearance is the quality attribute that consumers use to choose the foods that they will purchase (Faustman and Cassens 1990). The color of fresh meat is mainly determined by the amount and the oxidative states of the heme proteins, hemoglobin and myoglobin (Mancini and Hunt 2005). Myoglobin is a monomeric protein with a porphyrin ring. Hemoglobin is a tetrameric protein with each one of the subunits associated with a porphyrin ring. Each porphyrin has an iron atom that coordinates with four pyrrole nitrogen atoms in almost the same plane. The fifth coordination of iron perpendicular to the four-nitrogenous coordination plane is a nitrogen in the histidine residue. The sixth coordination of iron opposite to the fifth coordination is free to reversibly bind oxygen or other small molecules, thus forming the derivatives of myoglobin and hemoglobin. This binding of small ligands to the sixth position is greatly influenced by the valence state of the porphyrin iron.

The visible wavelength absorption properties or the color of the myoglobin and hemoglobin is determined by the binding of a ligand to the iron atom, and the interchangeable states of ferrous and ferric iron in the porphyrin ring. Deoxygenated myoglobin and hemoglobin, where, the sixth coordination position of the porphyrin iron is free from binding, as seen immediately after fresh cutting or vacuum-packaged meat, appear purple (Mancini and Hunt 2005). On the other hand, oxygenated myoglobin and

hemoglobin, with oxygen binding in their porphyrin irons, show a bright red color. This is the primary color characteristic of fresh meat and is contributed by the oxygenation of myoglobin and/or hemoglobin when meat is exposed to oxygen. In metmyoglobin and methemoglobin, the iron of porphyrin is in the ferric state, as commonly seen in meat passed its shelf life, is brown. The tendency for the ferrous iron to oxidize to ferric iron in the presence of oxygen is termed autoxidation. Molecular oxygen binds to myoglobin and hemoglobin only when the iron in the porphyrin ring is in the ferrous state. In the ferric state, the porphyrin iron loses oxygen binding capability and myoglobin and hemoglobin are no longer oxygen carriers. The reducing enzyme system in living cells limits this autoxidation by utilizing nicotinamide adenine dinucleotide (NADH) as a cofactor to reduce ferric iron to ferrous iron, thus, maintaining the oxygen binding capability of myoglobin and hemoglobin (Mancini and Hunt 2005). The loss of this nucleotide in postmortem muscle results in loss of metmyoglobin and methemoglobin reduction ability. Thus, meat gradually and permanently becomes brown (Mancini and Hunt 2005).

Apart from color and color changes, myoglobin and hemoglobin are also the potent promoters of lipid oxidation (Richards and Hultin 2002), thereby producing off-odor and off-flavor that negatively affect muscle meat quality (Kanner 1994, Richards and others 2005). In addition of lipid oxidation, the production of free radicals and hydrogen peroxide further accelerates the autoxidation of myoglobin and hemoglobin (Grunwald and Richards 2006). Deoxy forms of myoglobin and hemoglobin are stronger promoters of lipid oxidation than oxy forms due to their instability and more open configurations. This configuration renders the iron in the porphyrin ring more accessible

to oxidants (Mancini and Hunt 2005, Richards and others 2002, Richards and Hultin 2002). Thus, the distribution and stability of various derivatives of myoglobin and hemoglobin on the meat surface must be defined when color and lipid stability are studied. Furthermore, even though myoglobin and hemoglobin are similar proteins that have similar functions, they have marked differences, as well as coming from different sources. They must be differentiated and quantified separately when the causation of a color defect is analyzed. In addition, hemoglobin is a stronger promoter of lipid oxidation than myoglobin (Richards and Hultin 2002). Thus, the distribution of hemoglobin and myoglobin and their derivatives must be quantified and differentiated to elucidate their role in muscle color and impact on quality.

Numerous researchers have studied the distribution of hemoglobin and myoglobin in chicken, beef and pork muscle. Despite the fact that it was generally recognized that the major pigment in these meats is myoglobin (Fleming and others 1960, Kranen and others 1999, Han and others 1994, O'Brien and others 1992), the relative ratio of myoglobin and hemoglobin in these muscles was highly variable. Studies on the roles of myoglobin and hemoglobin in fish muscle present contradicting results. For example, Richards and Hultin (2002) reported that almost all of the heme pigments in either bled or unbled trout white muscle were hemoglobin. In addition, the myoglobin concentrations ranged from 35 to 44% of total heme pigments in dark muscle. However, O'Brien and others (1992) concluded that hemoglobin was only 19% of the heme pigments in rainbow trout skeletal muscle with the remains being myoglobin. This variation might be due to the incomplete extraction of heme-pigments from the muscle (Warriss 1979, Kranen and

others 1999), species or individual sample variation or dissociation of hemoglobin into subunits during extraction (Richards and others 2005).

Pre-slaughter handling has great impact on fish muscle color. Capture, transport, and killing may stress the fish and affect muscle quality including muscle color due to the fact that fish have no time to recover from the acute stress (Pottinger 2000). These factors affect muscle color by affecting the reserved glycogen and ATP levels, thus affecting the muscle pH and rigor development. The pH and rigor state have been found to influence the light scattering properties thus affecting muscle color (Robb 2000). Harvesting method also affects muscle activity and concentration of ATP in the muscle (Sigholt and others 1997). Moreover, killing affects the blood removal from muscle vessels and capillaries.

Pre-slaughter live-chilling in ice water is an alternative method for rendering fish unconscious for killing and further handling (Olsen and others 2006). This method has been adopted in the processing of salmon in Norway (Kiessling and others 2006), rainbow trout in Britain, and small-sized fish species in Mediterranean countries (Poli and others 2005). The temperature of live fish in warm water drops rapidly in ice water, thus, inhibiting the metabolism and activity (Poli and others 2005). Fish are asphyxiated and finally die of anoxia in 20 to 200 min depending on species (Poli and others 2005). Because fish die in a prolonged time, some researchers argue that it is not a humane way to kill fish (Kestin and others 1991, Wedermeyer 1997, Poli and others 2005). The effects of pre-slaughter chilling on the stress indicators and subsequent fish muscle quality are also controversial. Poli and others (2005) concluded that pre-slaughter, live-chilling

resulted in lower plasma glucose, muscle lactate levels and a prolonged pre-rigor state and shelf life of fish. In contrast, Kiessling and others (2006) reported that Atlantic salmon transferred from 12^{o}C to 4^{o}C for 2 h before slaughter showed more rapid onset of rigor. On the one hand, chilling minimizes the metabolic activity and microbial growth (post-slaughter); on the other hand, fish are stressed and exercised before losing conscious, thus speeding down the reserve energy. It seems there has not been conclusive evidence of the effect of pre-slaughter chilling on fish quality.

Fish have less blood volume than mammals (Smith 1966) and 60-70% of the blood is directed to muscle. This volume increases by 20% when fish are stressed (Thorarensen and others 1993, Farell and others 2001, Schultz and others 1999). Incomplete blood removal from fish results in blood spots on fillets or within fillets since residual blood is retained in the blood vessels (Olsen and others 2006). Low temperature postpones blood coagulation thus increasing the blood fluidity time in Atlantic salmon (Olsen and others 2006), whereas it induces stress that increases the coagulation rate of the blood in rainbow trout (Ruis and Bayne 1997).

Many studies have been conducted on antimicrobial and processing effects of catfish including color changes of fresh catfish muscle during the storage (Marshall and Kim 1996, Bal'a and Marshall 1998, Kim and others 2000, Silva and White 1994, Reed and others 1983, Przybylski and others 1989, Heaton and others 1972). However, there is no report concerning postmortem chilling effects on the color distribution of fresh catfish fillets. There is also no report regarding the role and distribution of myoglobin and hemoglobin in channel catfish muscle. A comparative study on the channel catfish

hemoglobin beta gene has been reported (Yeh and others 2006), but myoglobin gene characterization, cloning and expression have not been reported.

The objectives of this research were: 1). to investigate differences in color values of fresh catfish fillets affected by postmortem chilling and their changes during iced storage; 2) to quantify the total heme-protein in “white” and “reddish” colored catfish fillets; 3). to characterize channel catfish myoglobin and hemoglobin and their genes; and 4): to investigate bleeding and pre-slaughter acute chilling stress on channel catfish rigor, muscle color and muscle myoglobin gene expression.

CHAPTER II

LITERATURE REVIEW

1. Fresh fish color

Appearance, texture and flavor are the three major sensory properties that govern consumers' decisions regarding purchasing food product (Faustman and Cassens 1990). Among these traits, appearance is the crucial because it is the first trait that is perceived by the consumer and, if it is not satisfied, the other two properties may not have a chance to be considered (Francis and Clydesdale 1975). Color is a very important appearance attribute and is determined by the types and relative proportion of pigment components and their interactions. Two heme-proteins: hemoglobin and myoglobin play major roles in fresh meat color. Minor roles are carried out by cytochrome C and carotenoids. Cytochrome C only accounts for about 1- 4% of the total pigment present in fresh meat (Fleming and others 1991, Pikul and others 1986). Carotenoids play a major role in some fish species such as salmon and tuna. Between 48.5 and 99.8% of the flesh color in wild chinook salmon is contributed by astaxanthin (Bird and Savage 1990). Carotenoid concentrations in these species depend on the maturation stage (Torrissen and Naevdal 1988, Reid and others 1993, Love 1997), harvest season (Love 1997), and feed (Malak and others 1975, Love 1997, Torrissen 1989). But these only occur in some special circumstances. For the most part, fresh meat color is determined by myoglobin and

hemoglobin concentrations, oxidation state, and ligands that are bound to their heme groups.

When a beam of light strikes an object's surface, it is separated into three parts at different directions: reflective (scatter), absorptive and transmitted (Francis and Clydesdale 1975). The perception of color from a piece of meat is a physiological phenomenon for interpretation of reflective light and is determined by the spectra of incident light, and the spectra of reflective (scatter) light, absorptive light and transmitted light from meat surface. Meat is an opaque object with little transmitted light (AMSA 1991). The substances on the meat surface selectively absorb light at the wavelength based on their available energy difference (Francis and Clydesdale 1975). Myoglobin and hemoglobin selectively absorb most of the green light thus appearing red due to the reflectance of the red light. The physical properties of the meat surface determine the reflection of incident light (AMSA 1991). The relative proportion of myofibrils and sarcoplasm determine the degree of light scatter thus the reflective index (Offer and Trinick 1983). Shrinkage of myofibrils in rigor mortis increases light scattering by the sarcoplasm since it has a higher light scattering property than myofibrils (Offer and Trinick 1983). In dark, firm, and dry (DFD) meat, shrinkage of myofibrils is less obvious due to smaller pH decline, resulting in more transparent color than normal meat (Offer and Trinick 1983). In pale, soft, and exudative (PSE) meat, the shrinkage of myofibrils is severe and even the myofibrils expel water, thus the scatter of light is maximized (Offer and Trinick 1983). In addition, protein denaturation also increases light scatter (Offer and Trinick 1983). Thus, the color of meat in an observer's mind is the result of the

interpretation of the spectra of reflective light signal and his experience. This perception also depends on the illuminant and the background (AMSA 1991). However, less variation occurs among normal individuals in color vision under similar conditions (Francis and Clydesdale 1975).

2. Myoglobin and hemoglobin in fish

Myoglobin and hemoglobin are similar proteins both in spatial structure and physiological functions. Both proteins can bind or release small ligands such as oxygen or carbon dioxide, and have important physiological roles in biology. Hemoglobin is the major protein in blood and accounts for more than 90% of the total proteins in red blood cells (erythrocytes) (Antonini and Brunori 1971). Its main function is to transport oxygen from the lung in mammals or similar organ/tissue in other animals to any part of the body that requires it for respiration as the final electron acceptor, and carry carbon dioxide, the waste of respiration in the counter direction through the blood vessels and capillaries. Red blood cells are circulated in the cardiovascular system, in which hemoglobin releases oxygen and accepts carbon dioxide in the small blood capillaries that surround other cell types. Hemoglobin is an extracellular protein in muscle cells. Myoglobin is an intracellular protein that is mainly expressed in muscle cells within skeletal and heart muscle. Other organs and tissues such as liver, brain, kidney and gill in fish also express this hemoprotein or its isoforms upon the induction of hypoxia (Fraser and others 2006). Myoglobin functions to facilitate the diffusion of oxygen and carbon dioxide between the cell and mitochondrial membranes, where the electron transport chains are located. It also functions to store oxygen and buffer oxygen levels, thus maintaining a constant oxygen

level for respiration. In addition, myoglobin has other physiological functions not related to oxygen binding, such as protecting cell respiration from nitric oxide inhibition (Wunderlich and others 2003), and scavenging reactive oxygen species (ROS) (Brunori 2001).

Both myoglobin and hemoglobin are globular proteins with iron containing prosthetic groups: protoporphyrin (simply referred to as porphyrin or heme in the rest of this manuscript), which is a chromophore group (Stryer 1981). Myoglobin is a monomer with a porphyrin ring embedded in its hydrophobic packet; hemoglobin has four subunits with each one of them having one porphyrin ring inside its hydrophobic packet. Binding of small ligands occurs in porphyrin moiety, specifically, the iron atom in the porphyrin ring, but the globular protein moiety acts to stabilize the porphyrin ring and enhance its ligand transporting efficiency.

Each porphyrin ring has an iron atom that centrally coordinates to four pyrrole nitrogen atoms in almost the same plane. The fifth coordination of the iron atom that is perpendicular to the pyrrole nitrogenous plane is a nitrogen atom from the proximal histidine residue 93 in myoglobin apoprotein. The sixth coordination of the iron atom that is opposite to the fifth coordination is free to reversibly bind small ligands when the iron is in ferrous state (Fe^{2+}). When the iron atom is in ferric state (Fe^{3+}), the binding of molecular oxygen is limited. The binding of the ligand and the conversion of the ferrous state to the ferric state of iron in the porphyrin ring are influenced by the sixth coordination position proximal histidine 64 (distal histidine) of the globular protein because it affects the spatial structure (Mancini and Hunt 2005). These histidine residues

are highly conserved in all species that have functional myoglobin and hemoglobin (Suzuki and Imai 1998).

The wavelength absorption properties, thus the visible color of myoglobin and hemoglobin are determined by the ligands that are bound to the iron atom, and the redox states of the iron atom in the porphyrin ring. Deoxygenated myoglobin and hemoglobin are those in which their iron atoms are in the ferrous state and free from binding. Deoxygenated myoglobin and hemoglobin appear purple red in color, as typically seen in vacuum packaged or immediately cut fresh meat products (Mancini and Hunt 2005). Oxygenated myoglobin and hemoglobin bind to molecular oxygen at the iron in the porphyrin ring and are indicative of a bright red color. That fresh cut beef and pork change from purple to bright red color within minutes after cutting is the result of the oxygenation of deoxymyoglobin or deoxyhemoglobin (Ledward 1992). This reaction is reversible when the oxygen level is reduced. The oxygen partial pressure in the packaging environment determines the distributions of these two forms of myoglobin and hemoglobin on meat surfaces and thus affects the meat color. Metmyoglobin and methemoglobin, in which the iron in the porphyrin ring is in the ferric state, and binds to water, is revealed as a brown color. There is a tendency for ferrous iron to gradually oxidize to ferric iron in the presence of oxygen, resulting in a loss of oxygen binding capability for myoglobin and hemoglobin. The autoxidation of myoglobin and hemoglobin and the accumulation of metmyoglobin and methemoglobin on meat surfaces result in discoloration on the meat surface, thus, downgrading or totally losing the economical value of meat products. It was estimated that the United State beef industry

loses one billion US dollars annually due to meat discoloration (Mancini and Hunt 2005). Factors such as temperature, pH and meat reducing activity, and microbial growth influence the rate of myoglobin or hemoglobin autoxidation (Mancini and Hunt 2005). Autoxidation reactions of myoglobin and hemoglobin also occur in living biological systems; however, the reducing enzyme system in living cells limits this autoxidation. Reducing enzyme systems utilize NADH as a cofactor to reduce ferric iron to ferrous iron. This nucleotide maintains the oxygen binding capability of myoglobin and hemoglobin. The loss of this nucleotide in postmortem muscle results in the loss of metmyoglobin and methemoglobin reduction ability, which causes meat to gradually and permanently become brown.

Hemoglobin is expressed in red blood cells to shuttle oxygen and carbon dioxide for aerobic respiration. Hemoglobin presented in the muscle tissue is from the residual blood. The number of blood capillaries in muscle tissue depends on species and muscle type. White muscle tissue has less blood capillaries than dark muscle tissue (Soldatov 2006). Compared to mammalian hemoglobin, fish hemoglobin is made up of distinct cathodal and anodal components that differ in electrophoretic mobility and oxygen binding ability, which is an adaptive mechanism for diverse aquatic environments (Harrington 1986, Weber and others 2000). Anodal hemoglobin constitutes all the hemoglobin in king salmon fingerling (Harrington 1986) and 65% in adult trout hemoglobin (Richards and others 2005). The oxygen affinity of anodal hemoglobin is affected by pH and exhibits the Bohr Effect as normally found in mammalian hemoglobin and an extended Root Effect (a lower oxygen affinity at pH 6.3) that is

unique in fish anodal hemoglobin (Richards and others 2005, Harrington 1986). Oxygen binding affinity is also influenced by temperature and organic phosphate (Harrington 1986). Cathodal hemoglobin is present in adult king salmon and its oxygen affinity is independent of pH and organic phosphate level (Harrington 1986).

3. Myoglobin gene and its expression in muscle

In 1985, Varadarajan and others (1985) isolated a one-kilo base pair cDNA fragment from the human muscle cDNA library, cloned it into a phage λ gene, and expressed it in *Escherichia coli* cells as a fusion protein. After digestion of the fusion peptide with trypsin and reconstitution with heme, myoglobin had indistinguishable properties from isolated wild type myoglobin in spectrum absorption and amino acid sequence. The gene has 80 bp in the 5' and 530 bp in the 3' untranslated regions. The expressed apomyoglobin has a molecular weight of 17,000 Daltons. Lurman and others (2007) isolated a full length myoglobin cDNA from Atlantic cod, which contained 625 bp and coded for a 146 amino acid polypeptide.

It is well known that myoglobin is expressed in the heart and skeletal muscle of vertebrates to store oxygen and facilitate oxygen diffusion from erythrocytes to muscle cell mitochondria and buffer oxygen level. However, this theory was challenged by an experiment conducted by Garry and coworkers (1998) in which they knocked out the myoglobin gene in mice and determined that mice without myoglobin exhibited a normal exercise capacity and a normal ventilatory response in hypoxia. Some ice fish species from the Antarctic also have no functional myoglobin at all (Moylan and Sidell 2000). Francis and Clydesdale (1975) concluded that the quantity of myoglobin is related to

species, muscle tissue, activity, availability of blood and oxygen and animal age. Tissue with higher oxygen demand, such as the heart, express more myoglobin; whereas, highly efficient blood supply tissue such as bird wing muscle, expresses less myoglobin than other skeletal muscle since the oxygen supply is satisfied by hemoglobin (Francis and Clydesdale 1975). Myoglobin gene expression is associated with metabolic rate and oxygen demand (Lurman and others 2007, Wittenberg and Wittenberg 2007, Milliken 1939). A high metabolic rate requires an elevated myoglobin expression, along with the mitochondrial density and volume (Kanatous and others 1999, Lurman and others 2007). Increased myoglobin expression is also associated with elevated enzyme levels in the citric acid cycle (TCA cycle) such as citrate synthase and cyclooxygenase (Lurman and others 2007, Kanatous and others 1999). Underwood and Williams (1987) compared the expression of myoglobin mRNA between dark and white muscle in rabbit and found a 10-5 fold difference, but upon exercise, the mRNA expression increases 15 fold in both of them. Fraser and others (2006) reported that myoglobin gene expression was induced by hypoxia in heart and skeletal muscles of common carp only when they were acclimated at 30^{o}C, not at 17^{o}C. Gracey and others (2001) concluded that gene expression in hypoxia is tissue specific, reflecting different metabolic roles. Yamashita and others (1996) reported that the ferritin H three isoforms were over-expressed (2-3 fold) on five days cold stress at 4^{o}C, from 22^{o}C in the rainbow trout gonadal fibroblast cell line RTG-2 culture. Since ferritin functions to store iron and regulate iron level, and cold stress increases hemocrite in serum and initiates angiogenesis and erythropoiesis (Fraser and others 2006), an increase in myoglobin gene expression seems necessary to

meet the oxygen transport demand. Lurman and others (2007) acclimated either North Sea Atlantic cod or North East Arctic cod at 10 or 4^{o}C for two months and reported that the myoglobin mRNA and myoglobin in the heart increased 3.7 fold and 2.3 fold as the result of acclimation to 4^{o}C. They explained that these increases were required to meet the high metabolic demand at low temperature and were paralleled to increased mitochondrial capacities, in addition, to protect cells by scavenging the reactive oxygen species and nitric oxide.

Gracey and others (2000) reported that energy required processes such as protein synthesis and locomotion of skeletal muscle of *Gillichthys mirabilis* were shut down immediately at the onset of hypoxia. However, most of the genes for anaerobic ATP production and gluconeogenesis begin to induce in 24 h of hypoxia. In heart muscle, the mRNA levels of enolase and glyceraldehyde-3-phosphate dehydrogenase decreased. These researchers explained that this may be due to the initial levels of these enzymes meeting the requirements of reduced metabolism during hypoxia.

4. Myoglobin and hemoglobin concentrations in fish

Myoglobin is expressed in skeletal muscle to facilitate gas diffusion. The expression of myoglobin in muscle is varied with the different demands of oxygen in mitochondria among species and within species (Lurman and others 2007, Wittenberg and Wittenberg 2007, O'Brien and others 1992, Milliken 1939). Myoglobin content is also positively influenced by metabolic activity (Lurman and others 2007, Milliken 1939), and physical training can increase myoglobin content in the muscles of both humans and animals (Milliken 1939). In addition, living environmental factors such as

hypoxia induces the expression of myoglobin in muscle (O'Brien and others 1992, Fraser and others 2006). Muscle fiber diameter also plays an important role in determining the content of myoglobin, with less myoglobin in bird and fish due to their small muscle fiber diameter and a better diffusion of oxygen due to shorter diffusion distances (O'Brien and others 1992). Thus, the concentrations of myoglobin in fresh meat depend on species, sex, muscle type, development stage, activity and their environments (Hedrick and others 1994). The two types of skeletal muscle, white and dark, differ functionally and structurally and have different requirements for myoglobin and hemoglobin. White muscle has less mitochondria and blood capillaries (Bilinski 1975, Soldatov 2006). Dark muscle is a strip of brownish-red tissue lying beneath the skin of most fish (Love 1997). Dark muscle utilizes aerobic respiration thus requires a large amount of oxygen. Oxygen is provided through the abundant blood vessel and capillaries around these muscle cells (Soldatov 2006). A high amount of myoglobin inside these cells is also required to facilitate the oxygen diffusion within cells. Thus, dark muscle has higher amounts of both myoglobin and hemoglobin than white muscle (Peter and others 1972).

Richards and Hultin (2002) reported that trout muscle had 5.1-17.8 μmol/kg of hemoglobin in unbled fish and 4-13.9 μmol/kg in bled fish; Atlantic mackerel 4.8-8.8 μmol/kg in unbled and 2.7-4.4 μmol/kg in bled fish (Richards and Hultin 2002). Myoglobin was highly variable from fish to fish with either bled or unbled samples (Richards and Hultin 2002). Mackerel dark muscle contained 158.8 ± 21 and 121.8 ± 17 μmol/kg of hemoglobin in unbled and bled fish, respectively. Myoglobin in this muscle averaged 342 and 382.2 μmol/kg of myoglobin in unbled and bled samples, respectively.

Myoglobin accounted for 35% and 44% of the total pigments by weight in unbled sample and bled samples, respectively (Richards and Hultin 2002). Kranen and others (1999) reported that only hemoglobin was detected in chicken breast muscle and was 86% of the total pigments in the thigh. Brown (1962) reported that most of the pigment extracted from tuna dark and white muscle was myoglobin, and Naughton and others (1958) estimated that 95% of the pigment in tuna muscle is myoglobin. Han and others (1994) reported that total pigment concentrations of chicken and beef depended on species and muscle. Chicken drumstick was 0.12 mg/g; thigh was 0.21 mg/g; beef was around 4.2-4.9 mg/g. About 70-87% of the pigment components in beef were myoglobin.

5. Stress in fish

Stress is perceived by animals when they feel that their survival or ability to reproduce is threatened. Animals react to the stressor by eliciting a suite of physiological responses, trying to resist or overcome the stressor to prevent death and maintain their ability to reproduce. Selye (1950) noticed that an animal reacts in a similar manner to the stressors regardless of their sources. He postulated a general adaptation syndrome (GAS) to describe the response of the animal to stress. The syndrome is stressor independent and has three phases: the alarm phase, when the animal perceives the stressor; the resistant phase, when the response is initiated, and the exhausted phase, when the stress is not overcome. However, the adaptive theory was proven incomplete when it was discovered that chronic stress impairs immune system and reproduction ability.

The primary purpose of the stress response is to mobilize all available resources of the animal to overcome the stress and maintain homeostasis. This involves the

reallocation of the energy in the body from non-essential regions such as growth and reproduction to those essential for survival. The mobilization of the resources provides the animal with sufficient immediate energy substrate production, thus, increasing the body's metabolic activity. The stress response is a highly coordinated process and can be divided into three stages based on the rapidity of the response components: the primary response, the secondary response and the tertiary response (Wendelaar Bonga 1997). When animals perceive the stressor, it first triggers the neuroendocrine system to signal the mobilization of the energy resources. Two types of endocrine responses are activated, resulting in a series of hormone cascades (Sumpter 1997). The first endocrine response is the adrenergic response, which is initiated primarily through the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The initiation of the adrenergic response results in an increases in catecholamines including adrenaline and noradrenaline in the blood stream. The catecholamines, secreted from the adrenal gland in higher vertebrates or analog tissues (chromaffin cells) in fish, are transported through the blood stream to the liver, muscle and adipose tissues and impose their effects by regulating the expression of gene products. The catecholamines have widespread physiological functions. For example, they increase the blood output, increase blood oxygen transport capacity, and increase blood glucose and fatty acid levels (Wendelaar Bonga 1997). A second type of endocrine response is the hypothalamic-pituitary-interrenal (HPI) axis response (Sumpter 1997). The activation of the HPI axis culminates in an increase in cortisol in the blood stream. It has been reported that cortisol also participates in the mobilization of protein and fatty acid (Wendelaar Bonga 1997), but their functions are not yet clearly elucidated

(Pottinger 2000). A long-term elevation of cortisol from chronic stress, results in detrimental effects such as immunosuppression, susceptibility to disease, retardation of growth and suppression of reproduction activity (Pickering and Pottinger 1995).

When the stress response subsides, the animal invokes a replenishment process for energy storage to repay the debt incurred during the initial stress response (Mommsen and others 1999), and resumes normality in a few hours to a few days after the stressor is removed (Wang and others 1994), provided the stressors are within the limits. When the stress response is activated, the animal is switched from an anabolic state to a catabolic state (Pottinger 2000). However, if the animal suffered an acute pre-slaughter stress, because there is no sufficient time for the animal to recover from the stress response, a change of metabolism following the stress response will be expected, and the deterioration process is brought forward.

One important behavior feature of the animal, especially fish, in the stress response is abnormal exercise. This exercise reflects the animal trying to escape or avoid the stressor. Two types of muscles provide energy for exercise. In chronic and slow exercise, the animal utilizes energy from dark muscle that undergoes aerobic respiration through a sufficient supply of oxygen from the abundant blood vessels in the muscle tissue. Aerobic respiration completely degrades glucose to water and carbon dioxide and produces large amounts of energy for animal activity without by-product accumulation. Burst exercise relies on anaerobic respiration in the white muscle to provide energy. Anaerobic respiration provides less energy and causes accumulation of lactic acid as its end product, with a decrease in pH. Lactic acid is utilized for the resynthesis of glycogen

either directly in muscle tissue (Wang and others 1994) or in the liver through the Cori cycle (Nelson and Cox 2004) in post-exercise recovery. Fish is likely to preserve lactate for the resynthesis of glycogen in muscle after the stressor is removed, instead of transporting it to the liver through the Cori cycle (Wang and others 1994). Electrolyte imbalance is also overcome during the recovery. Since there is limited time for the animal to recover from stress when it is slaughtered immediately after post-harvest stress, the accumulation of lactic acid and the resulting decline in pH have pronounced effects on the postmortem biochemistry of muscle tissue. After postmortem, animal tissue continues to undergo respiration and provide energy to maintain the integrity and turgidity of the cells. This process continues until it is inhibited by the disappearance of substrates, the denaturation of enzymes, and/or the inhibition of enzyme activity by accumulated by-products. Animal tissue undergoes aerobic respiration by residual oxygen upon slaughter, but the process stops quickly as the residual oxygen is used up; thereafter, the tissue switches to anaerobic respiration. Anaerobic respiration results in diminishing of carbohydrates, accumulation of lactic acid and lowering of pH. The decline in pH inhibits energy production and accelerates the degradation of ATP by enhancing AMP deaminase activity (Dudley and Terjung 1985), therefore, influencing the energy pool in the tissues. An energy reserve is needed to maintain cell integrity and turgidity, and flesh elasticity. When the energy reserve decreases below a certain critical level, flesh muscle undergoes development of rigor mortis, and begins deteriorating.

The energy pool in the muscle is a group of high-energy phosphate compounds, including ATP, ADP, AMP and creatine phosphate. Direct energy is provided by the

hydrolysis of ATP or ADP in their high-energy phosphate bonds. When respiration ceases, creatine phosphate acts as an energy-buffering compound to maintain a stable energy pool by reacting with ADP and forming ATP, transferring its high energy phosphate bond to ATP. When ADP reaches ATP levels, ATP also begins to decline. The hydrolysis of ATP not only provides energy, but also contributes hydrogen ions to the muscle, thus, adding additional hydrogen ions to the muscle and accelerating the decline of pH in the muscle. Depletion of the energy pool induces the development of rigor mortis. The rate of pH decline and the final pH influences protein denaturation, which also influences the physicochemical properties such as appearance and water holding capacity. Pre-slaughter stress affects the respiration substrates, energy pool and pH of animal tissue at the point of slaughter, as well as postmortem development.

Pre-slaughter stress has been found to induce two types of defects in meat: PSE and DFD in cattle, pigs and poultry (Pottinger 2000). The DFD meat is derived from excessive struggle of the animal pre-slaughter, resulting in the depletion of muscle glycogen and abnormal high muscle pH postmortem (Braggins 1996). The PSE meat arises as a consequence of extremely rapid decline of pH in high temperature and this is also a result of stressors imposed to pre-slaughter animals (Pottinger 2000). The animal pre-slaughter stress also shortens the time to the ultimate pH of muscle and results in a rapid cessation of ATP synthesis. A pre-slaughter stressed animal always has less ATP in the muscle postmortem, subsequently, reducing the time from slaughter to onset of rigor mortis and decreasing the strength of muscle fibers and muscle connective tissue in rigor

(Pottinger 2000). As a general rule, the prevention of pre-slaughter stress not only improves the welfare of animals, but also provides desirable meat quality.

Proteins and lipids are the major energy reserves in fish (Love 1997). Fish have less reserve glycogen than their terrestrial counterparts (Huss 1994), but an acute stress causes even greater disturbances to fish than those observed in mammals because in burst exercise, the fish use all white muscle (Bone 1978, Pottinger 2000). Robb (2000) noticed a defect in stressed fish fillets similar to PSE in higher vertebrate meat. It has been reported that pre-slaughter stress, such as capture, transport, hypoxia, crowding and slaughter methods in salmon and trout, resulted in a rapid onset of rigor mortis due to the rapid depletion of their energy pool (Lowe and others 1993; Erikson and others 1999; Thomas and others 1999). Pre-slaughter stress also decreases strength of muscle fibers during rigor in salmon and trout (Mochizuki and Sato 1994; Jerrett and others 1996; Sigholt and others 1997) and increased muscle gapping (Robb 2000). Fish flesh color is also affected by pre-slaughter stress. Increase of the dropping rate of pH in postmortem results in higher Hunter 'L', hue and chroma values in rainbow trout fillets (Robb 1998) and a high 'L' value in striped bass (Eifert and others 1992). This may be the result of increased protein denaturation. Pre-slaughter stress decreased sensory scores in salmon (Sigholt and others 1997). Nunez (1997) reported that pre-slaughter stressed channel catfish had lower ATP in postmortem muscle, a shortened onset of rigor, a reduced duration of rigor and an increased rate of ATP degradation. Silva and others (2001) reported that pre-slaughter chilling of channel catfish resulted in a red, soft and exudative muscle (RSE), and that the fish achieved rigor mortis faster when exposed to lower pre-

slaughter chilling temperatures. Bosworth and Small (2004) compared the transport methods in channel catfish and concluded that an increase in transport stress increased drip-loss and surface L value in catfish fillets due to muscle protein denaturation.

6. Pre-slaughter temperature

Channel catfish are poikilothermin animals, and the body temperature is totally dependent on water temperature (Tucker and Robinson 1990). Channel catfish tolerate a wide range of environmental conditions. The optimum water temperature for growth is 24 to 29.5º C (Tucker and Robinson 1990), but fish can survive at temperatures from just above the freezing point to nearly 40º C. The fish survival is dependent on fish condition, rate of temperature change and other environmental factors such as dissolved oxygen level (Tucker and Robinson 1990). Fish acclimate to slow temperature changes by adopting the biochemical changes that can sustain the enzyme systems and cell membrane integrity (Tucker and Robinson 1990). A rapid change in temperature causes thermal shock in fish that result in stress or even death. Even though fish are stressed at lower temperatures, the oxygen level is sufficient because dissolved oxygen level is increased and oxygen consumption is decreased compared to optimum temperature (Tucker and Robinson 1990). Oxygen consumption of one-pound channel catfish at 1.6^{o}C was 12 times less than that at 35^{o}C (Boyd 1979).

Lowering the temperature in fish causes a decrease in the catalytic rate of enzymes and the rate of substrate diffusion, thus lowering the activity and locomotory performance (Egginton and others 2000). But an unfavorable temperature is a stressor to the fish, and fish evoke all available resources to cope with it. A compensation

mechanism is performed in fish that are acclimated to low temperatures and biochemical and physiological adaptation is initiated (Egginton and others 2000, Lurman and others 2007). The compensation mechanism requires a high metabolic rate thus an increase in oxygen demand is fulfilled by an increased peripheral oxygen supply in skeletal muscle and an expanded blood capillary bed (Egginton and others 2000, Johnston 1982). The mitochondrial volume, density of muscle cells also increase to facilitate the diffusion of substrates of acclimated fish at low temperatures (Egginton and others 2000). At the molecular level, the activities of citrate synthase, cyclooxygenase (COX), β-hydroxyacyl CoA dehydrogenase, phosphofructokinase increase for acclimated fish at low temperatures (Guderley and Gawlicka 1992, Lurman and others 2007). Myoglobin content in cold acclimated fish heart and skeletal muscle also increases to fulfill the increased oxygen demand (Lurman and others 2007). There is a positive correlation between the elevated citrate synthase and myoglobin content in cold acclimated fish (Lurman and others 2007).

7. Fish muscle pH

The most important factor that influences fish muscle pH postmortem is the production of lactic acid by anaerobic glycolysis from glycogen degradation (Sikorski and others 1990, Huss 1994, Rawdkuen and others 2008). Others factors include muscle buffering capacity and nitrogenous compounds produced by endogenous enzymes or microbial actions (Benjakul and others 2002). Buffering capacity is related to soluble protein level in muscle (Ferguson and others 1993, Somero and Childress 1990) but not insoluble protein level (Ferguson and others 1993). Some amino acids and peptides act as

buffering agents (Foegeding and others 1996). Since fish have less reserve glycogen than their terrestrial counterparts (Huss 1994) and muscle buffering capacity exists, the dropping of pH is not as significant as in terrestrial animals. Fish utilize muscle glycogen for bursts of exercise and produce lactic acid and tend to retain lactic acid for resynthesis of glycogen in muscle (Wang and others 1994). Therefore, postmortem muscle lactic acid and muscle pH of rested fish or fish that experienced pre-slaughter stress varies little provided the initial carbohydrate levels are the same (Love and Muslemuddin 1972, Love 1997, Skjervold and others 1999). However, initial carbohydrate reserve levels in muscle strongly influence the final muscle pH (Black and Love 1988). Non-stressed Atlantic salmon muscle pH is around 7.4 (Erikson and others 1997, Sorenson and others 2004). High pH in cod muscle results in a sloppy texture and can be solved by lower storage temperature (Love 1997). But the toughness of the texture of cod that has already been firm from the low pH may exceed the acceptability limit in cold storage (Love 1997). Following exercise, an increase in partial pressure of CO_2 is also noticed (Ferguson and others 1993), thus, a lower pH is expected. However, Butler and Day (1993) found no significant change in muscle pH of brown trout exposed to sublethal pH 4.5 demonstrating that acid-stressed fish had minor effect on intracellular pH (van Waarde and others 1990) but increased their sensitivity to other stressors (Barton and others 1985). Buffering capacity is related to muscle soluble protein level (Ferguson and others 1993, Somero and Childress 1990) but not insoluble protein level (Ferguson and others 1993). Drop in muscle pH after exercise is dependent on body size but conflicting results were reported (Ferguson and others 1993, Somero and Childress 1990).

Robb and Warriss (1997) reported that CO_2 anesthesia of fish resulted in a lower muscle pH and early onset of rigor. CO_2 anesthesia also resulted in fish muscle softening (Roth and others 2002). The pH values on the surface of chilled salmon fillets did not differ from those of control fish; however, those from CO_2 anesthesised fish initially decreased by 0.2-0.3 units and continued to decrease to a pH of 6.4 (initial 7.4-7.5) after six days of storage on ice (Erikson and others 2006). Yokoyama and others (1993) concluded that chilling (4^oC) before CO_2 anesthesia did not impair carp muscle quality. Channel catfish can tolerate higher carbon dioxide and lower oxygen levels than other fish (Tucker and Robinson 1990).

8. Rigor mortis in fish

After the animal is slaughtered, the elastic carcass will gradually become hard and stiff for a certain period of time. When the strength of the hard carcass reaches a maximum level, the stiffness is gradually resolved until the carcass becomes elastic again. This phenomenon is called rigor mortis and is brought on by the depletion of ATP in muscle tissue. Contraction and relaxation are the driving forces for muscle movement. In relaxation, ATP is required to drive the dissociation of the actin and myosin junction that is formed during contraction. The inability to relax causes the muscle to harden and stiffen. This stage lasts until the actin and myosin junction is weak or connective tissue is degraded (Erikson 2000a, Seki and Watanabe 1984, Montero and Borderias 1990, Ando and others 1993, Yamanoue and Takahashi 1988). Rigor mortis shortens muscle fibers and reduces fillet yield. Processing of fish during rigor mortis results in gapping of fillets, due to connective tissue breakage (Huss 1994). Rigor onset and resolution are the turning

points for fish quality. Harvest, transport and slaughter methods that stress the fish and accelerate the use of ATP result in a rapid onset and resolution of rigor mortis that accelerates the muscle deterioration process (Lowe and others 1993, Berg and others 1997, Erikson and others 1999, Thomas and others 1999). A slower ATP degradation at 5-15^{o}C than at 0^{o}C, resulted in retardation of rigor mortis of red seabream, plaice, and carp at 5-15^{o}C (Iwamoto and others 1985, Iwamoto and others 1987, Iwamoto and others 1988, Watabe and others 1991, Watabe and Itoi 2002). Intracellular calcium ion concentration is also associated with rigor mortis development. An accumulation of calcium ions in muscular cells due to a lower uptake efficiency of calcium ions by cells at lower temperatures is also part of the reason that rigor mortis development is accelerated at 0^{o}C when compared to 5-15^{o}C (Watabe and others 1989, Watabe and Itoi 2002). Watabe and Itoi (2002) reported that common carp can retard the onset of rigor mortis when acclimated to lower temperatures due to enhancement of ATP production. Fish captured during winter had a longer time period prior to the onset of rigor mortis (Tanaka 1991) when compared to fish that were captured at other times during the years. This is also due to the lower uptake efficiency of calcium ions from the cytoplasm to the sarcoplasmic reticulum, the calcium storage granule (Watabe and Itoi 2002).

9. Stress, plasma cortisol and fish quality

It has been well documented that stress induces plasma cortisol in many animal species, and thus plasma cortisol has been widely used as an indicator of the intensity of stress. However, elevated plasma cortisol levels that are reported in the literature are

difficult to compare, due to the different experimental protocols. Differences in species, sex, maturation state and rearing history may have an additional effect. Transport (Robertson and others 1987, Barton and Peter 1982, Bandeen and Leatherland 1997) induced an elevation of plasma cortisol and subsequent elevation of plasma glucose in red drum (*Sciaenops ocellatus*), rainbow trout and white suckers (*Catostomus commersoni*); however, loading density (McDonald and others 1993), dissolved oxygen level (Eriskon 2001b) and fasting (Eriskon 1997) had minor effect on plasma cortisol in salmon and white suckers. Skjervold and others (1999) reported that high density salmon induced an elevated cortisol level, but further chilling stress of these fish for 45 min did not further induce cortisol levels. However, chilling stress induced an elevated cortisol level in the low density fish group. This indicated that the effect of multiple stressors on cortisol level may not be additive.

Barnett and Pankhurst (1998) reported that wild greenback flounder that were rested had plasma cortisol levels of 3.9 ± 2.5 ng/ml, significantly lower than fish that were subjected to capture, confinement and transport (61.9 ± 3.1 ng/ml). The latency time of plasma cortisol response to stress was approximately 10 min. These researchers suspect that the cultured greenback flounder that were exposed to normal husbandry conditions had low plasma cortisol levels; however, three hours of crowding combined with five minutes chasing (stimulating grading) resulted in significantly elevated cortisol levels for up to 48 h. These researchers concluded that green flounder had higher aerobic respiration rate than other flatfish.

Lowe and others (1993) reported that snapper that were rested had a plasma cortisol level of 6.8 ng/ml, but when fish were subjected to 1 h exercise, the plasma cortisol level increased to 67.7 ng/ml. Fish subjected to 2 h netting stress had cortisol levels of 39.8 ng/ml; but 12 h netting stress decreased cortisol to that of rested fish. These researchers concluded that the decreasing level of cortisol from increased stress levels were the result of a recovery response during the late hours of stress. Full rigor was achieved in 4 h in 2 h stressed fish muscle; 8 h in 12 h stressed and exercised fish muscle; and 16 h in rested fish muscle. Controlled fish maintained a lower freshness indicator-K value than stressed fish until 72 h, indicating better quality. Erikson and others (1999) compared plasma cortisol, glucose and lactate levels of crowding Atlantic salmon for 5 d plus 1 h chasing versus rested salmon. These researchers reported that rested fish had a cortisol level of 20.9 ng/ml, as compared to 184.6 ng/ml for stressed fish. The depletion of ATP coincided with the onset of rigor mortis. In another experiment, these researchers studied the effect of slaughter methods on the post-mortem biochemistry of Atlantic salmon. They compared the effects of anaesthetized fish; non-stressed bled fish and stressed fish (chasing fish for 88 min) on pH level and rigor development postmortem. It was determined that the initial pH levels were 7.3 in anaesthetized fish muscle; 6.8 in non-stressed, bled fish muscle and 6.6 in stressed bled fish muscle. Rigor onset was 1-2 h in stressed fish, 2-5 h in non-stressed fish, and 23 h in anaesthetized fish muscle. Stressed fish muscle was stiffer in rigor. The authors concluded that slaughter can induce stress that can be minimized through proper handling, and that bleeding could not overshadow the stress effect.

Berg and others (1997) studied energy levels and postmortem rigor development of Atlantic salmon in non-stressed and stressed conditions. These researchers concluded that, handling stress resulted in a great drop of creatine phosphate; and the distribution of high-energy compound levels were affected by handling stress during slaughter rather than postmortem catabolism during ice storage. Non-stressed fish muscle attained full rigor at 20 h postmortem and after 50 h, stiffness began to decrease. After 80 h, rigor mortis of non-stressed fish muscle was fully resolved. However, full rigor of stressed fish occurred in 8 h. These researchers found there were great variations in the ratio of ATP and IMP in the different sample sites of muscle from the same fish carcass in non-stressed fish. The increase in IMP and decrease in ATP was stoichiometrical.

Atlantic salmon and rainbow trout were stressed (by lowering holding water level to the dorsal surface of fish for 30 min), exercised (by inducing exercise 0.1-0.3 body length/sec by increasing water flow from 3.3 cm/sec to 10 cm/sec for 30 min), stressed and exercised (by lowering holding water level and chasing fish for 30 min) (Thomas and others 1999). Plasma cortisol levels were: controlled: 49 ng/ml; exercised: 25 ng/ml; stressed: 69 ng/ml; stressed plus exercised: 83 ng/ml. Muscle pH of fish subjected to stress plus exercise was the lowest in all treatments. Rigor developed 12 h earlier in stressed plus exercised fish muscle than other fish muscle. In rainbow trout, plasma cortisol was controlled: 5 ng/ml; exercised: 12 ng/ml; stressed: 16 ng/ml; stressed plus exercised: 68 ng/ml. Plasma lactate followed the same pattern as cortisol. Muscle ATP at slaughter was the highest in the controlled (5.74μmol/g) and the lowest in the stressed

plus exercised treatment group. Rigor onset was the fastest in the stressed plus exercised group.

Jittinandana and others (2003) studied the influence of rearing temperatures (6.5 ^{o}C vs. 9.5^{o}C); fish size (small, middle, large), stress treatment (control, processing immediately stress, processing 24 h after stress, and processing 48 h after stress; fish were stressed by crowding with a net for 5 min) on plasma cortisol, chloride levels, flesh quality and smoking properties of Arctic char. Results indicated that hatching temperature and fish size did not affect plasma cortisol and chloride levels. Plasma cortisol levels in the stressed without recovery group (15.66 ng/ml) were almost five times that of the non-stressed group (3.64 ng/ml).

Small and Davis (2002) used a time resolved fluoroimmunoassay (TR-FIA) to measure the plasma cortisol concentration of channel catfish fingerlings that were rested or confined for 2 h. The averaged cortisol level was 13.2 ng/ml in rested fish and 40.2 ng/ml in stressed fish.

Bosworth and Small (2004) transported catfish with two different water temperatures (10^{o}C vs 20^{o}C); and three aeration type/dissolved oxygen (DO) levels (blower/DO at 4-5 ppm, pure O_2 diffuser/DO at 4-5 ppm or pure O_2 diffuser/DO at 9-10 ppm). Regardless of treatment, all fish were stressed as indicated by the elevation of plasma cortisol levels. However, no relationship was found between cortisol level and fish fillet quality. High transportation OD values resulted in lower Hunter L values and drip loss. Fillet drip loss was positively related with Hunter L values and pH. These researchers concluded that transport water OD levels affect catfish fillets Hunter L value

and drip loss, but have no effect on pH, a, and b values. Aaslyng and Barton-Grade (2001), Skjervold and others (2001) and Molette and others (2003) also reported that transport stress increased the drip loss and Hunter L value in poultry, swine and salmon, respectively.

Erikson and others (2006) analyzed water and quality of fillets from salmon that were live-chilled (refrigerated sea water, 2-4^{o}C) and carbon dioxide anaesthetized in a processing line. They reported that when carbon dioxide was increased, the pH was decreased in chilling tank. Fish were calm during live-chilling and fish mortalities were 100% at above 50 mg/L carbon dioxide levels with gaping mouths and flared operculums and brilliant cherry red color. Body temperature of the fish was about 1^{o}C higher than the water temperature. The pH values on the fillet surface of chilled fish did not differ from those of controlled fish; however, those from CO_2 anesthetized fish were 0.2-0.3 units lower. Chilled fish without carbon dioxide anesthesia (supposed to be non-stressed fish) began the onset of rigor at 20 h postmortem; maximal rigor was achieved at 48 h and rigor ended at 75 h. However, carbon dioxide anesthetized fish reduced the onset of rigor; and maximal rigor occurred at 25 h, and rigor ended after at 60-70 h. In addition, chilling and CO_2 anesthesia had no effect on fish muscle color. Skjervold and others (2001) reported that fish crowded at 16.8^{o}C before chilling at 1^{o}C for 1 h resulted in an early onset of rigor. Both crowding and non-crowding chilling reached maximal rigor at 18 h, which postponed maximal rigor for 10-12 h when compared to non-chilled fish. Skjervold and others (2001) reported that live-chilling at 1^{o}C for 45 min from 12.4^{o}C increased blood glucose levels by 20% and that crowding increased blood glucose levels

by 70%. Yokoyama and others (1989) concluded that live-chilling in ice slush at 0.5-0.8^{o}C is an inhumane method for killing fish because the anesthesia is too slow and fish vigorously attempt to escape.

CHAPTER III

EXPERIMENT I: EFFECTS OF POSTMORTEM CHILLING, STORAGE TIME ON CHANNEL CATFISH FRESH FILLET COLOR AND THE RELATIONSHIP OF COLOR AND TOTAL HEME-PROTEIN CONTENT

1. Introduction

Color is one of the most important quality attributes that impacts consumers purchasing decision regarding muscle food products (Faustman and Cassens 1990). Color of meat products is mainly derived from the two major pigments, myoglobin and hemoglobin. The concentrations and the oxidative states of these two heme-proteins determine the color of most meat products. Other pigments may also play major or minor roles to muscle color in specific species. For example, carotenoids are the major pigments in trout, shrimp, lobster, goldfish and especially in salmon (Nickell and Springate 2000). Factors other than these pigment molecules such as fat content, water content, pH and myofibril structure also affect the appearance of meat. Catfish muscle is characterized by a pale, grayish tint to a slightly red color. But variation of catfish muscle color may occur due to the difference of strain (Bosworth and others 2004) and cultural conditions including farming environment, water temperature and growing time (Hallier and others 2007). Farming conditions indirectly affect fish muscle color by affecting biochemical composition, muscle fiber development and composition (Hallier and others 2007).

Feeding regime has been found to affect catfish muscle color. For example, carotenoids such as xanthophylls in the diet can deposit into catfish skin and the anterior, dorsal part of the muscle resulting in yellow spots (especially in the fat). This is why carotenoids cannot exceed 11 mg/kg in the diet of channel catfish (Hwang 1987).

Chilling of fish fillets is a process step in which fillets are immersed into an ice slurry to lower the temperature of the fillets with agitation, thus minimizing the microbial growth and other chemical reactions. Chilling also removes blood spots and other contaminants, and may increase fillet weight due to water absorption. The bleaching effect and water absorption that is causing by chilling affect the muscle surface color. However, the extent of this bleaching effect on the surface color of catfish fillets is unknown.

During refrigerated storage, fish muscle is characterized by a loss of shininess (Suvanich and Marshall 1998), water loss, shrinkage of myofibrils, denaturation of protein (Offer and Knight 1988) and possible pH changes. In addition, autoxidation of myoglobin and hemoglobin occurs on the muscle surface. All these factors may affect the light scattering properties which would affect the color of fresh catfish fillets.

Even though most fresh catfish muscle is characterized by a pale and slightly red color, variations in fillet color sometimes occur that may affect the uniformity of fillets and consumer acceptability. Michie (2000) reported that about 40% of the downgraded salmon in secondary processing was derived from the poor visual color. However, this may not be as important in catfish muscle. Instrumental food color measurements are usually used, and are usually related to sensory color analysis (Setser 1984).

The objectives of this study were: 1) to correlate visual rating of catfish fillets with Hunter color parameters; 2) to quantify the postmortem chilling process effects on surface color and color distribution of catfish fillets on the bone-side; 3) to analyze the total heme-protein content from selected visual "white" and "reddish" catfish fillets and correlate with the instrumental color parameters; 4) to monitor the color change of fresh catfish fillets that were stored on ice.

2. Materials and Methods

(1). Establishment of color category of fresh fillets by visual rating and instrumental measurement

Fresh processed catfish fillets (within 1 h) were randomly chosen from processing plants, placed in polyethylene bags, placed in ice chests with ice and transported to the Food Processing Laboratory at Mississippi State University within 3 h. Bone-side color of the fillets was rated into five categories by three experts who have ten-year experience in determining fresh catfish quality and received training before the panel. The color of catfish fillets was divided into five categories and represented by five digital numbers. The color categories were described as follows: 1 – very white; 2 – off white; 3 – slightly pink; 4 – red to pink; 5 – extremely red. Before assessment, the experts discussed to set a uniform standard for each category. After assessment, the color of the fillets on bone-side was measured objectively with triplicate readings by a Labscan Model 6000 0/45 ^{o} spectrocolorimeter (Hunter Associates Laboratory, Inc. Fairfax, VA, USA) equipped with a computer with HunterLab Universal software version 1.4 (Hunter Associates Laboratory, Inc). The equipment was calibrated before measuring color and the fillets

were placed on a glass on top of a 2.54 cm port. Triplicate readings of each fillet from three parts were obtained and the average values were recorded. Hue angle, hue = arctan (b/a); chroma or saturation index, SI= $(a^2 + b^2)^{1/2}$ (Francis 1998); and whiteness, WI = $100-[(100-L)^2 + a^2 + b^2]^{1/2}$ (Reppond and Babbitt 1997) were calculated. Each category at least had five fillets. The CIELAB L*, a*, and b* values were calculated from Hunter L, a, b values according to the transfer equations provided by Hunter Associates Laboratory Inc (1996) based on a CIE 10 degree standard observer with a D6 illuminant. The experiment was repeated three times.

(2). Postmortem chilling effects on color of fillet on the bone-side

An onsite survey on fresh catfish fillet bone-side surface CIELAB color parameters before and after chilling in ice water was conducted in three commercial catfish processing plants with a portable chroma meter (CR-400, Konica Minolta Seneing, Inc. Japan) previously calibrated with a standard white plate (No. 18433006, Y: 94.5; X: 0.3134; y: 0.3198). The processing plants were located in west central Mississippi, northeast Mississippi and southwest Alabama. At each plant, 50 fillets were randomly selected either immediately before or after chilling and the CIE L*, a*, b* values were measured on the bone-side in the middle part of these fillets. The chilling procedures utilized in all three plants were similar. The fillets tumbled chilled in ice water from 1 to 5 min. The catfish were unloaded from the transport truck, stunned, conveyed to the deheader and filleted within 30-60 min at around 18^{o}C. The Hunter L, a, b values were calculated from CIE L*, a*, b* values by the transfer equations provided by Hunter Associates Laboratory Inc (1996). Hue angle, SI, whiteness were also

calculated from CIE L*, a*, b* with previously described equations. Surface pH was also taken in only one plant, using a portable pH meter (WTW pH 315i Model, Weilheim, Germany).

(3). Total heme-protein content in "white" and "reddish" fillets

Using visual observation on fillets bone-side, twenty "white" and twenty "reddish" fresh processed catfish fillets (50-150 g) were sampled from processing plants by visually observing their bone-side color and placed inside an ice chest containing ice and transported to the Food Processing Laboratory at Mississippi State University. Hunter color values of each fillet on the bone-side were measured as previously described method. The fillets were individually packed in polyethylene bags (Ziploc®, Johnson & Son Inc. Racie, SC, USA) and frozen at -20^{o}C immediately after Hunter color measurement. After thawing overnight at 4^{o}C, a 10-gram portion of grounded fillet from the middle portion, with the elimination of dark muscle, fat and connective tissue, was weighed and placed into 50 ml centrifuge tubes that contained 20 ml cold 50 mM Tris-HCl extraction buffer, pH 8.5. The fillets were homogenized with a homogenizer (Polytron®, Brinkmann Instruments Co. Westbury, NY, USA) for 1 min and centrifuged at 6,500 g for 10 min (CU-5000, IEC, Needham Heights WA, USA). Supernatants were filtered through No.4 Waterman filter papers (Waterman International Ltd, Maidstone, UK). Pellets were re-homogenized, centrifuged for 10 min with the same equipment as the first extraction and filtered. The two filtrates were combined and diluted to 100 ml volume with extraction buffer. Afterwards, a 990 µl sample filtrate was added to 10 µl of 60 mM $K_3Fe(CN)_6$ and 80 mM KCN, mixed and centrifuged at 14000 g in a small

centrifuge (Centrifuge 5415C, Eppendoff, Westbury, NY, USA) for 5 min to remove the haziness (Fleming and others 1960). The total heme-proteins, including myoglobin, hemoglobin, and cytochrome c, were converted to stable cyanmet-form derivatives (Han and others 1994). These cyanmet-form derivatives were monitored at 540 nm with a spectrophotometer (Spectronic Genesys^{TM} 5, Rochester, NY, USA) and the total heme-protein concentration was determined with a molar extinction coefficient of 11.3 x 10^3 and expressed as the milligrams of myoglobin (molecular weight 17000) per gram of fish muscle sample (Fleming and others 1960).

(4). Iced storage effect on color of fillets on the bone-side

Ten fresh processed catfish fillets were randomly selected from processing plant after postmortem chilling (within 1 h), placed inside ice chest with ice and transported to the Food Processing Laboratory at Mississippi State University. These fillets were individually packaged in polyethylene bags (Ziploc®, Johnson & Son Inc. Racie, SC, USA) and stored on ice inside a refrigerator. Hunter color parameters were monitored every two days until the fillets were spoiled and discarded. The experiment was repeated three times.

(5). Experimental design and statistical analysis

T-tests and multiple regression analysis were performed on Hunter color values and visual color rating scores by EXCEL (Microsoft 2000) and PROC GLM in SAS (SAS 1990). The postmortem chilling effects on CIE L*, a*, b* values of fresh catfish fillets on bone-side were analyzed by PROC MEANS and PROC GLM in SAS (SAS

1990). A t-test was also utilized to analyze total heme-protein content and Hunter color values between visual "white" and "reddish" fillets and a linear regression between Hunter a values and the total heme-protein contents was conducted by EXCEL (Microsoft, 2000). Ice storage effects on fresh catfish fillets on bone-side were analyzed with PROC GLM in SAS (SAS 1990). Except postmortem chilling experiment, which was conducted in a randomized complete block (RCB) design with each plant as a block; other experiments were conducted using completely randomized (CR) design. Means were separated by Fisher's least significant difference (LSD, $\alpha = 0.05$).

3. Results and Discussion

(1). Establishment of color category of fillets by visual rating and instrumental measurement

The Hunter L, a, b and CIE L*, a*, b* values and 95% confidence intervals of the five "visual bone-side color" categories of fresh catfish fillets are presented in Tables 3.1and 3.2. Both Hunter L, a, and CIE L*, a* values were different ($p \leq 0.05$) between the rating categories. Hunter L and CIE L* values were negatively related to rating scores whereas either Hunter a or CIE a* values were positively related to rating scores. Rating scores of 1-2 were considered to be "white" fillets and 3-5 were considered to be "reddish" fillets. However, either Hunter b or CIE b* values were not different ($p>0.05$) among the rating categories and had no correlation with visual rating using a rating scale of 5 points.

The correlation coefficient (R^2) between visual rating scores and Hunter a, L, hue angle, and whiteness values was 0.7997, 0.5446, 0.6989 and 0.5474, respectively

(Figures 3.1 to 3.4). The correlation coefficient R^2 was only 0.1431 between visual rating scores and Hunter b values and 0.0075 between visual rating scores and saturation indexes (SI), respectively. The t-test indicated that Hunter a values were highly ($p \leq 0.05$) correlated to the visual rating scores, but Hunter L and b were not ($p > 0.05$). Hunter hue angles and whiteness were also closely ($p \leq 0.05$) related to the visual rating scores. Multiple regression equations for visual rating scores on Hunter L, a, b values were: Rating score = 3.76 -0.0197 L + 0.3493a – 0.03b with an adjusted R^2 of 0.795. This indicates that visual rating scores were closely related with Hunter a, whiteness values and hue angles and were less affected by b values and SI.

Stevenson and others (1989) compared the visual color rating scores with a five point scales from bright fresh to extremely dark and CIELAB color parameters on venison color and reported that the color rating scores were highly related to CIE a* values, hue angles and saturation indexes and less related to L* and b* values. Eagerman and others (1977) reported that the correlation efficient between hue angles and saturation indexes and perceived color was lower for beef and lamb than for pork, and they contributed this to the marbling in these meats. Setser (1984) indicated that L, a, and b yielded a better relationship with perceived color. Stevenson and other (1989) concluded that CIE L*, a*, b* could be used to make the accept/reject decision due to the high correlation between color and acceptability of venison.

(2). Postmortem chilling effects on color of fillets on the bone-side

Onsite survey results showed there were differences ($p \leq 0.05$) in CIE L*, a*, b*, hue and whiteness values of fresh catfish fillets on bone-side before and after postmortem chilling (Tables 3.3 and 3.4). However, there were no differences ($p>0.05$) in SI between samples before and after chilling (Table 3.4). Chilling increased the L*, b*, whiteness values and hue angles, and decreased a* values. Thus, postmortem chilling rendered the fillets brighter and less red. This is expected since chilling acts as a washing step for removing blood, other surface debris, and also adds shine to the surface (Wiles and Green 2004). Muscle surface myoglobin and hemoglobin are easily removed by washing (Chaijan and others 2006), but other insoluble pigments such as cytochrome c may not be easily washed out (Park and others 1996). Ramirez-Suarez and others (2000) used 4-5 times cold water (less than 10^{o}C) to wash Monterey sardine minced flesh for 10-30 min with agitation. These authors reported that visual observation indicated that unwashed sardine mince was darker/redder/yellower than washed mince, but Hunter L, a, b, values and hue angles were not different.

The distribution of CIE L*, a*, and b* values of fresh catfish fillets on the bone-side are presented in Table 3.5. Before chilling, 33.3% of fillet CIE L* values were fitted in the 46.00-48.99 range, the largest group; the second largest group was 49.00-51.99 range, which contained 25.3%; and the third largest group was 43.00-45.99 range, which contained 16% of fillets. In the extreme, 2% of the fillets had L* values higher than 58.00 and 11.3% less than 42.00. After chilling, 33% of CIE L* values were fitted on 49.00-51.99 range, the second largest group was 52.00-54.99 range, which contained 19.3% of

the fillets; the third largest group was 18.7% of the fillets filled in the range of 46.00-48.99. The CIE L* values of the chilled fillets shifted 3 digits up from the non-chilled fillets. This should be noticeable to the consumer, thus it can be said that this is another advantage of chilling catfish fillets. The percentage of fillets that had CIE L* values higher than 58.00 increased from 2% before chilling to 9.3% after chilling; whereas the percentage of fillets that had CIE L* values of less than 42.00 decreased from 11.3% before chilling to 2.7% after chilling.

Before chilling, 63.3% of the CIE a* values in fresh catfish fillets were negative, indicating that less fillets appeared red in color. The largest group, 44% of the fresh fillets were in the 0.00 to -0.99 ranges, being grayish. The next two groups, comprising 36% of fillets, ranged from -1.00 to 1.00. On the other hand, 5.3% fillets had a* values higher than 3.00. After chilling, the majority (80.7%) of the fillets was in the negative range, with the largest group in the range of -1.00 to -1.99, which contained 47% of the fresh fillets. The percentage of fillets with CIE a* values higher than 3.00 decreased to 2% after chilling. Chilling decreased CIE a* values by 1 to 2 digits, indicating that chilling removed red pigments (Chaijan and others 2006, Wiles and Green 2004, Ramirez-Suarez and others 2000, Park and others 1996,). Before chilling, 67.4% of fresh fillets had negative CIE b* values increasing to 88.7% after chilling. The fillets decreased 1.5 digits in b* values after chilling. Jankowska and others (2007) reported that the CIE L*, a*, and b* values of European catfish (*Silurus glanis*) with natural feed were 48.78 ± 0.55, -0.73 ± 0.16, 10.58 ± 1.03, respectively. Whereas the CIE L*, a*, b* values of catfish with formulated feed were 47.44 ± 0.45, −2.55 ± 0.23, and 9.50 ± 0.99. Their report did not

indicate whether the fillets were chilled or not. The fillets in their report were less white and yellower than in ours.

To compare CIELAB color values to Hunter values, we did measurements on the same chilled fillets at the laboratory. The Hunter L, a, b values were (mean ± standard deviation): L: 58.11 ± 4.52; a: 0.37 ± 1.84, and b: 7.52 ± 2.05.

Nunez (1997) reported that bone side hand filleted channel catfish ranged from 43.4 to 53.1 in Hunter L values. These numbers were lower than our results due to the fact that our fillets were sampled from the mechanical processed fillets. Willits (1992) reported that Hunter L, a, b values of fresh channel catfish muscle from catfish steaks were 50.4, 1.8, and 3.5, respectively. Bosworth and others (2004) compared the Hunter color values of different strains of catfish. They reported that the average L values were 73.92 in USDA-103, 75.31 in Norris and 72.06 in Channel x Blue; the average a values were 8.35 in USDA-103, 8.81 in Norris and 9.25 in Channel x Blue; and the average b values were 22.01 in USDA-103, 25.74 in Norris and 22.75 in Channel x Blue. The numbers were higher than our results. The strain difference might contribute to this variation. Another explanation is that the scale of processing machines resulted in different quality of catfish fillets since fillets in our experiment were processed in a commercial processing line; whereas their fillets were processed in a pilot plant processing (Bosworth and others 2004).

Postmortem chilling of catfish fillets in ice water resulted in an increase in CIE L* values. This may be due to the washing of blood spots and debris, myofibrils' swelling due to changes in light scattering properties and slight denaturation of surface proteins.

Marroquin and others (2004) compared the color of fresh catfish fillets from hand cut and mechanical cut and reported that the hand cut fillets had higher L, and lower a and b values than mechanical cut fillets. They concluded that these differences were due to differences in the state of rigor of the fish and fillets from pre-rigor fish showed a gray discoloration. Forrester and others (2002) used water and 0.5% to 2% citric acid to vacuum-tumble catfish fillets and reported that citric acid treated fillets became brighter than water treated fillets (L value: 2% citric acid: 73.03; 0.5%: 68.52%; water: 65.83; control: 60.58). Such high L values seem likely due to protein denaturation by acid tumbling. They also reported that citric acid tumbling significantly decreased Hunter a and b values in catfish fillets. Bal'a and Marshall (1998) reported that dipping catfish fillet strips in 2% organic acid (acetic acid, lactic acid, hydrochloric acid, citric acid, malic acid or tartaric acid) resulted in a noticeable bleaching effect (higher 'L' value). They also reported that treatments rendered the catfish fillets redder and yellower than the untreated control.

The surface pH values of catfish fillets before and after chilling were 6.69 ± 0.13 and 6.58 ± 0.10, but did not differ significantly ($p > 0.05$). Yamaguchi and others (1976) reported that muscle pH of frozen-thawed catfish fillets was 6.28 to 6.39. Bosworth and others (2004) reported that the surface pH of three strains of catfish varied from 6.56-6.8 with little differences between strains. The small variation of muscle pH in channel catfish might be due to low glycogen level.

(3). Total heme-protein content in "white" and "reddish" fillets

Hunter L, a, and b values and the total heme-protein contents in "white" and "reddish" catfish fillets are presented in Table 3.6. "White" fillets had higher L values and lower a values ($p \leq 0.05$) than "reddish" fillets. These results indicate that the Hunter L and a values were in concordance with the visual observations/ratings. However, Hunter b values were not different ($p > 0.05$) between "white" and "reddish" fillets. Total heme-protein content in "white" fillets was 16.8% lower ($p > 0.05$) than that in "reddish" fillets. Multiple linear regression analysis indicated that there were no correlation ($p > 0.05$) between total heme-protein content and Hunter L ($R^2 = 0.047$), a ($R^2 = 0.026$), and hue angle (R^2 =0.0.013), but was slightly correlated with b ($R^2 = 0.306$) and SI ($R^2 = 0.209$). Multiple linear regression equations for total-heme-protein contents predicted by Hunter L, a, b values were: total-heme-protein content = -1.29 + 0.02L + 0.016a + 0.189b with an adjusted R^2 of 0.345.Our results were different from results reported by Fleming and others (1991), in which, they observed a negative correlation existed ($r > -0.97$) between the Hunter color L values and total heme pigments and a positive correlation between a values and heme pigments.

Fleming and others (1960) reported that the total heme-protein content in beef ranged from 3.08-3.69mg/g. Warriss (1979) used either phosphate buffer or acidified buffer to extract pigments from sheep meat and reported that the total heme-protein pigments in sheep meat was 5.22 ± 0.15 mg/g. By using Warriss' method, Krzymick (1982) reported that total heme-protein pigments in beef were 4.43 ± 1.2 mg/g. Brown (1962) reported that bruising of tuna by serine harvest increased the myoglobin and

hemoglobin content in muscle from 0.92 mg/g to 1.15 mg/g. Han and others (1994) reported that total heme-protein contents of chicken drumstick was 0.12 mg/g; thigh was 0.21 mg/g; beef was around 4.2-4.9 mg/g.

(4). Iced storage effect on color of fillets on the bone-side

Hunter L, a, and b values, hue angle and saturation index (SI) of catfish fillets on the bone-side on ice storage are presented in Figures 3.6 to 3.10. Hunter L mean values increase ($p \leq 0.05$) in the first two days of storage, and remained constant thereafter. Hunter a values increased ($p \leq 0.05$) in the first four days of refrigerated storage with no changes ($p > 0.05$) thereafter. Hunter b values increased ($p \leq 0.05$) in the first two days, and decreased ($p \leq 0.05$) at day eight. Hue angles did not change ($p > 0.05$) during storage. Saturation index (SI) increased ($p \leq 0.05$) at the first two days and decreased ($p \leq 0.05$) at the last two days. Suvanich and Marshall (1998) stored channel catfish frames (the backbone of catfish after the fillets removed) at 25^{o}C, 5^{o}C or 0^{o}C. They noticed that at 25^{o}C, Hunter L values decreased, but the values did not change at 5 or 0^{o}C. Hunter a values of frame stored at 25 and 5^{o}C increased at first two days then decreased. Hunter b values increased over the first 12 h at 25^{o}C and two days at either 5 or 0^{o}C, then decreased or kept unchanged thereafter. These authors explained that the slight decrease in Hunter L values or loss of shininess was due to autolysis or microbial action; the decrease in the redness (increased a values) in the last stage of storage to autoxidation of myoglobin and hemoglobin; and the decrease in yellowness (decreased b values) in the last stage of storage to the masking effect by brown material generated by microbial action. Marroquin and others (2004) observed that Hunter 'L' values of channel catfish

fillets that were stored on ice increased until the third day, but decreased by the sixth day during refrigerated storage. These results were similar to our results. Hunter L value is related to the physical property of the myofibrils (Offer and Trinick 1983). Myofibrils continue to shrink during storage, thus, increasing the contrast of myofibrils and the sarcoplasm. Consequently, the Hunter L values increase due to increased light scattering. Erikson and Misimi (2008) also observed that lightness of Atlantic salmon fillets increased during ice storage and coincided with the development of rigor. Increases in Hunter 'L' values over storage time for of refrigerated catfish fillets was correlated with a decrease in channel catfish surface pH (Silva and White1994). This decrease in pH might change the physical properties of muscle, thus the light scattering properties (Erikson and Misimi 2008). Erikson and Misimi (2008) noticed that CIE a* values of exhausted Atlantic salmon fillets increased until 60-70 h and kept constant to the end of ice storage, whereas the CIE a* values of rested fish fillets decreased until 30 h and leveled until 70 h. They also reported that CIE b* and saturation index (SI*) increased during storage, and a decrease and then increase of CIE Hue* values. These observations were similar to our results. The fillets in our experiment were presumably subjected to a certain degree of stress because they were sampled from commercial plants. Silva and White (1994) observed that Hunter a values decreased with storage time in high CO_2 packed catfish fillets, which was due to a low conversion percentage of oxymyoglobin to metmyoglobin. Przybylski and others (1989) found that irradiation of catfish fillets increased a values, but not L and b values. They explained that these changes were due to the shift in the deterioration process from putrefaction to rancidity.

4. Conclusions

Visual ratings of the bone-side color of fresh catfish fillets by experienced panelists using a five point scale were highly correlated with Hunter a values. In addition, visual ratings were also related to hunter L, whiteness and hue angle, but not Hunter b values and saturation index. Thus, the color of fresh catfish could be differentiated objectively by Hunter a values. The postmortem chilling process in catfish processing increased CIE L* values of the fresh catfish fillets by a three digit reading, indicating increased paleness; chilling also decreased CIE a* values and CIE b* values by 1 and 1.5 digits, respectively, and rendered the fillets less red and yellow. The majority of the fresh catfish fillets became paler, greener and bluer after postmortem chilling when compared to those before chilling. During eight days of refrigerated storage, the fillets became redder over time. The total heme-protein was slightly high in visually "reddish" fillets when compared to "white" fillets, but did not correlate well with Hunter a values. Surface color reading by instruments did not reflect the pigment scenario of the whole fillets.

Table 3.1 Visual color rating scale and Hunter color values of fresh catfish fillets on the bone-side

Rating	Description	Hunter L		Hunter a		Hunter b	
		Mean value	95% CI	Mean value	95% CI	Mean value	95% CI
1	Very white	59.48a	49.48 - 69.48	-2.09a	-(4.5) - 0.32	9.63a	6.56 - 12.70
2	Off-white	56.08b	51.93 - 60.23	-1.26b	-(3.24) – 0.72	8.95a	6.68 - 11.22
3	Slightly pink	53.08c	48.61 - 57.55	1.78c	-(0.65) – 4.21	7.15b	3.83 - 10.47
4	Red to pink	50.80d	45.79 - 55.81	4.04d	1.18 - 5.22	8.21a	6.78 - 9.64
5	Extremely red	50.51d	45.59 - 55.43	5.06e	3.84 - 6.28	7.71ab	6.24 - 9.18

Note: abcde: means of L, a, b values not followed by the same letter differ ($p \leq 0.05$)

Table 3.2 Visual color rating scale and CIE L*, a*, b* color values of fresh catfish fillets on the bone-side

Rating	Description	CIE L*		CIE a*		CIE b*	
		Mean value	95% CI	Mean value	95% CI	Mean value	95% CI
1	Very white	66.04	56.57 – 75.00	-2.42	-(5.60) - 0.35	13.51	9.59 – 17.16
2	Off-white	62.89	58.94 – 66.73	-1.48	-(3.94) – 0.82	12.78	9.59 – 15.94
3	Slightly pink	60.05	55.72 – 64.26	2.11	-(0.80) – 4.82	10.23	5.45 – 15.05
4	Red to pink	57.85	52.91 – 62.63	4.82	1.47 – 6.02	12.13	10.28 – 13.91
5	Extremely red	57.57	52.71 – 62.27	6.03	4.75 – 7.23	11.35	9.41 – 13.21

Table 3.3 CIE L*, a*, b* values of fresh catfish fillets before and after postmortem chilling process.

Chilling	L*	a*	b*
Before	48.48 ± 4.40b	0.35 ± 1.74a	-(1.51) ± 1.83b
After	51.11 ± 5.13a	-(0.64) ± 1.34b	-(0.21) ± 3.34a
LSD (0.05)	0.72	0.21	0.20
C.V%	8.99	887.34	145.17

Note: ab: means of L*, a*, b* value not followed by the same letter differ ($p \leq 0.05$)

Table 3.4 CIE hue*, SI*, WI* values of fresh catfish fillets before and after postmortem chilling process.

Chilling	hue (arctan (b*/a*))	SI $((a^{*2} + b^{*2})^{1/2}$	WI $(100-[(100-L^*)^2 + a^{*2} + b^{*2}]^{1/2}$
Before	177.13 ± 106.95b	2.01 ± 1.57	48.42 ± 4.39b
After	229.53 ± 59.81a	2.11 ± 1.05	51.06 ± 5.13a
LSD (0.05)	13.04	0.20	0.72
C.V%	39.97	59.98	9.00

Note: ab: means of L*, a*, b* value not followed by the same letter differ ($p \leq 0.05$)

Table 3.5 Distribution of CIE L*, a*, b* values of fresh catfish fillets before and after postmortem chilling process

CIE L* values							
Chilling	<42.99	43-45.99	46-48.99	49-51.99	52-54.99	55-57.99	58<
Before	11.3%	16%	33.3%	25.3%	10%	2%	2%
After	2.7%	6.7%	18.7%	33%	19.3%	11%	9.3%
CIE a* values							
	<-2	(-1.0)-(-1.99)	0-(-0.99)	0.01-1	1.01-2	2.01-3	<3
Before	0%	19.3%	44%	16.7%	8.7%	6%	5.3%
After	2.7%	47%	31%	11.3%	6%	0.6%	2%
CIE b* values							
	<-3	(-1.5)-(-2.99)	0-(-1.49)	0.01-1.5	1.51-3	3.01-4.5	<4.51
Before	10.7%	28.7%	28%	22.7%	7.3%	2.7%	0%
After	24%	38%	26.7%	7.3%	4%	0.6%	0%

Table 3.6 Hunter color values and total heme-protein content in "white" and "reddish" catfish fillets.

		White fillets	Reddish fillets
Hunter	L	55.95 ± 1.49a	52.80 ± 1.56b
	a	-0.93 ± 1.38a	3.51 ± 1.31b
	b	8.70 ± 1.02	8.24 ± 0.89
	hue (arctan (b/a))	96.41 ± 8.91a	67.26 ± 7.66b
	SI $((a^2 + b^2)^{1/2})$	8.86 ± 1.0	9.04 ± 1.04
CIE	L*	62.75 ± 1.72a	59.81 ± 1.46b
	a*	-1.09 ± 1.63b	4.12 ± 1.53a
	b*	11.53 ± 1.51	11.19 ± 1.32
	hue* (arctan (b*/a*))	95.13 ± 7.38a	71.34 ± 6.36b
	SI* $((a^{*2} + b^{*2})^{1/2})$	12.47 ± 1.65	12.89 ± 1.50
Total heme-protein (mg/g)		1.37 ± 0.51	1.60 ± 0.41

Note: ab: means of L, a, b, hue, a*, b* and hue* value not followed by the same letter differ ($p \leq 0.05$)

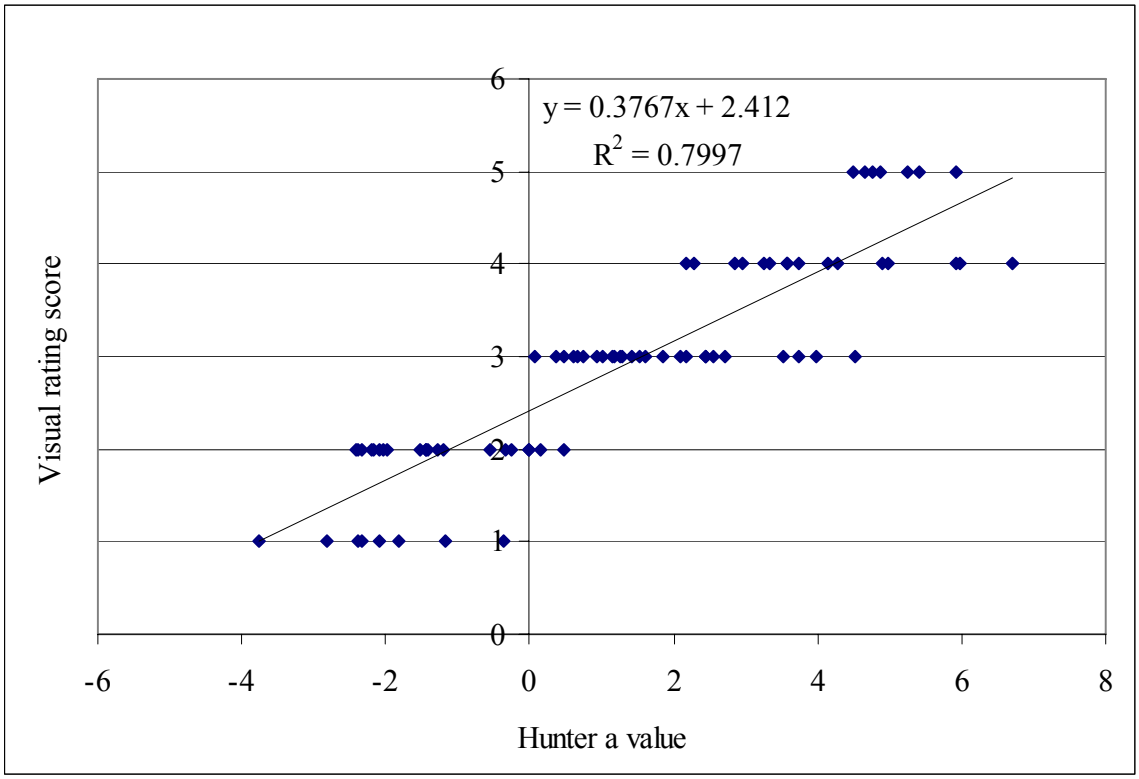

Figure 3.1 Linear correlation between visual rating scores and Hunter a values of fresh catfish fillets on the bone-side

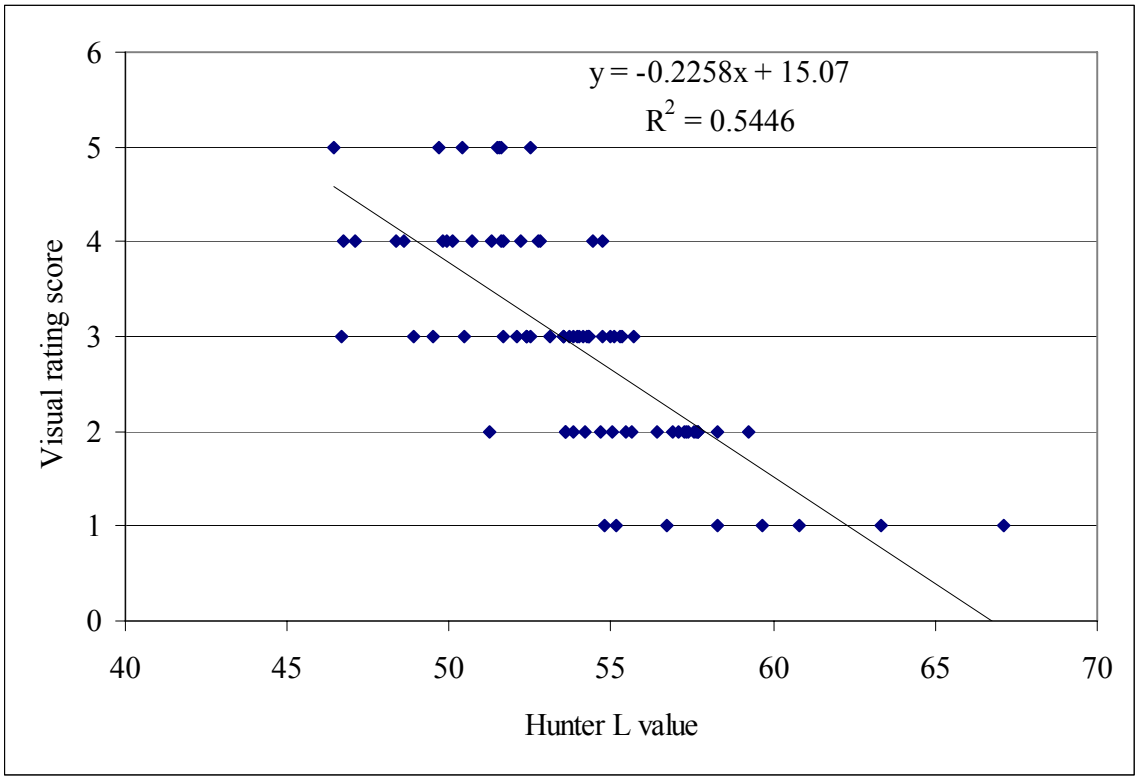

Figure 3.2 Linear correlation between visual rating scores and Hunter L values of fresh catfish fillets on the bone-side

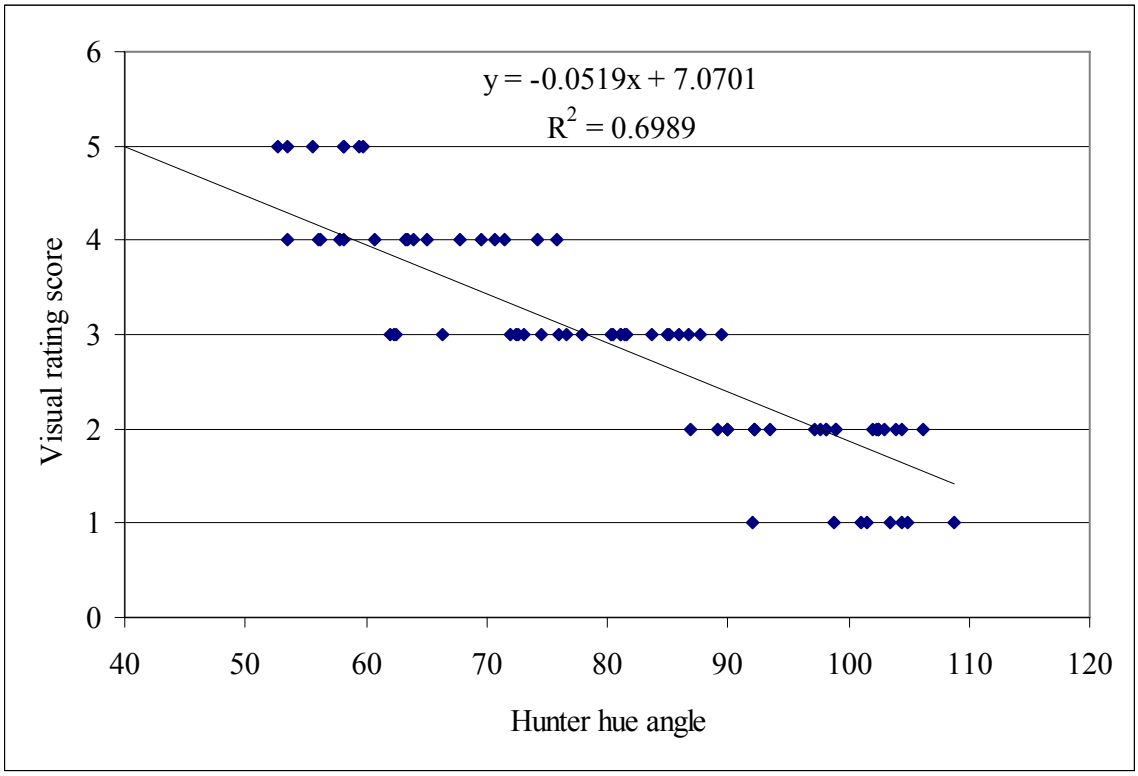

Figure 3.3 Linear correlation between visual rating scores and Hunter hue angle values of fresh catfish fillets on the bone-side

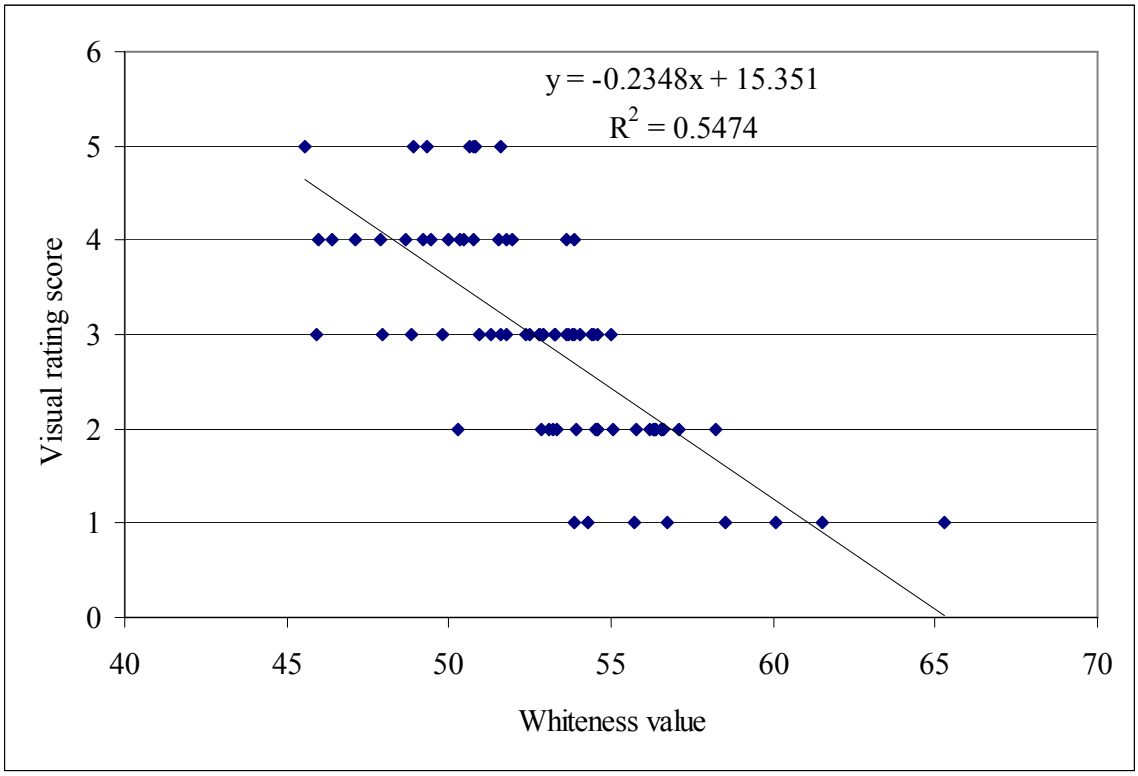

Figure 3.4 Linear correlation between visual rating scores and whiteness values of fresh catfish fillets on the bone-side

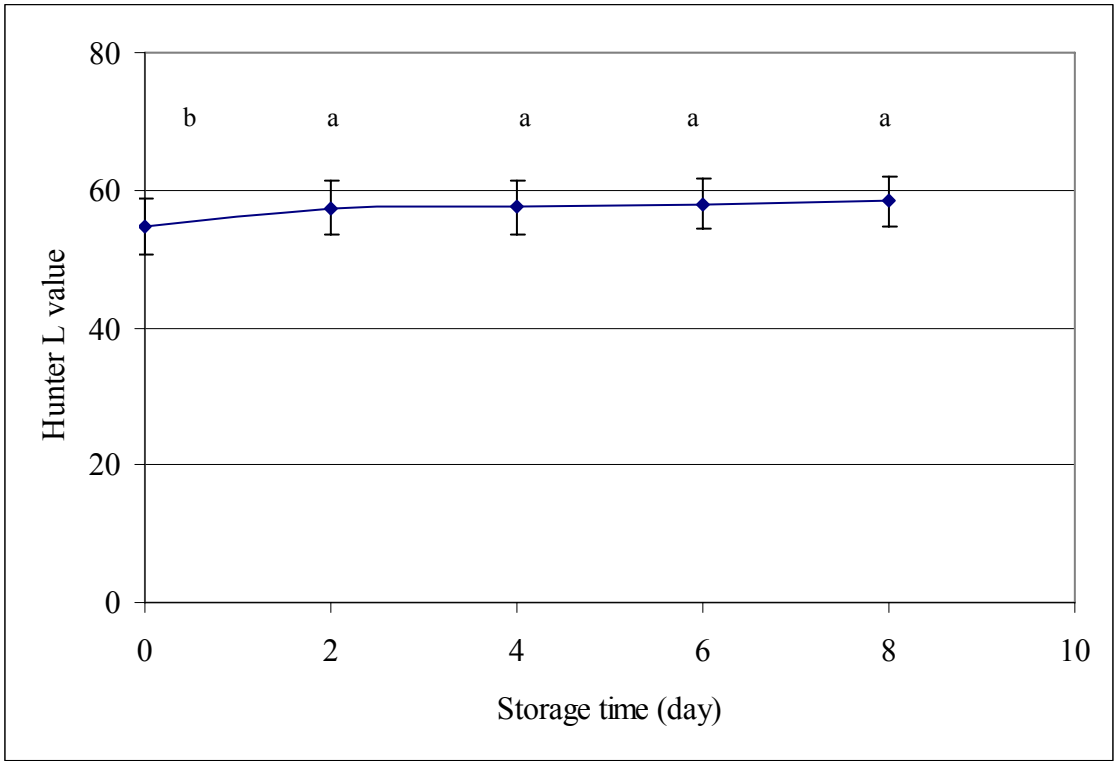

Figure 3.5 Hunter L values of fresh catfish fillets on the bone-side changed during ice-storage.

Note: ab: means of L value within storage time not followed by the same letter differ ($p \leq 0.05$)

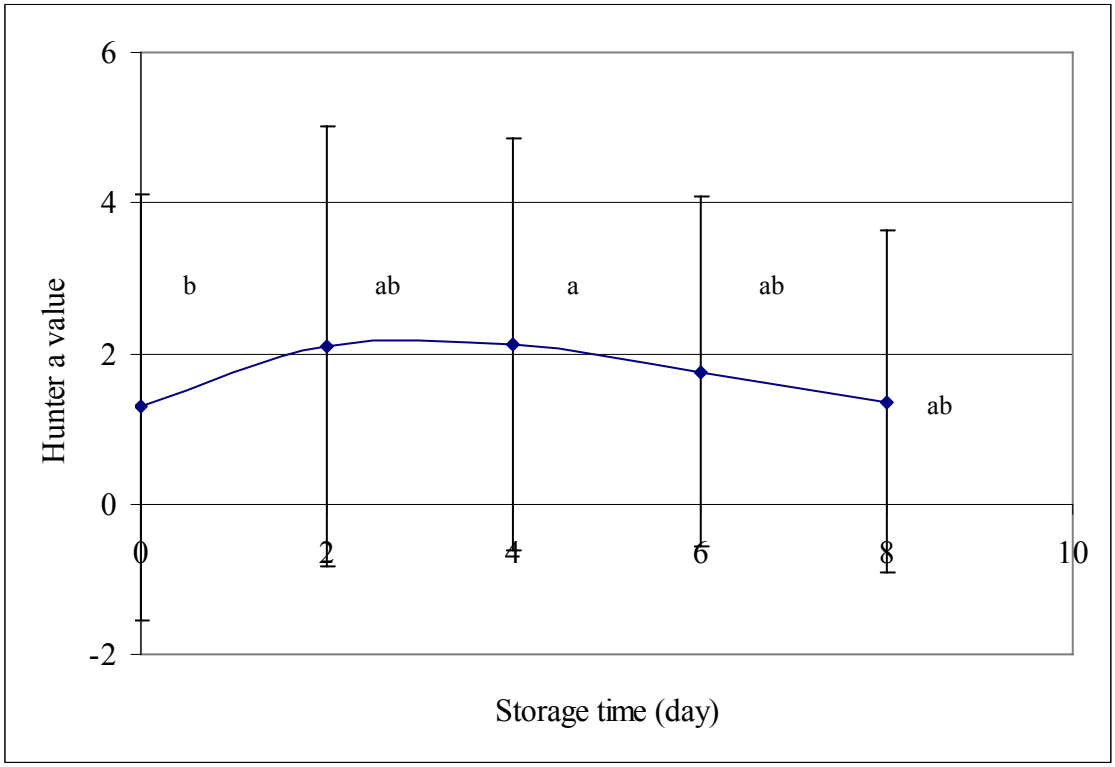

Figure 3.6 Hunter a values of fresh catfish fillets on the bone-side changed during iced storage.

Note: ab-means of a value within storage time not followed by the same letter differ ($p \leq 0.05$)

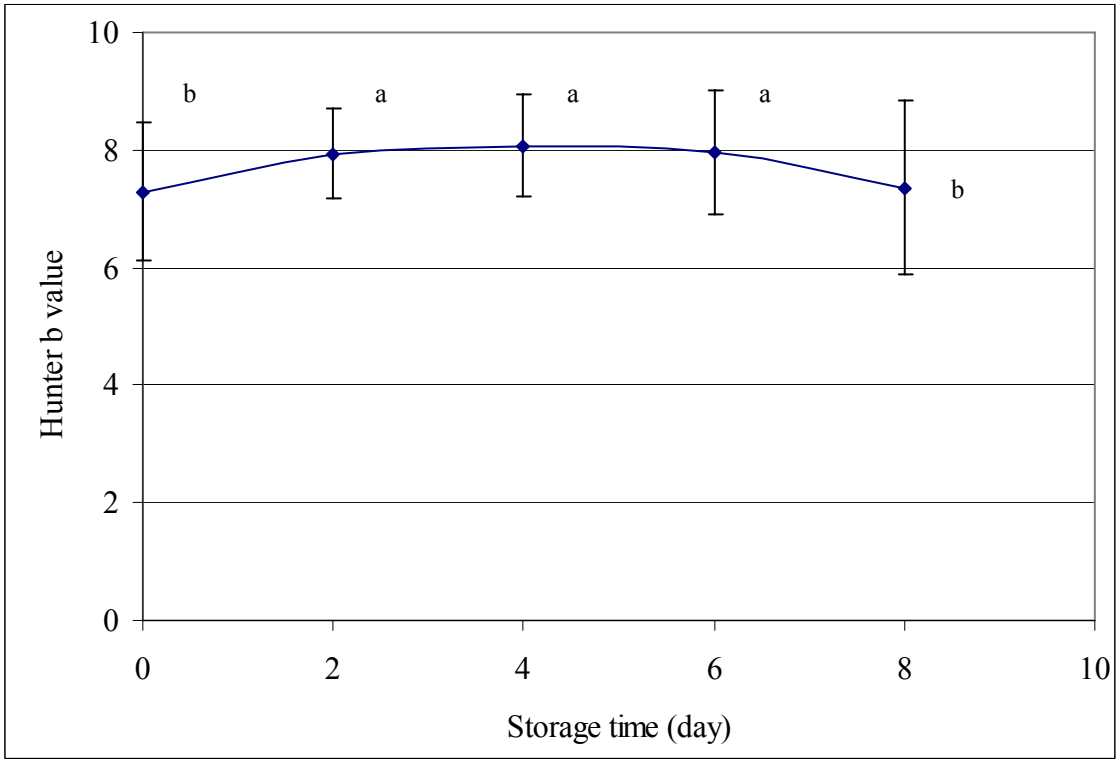

Figure 3.7 Hunter b values of fresh catfish fillets on the bone-side changed during iced storage.

Note: ab-means of b value within storage time not followed by the same letter differ ($p \leq 0.05$)

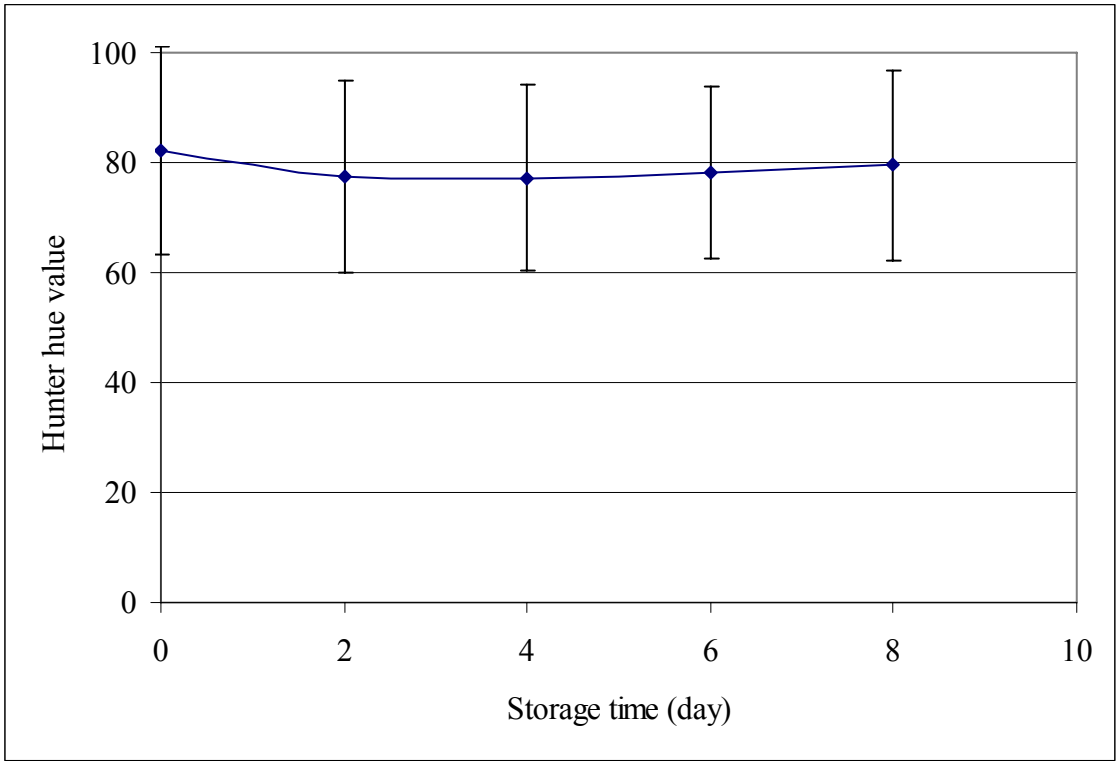

Figure 3.8 Hunter hue angle values of fresh catfish fillets on the bone-side changed during iced storage.

Note: no statistically significant differences were found in the mean of hue values among storage time.

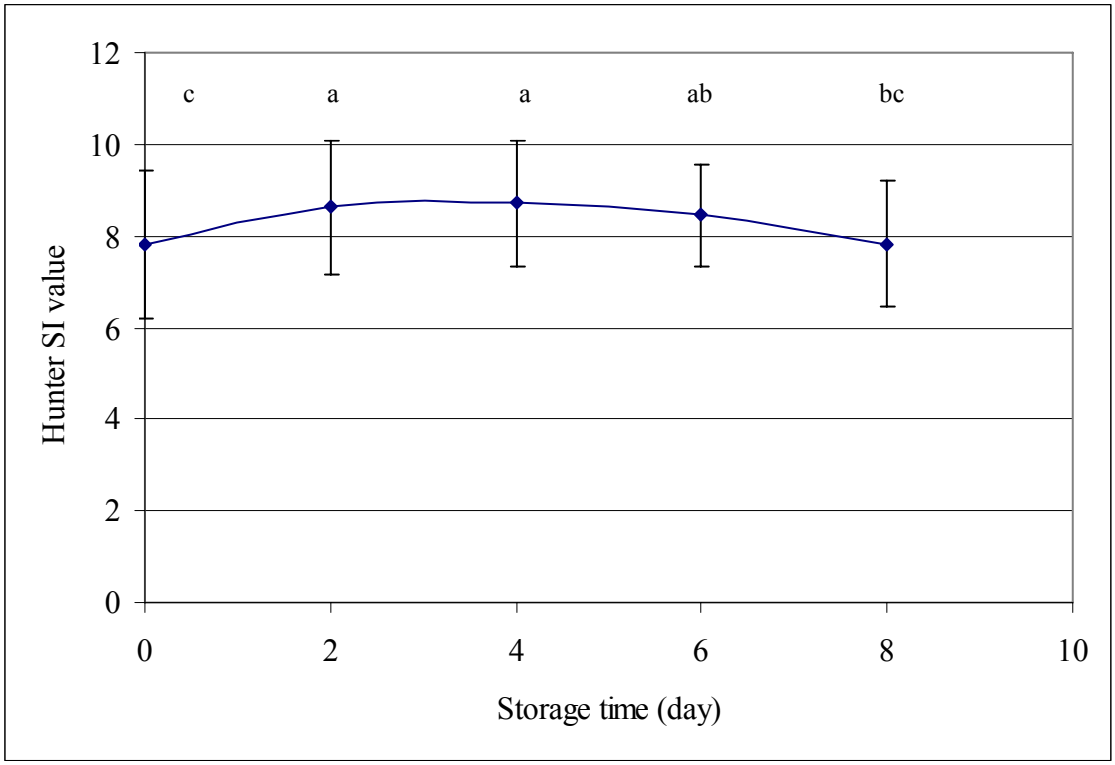

Figure 3.9 Hunter SI values of fresh catfish fillets on the bone-side changed during iced storage.

Note: ab: means of b value within storage time not followed by the same letter differ ($p \leq 0.05$)

CHAPTER IV

EXPERIMENT II: CHARACTERIZATION OF CHANNEL CATFISH HEMOGLOBIN AND MYOGLOBIN

1. Introduction

Since myoglobin and hemoglobin are responsible for meat color and color changes, and the autoxidation of these pigments from bright red to brown is considered to be a loss of the freshness (Faustman and Cassens 1990), myoglobin and hemoglobin contents are important aspects in muscle meat quality (Kranen and others 1999, O'Brien and others 1992, Han and others 1994, Sakai and others 2006). In addition, myoglobin and hemoglobin are differentiated by their expression in different cell types, variable rates of autoxidation and different mechanisms for promoting lipid oxidation (Richards and others 2005, Richards and others 2002, Richards and Hultin 2002).

Many protocols have been developed to separate hemoglobin and myoglobin (Kranen and others 1999, O'Brien and others 1992, Han and others 1994, Sakai and other 2006, Richards and Hultin 2002, Nishida and Nishida 1985). Separation is based on the size, charge, polarity and solubility of myoglobin and hemoglobin. Hemoglobin and myoglobin are similar proteins in structure and physiological functions. However, hemoglobin is a tetrameric protein with two α and two β polypeptide subunits, whereas

myoglobin is a monomeric polypeptide protein. The myoglobin and hemoglobin subunits are compact structures composed of eight α helixes. The eight α helixes are arranged in a hollow structure and the heme ring is embedded inside the helical structure. This arrangement limits the accessibility of other small ligands such as carbon monoxide and cyanide to the heme iron and reduces these toxic compounds' affinity to heme a 100-1000 fold compared to free heme. The globular structure of the protein also limits the autoxidation of ferrous iron to ferric iron, thus protecting the oxygen binding ability.

In the myoglobin and hemoglobin structures, the hydrophobic residues of amino acids embedded inside the hollow and hydrophilic residues are exposed to the surface. The subunits of hemoglobin interact with each other by hydrophobic interactions to form the tetramer. These hydrophobic interactions make hemoglobin more hydrophobic and less water soluble than myoglobin. This is confirmed by the fact that hemoglobin precipitates in a 40% saturation of ammonium sulfate solution, whereas, myoglobin precipitates in a minimum of 50% saturation of ammonium sulfate solution (Kranen and others 1999). Many researchers have utilized solubility properties to separate myoglobin and hemoglobin (Nishida and Nishida 1985, O'Brien and others 1992). But the co-precipitation of hemoglobin and myoglobin exists at 75% saturation of ammonium sulfate solution and the application of this method may not be able to precisely measure the concentration of myoglobin and hemoglobin in meat (Kranen and others 1999). Even though myoglobin and hemoglobin may be the most important appearance/color components in fresh catfish fillets and are critical in determining color and lipid stability,

the contents and their distribution in fresh channel catfish muscle have never been studied.

Autoxidation of hemoglobin and myoglobin leads to browning of the meat during storage. The rate of autoxidation of myoglobin and hemoglobin determines color stability and shelf life in meat products. Autoxidation of myoglobin and hemoglobin can also produce superoxide anion radicals which, forms the ferry radical with heme protein after dismutating to hydrogen peroxide (Grunwald and Richards 2006, Harel and Kanner 1985). Ferry radical species are the initiators of lipid oxidation (Harel and Kanner 1985).

Cloning and characterization of myoglobin and hemoglobin genes of human and other animals have been extensively studied (Varadarajan and others 1985, Lurman and others 2007, Ueki and Ochiai 2004). Yeh and others (2006) cloned and sequenced the hemoglobin beta gene of channel catfish from the head kidney cDNA library. They reported that the channel catfish hemoglobin beta gene is composed of 600 nucleotides with both 5'- and 3'- untranslated regions. The open reading flame encodes 148 amino acids, where the amino acids related to the heme iron are conserved. But they did not induce the expression of this protein. There is also no report regarding the characterization of the channel catfish myoglobin gene.

The objectives of this experiment were 1) to determine the rate of autoxidation of catfish hemoglobin at room and refrigeration temperatures; 2) to clone the myoglobin and hemoglobin beta genes from the channel catfish head kidney cDNA library and express these two proteins in *Escherichia coli* cells; 3) to sequence myoglobin cDNA; 4) to raise

myoglobin and hemoglobin beta antibodies; 5) to separate, identify and quantify myoglobin and hemoglobin from visual "white" and "reddish" catfish fillets.

2. Materials and Methods

(1). Hemoglobin preparation and its autoxidation

Channel catfish (*Ictalurus punctatus*) hemoglobin was prepared from blood. Sodium phosphates used for preparation of buffer were purchased form Sigma (Saint Louise, MO, USA). Other chemicals that were used in this experiment were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Catfish blood was collected into centrifuge tubes containing 20 ml heparin in 1.7% NaCl solution (50 µl of sodium heparin, 5000IU/ml) and stored immediately on ice. Blood was centrifuged at 6500 g (CU-5000, IEC, Needham Heights WA, USA) for 5 min to remove serum. A four-volume of ice cold saline (1.7% NaCl in 1 mM Tris, pH 8) was added to the packed red blood cells and centrifuged at 6500 g for 5 min in the same centrifuge. The cells were washed three times in 10 volumes of this saline solution and centrifuged at 6500g with the same centrifuge. Cells were lysed with 3 volumes of 1 mM Tris-HCl buffer (pH 8.0) for 1 h and centrifuged at 6500 g for 20 min. Total hemoglobin concentration with a molar extinction coefficient of 11.3×10^3 was determined by a spectrophotometric method (Fleming and others 1960). Blood hemolysate (hemoglobin) was diluted into 20 mM sodium phosphate buffer (pH 6.3) to a concentration of 80 µM heme basis and scanned between 500 nm to 700 nm for samples stored at either 4°C or room temperature (25°C) at 30 min intervals for 6 h. The hemoglobin oxidative components were calculated by the Benesch equation

(Benesch and others 1973). The rate of methemoglobin development was calculated as the rate of hemoglobin autoxidation at different temperatures. A Genesys^{TM} UV-visible spectrophotometer (Spectronic Instruments, Rochester, NY, USA) was used to measure all the absorption and spectrum scanning.

(2). Hemoglobin beta-subunit gene and myoglobin gene cloning from cDNA library

The channel catfish head kidney cDNA library was generously provided by Dr. Hung-Yueh Yeh (USDA-ARS, Aquatic Animal Health Research Unit, Auburn, AL, USA). The DNA fragment encoding Myoglobin cDNA was amplified from the cDNA library by *Taq* polymerases (Sigma, Saint Louis, MO, USA) in an Autorisierter Thermocycler (Mastercycler Gradient, Eppendorf, Westbury, NY, USA) using forward (5'-CCAATTCCATATGTCTGACTT TGACACCG-3') and reverse primers (5'-CCCAAGCTTCTAATGATGATGATGATGATGGCCGCGAAGCCGAGCTCC-3') (Sigma Genosys, Woodlands, TX, USA). The conditions for PCR were as follows: denaturation at 94^{o}C for 30 sec, annealing at 52^{o}C for 30 sec and amplification at 72^{o}C for 80 sec for 34 cycles. Since the yield was very low, the amplified PCR product was reused as the template for another amplification for 29 cycles by *Pfu* polymerase (Stratagene, La Jolla, CA, USA) with the same amplification conditions. In addition to the recommended reaction components, a 5% solution of dimethyl sulfoxide (DMSO, Sigma) was added to the mixture to eliminate any secondary structures of DNA. The gene encoding hemoglobin beta subunit was amplified with *Taq* polymerases (Sigma) by using a forward primer (5'-CCAATTCCATATGGTTCATTGGACAGAACGCC-3'); and a reverse primer (5'-

CCCAAGCTTTTAATGATGATGATGATGGTGGTATACCTGCTTTCCCAGAGC-3') (Sigma Genosys) with similar conditions as the first amplification of myoglobin cDNA. Forward primers for both the myoglobin and hemoglobin beta genes contained an *Nde*I restriction site, and reverse primers for these two genes contained a *Hind*III restriction site and a six-histidine tag at the N-terminus. The amplified PCR products were visualized in 2% agarose gel, excised from the gel under UV light and extracted with a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Extracted DNA and pET-29a (+) vector plasmid (Novagen, Madison, WI, USA) were digested with *Nde*I and *Hin*dIII restriction endonuclease (New England Biolabs, Ipswich, MA, USA) at 37^{o}C overnight. The restriction reaction was terminated by incubating the reaction mixture at 65^{o}C for 20 min. Digested samples were loaded onto a 1.5% agarose gel along with a 1 kb ladder (Promega, Madison, WI, USA). Gel slides with target DNA under visualization were excised and extracted again with a QIAquick gel extraction kit (Qiagen). After estimate the concentration of digested DNAs in 1.5% agarose gel, target cDNA fragments were ligated into a pET-29 a (+) vector at the molar ratio of 6:1 with T4 DNA ligase (New England Biolab) at 16^{o}C for 3h and transformed into *Escherichia coli* DH5α (Novagen). Transformation was performed on ice for 30 min, then heat shocking at 37^{o}C for 45 sec and icing again for another 2 min. Cells were incubated at 37^{o}C for 1 h after adding 1 ml Luria-Bertani Medium (LB medium, Difco^{TM}, Becton Dickinson and Co, Sparks, MD, USA). A 140 µl of cells was plated to an LB agar plate containing 100 µg/ml of ampicillin (Sigma). The plate was incubated at 37^{o}C for 12 h. A single colony was selected and inoculated into 3.5 ml of LB liquid medium with 100 µg/ml of

ampicillin (Sigma) and incubated in a 37°C shaker at 250RPM for 10 h. Cells were harvested and plasmids were isolated with a QIAprep plasmid isolation kit (Qiagen). Aliquots of isolated plasmids were subjected to restriction enzyme digestion with *Nde*I and *Hind*III. A 1.5% agarose gel electrophoresis indicated that either the gene encoding hemoglobin beta-subunit or myoglobin had been successfully inserted into the pET 29a (+) plasmids. Twelve µl isolated plasmids with either the gene of beta-subunit of hemoglobin or myoglobin were transformed to *E. coli* BL21 (DE3) (Novagen) with 50 µg/ml kanamycin (Sigma) by incubation on ice for 5 min, heating at 42°C for 30 sec and then on ice for another 2 min, followed by incubation at 37°C for 1 h after adding 1 ml LB liquid medium. After shaking at 37°C for 1 h, 200 µl of cells were plated onto LB plates with 50 µg/ml kanamycin and incubated at 37°C overnight. Single colonies were inoculated into 3.5 ml liquid LB medium with 50 µg/ml kanamycin and incubated in a 37°C shaker for 6h. About 50 µl of the cells were inoculated into 250 ml LB medium containing 2 mM hemin chloride (Sigma), 2 mM $FeCl_3$ (Sigma) and 1 mM isopropyl-D-thiogalactopyranoside (IPTG) (Sigma) and incubated at 37°C in a shaker for 12 h to express proteins. A preliminary experiment indicated that proteins can be expressed with or without the addition of IPTG.

(3). SDS-PAGE confirmation and purification of expressed hemoglobin beta-subunit and myoglobin

Cultured cells were harvested by centrifugation at 5500 g for 10 min at 4°C in a Sorvall Centrifuge (DuPont, Wilmington, DE, USA) and broken in 10 mM Tris-HCl buffer (pH 8.6) by sonication with a cell disruptor (Sonifier®, Branson, Danbury, CT,

USA) on ice in the presence of nitrogen gas. For each 1 min of sonication, a 1 min burst interval was done for a total of 5 min sonication. After centrifugation, the supernatant and pellet were sampled to analyze the presence of the expressed protein in a 15% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA, USA) gel in a minigel system (Bio-Red, Palo Alto, CA, USA) according to the conditions recommended by the manufacturer, 1 h in 200 V. The SDS-PAGE gel indicated that while half of the hemoglobin beta-subunit was expressed in a soluble form and half was expressed in insoluble form in inclusion body, all of the expressed myoglobin was in an insoluble form (inclusion body). The soluble form of the hemoglobin beta-subunit was purified from the supernatant using a zinc chelated affinity chromatography column (GE Healthcare Life Sciences, Piscataway, NJ, USA). The loading buffer was 20 mM Tris-HCl (pH 8.6) plus 0.5 M sodium chloride. The washing buffer was 20 mM Tris-HCl (pH 8.6) plus 0.5 M NaCl plus 10 mM imidazole (Sigma), and the eluting buffer was 250 mM imidazole in 20 mM Tris-HCl (pH 8.6) plus 0.5 M NaCl.

(4). Sequencing of myoglobin cDNA

Channel catfish myoglobin cDNA was sequenced by the chain-termination method from the amplified cDNA sequence with similar primers in myoglobin cloning. The PCR conditions to amplify cDNA for sequencing were as follows. It was first denatured at 96^{o}C for 2 min. Then reactions were conducted in 30 cycles with denaturation at 96^{o}C for 10 sec, annealing at 50^{o}C for 5 sec and chain extension at 60^{o}C for 4 min. The amplification reaction was performed in an Autorisierter Thermocycler (Mastercycler Gradient, Eppendorf) with an ABI Prism BigDye Terminator Cycle

Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). After amplification, the DNA was precipitated in ethanol, vacuum dried and resuspended in Hi-Di™ Formamide (Applied Biosystems). The sample was then loaded onto an ABI Prism Genetic Analyzer (Applied Biosystems) for sequence analysis.

(5). Antibody production

Due to failure to induce expression of the soluble form of myoglobin and the precipitation of the soluble form of hemoglobin beta after separation, we decided to raise antibodies against myoglobin and hemoglobin beta from the synthesized short peptides (15 amino acids). They were picked up by GenSript Corp (Piscataway, NJ, USA) from the amino acid sequences deduced from myoglobin gene sequence obtained by us, and the hemoglobin beta gene sequence reported by Dr. Yeh and others (2006). These sequences were the least conserved from other catfish. The synthesized peptides used to raise polyclonal antibodies against myoglobin and hemoglobin beta in rabbits were: peptide hemoglobin beta-subunit: KINHDEIGGQALARC; peptide myoglobin: PETQKLFEHPETQKC.

(6) Extraction of myoglobin and hemoglobin from "white" and "reddish" fillets and separation by HPLC

Randomly selected "white" and "reddish" catfish fillets (by visual observations) from the processing line after chilling were packed individually in Zipoc® bags, placed on ice, prior to transport to the Food Processing Laboratory at Mississippi State University. Myoglobin and hemoglobin were extracted from visual "white" and "reddish" catfish

fillets with the similar protocol as extraction of total heme-protein described in Chapter III.

A 500 µl extract was centrifuged at 14000 g for 5 min with an Eppendorf centrifuge (Centrifuge 5415C, Eppendoff) and filtered through a 0.4 µm syringe filter (Millipore, Bedford, MA, USA). A 10 µl of the sample was injected into a HPLC system (Agilent 1100 series, Agilent Technnologies, Santa Clara, CA, USA) with DAD (diode array detector). The mobile phase was solvent A: acetonitrile (Fisher Scientific) with 0.1% trifluoreic acetic acid (TFA, HPLC grade, Fisher scientific) and solvent B: water (ACS grade, Ricca Chemical Co, Arlington, TX, USA) with 0.1% TFA. A linear gradient suitable for myoglobin and hemoglobin separation was used as follows: at 0 min, 25% solvent A, 75% solvent B; at 25 min, 100% solvent A, 0% solvent B; at 30 min, 25% solvent A, 75% solvent B; at 36 min, 25% solvent A, 75% solvent B. The flow rate was 0.6 mL/min. A Gemini C8, 250 x 4.6 mm column (Phenomenex®, Lafayette, IN, USA) was used at 25°C. Myoglobin and hemoglobin were detected at 412 nm. Retention time and concentration were calculated from horse myoglobin (Sigma) and fish blood hemoglobin.

(7). **Native- and SDS-polyacrylamide electrophoresis**

Fish muscle extracts were analyzed with native- and SDS-polyacrylamide electrophoresis. After a 1:1 ratio mixing with sampling buffer, 20 µl of the sample were loaded onto the native polyacrylamide gel in a Mini-Gel System (Bio-Rad) and electrophoresis was performed. The sample with loading buffer for SDS-gel was boiled at 100°C for 10 min before loading on the gel. The native/SDS polyacrylamide gels used

15% polyacrylamide as a resolving gel and 5% polyacrylamide as the stacking gel. Both native gel and SDS gel followed the procedures of Laemmli (1970) with the elimination of SDS and mercathanol in the native gel. Native gel electrophoresis was performed at 200 V for 2.5 h inside a 7^{o}C low temperature incubator (Precision, GCA Corp, New York, NY, USA) to prevent overheating. SDS-PAGE was performed at 200 V power for 1 h at room temperature. Proteins in gels were visualized by either coosmassie brilliant blue method or silver staining method with the exception of those that were used for Western blot transformation.

(8). Immunoblotting of myoglobin and hemoglobin

Native proteins from the fish extracts in native gels after electrophoresis were transformed to a PVDF membrane (Immobilon^{TM}-P, Millipore, Billerica, MA, USA) overnight under 30 V at 7^{o}C in a Mini-Gel System (Bio-Rad). Blocking and antibodies attachment was performed with a TSB-T buffer following the company instructions (Amersham Bioscience, Piscataway, NJ, USA). ECL anti-rabbit IgG horseradish peroxidase linked whole from donkey (Amersham Bioscience) was used as the second antibody and the florescence signal was exposed to ECL X-ray film (Amersham Bioscience) and developed.

3. Results and Discussion

(1). Hemoglobin autoxidation

The rates of autoxidation of hemoglobin between room temperature (25^{o}C) and refrigeration temperature (4^{o}C) at pH 6.3 were compared (Figure 4.1). The rate of

autoxidation of hemoglobin was expressed as the percentage of methemoglobin formation in each minute. The rate of hemoglobin autoxidation at room temperature (25^{o}C) was 0.0126% methemoglobin formation per minute. The rate of hemoglobin autoxidation at refrigeration temperature (4^{o}C) was 0.0113% methemoglobin formation per minute. Thus, the rate of hemoglobin autoxidation at refrigeration temperature was only slightly different from that at room temperature. The formation of methemoglobin at room temperature increased linearly with time; however, the formation of methemoglobin at refrigeration temperature was highly variable. The variation of methemoglobin formation at refrigeration temperature might be due to the fact that lower temperature affected the spectrophotometric measurement.

Chaijan and others (2003) studied the autoxidation rate of sardine myoglobin at different temperatures. They reported that there was no obvious metmyoglobin formation in 10 min at temperatures lower than 20^{o}C; however, the rate of autoxidation increased with the increase of temperature from 20^{o}C to 70^{o}C, and the metmyoglobin formation increased by 9.81% in 10 min at 70^{o}C. Harrington (1986) studied the autoxidation rate of the total hemoglobin of king salmon at 25^{o}C in 0.1M phosphate buffer. He reported that at pH 6, autoxidation led to 38% methemoglobin formation in 150 min (0.253% per min). The rate of autoxidation was related to the molecular oxygen level and proportion of deoxygenated hemoglobin (Shikama 1998). The autoxidation of myoglobin and hemoglobin only occurs in the presence of oxygen; whereas, high level of partial pressure of oxygen prevents autoxidation (Shikama 1998). Deoxy-forms of myoglobin and hemoglobin are much more sensitive to oxidants for autoxidation than oxy-forms. Thus,

the autoxidation of oxymyoglobin and oxyhemoglobin is believed to be caused by the dissociation of molecular oxygen from oxymyoglobin and oxyhemoglobin and the oxidation of deoxymyoglobin and deoxyhemoglobin is molecular oxygen (Shikama 1998). Since fish anodal hemoglobin exhibits the Bohr Effect and the Root Effect at low pH (lower oxygen affinity thus increasing the proportion of deoxy form of hemoglobin at low pH), thus, the rate of autoxidation of hemoglobin is pH dependent with an increase in reaction rate with pH decrease. Furthermore, an increase of hydroxyl ions also enhances the autoxidation rate (Shikama 1998). Cathodal and anodal components in fish hemoglobin also have different autoxidation rates. The rate of autoxidation of myoglobin and hemoglobin is variable from species to species (Shikama 1998, Richards and Dettmann 2003). Richards and Dettmann (2003) reported that the autoxidation rate of trout hemoglobin was more rapid than those of chicken and beef hemoglobin, and that it was faster in chicken than beef hemoglobin.

(2). Cloning and expression of hemoglobin beta-subunit gene and myoglobin gene in *E. coli* BL21 (DE3) strain

The myoglobin gene was amplified from the channel catfish head kidney cDNA library by *Taq* polymerase. Because the yield was very low, the *Taq* polymerase amplified product was used as the template to amplify the gene again using *Pfu* polymerase with the same primers and similar reaction conditions. The amplified product was 462 nucleotides long (Figure 4.2) included an 18 nucleotide coding for a six-histidine tag. The open reading frame of the myoglobin gene was 444 nucleotides. The hemoglobin beta gene was directly amplified from the channel catfish head kidney cDNA

library by *Taq* DNA polymerase. The gene contained 526 nucleotides (Figure 4.3), which included an 18 nucleotide coding for a six-histidine tag. Both myoglobin and hemoglobin beta genes were cloned into pET 29a (+) plasmid system (Figure 4.4).

Several conditions were examined for the expression of myoglobin and hemoglobin beta. These conditions included culture temperature, with or without the IPTG, and lactose induction. Besides these conditions, 2 mM hemin chloride and 2 mM $FeCl_3$ were also added for the formation of functional hemoglobin beta and myoglobin with which, the heme moiety complexes with globular protein. Both the channel catfish beta-hemoglobin gene and the myoglobin gene with a histidine tag could be expressed in the BL21 host with or without the induction of IPTG or lactose (Figure 4.5). Large amounts of expressed myoglobin aggregated together and formed an insoluble inclusion body. There was no expressed myoglobin present in the water soluble fraction of cell lysate. A change of culture temperature from 37^{o}C to 15^{o}C that was used to induce water soluble expressed myoglobin did not change the expression pattern. Lower temperatures were supposed to decrease the expression of myoglobin and thus increased the expressed protein solubility; however, we did not achieve the expected result. Hemoglobin beta also formed an inclusion body at normal cultural temperature (25^{o}C); whereas, a fraction of hemoglobin beta subunits was also expressed in a soluble form along with the insoluble form. The ratio of water soluble form to water insoluble form was about 1:1. The water soluble form of hemoglobin beta could be purified with a zinc ion chelated affinity chromatographic column (Figure 4.6). However, it precipitated out during the subsequent frozen storage and could not be solubilized again.

(3). Myoglobin gene sequence

The channel catfish myoglobin cDNA sequence was composed of 444 nucleotides and this cDNA sequence is translated to 147 amino acids (Figure 4.7). The results of the blast algorithm (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (removing six-histidine tag sequence) with the database in the National Center for Biotechnology Information (NCBI) indicated that the channel catfish (*Ictalurus punctatus*) head kidney myoglobin cDNA has 97% similarity with the blue catfish (*Ictalurus furcatus*) heart myoglobin cDNA sequence. By using Compute pI/Mw online software (http://expasy.org/tools/pi_tool.html), the theoretical isoelectric point (pI) of this protein is 9.43 and the molecular mass is 16909. By using ProtPrarm online software (http://expasy.org/tools/protparam.html), the protein contains 17 negatively charged amino acid residues and 21 positively charged amino acid residues. The most abundant amino acids are: alanine, 12.2%; leucine, 10.3%; glycine, 9% and lysine, 8.3%. The protein contains 4 proline residues, occupied 2.6%. There is no cysteine residue presented in channel catfish heart kidney myoglobin cDNA, indicating no sulfhydryl residue in this protein. Sulfhydryl groups are found in some fish myoglobin and play an important role in myoglobin stability in these species (Dollars and others 1959, Chaijan and others 2007). By using online software Prosite (http://expasy.org/prosite/) to scan the amino acid sequence, results indicated that it is a globin family protein and the histidines 60 (the distal histidine) and 93 (the proximal histidine) are conserved for the interactions with iron atoms in porphyrin ring.

Ueki and Ochiai (2004) reported that the gene that encodes the myoglobin of bigeye tuna (*Thunus obesus*) was composed of a 5' non-coding region of 81 base pair and that an open reading frame contained 444 nucleotides and a 3' non-encoding region of 267 base pairs. The 444 nucleotides encode 147 amino acids and the deduced pI was 9.81 with a molecular mass of 15628. It also has the conserved proximal (His60) and distal (His89) histidine residues that contact with heme iron. In respect to the numbers of open reading frame nucleotide sequences and deduced amino acid sequences, those of the channel catfish myoglobin gene were similar to those of bigeye tuna, but the deduced pI and the molecular mass were higher than those of bigeye tuna.

(4). Myoglobin and hemoglobin separation from "white" and "reddish" fillets by HPLC and confirmed by SDS-PAGE and immunoblotting

By using a C8 column, myoglobin and hemoglobin could be separated by gradient washing of acetonitrile and TFA. In these conditions, myoglobin and hemoglobin in samples were denatured and could be monitored at 412 nm. Myoglobin was eluted at 10 min and hemoglobin was eluted at 11 min. Based on standard horse myoglobin and bovine hemoglobin, myoglobin content in "reddish" fillets was 0.43 ± 0.05 mg/g; hemoglobin was 1.65 ± 0.141mg/g. In "white" fillets, myoglobin was 0.51 ± 0.064 mg/g and hemoglobin was 1.47 ± 0.035 mg/g. There was no difference ($p > 0.05$) in both myoglobin and hemoglobin contents between "reddish" and "white" fillets. However, myoglobin content was 2 times lower ($p \leq 0.05$) than that of hemoglobin. This indicated that at least two thirds of the pigments in channel catfish muscle appear to be hemoglobin. SDS-PAGE (Figure 4.8) and Western blot analysis on native-PAGE (Figure

4.9) by antibodies against myoglobin and hemoglobin beta short peptides confirmed that most of the heme- proteins that were present in the muscles were hemoglobin. Myoglobin was undetectable. The HPLC results showed no differences ($p > 0.05$) in heme pigments between "reddish" and "white" fillets. SDS-PAGE and Western blot analysis showed similar results. However, visual and Hunter color values clearly indicated they had different expressions of visual color.

4. Conclusions

The channel catfish myoglobin gene and hemoglobin beta gene could be amplified from the head kidney cDNA library and inserted into a pET 29a (+) vector. The newly constructed recombinants of DNA could be expressed in *E. coli* BL 21 strain with or without the induction of IPTG or lactose. However, the myoglobin was expressed solely in the exclusion body and change of the incubation temperatures did not change the expression pattern. Hemoglobin beta could be expressed in a soluble form and purified with metal ion affinity chromatographic column if a histidine tag was inserted. However, the purified hemoglobin beta precipitated during storage so that it could not be used for antibody production. A sequence analysis indicated that the channel catfish myoglobin gene contained 444 nucleotides and could be translated into protein containing 147 amino acids. Even though the antibodies against channel catfish myoglobin and hemoglobin beta raised from short peptides picked up from deduced myoglobin and hemoglobin beta sequences were crossly reacted, the Western blot assay combined with SDS-PAGE still could determine the excessive pigments from "reddish" fresh catfish fillets were hemoglobin. Myoglobin concentration was low in both "white"

and “reddish” fresh catfish fillets when compared to hemoglobin level. In addition, the autoxidation rate of hemoglobin was only slightly different at room temperature compared to refrigeration temperature.

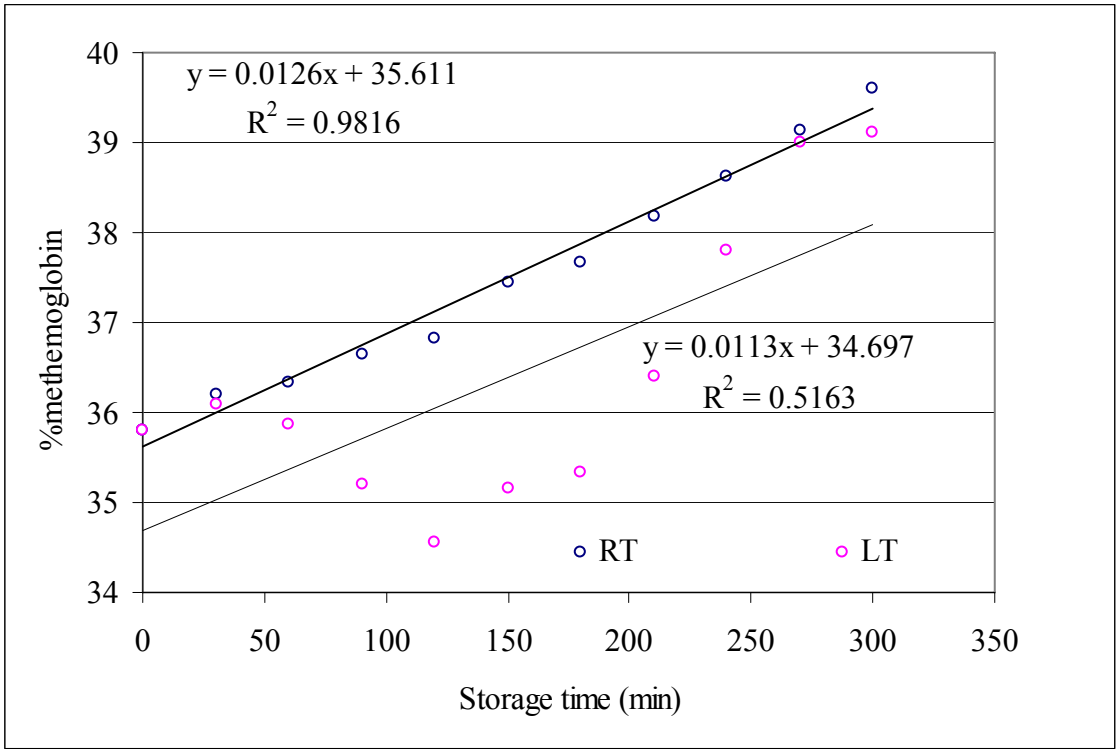

Figure 4.1 Autoxidation of hemoglobin at room temperature (25°C) and refrigeration temperature (4°C) at pH 6.3.

RT-room temperature; LT-refrigeration temperature.

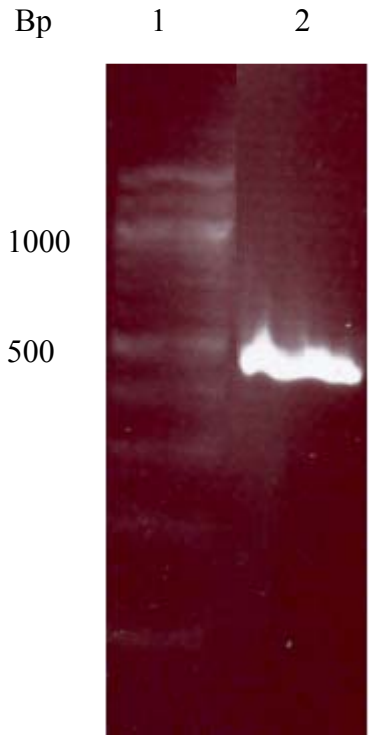

Figure 4.2 1.5% Agarose gel electrophoresis of PCR product of channel catfish myoglobin cDNA (with six-histidine tag sequence) from *Taq* amplified PCR product of head kidney cDNA library using *Pfu* polymerase.

Lane 1: 1 kb ladder; lane 2: myoglobin cDNA

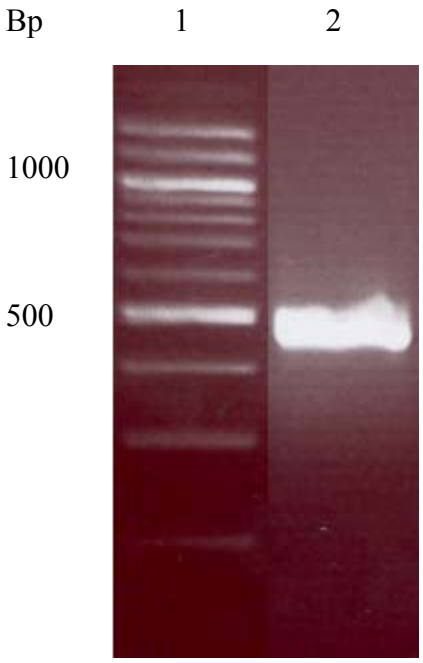

Figure 4.3 1.5% Agarose gel electrophoresis of PCR product of channel catfish hemoglobin beta cDNA (with six-histidine tag sequence) from head kidney cDNA library using *Taq* polymerase.

Lane 1: 1 kb ladder; lane 2: hemoglobin beta cDNA.

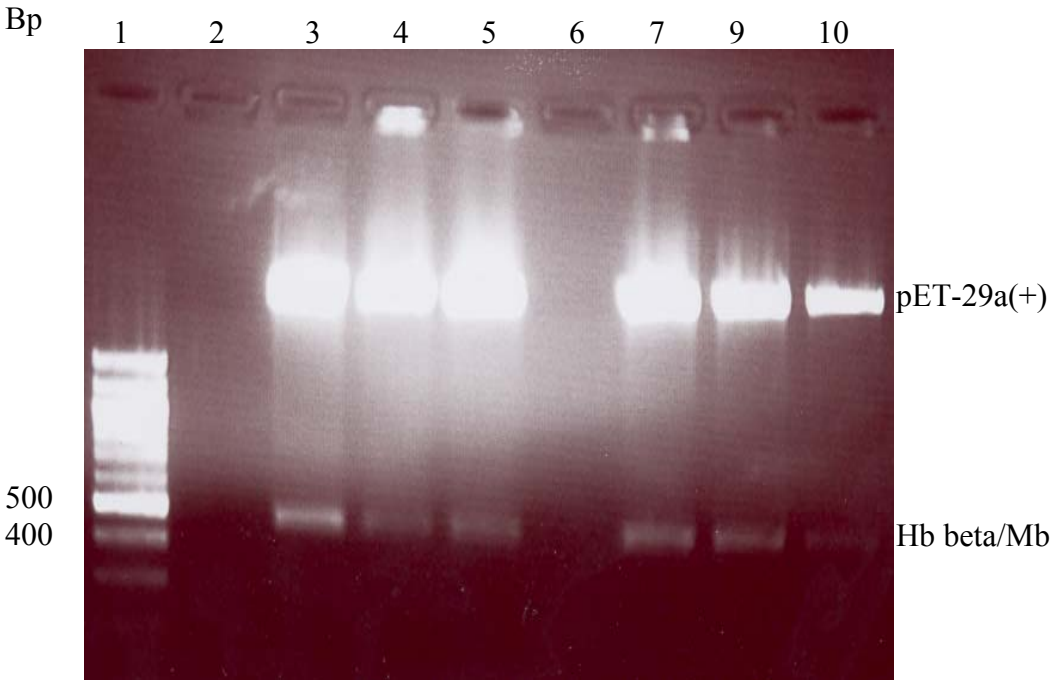

Figure 4.4 1.5% Agarose gel electrophoresis of *Ned*I and *Hind*III digested recombinant DNA of pET-29a (+) with either myoglobin or hemoglobin beta cDNA insert isolated from transformed *E. coli* DHα5, for successful cloning confirmation.

Lane 1: 100 bp ladder; lanes 3, 4, 5: recombinant with hemoglobin beta cDNA insert; lanes 7, 8, 9: recombinant with myoglobin cDNA insert

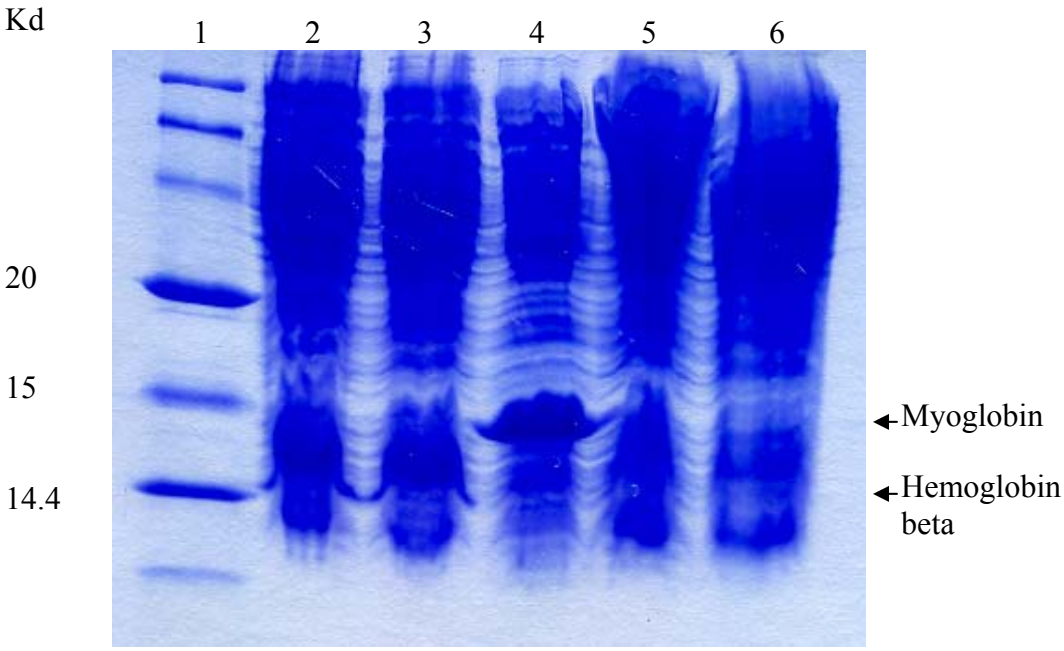

Figure 4.5 The expressions of channel catfish hemoglobin beta and myoglobin in *E. co*li BL21 (DE3) with either hemoglobin beta gene or myoglobin gene insert were confirmed by SDS-PAGE.

Lines 1: standard protein marker; 2: pellet fraction of BL21(DE3) cells with hemoglobin beta insert after cells subjected to sonication; 3: supernatant fraction of BL21 (DE3) cells with hemoglobin beta cDNA insert after cells subjected to sonication; 4: pellet fraction of BL21 cells with myoglobin cDNA insert after cells subjected to sonication; 5: supernatant fraction of BL21(DE3) cells with myoglobin cDNA insert after cells subjected to sonication; 6: BL21(DE3) cells without foreign cDNA insert (control). Note: myoglobin only was expressed in the pellet fraction (inclusion body); whereas, hemoglobin beta was expressed in both pellet and supernatant fractions.

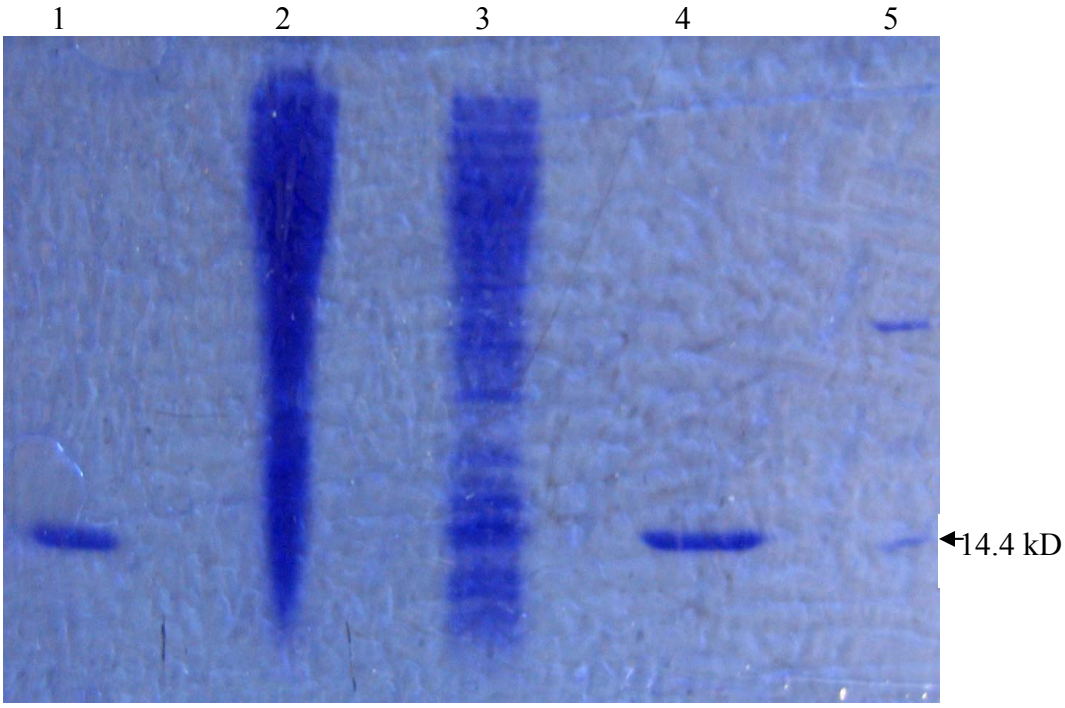

Figure 4.6 SDS-PAGE (15%) of expressed channel catfish hemoglobin beta-subunit before and after zinc ion chelated affinity chromatography with 250 mM imidazole elution.

Lane 1: diluted channel catfish hemolysate; 2: BL21(DE3) cells extract (without recombinant insert, control); 3: BL 21 cells extract (with recombinant insert, sonication breakdown, supernatant fraction); 4: zinc ion chelated column purified hemoglobin beta from lane 3; 5: protein marker, the lowest band indicates the molecular weight is 14.4 kD.

```
1   atgtctgactttgacaccgttctgacgagctggggcagcatggaagccaactatgctgcc
     M  S  D  F  D  T  V  L  T  S  W  G  S  M  E  A  N  Y  A  A
61  attggaggagaagttctgggccgtctgttcgtggagcaccctgaaacccagaaactcttc
     I  G  G  E  V  L  G  R  L  F  V  E  H  P  E  T  Q  K  L  F
121 cctaagtttgctgggatctccgctgctgatgcggctggaaatccggcggtcaaggcacat
     P  K  F  A  G  I  S  A  A  D  A  A  G  N  P  A  V  K  A  H
181 ggagaaaccgtcctgaaaaaactgggcgagctcatcaaggcaaaaggaaaccatgccgac
     G  E  T  V  L  K  K  L  G  E  L  I  K  A  K  G  N  H  A  D
241 atcctcaaaccactagctacatcccatgccaacatacacaagatcaccattaccaacttc
     I  L  K  P  L  A  T  S  H  A  N  I  H  K  I  T  I  T  N  F
301 aagctgatcagcgaaatcatcattaaggtgatggcagagaagggcctgctgaacagcggc
     K  L  I  S  E  I  I  I  K  V  M  A  E  K  G  L  L  N  S  G
361 gggcaggacgccatgagaagggtgttggctgccgtcatcaaacgacatagacgtctacta
     G  Q  D  A  M  R  R  V  L  A  A  V  I  K  R  H  R  R  L  L
421 caaggagctcggcttcgcggccatcatcatcatcattag
     Q  G  A  R  L  R  G  H  H  H  H  H  H  -
```

Figure 4.7 cDNA sequence and deduced amino acid sequence of channel catfish myoglobin.

The last codon TAG is the stop codon, and segment with underline from 442 to 459 is the continuous 6 CAT codons encoding a six-histidine tag that inserted for the purpose of facilitating the purification of expressed myoglobin by zinc ion affinity chromatography.

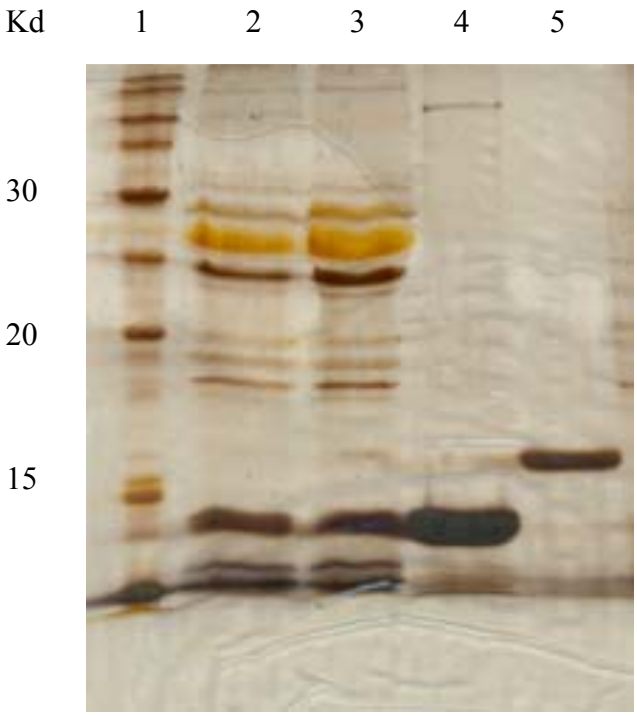

Figure 4.8 Channel catfish myoglobin and hemoglobin in SDS-PAGE from “white” and “reddish” fillets extracts by silver staining.

Lanes 1: protein marker; 2: “reddish” fillet extract; 3: “white” fillet extract; 4: catfish blood hemolysate; 5: horse muscle myoglobin

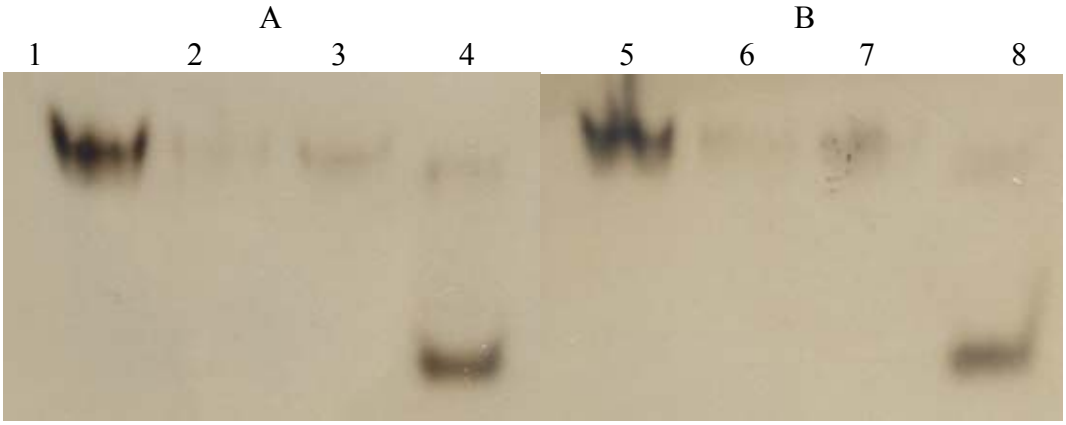

Figure 4.9 Immumoblotting of myoglobin and hemoglobin in "white" and "reddish" fillet extracts by polyclonal antibodies against short peptides selected from hemoglobin beta and myoglobin.

Proteins in extracts were separated in 15% polyacrylamide gel in native conditions and transferred to membrane. A: antibody against hemoglobin beta short peptide; B: antibody against myoglobin short peptide. Lanes 1, 5: catfish blood hemolysate; lanes 2, 6: "white" catfish fillet extracts; lanes3, 7: "reddish" catfish fillet extracts; lanes 4, 8: horse myoglobin.

CHAPTER V

EXPERIMENT III: EFFECT OF PRE-SLAUGHTER CHILLING AND BLEEDING ON PLASMA CORTISOL LEVEL, WHITE MUSCLE MYOGLOBIN GENE EXPRESSION, RIGOR DEVELOPMENT AND MUSCLE COLOR OF CHANNEL CATFISH

1. Introduction

Pre-slaughter live-chilling in ice water is an alternative method for rendering fish unconscious for killing and further handling (Olsen and others 2006) and has been adopted in the processing of salmon (Kiessling and others 2006), rainbow trout and small-sized species fish (Poli and others 2005). Chilling in ice water before slaughter is a stressor to fish (Skjervold and others 1999), especially tropical fish. Some reports have stated that ice-water chilling might not be as stressful as other slaughter methods such as piking, knocking, electric stunning and CO_2 narcosis (Poli and others 2005). When fish, normally grown at ambient temperature, are placed in ice water, the fish body temperature drops rapidly, which causes metabolism and activity to decrease rapidly (Poli and others 2005). Fish are asphyxiated and finally die of anoxia in 20 to 200 min depending on species (Poli and others 2005). Because fish die over a prolonged time, some researchers have argued that it is an inhumane way to process fish and have

suggested that live-chilling be prohibited as a method for killing fish (Kestin and others. 1991, Wedermeyer 1997, Poli and others 2005).

The effects of pre-slaughter chilling on the stress indicators and subsequent quality of fish muscle are also controversial. Poli and others (2005) added that live-chilling of fish pre-slaughter had low plasma glucose, muscle lactate levels and a prolonged pre-rigor state and shelf life. In contrast, Kiessling and others (2006) reported that Atlantic salmon transferred from 12^{o}C to 4^{o}C for 2h before slaughter had a more rapid onset of rigor due to stress and exercise prior to losing consciousness, thus accelerating the depletion of reserve energy. At low temperatures, the rates of enzyme activity and substrate diffusion are decreased. An increase in mitochondrion and blood capillary density has been observed in low temperature acclimated crucian carp as a compensation mechanism to surpass the low oxygen diffusion (Johnston 1982). A similar compensation mechanism was also reported in intertidal invertebrates subjected to rapid temperature change (Newell 1969, Hazel and Prosser 1974), but it might also present in some extent in vertebrate ectotherms such as fish (Crawshaw 1979).

An increase in myoglobin gene expression might be rational, based on the role that myoglobin has in facilitating oxygen diffusion. There is no report of an acute chilling effect on fish myoglobin gene expression and subsequent protein translation in muscle, even though stressors such as hypoxia have already been known to induce the expression of the myoglobin gene in muscle (Underwood and Williams 1987, Fraser and others 2006). Pre-slaughter acute live-chilling in ice water is sometimes used in catfish processing, while a method of holding fish on crushed ice was recommended for long

transport (haul) to the processing plant (Marshall 2004). Previously, Boggess and others (1973) covered the channel catfish with crushed ice for 3 h and reported that fish were hard to skin, poorly bled and had low sensory scores in appearance and color in baked whole fish.

There is no reported research regarding pre-slaughter acute live-chilling effect on processed catfish quality, except for that by Nunez (1997). However, a relatively higher proportion of "reddish" fillets in catfish (about 2%) were noticed by visual observation in commercial processing plants utilizing pre-slaughter live-chilling. These fillets are considered to be defective fillets for some markets. But it seems as if plants that did not chill live fish had a lower incidence of "reddish" fillets. It is necessary to know whether the excessive pigment molecules in those "reddish" fillets are blood hemoglobin or myoglobin, and whether there is any relationship between the acquiring of such pigments and pre-slaughter ice water chilling. Furthermore, the effects of pre-slaughter live-chilling on the stress and processing quality of catfish needs to be ascertained before this method can be recommended to the catfish processing industry. Bleeding and non-bleeding procedures were also compared to determine the bleeding effect on the muscle total heme-protein content and its influence on the production of "reddish" catfish fillets.

The objectives of this study were to investigate 1) the effects of pre-slaughter acute live-chilling of channel catfish on the stress indicator-plasma cortisol level, myoglobin gene expression in white muscle, and muscle rigor development; and 2) the effects of pre-slaughter acute chilling and bleeding on channel catfish fillet color, pH and total heme-protein contents.

2. Materials and Methods

(1). Live fish pre-slaughter chilling stress and bleeding protocol

Fifty commercial size (1-2 kg) channel catfish were raised in a concrete tank (275 cm x 90cm x 60cm, fish density: 40 kg/m^3) at the Mississippi Agriculture and Forest Experimental Station (MAFES) Aquaculture Unit at Mississippi State University, with a water flow rate of 0.3 m^3/min, natural light period and aeration. Water temperature was around 23^{o}C, pH was around 8.0 and dissolved oxygen level was 5 ppm. Previously, these fish were raised in outdoor commercial ponds for about 1.5 years, before being transferred to indoor raising tanks for 2-3 months for growth, under similar conditions to the above concrete tank. Fish were maintained undisturbed for 5 d prior to harvesting for the experiment. Fish were fed with two types of 32% protein flow feeds combined (Delta Western, Land of Lakes, MS, USA) every two days and feeding was stopped two days before harvest and slaughter.

Eight fish were randomly selected and quickly netted and anesthetized in a bucket containing 200mg/L solution (53cm x 28cm x 20 cm, fish density: 404.3kg/m^3) of tricaine methanesulfonate (MS-222, Finquel®, Fisheries Chemicals, Redmond, WA, USA) (Control group). The rest of the fish were netted and chilled, eight in each group in similar buckets containing ice water solutions (53 cm x 28 cm x 20 cm) for 20 min (20S), 40 min (40S), 60 min (60S), and chilled in ice water for 20 min then placed back in the raising tank for 1 h recovery (R) (fish density: 8.07 kg/m^3). The ice to water ratio was 1:1, and equilibrated for 10 min to a temperature lower than 5^{o}C before the fish were placed into the ice water. The controlled and recovered fish were anesthetized with 200

mg/L solution of MS-222 at room temperature for 5 min until the fish were sedated prior to slaughter. All the fish were killed by a blow (spiking) on the head immediately after removal from either ice water or the MS-222 solution.

Four fish from each group were bled through the tail. Blood was collected in 2.5 ml centrifuge tubes containing 1 ml of 1.7% sodium chloride solution with 50 IU sodium heparin. Blood samples were placed on ice for 30 min and centrifuged at 14000 g for 5 min. Blood plasma samples were transferred to sterile centrifuge tubes and quickly frozen in liquid nitrogen for cortisol analysis that was preformed at a later date. Plasma and packed blood cell volumes were recorded for the calculation of plasma cortisol level. About 10 g of white muscle from the lateral side of bled fish were excised and frozen immediately in liquid nitrogen for total RNA extraction and quantitative RT-PCR analysis of myglobin gene expression. The rest of the bled fish were placed on ice for bleeding. Unbled fish were killed by spiking on the head and placed on ice. Both bled and unbled fish were maintained on ice for 3-4 h. Then, a 10 g sample of muscle was excised from both bled and unbled fish and frozen at -20^{o}C for total heme-protein analysis. Another 30 g samples from each fish fillet were sectioned and packed individually in polyethylene bags and stored on ice for color measurement. Unbled fish with half muscle, bone and head intact were individually packed in large polyethylene bags and stored on ice for measurement of rigor index at three-hour intervals for 45 h.

(2). Rigor index measurement

Rigor mortis of the fish was assessed by the rigor index (RI) method described by Erikson (2000a). Fish were placed on a horizontal table and the vertical length from

dorsal fin to the tail when bent were recorded. The percentage ratio of the length at the initial stage (non-rigor), D_0, to the length difference, $(D_0 - D)$, at certain times was recorded and expressed as the rigor index:

$$\text{Rigor index (\%)} = 100\ (D_0 - D)/D_0$$

Rigor index was measured at every three hours interval.

(3). pH measurement

A five-gram white muscle was sectioned from the middle of each catfish fillet at 3-4 h of slaughter and frozen until analysis. A 20 ml of deionized water was added to the thawed muscle and homogenized for 30 sec with a homogenizer (Polytron®, Brinkmann Instruments Co.). The pH of the slurry was measured with a pH meter (Pinnacle M 530, Corning, Saint Louise, MO,USA) previously calibrated with pH 4 and pH 7 standard solutions.

(4). Color measurement and incident of "reddish" fillet determination

Hunter L, a, b values of fish muscle on the bone-side were measured with the similar method described in Chapter III. Hue angle, saturation index (SI) and whiteness were also calculated from Hunter L, a, b values based on the equations provided in Chapter III. The "white" and "reddish" fillets were determined and recorded by visual observation and the percentage of the incident of these "reddish" fillets was calculated.

(5). Total heme-protein determination

Total heme-protein extraction from treated catfish muscle and analysis used the similar methods described in Chapter III.

(6). Plasma cortisol determination

Plasma cortisol concentration was determined with an ELISA method (Neogen Co., Lexington, KY, USA) from 100 µl of plasma. The extraction and determination followed the procedures provided by the manufacturer. The development of color was recorded by a microplate reader (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA, USA) at the Life Science and Biotechnology Institute (LSBI) at Mississippi State University.

(7). Total RNA extraction and conversion to first-strand cDNA

Catfish white muscle that was quick frozen with liquid nitrogen was excised and homogenized in RTL buffer (Qiagen) with a sterile homogenizer (Polytron®, Brinkmann Instruments Co.) and digested with proteinase-k (Sigma). After centrifugation, the supernatant was transferred to a Qiagen RNeasy Kit (Qiagen) and RNA was extracted following the protocols that were provided by the manufacturer. The total RNA concentrations were determined by measuring absorbance at 260 nm with a spectrophotometer. Only the controlled, chilled 40 min, and recovered fish muscle were used to extract RNA. Total RNA were converted to first-strand cDNA with random primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). RNA fragments were digested with *E. coli* RNase H (Invitrogen).

(8). Quantitative RT-PCR for expression of myoglobin gene in white muscle

The relative expression level of myoglobin mRNA from cold induced catfish was compared to the expression of beta-actin mRNA was measured by quantitative real-time RT-PCR from the first stranded cDNA library. Primers were chosen by online Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Due to the non-specific amplification of the primers in the myoglobin gene selected from the transcription region, the following forward primer was chosen from the coding region and the reverse primer was chosen from the 3'-UTS. The primers (Sigma Genosys) are listed below:

Myoglobin: Forward: 5' CCGACATCCTCAAACCACT-3';

Reverse: 5'-CTCCACGGCTAAATAAACACC-3'.

Beta-actin: Forward: 5'-CCCATTGAGCACGGTATTG-3';

Reverse: 5'-TCTTCTCCCTGTTGGCTTTG-3'.

The PCR was initiated with a hot start reaction at 95^{o}C for 10 min, denatured at 95^{o}C for 10 sec, annealed at 58^{o}C for 10 sec and extended at 72^{o}C for 5 sec in a Light Cycler 2.0 (Roche, Mannheim, Germany) with Masterplus SybrGreen I (Roche). Relative myoglobin gene expression level was calculated using the following equation (Pfaffl 2001):

$$\text{Ratio} = \frac{(E_{target})^{\Delta CP_{target}^{(control-sample)}}}{(E_{ref})^{\Delta CP_{ref}^{(control-sample)}}}$$

Where: Ratio: the myoglobin gene expressed in 40 min chilled or recovered fish versus controlled fish in comparison with the beta-actin gene;

E_{target}: the real-time PCR efficiency of myoglobin mRNA;

E_{ref}: the real-time PCR efficiency of beta-actin mRNA;

ΔCP_{target}: CP deviation of control – sample of the myoglobin mRNA;

ΔCP_{ref}: CP deviation of control – sample of beta-actin mRNA.

(9). Experimental design and statistical analysis

The experiment was conducted as a two-factor factorial arrangement with three replications in a randomized completed block design (RCB). The two factors were pre-slaughter chilling methods (5 levels): non-chilled (C), chilled 20 min (20S), chilled 40 min (40S), chilled 60 min (60S), chilled 20 min then recovered 60 min (R); and bleeding method (2 levels): bleeding and non-bleeding. All data, except plasma cortisol level and relative myoglobin gene expression ratio, were analyzed in SAS by the GLM system (SAS 1991). Relative myoglobin gene expression ratio was measured by combining muscle samples from four fish in each treatment and conducted in two replications. Plasma cortisol level was conducted in one time experiment with individual fish as replications.

3. Results and Discussion

(1). Pre-slaughter chilling effect on blood plasma cortisol level

At the beginning of the experiment, eight catfish (controls) were harvested by netting three to four times (each time two to three fish). Upon harvest, the fish were placed into a MS-222 solution for anesthesia. Fish struggled for about one minute and lost activity in about 5 min. After finishing the harvest of the control fish, other fish were

netted three to four times in each group and placed on pre-measured ice water solutions in a group sequence of chilled 20 min, 40 min, 60 min and chilled 20 min plus recovered 60 min. The bodies of the chilled fish were stiff when removed from the ice solution for slaughter or placed back to the raising tank for recovery regardless of chilling time. The rigor-like stiffening was resolved in a few minutes after killing. Fish in the recovery (R) group resumed normal activity in about 10 min after being placed in the raised tank. Harvesting of recovered fish was more difficult and required more netting time due to lower fish density in the tank.

Lambooij and others (2006) placed African catfish (*Clarias gariepinus*) with averaged weights of 1738 g from 24^{o}C to 0^{o}C ice water and reported that fish became motionless after 5 min and lost consciousness when the body temperature was lower than 8.7^{o}C. They also noticed muscle clonic cramps when fish were chilled in ice water.

Non-chilled (controlled) fish had plasma cortisol levels of 14.4 ng/ml. Plasma cortisol levels were 46.3 ng/ml in fish chilled 20 min; 66.83 ng/ml in fish chilled 40 min; 54.9 ng/ml fish chilled 60 min; and 43.32 ng/ml in recovered fish. Channel catfish subjected to acute chilling in all groups, including the recovery group, had higher cortisol levels in blood plasma, when compared to the non-chilled fish. The elevation of blood plasma cortisol level was an indication of the stress response. This indicated that pre-slaughter acute chilling was a stressor to channel catfish in our experiment. Despite the fact that all the chilled fish had elevated plasma cortisol levels; the elevation of plasma cortisol levels did not linearly increase as chilling time increased. Recovery for 60 min

following the acute chilling for 20 min did not decrease the cortisol level in channel catfish plasma.

Small and Davis (2002) reported that plasma cortisol of rested channel catfish was 14.4 ng/ml, and after 2 h confinement stress, it increased to 46.5 ng/ml. This cortisol level of the rested fish was similar to our results. Bosworth and Small (2004) compared the effects of transport temperature of 10^{o}C versus 20^{o}C for channel catfish and reported that even though there was a trend that 10^{o}C transport elicited a higher cortisol level than 20^{o}C transport, they could not draw a clear conclusion due to the high variation in cortisol level among individual fish. The reaction of fish to chilling is variable depending on the species and the temperature to which fish is acclimated (Erikson 2000b). A temperature change of less than 10^{o}C is tolerable by most fish species, and a cold shock occurs and fish is stressed when the temperature change exceeds this range (Erikson 2000b). Elevation of plasma cortisol occurs following cold stress. Our fish were acclimated to 23^{o}C for 2-3 months. The water temperature of our raised tank was 23^{o}C, even though the experiment was conducted at the late fall and the winter and outdoor temperature might be lower than 5^{o}C. The temperature difference between the chilling tank and the raising tank was near 20^{o}C. Such a large temperature difference incurred a stress response in the channel catfish. This was confirmed by the elevation of plasma cortisol for all of the catfish that were chilled in ice water at various times. Even though chilling times increased linearly, cortisol level increase was not the same. Davis and others (2001) reported that channel catfish that were acclimated to 35^{o}C had a higher initial plasma cortisol level, indicating that 35^{o}C is a stressor to channel catfish, but this

level did not increase by further confinement stress; whereas, fish acclimated to 15^{o}C and 26^{o}C had low initial plasma cortisol levels but these levels increased after confinement. They contributed no further increase in plasma cortisol to additional stressors due to inadequate adrenal reserve for further synthesis of cortisol.

When a stressor is perceived, the anterior pituitary of the fish secretes corticotrophin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) to the adrenal cortex to stimulate catetholamine and cortisol release. Cortisol has a negative feedback inhibition on ACTH and CRH secretion (Porterfield 1996), thus, linear increase of stress intensity does not necessarily result in the same magnitude of cortisol elevation. After chilling for 20 min, eight fish from the recovery group were placed back to the raising tank to recover from stressed conditions for 60 min with similar temperature (23^{o}C) and aeration before chilling. Fish resumed normal activity after about 10 min. Even if the stress response might subside, the cortisol level still remains high since the half life of circulating cortisol is about 70 min (Porterfield 1996).

(2). Pre-slaughter chilling effect on expression of myoglobin gene in white muscle

Quantitative real-time RT-PCR analysis from the catfish white muscle cDNA library showed that beta-actin mRNA levels in the white muscle of fish that were chilled for 40 min were similar to those of non-chilled fish white muscle (CP values: 21.56 vs. 21.525). Based on the Pfaffl calculation method (Pfaffl 2001), the average calculated relative ratio of the expressed myoglobin mRNA level between fish chilled 40 min and non-chilled fish was 0.94. This means that white muscle from chilled fish was 0.94 times that of the myoglobin mRNA of non-chilled fish. The average calculated relative ratio of

the expressed myoglobin mRNA between 20 min chilled/60 min recovered fish and non-chilled fish was 1.21. This showed that pre-slaughter chilling fish did not result in the overexpression of myoglobin mRNA in white muscle from channel catfish.

Fish stress response comprises two hormone systems that trigger the biochemical and physiological processes to cope with fish homeostasis: one is catecholamines (adrenaline and noradrenaline) and another is the steroid cortisol (Pottinger 2000). The function of catecholamines is to increase the level of cardiorespiratory performance (Pottinger 2000). The high level of respiration manifests itself in an increase in gill vibration, heart beat and oxygen uptake (Poli and others 2005). The cardiac output and haematocrit value are also increased to fulfill the high oxygen demand (Poli and others 2005). The purpose of these physiological activities is to increase oxygen supply, otherwise, respiration will cease due to the low pH by lactic acid produced in anaerobic respiration under an insufficient oxygen level. Hypoxia was known to induce overexpression of myoglobin (Fraser and others 2006). The density of fish in chilling or anesthetic buckets was 10 times higher than fish in raising tanks, thus, the oxygen supply in fish in the chilled tanks might be reduced. But it seems unlikely that hypoxia for these fish occurred because low temperature also increases the dissolved oxygen level in water. Low temperature also lowers oxygen diffusion in tissue. Increases in mitochondrion and blood capillary density in crucian carp (*Carassius carassius*) muscle acclimated to low temperature were observed as a compensation mechanism from low oxygen diffusion (Johnston 1982). A similar compensation mechanism was also reported in fish subjected to rapid temperature changes. An increase in myoglobin expression seems to be rational,

based on the role of myoglobin in facilitating oxygen diffusion. However, the overexpression of the myoglobin gene did not occur in our experiment. If there had been any increase of pigmentation in channel catfish white muscle by exposing it to the acute chilling stress, most likely, the increased pigment molecule was not myoglobin.

(3). Pre-slaughter chilling effect on rigor development of iced stored catfish

Pre-slaughter chilling of channel catfish in ice water had a pronounced effect on postmortem rigor development (Figure 5.1). Pre-rigor times of catfish chilled in ice water for 40 min and 60 min was about 1 h, with full rigor attained in about 6 h postmortem. Rigor onset of non-chilled catfish was attained after 9 h and full rigor at about 21 h postmortem. The pre-rigor times in catfish chilled 20 min and recovered for 60 min, and those chilled 20 min without recovery were 5.5 h and 7.5 h, respectively; with full rigor attained in 9 and 12 h postmortem, respectively. Thus, chilled catfish in ice water for 40-60 min developed rigor in 8 h and achieved full rigor 15 h earlier than non-chilled catfish. Chilled catfish in ice water for 20 min developed rigor at 3.5 h and achieved full rigor 12 h earlier than non-chilled catfish. Catfish chilled for 20 min following recovery for 60 min developed rigor at 1.5 h and achieved full rigor 9h earlier than the non-chilled catfish. Catfish that were allowed to recover for 60 min after chilling in ice water for 20 min delayed the onset and full rigor for 2-3 h, when compared to catfish chilled for 20 min without recovery. There was no difference ($p > 0.05$) in rigor onset and full rigor times for catfish chilled for 40 and 60 min. Even though the onset times were different between chilled and non-chilled fish and among fish chilled for various times, the times

from slaughter to rigor resolution were almost the same. Fish chilled 40 min had lower ($p \leq 0.05$) rigor index in full rigor than other group fish (Figure 5.1).

The development of rigor mortis of postmortem fish is closely related to the depletion of the energy pool (Watabe and Itoi 2002), which is composed of a group of ready to use high energy phosphate compounds including ATP, ADP, AMP and creatine phosphate. Depletion of the energy pool is dependent on its initial level and the rate of its degradation. The initial energy pool level is determined by the pre-slaughter conditions of the animal. Animal in acute stress quickly uses up the available energy and initiates the rapid production of the energy to replenish the energy pool by breaking down glycogen or other resources such as fatty acids or proteins, due to the fact that animals in stress conditions require larger amounts of energy than in normal conditions. As a consequence, the postmortem initial energy pool level is low in animals subjected to pre-slaughter stress. Live-chilling animals may also have even lower initial energy pools postmortem because low temperature pre-mortem may also retard the replenishment of the energy by a lower enzyme activity (Skjervold and others 1999). The ATP depletion rate is influenced by storage temperature (Kiessling and others 2006).

Channel catfish can survive a wide range of temperatures from 40^{o}C to just above the freezing point if the fish is slowly acclimated (Tucker and Robinson 1990). However, channel catfish in Mississippi are usually acclimated to a 20-30^{o}C temperature range. Chilling in ice water from 23^{o}C to 0^{o}C in 5-10 min is an acute stress process for channel catfish. The elevation of cortisol level in chilled catfish plasma confirmed this. Catfish must mobilize all the available resources to cope with the acute chilling stress. The direct

result is that the available initial energy pool postmortem is limited and is used up rapidly, resulting in catfish developing rigor early when compared to the non-chilled fish.

Kiessling and others (2006) reported that Atlantic salmon that were acclimated to 12^{o}C and transferred to 4^{o}C for 2 d before slaughter had shorter pre-rigor time postmortem than fish acclimated to either 12^{o}C or 4^{o}C. Channel catfish recovered for 60 min following a 20 min chilling in ice water delayed pre-rigor time and the time from rigor onset to full rigor for 2-3 h compared to chilled 20 min without recovery, indicating that fish had partially replenished the loss of the energy pool, but 1 h is not enough time for full recovery from 20 min chilling.

It should be noted that there are differences between chilling and acclimation to low temperature with respect to rigor development of fish postmortem. It was noted that fish that acclimated to low temperatures might not have accelerated rigor development. Watabe and Itoi (2002) reported that temperate fish acclimated to 10^{o}C had slower rigor development than fish acclimated to 30^{o}C and stored at either 0^{o}C or 10^{o}C postmortem. They explained that two factors contributed to this difference. Firstly, ATP degradation in 5-10^{o}C is slower than in 0^{o}C due to the fact that calcium ion uptake by sarcoplasmic reticulum decreases with a decreasing Ca^{2+}-ATPase activity for the Ca^{2+} pump at low temperature, thus increasing the concentration of calcium ions in muscle cells. The increase in calcium ions in myofibrils could promote ATP consumption. Secondly, the content of ATP synthase (F_0F_1-ATPase) in mitochondria of fish acclimated to 10^{o}C is higher than that of fish acclimated to 25^{o}C. Nunez (1997) also reported on rigor development of channel catfish that were chilled in ice water, by placing catfish in water

(22^{o}C), then gradually adding ice to the water to lower the temperature with a temperature equilibrium (0^{o}C) time in 50-60 min. He indicated that the pre-rigor time of chilled fish was 12 h with no difference to that of anesthetized fish. He added that chilled fish muscle ATP was 3.0 μmol/g, lower than those of anaesthetized, transport stressed, and controlled fish. The chilling process in his protocol was gradual and the temperature decrease was slower than in our protocol. This was confirmed by the fact that plasma cortisol in chilled fish in his protocol was even lower than that of anesthetized fish. This indicated that 0.35- 0.4^{o}C/min decrease of temperature had allowed the acclimation process to occur for catfish and did not elicit the stress response.

(4). Pre-slaughter chilling effect on muscle total heme-protein content

The differences ($p \leq 0.05$) in the total heme-protein content between non-chilled and chilled fish white muscles only occurred in the 20 min chilled and recovery group (Figure 5.2). Non-chilled fish muscle had no differences ($p > 0.05$) in total heme-protein content with fish chilled 40 min and 60 min. Total heme-protein content of catfish chilled 20 min was the highest and differed ($p \leq 0.05$) with those of other fish, with the exception of recovered fish. Total heme-protein content of recovered fish was the second highest, different ($p > 0.05$) from either non-chilled fish muscle or those of chilled 60 min. These results were not consistent with either visual observations or Hunter color values. Stress increases the blood flow to the muscle in fish (Thorarensen and others 1993, Farell and others 2001, Schultz and others 1999) and increases hemoglobin level in fish white muscle (Olson and others 2006). Low temperature also reduces muscle blood flow rate due to the increased blood viscosity and sympathetic nerve activity (Egginton 1997).

Botta and others (1986) reported that an active heart influences the removal of blood from fish, but Roth and others (2002) concluded that heart beating was not necessary for complete removal of blood from fish fillets. The heart response to low temperature stressors either increase (tachycardia) or decrease (bradycardia) beating rate, depending on species sensitivity to cold (Lambooij and others 2006). Egginton (1997) concluded that low temperature induces bradycardia. Lambooij and others (2002) reported that eels decreased their heart beating rate when chilled in ice water. But Lambooij and others (2006) reported that an increase in heart beating rate at both conscious and unconscious states of African catfish chilled in ice water.

Even though we did not measure the heart rate, the heart rate response of channel catfish at low temperature in Mississippi may be similar to that of African catfish because they grow in similar temperature ranges. Stress may also result in an incomplete removal of blood from muscle and residual blood may remain in the blood capillaries (Thorarensen and others 1993, Farell and others 2001). However, there is a highly variable distribution of blood flow to white muscle in channel catfish (Schultz and others 1999) that might offset the thermal effect on blood removal upon bleeding. Collectively, these factors might contribute to the small difference in the total heme-protein levels between the different treatments.

(5). Pre-slaughter chilling effect on muscle pH

There was no difference ($p > 0.05$) in muscle pH between chilled and non-chilled catfish. The muscle pH values ranged from 6.56 to 6.63. Stressed fish always initiates anaerobic respiration with elevations in plasma lactic acid and muscle lactic acid. An

elevation of plasma and muscle lactic acid levels causes a decrease of pH and this is usually used as a stress indicator.

Plasma lactic acid and muscle lactic acid were neutralized in full recovered fish (Milligan 1997). A pre-slaughter stress on fish will cause a low pH in plasma and muscle due to the incomplete recovery of the fish from stress in limited time (Poli and others 2005); however, the muscle pH will be high if the recovery time is long enough (Poli and others 2005). Starvation of fish is required before slaughter and this causes a high muscle pH due to low energy resources if the fish is subjected to stress (Poli and others 2005). Initial glycogen levels in muscle strongly influence the final muscle pH (Black and Love 1988). Therefore, the similar pH levels in chilled and non-chilled fish muscle might be explained by the fact that a very limited muscle glycogen level was present in these fish starved for 2 d, thus, a high stress response might not be able to elicit a response in muscle pH.

(6). Pre-slaughter chilling effect on color of fillets on the bone-side

Catfish fillets in one replication are presented in Figure 5.3. On average, the percentage of the incident of "reddish" fillets from 40 min chilled fish was 31.25%, difference ($p \leq 0.05$) from that of recovered fish, which was 4.17%. The percentage of the incident of "reddish" fillets was 29.17% from 60 min chilled fish, 16.67% from non-chilled fish, 14.58% from the 20 min chilled fish, respectively. These values did not differ ($p > 0.05$) from each other and from either that of 40 min chilled fish or that of the non-chilled fish. Pre-slaughter chilling in ice water tended to increase the incident of "reddish" fillets. Recovered fish had lower incident of "reddish" fillet formation.

Hunter “L” values of bone-side fillets of non-chilled fish were not different ($p > 0.05$) from those catfish subjected to pre-slaughter chilling, except those for recovered catfish (Figure 5.4). The recovered catfish had higher ($p \leq 0.05$) Hunter “L” values than other fish with the exception of those chilled 20 min, which did not differ ($p > 0.05$) from those of all the other catfish. Hunter “a” values of bone-side fillets of fish chilled 40 and 60 min were higher ($p \leq 0.05$) than those of recovered fish, but not differed ($p > 0.05$) from the others (Figure 5.5). There were no differences ($p > 0.05$) in either Hunter “b” values (Figure 5.6) or saturation index (SI) (Figure 5.8) between different treated fish filets. However, recovered fish fillets had higher ($p \leq 0.05$) hue values than the other fish, which were not different ($p > 0.05$) from each other (Figure 5.7). These results indicated slight differences in Hunter color between some of the fillets from chilled fish. However, the differences were slight and the variations were large. The latter is the result of large variability on fillet appearance.

Erikson and Misimi (2008) reported that stressed Atlantic salmon had a darker muscle color than the rested one. They attributed this to the early rigor contraction and lower pH that affected the light scattering properties of the fillets. When the color parameters were measured, the chilled muscle was in the rigor stage, whereas, that of the non-chilled muscle was in the pre-rigor stage because non-chilled fish developed rigor 9 h later than chilled fish. The chilling stress effects on muscle lightness were masked by rigor contraction increasing the contrast of myofibril and the sarcoplasms and thus the light scattering (Offer and Trinick 1983, Erikson and Misimi 2008). The pH values of fish muscle, which were measured 3-4 h after slaughter, the similar time with the

measurement of muscle color, were almost the same in all groups, thus it seemed that there was no pH effect on the color reflection involved. Scherer and others (2005) evaluated the CIE L* a* b* space color parameter of grass carp subjected to either live-chilling 20 min or electrically stunned, prior to placement on ice and filleting at 93 h postmortem. These researchers found no difference between L* and a* values when comparing these harvesting methods. The rigor index of live-chilled fish was higher than stunned fish, but all lower than 50%. Boggess and others (1973) did not find any difference in fillet color of channel catfish that were slaughtered by either ice chilling or electrical killing. Hallier and others (2007) compared the color of fillet on skin-side of European catfish (*Silurus glanis*) from two different farming conditions (outdoor pond: less than 10^{o}C to higher than 25^{o}C vs. indoor pond: 27-31^{o}C) and reported that fish raised in the indoor pond had lighter, yellower and greener fillets. They attributed the lighter color from the indoor pond to the shorter growing time due to the fact that the fish from indoor pond were one year younger than fish from the outdoor pond. But they attributed the yellower and greener fillets from the indoor pond to the increased deposition of carotenoid pigments by high temperature. Jankowska and others (2007) reported that European catfish fed formulated feed resulted in lighter, less red, yellower and greener fillets compared to the fish fed natural feed that were grown for a longer time.

It seems that pre-slaughter chilling tends to increase the redness and decrease the lightness of catfish fillets, and chilling plus recovery decreased the redness and increased the lightness of the catfish fillets. The increased reddish pigment was hemoglobin rather than myoglobin, due to the fact that the content of myoglobin in catfish muscle is low

compared to hemoglobin and pre-slaughter chilling stress did not induce the overexpression of myoglobin. The increase of the pigmentation seems to be a surface phenomenon because total heme-protein did not significantly increase after chilling. The surface pigmentation was the hemoglobin from the blood.

(7). Bleeding effect on muscle total heme-protein content

Bled (1.39 mg/g) and unbled (1.56 mg/g) fish did not differ ($p > 0.05$) in total heme-protein. There was no significant interaction ($p > 0.05$) on the total heme-protein content of fish muscle between bleeding and chilling treatments. Roth and others (2007) reported the fillet color difference in farmed turbot (*Scophthalmus maximus*) by bleeding was contributed by the residual blood, which could be quantified by bloodspot counting (Roth and others 2005), hemoglobin quantification (Olsen and others 2006) and computer imaging analysis. They added that counting blood spots only quantified blood in the veins, whereas, measuring hemoglobin only quantified the blood in the capillaries. Richards and Hultin (2002) observed a variability of residual hemoglobin in trout and mackerel muscle between bled and unbled fish. Porter and others (1992) also concluded that there was no difference in hemoglobin content between bled and unbled sockeye muscle. Roth and others (2007) reported bleeding reduced the redness of fish fillets and this difference was contributed by the residual blood. Inconsistent results might be due to high variability in blood volume among species (Roth and others 2007) or individual fish within species (Schultz and others 1999).

(8). Bleeding effect on muscle pH and color of fillets on the bone-side

There was no difference ($p > 0.05$) in muscle pH of bled (6.59) and unbled (6.60) catfish. The percentage of the incident of "reddish" fillets in unbled fish was 23.44%, 14.29% in bled fish, with no differences ($p > 0.05$) each other. There were no significant differences ($p > 0.05$) in Hunter L (54.81 vs. 52.98), a, b, hue and SI values of bled and unbled catfish fillets. However, unbled fillets tended to be redder and darker than bled fillets, as noted by visual appearance. However, there was a high variability among fillets; thus, leading difficulty in finding the differences. Botta and others (1986) reported that the time of cod from harvest to slaughter significantly influenced the overall fillet color, but did not affect bruising/discoloration and blood spots. They stated that the color variability of fish bleeding/gutting by different methods and different times from harvest to slaughter was very high. Roth and others (2007) reported redder farmed turbot from unbled fish. Robb and others (2003) used various methods to evaluate small hemorrhage spots or blood spots in smoked salmon, either bleeding or non-bleeding. Even though they did find differences, they also thought factors other than bleeding might play a role in these differences.

4. Conclusions

Rapid chilling of channel catfish from 23^{o}C to ice water prior to slaughter was a stressor to fish, resulting in stress responses by increasing plasma cortisol level. Chilling stress of channel catfish slightly increased the heme-protein content and redness in white muscle. Acute chilling stress did not induce overexpression of myoglobin. This leads us to conclude that the slightly increased pigmentation in chilled fish is likely from residual

blood hemoglobin rather than myoglobin. Bleeding also tended to reduce the heme-protein content and redness, but since either chilling stress or bleeding of channel catfish could not prevent “reddish” fillet formation, other factors might be involved. The chilling stress also shortened the pre-rigor time to within 1 h postmortem, thus reducing the fillet yield and downgrading the quality. Therefore, chilling in ice water before slaughter as a means of anesthetization is not recommended for channel catfish processing.

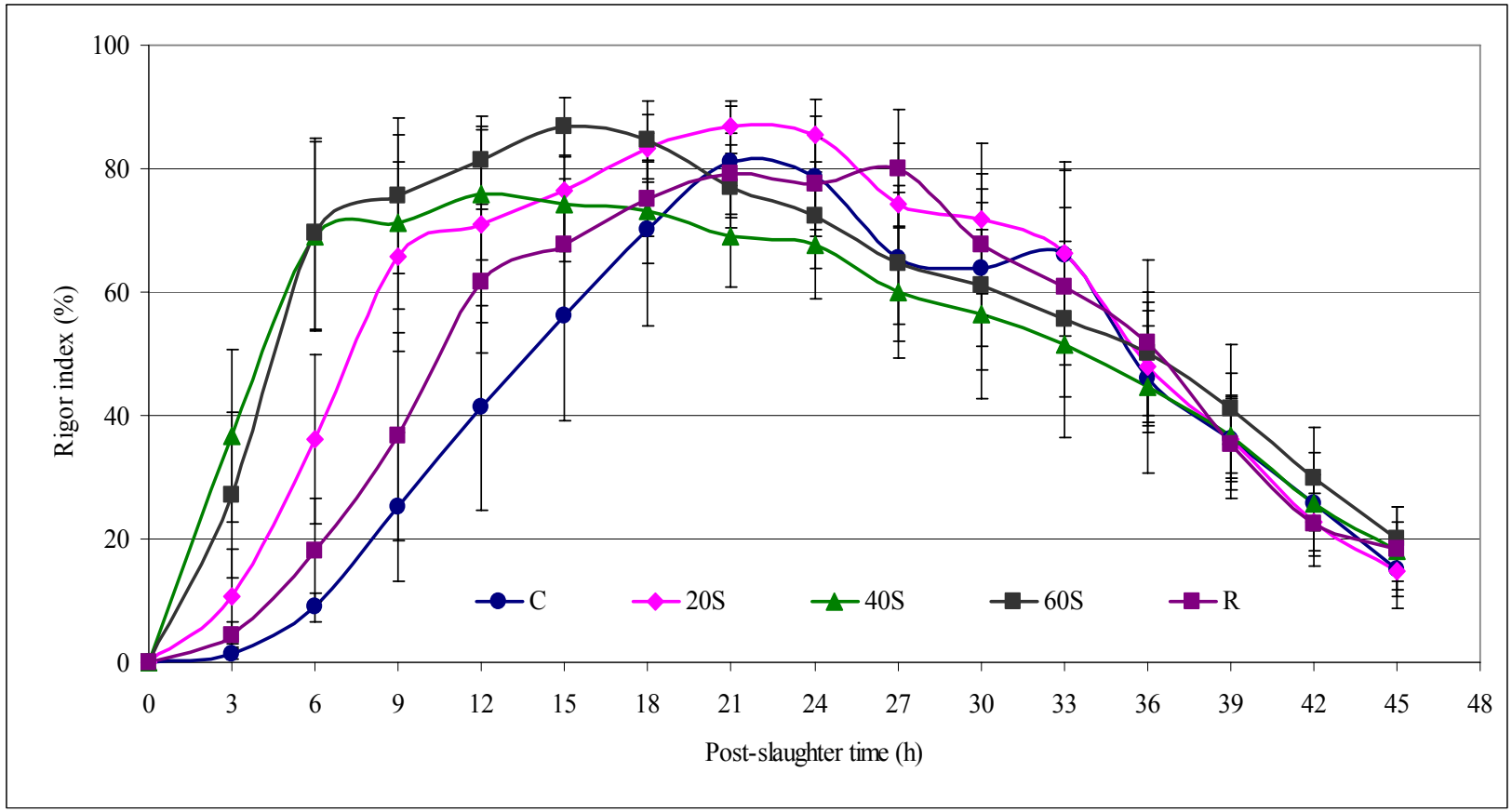

Figure 5.1 Pre-slaughter chilling effect on rigor development of ice-stored catfish.

C-controlled (non-chilled); 20S- pre-slaughter chilled 20 min; 40S- pre-slaughter chilled 40 min; 60S- pre-slaughter chilled 60 min; R- chilled 20 min then placed back to 23^{o}C raising tank for 1 h recovery before slaughter.

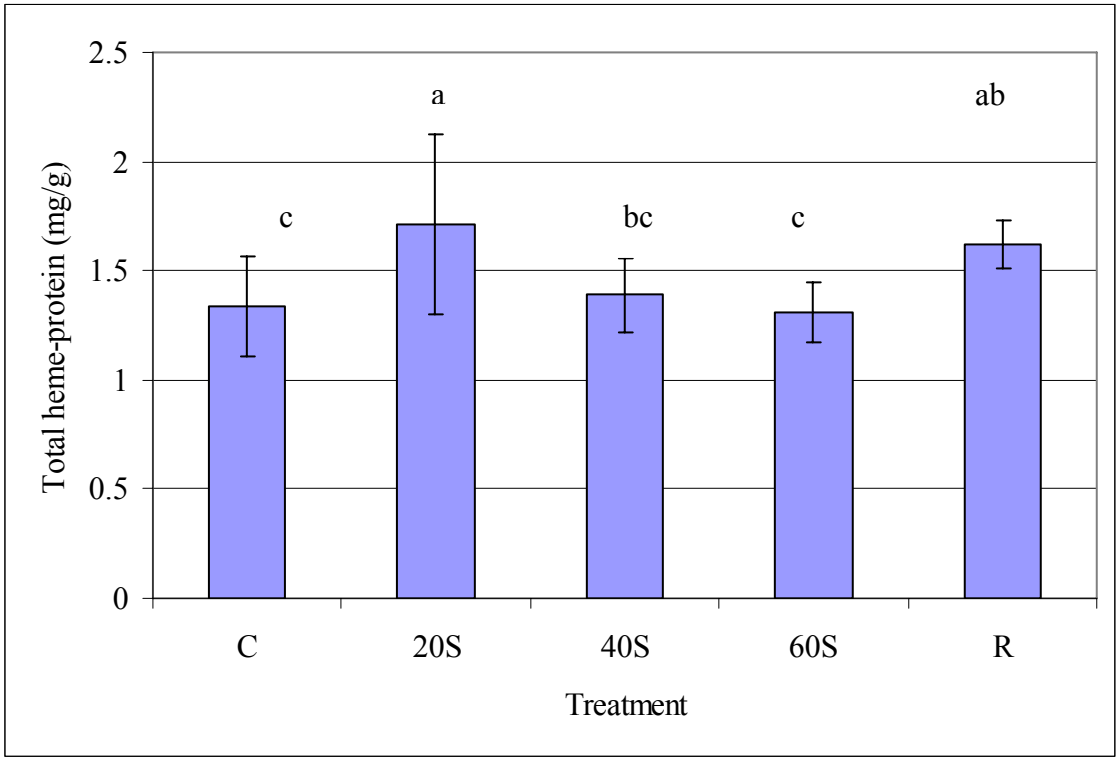

Figure 5.2 Pre-slaughter chilling effects on catfish muscle total heme-protein content.

C-controlled (non-chilled); 20S- pre-slaughter chilled 20 min; 40S- pre-slaughter chilled 40 min; 60S- pre-slaughter chilled 60 min; R- chilled 20 min then placed back to 23^{o}C raising tank for 1 h recovery before slaughter. Note: abc- mean values of total heme-protein content with the same letter did not differ significantly ($p > 0.05$)

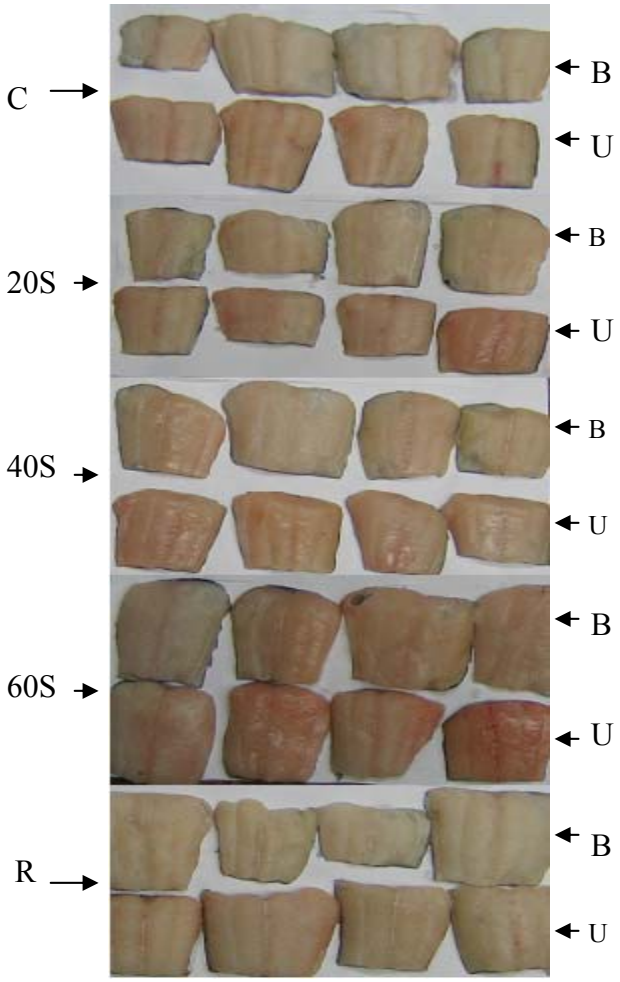

Figure 5.3 Picture of pre-slaughter chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter.

C-controlled (non-chilled); 20S- pre-slaughter chilled 20 min; 40S- pre-slaughter chilled 40 min; 60S- pre-slaughter chilled 60 min; R- chilled 20 min then placed back to 23^{o}C raising tank for 1 h recovery before slaughter. B-bled catfish muscle; U-unbled catfish muscle.

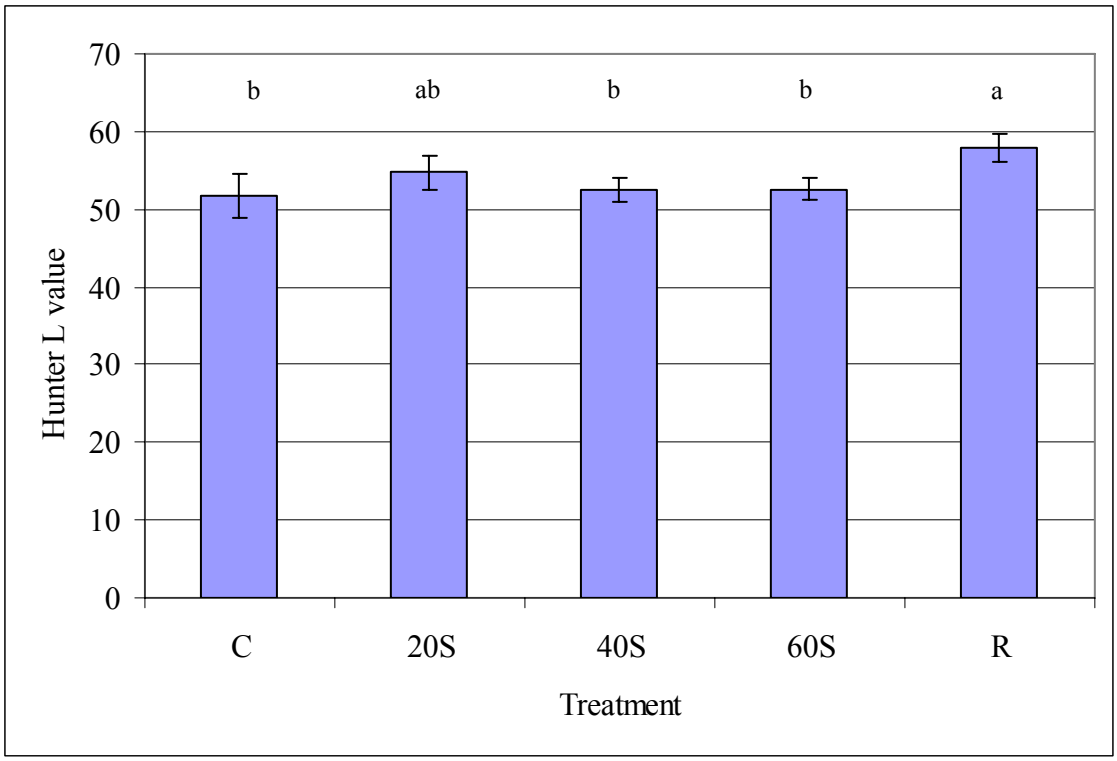

Figure 5.4 Hunter L values of pre-slaughter chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter.

C-controlled (non-chilled); 20S- pre-slaughter chilled 20 min; 40S- pre-slaughter chilled 40 min; 60S- pre-slaughter chilled 60 min; R- chilled 20 min then placed back to 23^{o}C raising tank for 1 h recovery before slaughter. Note: ab- mean values of Hunter L values with the same letter did not differ significantly ($p > 0.05$)

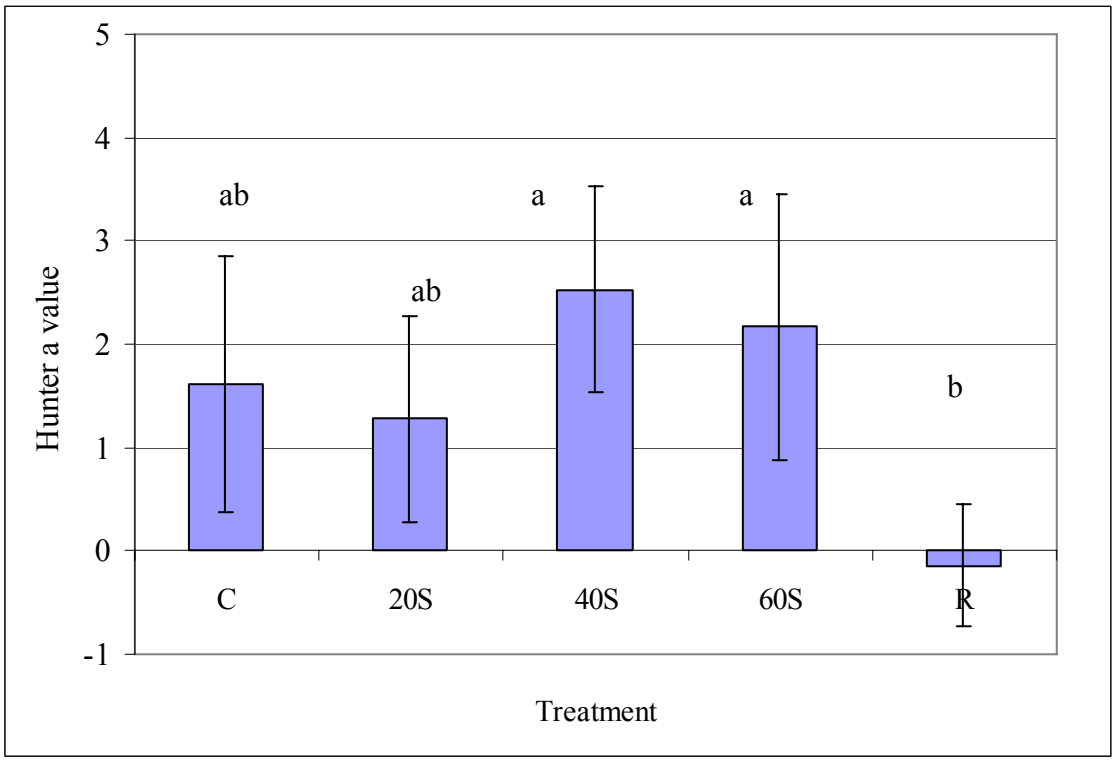

Figure 5.5 Hunter a values of pre-slaughter chilling stressed catfish fillets the bone-side trimmed at 3-4 h after slaughter.

C-controlled (non-chilled); 20S- pre-slaughter chilled 20 min; 40S- pre-slaughter chilled 40 min; 60S- pre-slaughter chilled 60 min; R- chilled 20 min then placed back to 23^{o}C raising tank for 1 h recovery before slaughter. Note: ab- mean values of Hunter a values with the same letter did not differ significantly ($p > 0.05$)

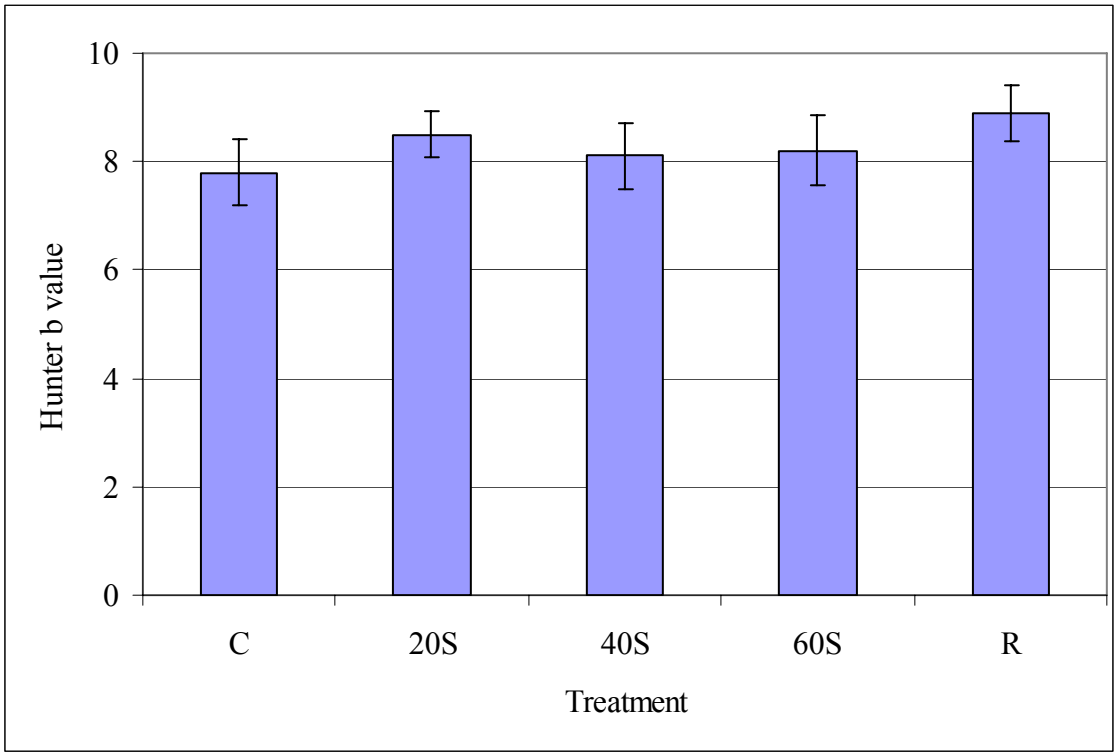

Figure 5.6 Hunter b values of pre-slaughter chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter.

C-controlled (non-chilled); 20S- pre-slaughter chilled 20 min; 40S- pre-slaughter chilled 40 min; 60S- pre-slaughter chilled 60 min; R- chilled 20 min then placed back to 23^{o}C raising tank for 1 h recovery before slaughter. Note: no significant difference ($p > 0.05$) was detected among the mean values of Hunter b values

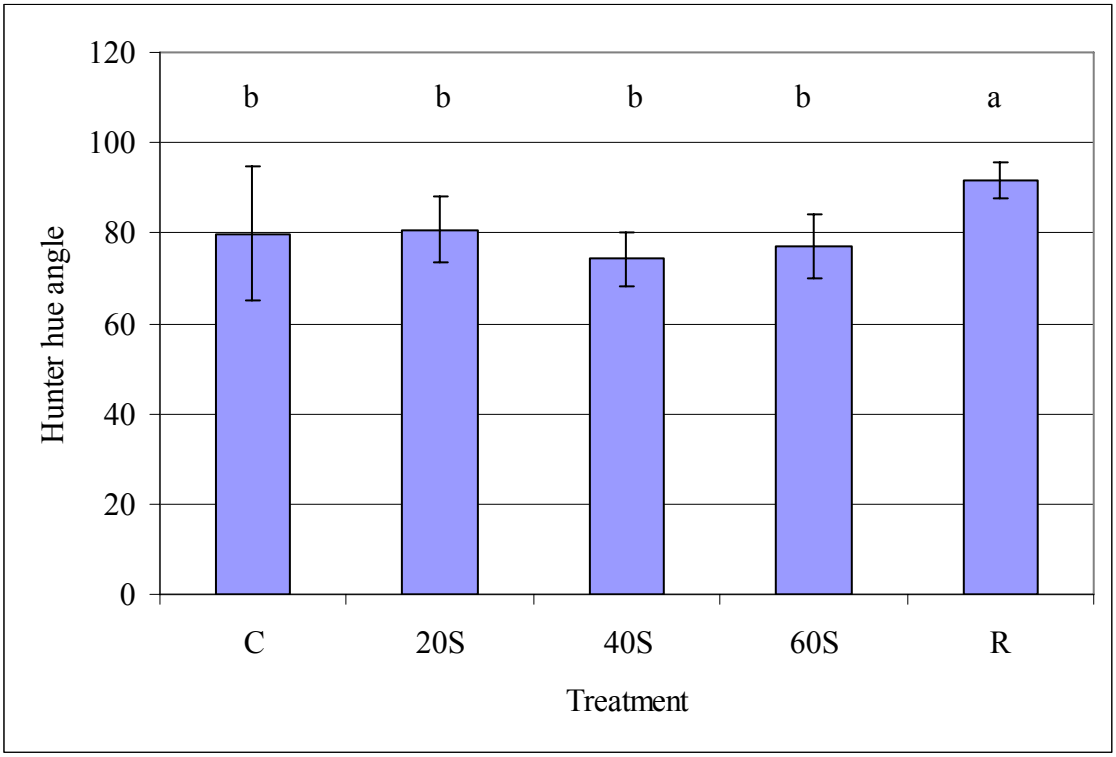

Figure 5.7 Hunter hue angle of pre-slaughter live-chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter.

C-controlled (non-chilled); 20S- pre-slaughter chilled 20 min; 40S- pre-slaughter chilled 40 min; 60S- pre-slaughter chilled 60 min; R- chilled 20 min then placed back to 23^{o}C raising tank for 1 h recovery before slaughter. Note: ab- mean values of Hunter hue angle with the same letter did not differ significantly ($p > 0.05$)

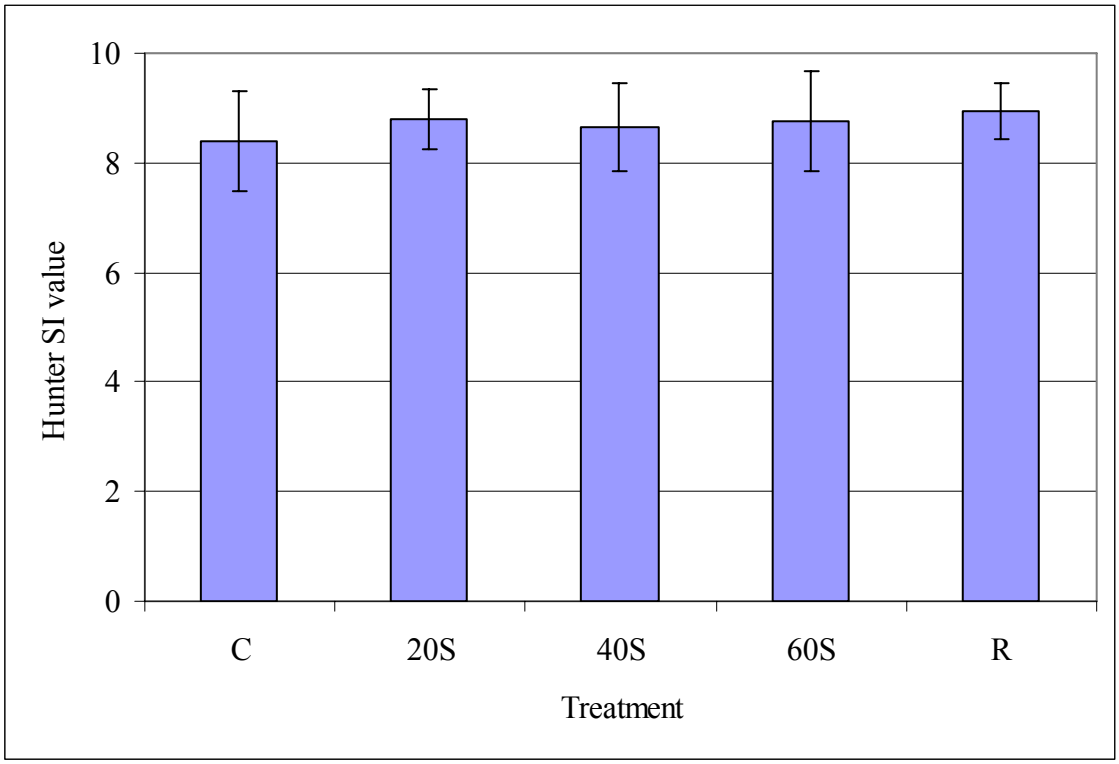

Figure 5.8 Hunter saturation index (SI) of pre-slaughter live-chilling stressed catfish fillets on the bone-side trimmed at 3-4 h after slaughter.

C-controlled (non-chilled); 20S- pre-slaughter chilled 20 min; 40S- pre-slaughter chilled 40 min; 60S- pre-slaughter chilled 60 min; R- chilled 20 min then placed back to 23^{o}C raising tank for 1 h recovery before slaughter. Note: significant difference ($p > 0.05$) was detected among the mean values of Hunter color saturation index (SI).

CHAPTER VI

SUMMARY AND CONCLUSIONS

We designed three experiments to study the color and color components in fresh catfish muscle. The first experiment was designed to study the instrumental color parameter distribution and its relationship with visual rating, instrumental color parameter changes as affected by postmortem chilling and ice storage; heme-protein contents in "white" and "reddish" fresh catfish fillets and their correlation with instrumental color parameters. Onsite survey of fresh catfish fillets indicated that postmortem chilling renders fresh catfish fillets pale and less red. The CIE L* values increased 3 digits, a* and b* decreased by 1.0 and 1.5 digits in chilled fillets. Color of fresh catfish fillets was affected by postmortem chilling. The fillets became slightly pale, redder on iced storage. Visual rating of fresh catfish fillets correlated well with Hunter L, a, hue and whiteness, but not with b and SI. That total heme-protein contents in "reddish" fillets were only slightly higher than those in "white" fillets and did not correlated well with Hunter a values indicated that the reddish on fillets was only a surface phenomenon. A CIE L* a* b* value distribution was given and this might be useful data for the future electronic monitoring of fresh catfish fillets color in industry.

The second experiment was aimed to study the oxidation and gene cloning and their ratio of the two major color components: myoglobin and hemoglobin in catfish

muscle. It involved the autoxidation rate of hemoglobin at either room or refrigeration temperatures; myoglobin gene sequencing, myoglobin gene and hemoglobin beta gene cloning and expression in *E. coli* cells. Myoglobin and hemoglobin in fresh catfish muscle were identified by SDS-PAGE and immunoblotting. Autoxidation rate of hemoglobin at room temperature was only slightly higher than at refrigeration temperature. The open reading frame of the myoglobin gene contains 444 nucleotides that are encoded for 147 amino acid polypeptide. The heme contacted histidines were conserved. Both the myoglobin and hemoglobin beta genes could be cloned to and expressed in *E. coli* cells without induction, but the proteins were expressed mostly in aggregated insoluble forms and could not be used for antibody productions. SDS-PAGE and immunoblotting by antibodies confirmed that the major red pigments in fresh catfish fillets were hemoglobin rather than myoglobin.

The third experiment was designed to study the pre-slaughter ice water chilling on the stress of catfish and this chilling and bleeding on muscle quality. Pre-slaughter acute chilling of live channel catfish was a stressor to the fish and the fish elicited a stress response. The chilling stress greatly influenced the rigor development in fish postmortem. Acute live-chilling of catfish slightly increased the total heme-protein content and the redness of muscle color. The slightly increased pigments were hemoglobin rather than myoglobin since myoglobin gene did not over-expressed by chilling. Bleeding also slightly increased the total heme-protein contents and the redness of fresh catfish muscle. But acute live-chilling and bleeding were not the major factors for the production of "reddish" fresh catfish fillets.

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