THE PRO-OXIDATIVE PROPERTIES OF TILAPIA OXY-, CARBOXY-AND MET-HEMOGLOBIN IN WASHED MINCED TILAPIA MUSCLE

By

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To my devoted parents, my siblings, my best friend and brother, Dr. Mohammed Aldaous

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Hemoglobin (Hb) plays an important role in quality deterioration of fish, affecting odor, flavor, and nutritional value. Autoxidation of oxy-Hb produces a very reactive form; met-Hb. Increasing stability of Hb can be done by binding carbon monoxide (CO) to the heme porphyrin group. The objective was to compare the pro-oxidative activity of oxy-, CO-, and met-Hb as a function of pH, Hb concentration, NaCl concentration, and storage temperature. Washed minced tilapia muscle (WMTM) was prepared and adjusted to pH 6.3, 6.8 and 7.3. Oxy-Hb was isolated from fresh tilapia blood. CO-Hb was prepared by flushing oxy-Hb with 100% CO for 2 min. Met-Hb was prepared by reacting oxy-Hb with $K_3Fe(CN)_6$. Concentrations of 6, 9 and 12 µM Hb forms and 150 and 450 mM NaCl were added to WMTM. Samples were stored at 3.7°C for 8 days and -25ºC for 24 weeks. Lipid oxidation was monitored by following thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH), and sensory analysis. Protein oxidation was measured by the DNPH method. Change in color was measured using Color Vision Machine System. Hb oxidation state was monitored spectrophotometrically. Loss of CO from the muscle system was detected using gas

chromatography. Development of off odor (rancidity) during storage at 3.7°C was evaluated by trained panelists and correlated with TBARS a*values. Dissociation rate of the hemin group from met-Hb was measured spectrophotometrically at 4° and 25°C. Statistical significance was reported as $p \leq 0.05$ using the General Linear Model and Least Square Means (SAS software 9.2).

The effect of pH superseded Hb form, Hb concentration, NaCl concentration, or temperature in influencing oxidation. Significant lipid, protein, and Hb oxidation was found to increase for all forms of Hb as pH decreased. More deoxy- (7%) and met-Hb (14%) forms were found at pH 6.3 compared to pH 7.3. TBARS and LOOH were significantly ($p \leq 0.05$) lower for CO-Hb samples compared to other Hb forms regardless of all other factors evaluated. CO-Hb samples had significantly higher red color ($p \leq 0.05$) after 24 weeks at -25ºC. As pH decreased the amount of protein carbonyls increased significantly. Low pH also resulted in loss of CO. As the concentration of NaCl increased, lipid/protein oxidation significantly ($p \leq 0.05$) increased at 3.7 and -25ºC. Met-Hb formation increased significantly ($p < 0.05$) with increasing concentration of NaCl at both temperatures. Treatment with CO preserved the red color significantly at -25ºC regardless of concentration of NaCl. Low pH (6.3) increased ($p \leq 0.05$) lipid oxidation at both temperatures regardless of all other factors evaluated. CO-Hb had significantly higher red color ($p \leq 0.05$) after 24 weeks at -25ºC. Freezing temperatures significantly ($p \leq 0.05$) enhanced stability of CO treated WMTM. TBARS formed at 3.7ºC were significantly higher than that at -25ºC for 24 weeks. Panelists rating of the intensity of the off odor developed during storage was strongly correlated ($r = 0.95$) with the oxidation measured with TBARS. This work suggests that effect of pH supplanted Hb form and

concentration, NaCl concentration or temperature in influencing oxidation. Low pH increased the susceptibility of WMTM to oxidation, perhaps due in part to the release of hemin at lower pH. Treatment of WMTM with CO significantly decreases lipid/protein oxidation.

CHAPTER 1
INTRODUCTION

Hemoglobin (Hb) plays an important role in quality deterioration of fish muscle by promoting lipid oxidation and leading to color and flavor changes. Hb possesses catalytic abilities that allow it to produce peroxy radicals, alkyl radicals and other products that make it a powerful catalyst of lipid oxidation in fish muscle causing oxidative rancidity (*1*). Additionally, the oxygen-transport proteins undergo autoxidation producing the met form which is believed to be far more reactive than the reduced form, since it is capable of reacting with peroxides to form compounds that cause oxidation.

To delay formation of the met form, it is important to increase the stability of Hb. This can be done by the binding of specific ligands such as carbon monoxide (CO) to the heme porphyrin group, which is a common practice in the seafood industry to stabilize red color. Little is known how this form of Hb (carboxy-Hb) in fish compares in function and structural properties to the other common forms, oxy- and met-Hb.

Lipids are chemically unstable food components, and can undergo free-radical chain reactions (*2, 3*) resulting in rancidity (*2, 3*) and degradation of the food product. Lipid oxidation in muscle foods is a complex process which is referred to as lipid peroxidation (*4, 5*). Unsaturated fatty acids react with molecular oxygen to undergo autoxidation. The exact mechanism of this reaction is disputed. The direct reaction of unsaturated fatty acids with molecular oxygen is not thermodynamically favorable. Simic and others (*6*) report that autoxidation may be initiated by H_2O_2, or the free radicals $O2^{\cdot -}$, $ROO^{\cdot}$, $HO^{\cdot}$, $NO^{\cdot}$, $GS^{\cdot}$ endogenous to the system, or exogenous initiators such as UV, ionizing radiation, and heat. Another source of lipid peroxidation within muscle involves metal ions including Fe^{++}, which can abstract a proton from unsaturated fatty

acids (*7*). Two major sources of Fe within both meat and fish muscle are found in Hb within red cells and myoglobin within the tissues.

Research Objectives

This research was to elucidate a better understanding of the pro-oxidative activity of oxy-, carboxy-, and met-hemoglobin on the stability of fish muscle and the development of lipid oxidation. Information obtained can be used to develop more effective strategies for preserving the quality of fish during storage. Specific objectives are:

1. To investigate the effect of different forms of Hb (oxy-, CO, and met-Hb) on the development of primary and secondary products of oxidation in tilapia washed muscle system.
2. To investigate the effect of different concentrations of added oxy-, CO-, and met-Hb (6, 9, and 12 µmol/kg muscle) on the development of primary and secondary products of oxidation in tilapia washed muscle system.
3. To investigate the effect of different pH values (6.3, 6.8, and 7.3) on the development of primary and secondary products of oxidation in tilapia washed muscle system and tilapia muscle
4. To investigate the effect of different concentrations of added NaCl (150 and 450 mM) on the development of primary and secondary products of oxidation in tilapia washed muscle system and tilapia.
5. To investigate the effect of storage at different temperatures (3.7 versus -25°C) on the development of primary and secondary products of oxidation in tilapia washed muscle system.
6. To investigate the effect of oxidation on the development of odor and color changes during storage of samples at 3.7°C.

Research Significance

In recent years CO has been used to increase the stability of foods against spoilage and loss of color. When CO binds to Hb and myoglobin (Mb) in fish muscle, it increases its stability by slowing its autoxidation reaction, and decreases its ability to participate in lipid oxidation. The binding of CO to the heme molecule is also affected by pH. It is also known that the presence of NaCl, which is commonly used as an additive during food processing, can promote lipid oxidation. The significance of this study could give a new direction for the use of CO in the prevention of lipid oxidation in fish muscle during storage. It will further shed light on how CO suppresses the oxidation of oxy-Hb to met-Hb. In addition, the interaction between lipid oxidation and heme oxidation will be evaluated in relationship to protein oxidation. Further, the effect of pH and salt concentration will be examined in relationship to lipid oxidation. An investigation into these conditions is expected to give a better understanding of oxidation processes mediated by Hb in fish muscle and thus allow for better control of lipid oxidation.

CHAPTER 2
LITERATURE REVIEW

Lipid Oxidation in Fish Muscle

Lipid oxidation is one factor contributing to food spoilage, affecting many aspects of food quality including nutritional value, odor, flavor, functionality and appearance (*1*). Muscle lipids and proteins are substrates for oxidation. The two major groups of fish muscle lipids are triacylglycerols and phospholipids (*8*). Triacylglycerols are found both within muscle cells, especially in fatty species of fish and outside the muscle cells, but the amount varies with species and environmental conditions. Triacylglycerols are composed of fatty acids with straight chains and 16 or 18 carbon atoms (*9*). Phospholipids give structure to the cell membrane and are found in white muscle of fish ~ 0.5%-1% (w/w) and in dark muscle of fish at higher levels due to the presence of more mitochondria. Phospholipids contain more C 20 and C 22 unsaturated fatty acids. The polyunsaturation is about 15 times greater than that of triacylglycerols, making them more susceptible to oxidation because oxygen attacks the double bonds of fatty acids to form peroxide linkages.

Lipid oxidation in muscle foods is a complex process which is referred to as lipid peroxidation (*4, 5*). Unsaturated fatty acids react with molecular oxygen to undergo autoxidation. The exact mechanism of this reaction is disputed but is believed to occur in three stages: initiation, propagation, and termination. The direct reaction of unsaturated fatty acids with molecular oxygen is not thermodynamically favorable. Simic and others (*6*) report that autoxidation may be initiated by H_2O_2, or the free radicals $O2^{\cdot -}$, $ROO^{\cdot}$, $HO^{\cdot}$, $NO^{\cdot}$, $GS^{\cdot}$, endogenous to the system or by other endogenous catalysts—Hb, Mb, low molecular weight transition metals, lipoxygenases, microsomal

enzymes and mitochondrial enzymes (*8*) or exogenous initiators such as UV, ionizing radiation, and heat.

Another source of lipid peroxidation within muscle involves metal ions including copper and Fe^{++}, which can abstract a proton from unsaturated fatty acids (*7*). Two major sources of Fe within both meat and fish muscle are Hb within red cells and Mb within the tissues. Hb transports oxygen, CO_2, and hydrogen. It is present in the blood of muscle in most meats and fish, and is considered responsible for lipid oxidation in muscle products (*10, 11*). Mb is more predominant in the dark muscle of fish, and can also lead to undesirable qualities in fish muscle when the heme iron is oxidized (*7, 11-13*). Cytochromes, which are also heme proteins, are associated with membrane systems, especially mitochondria. At post mortem, if H_2O_2 is high, iron may be released from heme proteins, and can initiate lipid peroxidation (*14*).

A number of protein complexes with sulphydryl groups also bind iron such as transferritin and ferritin. Ferritin is a non-heme iron that stores iron until it is needed by various processes within the cell. It can store up to 4500 iron atoms as Fe(O)OH (*15*) and this can be released by chelators. Superoxide anion, ascorbate and thiols release Fe(O)OH by reduction processes. The two atoms of iron per transferrin molecule is difficult to release (*14*).

Lipoxygenases and cyclooxygenases may play a role in lipid oxidation in seafood. Hultin (*14*) suggests that no direct evidence exists that these enzymes are important catalysts in post-mortem fish. Rather, he suggests that they may contribute to lipid oxidation by contamination of the muscle components from the skin of fish, occurring during deboning and deskinning of minced tissue. However, Frankel (*16*) says

lipoxgenases are recognized catalysts of lipid oxidation in fish, in which lower temperatures promote enzymatic oxidation. The lipoxygenase found in gill and skin tissues of fish produce hydroperoxides from polyunsaturated fatty acids in fish. This enzyme can be inactivated by heating above 60°C.

Lipid Composition in Fish

Two types of lipids are found in fish: phospholipids and triacyglycerides. Phospholipids, which are highly unsaturated, account for 1% (w/w) of lipids contained in the muscle of lean fish and are the major components of cell membranes (*8, 17*). Approximately 75% of the fatty acids within fish are mono and polyunsaturated fatty acids (*18*). Triacyglycerides, the other major lipid in fish, are found in adipocytes and act as a source of storage energy. Total lipids of fish vary not only among species, but also from one part of the fish to another. Lean fish species such as cod and hake store the majority of lipids in the liver. The light muscle of lean fish contains approximately 1% phospholipid (*19*).

Unsaturated fatty acids of fatty fish species (*20*) can undergo oxidative degradation, particularly the polyunsaturation found in membranes of muscle tissue (*21*). The amount of lipids present is not the controlling factor in lipid oxidation but rather the presence and amount of Hb (*8, 22*). Less than 0.1% levels of phospholipids are necessary for Hb mediated oxidation to occur (*23*). Although the percentage of phospholipids is low in washed mince muscle systems, phospholipids are considered the primary substrate for lipid oxidation because they have 100 times more surface area than tryacylglycerols and the fatty acids of phospholipids are more unsaturated than those in tryacylglycerols (*24*). Richards and Hultin (*23*) found that increasing membrane phospholipids six-fold did not affect the extent of lipid oxidation or the extent of rancidity

development during storage of washed cod containing added blood. When trout hemolysate (5.8 µmol Hb/kg washed cod) was added to cod myosin preparation at 2°C, rancidity and TBARS formation did not develop during six days of storage suggesting that at least trace amounts of lipid are required for rancidity to occur.

Inhibitors of Lipid Oxidation

Initiation inhibitors remove active reduction products of oxygen or convert transition metals to inactive forms (*14*). Catalase and peroxidases remove hydrogen peroxide, while superoxide dismutase removes superoxide anion. Phospholipases inhibit lipid oxidation by inhibiting the ability of the membranes to oxidize its lipids.

Propagation inhibitors convert free radicals to stable compounds. These inhibitors are either water-soluble or lipid-soluble. Ascorbate and the glutathione peroxidase system are water-soluble propagation inhibitors. Tocopherols in fish muscle are lipid soluble antioxidants and are found in the unsaturated fatty acids membranes. Both the initiation inhibitors and propagation inhibitors act by donating electrons and will be eventually oxidized. When this happens, rapid oxidation will occur.

Changes in Post-mortem Fish

Changes that take place in post-mortem storage of fish muscle make it more susceptible to lipid oxidation (*14*). During storage at refrigerator temperature, low molecular weight iron in light muscle almost doubled from 155 ppb to 225 ppb (*25*), most likely coming from ferritin. Within 10 days post-mortem, the reduced forms of Hb and Mb were oxidized to met-Hb and met-Mb. Loss of reducing compounds over time such as ascorbate (*25*), NAD(P)H (*26*) and glutathione (*27*) decreased the reducing capacity of fish muscle. Muscle cells lose the ability to maintain ion gradients, especially calcium ions, which can activate enzymes such as lipases, phospholipases, and

proteases. Mincing of fish muscle exposes the tissue to more oxygen than the muscle receives with the living fish. Removal of oxygen will inhibit or stop lipid oxidation (*28*) (*29*). There is also loss of energy sources such as ATP. The post mortem pH of fish muscle can be 7.0 or higher in white fish such as cod to as low as 5.5 in some red meat fish. Lower pH is associated with increased lipid oxidation due to higher deoxy-Hb content.

Protein Oxidation

One of the common ways to increase the shelf life of fish is frozen storage. However, frozen storage and fluctuating temperatures can have a negative effect on the quality of fish (*30*). Some quality changes which cannot be attributed to lipid oxidation solely during frozen storage are toughness, changes in texture, loss of juiciness, and loss of protein functional properties (*31*). Little is known about the interaction between protein and lipid oxidation. It has been shown that the products of lipid oxidation can interact with proteins to produce oxidation (*32*).

Dalle-Donne and others (*33*) describe the production of protein carbonyl derivatives (aldehydes and ketones) as occurring in four ways: (1) direct oxidation of amino side chains, (2) oxidative cleavage by the α-amidation pathway or through oxidation of glutamine side chains, (3) Michael addition reactions of α,β-unsaturated aldehydes from lipid peroxidation and (4) reaction of reducing sugars or their oxidation products to the amino group of lysine residues (glycation and glycoxidation). Studies of protein carbonyl derivatives cannot distinguish how they have been formed, and protein carbonyl derivatives are therefore considered a broad marker of oxidation.

Ostdal and others (*34*) have demonstrated that proteins can transfer free radicals to other molecules such as lipids. Baron and others (*31*) investigated prolonged frozen

storage of rainbow trout to determine if oxidative changes in the protein fraction correlated with changes observed in the lipid fraction. The authors measured lipid oxidation using peroxide values, and protein oxidation was assessed with UV spectroscopy of protein carbonyl groups and SDS-PAGE and immunoblotting. No significant levels of protein carbonyls were found in rainbow trout stored for 13 months at -80°C and -30°C, indicating that little protein oxidation had occurred. However, fish stored at -20°C showed a significant increase in protein carbonyls (7.7 nmol/mg of protein) after 8 months of storage, demonstrating that protein oxidation had occurred. Immunoblotting of fish stored at -20°C revealed oxidation in bands that were identified as myosin and actin. The three storage temperatures (-20°C, -30°C, -80°C) showed small differences in protein oxidation patterns and levels with samples at -20°C slightly more oxidized compared to the other two temperatures. Levels of lipid hydroperoxides after 8 months of storage at -20°C were significantly increased with an even greater increase after 13 months of storage. Samples stored at -80 and -30°C did not show any significant increases in peroxides. Comparison of samples stored at -20°C indicated that lipid and protein oxidation followed the same pattern and suggests that protein and lipid oxidations were simultaneous. However, quantification of secondary volatile oxidation products using headspace GC-MS revealed that lipid oxidation proceeded differently in fish stored at -30°C compared to fish stored at -80°C. After 8 months of storage at -30°C significant increases in volatile oxidation products were detected. At this temperature, similar increases in protein carbonyls were not detected.

Eymard and others (*35*) investigated the link between lipid oxidation and protein oxidation during processing and storage of horse mackerel. Using fish minces with

differences in lipid and protein fractions and different oxidative levels, the authors were able to compare lipid and protein oxidation development. Protein carbonyls were measured as described by Levine and others (*36*). Horse mackerel fillets were processed into mince samples. The four samples included (MO) mince after the grinding, (M1) mince after the first dewatering, (M2,) mince obtained after the second dewatering, and (M3) mince obtained after the third dewatering. The authors found that the protein carbonyl was lowest in MO, with protein oxidation developing very rapidly during storage at 5°C and reaching the maximum after 12 hours of storage. For the washed minces, high carbonyl levels were detected at times zero and did not increase significantly. The authors also demonstrated with that at time zero, myosin and other high molecular weight proteins were already oxidized in all four products including the unwashed mince. The authors further demonstrated that the loss of Hb and Mb was most pronounced after the first washing. Some iron remained after the third washing and it was speculated that heme, which is very hydrophobic, may have been incorporated into the membranes. Since iron and heme proteins are pro-oxidative, their removal is a crucial step in obtaining stability against oxidation. Further, the authors found little lipid oxidation at times zero but significant oxidation in the washed samples. The authors also found that washing removed storage proteins more easily than the membrane phospholipids which are very sensitive to oxidation, which increased significantly after the second wash. The authors conclude that lipid and protein oxidation developed simultaneously but it was difficult to determine how they are linked. Lipid and protein oxidation share the same catalysts, and they can develop independently of one another, or in parallel, or they can interact with each other.

Kjærsgård and others (*30*) sought an understanding of the nature of protein oxidation by conducting proteome analysis of rainbow trout muscle proteins fractionated as low-salt (LS) and high-salt (HS) soluble, and identified oxidized proteins by LC-MS/MS. Analyses of fish, fresh or stored for 3 years at -80°C, showed no difference in the total level of carbonyl protein. Comparison of storage temperatures at -20°C versus -80°C over a two-year period revealed that frozen storage at -20°C produced two times higher levels of protein carbonylation and 10 times higher levels of peroxides. Immunoblots of proteins soluble in LS and HS buffer showed no clear differences in carbonylation at -80 and -20°C. High levels of carbonyls were found in muscle proteins fractionated as LS and HS. In the HS soluble protein fraction, three proteins with a size around 16 kDa, all identified as nucleoside diphosphate kinase (NDPK), showed alterations in concentration with concentrations 10 times higher for fish stored at -20°C compared to -80°C. The authors conclude that frozen storage at -20°C of rainbow trout results in increased protein carbonylation and induces changes in protein solubility.

Role of Heme Proteins in Oxidation

Hb distributes oxygen to the tissues of the body, and MB gives muscle meat its red pigment (along with Hb) and is located in the muscle cell (*5*). Both of these proteins bind oxygen and contain Fe(II) in the center of the heme group (*37*). Mb contains one polypeptide chain with one porphyrin ring containing one iron atom in the heme pocket (*38*); Hb is made up of four polypeptide chains, each containing a heme group. (Heme refers to the porphyrin ring with ferrous [Fe^{2+}], hemin refers to the porphyrin ring containing ferric [Fe^{3+}]). Fe is bound to four nitrogen molecules in the heme pocket. The bright red color of muscle tissue is due to iron in its reduced form (Mb—Fe^{+2}—O_2). When Fe^{+2} (ferrous) is converted to Fe^{+3}, autoxidation occurs. Cutting, freezing and

thawing of fish dark muscle makes it more susceptible to autoxidation, resulting in the formation of met-Mb (Mb—Fe^{+3}—H_2O), which is brown in color (*37*). During autoxidation, oxygen is released from oxy-Hb to form the superoxide anion radical, $O_2^{\bullet -}$ and ferric met-Hb which is converted to hydrogen peroxide which increases the ability of heme proteins to promote lipid oxidation. As demonstrated in Diagram 1, an adaptation of Baron and Andersen's (*5*), dynamic of Mb which reacts similarly to Hb, two active forms of Hb are present. Both deoxy-Hb (HbFe(II)) and oxy-Hb (HbO_2Fe(II)) exist in the reduced state and thus are susceptible to autoxidation. As can be seen from the diagram these two reduced forms are oxidized to the met-Hb (Hb-Fe(III)) which in turn is reduced again to the deoxy-Hb (Hb-Fe(II)). Met-Hb, in the presence of H_2O_2, can also be converted to the very pro-oxidative forms, perferryl-Hb ($^{\bullet}$Hb-Fe(IV)=O) and ferryl-Hb (Hb-Fe(IV)=O).

However, Richards and Hultin (*4*) reported that in the presence of 3.4 µM of lipid peroxide, reduced Hbs (oxy/deoxy-Hbs) produced high levels of lipid peroxide formation, but met-Hb caused little peroxidation of linoleic acid. A reason for this is met-Hb cannot autoxidize but the reduced Hbs form superoxide anion radicals during autoxidation which can form hydrogen peroxide. The hydrogen peroxide will then activate met-Hb (*39*). If met-Hb is the initial reactant there is no source of oxygen to form hydrogen peroxide. In addition, reduced Hbs but not met-Hb can produce hydroxyl radicals (*40*). Met-Hb forms hemichromes which are not considered catalysts of lipid peroxidation (*41*). If deoxy-, oxy-, or met-Hb is denatured or their structures are altered, low spin iron(II)-hemochromes or iron(III) hemichromes may form which can be

reversible or irreversible reactions but their role in lipid oxidation has received little attention (*5, 42*).

Deoxy-Hb content affects heme protein autoxidation. Deoxy-Hb, in the presence of O_2, is susceptible to rapid oxidation (*1, 4*). However, fully oxygenated Hb was found to react slower with hydrogen peroxide because the accessibility of the heme iron is inhibited by the O_2 ligand in oxygenated molecules (*43*).Oxy-Hb is also more compact compared to deoxy-Hb which has greater flexibility in the heme pocket (*43, 44*). However, despite the low deoxy-Hb content found for perch Hb, Richards and Dittman (*1*) found that perch Hb had a high autoxidation rate. This could be explained by the amino acid sequences near the heme crevice. It has been found that disrupting the hydrogen-bonding network of His^{97} created easier accessibility of H_2O into the heme crevice (*5, 45*). This would then accelerate the formation of Met-Hb, which would also increase lipid oxidation.

Met-Hb studies have not demonstrated a pro-oxidative activity at physiological pH (*46-48*). Sato and Hegarty (*49*) were not able to demonstrate the catalytic activity of met-Hb in meat and other studies have supported this (*50-52*). However, these studies used washed muscle systems which could underestimate the pro-oxidative activity of met-Hb by washing away compounds such as hydroperoxides which could be important for the pro-oxidative activity of met-Hb (*7*). Recent research has found that met-Hb is a potential pro-oxidant at the pH found in fresh meat (between 5.3 and 6.2) which emphasizes the importance of hydroperoxides in met-Hb initiated lipid oxidation (*53*). Electrostatic and hydrophobic interactions are involved when Hb binds to phospholipids. Met-Hb affects the structural and physiochemical parameters of the lipid-water interface

(*54*). This results in the formation of hemichrome, a poor initiator of lipid oxidation (*41*) at physiological pH in model systems containing long-chain free fatty acids, resulting in non-catalytic activity. At lower pH values, the electrostatic and hydrophobic interactions are not involved, most likely due to the different charge distribution on both the fatty acid and the heme protein (*5*). Thus, in the presence of lipids, met-Hb at physiological pH, due to the formation of the non-catalytic hemichrome, can undergo rapid neutralization. However, in a high lipophilic environment, denaturation of the heme protein may result in exposure of the heme group to the surrounding lipids and induce lipid peroxidation. However, at lower pH values, met-Hb is able to initiate lipid oxidation in a lipid hydroperoxide-dependent mechanism.

Richards and Hultin (*11*) found that increasing the concentration of Hb in washed mince cod increased lipid oxidation. Hemolysate containing 0.06 and 0.50 µmol Hb/kg tissue (pH 6.3) did not develop rancidity during storage at 2°C as measured by sensory scores and TBARS. However, rancidity developed at day 1 and day 2 for hemolysate containing 5.8 and 1.8 µmol, respectively. These authors also found that although Hb concentration was higher in whole trout muscle than in light muscle of mackerel, more oxidation occurred in the light muscle during storage on ice. It was postulated that this difference in oxidation susceptibility is based upon different types of Hb. At post mortem pH, anodic Hbs bind oxygen poorly while cathodic Hbs retain a high affinity for oxygen. This is important because deoxy-Hb is a more effective catalyst of lipid oxidation as compared to oxy-Hb and met-Hb. Richard and others (*55*) demonstrated that deoxy-Hb is more pro-oxidative than oxy-Hb in washed cod muscle. From their data, the reason for this could not be determined but the authors speculated that it could be due to ferryl-

Hb forming more readily, or the release of hemin, or better access of lipid hydroperoxides to the heme crevice in the oxy-Hb, or a combination of these mechanisms.

Richards and others (*56*) compared the oxidative characteristics of trout Mb versus trout Hb and found that Mb was a weaker promoter of lipid oxidation. This was explained in part, by the fact that the reactive heme group in Met-Hb is more loosely anchored in the globin and thus more capable of initiating lipid oxidation. Additionally, the met-Hb protein can react with hydrogen peroxide or lipid peroxides and form the ferryl protein cation radical, which in turn, can initiate lipid oxidation. Further, the authors measured heme dissociation from trout Mb and Hb and found that heme dissociated much faster from anodic trout Hb than from trout Mb, suggesting that heme dissociation plays a role for various Hbs to promote lipid oxidation. In an earlier study, Richards and others (*55*) found a 14-fold difference in the rate of TBARS formation during 1.5 days of storage (pH 6.3, 2°C) for anodic Hb as compared to cathodic Hb. This provided further evidence that heme dissociation is a major contributing factor for different Hbs to promote lipid oxidation.

The heme group within both Hb and Mb contains iron in its reduced state when muscle is fresh. When iron is oxidized, it contributes to the brown color of fish muscle (*57*). This oxidation also produces more active forms of Hb and Mb, the met, ferryl and perferryl forms, which leads to more lipid oxidation (*11*). One way to reduce the pro-oxidative activity of these heme proteins is to maintain the proteins in their reduced state. For example, Kristinsson and others (*57*) investigated the effect CO gas treatment had on tilapia Hb and found that treatment with CO greatly stabilized the protein with

respect to its pro-oxidative potential (in a model linoleic acid system) since it was maintained in the reduced state.

Loss of Hemin

Mechanisms of hemin loss using Hb and Mb variants were investigated by Grunwald and Richards (*58*). Comparing human Hb, which was genetically cross-linked to prevent tetrameric Hb from dissociating into subunits, and a Mb variant from sperm whale in which the native valine residue was substituted with threonine (68^{th} site) to provide high hemin affinity versus the native variants of both Hb and Mb, the authors found that higher hemin affinity in the genetically altered variants resulted in decreased lipid oxidation. TBARS formation occurred more rapidly with the native Hb at pH 5.7 in washed cod muscle during storage at 2°C. Similarly, the genetically altered Mb was less effective in promoting lipid oxidation than the native Mb as measured by TBARS formation in washed cod. To further determine the effect of hemin on lipid oxidation, addition of hemin and hemin with bovine albumin to washed cod was examined during storage at 2°C. TBARS reached a maximum formation after two days of storage with hemin alone. The addition of albumin to hemin increased the onset of TBARS formation. The authors suggest albumin delivers hemin into the lipid phases which increases the ability of hemin to oxidize the lipids.

Hargrove and others (*59*) develop an assay for hemin dissociation. His^{64} in sperm whale myoglobin was replaced by Tyr, producing a holoprotein with the discrete green color. Val^{68} was replaced with Phe in the same protein to increase its stability while retaining high affinity for hemin. This protein can then be used for complete extraction of hemin from Hb and Mb, giving absorbance changes to allow reactions at low hemin concentrations. When this protein (apoprotein Tyr^{64}•Val^{68}) is mixed in excess with

mutant Hbs, the solution turns from brown to green, and the absorbance changes can be used to measure the rate of dissociation of hemin. Mutant hybrids of human Hb were prepared by substituting Gly at His^{64} (E7) and oxidized with ferricyanide and then reacted quickly with apoprotein Tyr^{64}•Val^{68}. Hemin dissociation from the Gly^{64} mutants and subsequent uptake by apoprotein Tyr^{64}•Val^{68} resulted in rapid absorbance increases. Dissociation of hemin from Tyr^{64} mutant Mb at pH 5.0, 37°C is attributed to a protonation of the proximal His^{93} imidazole side chain, which disrupts the Fe^{3+} -His^{93} bond. The authors conclude that the results presented indicate that apoprotein Tyr^{64}•Val^{68} can be used reliably for measuring hemin loss.

Hemin loss from oxidized trout and perch Hb and bovine Hb were measured by mixing them with an apoglobin form of H64Y sperm whale Mb at 25°C and pH 5.7, 6.3, and 8.0 (*60*). The authors found that perch Hb had approximately 50 fold higher hemin loss rates at pH 5.7 and 6.3 compared with bovine Hb. Hemin loss rates from trout Hb were 10 to 30 fold faster than bovine Hb at the same low pH values.

Aranda and others (*60*) suggests that there are four mechanisms by which auto oxidation and hemin loss occur. These include steric displacement of bound ligands, weak anchoring of the heme propionate to the globin, larger channels for solvent entry into the heme pocket, and weakened interactions with the distal histidine. The rate of autoxidation is increased 15-fold when Val (E11 side chain) is replaced by Ile (E11) in sperm whale Mb. This increase is explained by the fact that the δ methyl group of Ile is located closer to the heme iron atom in the heme pocket than Val (E11). When the distance between the Cδ atom of the sec-butyl side chain and the bound ligand is shorter (1.8-2.6 Å), strong steric clashes occur with the bound ligand. Thus, the

presence of Ile in fish Hb subunits hinders the bound ligand, and enhances the rate of •OOH dissociation and autoxidation in perch and trout Hbs (*60*).

In perch and trout Hbs, the side chains Thr E10 and α CE3 results in weak anchoring of the heme propionate. Thr E10 in the subunits of both fish are too short to hydrogen bond through the water molecule to the 7-propionate so there are no favorable electrostatic interactions with the heme group. Hemin loss increases when this type of bonding is not present (*61*). Trout IV met-Hb alpha chains at pH 5.7 and 6.3 have a much shorter distance (2.7-2.8Å) between Nε of His(CE3) and a proprionate O atom than perch met-Hb (3.2 and 4.7 Å) at pH 6.3 and 5.7, which indicates little or no electrostatic stabilization of the heme-6 propionate. Previous studies have shown that if the heme 6 propionate cannot be stabilized, a higher rate of hemin loss from human beta subunits occurred (*62*).

Liong and others (*45*) have shown that increased access of water molecules to the heme pocket increases hemin release in mutants of Mb. The amino acid residue at the CD3 position is not able to hydrogen bond to the heme-6-propionate O atoms. The further away this residue is from the heme pocket, the easier it is for water to enter the heme pocket, increasing auto oxidation and hemin loss. These distances will vary with pH. Compared to bovine Hb at lower pH, perch and trout IV β subunits have a greater distance between the CD3 side chain and the heme-6-proprionate O atom. This increase in distance increases autoxidation and hemin loss (*60*). The electrostatic interaction of the hydrogen bonding of the distal His to bound O_2 inhibits auto oxidation by limiting access of solvent to the heme crevice (*63*). The rate of hemin loss will also

be decreased if hydrogen bonding of the distal His to water in oxidized Hb occurs (*45, 61*).

Auto oxidation and hemin loss for Perch, trout IV, and bovine Hb are pH dependent. As the pH decreases, auto oxidation and hemin loss increase. Aranda and others (*60*) suggested that the reason for the pH dependence may involve the protonation of distal His resulting in disruption of the bound O_2. This disruption might cause a rotation of the side chain. Another reason is the dissociation of $^{\bullet}OOH$ by the bound O_2, which result in the formation of met-Hb. It could also be due to the interruption of the electrostatic forces with amino acids at E10 and CD3 positions. Higher rates of auto oxidation and hemin loss occur at higher pH, for trout and perch Hb as compared to bovine Hb, indicating higher rates (*pKa* values) for protonation of HisE7, bound O_2, HisF8, and the heme propionates.

Met Hemoglobin Reduction

Hb and Mb share structural properties and physiological functions (*64*). However, the mechanism for reducing met-Hb is not the same for both of these proteins. Work by Arihara and others (*64*) has shown that met-Mb reduction occurs in the mitochondrial fraction skeletal muscle and at the sarcoplasmic reticulum. OM cytochrome *b* or cytochrome b_5, both of which act as electron transfer mediators, are required for NADH-cytochrome b_5 reductase to reduce met-Mb, the former at the mitochondria, and the latter at the sarcoplasmic reticulum. However, in humans, it has been shown that NADH-cytochrome b_5 reductase uses cytochrome b_5 to reduce met-Hb. The absence of the enzyme NADH-cytochrome b_5 reductase, causes an accumulation of met-Hb (met hemoglobinemia) in humans. NADH-cytochrome b_5 reductase pathway has been the only one found as an electron transfer mediator in the erythrocytes of humans as an

electronic transfer mediator (*64*). OM cytochrome *b* has not been found in the erythrocytes.

Although the reduction pathways for met-Mb and met-Hb are not same, these two heme proteins share some factors that affect their reducing activity. In their review article (Bekhit and Faustman (*65*)) several studies indicate that met-Mb reducing activity during assay is temperature dependent for different species. For example, the optimal temperature for met-Mb reductase in the muscle of tuna is 25°C whereas that for bovine cardiac muscle is 37°C. Further, at low pH temperature effects are small, but Reddy and Carpenter (*66*) reported that met-Mb reducing activity increased markedly with increasing the temperature from 4 to 30°C.

Similar to the effect of temperature at time of assay, met-Mb reducing activity is pH dependent and also related to purified preparations versus extracts and assay conditions (*65*). Conflicting studies report optimal pH levels. For example, using methylene blue and NADH, Taylor and Hochstein (*67*) reported that the optimum pH for bovine cardiac met-Hb reductase activity was 7.0, but Hagler and others (*68*) reported the met-Hb reductase activity with purified bovine cardiac muscle was greatest at pH 6.5. Differing techniques and methodologies from different researchers' results in different conclusions. Bekhit and Fauustman (*65*) further suggest met-Hb reducing activity during storage time gives varying results due to different methodologies. Additionally, the actual storage time was different between studies presented so no general conclusion can be drawn about how storage time affects met-Hb reduction.

Role of Carbon Monoxide (CO)

The autoxidation and the resultant brown coloring can be prevented by treatment of the fish muscle with CO which has a greater affinity (>240 times more than oxygen)

for the Fe(II) binding site, forming carboxy-Hb (CO-Hb) and carboxy-Mb (CO-Mb), resulting in a cherry red color that is stable over longer periods. When CO is bound to heme in Hb, it gives stability to the protein, and the protein will resist autoxidation on heating, freezing, and thawing (*69, 70*). To improve the quality and preserve the attractive red color of dark muscle, CO or tasteless smoke (TS) which contains CO gas, have been used by the seafood industry (*37*) to preserve the red "fresh" appearance of seafood products. Kristinsson and others (*69*) have shown that treating yellowfin tuna significantly enhanced the red color, reduced lipid oxidation, and lowered aerobic bacterial growth. Anderson and Wu (*37*) used GC/MS to determine CO in tuna and mahi-mahi tissues in vacuum-packaged products and CO-treated frozen mahi-mahi samples. Samples of vacuum packaged tuna showed CO levels about 1 mcg/g while the CO treated frozen mahi-mahi samples had 500 ng/g. This quantitative determination of CO can be useful for regulatory purposes in determining exposure to CO.

Replacing oxygenated Hb and Mb with CO-Hb and CO-Mb in the dark muscle of fish may offer some protection against lipid oxidation (*69, 70*). High concentrations of unsaturated fatty acids in dark muscle of fish, in the presence of oxy-Hb and deoxy-Hb, may result in increased levels of lipid peroxides. This is due mainly to the autoxidation and deoxygenation of Hb as pH was reduced. Kristinsson (*69*) reported that, at lower pH, the protein is partly unfolded, giving the heme portion greater ability to participate in oxidation. Autoxidation of the heme protein to the met form is a critical step in lipid oxidation.

CO treatment retards autoxidation of Hb and Mb to the ferric form, and thus plays a role in decreasing lipid oxidation. Danyali (*71*) found that CO and filtered smoke (FS)

treatment can be effective in retarding lipid oxidation. FS, which contains phenolics that have the potential to serve as antioxidants, was not more effective than the other gas treatments in retarding oxidation development. After tuna steaks were treated with FS, 4%, 18% and 100% CO, Kristinsson and others (*72*) measured lipid oxidation using TBARS (thiobarbituric acid reactive substances). They found that the 4% CO treated tuna had higher TBARS than untreated tuna. The authors theorized that the higher levels of O_2 and CO_2 in the 4% CO may have promoted oxidation of heme protein. The 18% CO and FS (containing approximately 18% CO) may have protected the heme molecule against oxidation by CO_2. After freezing and cold storage (4°C) for 4 days, TBARS gradually decreased for all treated samples. The authors attributed the reduction in TBARS to muscle proteins binding the MDA (malondialdehyde) making less available in the TBARS assay. Garner and others (*73*) described decreased lipid oxidation in the white and dark muscle of Spanish mackerel that had been treated with CO and FS. Additionally, Garner and others (*73*) reported that 100% CO was more effective than 100% nitrogen in retarding lipid oxidation, which suggests that the effect is not due solely to the absence of oxygen. Kristinsson and others (*72*) proposed that treating yellowfin tuna with medium to high levels of CO may stabilize the heme protein molecule against lipid oxidation and preserve the red color of muscle foods by binding with Fe^{+2} of muscle heme, forming a cherry red carboxy Mb/Hb.

Levels of CO found in fish muscle after treatment with CO are not considered hazardous to the health of humans. During cooking, approximately 85% of CO is lost (*74*). However, the stable red color produced by CO can mask spoilage beyond the

recommended shelf life of meat (*75*). In United States, exact determination of the legality of using CO to preserve fish is yet to be ascertained.

Modified Atmosphere Packaging (MAP) Using CO

Modified atmosphere packaging (MAP) replaces air with a single gas or a mixture gases. These gases include oxygen, nitrogen, carbon dioxide and carbon monoxide, depending upon color stability requirement, growth of microbial agents and the sensitivity of the product to the particular gases that are used (*76*). FDA has reviewed the use of CO in packaging for meat products and has classified it as Generally Recognized as Safe (GRAS). Low levels of CO in MAP cause no risk to humans. Further, the USDA Food Safety and Inspection Service (FSIS) does not require labeling for modified atmosphere gases, including CO. The low levels of CO (0.4%) in MAP stabilize the natural red color of fish and meat. Caution must be taken by consumers because the red color, which indicates “freshness” to the consumer, may last longer than the shelf life of the product. Thus, it may mask the spoilage of the product. Sørheim and others (*74*) reviewed the use of CO in the modified-atmosphere packaging of meat and found that for the past 10 years the Norwegian meat industry has been using a combination of gases in MAP that included 0.3-0.4% CO which increased the microbial shelf life and maintained the cherry red color. Evaluation of the stability of this color (*74*) in ground beef patties indicated that a mixture of air and CO in MAP stabilized the color for 15 d compared with 5 d for air. Recently, the FDA has declared (*77, 78*) that the use of CO in MAP is a “processing aid” and does not require product labeling. The 0.4% of CO used in MAP for meats met the legal definition of a processing aid and there would not be significant amounts of CO in the finished product. This declaration paves the way for the use of CO in MAP of fresh fish (*79*). Consumers should not rely

exclusively upon the color of the meat to determent its suitability to consume, but should also check the “use by date”, strong spoilage odor, slippery texture, or packaging that has begun to swell.

Effect of pH on Oxidation

Most post mortem fish muscle and muscle systems have pH values below 7.0. The role of pH under these conditions is important in understanding lipid oxidation. Richards and Hultin (*4*) investigated the effect of pH (7.6, 7.2, and 6.0) on lipid oxidation using trout Hb as a catalyst in a washed minced cod muscle system and found that at lower pH values formation of met-Hb occurred more rapidly and the level of deoxy-Hb increased sharply. Lipid oxidation of the washed cod muscle induced by trout hemolysate occurred more rapidly at pH 6.0 compared to pH 7.2, using TBARS and sensory scores to measure oxidation. Lowering the pH below neutrality decreases the oxygenation of Hb. This is known as the Bohr effect (*43*).The Bohr effect can explain the high levels of deoxy-Hb at postmortem due to the low pH levels. When pH levels are decreased below 6.5, further deoxygenation occurs and this is known as the Root effect. The authors proposed that these results suggest that deoxy-Hb may play the role of a catalyst in lipid oxidation. Undeland and others (*22*) studied the effect of pH in a washed, minced cod model system using menhaden, mackerel, flounder, and Pollock Hb as catalysts. At pH 6.0 TBARS and painty odor showed that at all four Hbs were equally active proxidants. However, at pH 7.2, the proxidative activity of Hb was slowed except that from pollock. The authors found that the higher rate of oxidation at pH 6.0 corresponded to a greater formation of deoxy-, and met-Hb.

Richards and others (*55*) added Hbs obtained from beef, chicken, and trout to washed cod minced muscle to give a final concentration of 5.8 µmol/kg at pH 6.3. These

Hbs were used because there was a high variation in the oxygenation of each. At postmortem pH values, compared to bovine Hb, trout Hb is largely deoxygenated and chicken has an intermediate level of deoxy-Hb content. The authors speculated that the poorly oxygenated levels of trout Hb would be a better catalyst for lipid oxidation then either chicken or beef Hb. During seven-day storage at 2°C, TBARS developed more quickly using trout Hb in the cod mince as opposed to the chicken or beef Hb. The same was also true for lipid peroxidation development of washed cod muscle.

Richards and others (*80*) added separately trout Hb and tilapia Hb to washed cod muscle at pH 6.3 and 7.4 to assess the onset of lipid oxidation of samples that were stored at 2°C. Indicators of lipid oxidation included formation of TBARS, painty odor, and lipid peroxides. At day 2, trout Hb-containing samples (pH 6.3) had higher TBARS values compared to those containing tilapia Hb ($p < 0.001$). At pH 7.4, trout Hb-containing samples did not show an increase in TBARS until day 8 of storage while TBARS for tilapia Hb-containing samples remained low during the 14 days of storage. The authors also added trout and tilapia Hb to washed tilapia at pH 6.3 and 7.4. They found no difference in TBARS values at pH 6.3, but at pH 7.4 TBARS were significantly depressed for both Hb samples. The trout and tilapia Hbs (pH 6.3) in the tilapia muscle system had higher TBARS compared to the washed cod. During storage at 2°C, pH 6.3, trout Hb samples developed a painty odor by day 2, and by day 3 tilapia Hb samples. At pH 7.4, no painty odor was detected in tilapia Hb samples at day 14 but trout Hb samples had moderate rancid odor at day 12. Lipid peroxide values obtained during storage at 2°C (pH 6.3) showed that tilapia Hb samples in washed cod had lower peroxide values on day 2 than trout Hb samples. At pH 7.4, low lipid peroxide values

were found for both trout and tilapia Hb samples on day 8 in the washed cod system. Using the same Hb samples in the tilapia washed system showed rapid formation of lipid peroxides at pH 6.3 similar to that observed for TBARS. Both types of Hb in the tilapia system at pH 7.4 showed significantly slower and less peroxide formation. At pH 6.3 and 7.4, each Hb formed lipid peroxide more extensively in washed tilapia compared to washed cod.

In another study, using washed cod muscle and cod Hb (3µmol Hb/kg of mince), decreasing the pH from 7.8 to 6.3 greatly decreased the lag phase and increased the rate of lipid oxidation (*81*). Phospholipids constituted approximately 80% of the total lipids. The lag phase at pH 7.8 was ~40 h and the oxidation rate was slower compared to pH 6.8 and 3.5, which had faster rates of oxidation and lag times of 6 and 3 h, respectively. At pH values above 7.8, slower rates of oxidation were observed. The authors conclude that the rate of oxidation as a function of pH was determined by a decrease Hb-catalyzed activity at high pH and decreased susceptibility of the membranes to oxidation at low pH values.

Role of Sodium Chloride (NaCl) in Oxidation

Sodium chloride ions are found in all living cells and can act as a pro-oxidant of muscle lipid peroxidation (*82*). Kanner and others (*83*) found that presence of NaCl initiates reactions producing superoxide anion radical ($O_2^{\cdot-}$) which results in formation of hydroxyl radical. The pro-oxidative effect and the increase of lipid peroxidation by NaCl in model systems were studied and the elevation of free iron in tissues was ascribed to NaCl (*82*). Wallace and others (*84*) found that the stability of oxy-Hb and its oxidation to met-Hb may have been affected by the presence of NaCl by shifting ferrous ions from

interaction with oxygen to reaction with hydroperoxides and decomposing these compounds to free radicals, accelerating the peroxidation process.

Harel (*85*) investigated the effect of NaCl on autoxidation of ferrous and cuprous ions in the presence of ascorbic acid and iron chelators. The generation of hydroxy radicals by ascorbic acid and metal ion's was inhibited by NaCl. NaCl also inhibited the oxidation of ascorbic acid by preventing the interaction of Fe or Cu with oxygen. The chloride anion interacts with the iron ion inhibiting the ferrous ion oxidation. Calcium chloride, magnesium chloride, and Lithium chloride showed similar results. Wallace and others (*84*) demonstrated that NaCl accelerates the decomposition of oxy-Hb to met-Hb. Harel (*85*), postulating that NaCl prevents or disturbs the interaction between heme iron and oxygen in the same way.

Thus, a review of the literature revels that lipid/protein oxidation are complex mechanisms in determining the fish muscle. Additionally, the role of heme has been more carefully clarified. Use of CO treatment has had a significant effect on the stability of Hb, thereby reducing oxidative processes. It has also been shown that the effect of pH on oxidation supersedes all other factors when controlling for deterioration of fish muscle. Finally, concentrations of NaCl also affect oxidation negatively. The interaction of these various factors contributes considerably to the overall stability of fish muscle.

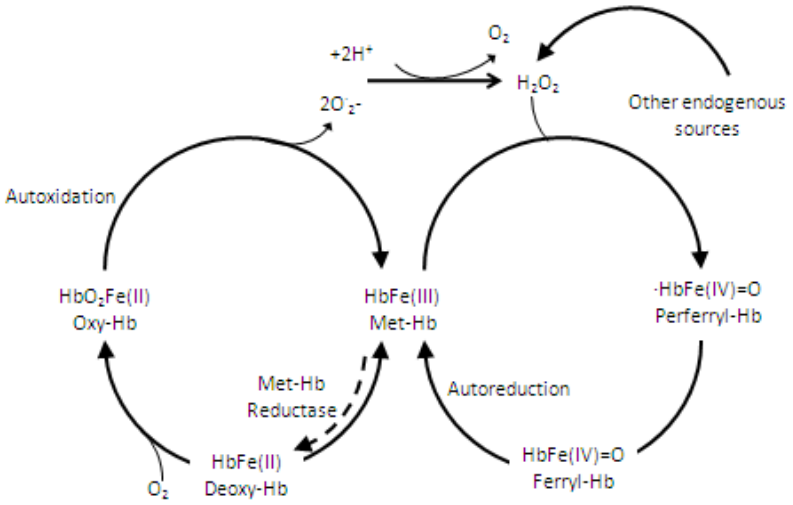


Figure 2-1. The dynamic conversion between different Hb forms (adapted from Baron and Andersen (2002)).

CHAPTER 3
CARBOXY-, OXY- AND MET-HEMOGLOBIN: A COMPARATIVE STUDY OF HEMOGLOBIN MEDIATED LIPID/PROTEIN OXIDATION IN WASHED MINCED TILAPIA MUSCLE AT TWO DIFFERENT STORAGE TEMPERATURES

Introduction

Quality deterioration of seafood products caused by lipid oxidation results in off-odors, off-flavors, and color defects. Two heme proteins play an important role in the quality of seafood products; hemoglobin (Hb), located in red blood cells and myoglobin (Mb) located in muscle cells (*5*). Both of these proteins bind oxygen and contain Fe(II) in the center of the heme group (four in Hb and one in Mb) (*37*) which can bind to different ligands. The bright red color of muscle tissue is due to iron in its reduced form (Mb—Fe^{+2}—O_2). When Fe^{+2} (ferrous) is converted to Fe^{+3}, autoxidation occurs, resulting in the formation of met-Mb (Mb—Fe^{+3}—H_2O), producing an undesirable brown color (*37*) and ultimately, quality deterioration of the seafood product.

Both deoxy-Hb (HbFe(II)) and oxy-Hb (HbO_2Fe(II)) exist in the reduced state and thus are susceptible to autoxidation. Reduced Hbs form superoxide anion radicals during autoxidation which can form hydrogen peroxide (H_2O_2). Met-Hb, in the presence of H_2O_2, can be activated (*39*), and converted to the very pro-oxidative forms, perferryl-Hb (·Hb-Fe(IV)=O) and ferryl-Hb (Hb-Fe(IV)=O).

Deoxy-Hb content affects heme protein autoxidation. Deoxy-Hb, in the presence of oxygen, is susceptible to rapid oxidation (*1, 4*). However, fully oxygenated Hb was found to react slower with H_2O_2 because the accessibility of the heme iron is inhibited by the O_2 ligand in oxygenated molecules (*43*). Oxy-Hb is also more compact compared to deoxy-Hb which has greater flexibility in the heme pocket (*43, 44*). It has been found that disrupting the hydrogen-bonding network of His^{97} created easier accessibility of

H_2O into the heme crevice (*5, 45*). This would then accelerate the formation of met-Hb which would also increase lipid oxidation.

Richards and Hultin (*11*) found that increasing the concentration of Hb in washed minced cod increased lipid oxidation. Hemolysate containing 0.06 and 0.50 µmol Hb/kg tissue (pH 6.3) did not develop rancidity during storage at 2°C as measured by sensory scores and TBARS. However, rancidity developed at day 1 and day 2 for hemolysate containing 5.8 and 1.8 µmol, respectively.

One way to reduce the pro-oxidative activity of these heme proteins is to maintain the proteins in their reduced state. Kristinsson and others (*57*) investigated the effect of CO gas treatment on tilapia Hb and found that treatment with CO greatly stabilized the protein with respect to its pro-oxidative potential (in a model linoleic acid system). CO binds to heme with greater affinity and easily replaces oxygen from the heme pocket. The CO binding produces a cherry red color, which is stable during refrigeration and frozen storage temperatures.

The objective of this study was to compare the pro-oxidative activity of oxy, CO-, and met-Hb in a washed model system. It is hypothesized that tilapia Hb treated with CO will be less pro-oxidative than oxy or met-Hb during storage at 3.7°C and -25°C in a washed minced model system.

Materials and Methods

Preparation of Washed, Minced Tilapia Muscle (WMTM)

Fresh skinless tilapia fillets were obtained locally from Rainforest Aquaculture (Sunrise, FL). All dark muscle, fat, blood spots and excess connective tissue were removed. The remaining fillets were ground (Oster® Heavy Duty Food Grinder, Sunbeam Corporation, Inc.) and washed twice with distilled deionized water at 1:3

mince to water ratio (at <5°C). The mince was stirred for 2 min with a glass rod, and allowed to stand for 15 min on ice. The minced muscle was then dewatered on a nylon screen. Finally, the washed muscle was mixed with a 50 mM sodium di-phosphate buffer at the pH of interest (6.3, 6.8, and 7.3) at a ratio of 1:3 mince to buffer and stirred for 2 min with a glass rod, then allowed to stand for 15 min on ice. The washed muscle was centrifuged at 15000g for 20 min at 3.7ºC using an Eppendorf 5702 centrifuge (Eppendorf North America Inc., New York, NY). Streptomycin (200 ppm) was added to prevent microbial growth and samples were vacuum packed (~100 g) and stored at -80ºC until used. The final moisture content of the samples was ~83% measured using a moisture balance (CSI Scientific Company, Inc., Fairfax, VA).

Collection of Fish Blood

Live tilapia were obtained from a pond at the UF Department of Fisheries and Aquatic Sciences and transported in an aerated water tank to the Aquatic Food Pilot Plant, University of Florida. Upon arrival, the fish were placed on ice for 1 minute with the pectoral side facing up. The bleeding procedure was conducted according to Kristinsson and others (*57*). Blood was drawn through the caudal vein using syringes (25 gauge, 1-inch needles). Syringes were pre-loaded with heparin solution (150 units/mL) according to Fyhn and others (*86*).

Preparation of Hemolysate

The hemolysate was prepared according to a modified procedure of Fyhn and others (*86*). Heparinized blood was washed with four volumes (1:4) of ice cold 1.7% NaCl in 1mM Tris buffer, then centrifuged using a tabletop centrifuge (Eppendorf Centrifuge 5702, Brinkman Instruments. Inc., Westbury, NY) at 1000g for 10 min at 3.7ºC. Plasma was removed and discarded. Red cells were washed three times with 10

volumes (1:10) of ice cold 1.7% NaCl in 1mM Tris buffer and centrifuged at 1000g for 10 min. Supernatant was removed and discarded. The red cells were lysed in Tris-HCl buffer at pH 8.0 for one hour. One-tenth volume of 1M NaCl was added to aid in stromal removal (pellets) before centrifugation at 30000g for 15 min at 3.7°C. The hemolysate, obtained as the supernatant, was stored in 1.5 mL cryogenic tubes in a freezer at -80º C until used.

Quantification of Hemoglobin Levels in Hemolysate

Hb levels were quantified spectrophotometrically according to the Bradford procedure(*87*) using Coomassie® Plus Protein Assay Reagent Kit (Pierce Technology, Rockford, IL). An approximate Hb concentration level was calculated using a BSA standard curve and the average molecular weight of Hb (ca. 66,000).

Oxy-, CO- and Met-hemoglobin Preparation

Oxy-Hb was obtained from the lysis of red cells (hemolysate) and purified according to Fyhn and others (*86*), as modified by Richards and Hultin (*4*), described in the preparation of the hemolysate. Oxy-Hb was identified using UV-Vis spectra of heme proteins according to the method of Kristinsson and others (*57*). A diluted aliquot of the hemolysate was used to determine the presence of oxy-Hb. A peak at 414 nm was observed indicating that the heme protein was bound to oxygen.

CO-Hb was prepared according to Kristinsson and others(*57*) by placing a 5 mL aliquot of oxy-Hb in 50 mL Falcon tubes on ice and passing a stream of 100% CO over the solution for 2 min. The tube was capped immediately until used. The CO-Hb protein aliquot showed a peak at 419 nm suggesting that the muscle sample was bound to CO.

Met-Hb was prepared according to DeYoung and others (*88*) by reacting oxy-Hb with potassium ferricyanide ($K_3Fe(CN)_6$). $K_3Fe(CN)_6$ was added at three times the molar

concentration of the Hb and the mixture allowed to stand for 30 min on ice. Unreacted ferricyanide was removed using Centricon centrifugal filter devices using a tabletop centrifuge (Eppendorf Centrifuge 5702, Brinkman Instruments. Inc., Westbury, NY) at 4000g for 30 min at 3.7ºC. Sodium phosphate buffer (20 mM) was used twice to remove potassium ferricyanide and centrifuged again for 30 min. Changes in Hb levels after preparation of met-Hb were monitored spectrophotometrically according to Bradford (*87*). The sample peaked at 406 nm and thus was identified as fully oxidized met-Hb.

Sample Preparation: Addition of Hb and NaCl

Samples of tilapia washed model system previously prepared and stored at -80°C were thawed rapidly under running water (20°C) and kept on ice. The pH was adjusted to the desired pH of (6.3, 6.8, or 7.3) using 2N NaOH or 2N HCl. After the desired pH was established, moisture content was determined using a moisture balance (CSI Scientific Company, Inc., Fairfax, VA).

Oxy-Hb previously prepared was thawed under running water and added to the muscle system to give a final concentration of ~6, 9, and 12 µmol/kg washed muscle. Hb was mixed manually into the WMTM system using a plastic spatula. The homogenous color of the minced muscle indicated adequate mixing. Each of these Hb concentrations was added to the system at three pH levels (6.3, 6.8, and 7.3). NaCl was added to the samples at a concentration of 150, and 450 mM with constant manual mixing. Samples were plated in Petri dishes (~25 g), covered with a lid, and stored at 3.7ºC for 8 days and at -25ºC for 24 weeks. All samples were stored in duplicate at both temperatures. The various combinations were conducted as presented in Table 3-1.

CO-Hb samples were prepared by gassing the WMTM with 100% CO for two hours on ice. The WMTM was placed in gastight vacuum bags equipped with a silicon

septum valve obtained from LabPure® Instruments. CO-Hb was added to the muscle after removal from the bags to give a final concentration of ~6, 9, and 12µmol/kg WMTM. CO-Hb was mixed manually using a plastic spatula. Each of these CO-Hb concentrations was added to the system at three pH levels (6.3, 6.8, and 7.3). Samples were placed in Petri dishes (~25 g), covered with a lid, and stored at 3.7ºC for 8 days and at -25ºC for 24 weeks. All samples were stored in duplicate at both temperatures (Table 3-1).

Met-Hb, previously prepared, was mixed manually with the washed system using a plastic spatula to final concentrations of ~6, 9, and 12 µmol/ kg washed muscle. Each of these met-Hb concentrations was added to the system at three pH levels (6.3, 6.8, and 7.3). Samples were plated in Petri dishes (~25 g), covered with a lid and stored at 3.7ºC for 8 days and at -25ºC for 24 weeks. All samples were stored in duplicate at both temperatures (Table 3-1).

One g of sample was taken from each Petri dish every other day from samples at pH 6.3, 6.8, and 7.3. The remainder of the samples were returned to 3.7ºC. For samples stored at -25ºC, 1g was taken every 4 weeks and analyzed while frozen. The remainder of the samples was returned to the freezer. Due to the difficulty of mixing, the frozen samples and the samples containing 450 mM NaCl, samples were taken from the core and side of the dishes. These samples were then stored in aluminum foil at -80ºC until analysis was performed.

Determination of Protein Content

Protein content was quantified spectrophotometrically using the Biuret method described by Robinson and Hodgen (*89*). A bovine serum albumin (BSA) standard curve was constructed to calculate protein concentration. One g of sample was

homogenized with 10 mL of 0.1N NaOH for 1 min. 100µL of the homogenate in a tube (culture tubes) was added to 900 µL 0.1 N NaOH. To this, Biuret reagent was added (4 mL) and incubated at room temperature for 30 min. Absorbance was measured at 540 nm against a blank containing 1 mL of 0.1 N NaOH + 4 mL Biuret reagent. Protein concentration was calculated using the BSA standard curve.

Determination of Total Lipids

Total lipid content was determined using the method of Lee (*90*). Ten grams of tilapia muscle system was homogenized with 50 mL of 1:1 (v/v) chloroform: methanol for 60 s using a hand held homogenizer (Biospec products Inc., Bartlesville, OK). The homogenate was filtered using Whatman filter paper number 4 into separating funnels. Twenty mL of 0.5% NaCl solution was added to increase phase separation and the solution gently shaken manually. The mixture was stored at 4°C for 3 h. A known volume of the chloroform layer was separated into three aliquots of 4 mL each in previously weighed beakers. The total lipid content was obtained gravimetrically by evaporation of the solvent using a hot plate at low temperature (65-70°C).

Determination of Phospholipids Content

Measurement of organic phosphorous in the extracted lipid phase (from previous total lipid determination step) was used to estimate total phospholipid content. Lipid phosphorous was converted to phospholipid based on the assumption that 31 g atoms of phosphorous is equivalent to 750 g atoms of phospholipid.

According to a modified procedure of Anderson and others (*91*), a 100 µL sample of the chloroform layer was obtained from the lipid analysis step and placed in test tubes. These were heated at 105°C to evaporate the chloroform using the metal blocks. Samples were cooled to room temperature, 100 µL of concentrated sulfuric acid

(H_2SO_4) were added, and heated again in the metal blocks at 155°C for 10 min. After cooling to room temperature, 200 µL of 6% H_2O_2 (hydrogen peroxide) were added and samples were heated in the metal blocks at 155°C for 40 min. Two mL of distilled water were added and vortexed well. To this was added 0.8 mL of a mix of 10.1 mM ammonium molybdate and freshly prepared 0.28 M ascorbate solution and vortexed well. Samples were finally heated in a water bath for 7 min then cooled to room temperature. Absorbance was recorded at 797 nm against sodium phosphate buffer. A standard of sodium phosphate (NaH_2PO_4) was used to determine the amount of phospholipids.

Determination of Peroxide Value (PV)

Lipid hydroperoxides (primary products of oxidation) were determined according to Shantha and Decker (*92*) with modifications by Undeland and others (*8*). Samples were prepared in duplicate. Total lipids were extracted using 10 mL of a chloroform/methanol (1:1) mixture with vortexing. Sodium chloride, 3 mL (0.5%), was added to the sample, and the sample was vortexed for 30 seconds, and centrifuged (Ependorf, model 5702, Brinkman Instruments, Inc., Westbury, NY) at 4ºC for 10 min at 2,000 g. Two mL of the bottom layer were obtained using a 2 mL glass syringe and needle (Micro-Mate® Interchangeable glass syringe, stainless steel needle, 20G x18", Popper and Sons, Inc., New Hyde Park, NY). Two mL of the chloroform layer were mixed with 2 mL of chloroform/methanol (1:1). Fifty µL of ammonium thiocyantate (3.94M) were added to the sample, followed by addition of 50 µL iron chloride (prepared by adding equal amounts of 0.144 M $FeSO_4$ and 0.132 M $BaCl_2$). Following each reagent addition, the mixture was vortexed for 2-4 seconds. After an incubation period of 5 min at room temperature, samples were read at 500 nm using a spectrophotometer (Agilent 8453

UV-Visible, Agilent Technologies, Inc., Palo Alto, CA). A blank contained all reagents except the sample. A standard curve was prepared using cumene peroxide.

Determination of Thiobarbituric Acid Reactive Substances (TBARS)

TBARS, which measures secondary oxidation products, were determined according to a modified procedure of Lemon (*93*). All samples were prepared in duplicate. One gram of sample was homogenized with 6 mL of trichloracetic acid (TCA) solution composed of 7.5 %TCA, 0.1% propyl gallate, and 0.1% EDTA (ethlenediaminetetraacetic acid 99%) dissolved in deionized water. The homogenate was filtered through Whatman No. 1 filter paper. The TCA extract (2 mL) was added to 2 mL of thiobarbituric acid (TBA) (0.23% dissolved in deionized water). The TCA and TBA solutions were freshly prepared and heated as needed. The TCA/TBA solution was heated for 40 min in boiling water at 100ºC. Samples were cooled immediately on ice and absorbance measured at 530 nm using a spectrophotometer (Agilent 8453 UV-Visible, Agilent Technologies, Inc., Palo Alto, CA). Amount of oxidation was determined from a standard curve of 1, 1, 3, 3-tetraethoxypropane.

Determination of Carbonyl Groups

Protein carbonyl content was determined using 2, 4-Dinitrophenylhydrazine (DNPH) following a procedure by Levine and others (*36*) as modified by Kj and others (*94*). The 2, 4-Dinitrophenylhydrazine reacts with the carbonyl group of the protein, giving a hydrazone, which is quantified spectrophometrically. Carbonyl groups, which have formed a Schiff base with amino acids, also react with DNPH. This method is based on the reaction of hydrazyne and carbonyl groups forming a hydrazone. The hydrazone formed can be measured spectrophotometrically at 370 nm and total protein at 280 nm. One g sample was homognized with 12 mL 50 mM phosphate buffer of a pH

corresponding to the sample pH using a hand held homogenizer. The final concentration was 1 mg protein/mL sample. To three 1.5 mL cryogenic (amber) tubes, 1 mL protein solution was added (2 samples and 1 blank). Protein was precipitated with 100% trichloroacetic acid (TCA) to a final 10%-20% TCA (v/v). Samples were centrifuged for 3 min at 12,000 rpm and the supernatant was discarded. One mL of 2 M HCl was added to the protein blank and 1 mL DNPH (2, 4-dinitrophenylhydrazine in 2 M HCl) was added to the samples. Samples were kept for 1 hour at room temperature in the dark and then precipitated with an additional 50 µL TCA. Samples were centrifuged for 3 min at 12,000 rpm. The supernatant was discarded and the pellet was washed with 1 mL (1:1) Ethanol/Ethyl acetate. Samples were centrifuged for 3 min at 11,000 rpm, the supernatant was discarded and this step was repeated twice. The pellets were re-dissolved in 1 mL of 6 M guanidine-HCl. Samples were incubated at 4ºC overnight. Samples were left at room temperature for 1 hour and centrifuged for 6 min at 12,000 rpm to remove any insoluble material. Absorbance was recorded at 370 nm and 280 nm against guanidine-HCl as a blank.

Heme Group Autoxidation

Relative oxygenation of heme group within the Hb was monitored during oxidation. Hb was extracted from the washed system (2 g) with 4 mL 50 mM phosphate buffer having the same pH (6.3, 6.8, and 7.3) as the samples. The final ratio of buffer to muscle in extracts was 2:1. Samples were homogenized for 10 s using a hand held homogenizer (Biospec products Inc., Bartlesville, OK) and centrifuged at 4,000 rpm for 20 min using a table top centrifuge (Eppendorf North America Inc., New York, NY). The supernatant was scanned in the visible range from 350 to 700 nm using a spectrophotometer (Agilent Technologies, Palo Alto, CA) according to the procedure of

Krzywicki (*95*). Changes in the heme peak environment indicates how oxygenated the heme group was. Autoxidation of the heme proteins was calculated using Krzywicki's modified equation (*96*).

Color Analysis

Deterioration of color during development of oxidation processes in the washed muscle samples was measured using a digital Color Machine Vision System (CMVS) (*97*). The CMVS measures the average lightness (L*value), redness (a*value), and yellowness (b*value) for each sample. Pictures of samples were taken at the same intervals that samples were taken for chemical and sensory analysis.

A 10 g sample was placed in a closed chamber (impermeable to stray light). A digital camera (Nikon D200 Digital Camera, Nikon Corp., Japan) facing bottom of the chamber was used to capture the pictures of the samples. Two fluorescent lights (top of the chamber), each to simulate illumination by noonday summer sun (D65 illumination), were used to obtain uniformity of light. The Nikon D200 Settings used are shown in Table 2. A red reference tile was placed with each picture and used as a standard for redness (a*value) (Figure 5-27).

Results

Tilapia was chosen as the raw material for the oxidation system because of its fresh availability all year round. Washing the minced tilapia white muscle removes much of the endogenous pro- and anti-oxidants, retaining the muscle proteins and cellular membranes. This model system therefore allows for controlled and detailed manipulation of pH values, sodium chloride (NaCl) concentrations, and amount of Hb added. The composition of tilapia whole muscle was tested and contrasted with the washed muscle at three levels of pH (Table 3-3, Table 3-4). Whole muscle had

significantly less H_2O, and significantly ($p<0.05$) less protein (dry WT basis, Table 3-4). The % H_2O significantly ($p<0.05$) increased as the pH increased in minced muscle (Table 3-3). If the pH drops quickly at post-mortem, the net surface charge is reduced, causing the protein to denature. When denaturation of sarcoplasmic (contractile) protein occurs (*98*), this decreases the ability of contractile proteins to bind water. As shown is Table 3-3, the lower the pH, significantly ($pH<0.05$) less % H_2O is present in minced muscle. This is associated with poor water-holding capacity and pale color (*98*). The lower the pH, the greater the toughness of texture due to denaturation of myosin at lower pH causing shrinkage. The % of total lipids was significantly ($pH<0.05$) lower in the tilapia minced muscle at only pH 7.3 compared to tilapia muscle. The % phospholipid was also significantly ($pH<0.05$) lower at higher pH levels compared to tilapia muscle. At higher pH levels, the % H_20 is higher, which ultimately results in less protein and phospholipids.

Lipid Oxidation Analysis

Lipid hydroperoxide values (Figure 3-1) revealed significant differences for the effect of storage at 3.7ºC regardless of pH, Hb, and NaCl concentrations. At day 2 and 4 of storage, oxy-Hb had significantly higher PV values than either CO- or met-Hb. By day 8, CO-Hb had significantly ($p<0.05$) lower PV values than oxy- and met-Hb which did not differ significantly ($p<0.05$) from each other. Lipid hydroperoxide values for the effect of storage at -25°C (Figure 3-2) revealed no significant differences between oxy- and met-Hb. CO-Hb had however significantly ($p<0.05$) lower lipid peroxide values at week 20 and 24 compared to oxy- and met-Hb consistent with the observations at 3.7°C.

The formation of TBARS was used to assess lipid oxidation during storage at 3.7°C for 8 days and -25°C for 24 weeks. Regardless of pH, Hb and NaCl

concentration, the effect of storage for 8 days at 3.7°C showed extensive formation of TBARS by day 6 for met-Hb, but not for oxy- and CO-Hb forms (Figure 3-3). By day 8 of storage, met-Hb continued to have significantly ($p<0.05$) higher TBARS than oxy- and CO-Hb. On day 8, although oxy-Hb had slightly lower TBARS than CO-Hb, these differences were not significant ($pH<0.05$). On the other hand, at -25°C, oxy-Hb had significantly ($p<0.05$) higher TBARS by week 12 than CO- and met-Hb which did not differ from each other (Figure 3-4). After 20 weeks of storage at -25°C, oxy-Hb had significantly ($p<0.05$) higher TBARS than CO- and met-Hb. Throughout the 24 weeks of storage, CO- and met-Hb did not differ significantly ($p<0.05$) from each other but continued to have significantly ($p<0.05$) lower TBARS formation than oxy-Hb.

Protein Oxidation Analysis

The effect of storage for 8 days at 3.7ºC regardless of pH, Hb and NaCl concentrations showed that oxy-Hb had significantly ($p<0.05$) higher formation of carbonyls than CO- and met-Hb throughout the 8 days of storage (Figure 3-5). The lower carbonyl values for met-Hb on day 0 differed significantly ($p<0.05$) from CO- and oxy-Hb, which did not differ from each other significantly ($p<0.05$) on day 0. Carbonyl values (Figure 3-6) for the effect of storage for 24 weeks at -25°C were similar to, although higher in value, to that of those found at 3.7°C. oxy-Hb had significantly ($p<0.05$) higher carbonyl values than CO- and met-Hb through week 16 of storage at -25°C. At week 24, oxy-Hb had significantly ($p<0.05$) lower hydroperoxide values than CO- and met-Hb. CO-Hb had a slight but significantly ($p<0.05$) higher carbonyl values than met-Hb at week 24.

Color Analysis

Color analysis of the effect of storage for 8 days at 3.7°C regardless of pH, Hb and NaCl concentrations revealed that the three forms of Hb (oxy-, CO-, and met-Hb) differed significantly ($p<0.05$) from each other. During the 8 day storage period, a*values (redness) declined significantly ($p<0.05$) (Table 3-5) for all three forms. Met-Hb had a slight increase in a*value on day 2 which differed significantly ($p<0.05$) from the rest of the storage days for met-Hb. CO-Hb had significantly ($p<0.05$) higher a*values during the first four days of storage compared to oxy- and met-Hb. By day 6, oxy-Hb had significantly ($p<0.05$) higher a*value than CO- and met-Hb. At -25°C storage (Table 3-6) the a*values differed significantly ($p<0.05$) for the three forms of Hb. CO-Hb maintained higher values than oxy- and met-Hb, with a significant increase in a*value at week 4. Met-Hb also had a significantly ($p<0.05$) higher a*value at week 4 of storage but the remaining 24 week storage period for met-Hb showed no significant change in a*value. The a*values for oxy-Hb began to decline at week 4 and by week 12 no further significant decline in a*value was noted.

L*values (lightness) (Table 3-5) differed significantly ($p<0.05$) for the three forms of Hb at 3.7ºC, with met-Hb having significantly ($p<0.05$) higher L*values than CO- and oxy-Hb throughout the 8 days of storage with its highest L*value evident at day 8. The L*values for CO-Hb did not differ significantly ($p<0.05$) from day to day during the 8 day storage period. Oxy-Hb had slightly but significant higher L*values beginning on day 4. At -25°C storage, L*values for met-Hb differed significantly ($p<0.05$) from oxy- and CO-Hb (Table 3-6). However, there was no significant difference in L*values for met-Hb beginning with week 4 and throughout the remaining 24 week storage period. CO- and met-Hb had significantly ($p<0.05$) lower L*values after 24 weeks of storage whereas

oxy-Hb had higher L*values at week 24 but significantly ($p<0.05$) stable L*values from week 4 to week 16.

b*values (yellowness) (Table 3-5) revealed that the three forms of Hb (oxy-, CO-, and met-Hb) differed significantly ($p<0.05$) from each other regardless of pH, Hb and NaCl concentrations. All three forms of Hb increased in yellowness over time, with day 8 having significantly ($p<0.05$) higher b*values compared to day 0. b*values for oxy-Hb were significantly ($p<0.05$) higher by day 6 and remained similar throughout the rest of the 8 day storage period. At -25°C b*values differed significantly ($p<0.05$) during storage for the three forms of Hb. All three forms had significantly ($p<0.05$) higher b*values at week 24 compared to week 0. CO-Hb had significantly ($p<0.05$) lower b*values than oxy- and met-Hb throughout the 24 weeks of storage.

Heme Group Autoxidation

The absorption spectra (Figure 3-11) identified the absorption peak for met-Hb at 503 nm, oxy-Hb at 582 nm, and CO-Hb at 542 nm. The isobestic point was identified at 582 nm. These results are similar to that of Tang (*96*). Mb coefficients are quite similar to Hb. Some of the traditional methods (*95*) to estimate met-Hb have not included CO-Hb. They are based on the maxima wavelengths for oxy-, deoxy- and met-Hb which does not account for the extinction coefficients and wavelength maxima CO-Hb and calculations for the three forms rarely came close to 100%. Tang and others (*96*) modification yielded more consistent and logical results.

The % oxy-Hb formed (Figure 3-9) during storage at 3.7°C for 8 days differed significantly ($p<0.05$) for the three forms of Hb regardless of pH, Hb and NaCl concentrations. There was a significant decrease in the percentage of oxy-Hb during storage for all three forms of Hb. CO-Hb had a significantly ($p<0.05$) higher % oxy-Hb

throughout the storage period than oxy- and met-Hb. The Tang's formula (*96*) dose not take % CO-Hb into consideration; however, when calculating % oxy-Hb in CO-Hb samples, % oxy-Hb is used to report relative % CO-Hb in the sample. At -25°C, % oxy-Hb formed was significantly ($p<0.05$) different (Figure 3-10) for all three forms of Hb with decreasing % oxy-Hb over the 24 week storage period. CO-Hb had significantly ($p<0.05$) higher % oxy-Hb than met- and oxy-Hb. Met-Hb had the lowest % oxy-Hb and differed significantly ($p<0.05$) from oxy-Hb on week 16, 20, and 24.

As expected, met-Hb had significantly ($p<0.05$) higher % met-Hb during storage at 3.7°C for 8 days than oxy- and CO-Hb. However, during the 8 day storage period both oxy- and CO-Hb had significant increases in the percentage of met-Hb. At -25°C (Figure 3-10), the three forms of Hb differed significantly ($p<0.05$) in % met-Hb, with increasing amounts of % met-Hb with increased storage time. As would be expected, the met-Hb sample had significantly ($p<0.05$) higher percent of its Hb in met form compared to CO- and oxy-Hb throughout the 24 week storage period at -25ºC.

Percent deoxy-Hb formed during storage at 3.7°C for 8 days differed significantly ($p<0.05$) for the three forms of Hb. All three forms showed a significant increase in % deoxy-Hb formed through day 4. CO- and oxy-Hb did not differ in % deoxy-Hb formed on day 6 and 8. At -25°C, the three forms of Hb differed significantly ($p<0.05$) over the storage period (Figure 3-10). The % deoxy-Hb did not differ significantly ($p<0.05$) for met-Hb up to week 12, but significant differences were found on week 12 and throughout the remaining 24 weeks of storage. The % deoxy-Hb increased significantly ($p<0.05$) over the 24 weeks for the three forms of Hb with CO-Hb having the greatest

significant increase. By week 16 and for the remaining weeks of storage, oxy- and CO-Hb did not differ in the % deoxy-Hb formed.

Discussion

In the present study pro-oxidative activity of three Hb (oxy-, CO-, and met-Hb) in a WMTM system were examined at two storage temperatures, 3.7ºC, and -25ºC. Oxidative activities of every sample were determined by quantifying products of lipid oxidation (hydroperoxide values and TBARS values), as well as protein oxidation (carbonyl groups). Color changes and oxidation state of the three forms of Hb were also monitored and recorded throughout the experiment to establish possible correlation with the oxidative process.

Heme proteins are considered to be potent catalysts of lipid oxidation in muscle food systems (*1, 22*). From the hydroperoxide value results it is evident that oxy-Hb oxidizes lipids significantly more rapidly than either CO- or met-Hb, irrespective of environmental factors, reaching the peak and plateau by day 4 of refrigerated storage. In contrast, lipid oxidation by CO- and met-Hb follows somewhat similar patterns, oxidizing lipids at a slower rate, and reaching the peak and plateau by day 6. Interestingly, by day 8 both oxy- and met-Hb exhibited comparable oxidative values, with CO-Hb being the slowest catalyst of lipid oxidation (Figure 3-1). It is well-established that Hb can exist in different redox states. It was proposed that maintaining Hb in the reduced state will delay/decrease autoxidation and lipid oxidation, thus preserving freshness and improving the shelf-life of fish products (*57*). Oxy-Hb is believed to be less pro-oxidative, possibly due to its reduced state. Despite that, previous research studies suggest that oxy-Hb might in fact be a strong promoter of lipid oxidation under certain conditions (*57*). It may be explained, in part, by increased rate of

deoxygenation and deoxy-Hb formation in the post-mortem muscle system. It has been demonstrated in numerous previous studies that increased deoxygenation can enhance pro-oxidative activity of Hb, promote Hb autoxidation and hemin loss, which in turn leads to a formation of met-Hb and increased lipid oxidation (*22, 56*).

Formation of met-Hb occurs by the conversion of ferrous iron (Fe^{2+}) of the Hb heme group to the ferric (Fe^{3+}) form, which is less stable, readily reactive, and susceptible to oxidation. Furthermore, in the presence of preformed lipid hydroperoxides met-Hb can oxidize to two extremely reactive species, or perferryl-Hb (Fe^{5+}) and ferryl-Hb (Fe^{4+}) (*99, 100*). The presence of CO bound to Hb may considerably delay the deoxygenation process due to its higher affinity to heme proteins, increasing its stability which, in turn, may retard lipid oxidation. CO is extensively used in the food industry to stabilize the red color and preserve freshness of seafood due to its ability to maintain heme proteins in their reduced state for relatively long periods of time. It is thought that CO exerts its action by displacing O_2 from the heme, apparently making it more compact and possibly changing conformation of the molecule, hence stabilizing the structure of Hb, making it less susceptible to autoxidation (*57*). To determine the rate and pattern of autoxidation of different Hb, we examined relative deoxygenation, expressed as the formation of deoxy- and met-Hb in all samples using Tang's equation (*96*). Oxygen molecules are replaced by CO in CO-Hb, therefore, the above method is considered useful in determining the relative rates of heme protein autoxidation. It appears that at storage temperatures (3.7ºC) the process of deoxygenation, formation of deoxy- and met-Hb, or autoxidation was significantly retarded in CO-Hb and oxy-Hb treated samples throughout the storage period as compared to met-Hb. Although both CO- and

oxy-Hb exhibited similar trends and rates of formation of deoxy- and met-Hb, CO-Hb was significantly ($p<0.05$) less oxidized until day 6 (Figure 3-7).

During frozen storage (-25ºC) slower lipid oxidation with lesser hydroperoxide formation was observed for all three types of Hb. However, CO-Hb exhibited greater oxidative stability than oxy-and met-Hb (Figure 3-4). This correlated with a slower rate of deoxygenation at -25ºC. This may be attributed to the ability of low temperatures to preserve muscle systems from lipid oxidation which is in agreement with previous studies (*31*).

The results of the different Hb forms were different when TBARS values were examined. While oxy-Hb demonstrated higher oxidation activity at the beginning, it reached peak TBARS formation sooner than CO- and met-Hb, with met-Hb reaching the significantly ($p<0.05$) highest score by day 8 at 3.7ºC (Figure 3-3). On the other hand, frozen storage conditions retard TBARS formation in met- and CO-Hb systems, but not in the oxy-Hb system (Table 3-5). Met-Hb requires preformed hydroperoxides for oxidative activity. Under low storage temperatures the formation of hydroperoxides is retarded, which may, to a degree, suppress the oxidative activity of met-Hb. It is possible that continuous deoxygenation and autoxidation of oxy-Hb even under frozen storage conditions could provide enough substrate for continuous oxidation in this Hb system.

Recent studies have associated the quality deterioration of muscle food systems during processing and storage with protein oxidation (*31, 94*). However, very few studies investigating protein oxidation in muscle food systems have been conducted to date. In this study, comparing different forms of Hb, protein oxidation in WMTM was

monitored by formation of carbonyls. Previous observations demonstrated great variability in basal levels of protein carbonyls in different tissues (*33*). Furthermore, certain processing manipulations and storage conditions may alter protein structures making them more susceptible to oxidation, and thus affect their functionality and changes in quality (*94*). Additionally, prior experiments pointed out the possibility that carbonyl quantification might be affected by heme-containing compounds, including Hb, since these compounds exhibit high absorbance at the same length as DNPH (*101*). All of this may explain the fact that increased levels of carbonylation was detected on day 0 in all tested samples in the present study. Moreover, initial carbonyl content was similar for all Hb systems. Striking similarity was observed in carbonyls formation in oxy-Hb system at both storage temperatures, reaching the peak mid-way throughout storage, and then subsiding (Figure 3-5, 3-6). On the contrary, additional formation of the carbonyls was not detected in CO- and met-Hb systems at 3.7°C, while at -25ºC steady increase in carbonyls development was observed in CO- and met-Hb systems. This finding is consistent with previous studies (*31*). Frozen storage of rainbow trout fillets at lower freezing temperatures (-20ºC) was found to increase protein carbonylation (*94*). Mincing the fish tested in this study could potentially accelerate carbonyl formation during frozen storage due to possibly more protein denaturation and more exposure of proteins by catalysts and oxygen. Nevertheless, it is unclear why protein carbonylation in the oxy-Hb system was increased at first and subsequently subsided to the initial level. It may be speculated that increased oxygenation of the oxy-Hb could initially accelerate protein oxidation, with subsequent reduction in protein oxidation following oxy-Hb deoxygenation.

To determine deterioration of the color in all tested Hb systems during storage, a*values, L*values, and b*values were obtained and monitored throughout the study. At 3.7ºC CO-Hb treated samples maintained higher a*value until day 4 of the study as compared to oxy- and met-Hb, with subsequent deterioration in color by day 8 in all Hb. Mantilla (*102*) found that treating the red muscle of tilapia fillets post mortem with CO increased the red color which remained significantly higher until day 9 compared to untreated fillets and fillets obtained from fish euthanized with CO treatments. Huo (*103*) found that a*values declined after 9 days of storage for untreated samples compared to samples of tuna treated with CO. Based on peak wavelengths, Huo found that there was some CO still bound to Hb. Danyali (*71*) and Garner and Kristinsson (*73*) found that the decrease in red color was associated with a decrease of CO binding to heme proteins.

In contrast, at -25ºC red color deterioration, expressed as reduction in a*value, was almost undetected in CO-Hb, while oxy- and met-Hb sustained significant decrease in a*value. Interestingly, red color appeared to be enhanced for the first 8 weeks, and by week 24 a*value of CO-Hb system was comparable with that of oxy-Hb at day 0. Mantilla (*102*) found that a*values decreased significantly after freezing. However, he found no significant difference between a*values of fresh fish and that of tuna after 2 months of storage, suggesting that freezing preserved the red color. After 4 months of storage, a*values of CO euthanized fillets were not significantly different from initial, fresh controls, desirable red color of meat muscle systems was linked with higher levels of heme proteins. Ability of CO to maintain Hb in the reduced state longer, preventing heme loss, and thus stabilizing red color was previously reported and in agreement with

current findings (*69, 73*). The increase in a*value of CO-Hb after 8 weeks of frozen storage found in this study is consistent with Mantilla (*102*) findings. Danyali (*71*) also found an increase in a* values of yellowfin tuna steaks that were treated with CO and stored at -25°C for 30 days. Huo (*103*) noted that tuna steaks treated with CO at -20°C had higher a* values and CO levels than those treated at 4°C indicating more CO binding to heme protein (*79*).

Conclusion

The results of this chapter suggest that tilapia minced muscle treated with CO could have greater stabilities to oxidation, thus resulting in increased color stability and oxidative stability. Oxy-Hb maintained its catalytic effect and is believed to catalyze oxidation by the breakdown of preformed lipid hydroperoxides. The greater the concentration of preformed hydroperoxides, the more the pro-oxidative activity of oxy-Hb. met-Hb may also form ferryl-Hb which may initiate lipid peroxidation. However, low pro-oxidative activity of the oxidized form (met-Hb) could be a result of the absence of oxygen to form hydrogen peroxide required for met-Hb oxidation. Washing fish muscle could reduce the amount of hydroperoxides which are important for the pro-oxidative activity of met-Hb.

The low pro-oxidative activity of CO-Hb is due in part to CO increasing the stability of heme protein structure and slowing down autoxidation. The reduced reactivity of CO-Hb with hydrogen peroxides may be due to the strong affinity of CO to Hb. More studies on how CO affects fish Hb will assist in the advancement of CO based methods to stabilize sea-food products.

Table 3-1. Sample preparation of the three forms of hemoglobin (Oxy-, CO-, and Met-Hb) at three different pH values, Hb Concentrations, NaCl added, and two storage temperatures*.

Temperature	pH	Hb Concentration (µmol/kg)	NaCl added (mM/Kg)
3.7ºC	6.3	6	0
			150
			450
		9	0
			150
			450
		12	0
			150
			450
	6.8	6	0
			150
			450
		9	0
			150
			450
		12	0
			150
			450
	7.3	6	0
			150
			450
		9	0
			150
			450
		12	0
			150
			450

*Storage temperatures were 3.7º and -25ºC

Table 3-2. Nikon D200 camera settings used for measurement of change in color during storage period.

Setting	Specification
Lens	VR 18-200 mm F 3.5-5.6 G
Focal length	36 mm
Sensitivity	ISO 100
Optimize image	Custom
High ISO NR	Off
Exposure mode	Manual
Metering mode	Multi-pattern
Shutter speed and aperture	1/3s-F/11
Exposure compensation (in camera)	-1.0 EV
Focus mode	Manual
Long exposure NR	Off
Exposure compensation (by capture NX)	0 EV
Sharpening	Normal
Tone compensation	Normal
Color mode	Mode I
Saturation	Normal
Hue adjustment	0°
White balance	Direct sunlight
Zoom	Manual

Table 3-3. Composition of tilapia muscle and washed tilapia muscle model system.

Sample	%H_2O	%Protein	%TL[a]	%PL[b]	%PL/TL
Tilapia muscle	78.73±0.3^{d}	13.83±0.8^{a}	0.21±0.04^{a}	0.2±0.02^{a}	93.5±0.154^{a}
WMTM					
pH6.3	83.10±0.1^{c}	13.49±1.3^{ab}	0.19±0.001^{a}	0.18±.001^{a}	93.3±0.070^{a}
pH6.8	84.27±0.1^{b}	12.86±0.2^{bc}	0.18±0.002^{a}	0.11±0.04^{b}	61.7±0.251^{c}
pH7.3	85.80±0.2^{a}	12.14±0.6^{c}	0.16±0.29^{b}	0.11±0.04^{c}	68.7±0.043^{b}

[a] TL, total lipids. [b] PL, phospholipid. Values are Means ± standard deviations (n=3). Different letters within the same columns indicate significant differences at $P < 0.05$ separated by Tukey's HSD.

Table 3-4. Composition of tilapia muscle and washed tilapia muscle model system on dry weight basis.

Sample	%Protein	%TLa	%PLb
Tilapia muscle	65.02±0.023d	1.00±0.053a	0.93±0.200b
WMTM			
pH6.3	79.79±0.043[c]	1.16±0.062[a]	1.08±0.052[a]
pH6.8	81.74±0.004[b]	1.17±0.112[a]	0.72±0.021[c]
pH7.3	85.48±0.006[a]	1.12±0.074[a]	0.77±0.004[b]

a TL, total lipids. b PL, phospholipid. Values are Means ± standard deviations (n=3). Different letters within the same columns indicate significant differences at $P < 0.05$ separated by Tukey's HSD.

Table 3-5. Changes in a* value, L* value and b* value in washed tilapia muscle containing different forms of Hb at 3.7ºC, averaged across pH, Hb concentration and added NaCl.

a* value

Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	19.9±4.7[b1]	21.9±4.1[a1]	8.1±1.7[c2]
2	16.6±7.9[b2]	20.8±5.0[a1]	10.2±2.2[c1]
4	15.2±9.3[b2]	17.2±7.6[a2]	8.1±2.3[c2]
6	13.5±10.3[a3]	11.3±8.2[b3]	6.1±2.7[c3]
8	12.1±9.8[a3]	9.8±7.3[b3]	4.8±2.7[c3]

L* value

Storage time (day)	Oxy-Hb[c]	CO-Hb[b]	Met-Hb[a]
0	71.2±3.4[c2]	73.3±3.0[b1]	74.9±3.1[a2]
2	71.4±3.3[c2]	72.9±3.3[b1]	73.9±3.3[a3]
4	72.3±3.5[b1]	72.9±3.3[b1]	73.5±3.1[a3]
6	72.6±4.0[b1]	73.2±3.5[b1]	73.9±3.1[a3]
8	72.6±4.3[b1]	72.8±4.0[b1]	75.1±3.3[a1]

b* value

Storage time (day)	Oxy-Hb[b]	CO-Hb[c]	Met-Hb[a]
0	3.3±2.2[ab3]	2.9±1.8[b4]	3.8±1.6[a5]
2	4.4±2.3[b2]	4.4±1.9[b3]	5.3±1.5[a4]
4	6.2±2.4[a1]	5.0±2.4[b3]	6.1±2.0[a3]
6	6.8±2.7[a1]	6.1±2.8[b2]	7.2±2.5[a2]
8	6.2±3.4[c1]	7.0±3.1[b1]	8.9±2.4[a1]

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=54). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD. Numbers within the same columns indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table 3-6. Changes in a* value, L* value and b* value in washed tilapia muscle containing different forms of Hb at -25ºC, averaged across pH, Hb concentration and added NaCl.

a* value

Storage time (week)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	19.9±4.7[b1]	21.9±4.1[a2]	8.1±1.7[c3]
4	17.2±6.4[b2]	24.9±4.9[a1]	12.3±2.8[c1]
8	15.8±6.3[b23]	23.0±5.9[a2]	11.1±2.4[c12]
12	13.5±7.3[b34]	21.4±5.7[a2]	10.3±2.4[c2]
16	14.5±7.6[b4]	20.6±6.0[a2]	10.2±2.7[c2]
20	15.2±8.9[b4]	19.2±6.2[a3]	9.8±2.8[c2]
24	13.4±9.5[b34]	18.4±6.7[a3]	9.5±3.2[c2]

L* value

Storage time (week)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	71.2±3.4[c2]	73.3±3.0[b1]	74.9±3.2[a1]
4	69.4±3.3[b3]	69.7±3.5[b234]	71.9±3.2[a2]
8	69.8±3.4[b3]	70.0±3.6[b234]	71.3±3.2[a2]
12	69.0±3.4[b3]	70.7±3.1[a2]	71.5±3.4[a2]
16	69.7±3.6[b3]	70.1±3.4[b234]	72.1±3.3[a2]
20	71.5±3.8[a2]	70.0±3.5[b234]	72.1±3.5[a2]
24	72.8±4.4[a1]	69.5±3.6[b4]	71.9±3.7[a2]

b* value

Storage time (week)	Oxy-Hb[a]	CO-Hb[c]	Met-Hb[b]
0	3.3±2.2[ab7]	2.9±1.8[b7]	3.8±1.6[a7]
4	11.1±2.7[a3]	6.7±2.2[c2]	7.8±2.1[b6]
8	12.1±2.7[a2]	6.7±2.2[c1]	8.6±2.5[b54]
12	12.9±4.3[a125]	5.8±2.2[c3]	8.8±3.0[b4523]
16	9.9±4.6[a4256]	4.6±2.4[b4]	9.4±3.0[a24]
20	8.4±3.0[b5246]	3.7±2.4[c6]	9.3±3.0[a34]
24	8.4±3.3[b645]	4.2±1.9[c456]	9.9±3.3[a1]

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=54). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD. Numbers within the same columns indicate statistically (<0.05) significant differences separated by Tukey's HSD.

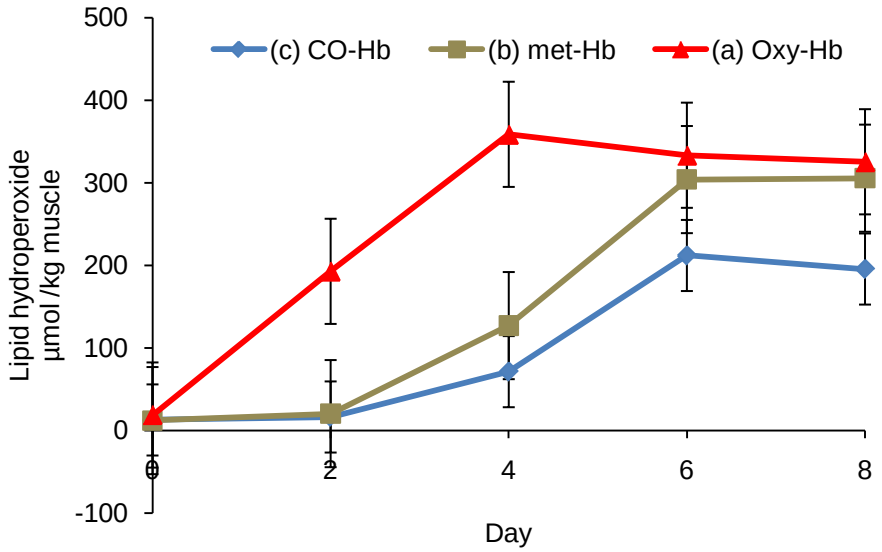


Figure 3-1. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at 3.7ºC, averaged across pH, Hb concentration and added NaCl. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

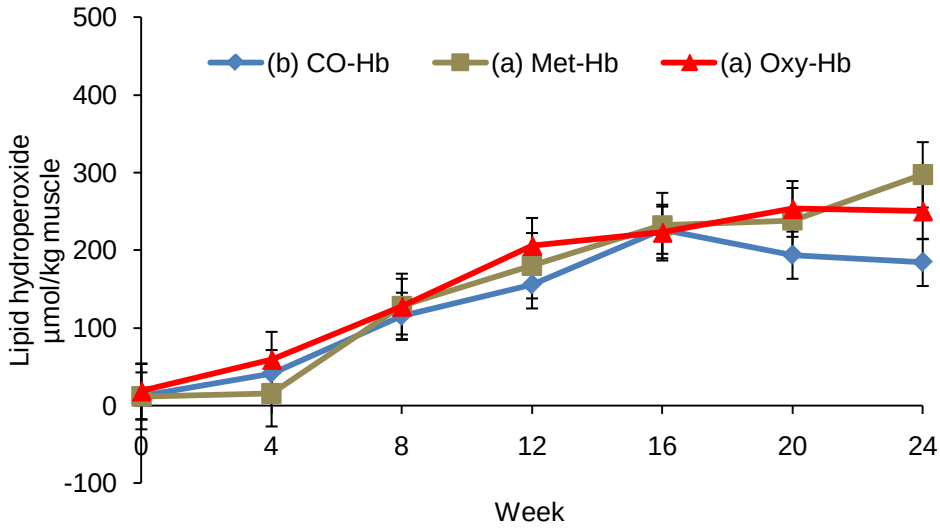


Figure 3-2. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at -25ºC, averaged across pH, Hb concentration and added NaCl. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

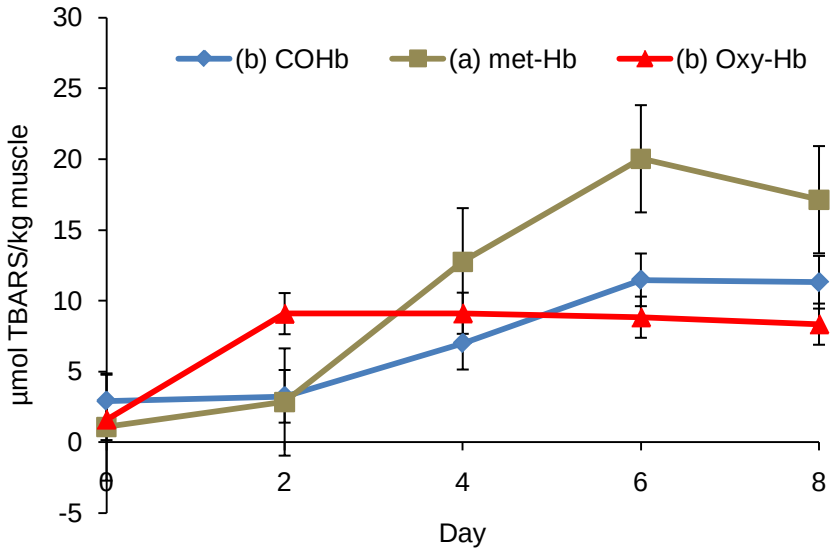


Figure 3-3. TBARS values in washed tilapia muscle containing different forms of Hb at 3.7ºC, averaged across pH, Hb concentration and added NaCl. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

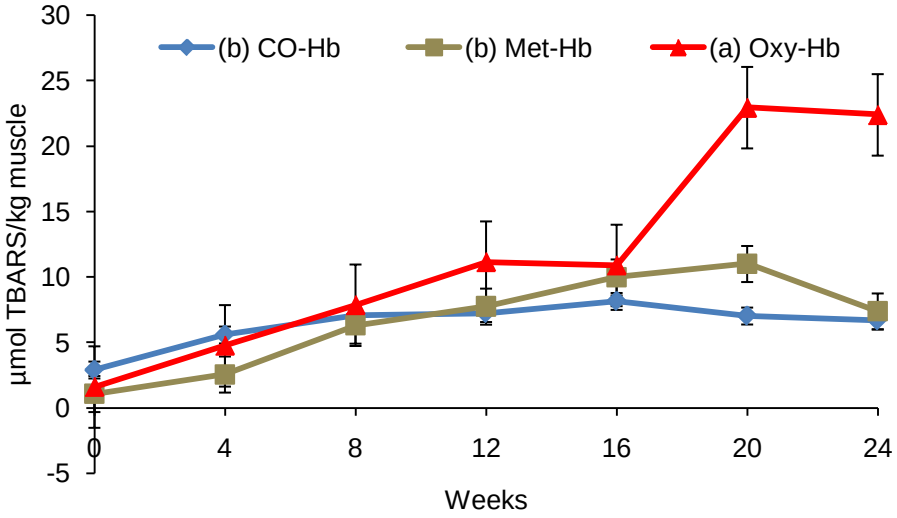


Figure 3-4. TBARS values in washed tilapia muscle containing different forms of Hb at -25ºC, averaged across pH, Hb concentration and added NaCl. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

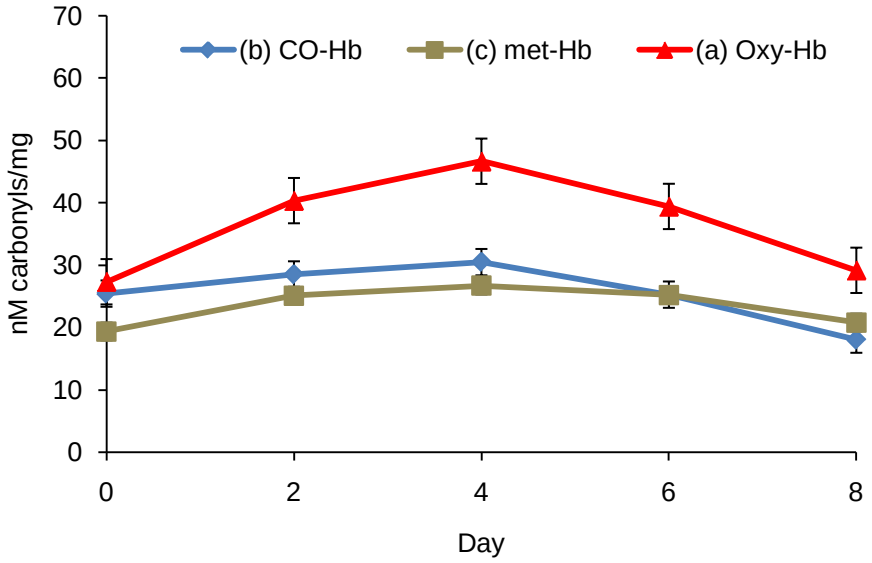


Figure 3-5. Carbonyl values in washed tilapia muscle containing different forms of Hb at 3.7ºC, averaged across pH, Hb concentration and added NaCl. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

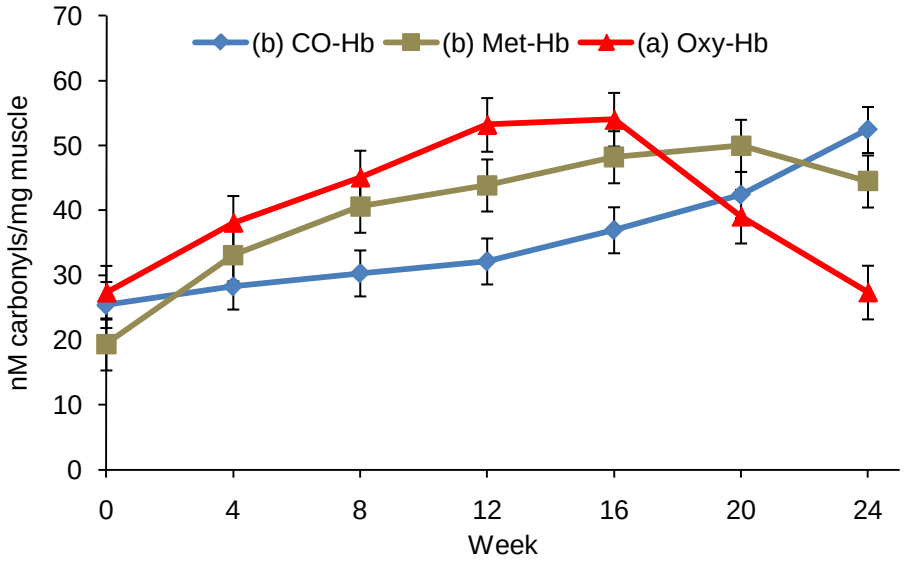


Figure 3-6. Carbonyl values in washed tilapia muscle containing different forms of Hb at -25ºC, averaged across pH, Hb concentration and added NaCl. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

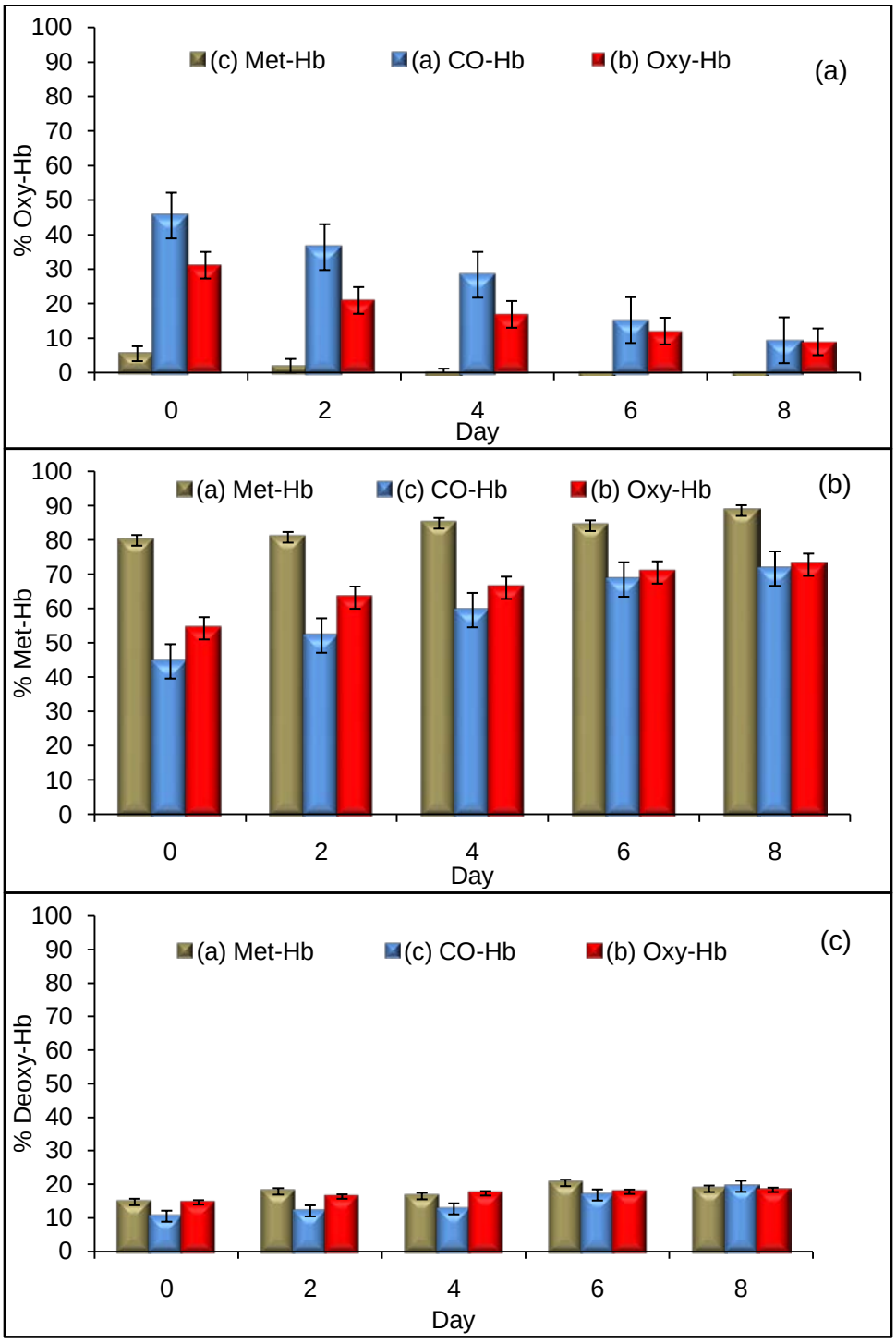


Figure 3-7. % of a) Oxy-, b) Met-, and c) Deoxy-Hb in washed tilapia muscle containing different forms of Hb at 3.7ºC, averaged across pH, Hb concentration and added NaCl. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

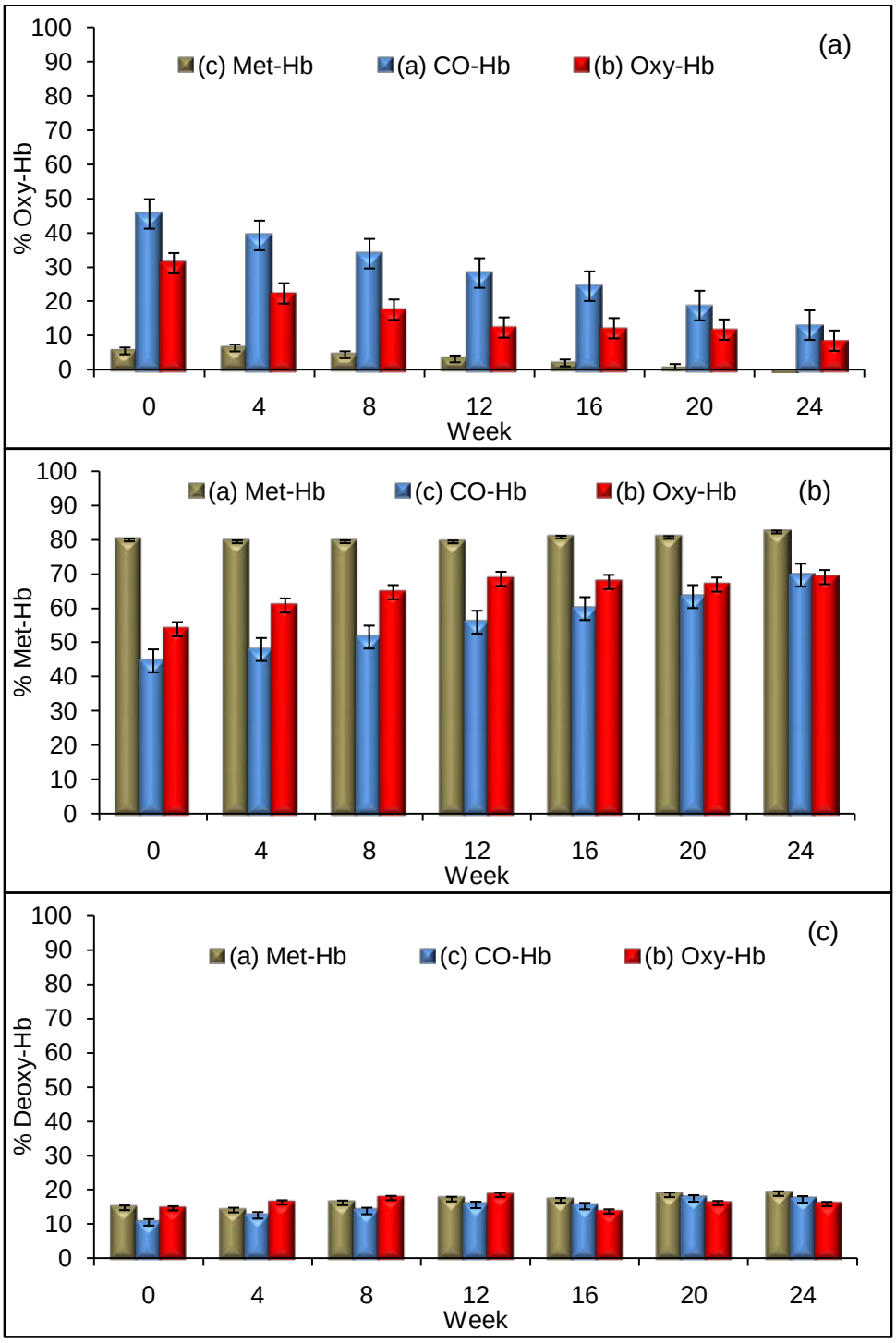


Figure 3-8. % of a) Oxy-, b) Met-, and c) Deoxy-Hb in washed tilapia muscle containing different forms of Hb at -25ºC, averaged across pH, Hb concentration and added NaCl. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

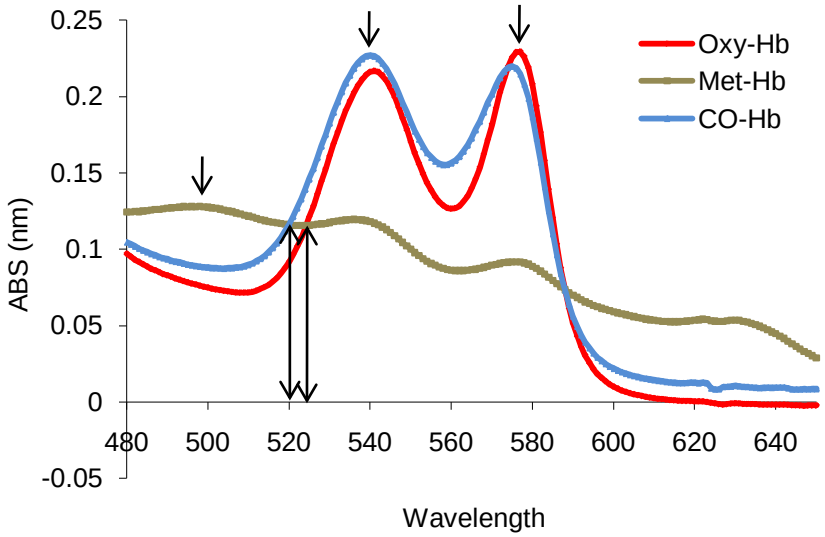


Figure 3-9. Absorption spectra of met-Hb, oxy-Hb, and CO-Hb solutions containing equivalent hemoglobin concentrations. The arrows indicate the isobestic point at 525 nm, Met-Hb absorption peak at 503 nm, Oxy-Hb absorption peak at 582 nm, and CO-Hb absorption peak at 542 nm.

CHAPTER 4
EFFECT OF LOW AND HIGH CONCENTRATIONS OF HEMOGLOBIN ON THE PRO-OXIDATIVE ACTIVITY OF OXY-, CO-, AND MET-HEMOGLOBIN IN A WASHED MINCED TILAPIA MUSCLE SYSTEM AT TWO DIFFERENT STORAGE TEMPERATURES

Introduction

Fish muscle contains several components that can serve as possible catalysts for lipid oxidation including Hb, Mb, low molecular weight transition metal complexes and lipoxygenase (*81*) resulting in undesirable sensory characteristics. Of interest to this experiment is the amount of Hb present in fish muscle and the concentration levels that are conducive to lipid oxidation. Richards and Hultin (*11*) found that Hb concentrations were higher in trout muscle that had not been tail or gill bled versus those that had been bled. To further investigate the contributions of Hb to lipid oxidation, the authors used a washed cod model system to which varying concentrations of hemolysate (0.06, 0.50, 1.8, and 5.8 µmol/kg washed cod) was added in the presence or absence of plasma. Plasma delayed development of TBARS at all four concentrations of hemolysate. It also delayed rancidity development at the two higher concentrations of hemolysate. No rancidity occurred during storage (2°C /6 days) in the two lower levels of hemolysate. TBARS increased with increasing concentrations of hemolysate (R^2= 0.99). An unexpected finding was the higher Hb concentration in trout whole muscle. Yet, mackerel light muscle was more disposed to lipid oxidation.

The authors considered the different types of Hb, i.e., anodic or cathodic to explain the differences between the behavior of trout whole muscle and mackerel light muscle. Anodic Hbs have low oxygen affinity at low pH post mortem while cathodic Hbs retain high oxygen affinity. Mackerel Hbs are known to have lower oxygen affinity (*104*).

Low oxygen affinity results in greater formation of deoxy-Hb, which is a potent catalyst for lipid oxidation. The purpose of this study is to investigate the effect of varying concentrations of tilapia oxy, CO, and met Hb on oxidation in a WMTM.

Materials and Methods

The following methods have been described in detail in chapter 3: Preparation of Washed Minced Tilapia Muscle (MTWM), Collection of Fish Blood, Preparation of Hemolysate, Quantification of Hb Levels in Hemolysate, Oxy-, CO- and Met-Hb Preparation, Sample Preparation: Addition of Hb and NaCl, Determination of Peroxide Value (PV), Determination of Thiobarbituric Acid Reactive Substances (TBARS), Determination of Carbonyl Groups, Heme Group Autoxidation, and Color Analysis.

CO Calibration Curve by the Gas Chromatography Method

A CO calibration curve was established by injecting 100% CO (CP-Grade obtained from Airgas®) in various concentrations (0.2-1.0 µL). The areas under the resultant peaks were plotted against the injected quantity of gas (µL) to obtain a linear regression equation. The Ideal Gas Law was used to determine the actual CO mass. $PV=nRT^o$, where P (atm) is the barometric pressure, V (µL) is the volume of CO gas, n (mol) is the number of moles of CO, R (0.08206 $L \cdot atm \cdot K^{-1} \cdot mol^{-1}$) is a universal gas constant, and T^o is the temperature in Kelvin (298K).

Sample Analysis by the Gas Chromatography (GC) Method

CO loss from the system during oxidation was determined using gas chromatography. Three g of sample (CO-Hb mixed with washed muscle) were transferred into a headspace 20 mL vials (Fisher Scientific, Fair Lawn, NJ), and 2 drops of octanol (antifoaming) were added. To this mix, 6 mL of 10% sulfuric acid (H_2SO_4) was added and the vials were sealed immediately with a Teflon-fluorocarbon-resin/silicone

septum lid. The mix was hand shaken for 1 min and incubated for 20 min at 40°C. Vials were shaken using a table top shaker (American Optical CO. Scientific Instrument Division, US.) for 15 min at room temperature. Then 500 µL of the headspace was taken for GC injection.

Gas Chromatography Conditions for CO Analysis

An Agilent 6890N GC system (Agilent Technologies, Palo Alto, CA) was used equipped with a flame ionization detector (FID) (Agilent Technologies, Palo Alto, CA), a Porapak Q column, 80/100 mesh, 6 FT x 1/8 IN (Supelco, Bellefonte, PA), and a hydrogen aided Nickel Catalyst (Agilent Technologies, Palo Alto, CA) (to convert CO and CO_2 into 42 methane) was used for analysis. The samples were manually injected using a gastight® syringe (Hamilton CO., Reno, Nevada). The oven temperature was set at 35°C for 2 min. Nitrogen was used as carrier gas and held at a constant flow of 30 ml/min. The injector temperature, nickel catalyst temperature and FID temperature were held at 100°C, 375°C and 200°C respectively.

Results

Lipid Oxidation Analysis

Washed tilapia muscle containing Oxy-Hb at 6 µmol/kg muscle and stored at 3.7°C developed significantly ($p \leq 0.05$) higher lipid hydroperoxide values than samples with met- and CO-Hb, which did not differ significantly from each other (Figure 4-1). Oxy-Hb had significantly ($p \leq 0.05$) higher hydroperoxide values than CO-Hb on day 2, 4, and 8, and significantly higher values than met-Hb on day 2 and 4. CO-Hb and met-Hb did not differ significantly on any day during storage. The hydroperoxide value for CO-Hb was lower than met-Hb on day 4, 6, and 8 but this did not reach statistical significance ($p \leq 0.05$). For lipid hydroperoxide values obtained at -25°C and 6 µmol/kg of

CO-, oxy-, and met-Hb (Figure 4-2). Oxy-Hb differed significantly from met-Hb but not CO-Hb. CO-Hb and met-Hb did not differ significantly from each other.

Washed tilapia muscle containing 9µmol/kg muscle of Hb and stored at 3.7°C (Figure 4-3) CO-Hb had significantly lower hydroperoxide values than met- and oxy-Hb. Oxy-Hb had significantly higher hydroperoxide values than CO- and met-Hb on day 2 and 4 but did not differ significantly from met-Hb on day 6 and 8. CO-Hb had significantly ($p \leq 0.05$) lower hydroperoxide values than oxy- and met-Hb on day 6 and 8. CO-Hb developed the least amount of lipid hydroperoxide at -25°C (Figure 4-4).

At 12µmol/kg muscle, oxy-Hb developed significantly ($p \leq 0.05$) higher hydroperoxides values than CO- and met-Hb at 3.7°C. CO-Hb had significantly ($p < 0.05$) lower hydroperoxide values than met- and oxy-Hb on day 6 and 8. Lipid hydroperoxide values obtained at -25°C and 12 µmol/kg of CO-, oxy-, and met-Hb (Figure 4-6) showed that met- and oxy-Hb had significantly ($p \leq 0.05$) higher hydroperoxide values than CO-Hb.

Washed tilapia muscle containing 6 µmol/kg muscle of either oxy-, CO-, and met-Hb and stored at 3.7°C (Figure 4-7) did not develop significantly different TBARS values ($pH < 0.05$). Examination of each storage day also did not show significant differences. At the end of storage on day 8 oxy-Hb appeared to be less pro-oxidative but these findings were not significant. TBARS values obtained at -25°C and 6µmol/kg of CO-, oxy-, and met-Hb (Figure 4-8) revealed that oxy-Hb was most pro-oxidative. CO- and met-Hb did not differ from each other. However, oxy-Hb was more pro-oxidative than CO-Hb at week 8, 12, and 16. Oxy-Hb was more pro-oxidative than CO- and met-Hb beginning at week 12 and continuing throughout the remaining weeks of storage.

Washed tilapia containing met-Hb at 9µmol/kg muscle and stored at 3.7°C developed significantly ($p < 0.05$) higher TBARS values than oxy-and CO-Hb (Figure 4-9) which did not differ significantly from each other. At days 4, 6, and 8 CO-Hb was less pro-oxidative than met-Hb. Oxy-Hb had significantly ($p \leq 0.05$) higher TBARS values than met-Hb on day 6 and 8. TBARS values obtained at -25°C and 9 µmol/kg muscle of CO-, oxy-, and met-Hb (Figure 4-10). Oxy-Hb was significantly ($p \leq 0.05$) more pro-oxidative than CO- and met-Hb at week 20 and 24. Met-Hb had significantly higher TBARS than CO-Hb at week 20 only.

Washed tilapia containing met-Hb at 12µmol/kg muscle and stored at 3.7°C developed significantly ($p < 0.05$) higher TBARS values than oxy-and CO-Hb (Figure 4-11) which did not differ significantly from each other. On day 2 oxy-Hb was significantly ($p \leq 0.05$) more pro-oxidative than met-Hb and CO-Hb but remained level throughout the rest of the storage period. TBARS values obtained at -25°C and 12 µmol/kg of CO-, oxy-, and met-Hb (Figure 4-12) indicated that oxy-Hb was more pro-oxidative than CO- and met-Hb. CO-Hb was significantly ($p \leq 0.05$) different from met-Hb at week 2 only. Oxy-Hb was significantly different from CO- and met-Hb at week 20 and 24.

Protein Oxidation Analysis

Washed tilapia muscle containing oxy-Hb at 6 µmol/kg muscle developed significantly ($p \leq 0.05$) more carbonyls at 3.7°C compared to CO- and met-Hb, which did not differ significantly from each other throughout the 8 days of storage (Figure 4-13). Oxy-Hb was significantly more pro-oxidative than met- and CO-Hb on day 2, 4, and 6 but not day 8 where there was no significant difference among the three forms. Carbonyl values obtained at -25°C and 6 µmol/kg of CO-, oxy-, and met-Hb (Figure 4-14) showed CO-Hb was less pro oxidative than met- and oxy-Hb which did not differ

significantly ($p \leq 0.05$) from each other. Oxy-Hb was significantly more pro-oxidative than met- and CO-Hb at week 12 but at week 20 and 24 met-Hb was more pro-oxidative than oxy-Hb. CO- and met-Hb were both significantly ($p \leq 0.05$) more pro-oxidative at week 20 and 24 than oxy-Hb.

Washed tilapia muscle containing oxy-Hb at 9 µmol/kg muscle developed significantly more carbonyls at 3.7°C compared to CO- and met-Hb, which did not differ significantly ($p \leq 0.05$) from each other throughout the 8 days of storage (Figure 4-15). There was no significant ($p \leq 0.05$) difference on day 0 for the three forms but for the remaining storage days oxy-Hb had significantly higher carbonyl values than CO- and met-Hb which did not differ on any day. Carbonyl values obtained at -25°C and 9 µmol/kg of CO-, oxy-, and met-Hb (Figure 4-16) showed a pattern similar to that at 3.7°C. CO-Hb was significantly ($p \leq 0.05$) less pro-oxidative than met- and CO-Hb.

Washed tilapia muscle containing oxy-Hb at 12 µmol/kg muscle developed significantly ($p \leq 0.05$) more carbonyls at 3.7°C compared to CO- and met-Hb, which did not differ significantly from each other throughout the 8 days of storage (Figure 4-17). These significant differences were evident for each day of storage. Carbonyl values obtained at -25°C and 12 µmol/kg of CO-, oxy-, and met-Hb (Figure 4-18) found that all three forms of Hb differed significantly. Oxy-Hb had significantly ($p \leq 0.05$) higher carbonyl values at week 0, 4, 12, and 24 than met-Hb. CO-Hb had significantly lower carbonyl values than both oxy- and met-Hb at week 4, 8, 12, and 16. At week 20 there was no significant ($p \leq 0.05$) difference found for the three forms. However, at week 24 CO-Hb was significantly more pro-oxidative than met- and oxy-Hb.

CO Release

Results from washed tilapia muscle with CO-Hb show that all concentrations tested released significantly greater % CO at 3.7°C on day 0 than day 2, 4, and 6 (Figure 4-19). The sample containing 6 µmol/kg of CO-Hb released significantly less than the 9 and 12 µmol/kg samples, on day 0. At day 8 all three Hb concentrations tested showed an increase in release of % CO. At - 25°C samples had released significant % CO by week 16 compared to earlier weeks, irrespective of Hb concentration (Figure 4-20). Unlike the findings at 3.7°C, the samples with 6 µmol/kg CO-Hb released more CO compared to 9 and 12 µmol/kg muscle.

Color Analysis

Samples with Hb at 6 µmol/kg muscle, stored at 3.7°C, showed a significant ($p \leq 0.05$) difference in a*values for the three forms of Hb tested (Table 4-1). CO-Hb had significantly higher values than met-Hb throughout the 8 days of storage. CO-Hb also had significantly higher a*values than oxy-Hb on day 0, 2, and 6 only. Oxy-Hb had significantly higher a*values than met-Hb throughout the 8 days of storage. At -25°C, a*values differed significantly for all three forms of Hb (Table 4-2). CO-Hb had significantly higher a*values than oxy- and met-Hb throughout each of the 24 weeks of storage. Oxy- Hb had significantly higher a*values than met-Hb for week 0, 12, and 20.

Samples with Hb at 9 µmol/kg muscle, stored at 3.7°C, showed a significant difference ($p \leq 0.05$) between CO- and oxy-Hb versus met-Hb (Table 4-1), which had significantly lower a*values throughout each of the 8 days of storage. CO-Hb had significantly ($p \leq 0.05$) higher a*values than oxy-Hb on day 0, 2, and 4. At -25°C storage, a*values differed significantly for all three forms of Hb (Table 4-2). CO-Hb had significantly ($p \leq 0.05$) higher a*values than oxy- and met-Hb throughout each of the 24

storage weeks. Oxy-Hb had significantly ($p \leq 0.05$) higher a*values than met-Hb for each storage week, except the last where it did not differ significantly.

Samples with Hb at 12 µmol/kg muscle, stored at 3.7°C, showed a significant ($p \leq 0.05$) difference between the three forms of Hb (Table 4-1). CO-Hb had significantly higher a*values than oxy-Hb on day 0, 2, and 4, and significantly ($p \leq 0.05$) higher than met-Hb on each of the storage days except day 8. Met-Hb had significantly lower a*values than oxy-Hb throughout each of the 8 days of storage. At -25°C, a*values differed significantly ($p \leq 0.05$) for all three forms of Hb (Table 4-2). CO-Hb had significantly ($p \leq 0.05$) higher a*values than met-Hb for each week during the storage period. Oxy-Hb had significantly lower a*values than CO-Hb at week 14 only.

Heme Group Autoxidation

At 3.7°C storage, the % oxy-Hb in washed tilapia muscle containing 6µmol/kg muscle of Hb was significantly ($p \leq 0.05$) different for the three forms of Hb (Figure 4-21a). CO-Hb had significantly greater % oxy-Hb than oxy- and met-Hb. On day 0, 2, and 4 CO-Hb differed significantly ($p \leq 0.05$) from oxy-Hb in % oxy-Hb, but not on day 6 and 8. The % oxy-Hb in met-Hb samples was significantly different from oxy- and CO-Hb throughout the 8 days of storage. At -25°C, all three forms differed significantly ($p \leq 0.05$) in % oxy-Hb (Figure 4-22a). Samples containing 6 µmol/kg muscle of CO-Hb had significantly greater % oxy-Hb than met-Hb throughout each week of storage. Met-Hb had significantly ($p \leq 0.05$) less % oxy-Hb than CO- and oxy-Hb.

At 3.7°C storage, the % oxy-Hb in washed tilapia muscle containing 9µmol/kg muscle of Hb was was significantly ($p \leq 0.05$) different for the three forms of Hb (Figure 4-21b), the same pattern of % oxy-Hb as that seen with 6 µmol/kg muscle of Hb. Although the % oxy-Hb in samples with 9 µmol/kg muscle was greater, days 0, 2, and 4

showed the same significant difference ($p \leq 0.05$) between oxy- and CO-Hb with met-Hb having significantly less % oxy-Hb throughout the 8 days of storage. At -25°C, all three forms differed significantly in % oxy-Hb (Figure 4-22b). For each week of storage, except week 24, CO-Hb had significantly greater % oxy-Hb than oxy- and met-Hb.

At 3.7°C storage, the % oxy-Hb in washed tilapia muscle containing 12µmol/kg muscle of Hb was was significantly ($p \leq 0.05$) different for the three forms of Hb (Figure 4-21c), and the significant differences were similar to those described for 6 and 9 µmol/kg muscle. At -25°C, all three forms differed significantly ($p \leq 0.05$) in % oxy-Hb (Figure 4-22c). For each week of storage, except week 24, CO-Hb had significantly greater % oxy-Hb than oxy- and met-Hb, the pattern being similar to that at 6 µmol/kg muscle with CO-Hb having slightly higher % oxy-Hb at 12 µmol/kg muscle.

At 3.7°C storage, the % met-Hb in washed tilapia muscle containing 6µmol/kg muscle Hb was was significantly ($p \leq 0.05$) different for the three forms of Hb (Figure 4-23a). of Met-Hb had, as expected, significantly higher % met-Hb than CO- and oxy-Hb on each day of storage. Oxy-Hb had significantly ($p \leq 0.05$) greater % met-Hb than CO-Hb. At -25°C, all three forms differed significantly in % oxy-Hb (Figure 4-24a). Met-Hb had significantly ($p \leq 0.05$) higher % met-Hb than oxy- and met-Hb throughout each day of the 24 week storage period. Oxy-Hb had greater % met-Hb than CO-Hb throughout the 24 weeks.

At 3.7°C storage, the % met-Hb in washed tilapia muscle containing 9µmol/kg muscle Hb was was significantly ($p \leq 0.05$) different for the three forms of Hb (Figure 4-23b). Met-Hb had significantly greater % met-Hb on each day of storage than oxy- and CO-Hb. Oxy-Hb had significantly ($p \leq 0.05$) greater % met-Hb than CO-Hb on day 0, 2,

and 4 but was not significantly (p≤0.05) different on day 6 and 8. At -25°C, all three forms differed significantly in % oxy-Hb formed (Figure 4-24b). Met-Hb had significantly (p≤0.05) higher % met-Hb than oxy- and met-Hb throughout each day of the 24 week storage period. Oxy-Hb had significantly greater % met-Hb than CO-Hb at week 0, 4, 8, 12, 16, and 20; they did not differ significantly week 20.

At 3.7°C storage, the % met-Hb in washed tilapia muscle containing 12µmol/kg muscle Hb was was significantly (p≤0.05) different for the three forms of Hb (Figure 4-23c). Met-Hb had greater % met-Hb than oxy- and CO-Hb on each storage day. Oxy-Hb had greater % met-Hb than CO-Hb day 0, 2, and 4 but these did not differ significantly (p≤0.05) on day 6 and 8. At -25°C, all three forms differed significantly in % deoxy-Hb (Figure 4-24c). Met-Hb had significantly higher % met-Hb than oxy- and met-Hb throughout each day of the 24 week storage period. Oxy-Hb had significantly greater % met-Hb than CO-Hb at weeks 0, 4, 8, 12, and 16 but not at weeks 20 and 24.

At 3.7°C storage, the % deoxy-Hb in washed tilapia muscle containing 6µmol/kg muscle Hb showed that met- and oxy-Hb had significantly (p≤0.05) greater % deoxy-Hb than oxy-Hb (Figure 4-25a). CO-Hb had significantly less % deoxy-Hb compared to met-Hb and oxy-Hb on day 0, 2, and 4, but they were not significantly different day 6 and 8. At -25°C CO-Hb had lower % deoxy-Hb had than met- and oxy-Hb (Figure 4-26a). CO-Hb and met-Hb differed significantly in % deoxy-Hb at week 0 only, while CO-Hb and oxy-Hb differed significantly (p≤0.05) at each week of storage except week 16.

At 3.7°C storage, the % deox-Hb in washed tilapia muscle containing 9µmol/kg muscle Hb showed that met- and oxy-Hb had significantly greater % deoxy-Hb (Figure 4-25b) than CO-Hb day 0, 2, 4, and 6. CO-Hb had significantly (p≤0.05) less % deoxy

than oxy-Hb day 0, 2, 4, and 6. On day 8 there were no significant differences ($p \leq 0.05$) for the three forms of Hb. At -25°C CO-Hb had significantly less % deoxy-Hb than oxy-Hb at week 0, 4, and 8, and significantly ($p \leq 0.05$) less than met-Hb at week 0 and 8 (Figure 4-26b). The remaining days of storage showed no significant differences ($p \leq 0.05$) for the three forms of Hb.

At 3.7°C storage, the % deoxy-Hb in washed tilapia muscle containing 9µmol/kg muscle Hb showed that met- and oxy-Hb had significantly ($p \leq 0.05$) greater % deoxy-Hb (Figure 4-25c). CO-Hb had significantly lower % deoxy-Hb than oxy- and met-Hb on each day of storage, except day 8 where there were no significant differences for the three forms. At -25° CO-Hb had significantly ($p \leq 0.05$) less % deoxy- than met- and oxy-Hb at week 0 and less than oxy-Hb at week 8 (Figure 4-26c). There were no significant differences ($p \leq 0.05$) for the remaining weeks of storage.

Discussion

The pro-oxidative activity of fish Hb has been well established in numerous previous studies (*56*). The form of Hb present in muscle meat was shown to be one of the important determinants of the rate and magnitude of oxidation during the storage (*1, 105*). Based on accumulated evidence, it appears reasonable to hypothesize that the concentration of the Hb present in fish muscle system may also affect its oxidation pattern. However, to our knowledge, little research is available determining possible associations between Hb concentrations and their catalytic capacity to oxidize lipids and proteins in a WMTM system.

In this study, the pro-oxidative activities of oxy-, CO-, and met-Hb as a function of different concentrations were examined under two storage conditions. Formation of TBARS, lipid hydroperoxides, and protein carbonyl was used as indicators of oxidative

activities. At 3.7ºC the concentration of met-Hb significantly influenced production of TBARS with lower TBARS correlating with lower met-Hb concentrations (Figure 4-7, 4-9, 4-11). Increased oxidation with increasing Hb concentrations is consistent with the findings of Richards and Hultin (*11*) who found increasing the µmol of Hb added to cod washed muscle produced higher levels of TBARS earlier and rancidity was also detectable earlier in the higher concentrations (1.8 and 5.8 µmol) versus the lower concentrations (0.06 and 0.50 µmol). In contrast to met-Hb, concentrations of oxy- and CO-Hb did not appear to affect TBARS formation to any considerable extent (Figure 4-7, 4-9, and 4-11). Previous reports have established that met-Hb will readily react with preformed hydrohydroperoxides to generate further oxidation (*38*). Increased availability of met-Hb for reaction with hydroperoxides with increased concentration may partially explain the observed results.

Very different trends were seen for oxidation measured by lipid hydroperoxides. Each increase in concentration of oxy-Hb showed a significant rise in lipid hydroperoxides (Figure 4-1, 4-3, and 4-5). Rapid deoxygenation as oxy-Hb concentrantion are increased could potentially increase lipid oxidation. Of note, at all concentrations, the rate and pattern of lipid oxidation for each form of Hb was similar, differing only in magnitude (Figure 4-1, 4-3, and 4-5).

Results at -25ºC were different than those at 3.7°C. Overall TBARS values were not significantly affected by Hb concentration (Figure 4-8, 4-10, 4-12). Interestingly, during frozen storage oxy-Hb led to the highest TBARS value, regardless of concentration. Lipid oxidation increased significantly for all forms of Hb as their concentration increased (Figure 4-2, 4-4). However, CO-Hb led to less lipid

hydroperoxides compared to oxy- and met-Hb regardless of concentration (Figure 4-2, 4-4, 4-6). TBARS and lipid hydroperoxide data support the hypothesis that CO-Hb is least pro-oxidative of the three forms, regardless of Hb concentrations in the washed system.

Protein oxidation (carbonyls) appeared not to be affected by Hb concentration at 3.7ºC (Figure 4-13, 4-15, 4-17), but was affected more by temperature. Although oxy-Hb remained the most pro-oxidative with respect to protein oxidation of the three forms at both temperatures, at -25°C a significant difference in protein oxidation emerged between CO-Hb and met-Hb. There was no significant difference between the two forms at 3.7°C but at -25ºC a significant difference emerged with CO-Hb being the least pro-oxidative, i.e. leading to the least protein oxidation.

Effects of different Hb concentrations on red color deterioration were also evaluated. At 3.7ºC, the higher concentration of oxy-Hb was associated with more stable red color (Table 4-1). Contrary to what was expected, increasing the concentration of CO-Hb did not lead to a more stable red color. On the contrary, the higher concentration of CO-Hb led to lower a*value by the end of the storage time (Table 4-1). In contrast, at -25ºC, red color deterioration was significantly retarded by higher concentrations of all of the forms of Hb (Table 4-2). CO-Hb samples showed the most red color stability at -25°C. Unfortunately, accumulated research data is insufficient to explain these obtained results. More studies need to be conducted to further explore the effects of concentrations on Hb pro-oxidative activities.

Conclusion

CO-Hb was significantly less pro-oxidative compared to other forms of Hb regardless of concentration used. This work suggests fish with CO bound to Hb may be more stable with respect to lipid oxidation. Temperature significantly affected the stability of CO-Hb and its oxidation to Met-Hb.

Table 4-1. Changes in a*value in washed tilapia muscle containing different forms of Hb at a concentration of 6, 9, and 12µmol/kg at 3.7ºC, averaged across pH level and NaCl added.

a* value	6µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	15.4±2.2	19.9±4.5	6.5±1.1
2	12.9±5.5	19.4±5.7	8.3±1.4
4	11.9±7.3	18.1±7.3	6.9±1.4
6	10.7±7.7	13.3±7.9	5.0±1.8
8	9.5±8.2	12.4±8.3	3.8±2.1

a* value	9µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[a]	CO-Hb[a]	Met-Hb[b]
0	20.4±3.1	22.0±3.5	8.2±1.4
2	16.8±7.5	20.7±4.7	10.2±1.7
4	15.3±9.0	16.5±8.1	8.0±2.2
6	13.5±10.4	9.6±7.7	6.0±2.5
8	12.4±10.0	9.3±7.1	4.8±2.7

a* value	12µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[a]	CO-Hb[b]	Met-Hb[c]
0	24.0±3.8	23.8±3.5	9.5±1.2
2	20.1±9.0	22.1±4.5	12.2±1.1
4	18.3±10.6	17.0±7.8	9.3±2.6
6	16.2±12.0	11.0±9.1	7.4±3.2
8	14.3±11.0	7.8±5.8	5.8±3.1

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table 4-2. Changes in a*value in washed tilapia muscle containing different forms of Hb at a concentration of 6, 9, and 12µmol/kg at -25ºC, averaged across pH level and NaCl added.

a* value	6µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	15.4±2.2	19.9±4.5	6.5±1.1
4	12.8±3.9	24.0±5.3	9.5±1.6
8	11.7±3.8	22.1±7.0	8.7±1.2
12	12.5±5.5	21.0±6.6	7.8±1.3
16	11.3±6.0	19.6±7.3	7.6±1.4
20	11.3±6.8	19.0±7.1	7.2±1.6
24	9.9±7.3	19.0±8.4	6.3±1.6

a* value	9µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	20.4±3.1	22.0±3.5	8.2±1.4
4	17.0±4.9	25.7±4.8	12.6±1.8
8	15.8±5.4	24.7±5.0	11.2±1.3
12	13.5±5.9	22.6±5.3	10.3±1.4
16	15.3±7.9	21.5±5.5	10.2±1.7
20	15.4±8.6	20.4±5.6	9.7±1.8
24	13.5±9.3	19.3±5.9	9.7±1.9

a* value	12µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	24.0±3.8	23.8±3.5	9.5±1.2
4	21.8±6.6	25.1±4.8	14.8±1.7
8	19.9±6.6	22.2±5.4	13.4±1.9
12	14.6±10.0	20.6±5.4	12.7±1.6
16	17.1±7.9	20.8±5.9	12.8±1.9
20	18.7±10.0	18.3±5.2	12.4±1.9
24	16.8±10.8	16.8±5.5	12.4±2.5

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

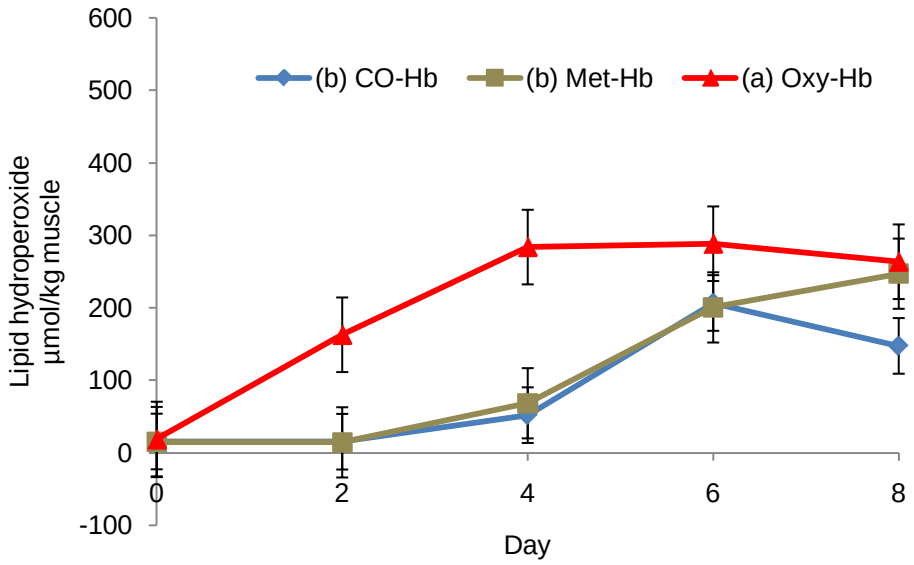


Figure 4-1. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at a concentration of 6 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

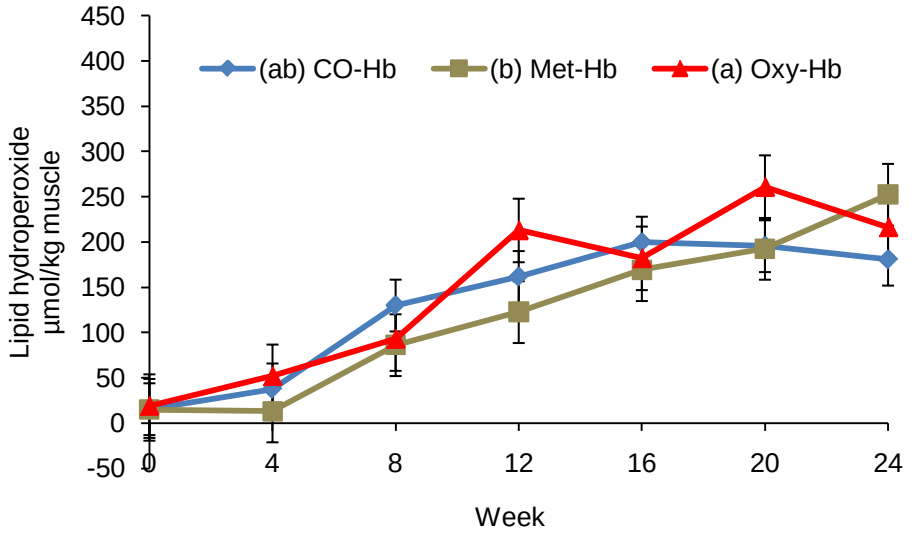


Figure 4-2. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at a concentration of 6 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

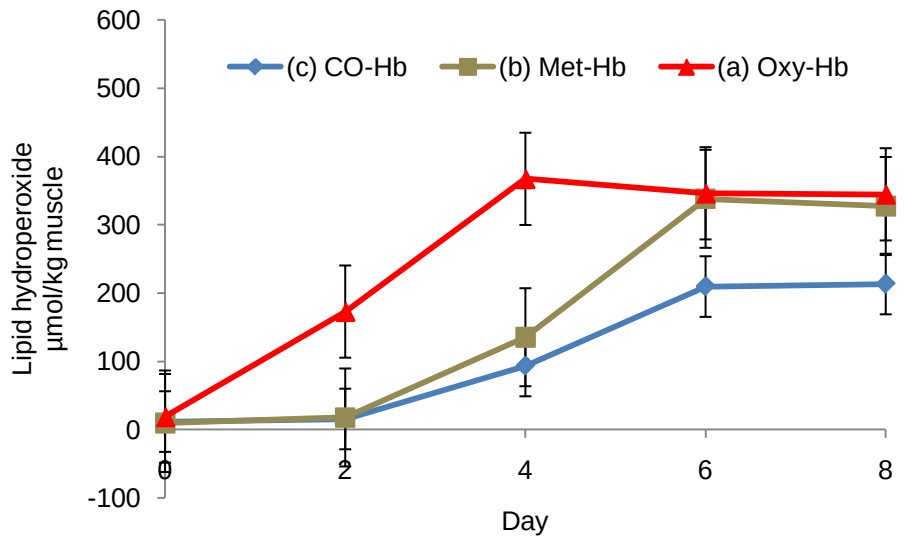


Figure 4-3. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at a concentration of 9 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

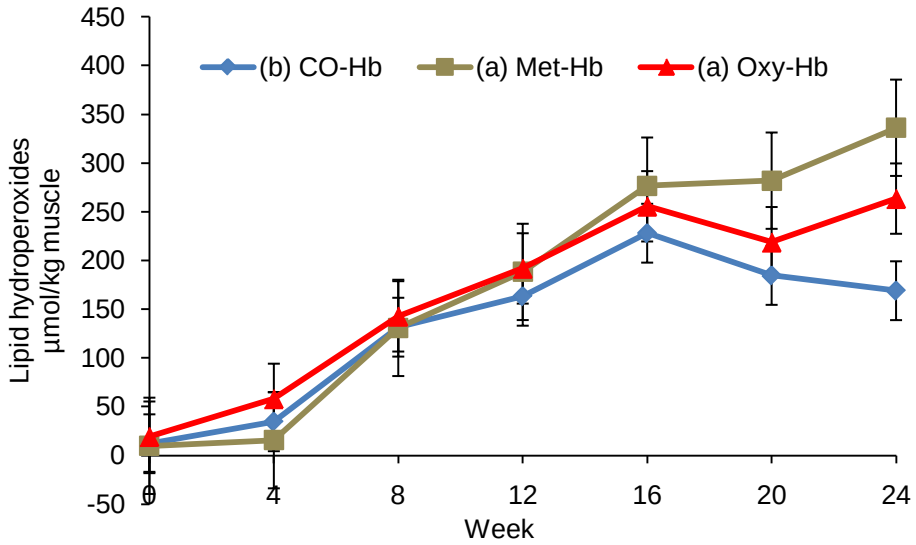


Figure 4-4. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at a concentration of 9 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

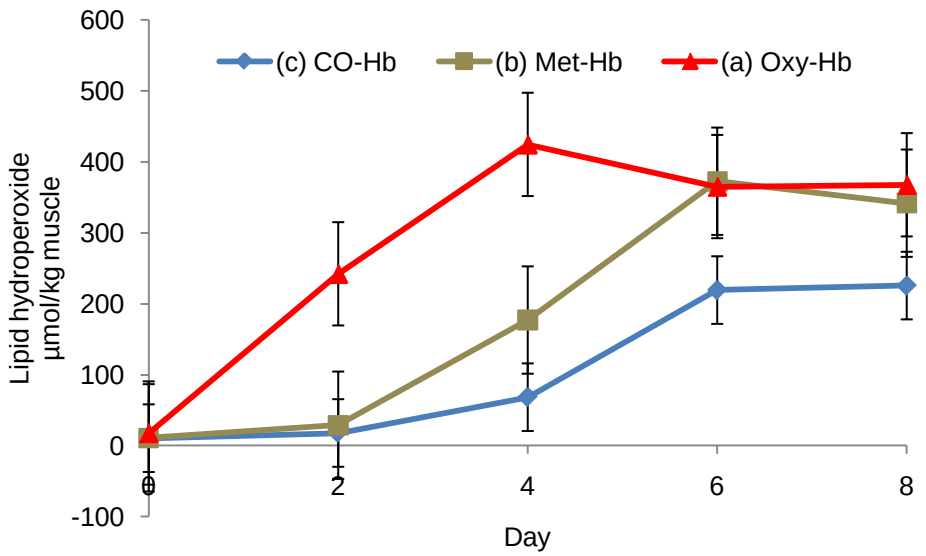


Figure 4-5. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at a concentration of 12 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

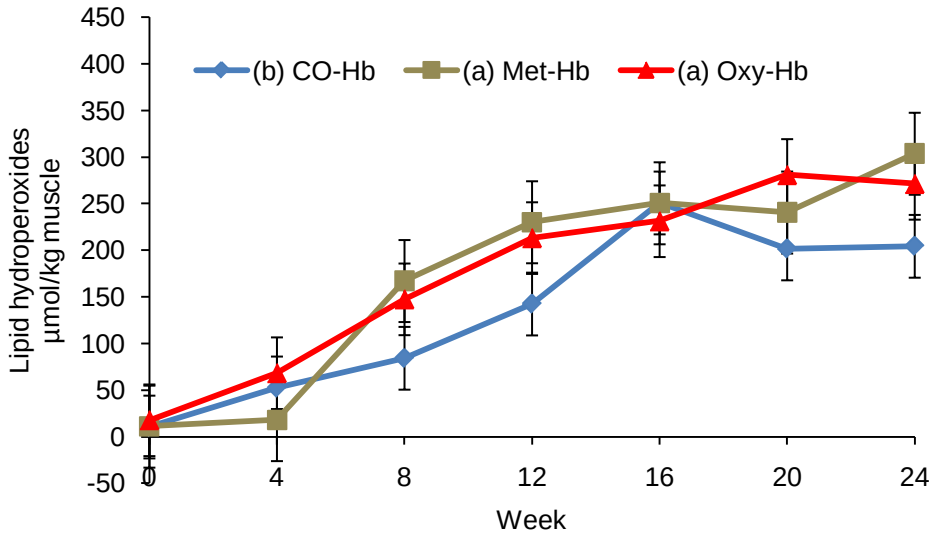


Figure 4-6. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at a concentration of 12 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

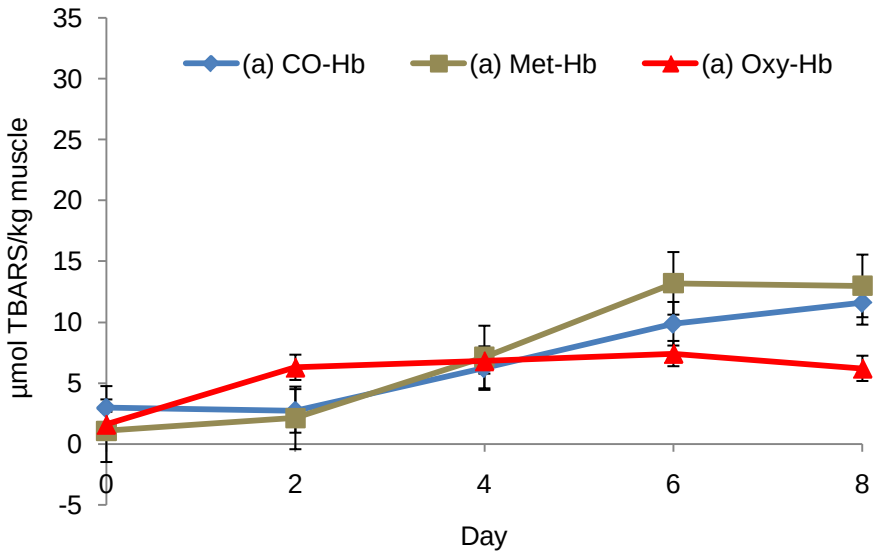


Figure 4-7. TBARS in washed tilapia muscle containing different forms of Hb at a concentration of 6 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

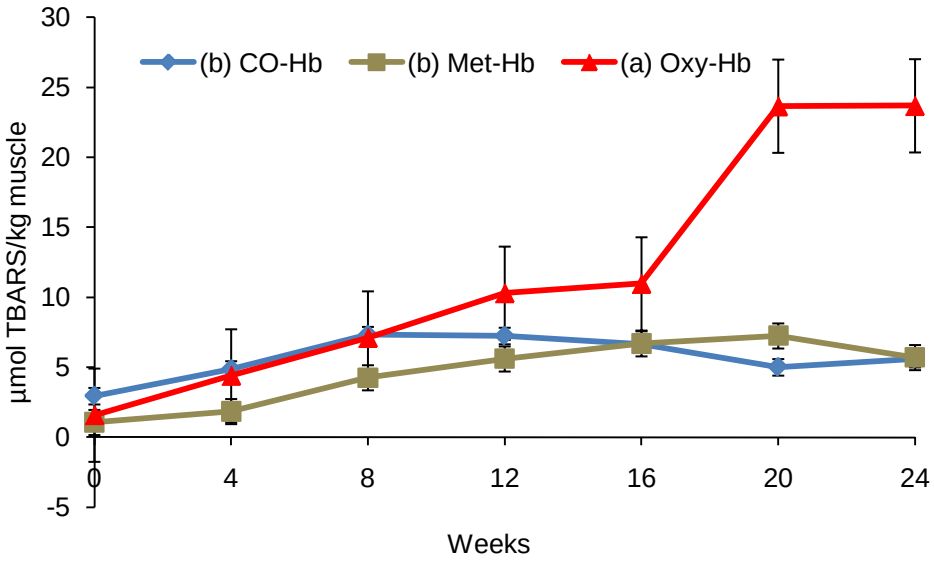


Figure 4-8. TBARS in washed tilapia muscle containing different forms of Hb at a concentration of 6 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

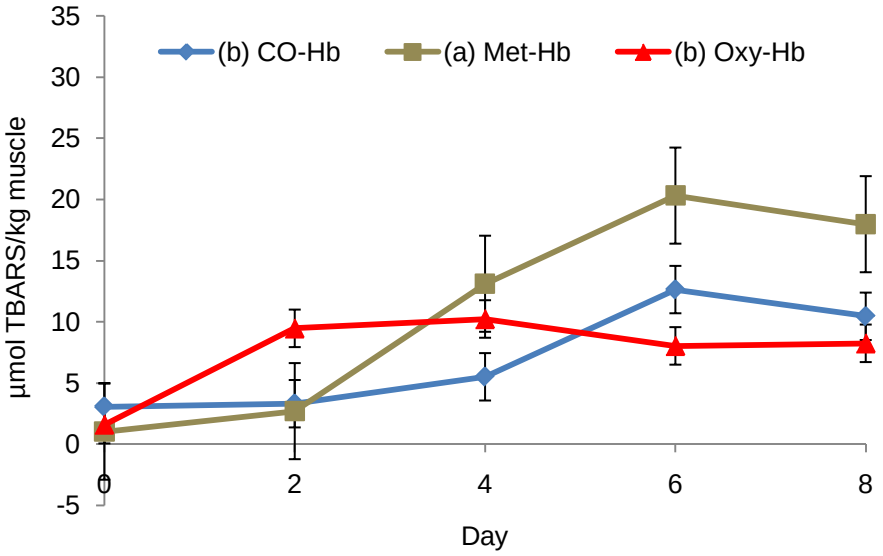


Figure 4-9. TBARS in washed tilapia muscle containing different forms of Hb at a concentration of 9 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

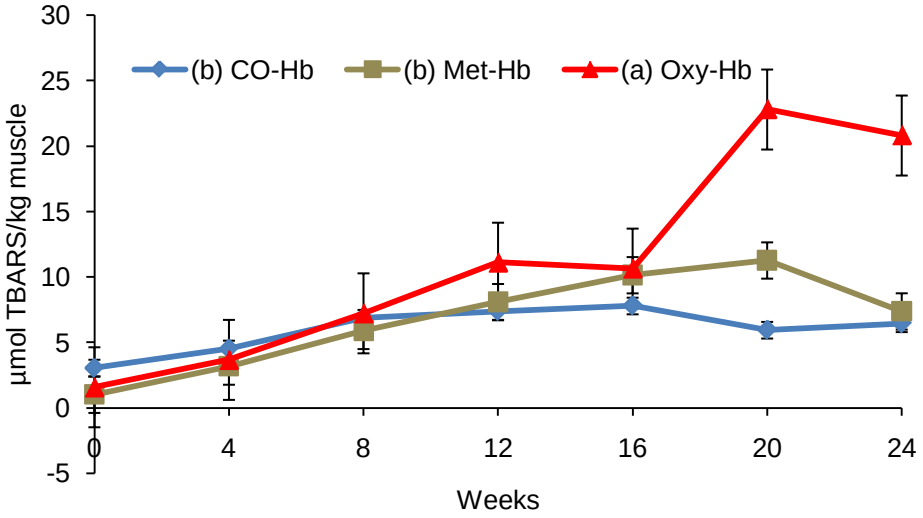


Figure 4-10. TBARS in washed tilapia muscle containing different forms of Hb at a concentration of 9 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

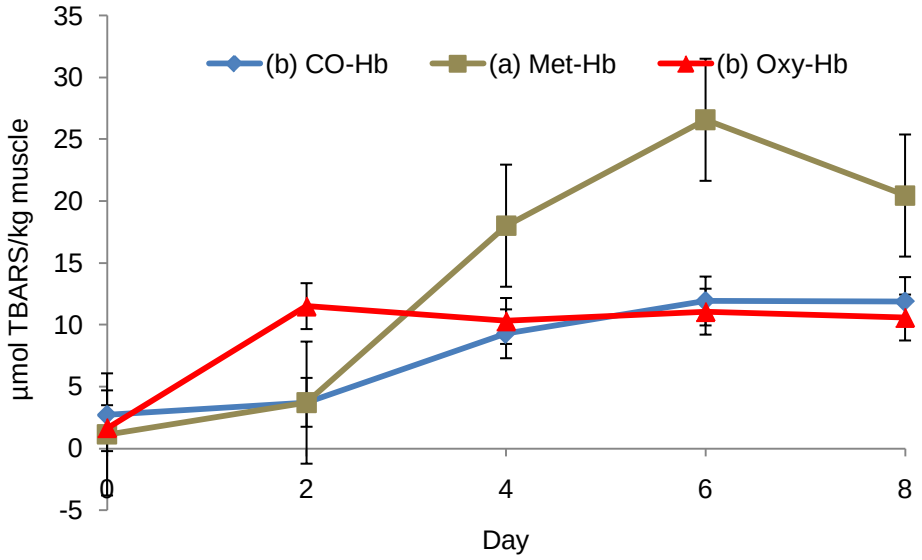


Figure 4-11. TBARS in washed tilapia muscle containing different forms of Hb at a concentration of 12 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

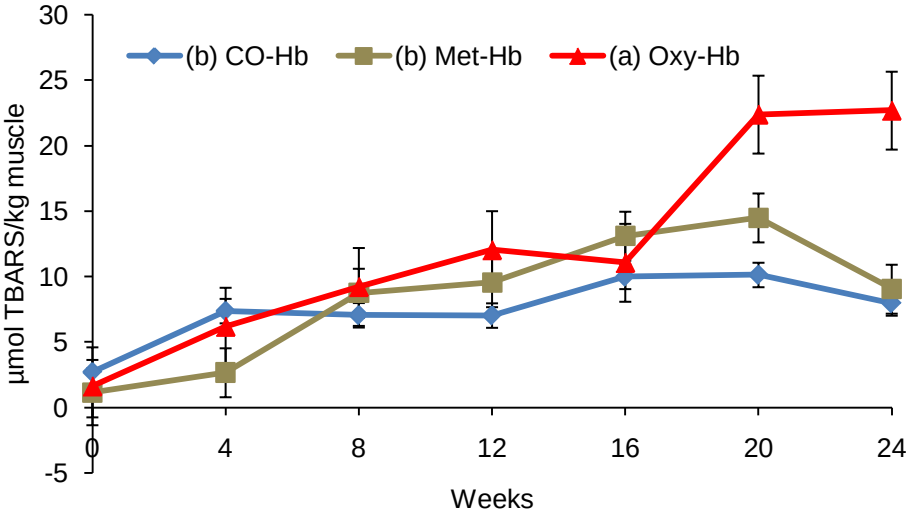


Figure 4-12. TBARS in washed tilapia muscle containing different forms of Hb at a concentration of 12 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

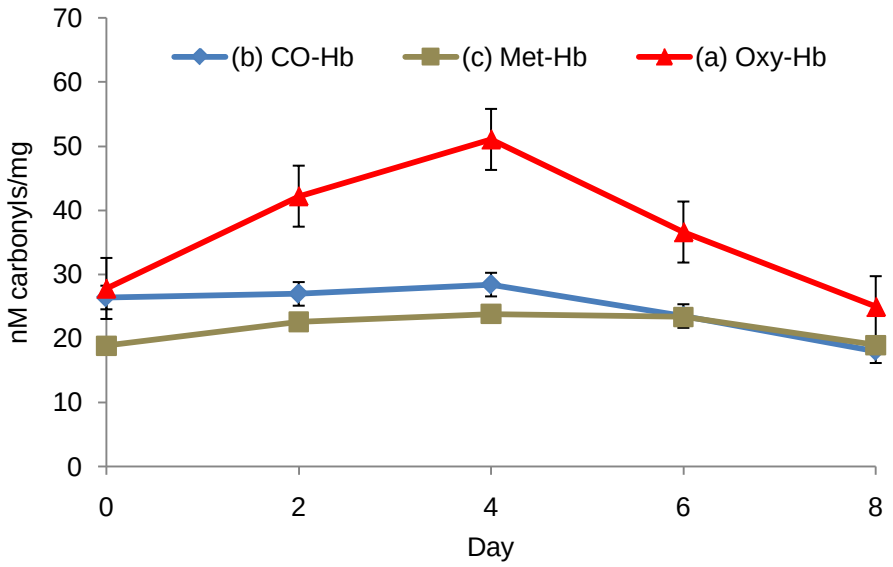


Figure 4-13. Carbonyl values in washed tilapia muscle containing different forms of Hb at a concentration of 6 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

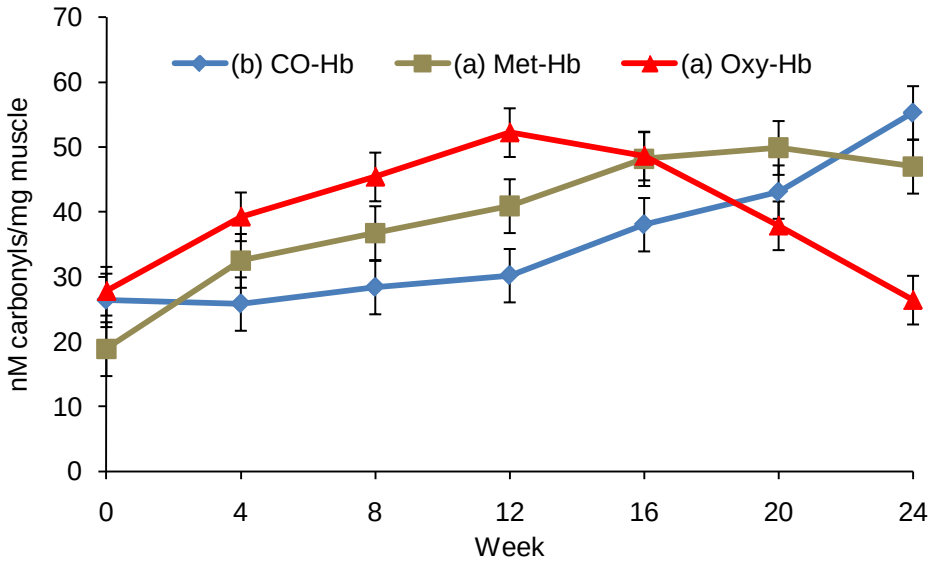


Figure 4-14. Carbonyl values in washed tilapia muscle containing different forms of Hb at a concentration of 6 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

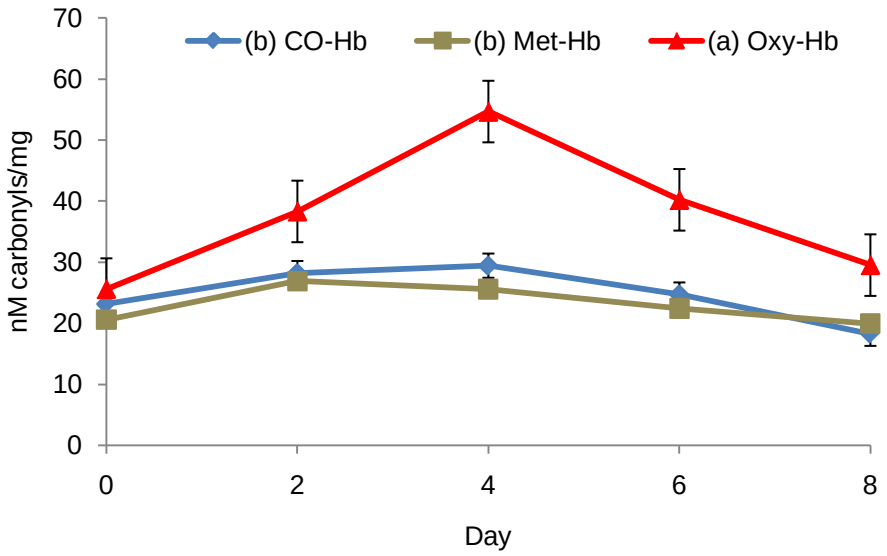


Figure 4-15. Carbonyl values in washed tilapia muscle containing different forms of Hb at a concentration of 9 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

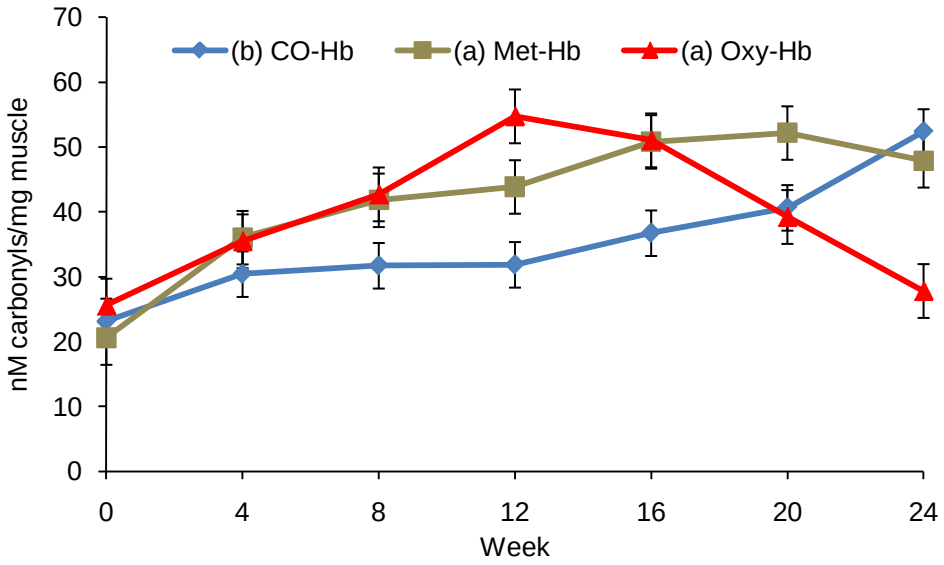


Figure 4-16. Carbonyl values in washed tilapia muscle containing different forms of Hb at a concentration of 9 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

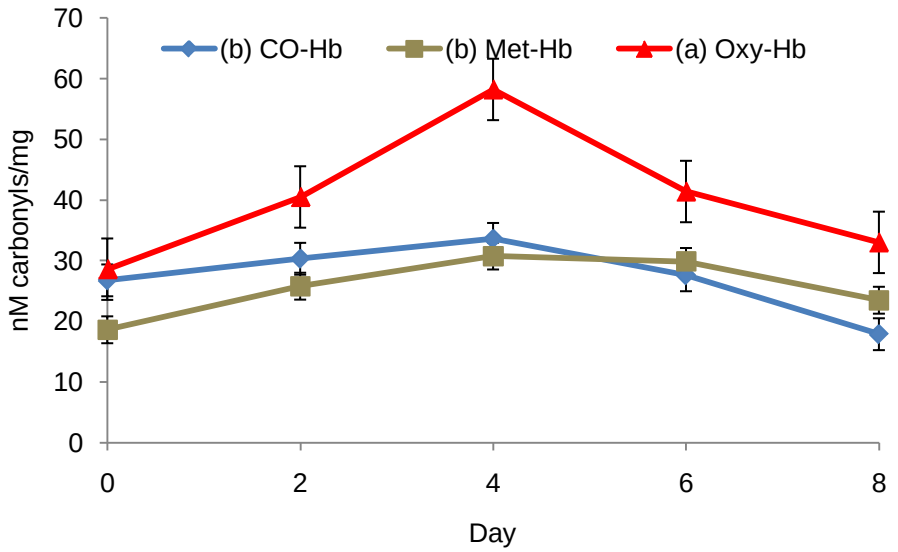


Figure 4-17. Carbonyl values in washed tilapia muscle containing different forms of Hb at a concentration of 12 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

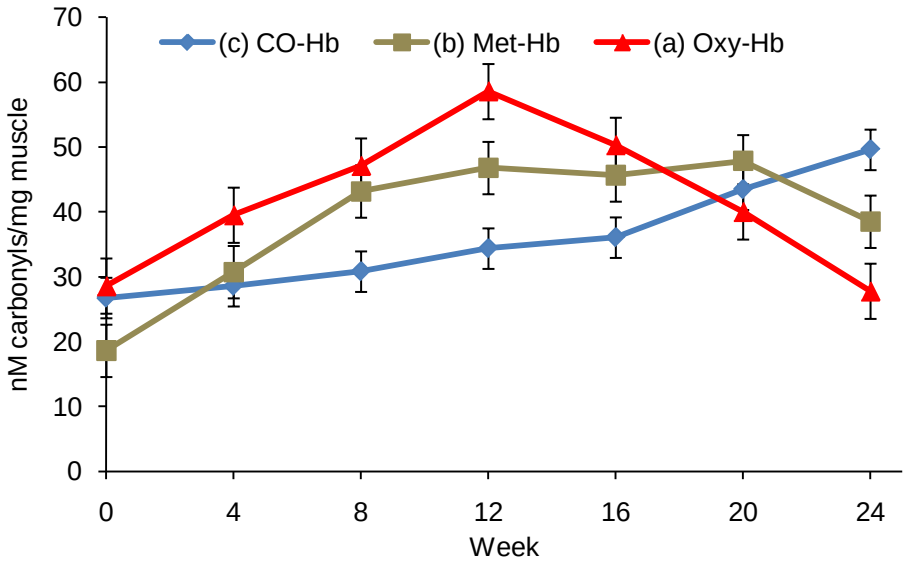


Figure 4-18. Carbonyl values in washed tilapia muscle containing different forms of Hb at a concentration of 12 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

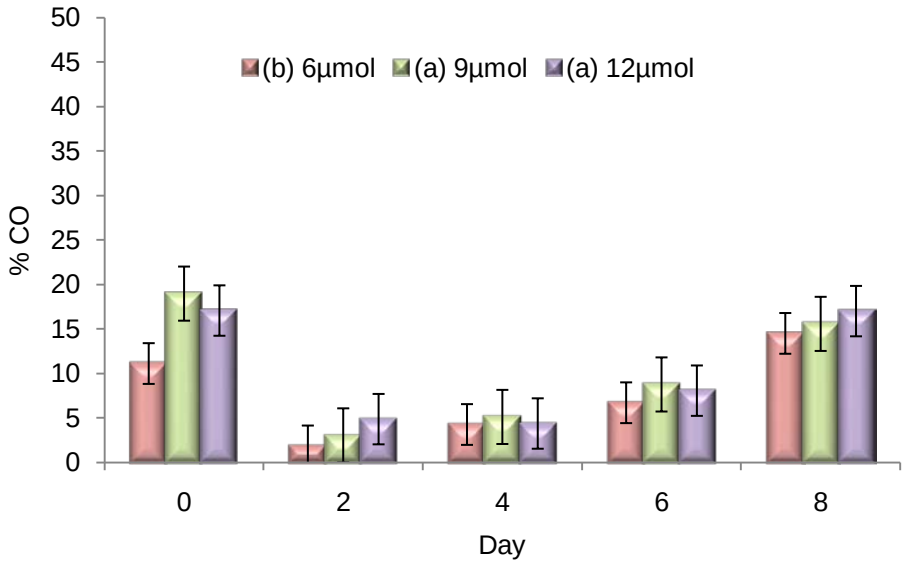


Figure 4-19. %CO released during 3.7ºC storage in washed tilapia muscle containing CO-Hb at a concentration of 6, 9, and 12µmol/kg muscle. The effect of forms of Hb (Oxy-, CO-, and Met-Hb) by storage interaction averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

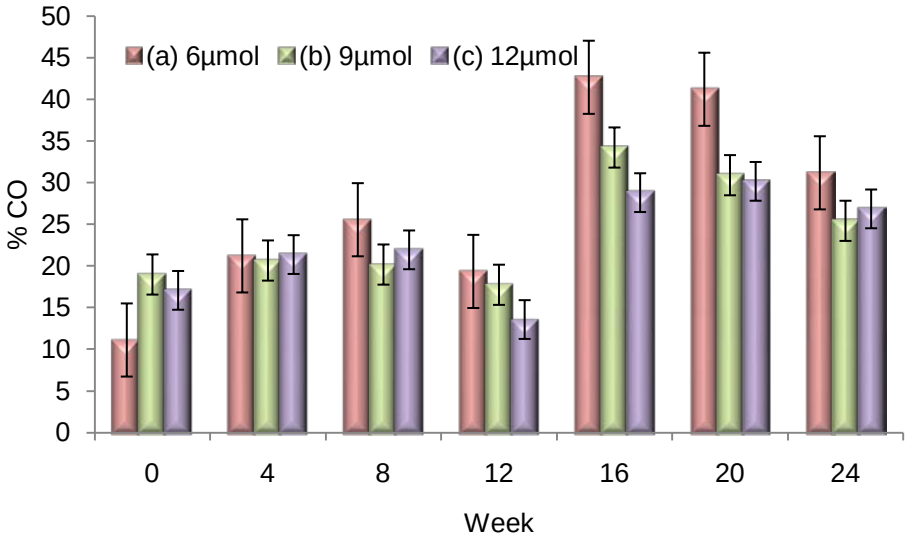


Figure 4-20. %CO released during -25ºC storage in washed tilapia muscle containing CO-Hb at a concentration of 6, 9, and 12 µmol/kg muscle. The effect of forms of Hb (Oxy-, CO-, and Met-Hb) by storage interaction averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

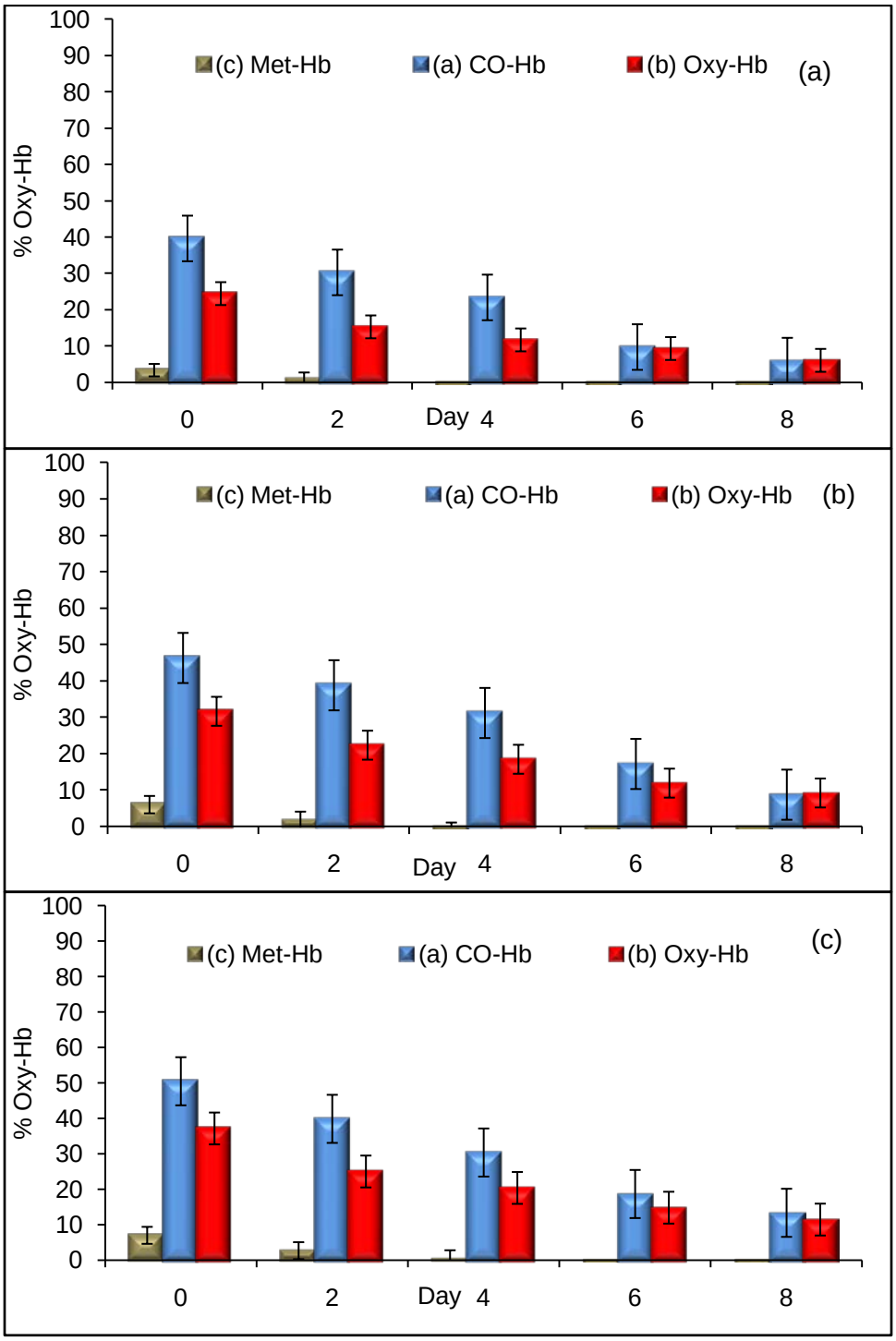


Figure 4-21. %Oxy-Hb in washed tilapia muscle containing different forms of Hb at a concentration of a) 6, b) 9, and c) 12 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

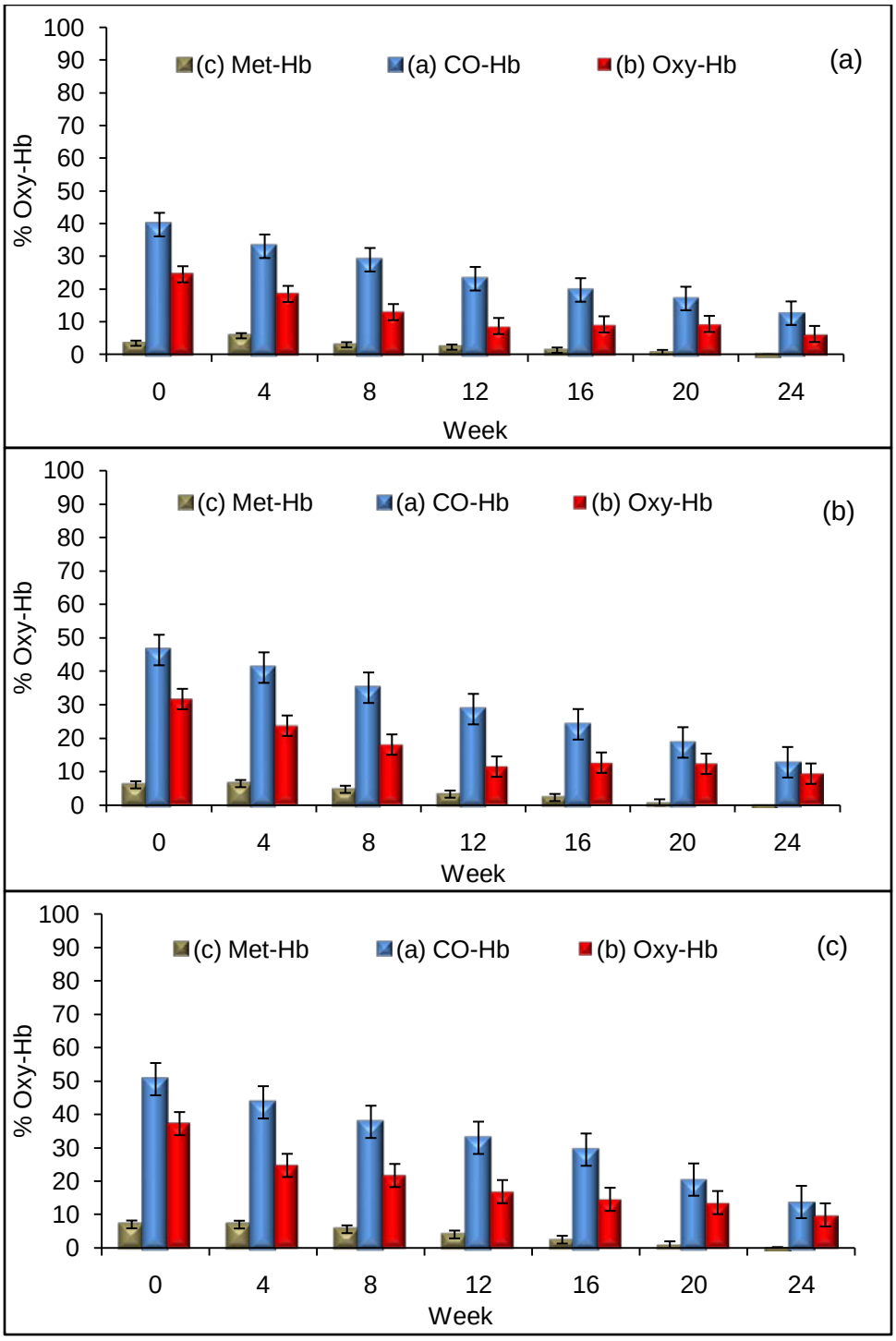


Figure 4-22. %Oxy-Hb in washed tilapia muscle containing different forms of Hb at a concentration of a) 6, b) 9, and c) 12 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

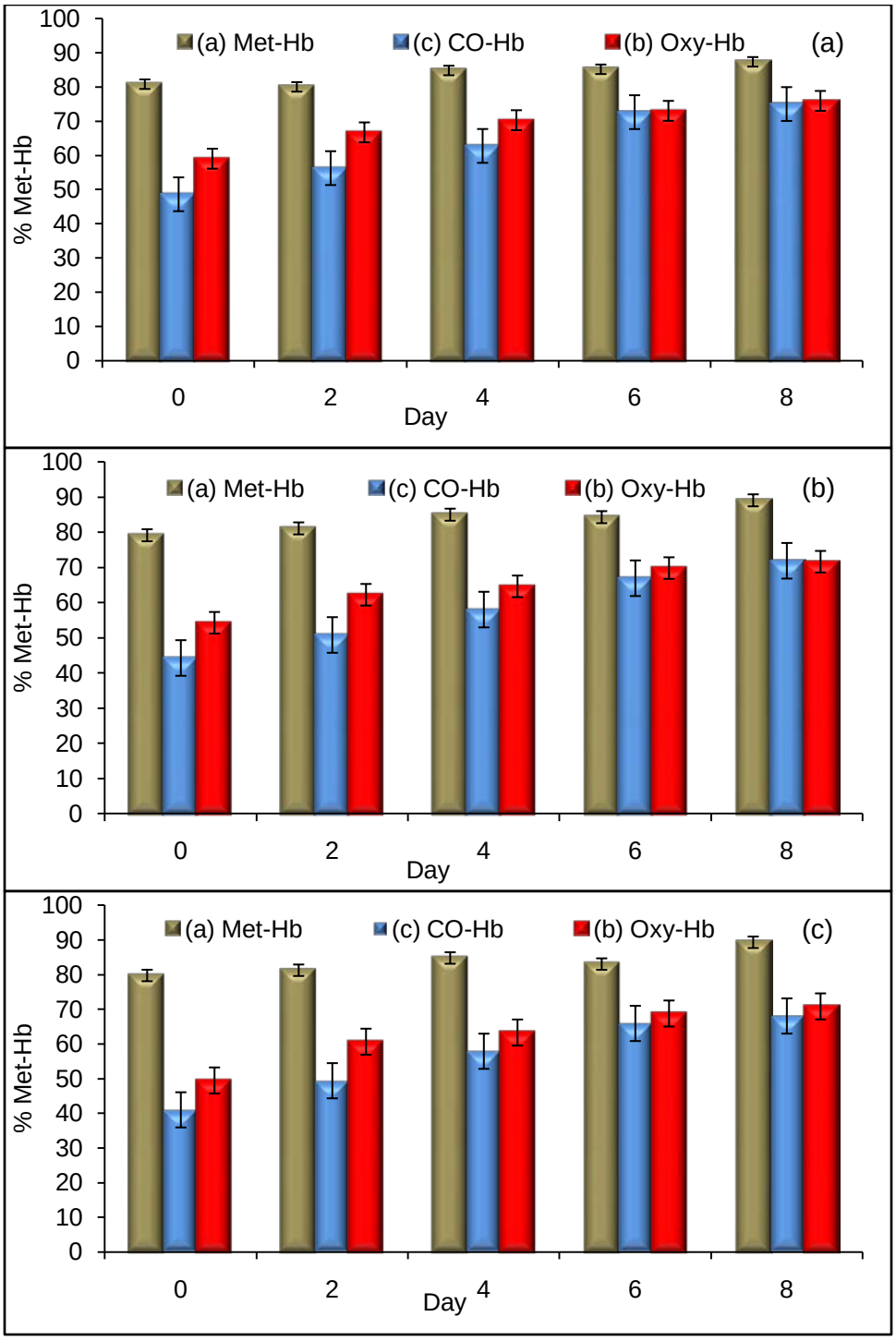


Figure 4-23. %Met-Hb in washed tilapia muscle containing different forms of Hb at a concentration of a) 6, b) 9, and c) 12 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

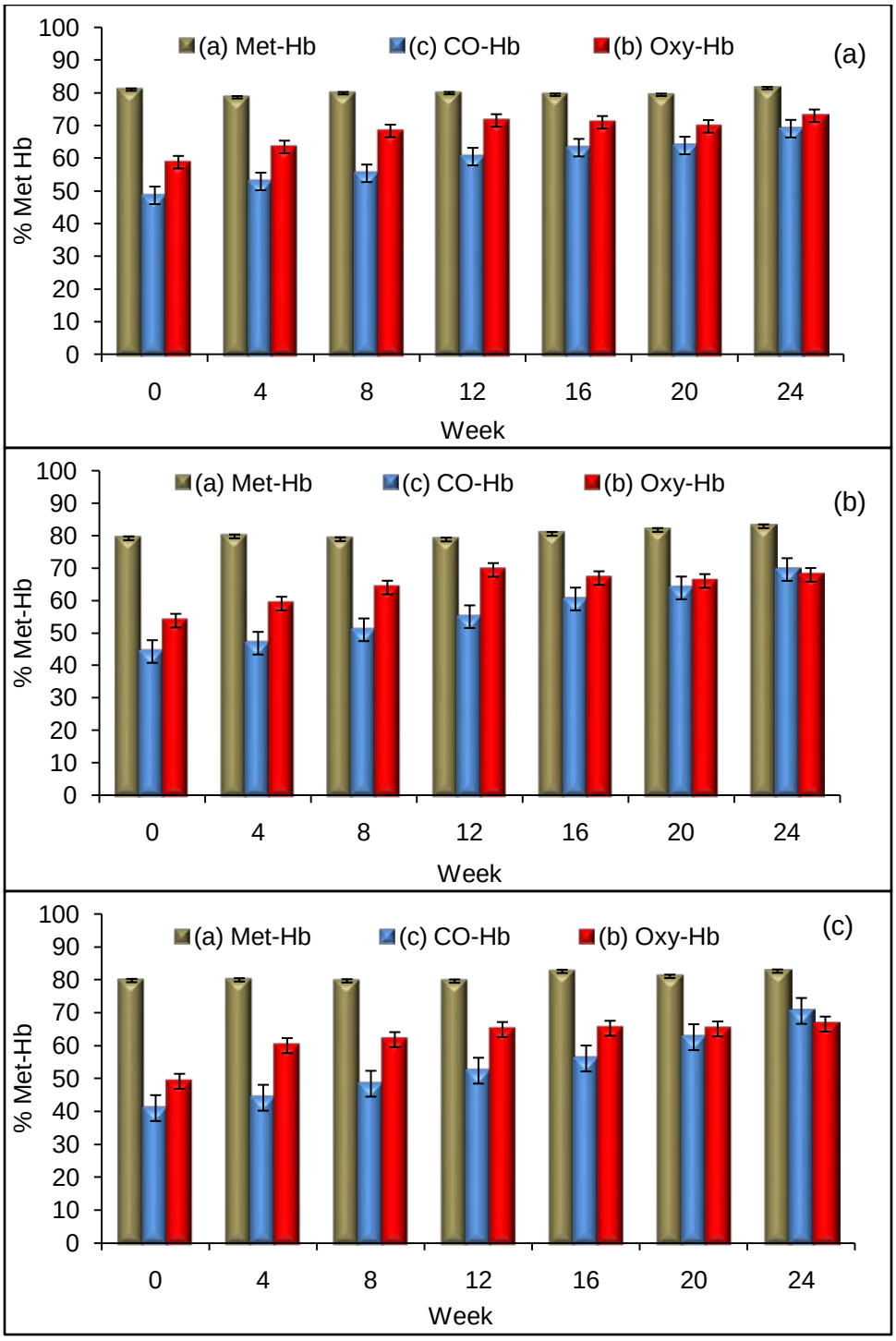


Figure 4-24. %Met-Hb in washed tilapia muscle containing different forms of Hb at a concentration of a) 6, b) 9, and c) 12 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

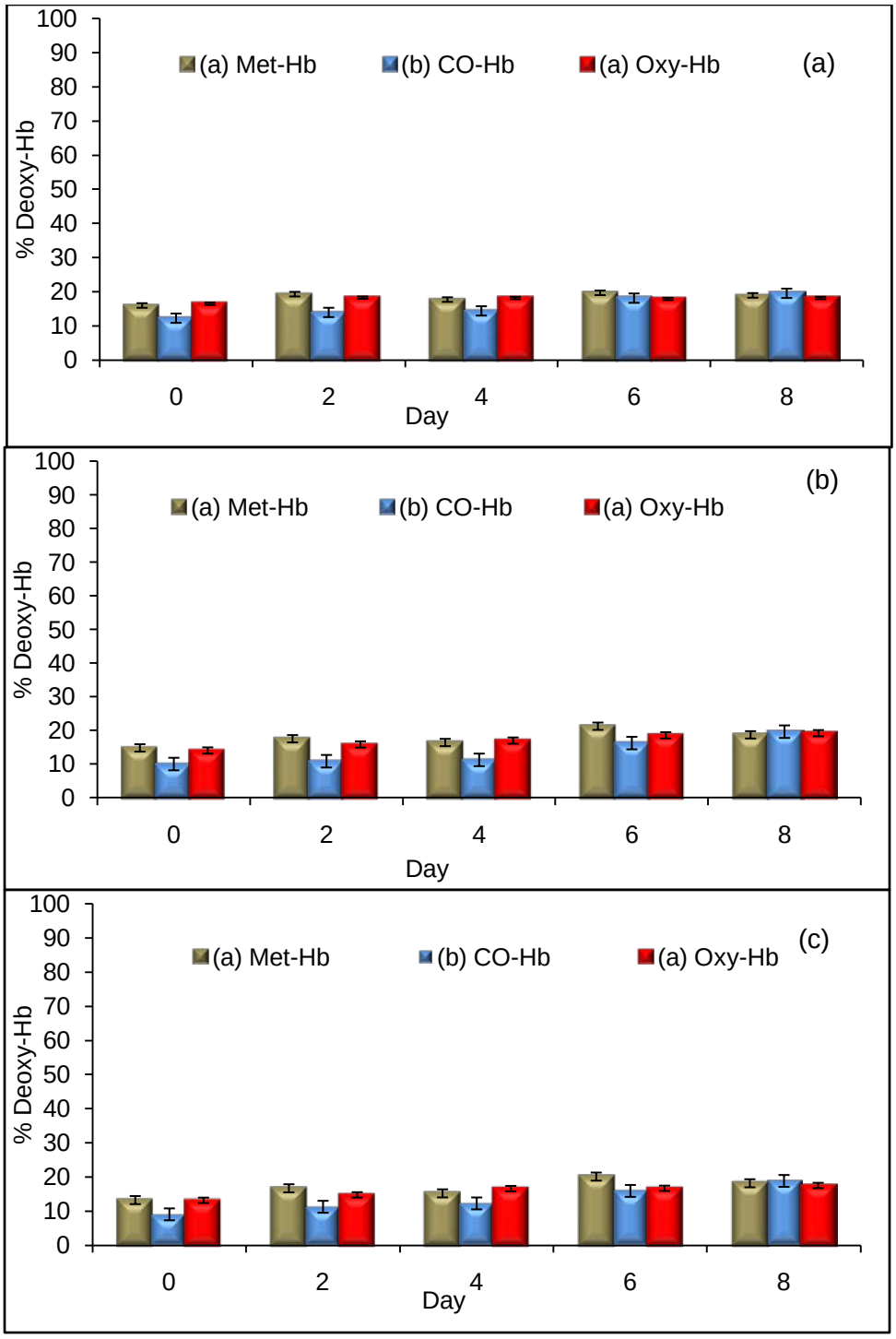


Figure 4-25. %Deoxy-Hb in washed tilapia muscle containing different forms of Hb at a concentration of a) 6, b) 9, and c) 12 µmol/kg at 3.7ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

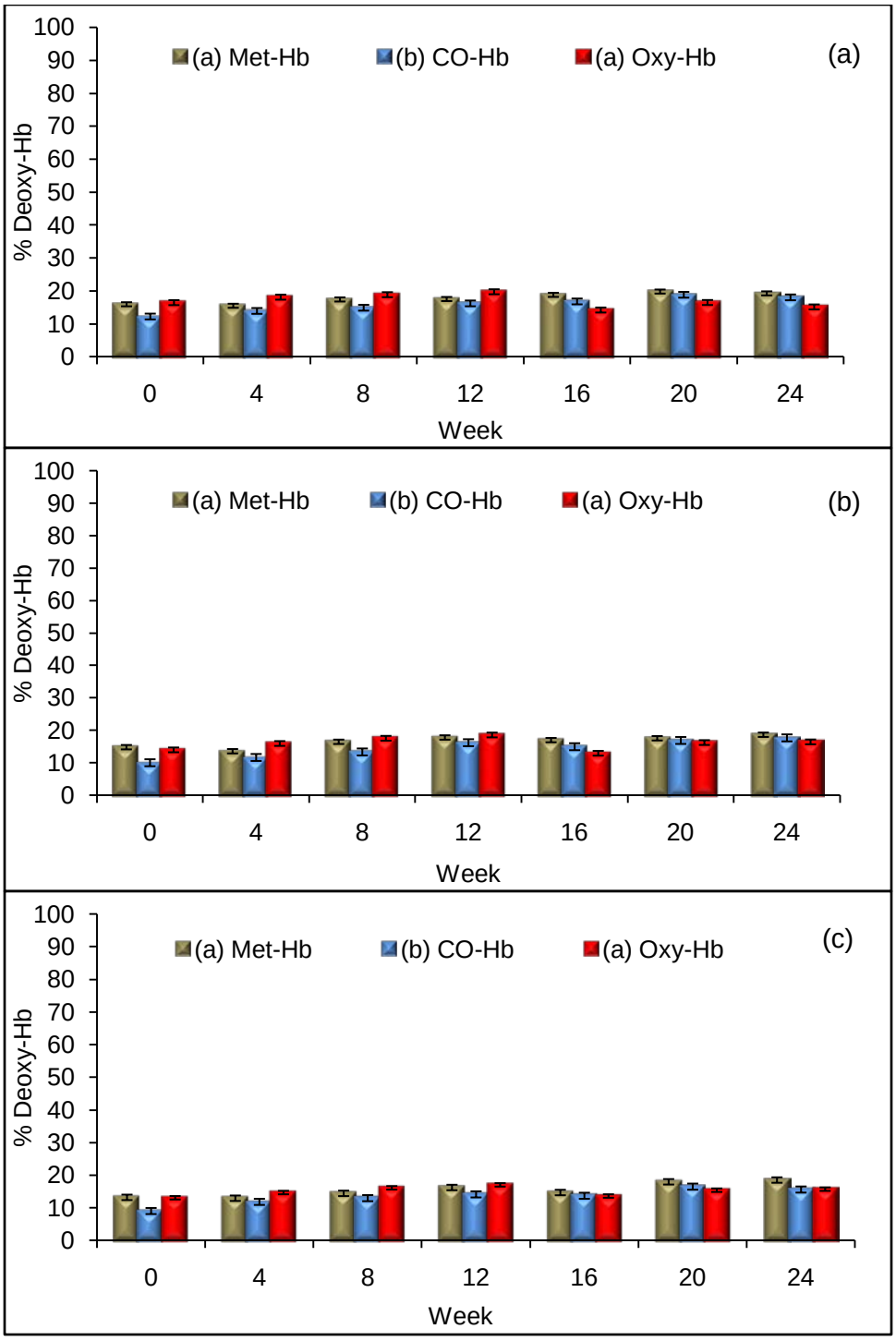


Figure 4-26. %Deoxy-Hb in washed tilapia muscle containing different forms of Hb at a concentration of a) 6, b) 9, and c) 12 µmol/kg at -25ºC, averaged across pH level and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

CHAPTER 5
EFFECT OF pH ON THE PRO-OXIDATIVE ACTIVITY OF OXY-, CO-, AND MET-HEMOGLOBIN IN A WASHED MINCED TILAPIA MUSCLE SYSTEM AT TWO DIFFERENT STORAGE TEMPERATURES

Introduction

Most post mortem fish muscle and muscle systems have pH values below 7.0. The role of pH on lipid oxidation under post-mortem conditions is important to understand. At lower pH values formation of Met-Hb occurs more rapidly and the level of deoxy-Hb increases sharply (*4*).

Researchers have demonstrated that trout and tilapia Hb added to washed cod muscle at 2°C are more pro-oxidative at pH 6.3 compared to 7.4. Washed cod muscle containing trout Hb showed more pro-oxidative activity than tilapia Hb at both pH levels (*80*). However, trout and tilapia Hb added to washed tilapia developed significant levels of TBARS at pH 6.3, while at pH 7.4 TBARS values were significantly depressed for both Hb samples.

Using washed cod muscle and cod Hb, Pazos (*81*) established that decreasing the pH from 7.8 to 6.3 greatly decreased the lag phase and increased the rate of lipid oxidation (*81*). The lag phase at pH 7.8 was ~40 h and the oxidation rate was slower compared to pH 6.8 and 3.5 which had faster rates of oxidation and lag times of 6 and 3 h, respectively. At pH values above 7.8, slower rates of oxidation were observed.

Lowering the pH below neutrality decreases the oxygenation of Hb. This is known as the Bohr Effect (*43*).The Bohr Effect can explain the high levels of deoxy-Hb at postmortem. When pH levels are decreased below 6.5, further deoxygenation occurs and this is known as the Root effect. Richards and Hultin (*4*) proposed that deoxy-Hb may play the role of a catalyst in lipid oxidation. Because its heme crevice is more

accessible, deoxy-Hb acts as a stronger oxidation catalyst than oxy-Hb (*44*). Undeland and others (*22*) found that higher rates of oxidation at pH 6.0 corresponded to a greater formation of deoxy-, and met-Hb when studying Hb from menhaden, mackerel, flounder, and pollock. At pH 6.0 all the Hbs were equally active pro-oxidants; however, at pH 7.2, the pro-oxidative activity of Hb was reduced except Hb from pollock. At both pH levels, pollock had greater formation of deoxy and met-Hb. The acceleration of lipid oxidation at pH 6 compared to 7.2 may be related to lower oxygenation (Bohr or Root Effect) or to increased oxidation of these Hbs (*11*).

Maintaining heme proteins in the reduced state has important implications for the quality of seafood products. Treating seafood with carbon monoxide at post-mortem pH levels (pH 6.5) maintains the heme protein in the reduced state because carbon monoxide (CO) binds with the heme protein more readily than O_2, replacing it from the heme. It is hypothesized that CO-Hb will be less pro-oxidative at lower pH levels than oxy- or met-Hb. As pH is decreased, hemin loss rates increase dramatically (*60*). Aranda and others (*60*) suggested that there are four mechanisms by which auto oxidation and hemin loss occur. These include steric displacement of bound ligands, weak anchoring of the heme propionate to the globin, larger channels for solvent entry into the heme pocket, and weakened interactions with the distal histidine.

Hargrove and others (*59*) developed an assay for hemin dissociation where His^{64} in sperm whale Mb was replaced by Tyr, producing a holoprotein with a discrete green color. Phe replaced Val^{68} in the same protein to increase its stability, while retaining high affinity for hemin. This protein can then be used for complete extraction of hemin from Hb and Mb, giving absorbance changes to allow reactions at low hemin

concentrations. When this protein (apoprotein Tyr^{64}•Val^{68}) is mixed in excess with mutant Hb, the solution turns from brown to green, and the absorbance changes can be used to measure the rate of dissociation of hemin. Mutant hybrids of human Hb were prepared by substituting Gly at His^{64} (E7) and oxidized with ferricyanide and then reacted quickly with apoprotein Tyr^{64}•Val^{68}. Hemin dissociation from the Gly^{64} mutants and subsequent uptake by apoprotein Tyr^{64}•Val^{68} resulted in rapid absorbance increases. The authors concluded that the results presented indicate that apoprotein Tyr^{64}•Val^{68} can be used reliably for measuring hemin loss.

Materials and Methods

The following methods have been described in detail in chapter 3: Preparation of Washed Minced Tilapia Muscle (MTWM), Collection of Fish Blood, Preparation of Hemolysate, Quantification of HB Levels in Hemolysate, oxy-, CO- and met-Hb Preparation, Sample Preparation: Addition of Hb and NaCl, Determination of Peroxide Value (PV), Determination of Thiobarbituric Acid Reactive Substances (TBARS), Determination of Carbonyl Groups, Heme Group Autoxidation, and Color Analysis. The Gas Chromatography (GC) Method was described in Chapter 4.

Determination of Hemin Loss

The dissociation of met-Hb was determined according to the method of Hargrove and others (*106*) modified by Richards and Grunwald (*58*). In this procedure, previously prepared and frozen at -80ºC holoprotien H64Y with distinct green color was used to prepare the apomyoglobin. The determination of hemin loss was conducted in the following manner.

Preparation of buffer

In a 1000 ml beaker, 150mM of Bis-tris was dissolved in distilled water containing 450mM sucrose. Once dissolved, the pH of the solution was adjusted to the desired pH value of 6.3, 6.8, or 7.3. The solution was then transferred to a graduate cylinder, diluted to a final volume of 500 ml with distilled water, filtered by vacuum filtration using 0.45 µm, Millipore filter paper (Fisher Scientific), and then transferred to a bottle and stored at 2-4 °C overnight.

Preparation of FPLC (fat protein liquid chromatography)

Before using the desalting column of the FPLC, it was cleaned of any potential bacteria growth and conditioned by pumping initially 20% ethanol followed by degassed distilled water, and then the first buffer of pH 7.3 at a rate of 5 ml/min.

Sample preparation

Frozen Holoprotein –H64Y (Mb of sperm whale) was thawed under running water. In an ice cold culture tube (tube 1), 1 ml of the thawed Holoprotein was mixed with 1 ml maleic acid (0.2M) and stirred gently for 1 min till the color changed from brown-green to brown-red. The sample was incubated in an ice bath for 10 min before 2 ml of ice cold extraction solvent (methyl ethyl ketone) was added, followed by vortexing for 10 seconds, and then incubated in ice for another 10 min.

The lower layer in tube-1, a gold-green phase, was transferred to another tube (tube 2) where 2 ml of ice cold extraction solvent was added, vortexed for 20 sec, and then incubated for 10 min in an ice bath.

The lower layer in tube-2, a golden phase, was transferred to another tube (tube 3). Using a 5 ml syringe, the golden phase in tube 3 was injected into the desalting column and run for 25 min in order to collect the protein fraction (Apoprotein) in a buffer

at pH 7.3. After the 25 min run, sample collection began with the first appearance of the chromatogram peak, and stopped before the endpoint of the peak in order to avoid any residual ketone fraction. This same procedure was followed for pH 6.8 and 6.3.

Verification and analysis of extracted samples

UV-vis absorbance spectroscopy in the range of 200-700 nm was used to ascertain the success of the preparation and to determine the concentration of the Apo sample prepared.

The rate of hemin loss

The dissociation rate of hemin was determined spectrophotometrically. In a quartz cuvtte, 1 mL mixture of 10 µM met-Hb (heme base) was added to 40 µM ApoMb with 150 mM buffer of the desired pH and 450 mM sucrose. The absorbance was recorded at 600-700 nm against a blank. The blank contained 1 mL 150 mM buffer with 450 mM sucrose and 12.5 µl 10 mM Tris buffer at pH 8.0. Concentrations of met-Hb and ApoMb were calculated using a previously prepared standard curve in Dr. Mark Richards's lab in the Department of Animal Science at the University of Wisconsin, Madison (*58*). The cuvettes were transferred to a 6 cell holder and the absorbance was recorded for 24 hours. This procedure was performed at two different temperatures (4 and 25ºC).

Calculation of Dissociation Rate

Heme that was released from the samples was gathered by the globin since globin with tyrosine replacement has a strong affinity for heme. Heme binds to the mutant globin upon release, resulting in a green color formation that has a strong absorbance at 600 nm. Rate of hemin loss was calculated using the Igor Pro software (WaveMetrics Inc., Portland, OR) and the exponential function (*58*). This procedure was conducted in collaboration with Dr. Richards's lab.

Results

Lipid Oxidation Analysis

Lipid hydroperoxide values obtained at 3.7°C and pH 6.3 (Figure 5-1) showed that oxy-Hb oxidized quickly and more ($p<0.05$) than met- and CO-Hb, reaching a peak at day 4 and then decreasing until day 8. CO-Hb and met-Hb did not differ significantly in hydroperoxide values until day 8, where CO-Hb was significantly less pro-oxidative than met- and oxy-Hb. At pH 6.3 and -25°C (Figure 5-2), oxy- and met-Hb were significantly more pro-oxidative than CO-Hb but were not significantly different ($p\leq0.05$) from each other. On week 4 met-Hb was significantly ($p\leq0.05$) less pro-oxidative than CO-Hb, but by week 20 and 24 CO-Hb was significantly less pro-oxidative than met- and oxy-Hb.

At 3.7°C and pH 6.8 (Figure 5-3), lipid hydroperoxides differed significantly ($p\leq0.05$) for all three forms of Hb, with met-Hb being significantly more pro-oxidative. Less lipid hydroperoxides formed at pH 6.8 compared to pH 6.3. CO-Hb was significantly less pro-oxidative than met-Hb on day 4 and day 6, but by day 8, there were no significant differences between them. At pH 6.8 and -25°C (Figure 5-4), met-Hb was significantly more pro-oxidative than CO-and oxy-Hb. On week 24, CO-Hb was significantly ($p\leq0.05$) less pro-oxidative than met- and oxy-Hb.

Lipid hydroperoxide values at 3.7°C and pH 7.3 (Figure 5-5) were very low and did not show significant differences ($p\leq0.05$) between the three forms until day 6 when met-Hb had higher values ($p<0.05$) than oxy- and CO-Hb. Throughout the 8 days of storage CO- and met-Hb did not differ in lipid hydroperoxide values. At pH 7.3 and -25°C (Figure 5-6), there were no significant differences for the three forms of Hb and very low levels of lipid hydroperoxides formed.

TBARS development at 3.7°C (Figure 5-7) and pH 6.3 showed CO-Hb was the least pro-oxidative of all forms tested. Oxy-Hb oxidized significantly ($p \leq 0.05$) faster than CO-Hb. Met-Hb did not differ significantly from oxy-Hb. By day 6, met-Hb was significantly more pro-oxidative than oxy-Hb, as samples with oxy-Hb were on a decline. By day 8 there were no significant differences ($p \leq 0.05$) for the three forms. At -25°C and pH 6.3, oxy-Hb (Figure 5-8) was also significantly more pro-oxidative than met- and CO-Hb. Although CO-Hb appeared to be the least pro-oxidative it did not differ significantly from met-Hb until week 16 and on.

TBARS development was reduced at pH 6.8 at 3.7°C compared to pH 6.3. Significant oxidation of samples did not occur until day 4 for all forms. Contrary to results at pH 6.3, where oxy-Hb was most pro-oxidative, met-Hb was overall significantly ($p \leq 0.05$) more pro-oxidative at pH 6.8 than oxy- and CO-Hb (Figure 5-9), reaching maximum oxidation on day 6. CO- and oxy-Hb were, however, not significantly different from each other, except for day 8.Results for samples stored at -25°C and pH 6.8 (Figure 5-10) showed less TBARS formation compared to pH 6.3.Not much difference was found between the samples. However, at the end of the study, oxy-Hb had the highest oxidation. At week 16, 20, and 24 significant differences ($p \leq 0.05$) were seen between the three forms, with CO-Hb being least pro-oxidative.

Samples at pH 7.3 and 3.7°C (Figure 5-11) developed even less TBARS than at pH 6.8. Like at pH 6.8, met-Hb was most pro-oxidative, however significant ($p \leq 0.05$) oxidation didn't occur until day 6 and on. Oxy- and CO-Hb caused very little oxidation during the 8 day storage period and did not differ significantly from each other. At -25°C and pH 7.3 (Figure 5-12) even lower levels of TBARS were seen than at ph 6.8,

although oxy-Hb developed similar levels of TBARS at weeks 20 and 24, significantly more than met- and CO-Hb. Throughout the 24 weeks of storage, CO- and met-Hb led to very little TBARS formation and did not differ significantly from each other.

Protein Oxidation Analysis

Carbonyl values obtained at 3.7°C and pH 6.3 (Figure 5-13) revealed that CO-Hb was significantly less pro-oxidative, with respect to protein oxidation, than met- and oxy-Hb. CO-Hb did however have double the carbonyl values ($p<0.05$) on day 0 compared to the other forms. Protein oxidation was reduced in CO-Hb containing samples and at days 4-8 it had significantly lower carbonyl values than oxy-Hb. On day 6 and 8, CO- and met-Hb did not differ significantly ($p\leq0.05$). Oxy-Hb developed the highest level of carbonyl groups at day 4. Oxy- and met-Hb differed significantly on day 2 and 4 only. At -25°C and pH 6.3 (Figure 5-14), the three forms of Hb differed significantly. CO-Hb had the lowest carbonyl values overall throughout frozen storage, while oxy-Hb led to the highest carbonyl value at week 16. All three forms of Hb differed significantly ($p\leq0.05$) at each week of storage, except for week 24, which showed no significant difference.

At pH 6.8 and 37°C (Figure 5-15), the three forms of Hb differed significantly ($p\leq0.05$) in carbonyl values, with oxy-Hb again being the most pro-oxidative, and CO-Hb having the lowest carbonyl value (until day 6, where Met-Hb has equal values). CO- and met-Hb did not differ significantly ($p\leq0.05$) on days 4-8. Samples containing CO- and met-Hb had lower carbonyl values at pH 6.8 compared to pH 6.3, while values were similar for oxy-Hb. A different protein oxidation trend was seen at -25°C and pH 6.8 (Figure 5-16) compared to pH 6.3 for all samples. Oxy-Hb was significantly more pro-oxidative than the other forms until week 12, where carbonyls peaked and then decreased rapidly until week 24 where values were similar to values at week 0. CO- and

met-Hb did not differ significantly on week, 4, 8, 12, and 20, but differed significantly ($p \leq 0.05$) on week 0, 16, and 24, with CO-Hb being less pro-oxidative.

Samples at pH 7.3 and 37°C (Figure 5-17) developed fewer carbonyl groups than at ph 6.8 and 6.3. However, like those seen at the lower pH values, oxy-Hb was the most pro-oxidative of all forms, while met-Hb led to the lowest carbonyl values. CO- and met-Hb differed significantly in carbonyl values on day 4 only. A similar trend in the development of carbonyls was seen at -25°C and pH 7.3 (Figure 5-18) as was seen at pH 6.8, where oxy-Hb samples peaked at week 12, met-Hb at week 20 and CO-Hb was still increasing at week 24. Overall, there was little significance difference ($p \leq 0.05$) between the three Hb forms. However, at week 0 and 4, oxy-Hb had significantly higher carbonyl levels than CO- and met-Hb. At week 8, met-Hb had significantly ($p \leq 0.05$) higher carbonyl values than oxy-Hb but on week 24 CO-Hb had significantly higher values than both met- and oxy-Hb.

Hemin Loss Rate

Analysis of the rate of hemin loss (Table 5-1) showed hemin loss occurred at a faster rate at 25°C compared to 4°C. The rate of hemin loss was also affected by pH; the higher the pH, the slower the rate of hemin loss. Tilapia Hb, which was used in the current study, was compared to trout Hb and it was found that at pH 6.3 and both temperatures (25°C and 4°C), the rate of hemin loss was faster for trout Hb, suggesting a stronger heme-protein interaction of the tilapia Hb.

CO Release

Amount of CO loss from CO-Hb containing samples varied by storage day and pH (Figure 5-19). Samples at pH 6.3 and 6.8 released significantly greater amount of CO on day 0 than any other day, and released significantly more than samples at pH 7.3.

Samples at pH 7.3 overall had CO more strongly bound to Hb, while at day 8 they showed a significant amount released. As storage time increased, the amount of CO released per day decreased, until day 6 and 8 when increasing amounts of CO were released. Samples stored at -25°C for 24 weeks (Figure 5-20) overall showed significant amounts of CO released on each week of storage. Samples at pH 6.3 and 6.8 lost more CO than samples at ph 7.3 and showed a sharp increase in CO loss at week 16. All samples lost significantly more CO at weeks 16-24 compared to weeks 0-12.

Color Analysis

Significant differences in a*value were seen in samples containing CO-, oxy- and met-Hb at 3.7°C and pH 6.3 (Table 5-2). Samples containing CO-Hb had significantly ($p \leq 0.05$) higher a*values than oxy- and met-Hb on days 0, 2, and 4. As expected, on day 0, oxy-and CO-Hb had significantly ($p \leq 0.05$) higher a*values than met-Hb, while on on day 2 and 4 Met-Hb had significantly ($p \leq 0.05$) higher a* values. At -25°C and pH 6.3 (Table 5-3) CO-Hb had significantly higher a*values than both oxy- and met-Hb during the entire 24 weeks. Only at week 0 and 24 did oxy-Hb have significantly different ($p \leq 0.05$) a*values than met-Hb, higher at week 0 and lower at week 24.

At pH 6.8 and 3.7°C (Table 5-2) CO-Hb and oxy-Hb did not have significantly different ($p \leq 0.05$) a*values on days 0, 2, and 4 but both were significantly different from met-Hb. Both CO- and oxy-Hb had higher a*values at pH 6.8 compared to pH 6.3. Met- and CO-Hb did not differ in a*values on day 6 and 8, but oxy- and CO-Hb differed significantly ($p \leq 0.05$) with oxy-Hb having higher a* values. At -25°C and pH 6.8 (Table 5-3), the three forms of Hb differed significantly ($p \leq 0.05$). Throughout the 24 weeks CO-Hb had the highest a*values of all forms, except for week 0 where it did not differ

significantly from oxy-Hb. Oxy-Hb had significantly ($p \leq 0.05$) higher a*value than met-Hb on weeks 0, 4, 8, 20, and 24.

At pH 7.3 and 3.7°C (Table 5-2), samples with CO- and oxy-Hb differed significantly ($p \leq 0.05$) from met-Hb but did not differ significantly from each other. Throughout the 8 day storage period, CO-Hb differed significantly from met-Hb but only differed significantly ($p \leq 0.05$) with oxy-Hb on day 8 where it's a*values were lower. At -25°C and pH 7.3 (Table 5-3), both oxy- and CO-Hb had higher a* values than at the other pH values tested, and also demonstrated more color stability. On week 0, 16, 20, and 24, CO- and oxy-Hb did not significantly ($p \leq 0.05$) differ in a* values, but were significantly different at weeks 4, 8, and 12, where CO-Hb samples had higher a*values. Both oxy- and CO-Hb differed significantly from met-Hb throughout the 24 week storage period. The results for the change in the L*value (lightness) and b* value (yellowness) in washed tilapia muscle containing different forms of Hb at pH 6.3, 6.8, and 7.3 during 3.7ºC and -25ºC storage are presented in Appendix B.

Heme Group Autoxidation

Results at 3.7°C showed that as pH levels increased, more oxy-Hb was present in all samples (Figure 5-21). There were also significant ($p \leq 0.05$) differences for the three forms of Hb. At all pH levels tested, CO-Hb had a greater amount of oxy-Hb compared to the other forms. All pH forms tested showed a decline in % oxy-Hb during storage, but had the most stability to autoxidation at pH 7.3. Samples stored at -25°C (Figure 5-22) demonstrated a very similar trend in % oxy-Hb as the samples at 3.7°C. Level of oxy-Hb decreased over time for all samples and was more stable as pH increased. Under all three pH conditions, CO-Hb had higher % oxy-Hb. Oxy-Hb had significantly ($p \leq 0.05$) less % oxy-Hb than CO-Hb but significantly more than met-Hb.

As expected, the level of met-Hb at 3.7°C was highest for samples containing met-Hb (Figure 5-23). Met-Hb samples showed a slight increase in % met-Hb over the storage period at pH 6.3 and 6.8, while at pH 7.3 they remained stable. Both CO- and oxy-Hb showed a significant ($p \leq 0.05$) increase in % met-Hb over the 8 day storage period, regardless of pH, however levels of met-Hb were lower as pH was higher. Very similar trends were seen in samples stored at -25°C (Figure 5-24) with % met-Hb at all three pH levels being highest for met-Hb samples, and CO-Hb having the least % met-Hb. % met-Hb significantly ($p \leq 0.05$) increased for CO- and oxy-Hb during the 24 weeks of frozen storage, while met-Hb samples remained stable.

Since samples were kept aerobically relatively low levels of deoxy-Hb were seen for all samples at either storage temperatures. At 3.7°C and pH 6.3, there were no significant ($p \leq 0.05$) differences between oxy-, CO-, and met-Hb in % deoxy-Hb (Figure 5-25). At pH 6.8 and 7.3, met-Hb had significantly more % deoxy-Hb than oxy- and CO-Hb, while CO-Hb samples had the lowest levels. At both pH 6.3 and pH 6.8, significant increases in % deoxy were seen for CO- and oxy-Hb over time. At pH 7.3, met-Hb samples showed significant ($p \leq 0.05$) increases in % deoxy-Hb throughout the storage period. At -25°C and pH 6.3 there were no significant differences found between met-Hb and CO-Hb in % deoxyHb (Figure 5-26). Oxy-Hb had significantly ($p \leq 0.05$) greater % deoxy-Hb than met- and CO-Hb. At pH 6.8 and 7.3, CO- and oxy-Hb did not differ significantly in % deoxy-Hb. Met-Hb had greater % deoxy-Hb than both CO- and oxy-Hb at pH 6.8 and 7.3.

Discussion

There is a sufficient body of evidence accumulated to date suggesting that lowering pH below neutrality may enhance pro-oxidative activities of Hbs present in

muscle foods including fish (*10, 22, 107*). This phenomenon is attributed to increased rate of deoxygenation of Hbs in acidic environment (e.g., the post-mortem system) that promotes Hb autoxidation and subsequently, lipid oxidation, leading to muscle food deterioration and loss of quality. The change in oxygen affinity of Hb with changes in pH was first discovered and described at the beginning of 20th century by Bohr, and in later years was established as a concept named “Bohr Effect”. Extensive research has been done since to determine possible mechanism(s) responsible for this effect. It is believed that alterations in Hb structure at different pH levels may, at least partially, be accountable for the Bohr Effect. It was generally proposed that with lowering of pH, Hb unfolds that accelerates release of hemin. Rapid release of hemin in turn can stimulate lipid peroxidation promoting food spoilage (*58*).

In the current research study we investigated effects of various physiological pH levels on pro-oxidative activities of different types of Hb (oxy-, CO- and met-Hb) under two storage conditions (3.7ºC and -25ºC). Three pH levels (6.3, 6.8, and 7.3) were chosen to address common levels of pH seen in post-mortem fish muscle systems and fish products.

At pH 6.3, rapid oxidation of all types of Hb was observed. As % oxy-Hb was depleated at day 8 (Figure 5-21a), formation of met-Hb (Figure 5-23a) and % deoxygenation (Figure 5-25a) increased significantly reaching virtually full oxidation by the end of experiment (day 8); this is in accordance with the Bohr Effect. This is further exemplified by the negative correlations between % oxy-Hb and % deoxy-Hb (r=-0.85, Appendix C, Table C-1). Comparable results were obtained by previous investigators for different Hbs (*22*). As expected, the rate of autoxidation of CO-Hb was notably slower

than that of other forms of Hb, suggesting that the CO molecule stabilized the heme group by replacing oxygenated Hb with CO-Hb (*69, 70*). During autoxidation, oxygen is released from oxy-Hb to form the superoxide anion radical, $O_2^{\bullet -}$ and ferric met-Hb which is converted to hydrogen peroxide which increases the ability of heme proteins to promote lipid oxidation. Both deoxy-Hb(HbFe(II)) and oxy-Hb ($HbO_2Fe(II)$) exist in the reduced state and thus are susceptible to autoxidation. These two reduced forms can oxidize to the met-Hb(Hb-Fe(III)). Met-Hb, in the presence of H_2O_2, can also be converted to the very pro-oxidative forms, perferryl-Hb ($^{\bullet}$Hb-Fe(IV)=O) and ferryl-Hb (Hb-Fe(IV)=O) (*5*). The autoxidation and the resultant brown coloring can be prevented by treatment of the fish muscle with CO which has a greater affinity for the Fe(II) binding site, forming CO-Hb. When CO is bound to heme in Hb, it gives stability to the protein, and the protein will resist autoxidation on heating, freezing, and thawing (*69, 70*).

Autoxidation was also highly influenced by pH, with higher pH resulting in less autoxidation. These results are consistent with the findings of Richards and Hultin (*4*) who investigated the effect of pH (7.6, 7.2, and 6.0) on lipid oxidation using trout Hb as a catalyst in a washed minced cod muscle system. The authors found that at lower pH values formation of met-Hb occurred more rapidly and the level of deoxy-Hb increased sharply. Lowering the pH below neutrality decreases the oxygenation of Hb (*43*).The Bohr effect can explain the high levels of deoxy-Hb at postmortem due to the low pH levels (*43*). When pH levels are decreased below 6.5, further deoxygenation occurs (Root effect). This further suggests that deoxy-Hb may play the role of a catalyst in lipid oxidation (*43*). Undeland and others (*22*) studied the effect of pH in a washed, minced

cod model system and found that the higher rate of oxidation at pH 6.0 corresponded to a greater formation of deoxy-, and met-Hb.

Both lipid hydroperoxide and TBARS values followed a similar trend as a function of pH, where more rapid and in general higher levels of oxidation were seen as pH was lower (Figures 5-1 – 5-12). Higher levels of % met-Hb and lower levels of %oxy-Hb were seen as pH was lower (Figures 5-21 – 5-26), suggesting a connection between autoxidation and lipid oxidation. Hydrogen peroxide formed during autoxidation of reduced Hbs, increases the ability of heme proteins to promote lipid oxidation (*5*). Richards and Dittman (*1*) found a high autoxidation rate in perch Hb despite the low deoxy-Hb content. This could be explained by the amino acid sequences near the heme crevice. It has been found that disrupting the hydrogen-bonding network of His^{97} created easier accessibility of H_2O into the heme crevice (*5, 45*). This would then accelerate the formation of met-Hb, which would also increase lipid oxidation.

It was of interest to see that at 3.7°C and pH 6.3, oxy-Hb was the most pro-oxidative, while met-Hb was the most pro-oxidative at pH 6.8 and 7.3. Richards and Hultin (*4*) reported that in the presence of lipid peroxide, reduced Hbs (oxy/deoxy-Hbs) produced high levels of lipid peroxide formation, but met-Hb caused little peroxidation. Met-Hb cannot autoxidize but the reduced Hbs form superoxide anion radicals during autoxidation, which can form hydrogen peroxide. The hydrogen peroxide will then activate met-Hb (*39*). If met-Hb is the initial reactant, there is no source of oxygen to form hydrogen peroxide. In addition, reduced Hbs but not met-Hb can produce hydroxyl radicals (*40*). Met-Hb forms hemichromes which are not considered catalysts of lipid peroxidation (*41*).

CO-Hb exhibited a similar lipid oxidation pattern as met-Hb at ph 6.8, but showed significantly slower rate for TBARS formation (Figure 5-7 and 5-1). Of note, by day 8 TBARS scores and carbonyl values (Figure 5-13) were similar for all forms of Hb, while lipid hydroperoxides formation (Figure 5-1) was highest for oxy-, and lowest for CO-Hb. The production of carbonyls actually diminished in CO-Hb samples by day 8 of the experiment (Figure 5-13). That confirms the hypothesis that treatment with CO may, to some extent, stabilize Hb structure, thus retarding deterioration of muscle food even under unfavorable acidic conditions. From the earlier reports it is evident that Hb has an ability to stimulate lipid oxidation at lower (more acidic pH), and this ability is in correlation with increasing in deoxy-Hb formation (*55*). These results are in agreement with present findings. Frozen storage (-25ºC) in general led to lower TBARS (Figure 5-8) and lipid hydroperoxide formation (Figure 5-2) in all forms of Hb. However under the same conditions enhanced carbonyl formation was observed for oxy- and met-Hb (Figure 5-14). The latter finding may be attributed to muscle protein denaturation due to a low temperature.

The lag time prior to development of TBARS (Figure 5-9) and lipid hydroperoxide formation (Figure 5-3) for all Hb forms at pH 6.8 and 3.7°C increased compared to pH 6.3. However, CO-Hb was found to have the most reduced lag time for lipid hydroperoxidation of all forms (Figure 5-3). These results are in agreement with autoxidation (Figure 5-21b) and deoxygenation (Fig 5-25b) observed for the Hbs. The correlations between lipid hydroperoxides and TBARS formation with % oxy-, % deoxy-, and % met-Hb, also reflect that as % deoxy- and % met-Hb increased, lipid oxidation increased (Appendix C, Table C-1). Although the increase in pH from 6.3 to 6.8 led to

lower levels of carbonyls for met- and CO-Hb, it did not for oxy-Hb (Figure 5-15). It is speculated that oxy-Hb (reduced form of Hb) is a main promoter of lipid oxidation. Richards and Hultin (*4*) reported that reduced Hbs (oxy/deoxy-Hbs) produced high levels of lipid peroxide formation, whereas met-Hb caused little peroxidation. These findings might indicate that oxy-Hb could also play an important role as a catalyst in promoting protein oxidation. Eymard and others (*35*) investigated the link between lipid oxidation and protein oxidation during processing and storage of horse mackerel using fish minces with differences in lipid and protein fractions and different oxidative levels. The authors concluded that lipid and protein oxidation developed simultaneously but it was difficult to determine how they are linked. Lipid and protein oxidation share the same catalysts, and they can develop independently of one another, or in parallel, or they can interact with each other (*35*).

Lower levels of oxidation at pH 6.8 compared to pH 6.3 were also seen during frozen storage (Figures 5-4 and 5-8) regardless of Hb form, and also correlated (Appendic C, Table C-2) with a drop in % oxy-Hb and increase in % met-Hb (Figure 5-22b and 5-24b). Formation of carbonyl groups of all samples (Figure 5-16) was in fact enhanced at -25ºC (Figure 5-16). There was not a direct relationship seen with lipid oxidation levels and carbonyl levels. This implies that some other mechanisms might be present in fish muscle system under freezing conditions, which leads to more protein oxidation, for example increased protein denaturation and cross-linking due to the activity of the trimethylamine-N-oxide (TMAO) or other enzymes such as lipases and phospholipases. These enzymes can cause denaturation of fish muscle during frozen storage (*98*). Free fatty acides released by Lipases and phospholipases activity can

react with proteins. The reaction of oxidized lipids with proteins may result in toughening of fish muscle during frozen storage (*98*).

A very significant retardation of lipid oxidation (Figure 5-5, 5-6, 5-11 and 5-12) was seen for all Hb forms at pH 7.3, regardless of storage temperature. These findings were correlated (Appendix C, Table C-1 and C-2) with a decreased rate of autoxidation at pH 7.3 (Figure 5-21, 5-22). Carbonyl levels still increased at pH 7.3, unlike lipid oxidation (Figure 5-17). The fact that carbonyl formation was not affected by pH, unlike the Hb catalysts, implies that there may be some other mechanism(s) involved in protein oxidation in the WMTM system. It may be speculated that manual manipulations with tilapia muscles (such as mincing) induces certain protein denaturation that predispose to enhanced protein oxidation independently of pH levels. Eymard and others (*35*) investigated the effect of mincing and washing horse mackerel muscle on protein oxidation and found that at time zero, myosin and other high molecular weight proteins were already oxidized in all products including the unwashed mince. It may be further proposed that higher rate of oxy-Hb autoxidation may to a degree promote protein oxidation. Detailed mechanism(s) of protein oxidation should be elucidated in future studies.

The effects of the three pH levels on the rate of hemin release in tilapia met-Hb samples were tested at both 4°C and 25°C (Table 5-1). This was of interest since heme release from Hb is believed to play an important role in the pro-oxidative activity of Hb. The rate of hemin loss in trout Hb under both temperatures at pH 6.3 was also measured to provide a relevant comparison. The results show that lower pH may accelerate hemin loss, which is in agreement with the hypothesis that acidic

environment may affect the structure of Hb promoting its rapid unfolding that in turn triggers rapid hemin loss and increased lipid oxidation (*38, 58*). As the pH decreases, auto oxidation and hemin loss increase. Aranda and others (*60*) suggested that the reason for the pH dependence may involve the protonation of distal His resulting in disruption of the bound O_2. This disruption might cause a rotation of the side chain. Another reason is the dissociation of $^{\bullet}OOH$ by the bound O_2, which result in the formation of met-Hb. It could also be due to the interruption of the electrostatic forces with amino acids at E10 and CD3 positions (*60*). A faster hemin loss rate was noted at 25°C compared to 4°C. Moreover, at pH 6.3 trout Hb released hemin more rapidly than tilapia Hb, suggesting a stronger heme-globin interation for tilapia Hb (Table 5-1). It may indicate that different species may respond in different ways to changes in acidity/alkalinity. Precise mechanism(s) of the observed events should be determined in future more detailed investigations.

Deterioration of desirable red color of fish samples (Figure 5-27) represented as a*value into undesirable brown color was followed throughout the storage period. The results show that the red color was highly influenced by pH, being more unstable as pH levels decreased. Treatment of fish muscle with CO improved the stability of red color in all CO-Hb samples at the three pH levels tested (Table 5-2 and 5-3) compared to oxy-, and met-Hb. This suggests that the autoxidation and the resultant brown coloring can be prevented by treatment of the fish muscle with CO forming CO-Hb, resulting in a cherry red color that is stable over longer periods(*69, 70*). The stability of the red color may also be a result of replacing oxygenated Hb CO-Hb that may offer some protection against lipid oxidation (*69, 70*). Moreover, under freezer conditions a*value in CO-Hb

samples was found to increase significantly ($p \leq 0.05$) after one week of storage (Table 5-3). The pH 7.3, however, delayed red color deterioration in both oxy- and CO-Hbs at both tested temperatures (Table 5-2, 5-3) compared to met-Hb. It has been reported that under alkaline pH conditions, deoxygenation of Hbs is maintained in the reduced state, thus slowing down the autoxidation and deterioration of fish muscle system. This might, in turn, delay formaton of undesirable color (*4, 81*).

Conclusion

Low pH increased the susceptibility of the washed minced tilapia muscle to oxidation and promoted loss of hemin. Fish muscle treated with CO may have better stability to oxidation conditions, thus resulting in red color stability. Treatement with CO delayed the onset of lipid and protein oxidation during cold and freezer storage temperatures.

Table 5-1. Hemin loss rate for met-Hb (tilapia and trout)

Hb	pH	T° (C)	$K_{dissociation}/h^{-1}$
Tilapia	6.3	25	2.87±0.32
	6.8	25	0.54±0.07
	7.3	25	0.17±0.01
	6.3	4	0.59±0.01
	6.8	4	.02±0.001
	7.3	4	0.0036±0.0006
Trout	6.3	25	5.55±0.31
	6.3	4	0.76±0.07

Values are means ± Standard Deviations for all samples (n=3).

Table 5-2. Changes in a* value in washed tilapia muscle containing different forms of Hb at pH 6.3, 6.8, and 7.3 at 3.7ºC, averaged across Hb concentration and NaCl added.

a* value	pH 6.3		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	16.3±3.4	19.6±3.8	9.1±1.5
2	6.9±3.4	16.2±2.9	11.0±1.5
4	3.0±0.9	8.7±4.6	6.9±2.3
6	1.6±0.7	4.6±2.0	3.8±1.2
8	1.4±0.9	4.0±1.6	2.4±0.9

a* value	pH 6.8		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	21.3±4.3	22.9±4.0	8.4±1.4
2	21.0±4.3	21.8±4.0	11.2±1.8
4	20.1±3.9	19.0±3.9	9.2±1.7
6	16.2±8.0	8.1±3.2	6.5±2.3
8	13.5±8.6	8.3±3.5	5.8±2.3

a* value	pH 7.3		
Storage time (day)	Oxy-Hb[a]	CO-Hb[a]	Met-Hb[b]
0	22.2±4.0	23.2±3.7	6.7±1.4
2	21.9±4.1	24.3±4.2	8.5±2.0
4	22.3±3.9	23.9±4.1	8.1±2.4
6	22.6±4.1	21.3±5.8	8.1±2.4
8	21.3±3.5	17.1±7.5	6.2±2.9

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table 5-3. Changes in a* value in washed tilapia muscle containing different forms of Hb at pH 6.3, 6.8, and 7.3 at -25ºC, averaged across Hb concentration and NaCl added.

a* value	pH 6.3		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[b]
0	16.3±3.4	19.6±3.8	9.1±1.5
4	11.6±3.5	21.6±4.6	13.0±2.1
8	10.5±3.7	19.5±5.5	10.4±2.0
12	9.0±3.7	17.2±4.5	9.6±2.1
16	8.7±3.2	15.8±5.3	9.2±2.5
20	6.0±2.6	14.5±4.4	8.4±2.1
24	2.9±2.9	12.6±4.9	7.6±1.9

a* value	pH 6.8		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	21.3±4.3	22.9±4.0	8.4±1.4
4	19.2±4.5	25.8±4.3	12.7±2.8
8	16.0±5.2	23.2±5.9	10.6±2.4
12	10.5±5.7	21.5±5.5	10.9±2.8
16	12.4±5.4	22.1±6.0	10.6±2.8
20	14.7±5.0	19.7±5.7	9.8±2.9
24	13.8±4.5	19.3±6.4	9.4±3.3

a* value	pH 7.3		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	22.2±4.0	23.2±3.7	6.7±1.4
4	20.9±4.9	27.4±4.2	11.0±3.0
8	20.8±5.0	26.3±4.1	12.2±2.6
12	21.0±5.5	25.5±4.1	10.4±2.3
16	22.5±5.6	24.1±4.1	10.9±2.6
20	24.8±5.2	23.5±3.9	11.1±2.7
24	23.6±5.1	23.2±3.8	11.4±3.1

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

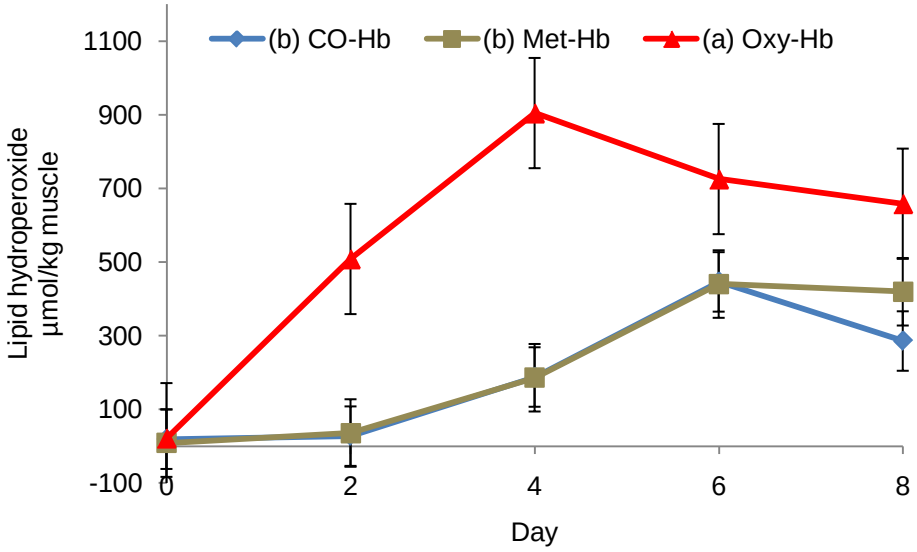


Figure 5-1. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at pH 6.3 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

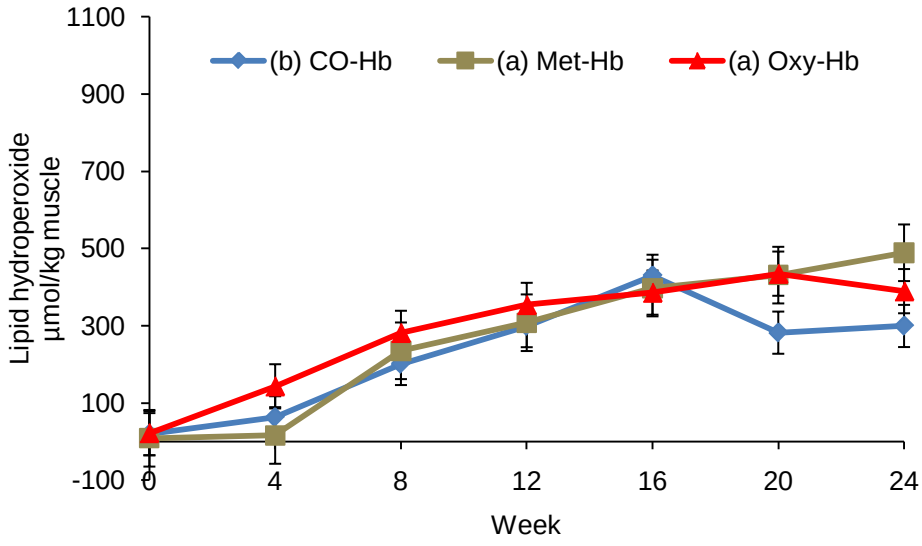


Figure 5-2. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at pH 6.3 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

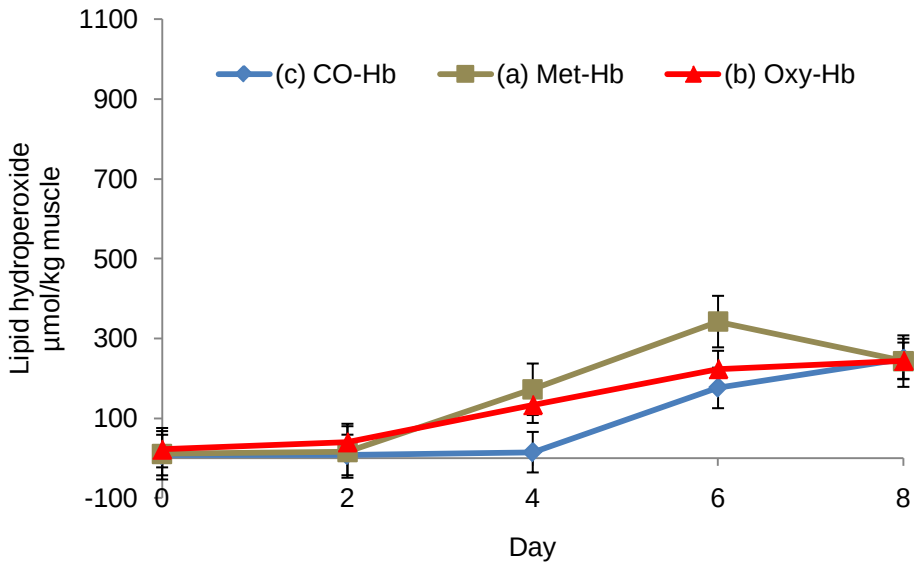


Figure 5-3. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at pH 6.8 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

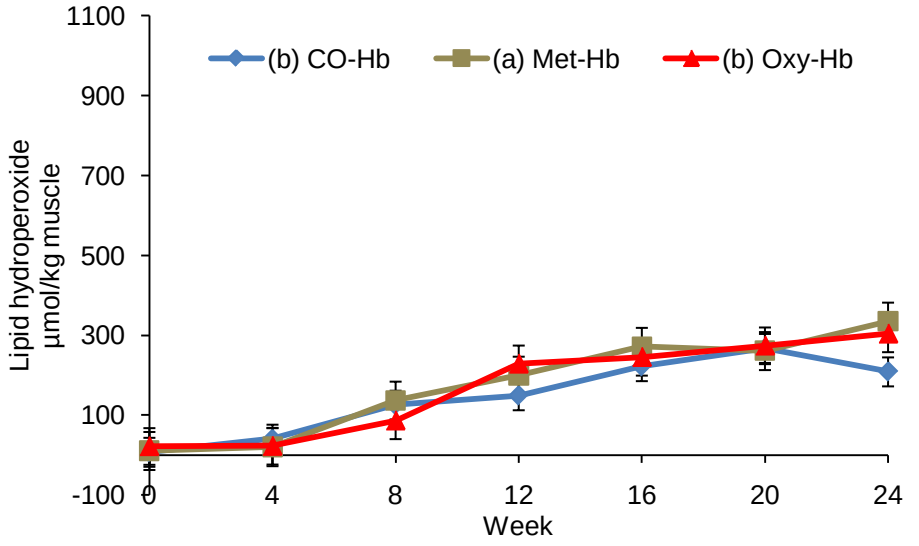


Figure 5-4. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at pH 6.8 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

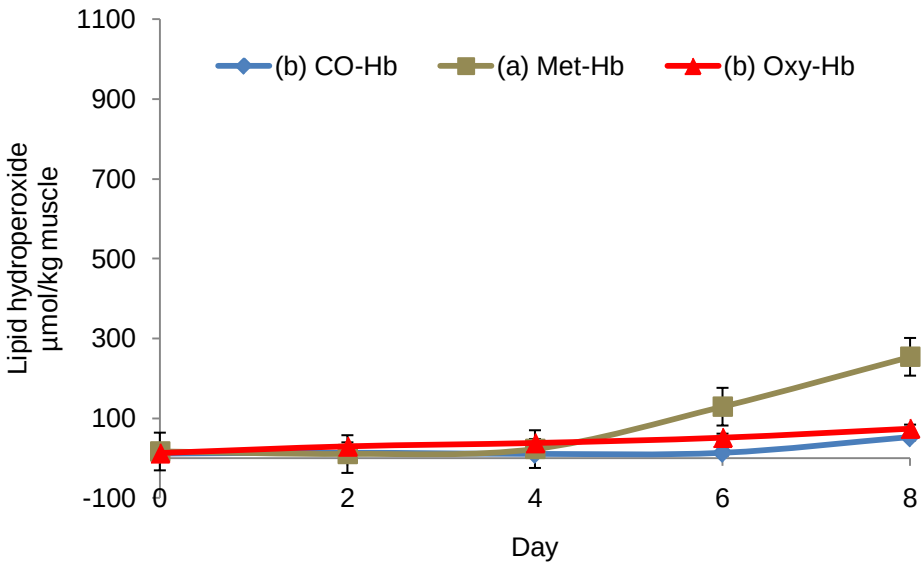


Figure 5-5. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at pH 7.3 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

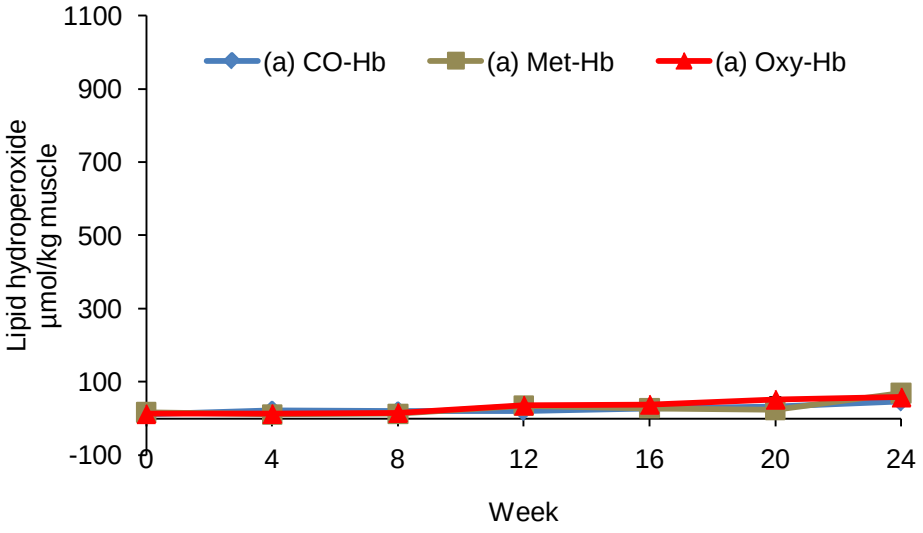


Figure 5-6. Lipid hydroperoxide values in washed tilapia muscle containing different forms of Hb at pH 7.3 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

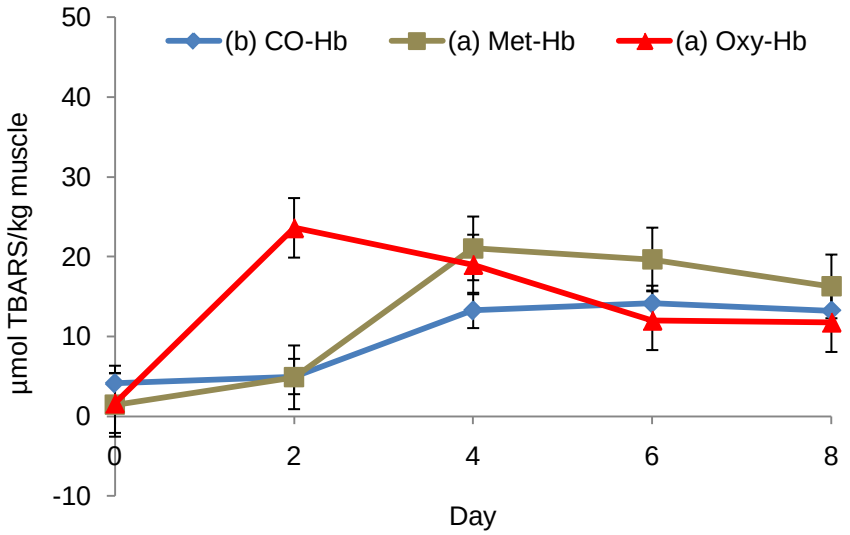


Figure 5-7. TBARS values in washed tilapia muscle containing different forms of Hb at pH 6.3 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

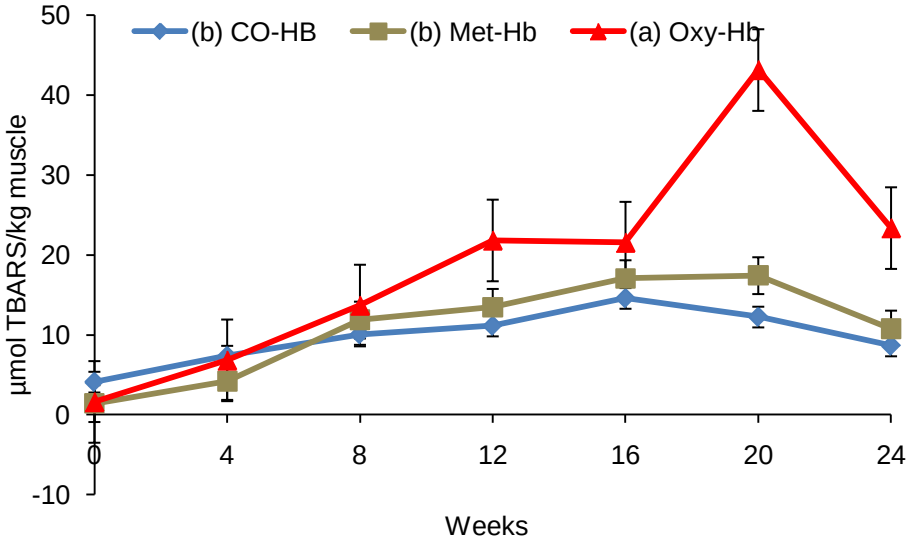


Figure 5-8. TBARS values in washed tilapia muscle containing different forms of Hb at pH 6.3 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

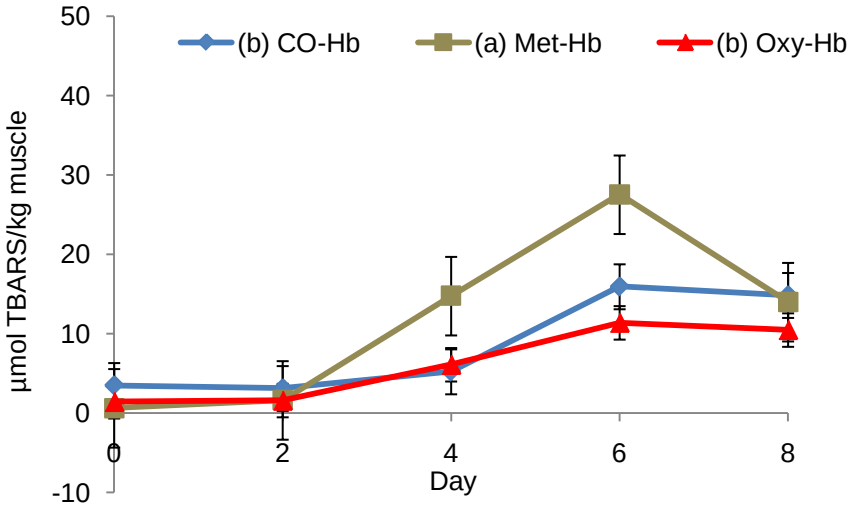


Figure 5-9. TBARS values in washed tilapia muscle containing different forms of Hb at pH 6.8 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

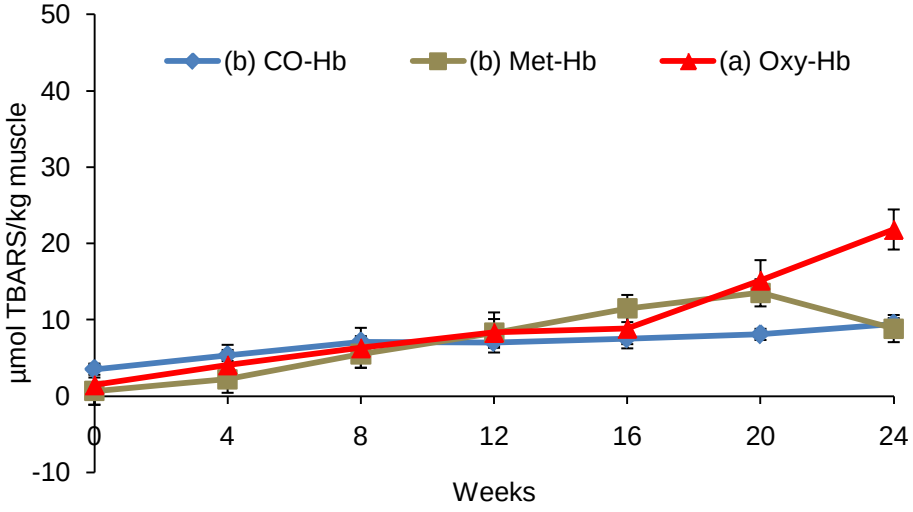


Figure 5-10. TBARS values in washed tilapia muscle containing different forms of Hb at pH 6.8 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

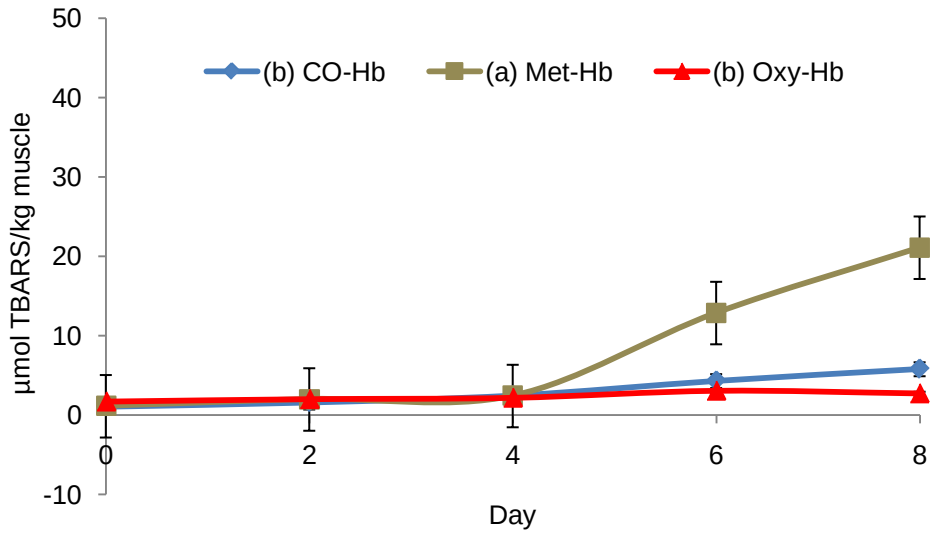


Figure 5-11. TBARS values in washed tilapia muscle containing different forms of Hb at pH 7.3 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

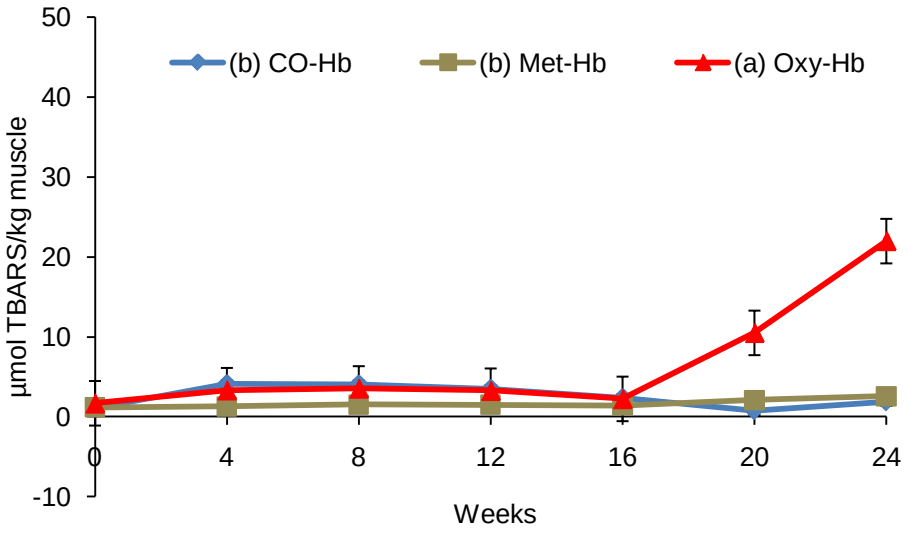


Figure 5-12. TBARS values in washed tilapia muscle containing different forms of Hb at pH 7.3 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

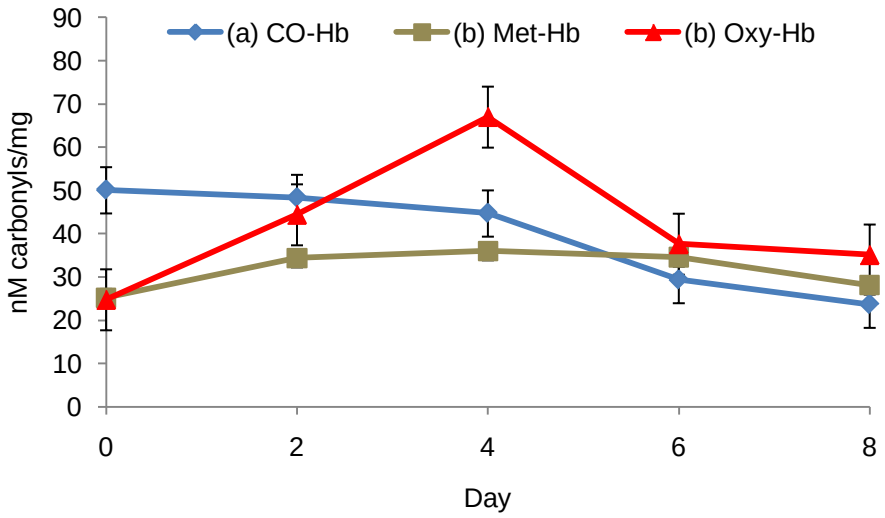


Figure 5-13. Carbonyl values in washed tilapia muscle containing different forms of Hb at pH 6.3 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

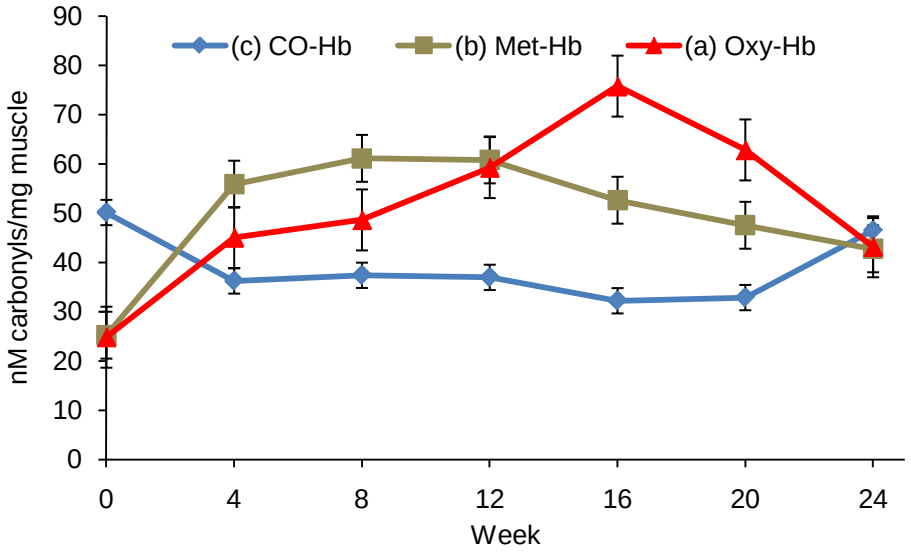


Figure 5-14. Carbonyl values in washed tilapia muscle containing different forms of Hb at pH 6.3 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

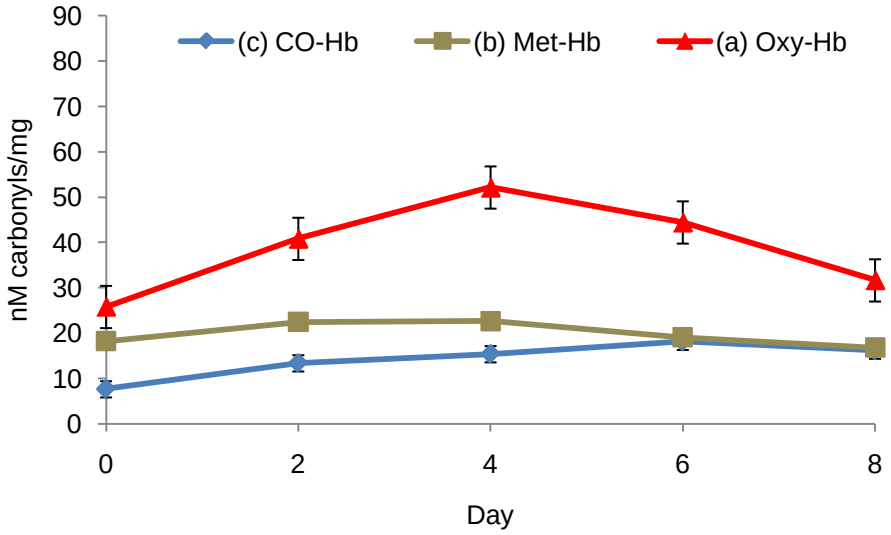


Figure 5-15. Carbonyl values in washed tilapia muscle containing different forms of Hb at pH 6.8 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

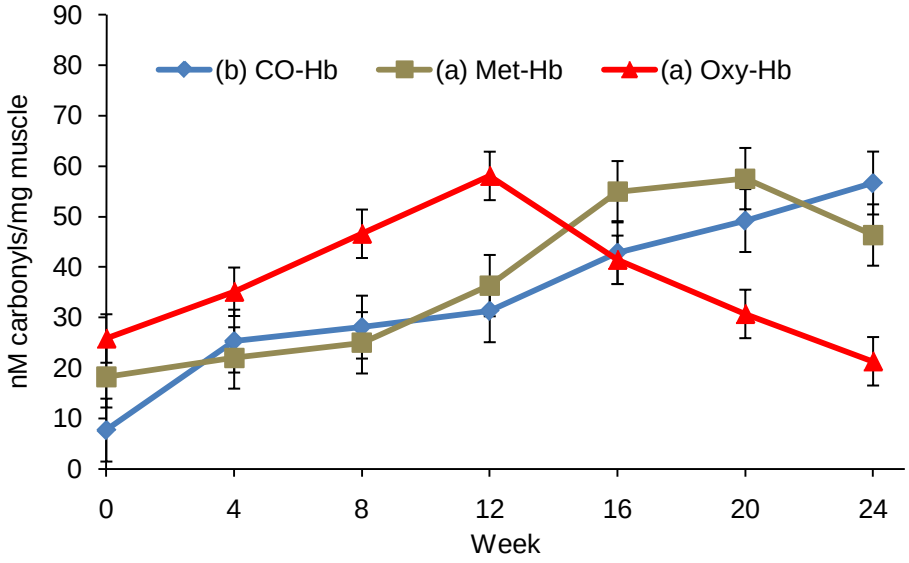


Figure 5-16. Carbonyl values in washed tilapia muscle containing different forms of Hb at pH 6.8 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

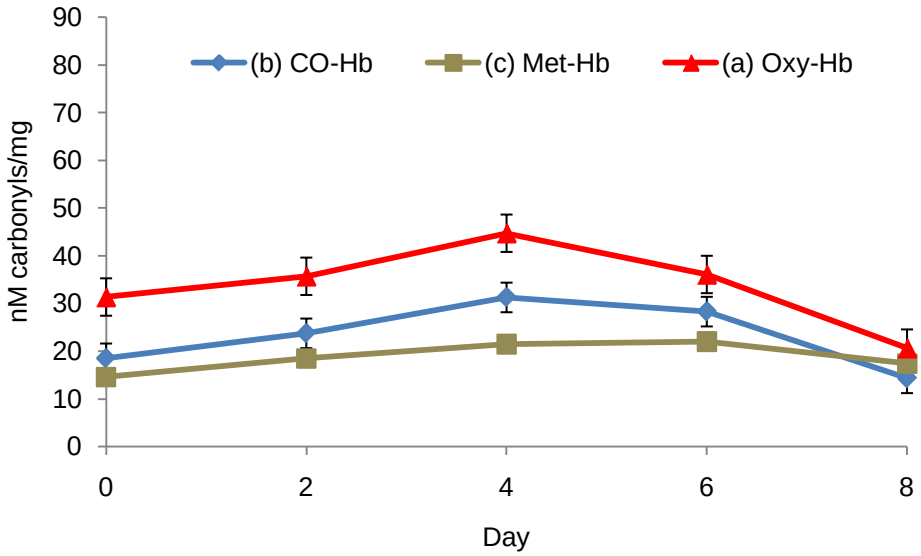


Figure 5-17. Carbonyl values in washed tilapia muscle containing different forms of Hb at pH 7.3 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

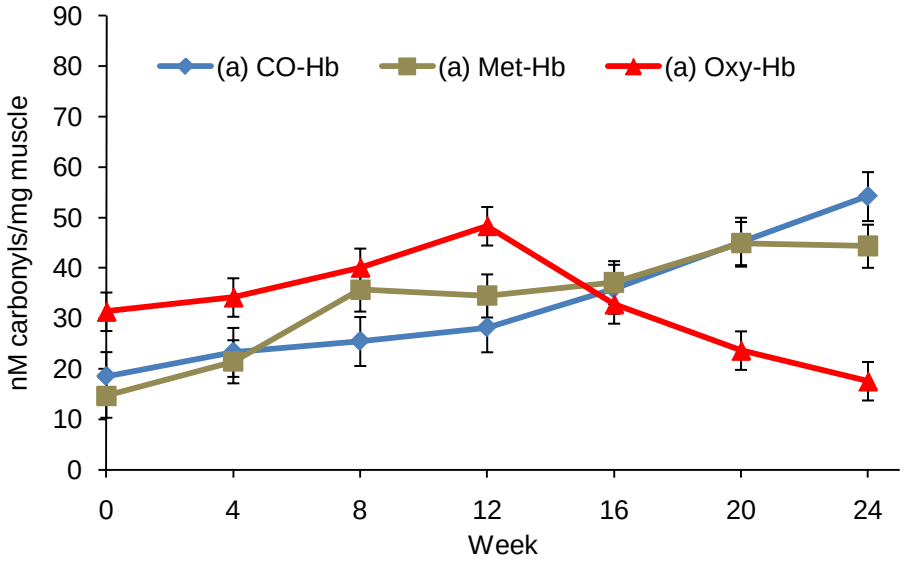


Figure 5-18. Carbonyl values in washed tilapia muscle containing different forms of Hb at pH 7.3 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

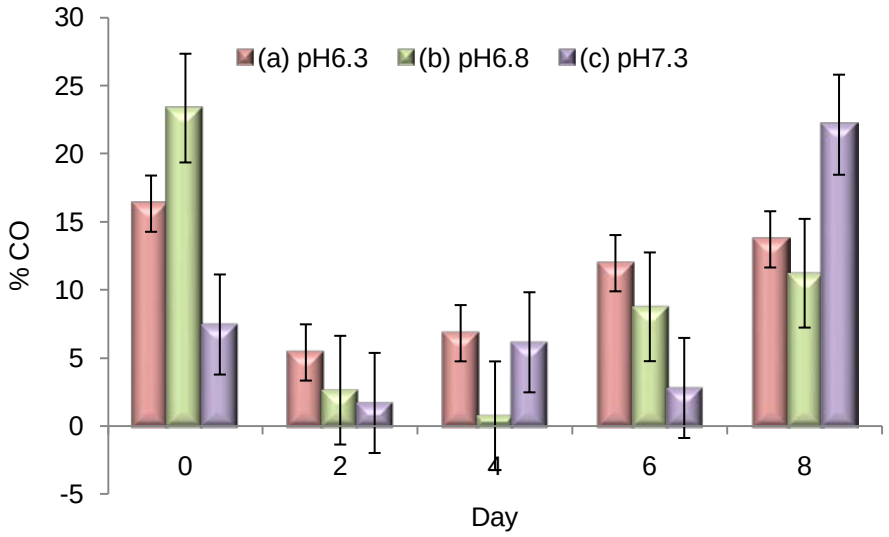


Figure 5-19. %CO released during 3.7ºC storage at different pH in washed tilapia muscle containing CO-Hb, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

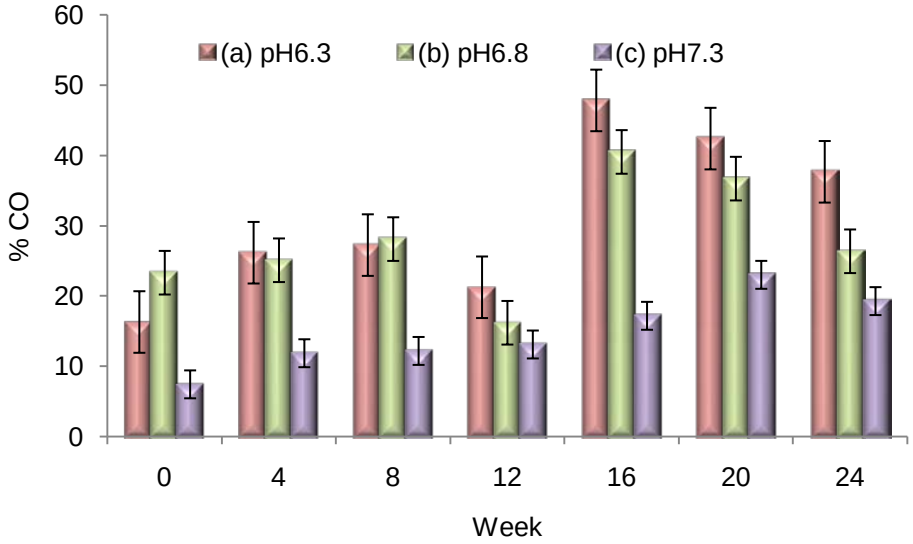


Figure 5-20. %CO released during -25ºC storage at different pH in washed tilapia muscle containing CO-Hb, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

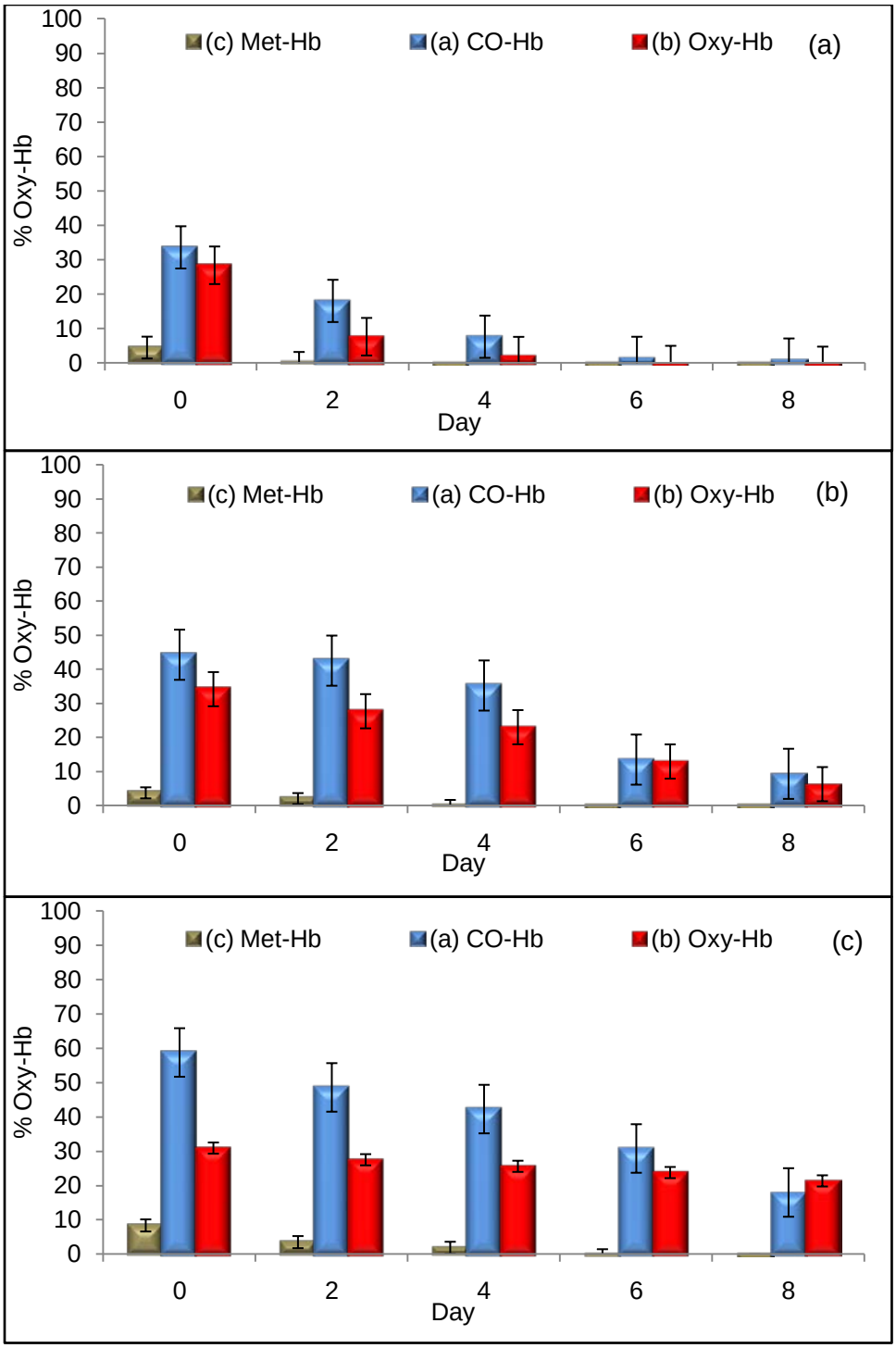


Figure 5-21. %Oxy-Hb in washed tilapia muscle containing different forms of Hb at pH a) 6.3, b) 6.8, and c) 7.3 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

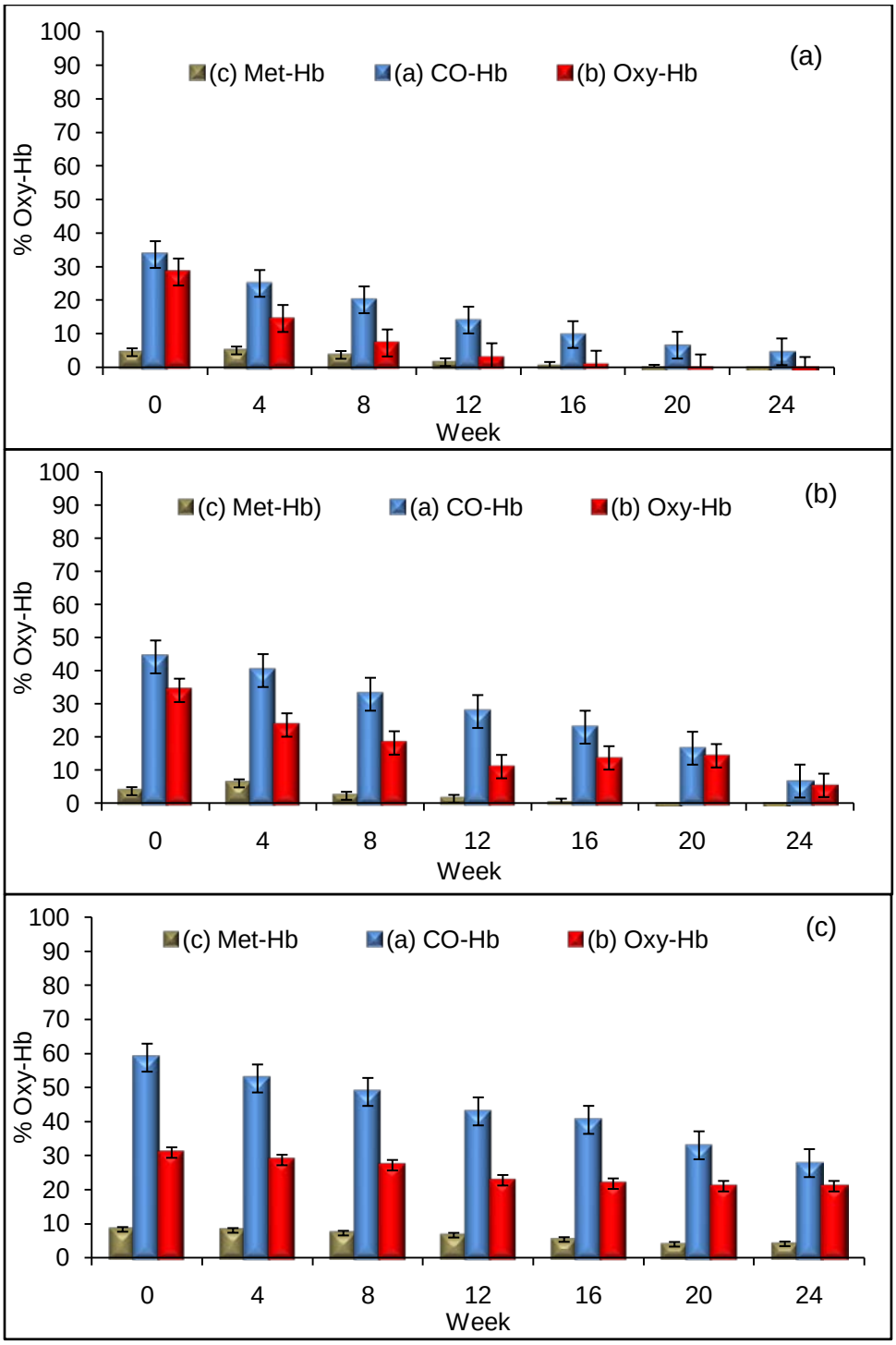


Figure 5-22. %Oxy-Hb in washed tilapia muscle containing different forms of Hb at pH a) 6.3, b) 6.8, and c) 7.3 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

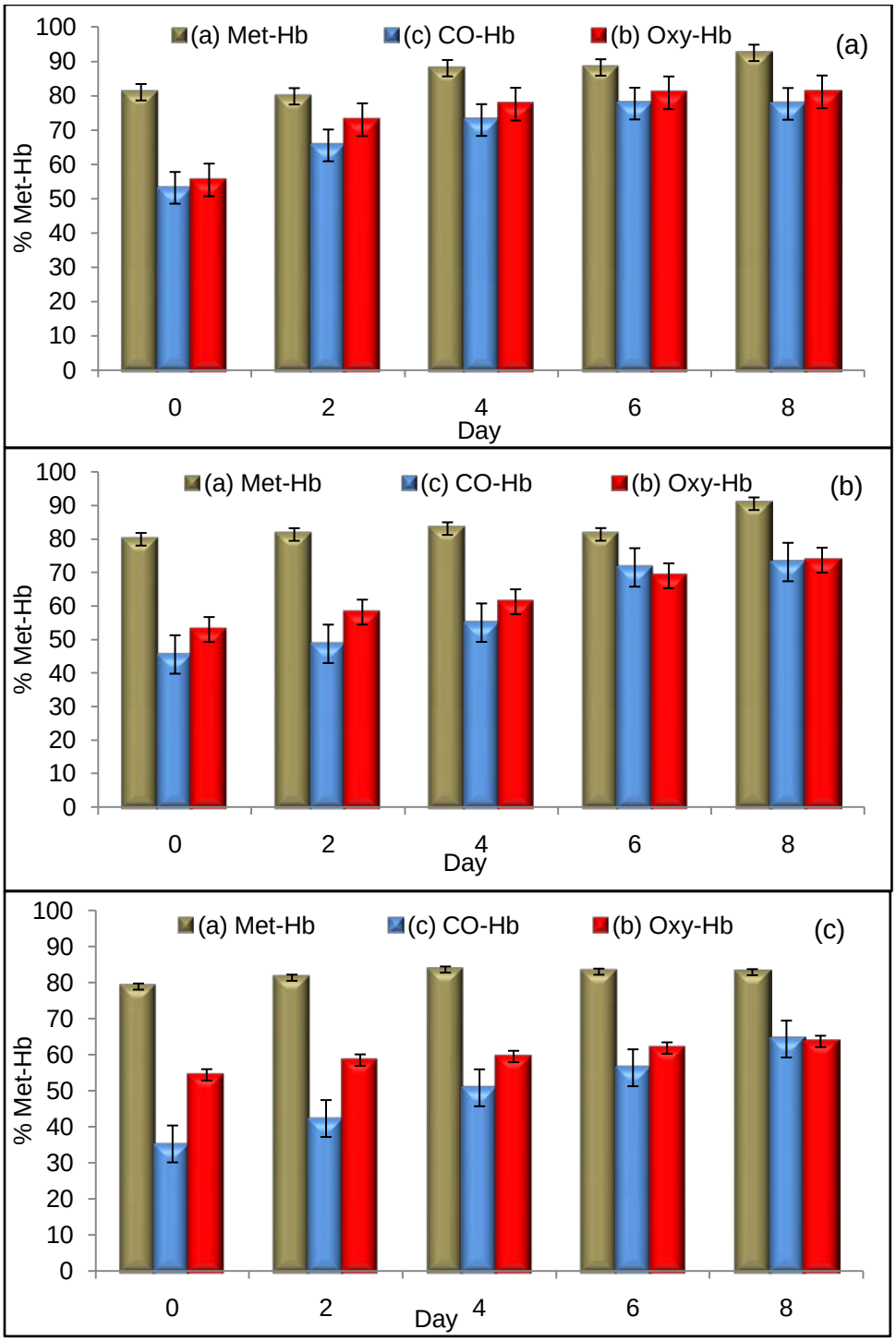


Figure 5-23. %Met-Hb in washed tilapia muscle containing different forms of Hb at pH a) 6.3, b) 6.8, and c) 7.3 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

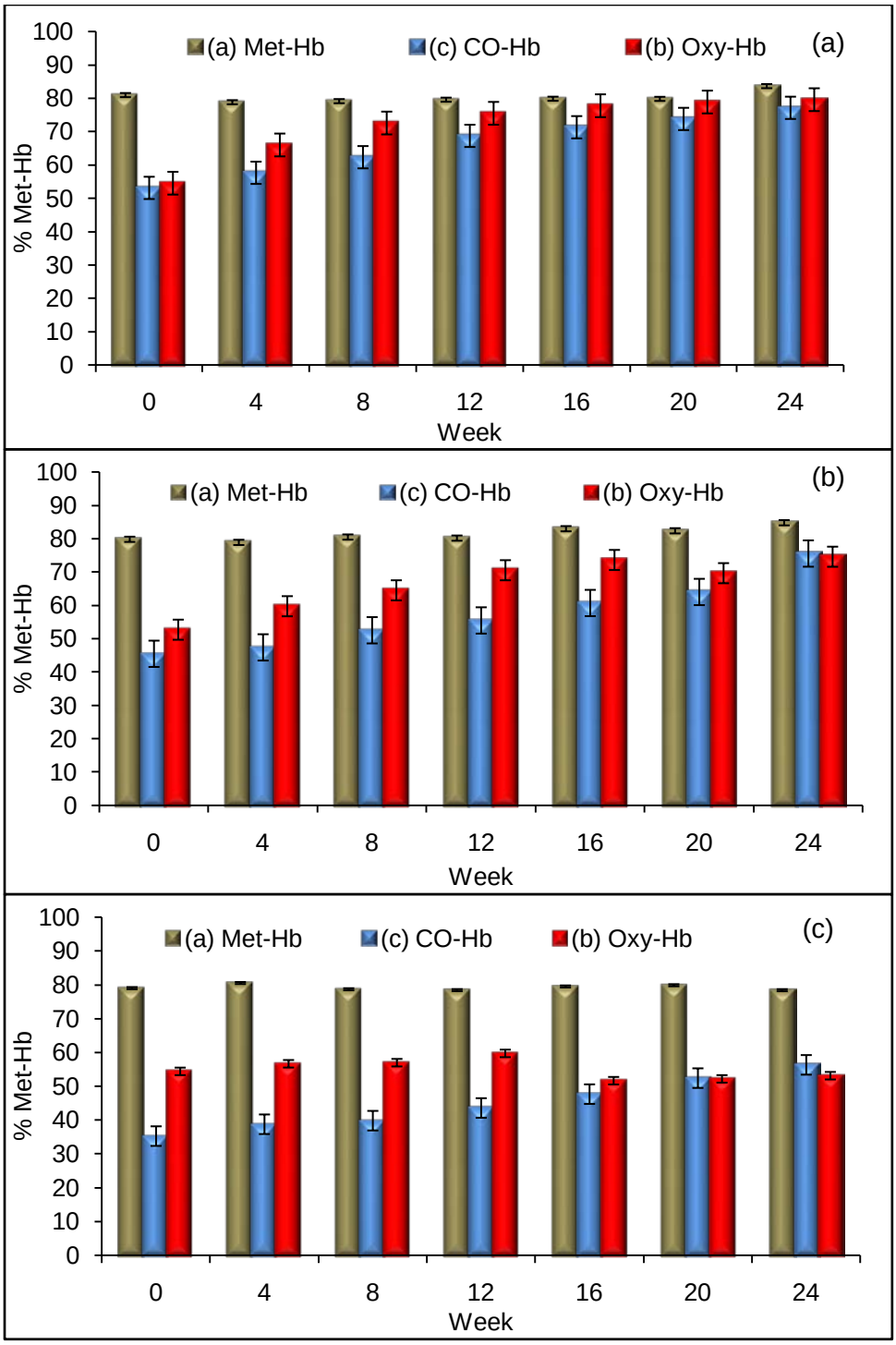


Figure 5-24. %Met-Hb in washed tilapia muscle containing different forms of Hb at pH a) 6.3, b) 6.8, and c) 7.3 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

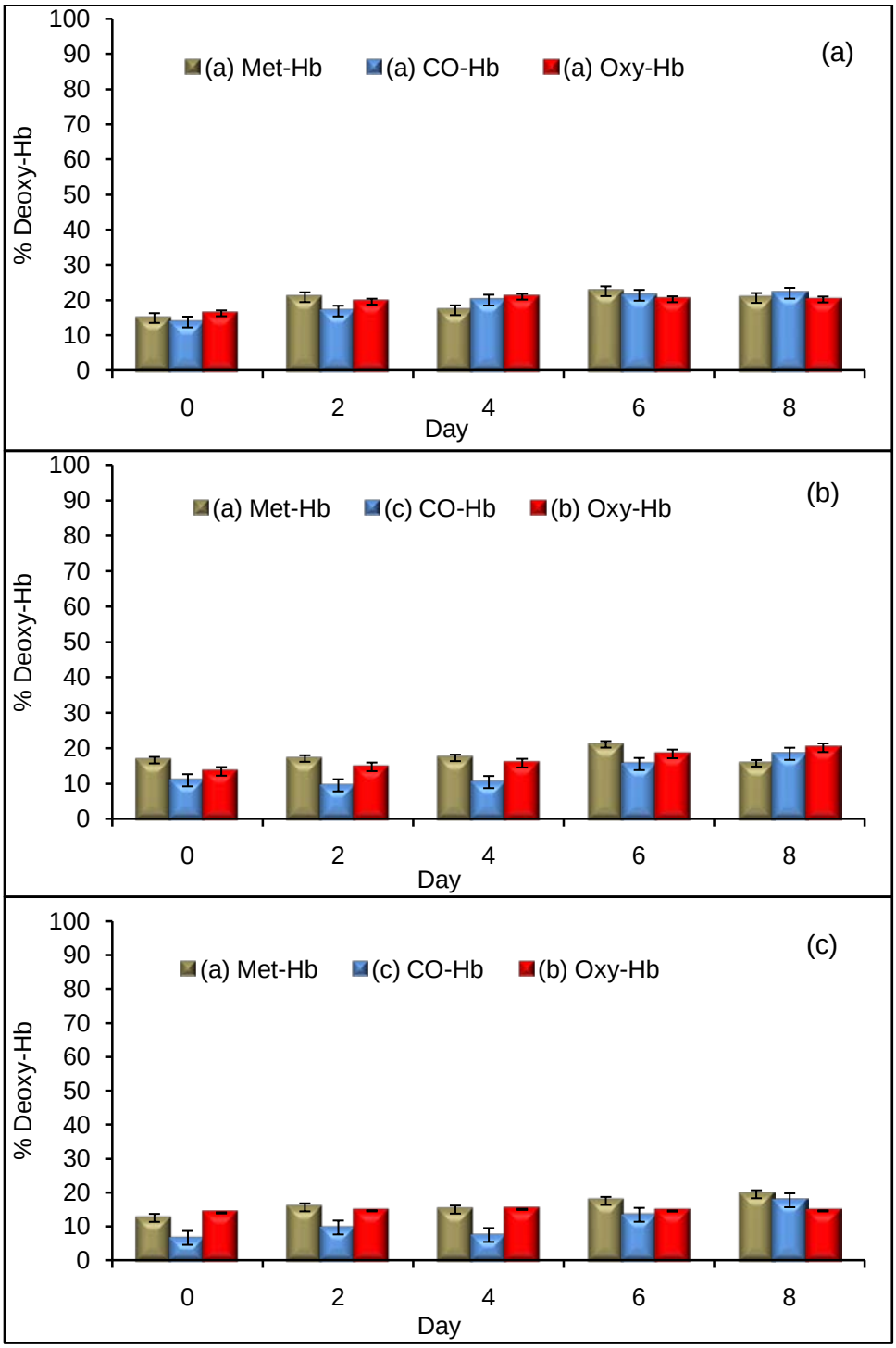


Figure 5-25. %Deoxy-Hb in washed tilapia muscle containing different forms of Hb at pH a) 6.3, b) 6.8, and c) 7.3 at 3.7ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

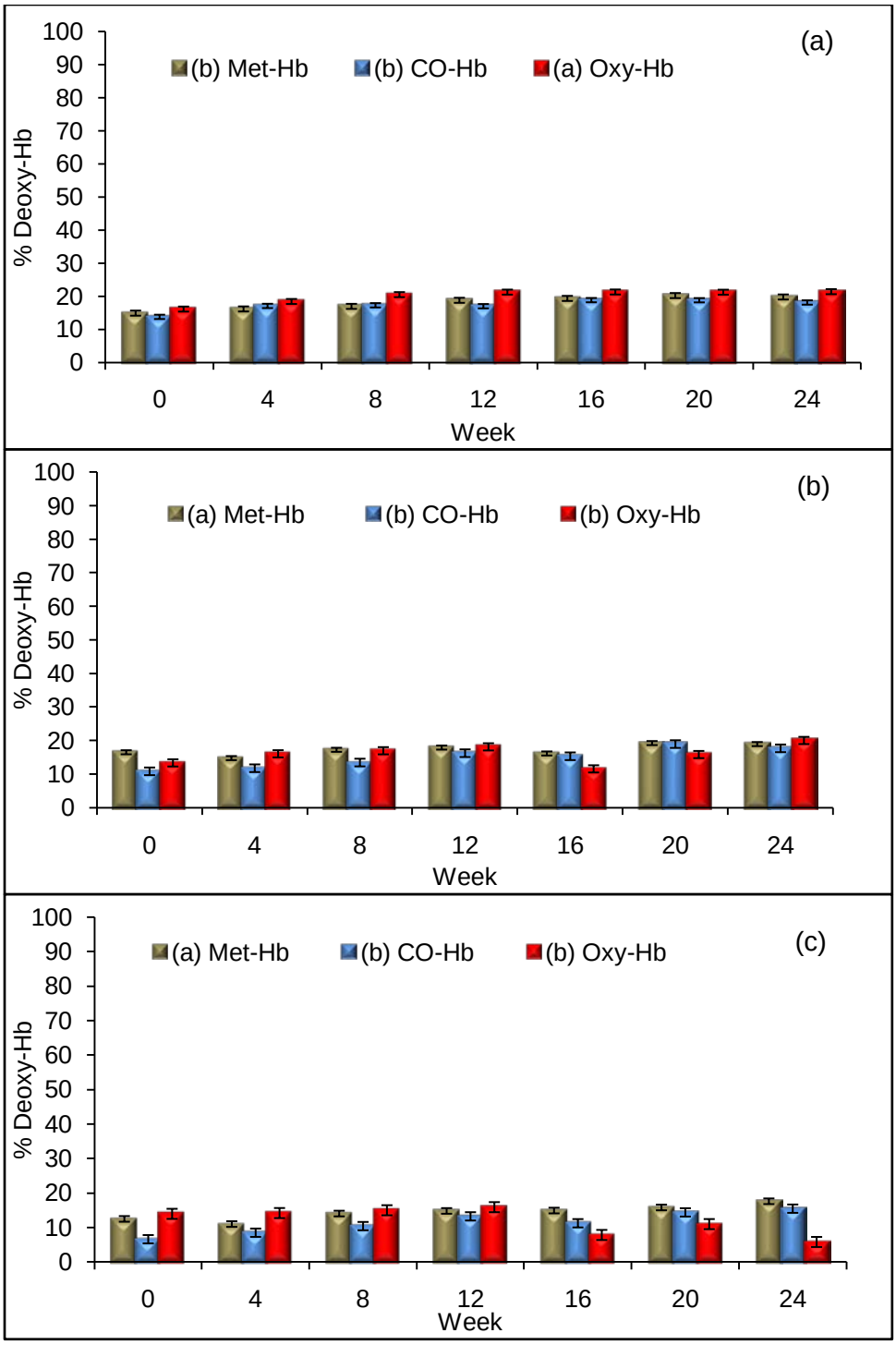


Figure 5-26. %Deoxy-Hb in washed tilapia muscle containing different forms of Hb at pH a) 6.3, b) 6.8, and c) 7.3 at -25ºC, averaged across Hb concentration and NaCl added. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

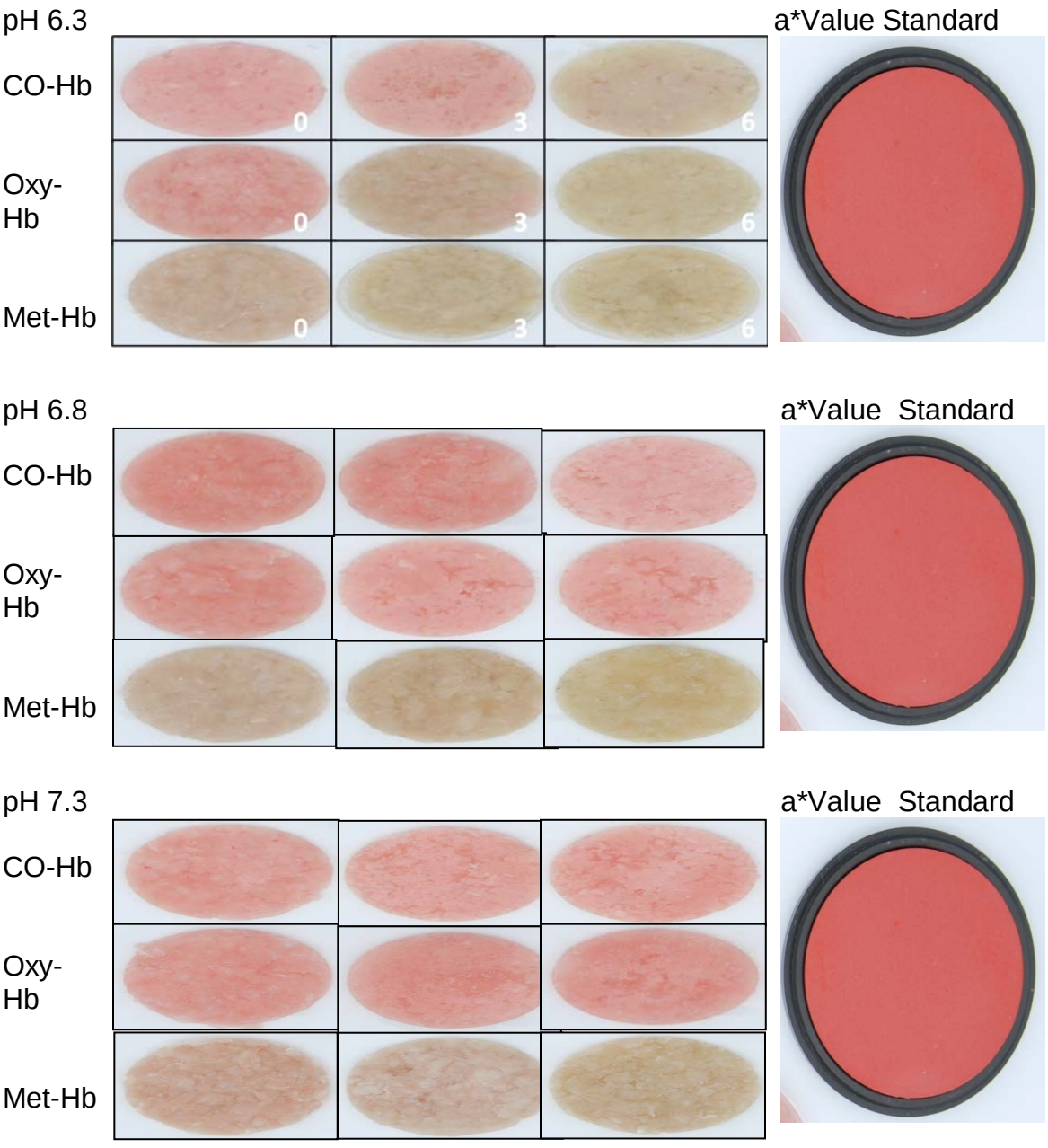


Figure 5-27. Images of washed tilapia muscle containing oxy-, CO-, and met-Hb at a concentration of 12µmol Hb/kg muscle. a) pH 6.3, Day 0, 3, and 6 b) pH 6.8, Day 0, 3, and 6, and c) pH 7.3, Day 0, 3, and 6, Obtained during storage for six days.

CHAPTER 6
THE ROLE OF SODIUM CLORIDE ON THE PRO-OXIDATIVE ACTIVITY OF OXY-, CO-, AND MET-HEMOGLOBIN ON IN A WASHED MINCED TILAPIA WASHED SYSTEM

Introduction

Sodium chloride ions are found in all living cells and can act as a pro-oxidant of muscle lipid peroxidation (*82*). Kanner and others (*83*) found that presence of NaCl initiates reactions producing superoxide anion radical ($O_2^{\cdot-}$) which results in formation of hydroxyl radical.

The pro-oxidative effect and the increase of lipid peroxidation by NaCl in model systems were studied and the elevation of free iron in tissues was ascribed to NaCl (*82*). Wallace and others (*84*) found that the stability of oxy-Hb and its oxidation to met-Hb may have been affected by the presence of NaCl by shifting ferrous ions from interaction with oxygen to reaction with hydroperoxides and decomposing these compounds to free radicals, accelerating the peroxidation process.

Harel (*85*) investigated the effect of NaCl on autoxidation of ferrous and cuprous ions in the presence of ascorbic acid and iron chelators. The generation of hydroxy radicals by ascorbic acid and metal ion's was inhibited by NaCl. NaCl also inhibited the oxidation of ascorbic acid by preventing the interaction of Fe or Cu with oxygen. The chloride anion interacts with the iron ion inhibiting the ferrous ion oxidation. Calcium chloride, magnesium chloride, and Lithium chloride showed similar results. Wallace and others (*84*) demonstrated that NaCl accelerates the decomposition of oxy-Hb to met-Hb. Harel (*85*), postulating that NaCl prevents or disturbs the interaction between heme iron and oxygen in the same way.

Materials and Methods

The following methods have been described in detail in chapter 3: Preparation of Washed Minced Tilapia Muscle (MTWM), Collection of Fish Blood, Preparation of Hemolysate, Quantification of Hb Levels in Hemolysate, Oxy-, CO- and Met-Hb Preparation, Sample Preparation: Addition of Hb and NaCl, Determination of Peroxide Value (PV), Determination of Thiobarbituric Acid Reactive Substances (TBARS), Determination of Carbonyl Groups, Heme Group Autoxidation, and Color Analysis. The Gas Chromatography (GC) Method was described in Chapter 4.

Results

Lipid Oxidation Analysis

Lipid hydroperoxide results obtained at 3.7°C with no added salt demonstrated, as shown previously, that oxy-Hb was significantly more pro-oxidative than CO-Hb and met-Hb. Oxy-Hb did not only lead to significant oxidation two days before the other forms but also resulted in higher maximum hydroperoxide values (Figure 6-1). CO-Hb and met-Hb led to a similar development of oxidation, with CO-Hb having significantly lower TBARS than met-Hb on day 8 only. At -25°C storage over 24 weeks (Figure 6-2) there was little difference between the three forms, although CO-Hb at week 20-24 was significantly less pro-oxidative than oxy-Hb.

Lipid hydroperoxides formed faster in the presence of 150 mM NaCl at 3.7°C than in the absence of salt (Figure 6-3). Oxy-Hb samples were already significantly oxidized on day 2, and hydroperoxide values were significantly ($p \leq 0.05$) higher at day 2 and 4 compared to CO-Hb and met-Hb. Samples with CO- and met-Hb did not differ significantly ($p \leq 0.05$) in lipid hydroperoxides throughout storage at 3.7°C. At -25°C, no

significant differences were found between the three forms of Hb, except CO-Hb was significantly ($p \leq 0.05$) less pro-oxidative than met- and oxy-Hb at week 24 (Figure 6-4).

At 3.7°C storage in the presence of 450mM NaCl a similar development of lipid hydroperoxides was seen for the three forms of Hb as in the presence of 150 mM NaCl (Figure 6-5). CO-Hb, however, gave significantly ($p \leq 0.05$) lower hydroperoxide values than oxy- and met-Hb on day 4 and 6. At -25°C storage overall no significant differences were noted between the three forms of Hb, except at week 4 where oxy-Hb was significantly more pro-oxidative than met-Hb and week 24 were oxy-Hb had significantly higher values than CO- and met-Hb (Figure 6-6). More lipid hydroperoxide formation was seen at -25°C in the presence of 450 mM NaCl, compared to the lower salt levels tested.

At 3.7°C storage with no added salt, washed tilapia muscle containing met-Hb developed higher TBARS values sooner than CO- and oxy-Hb, which did not differ from each other except on day 8 when CO-Hb had significantly higher TBARS than oxy-Hb (Figure 6-7). Met-Hb differed significantly from CO-Hb only on day 6, being the more pro-oxidative. Met-Hb differed significantly from oxy-Hb day 6 and 8. At -25°C, oxy-Hb led to the most oxidation, having significantly higher TBARS than CO-Hb from week 16-24 met-Hb from week 12-24 (Figure 6-8). CO- and met-Hb did not differ significantly during frozen storage.

A sooner onset of lipid oxidation (TBARS) was seen for all forms in the presence of 150 mM NaCl 3.7°C compared to samples with no added salt (Figure 6-9), which is in agreement with the lipid hydroperoxide data. Oxy-Hb samples peaked in TBARS values at day 0, having significantly higher values than CO- and met-Hb. However, by day 6

oxy-Hb had significantly significantly lower TBARS than CO- and met-Hb. Although CO-Hb lagged in oxidation behind met-Hb, the two forms did not differ significantly from each other. Met-Hb did however lead to the highest level of TBARS formed of all three forms tested. Very similar trends in the formation of TBARS was seen at -25°C in the presence of 150 mM NaCl compared to no added salt, although oxidation values were a bit higher in the system with salt (Figure 6-10). CO- and met-Hb did not differ significantly in pro-oxidative activity, while oxy-Hb was significantly more pro-oxidative than CO-Hb at week 12 and both CO- and met-Hb from weeks 20-24.

TBARS development in washed tilapia muscle at 3.7°C with 450 mM added NaCl (Figure 6-11) was similar to samples with 150mM NaCl. Although oxy-Hb had the highest level of TBARS at day 2, it did not differ significantly from CO-Hb, but both differed from met-Hb, which was significantly more pro-oxidative on day 6 only. Higher TBARS values were formed in samples with 450 mM NaCl at -25°C compared to the other salt levels tested (Figure 6-12). Samples with CO- and met-Hb did not differ significantly in TBARS values except for week 20 where met-Hb was significantly more pro-oxidative than CO-Hb. Oxy-Hb was significantly more pro-oxidative than met- and oxy-Hb from weeks 20-24.

Protein Oxidation Analysis

Formation of carbonyls as a function of Hb type was similar at all added salt levels tested at 3.7°C, where oxy-Hb led to an increase in carbonyl groups formed and also gave the highest values (Figures 6-13, 6-15 and 6-17). In the absence of added salt oxy-Hb had significantly higher carbonyl values than met- and oxy-Hb on days 2-6 (Figure 6-13). CO-Hb had higher carbonyl values than met-Hb only on day 0. In the presence of 150mM NaCl found no significant differences in carbonyl values were found

between CO- and met-Hb. Oxy-Hb had however significantly higher carbonyl values than CO- and met-Hb from days 2-6 and significantly higher values than CO-Hb on day 8. Oxy-Hb was significantly more pro-oxidative with respect to protein oxidation than met- and oxy-Hb in the presence of 450mM NaCl (Figure 6-17). CO-Hb and met-Hb differed significantly on day 2 only, CO-Hb giving higher carbonyl values.

Similar trends in formation of carbonyl values were observed at -25°C regardless of salt concentration of the washed tilapia muscle system with added Hb (Figures 6-14, 6-16 and 6-18). Oxy-Hb was significantly more pro-oxidative than CO- and met-Hb from week 4-12 in the presence of no added salt (Figure 6-14). However, carbonyls declined after 16 weeks, and from week 20-24, oxy-Hb was less pro-oxidative than the other Hbs. CO-Hb had significantly ($p \leq 0.05$) lower carbonyl values than met-Hb from week 8-12, but significantly higher values at week 24, where met-Hb had leveled off. In the presence of 150mM NaCl at -25°C (Figure 6-16), oxy- and met-Hb led to significantly ($p \leq 0.05$) higher level of carbonyls than CO-Hb. However, it declined rapidly after week 12 and at week 24 had significantly lower values than both CO- and met-Hb forms (Figure 6-16). Met-Hb was more pro-oxidative than CO-Hb at weeks 12-20. In the precence of 450mM NaCl at -25°C, samples with CO-Hb were significantly less pro-oxidative than met- and oxy-Hb throughout storage, except for week 24 where it had significantly higher carbonyl values (Figure 6-18). Met-Hb had significantly lower carbonyls than oxy-Hb at week 12, but significantly higher values week 24.

CO Release

The % of CO released during storage at 3.7°C of washed tilapia muscle containing CO-Hb with no added, 150, and 450mM of NaCl (Figure 6-19) showed that a greater % of CO was released from the samples that contained 150mM NaCl. A greater

% of CO was released on day 0 by samples containing all three forms of Hb, but the following two days the % of CO released was minimal but began to increase on day 6 and 8. At -25°C storage (Figure 6-20), the greatest % of CO was released by the samples containing 0.0mM NaCl. The % of CO released increased with increasing weeks of storage.

Color Analysis

Changes in a*values (redness) during 3.7°C storage of washed tilapia muscle containing oxy-, CO-, and met-Hb and no added NaCl demonstrated significant differences for the three forms of Hb (Table 6-1). Oxy-Hb had significantly higher a*values than met-Hb throughout fresh storage, and significantly higher a*values than CO-Hb on days 6-8. CO-Hb had significantly higher a*values than met-Hb on all days except day 8, where they were equal. At -25°C, samples with CO-Hb had significantly higher a*values than met-Hb throughout each week of storage and significantly higher a*value than oxy-Hb at week 12 (Table 6-2). Oxy-Hb had significantly higher a*values than met-Hb at all weeks, except week 12.

In the presence of 150mM NaCl at 3.7°C, initial a*values were higher than in the absence of added salt (Table 6-1). Samples with CO-Hb and oxy-Hb had both significantly higher a*values than met-Hb throughout the storage period, while CO-Hb had significantly higher a*values than oxy-Hb on day 2 only. CO-Hb had significantly higher a*values than met-Hb throughout the 24 weeks of storage at -25°C and significantly higher values than oxy-Hb, except for week 0. Oxy-Hb had significantly higher a*values than met-Hb at weeks 0, 4, 8, 16, and 20 (Table 6-2).

Initial a*values of all samples increased even more in the precence of 450 mM NaCl than the other levels studied (Table 6-1). CO-Hb had significantly higher a*values

than met-Hb throughout the 8 day storage period and significantly higher a*values than oxy-Hb on all days of storage except day 6. Oxy-Hb had significantly higher a*values than met-Hb on all days of storage. CO-Hb had significantly higher a*values than both met-Hb and oxy-Hb throughout the 24 weeks of storage at -25°C (Table 6-2). Oxy-Hb had significantly higher a*values than met-Hb at weeks 0, 4, 12, and 20.

Heme Group Autoxidation

% oxy-Hb declined over the 8 days at 3.7°C for all Hb forms tested regardless of salt level in the model system. Oxy- and CO-Hb had significantly greater % oxy-Hb than met-Hb throughout each day of storage at 3.7°C, regardless of salt concentration tested (Figure 6-21). Both in the absence of added salt and in the presence of 150 mM NaCl, CO-Hb had greater % oxy-Hb on days 0-4 than oxy-Hb (Figure 6-21a,b). However, in the presence of 450 mM NaCl, CO-Hb had greater % oxy-Hb than oxy-Hb on days 0, 4 and 6.

% oxy-Hb values also declined over time at -25°C, regardless of Hb-form tested and salt concentration. Results at -25°C were similar to the results at 3.7°C where CO- and oxy-Hb had significantly greater % oxy-Hb throughout the 24 weeks of storage than met-Hb regardless of salt concentration (Figure 6-22). In the absence of added salt, CO-Hb had significantly more % oxy-Hb than oxy-Hb from week 0-16, which was extended to week 20 in the presence of 150 mM and 450 mM NaCl.

% met-Hb increased during storage at 3.7°C for all samples, irrespective of salt levels tested (Figure 6-22). As expected, met-Hb had significantly greater % met-Hb than CO- and oxy-Hb at all salt levels tested. CO-Hb had significantly less % met-Hb than oxy-Hb day 0, 2, and 4 in the absence of salt and in the presence of 450 mM NaCl, but was only lower in % met-Hb at days 0 and 2 in the presence of 150 mM NaCl

During -25°C storage there were minor changes in % met-Hb for samples with met-Hb, while the two other forms increased in % met-Hb over time (Figure 6-24). Met-Hb had significantly higher % met-Hb than CO- and oxy-Hb throughout the 24 weeks of storage, regardless of salt levels tested. CO-Hb had significantly less % met-Hb formed than oxy-Hb from weeks 0-16, at no added salt and in the presence of 150 mM NaCl, while in the presence of 450 mM NaCl the time was reduced to 12 weeks.

No significant differences were seen between samples with oxy- and met-Hb in terms of % deoxy-Hb at 3.7°C, regardless of salt concentration (Figure 6-25). In the absence of salt and in the presence of 150 mM NaCl, CO-Hb had less % deoxy-Hb than met-Hb from days 0-4, and less than oxy-Hb from days 2-4. In the presence of 450 mM NaCl the values were lower for CO-Hb compared to met-Hb on days 0, 4 and 6, and days 0 and 4 compared to oxy-Hb. During the entire -25°C storage period % deoxy-Hb did not differ significantly for oxy- and met-Hb, regardless of salt concentration tested with the exception for samples with 450 mM NaCl where met-Hb had significantly greater % deoxy-Hb from weeks 16-24 (Figure 26a). Samples with CO-Hb were however, affected somewhat by salt concentration. In the absence of salt, CO-Hb had significantly less % deoxy-Hb than met-Hb at weeks 0 and 8, and significantly less than oxy-Hb at week 8. In the presence of 150 mM NaCl, CO-Hb had significantly less % deoxy-Hb than oxy-Hb from weeks 0-12, and significantly less than met-Hb at week 0 only. In the presence of 450 mM NaCl, CO-Hb had significantly less % deoxy-Hb than oxy-Hb from weeks 0-12 and at week 24, while it was significantly less than met-Hb week 0.

Discussion

NaCl is a common additive used in the food industry for a variety of purposes, including inhibition of microbial growth and flavor enhancement (*98*). The extensive use of NaCl in food preparation and preservation dictates the necessity for thoroughly examining its effects on food quality. Conflicting data exists among previous research scientists investigating the influence of addition of salt on the oxidative properties of muscle food systems. While results of some observations suggest that NaCl may exhibit pro-oxidative activities, accelerating lipid and protein oxidation in muscle food systems, including fish (*82, 108*) others report opposite effects (*10, 109*), which makes further exploration of this topic important. Ability of various salt concentrations to induce changes in protein solubility and gelation was previously described (*98*), and may affect protein oxidation.

In this experiment the effects of NaCl (150 and 450mM) concentrations on the properties of different forms of Hbs (oxy-, CO-, and met-Hbs) in a washed tilapia muscle system were investigated under refrigeration (3.7ºC) and frozen (-25ºC) storage conditions. The results of the present investigation strongly support previous suggestions that higher concentration of NaCl may enhance pro-oxidative activities of Hb that is confirmed by TBARS scores and lipid peroxidation analysis (*110*). It appears that both rate and magnitude of Hb oxidation increases with increase in NaCl concentration regardless of storage conditions, although under frozen storage conditions the rate of oxidation was somewhat retarded (Figure 6-21, 6-22). The significant finding of this study was that the oxidative activities of CO-Hb were overall less affected by increased concentrations of NaCl as compared to oxy- and met-Hb (Figure 6-1- 6-6). It was previously reported that NaCl may disrupt the stability of oxy-

Hb, accelerating its deoxygenation and formation of met-Hb (*84*). From accumulated data it appears that the pro-oxidative activities of NaCl may be due, in part, to its ability to hasten release of iron from heme (*82*). In particular, the chloride anion was implicated in Hb autoxidation, serving as a substrate for a chloroperoxidase in the presence of preformed hydrogen peroxide (*84, 111*). In addition, it was proposed that NaCl may have the ability to accelerate lipid peroxidation by shifting iron-oxygen to iron-hydroperoxide interactions. A significantly higher rate of lipid peroxidation and TBARS formation was observed for oxy-Hb at 3.7°C with NaCl present compared to the other forms (Figure 6-7 – 6-12). It may be hypothesized that rapid deoxygenation of oxy-Hb under these conditions may be further augmented by the presence of NaCl, leading to increased oxidation. % oxy-Hb decreased significantly for all samples during 8 days of storage (Figure 6-21a, b, and c). CO-Hb autoxiation was least affected by NaCl addition (Figure 6-23a, b, and c). Moreover, it was also suggested that addition of NaCl may decrease the pH level and Hb affinity to oxygen which would contribute to increased Hb autoxidation and subsequently lipid peroxidation (*112*). In addition, other factors besides Hb autoxidation may influence lipid peroxidation under described conditions. Previous studies indicate that the addition of salt may suppress glutathione peroxidase activities in refrigerated ground pork muscle that may result in increase of lipid peroxidation (*113*). Another study on oxidative changes in salted herring found that the decreased levels of alpha-tocopherol in the ripening salted herring may further enhance oxidative processes (*114*).

At -25°C however, the same rate of formation of TBARS and lipid hydroperoxides was observed for all forms of Hb, with oxy-Hb producing significantly higher levels of

TBARS by the end of storage at any NaCl concentration (Figure 6-8, 6-10, and 6-12). CO-Hb was least susceptible to oxidation without added NaCl and with the 150 mM of NaCl under frozen storage conditions (Figure 6-2, 6-4, 6-8, and 6-10). This implies that lower temperatures may slow the oxidation rate of muscle foods containing Hb independently of the presence or absence of NaCl and type of Hb.

Protein oxidation (assessed by carbonyl formation) catalyzed by all forms of Hb was not significantly affected by addition of NaCl at either storage temperature (Figure 6-13- 6-18). However, at 3.7°C and -25°C, the pattern of carbonyl production was different for each form of Hb and the days/weeks on which oxidations occurred. Samples with oxy-Hb did however result in the highest levels of carbonyls formed. It is speculated that oxy-Hb is a main promoter of lipid oxidation. Richards and Hultin (*4*) reported that oxy- and deoxy-Hb produced high levels of lipid peroxide formation, whereas met-Hb caused little peroxidation. These findings might indicate that oxy-Hb could also play an important role as a catalyst in promoting protein oxidation. Eymard and others (*35*) investigated the link between lipid oxidation and protein oxidation during processing and storage of horse mackerel using fish minces with differences in lipid and protein fractions and different oxidative levels. The authors concluded that lipid and protein oxidation developed simultaneously but it was difficult to determine how they are linked. Lipid and protein oxidation share the same catalysts, and they can develop independently of one another, or in parallel, or they can interact with each other (*35*).

Our results are consistent with a recent report on the effects of NaCl on protein oxidation in frozen yellowtail meat, which found that the addition of NaCl did not have any significant effect on the protein carbonyls content in the yellowtail meats (*110*).

Further exploration of effects of NaCl on protein oxidation with emphasis on the detailed mechanism(s) involved in this process is warranted.

Changes in a*values observed during 3.7ºC storage were unexpected. Without addition of salt and with intermediate (150 mM) concentration, oxy-Hb demonstrated overall higher color stability as compared to CO-Hb and, as expected, met-Hb (Table 6-1). The above results are consistent with the loss of oxy-Hb and the rate of deoxygenation and Hb autoxidation presented in Figures 6-21, 6-23, 6-25. Although, at the highest concentration of NaCl (450 mM). Red color at 3.7ºC significantly correlated positively with % oxy-Hb and negatively with % deoxy- and met-Hb (Appendix C, Table C-1). However, at the highest concentration of NaCl (450 mM) red color was more stable for CO-Hb at day 1 (Table 6-1). During -25ºC storage, a*value was significantly higher for CO-Hb with and without salt (Table 6-2). These results suggest that treatment of fish muscle with CO may delay color deterioration under specific NaCl concentrations and temperature conditions. These findings support the results of earlier studies that evaluated color stability of different kinds of meat exposed to CO treatment (*115*). This may be explained by CO replacing oxygen; it slowes the oxidation and thus deterioration of color in muscle meat systems including fish. It was also interesting to note that as salt levels was increased, the initial a*value of all samples was increased.

Conclusion

This work suggests that NaCl is highly pro-oxidative in a system containing oxy-Hb and membrane lipids, thus providing insights into how oxidation in seafood based products containing salt could be potentially controlled. Oxy-Hb maintained its catalytic effect and is believed to catalyze oxidation by the breakdown of preformed lipid hydroperoxides. The low pro-oxidative activity of CO-Hb is due in part to CO increasing the stability of heme protein structure and slowing its autoxidation. CO treatment enhanced the red color of fish muscle stored at freezer temperatures with and without added NaCl.

Table 6-1. Changes in a*value in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl (0, 150, and 450 mM) at 3.7ºC, averaged across pH level and Hb concentration.

a* value	0 added NaCl		
Storage time (day)	Oxy-Hb[a]	CO-Hb[b]	Met-Hb[c]
0	18.5±4.8	18.3±2.0	7.4±1.3
2	17.6±5.9	17.5±2.1	9.9±2.0
4	15.3±9.5	16.0±2.7	10.0±1.8
6	15.0±10.5	9.2±4.3	7.9±2.5
8	14.9±10.5	6.9±3.0	6.9±3.0

a* value	150.0mM NaCl		
Storage time (day)	Oxy-Hb[a]	CO-Hb[a]	Met-Hb[b]
0	19.7±4.3	22.0±3.0	8.1±1.8
2	16.0±8.2	21.2±3.7	10.3±2.3
4	15.5±9.6	16.7±7.3	7.7±2.0
6	15.1±10.3	11.9±8.6	5.7±2.6
8	13.4±9.2	10.8±7.3	4.5±2.2

a* value	450.0mM NaCl		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	21.5±4.6	25.4±3.6	8.7±1.9
2	16.2±9.6	23.5±6.5	10.5±2.3
4	14.7±9.3	18.9±10.8	6.5±1.6
6	10.4±9.9	12.9±10.6	4.8±2.0
8	8.0±8.8	11.8±9.4	2.9±1.0

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table 6-2. Changes in a* value in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl (0, 150, and 450 mM) at -25ºC, averaged across pH level and Hb concentration.

a* value	0 added NaCl		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	18.5±4.8	18.3±2.0	7.4±1.3
4	19.8±4.4	22.9±3.2	13.4±2.4
8	18.0±4.4	21.1±4.3	11.9±2.5
12	13.2±6.4	19.6±3.5	11.4±2.3
16	17.2±5.9	18.6±3.7	11.2±2.4
20	17.3±7.2	17.3±3.5	10.8±2.2
24	15.5±7.7	16.9±3.9	10.1±2.7

a* value	150.0mM NaCl		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	19.7±4.3	22.0±3.0	8.1±1.8
4	16.6±6.7	24.8±3.2	12.3±3.0
8	15.9±6.5	22.8±4.4	11.5±2.2
12	12.8±7.3	21.4±4.7	10.2±1.9
16	14.4±7.0	20.3±5.1	10.2±2.4
20	14.2±9.1	19.5±4.9	9.9±2.9
24	12.6±9.6	18.5±5.7	9.7±3.2

a* value	450.0mM NaCl		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[c]
0	21.5±4.6	25.4±3.6	8.7±1.9
4	15.2±7.1	27.1±6.8	11.1±2.5
8	13.5±7.0	25.0±7.8	9.7±2.1
12	14.6±8.4	23.3±7.8	9.3±2.6
16	12.0±9.0	23.1±8.4	9.2±2.9
20	14.0±10.3	21.0±8.2	8.7±3.0
24	12.2±11.1	19.7±9.4	8.6±3.6

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

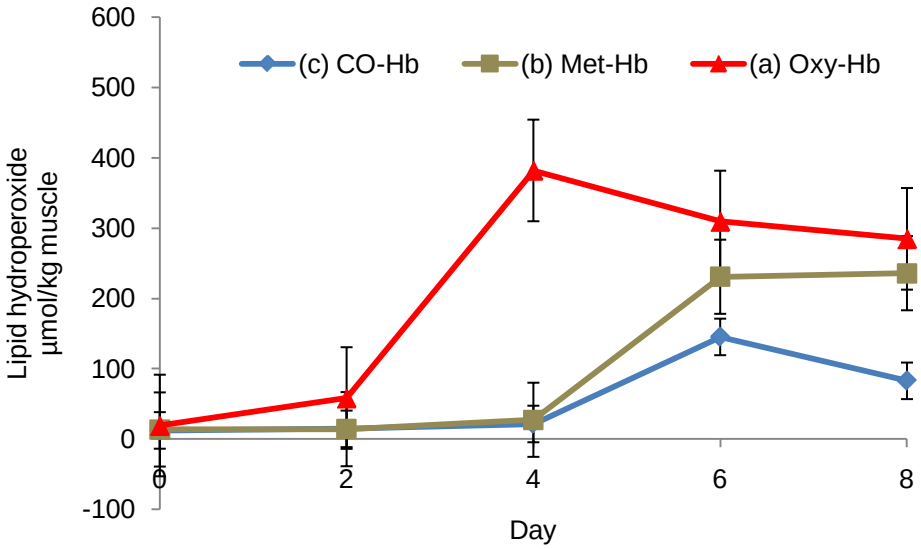


Figure 6-1. Lipid hydroperoxide values in washed tilapia muscle at 3.7ºC with no added NaCl and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

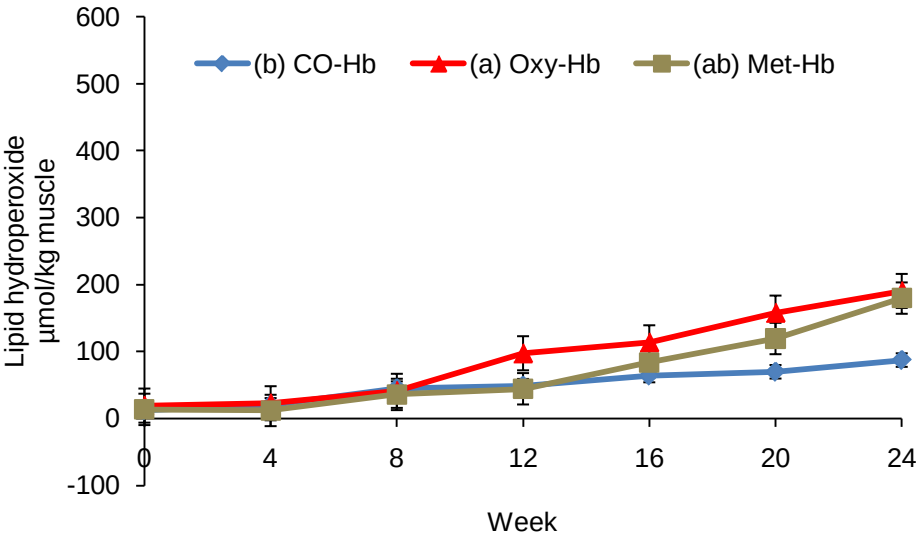


Figure 6-2. Lipid hydroperoxide values in washed tilapia muscle at -25ºC with no added NaCl and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

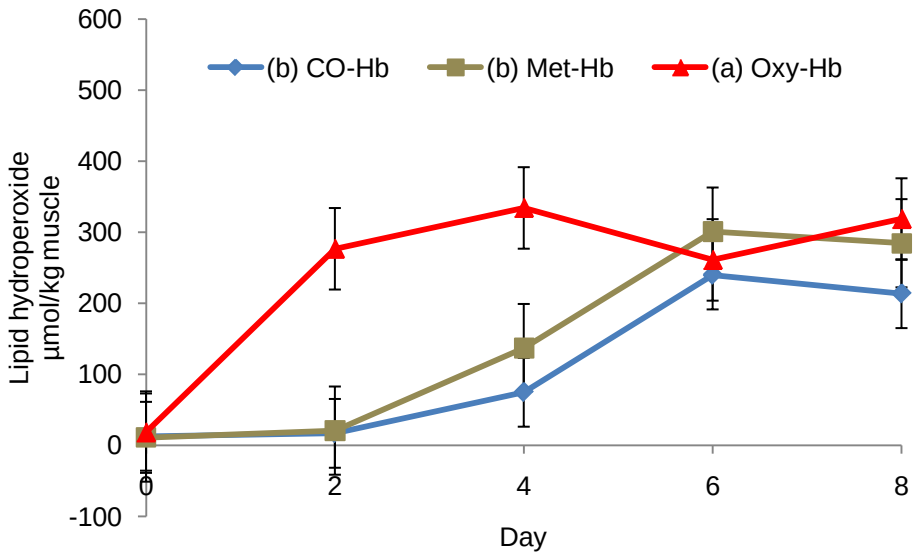


Figure 6-3. Lipid hydroperoxide values in washed tilapia muscle at 3.7ºC with 150 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

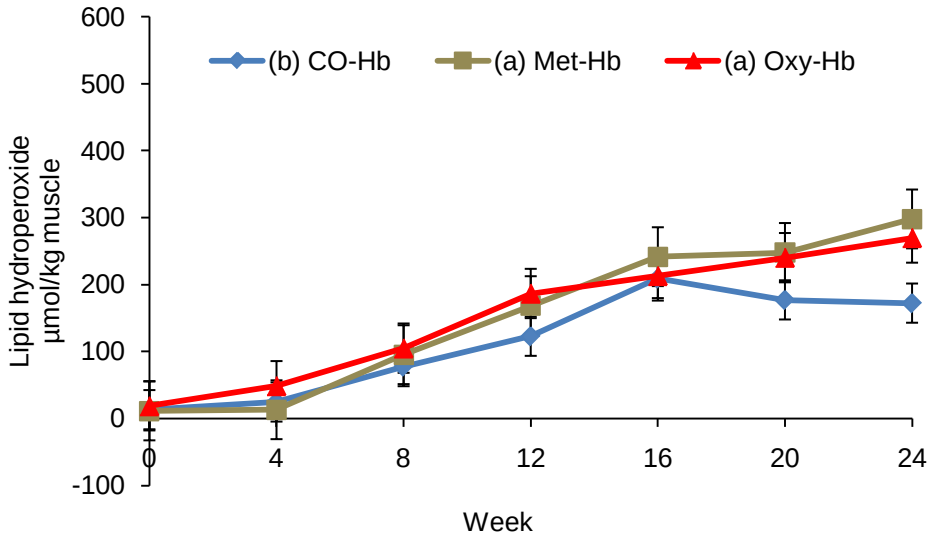


Figure 6-4. Lipid hydroperoxide values in washed tilapia muscle at -25ºC with 150 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

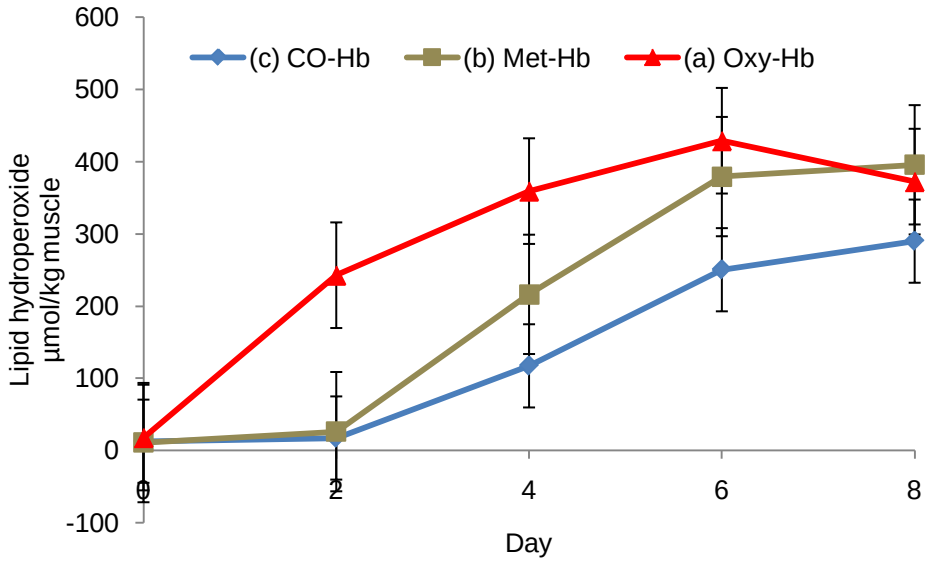


Figure 6-5. Lipid hydroperoxide values in washed tilapia muscle at 3.7ºC with 450 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

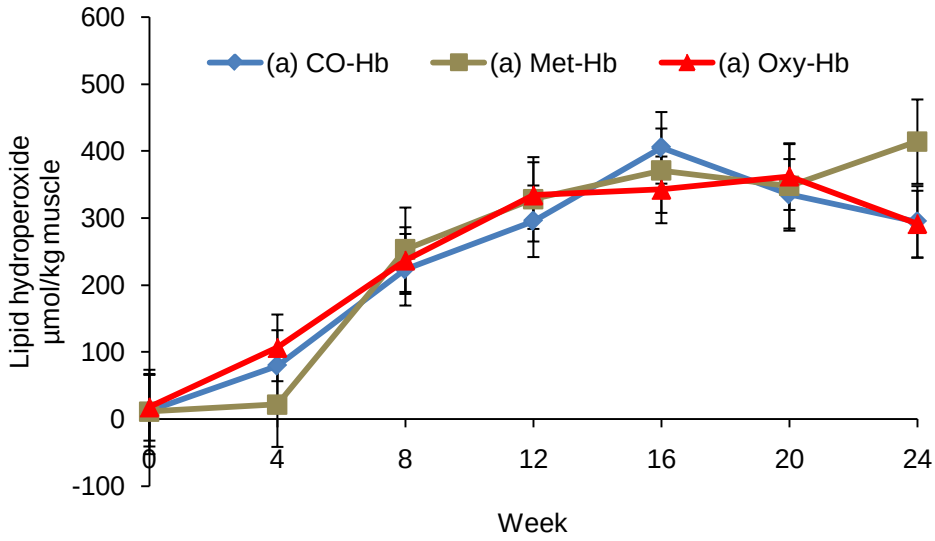


Figure 6-6. Lipid hydroperoxide values in washed tilapia muscle at -25ºC with 450 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

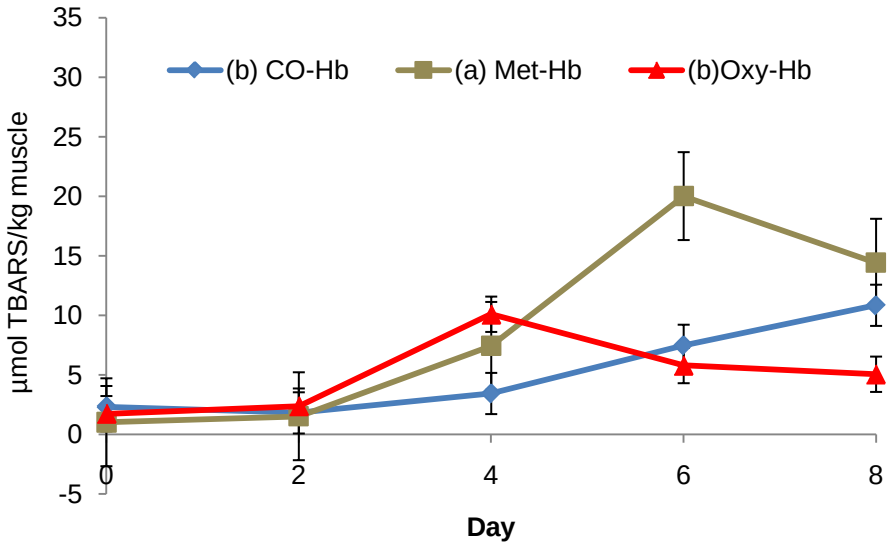


Figure 6-7. TBARS values in washed tilapia muscle at 3.7ºC with 150 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

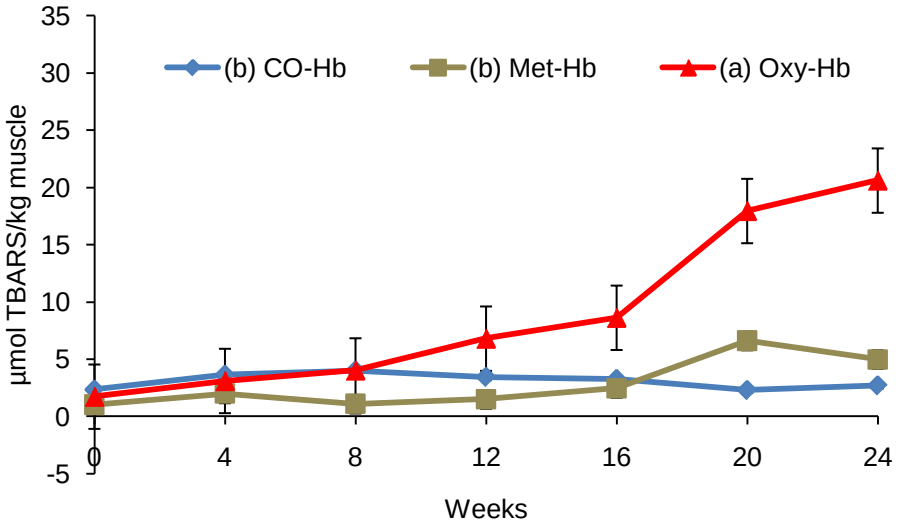


Figure 6-8. TBARS values in washed tilapia muscle at -25ºC with no added NaCl and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

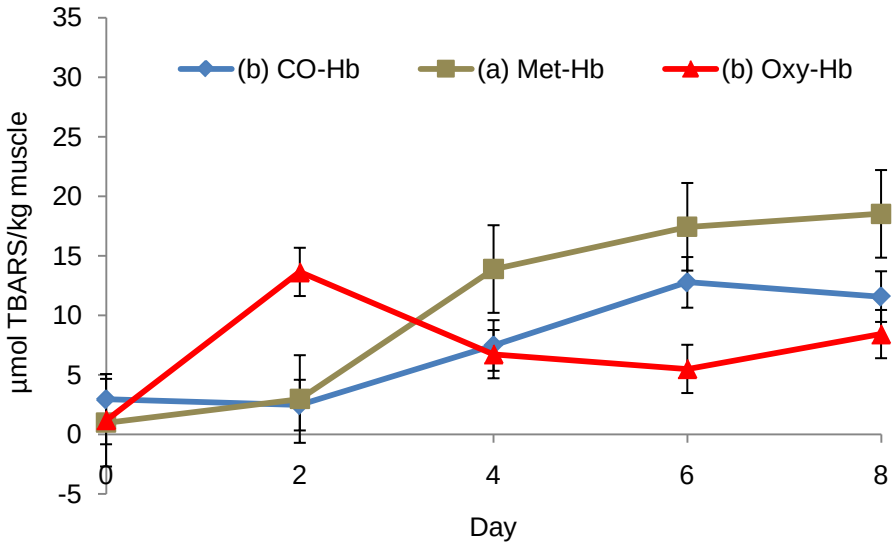


Figure 6-9. TBARS values in washed tilapia muscle at 3.7ºC with 150 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

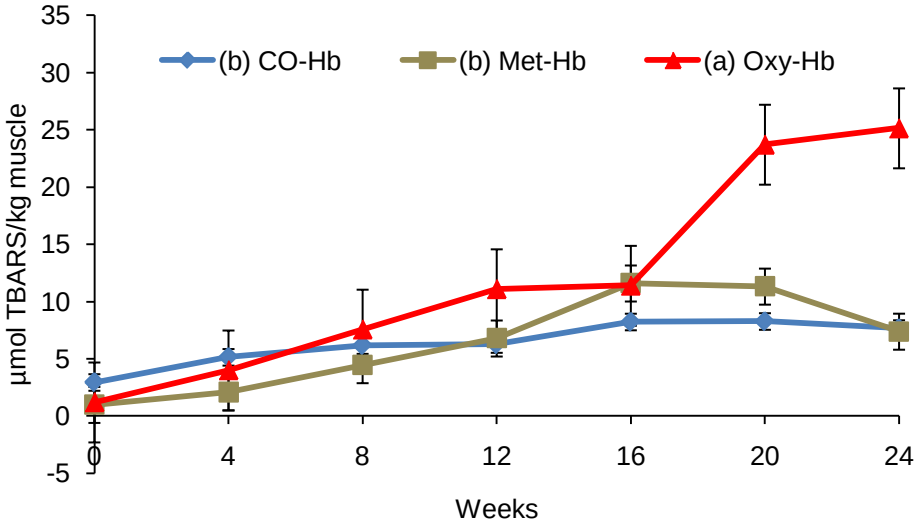


Figure 6-10. TBARS values in washed tilapia muscle at -25ºC with 150 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

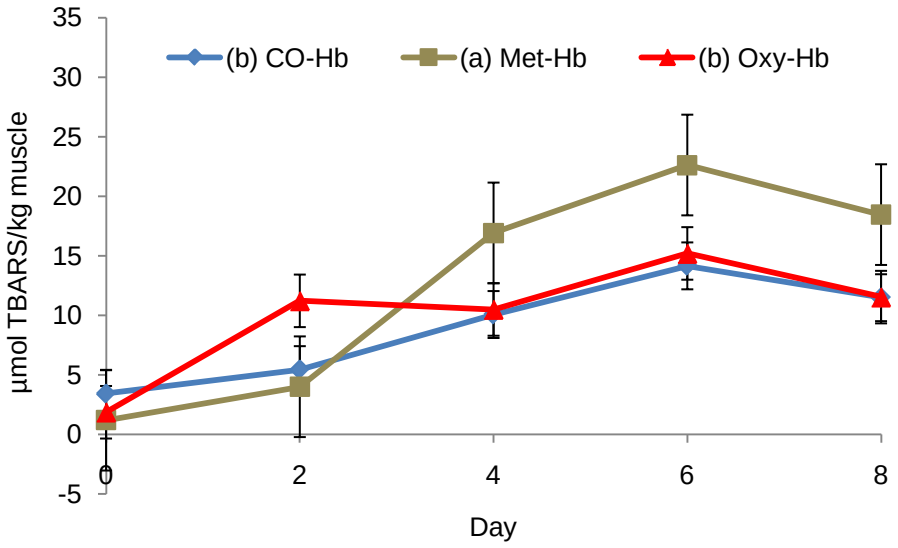


Figure 6-11. TBARS values in washed tilapia muscle at 3.7ºC with 450 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

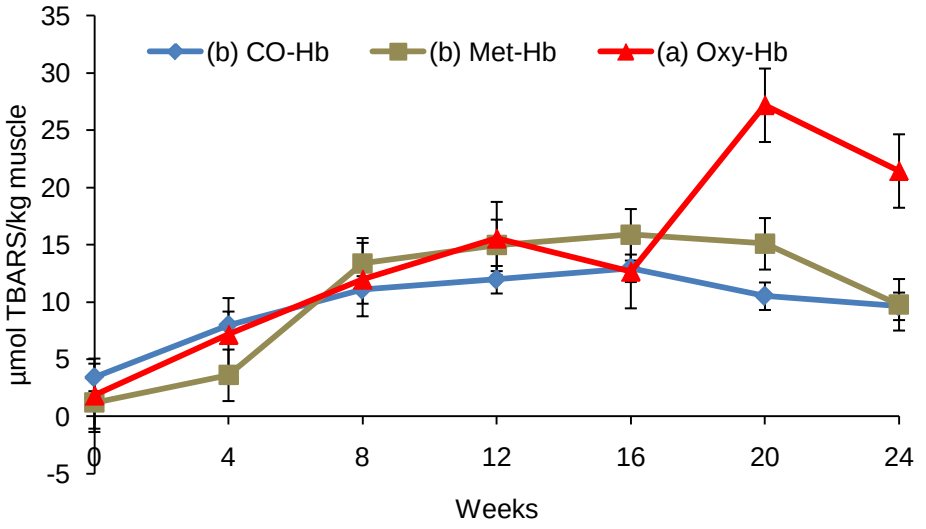


Figure 6-12. TBARS values in washed tilapia muscle at -25ºC with 450 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

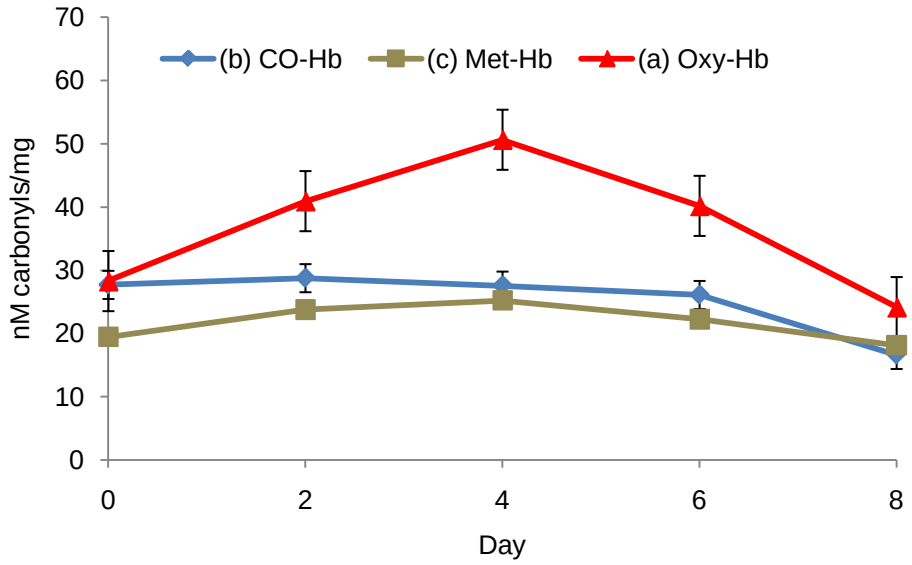


Figure 6-13. Carbonyl values in washed tilapia muscle at 3.7ºC with 150 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

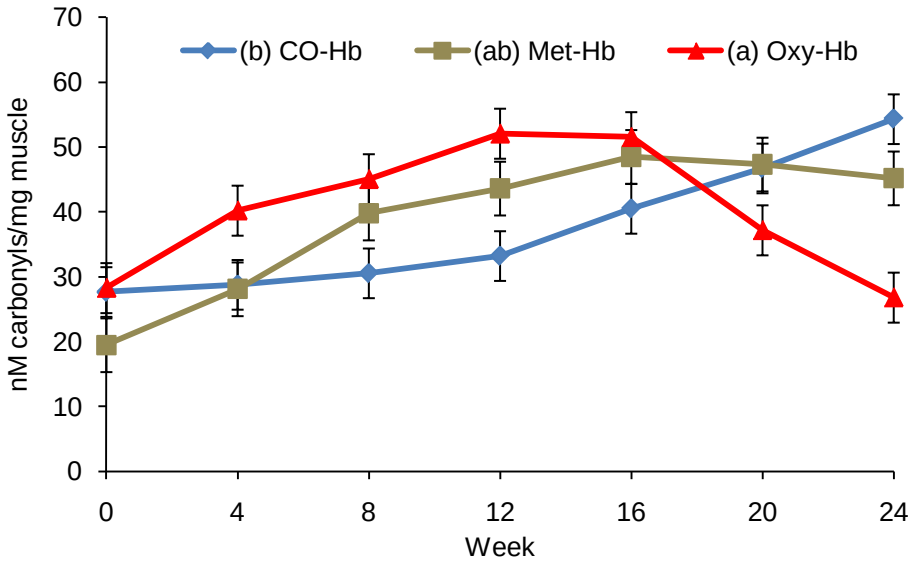


Figure 6-14. Carbonyl values in washed tilapia muscle at -25ºC with no added NaCl and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

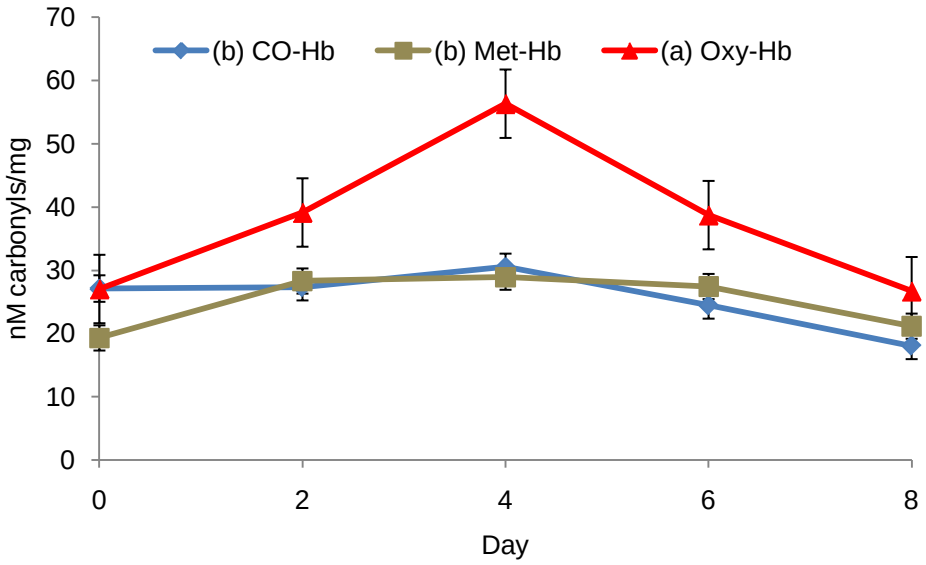


Figure 6-15. Carbonyl values in washed tilapia muscle at 3.7ºC with 150 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

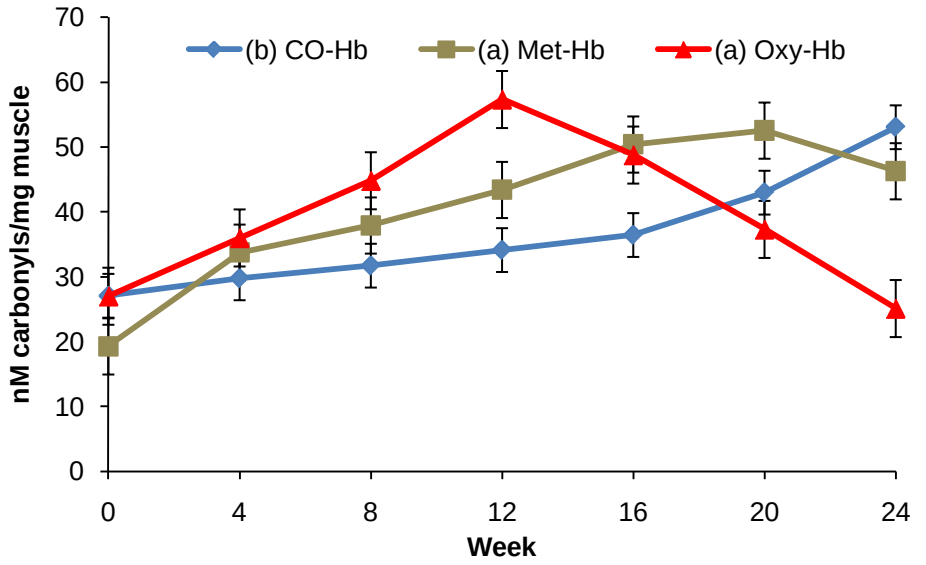


Figure 6-16. Carbonyl values in washed tilapia muscle at -25ºC with 150 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

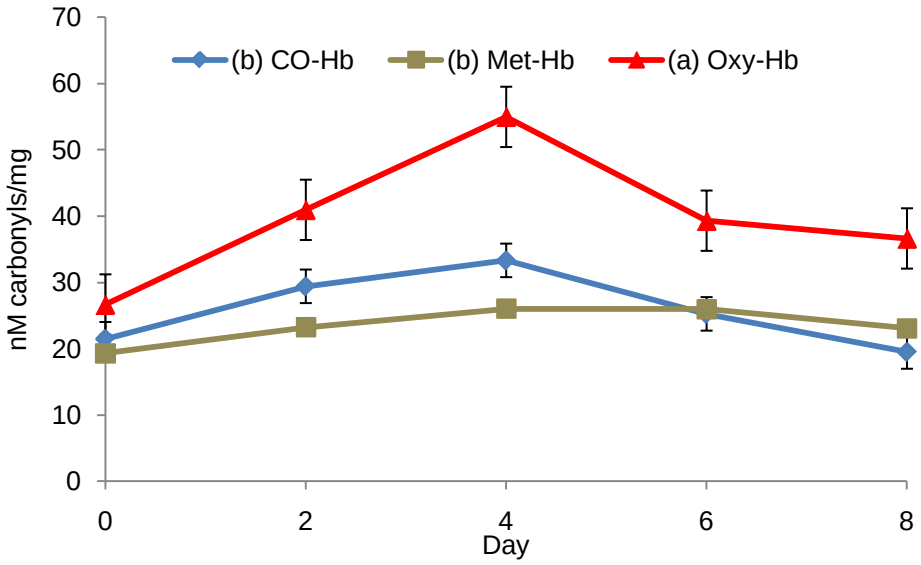


Figure 6-17. Carbonyl values in washed tilapia muscle at 3.7ºC with 450 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

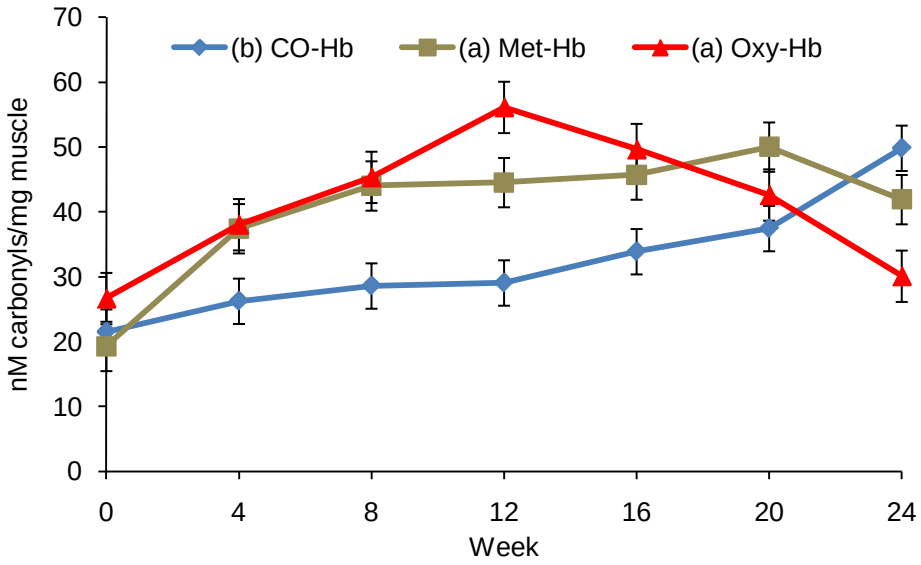


Figure 6-18. Carbonyl values in washed tilapia muscle at -25ºC with 450 mM NaCl added and containing different forms of Hb, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

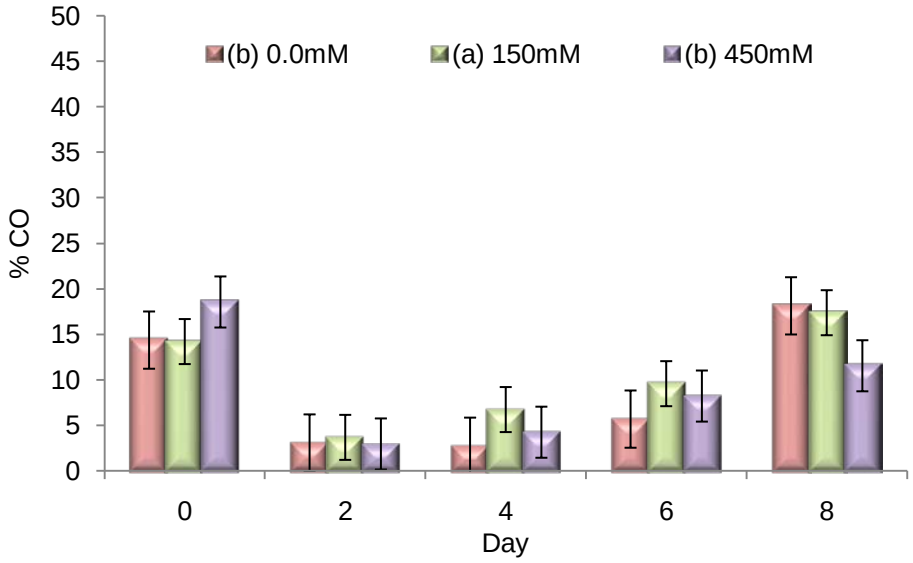


Figure 6-19. %CO released during 3.7ºC storage of washed tilapia muscle containing CO-Hb with 0, 150, and 450 mM NaCl added to the system. The effect of forms of Hb (Oxy-, CO-, and Met-Hb) by storage interaction averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

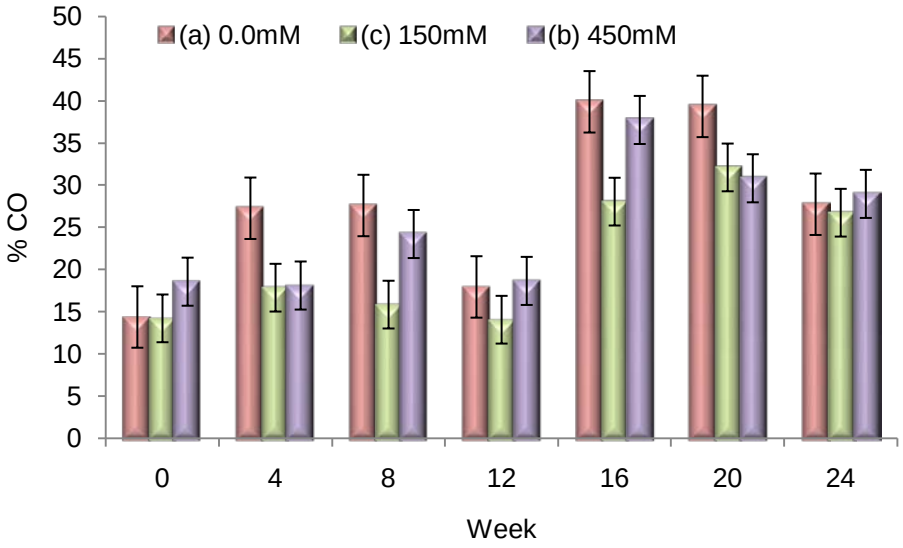


Figure 6-20. %CO released during -25ºC storage of washed tilapia muscle containing CO-Hb with 0, 150, and 450 mM NaCl added to the system. The effect of forms of Hb (Oxy-, CO-, and Met-Hb) by storage interaction averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

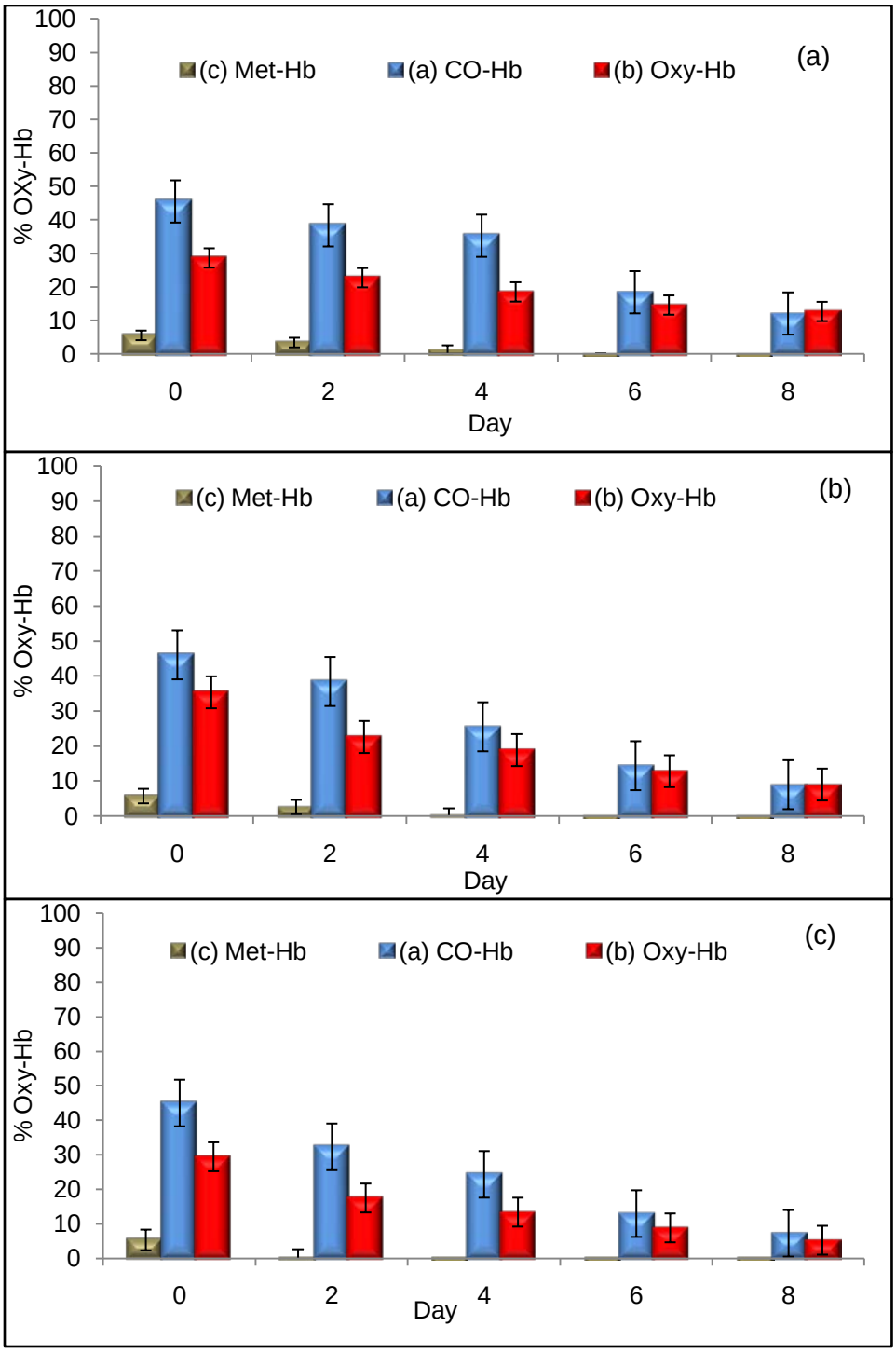


Figure 6-21. %Oxy-Hb formed in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl a) 0, b) 150, and c) 450 mM at 3.7ºC, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

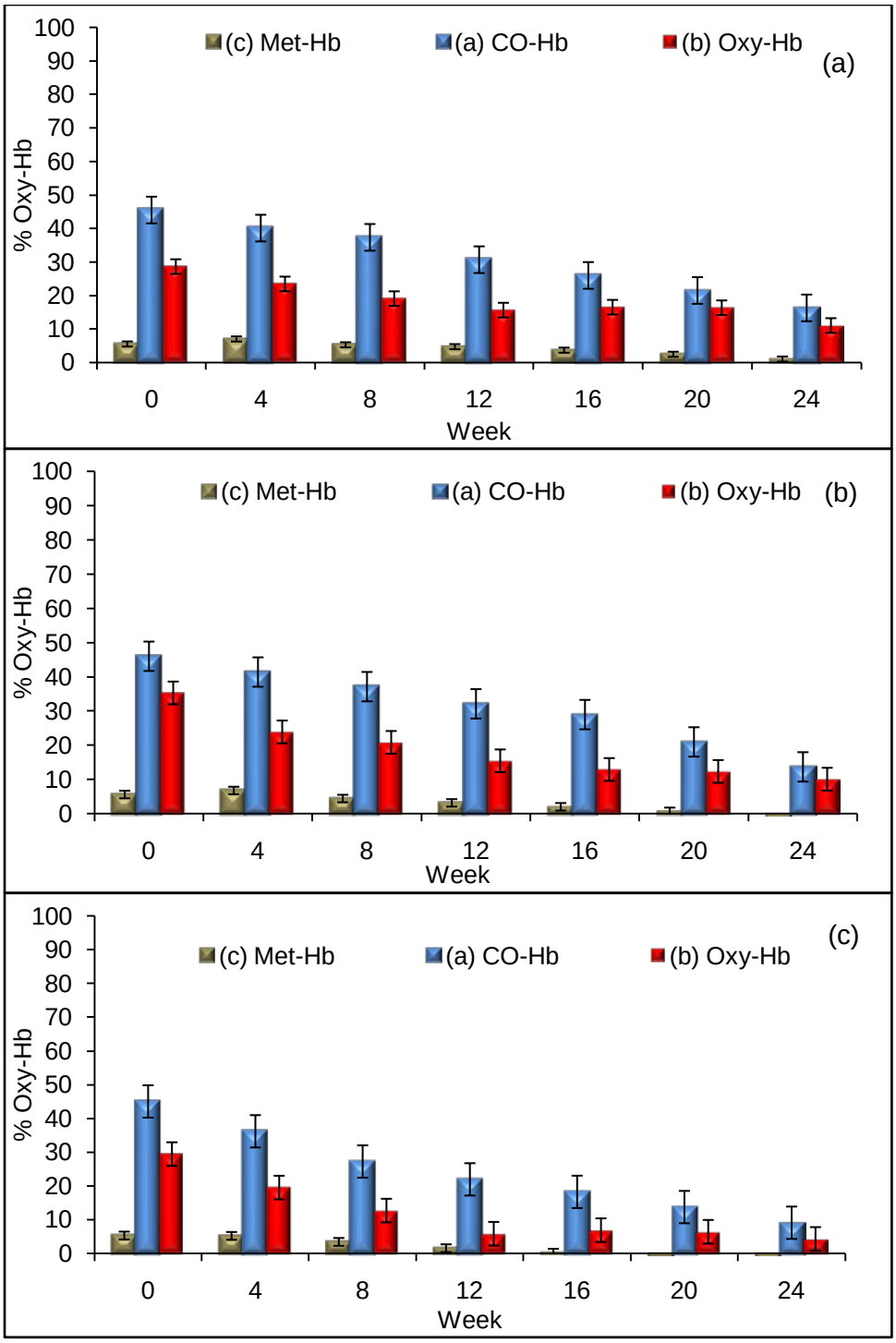


Figure 6-22. %Oxy-Hb formed in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl a) 0, b) 150, and c) 450 mM at -25ºC, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

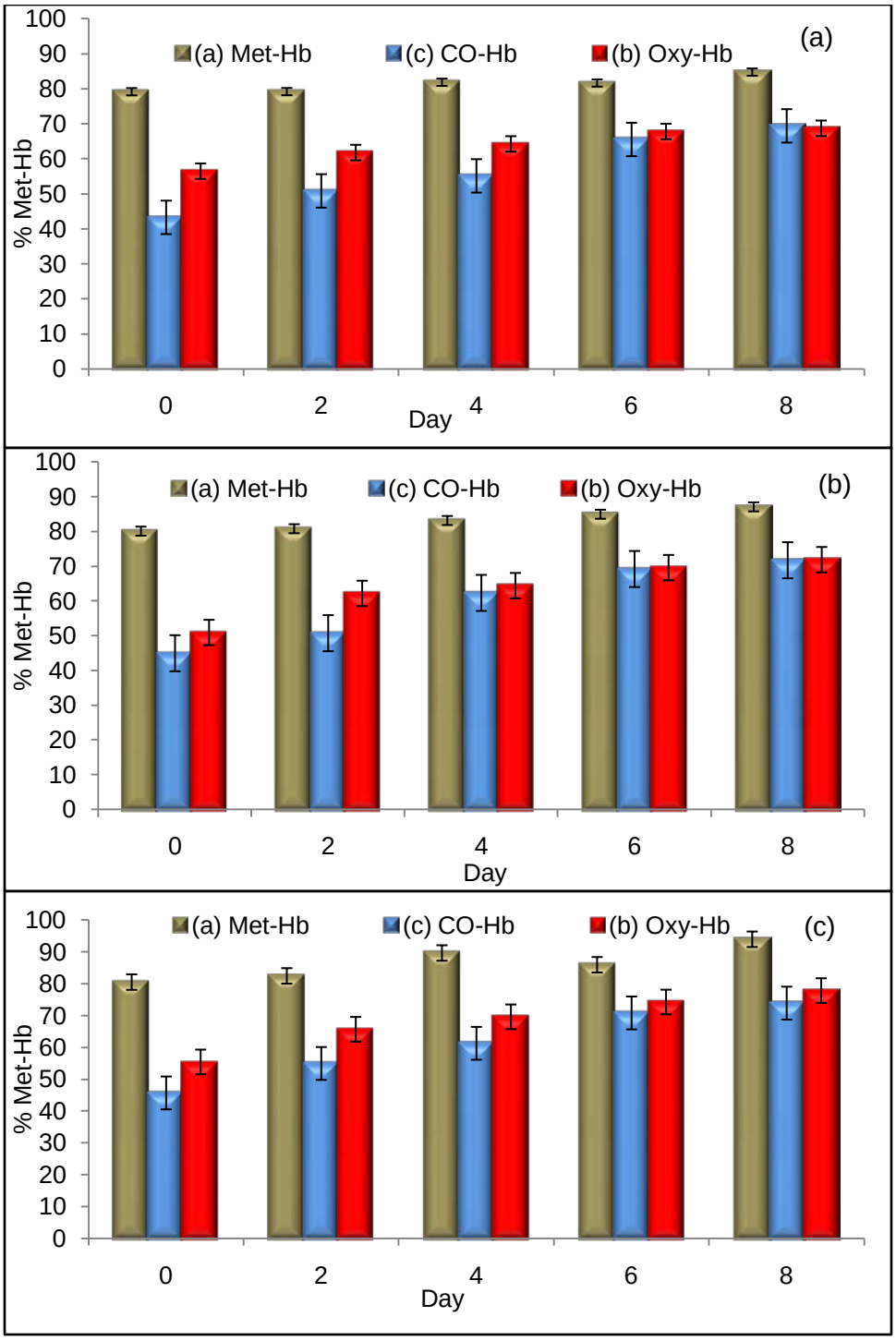


Figure 6-23. %Met-Hb formed in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl a) 0, b) 150, and c) 450 mM at 3.7ºC, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

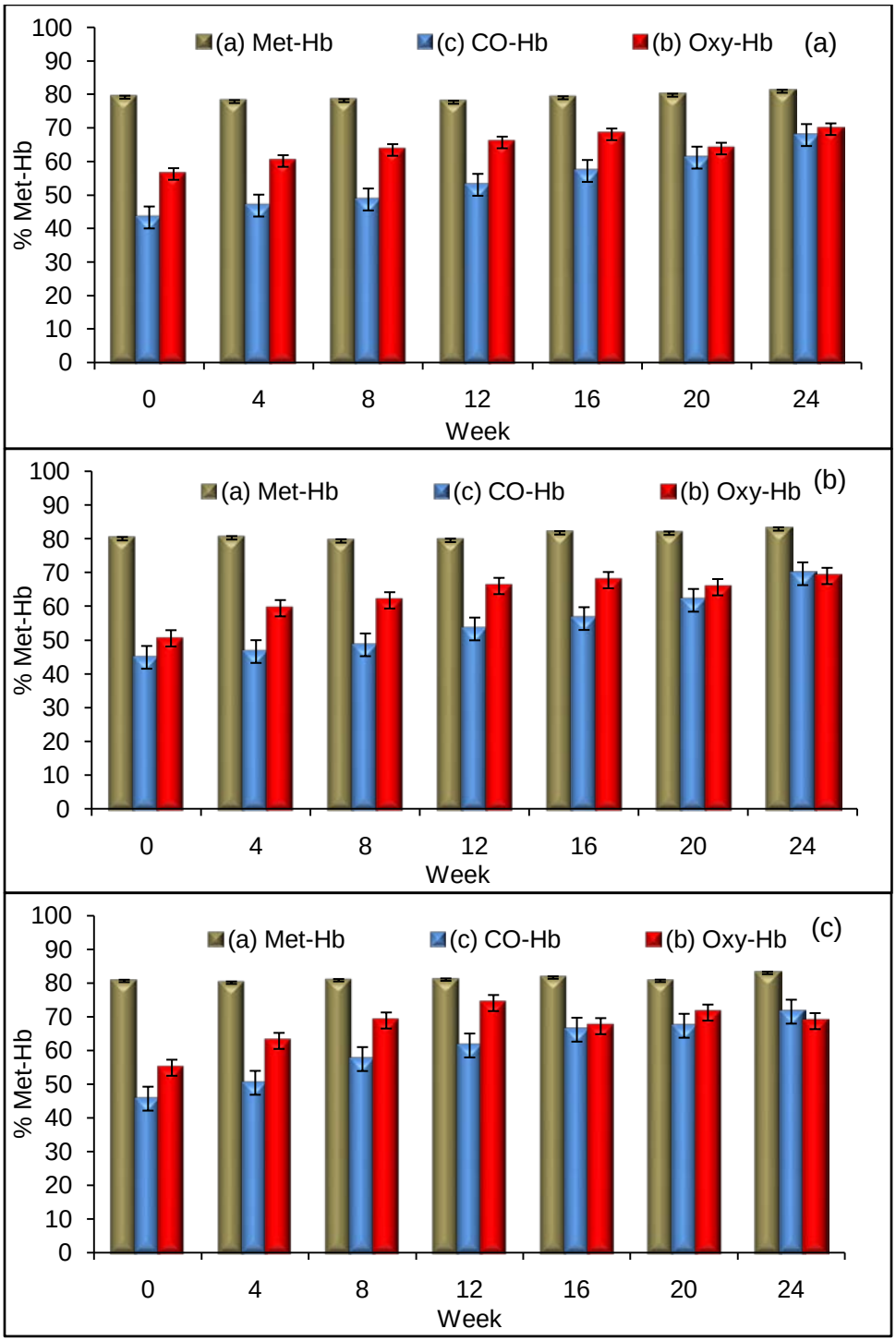


Figure 6-24. %Met-Hb formed in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl a) 0, b) 150, and c) 450 mM at -25ºC, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

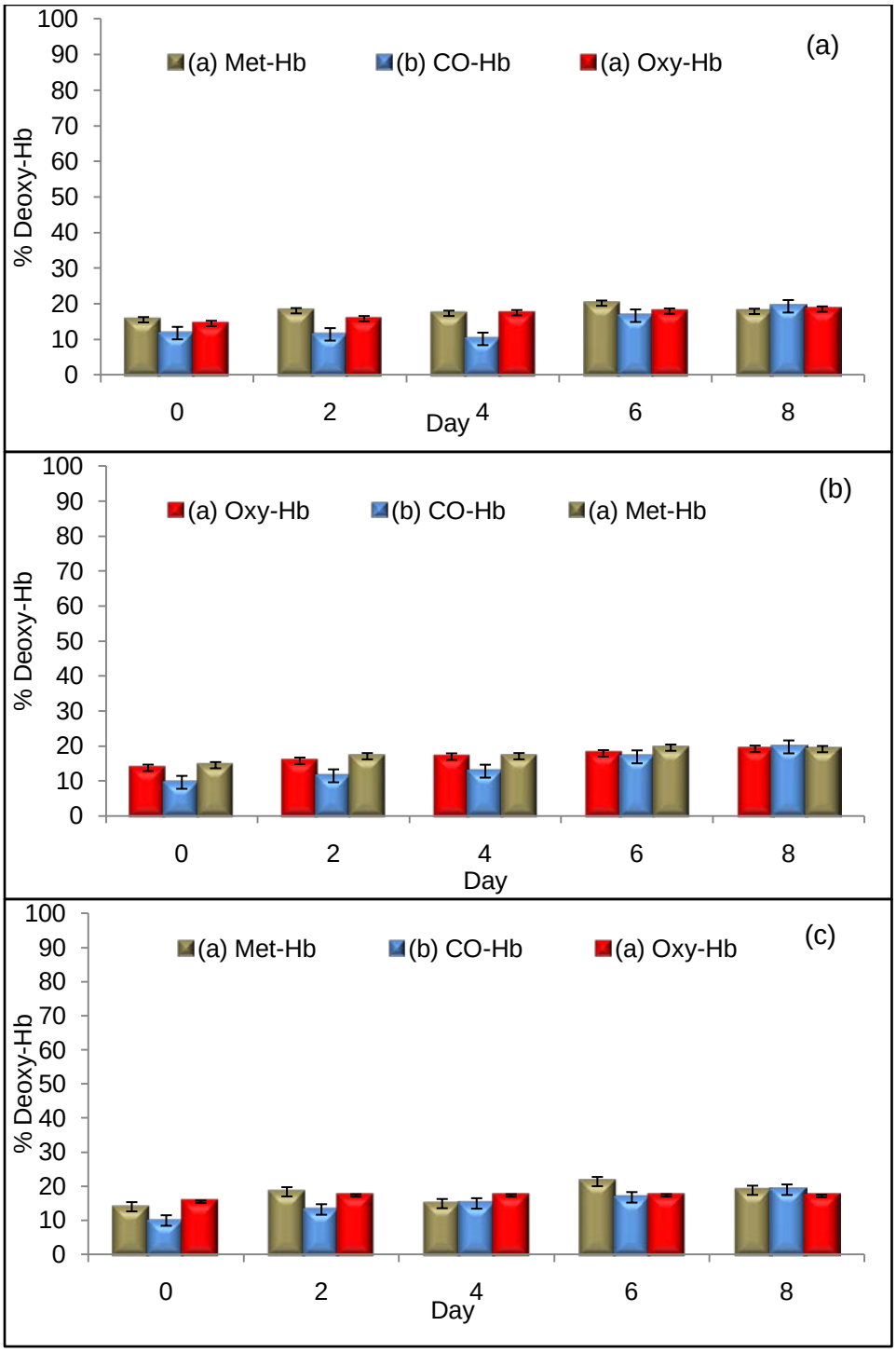


Figure 6-25. %Deoxy-Hb formed in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl a) 0, b) 150, and c) 450 mM at 3.7ºC, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

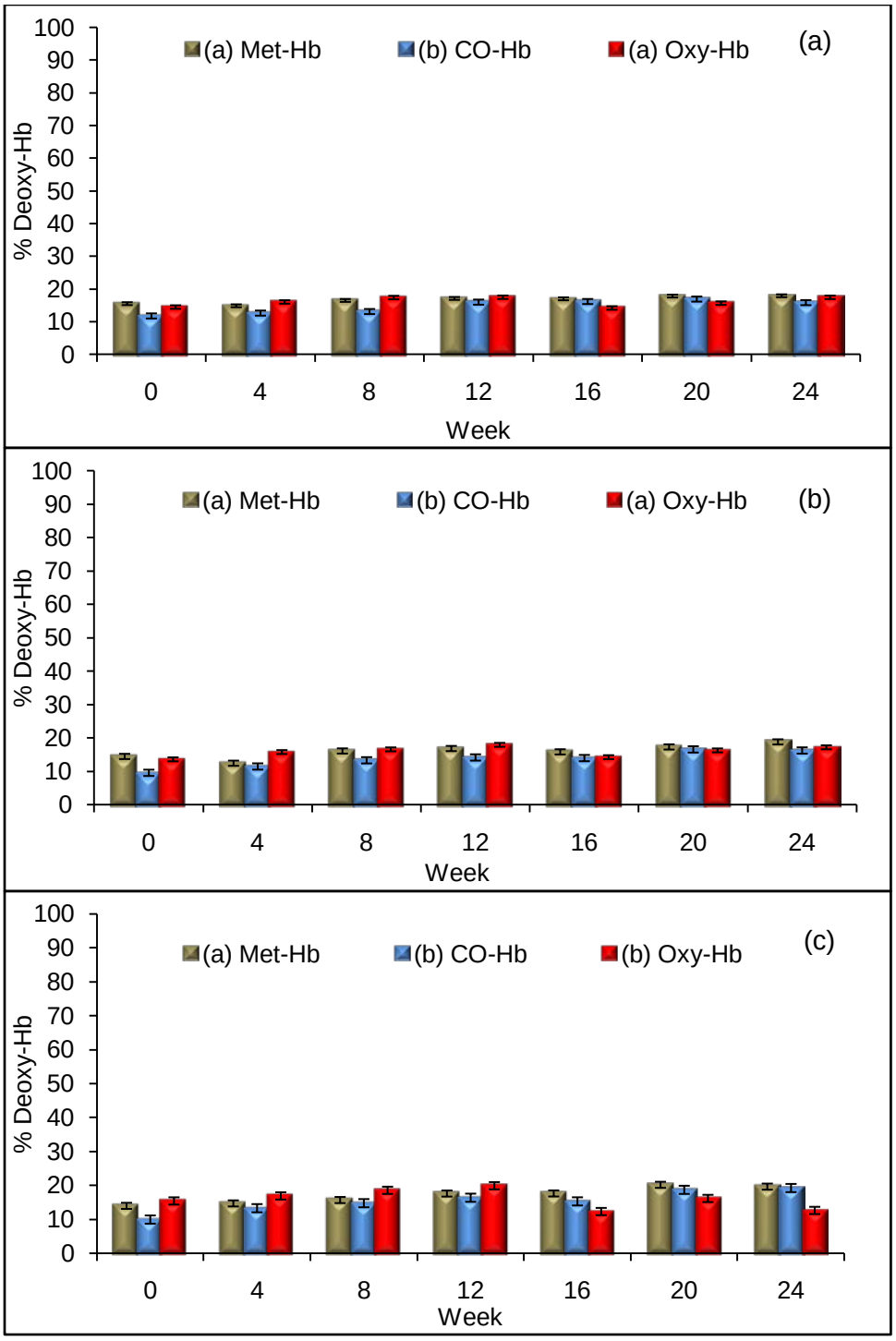


Figure 6-26. %Deoxy-Hb formed in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl a) 0, b) 150, and c) 450 mM at -25ºC, averaged across pH level and Hb concentration. Letters within the legend of the figure indicate statistically (<0.05) significant differences separated by Tukey's HSD.

CHAPTER 7
EFFECT OF THE OXIDATION OF OXY-, CO-, AND MET-HEMOGLOBIN ON THE QUALITY OF REFRIGERATED WASHED MINCED TILAPIA MUSCLE

Introduction

World consumption of fish has increased dramatically over the last 50 years and this increase is now only met with increased capacity of aquaculture (*116*). The stability of seafoods and the extension of the shelf life of seafood products has become crucial for the seafood industry. Of prime importance is the red color found in the dark muscle of fish like mahi mahi, tuna, and tilapia. The presence of this red color influences the consumer and is interpreted as “freshness”, thereby increasing its market value. The brown or dark color of fish suggests lack of freshness and poor quality (*79*). Maintaining this red color has been achieved in the past through refrigeration, freezing and modified packaging. More recently, the use of carbon monoxide and filtered wood smoke has been used to enhance this red color in fish muscle (*69*).

Carbon monoxide (CO) has been used to stabilize the red color in fish muscle. However, few studies have been published on the effect of CO on the oxidative stability of seafood products by stabilizing the heme protein, a key pro-oxidant, and preventing oxidation of oxygenated hemoglobin and myoglobin, (red color) to methemoglobin and myoglobin (brown color).

Quality of fish may have different meanings for different people. Furthermore, quality can be assessed by instrumental and sensory evaluations. Since the consumer is the ultimate judge of quality, correlation of instrumental and sensory evaluations will provide the best evidence of quality.

In the present study we examined effects of different degrees of oxidation on the development of painty/off odor and color changes in a washed tilapia muscle model

system at 3.7°C , assessed by a comparison between subjective (sensory evaluation), chemical (TBARS), and physical (a*value) analysis. Sensory evaluation is commonly utilized to assess the quality and freshness of fish and fish products during processing and storage. However, it is believed that various processing methods may mask (alter, modify to a certain degree) the subjective perception of freshness of seafood (*117*). For instance, some manipulations aimed to stabilize and prevent deterioration of the color may give false impression on quality and freshness of muscle foods to a consumer. In this part of the experiment, we aimed to determine the correlations between subjective (sensory evaluation) and objective methods (chemical presented by TBARS scores, and physical presented by a*values), and therefore verify the validity of descriptive analysis in rating of freshness of seafood (particularly tilapia) under different processing and storage conditions.

Materials and Methods

The following methods have been described in detail in chapter 3: Preparation of Washed Minced Tilapia Muscle (MTWM), Collection of Fish Blood, Preparation of Hemolysate, Quantification of Hb Levels in Hemolysate, Oxy-, CO- and Met-Hb, and Determination of Thiobarbituric Acid Reactive Substances (TBARS).

Sample Preparation: Addition of Hb and NaCl

Samples of tilapia washed model system previously prepared and stored at -80°C were thawed rapidly under running water (20°C) and kept on ice. The pH was adjusted to the desired pH of (6.3, 6.8, or 7.3) using 2N NaOH or 2N HCl. After the desired pH was established, moisture content was determined using a moisture balance (CSI Scientific Company, Inc., Fairfax, VA).

Oxy-hemoglobin previously prepared was thawed under running water and added to the muscle system to give a final concentration of 12 µmol/kg washed muscle. Hb was mixed manually into the WMTM system using a plastic spatula. The homogenous color of the minced muscle indicated adequate mixing. Hb was added to the system at three pH levels (6.3, 6.8, and 7.3). Concentrations of added NaCl to the samples were 0 and 450 mM. NaCl was mixed manualy. Samples were plated in Petri dishes (~25 g), covered with a lid, and stored at 3.7ºC for 6 days. All samples were stored in duplicate. The various combinations were conducted as presented in Table 7-1.

CO-Hb samples were prepared by gassing the washed system with 100% CO for two hours on ice. The WMTM was placed in a gastight vacuum bags equipped with a silicon septum valve obtained from LabPure® Instruments. CO-Hb was added to the muscle after removal from the bags to give a final concentration of ~12µmol/kg washed muscle. CO-Hb was mixed manually using a plastic spatula. CO-Hb was added to the system at three pH levels (6.3, 6.8, and 7.3). Samples were placed in Petri dishes (~25 g), covered with a lid on, and stored at 3.7ºC for 6 days. All samples were stored in duplicate (Table 7-1).

Met-Hb, previously prepared, was mixed manually with the washed system using a plastic spatula to a final concentration of ~12 µmol/ kg washed muscle. Met-Hb was added to the system at three pH levels (6.3, 6.8, and 7.3). Samples were plated in Petri dishes (~25 g), covered with a lid on, and stored at 3.7ºC for 6 days. All samples were stored in duplicate (Table 7-1).

One g of sample was taken at day 0, 3, and 6 for chemical analysis. The remainder of the samples were returned to 3.7ºC. For color analysis, 12 g of samples

were stored with a lids on. Due to the difficulty of mixing samples containing 450 mM NaCl, samples were taken from the core and side of the dishes. These samples were then stored in aluminum foil at -80ºC until analysis was performed.

Descriptive Sensory Analysis

Sensory analysis was conducted with modification of the procedure of Richards and others (*118*) to assess the qualitative and quantitative off odor of oxidation. A descriptive analysis sensory test was used. Thirteen panelists were chosen based on their ability to detect the rancid odor. Panelists were screened using a simple triangle test. Panelists were asked to sniff the samples in dark cups and identify the odd sample. The design of this Triangle Test is presented in Appendix D. Based upon the results of the triangle test, 13 panelists were then selected to be trained to rate the off odor (painty/rancid odor) using a 15 point scale, 1 being no off odor and 15 the strongest (Appendix E). Three sessions, 60 min each, were used to train the panelists to correctly rate the intensity of the off odor (painty/rancid odor). A 10-15 min break was taken between each set of samples. Panelist were trained using a set of samples previously prepared as a standard at pH 6.3 oxy-Hb day 0, 2, 4, and 6 with and without NaCl. Table 7-2 represents treatments used to train panelists.

Six evaluation sessions followed the training sessions. Table 7-3 represents the combinations of treatments evaluated by panelists. The samples within a session were randomly presented. For each sensory session, samples stored at 3.7°C for 0, 3, and 6 days were used. Oxy, CO and Met Hb at a Hb concentration of 12 µL/kg muscle were evaluated in duplicate in each session. Hemolysate was thawed under running water and mixed with the tilapia minced wash muscle in the form of Hb (oxy-, CO-, and met-Hb) and pH (6.3, 6.8, and 7.3) desired (Table 7-4). Sample were placed in capped dark

cups (11 g) and stored at 3.7°C for six days. Day zero samples were vacuum packed and stored at -80°C immediately followed by day 3 and day 6.

The sensory testing was conducted in the FSHN sensory lab where panelists were seated in separate booths. Color of the samples was masked using red light to avoid the influence of color on the rating of the odor intensity. Samples were thawed to 3.7°C 2 h prior to the test and kept on ice until presented to the panelist. Samples were assigned three random digit numbers. Panelists were asked to sniff the samples in the order presented from left to right and rate the intensity of the off odor. Test ballots are presented in Appendix E. Total number of samples evaluated in each session was 9 and each sample was evaluated in duplicate. Panelists were asked to take a 10-15 min break between each replication to avoid fatigue.

Color Analysis

Deterioration of red color (a*value) during oxidation in the washed muscle samples was measured using a digital Color Machine Vision System (CMVS) (*97*). The CMVS measures the average (a*value) for each sample. Pictures of samples were taken at the same intervals that samples were taken for chemical and sensory analysis. Change in color was correlated with TBARS and sensory results.

In a closed chamber (impermeable to stray light), a 12 g sample was placed. A digital camera (Nikon D200 Digital Camera, Nikon Corp., Japan) facing the bottom of the chamber was used to capture pictures of the samples. Two fluorescent lights (top of the chamber), each to simulate illumination by noonday summer sun (D65 illumination), were used to obtain uniformity of light. The Nikon D200 Settings used are shown in Table 7-4. A red reference tile was placed with each picture and used as a standard for the redness (a*value).

Statistical Analysis

The mean and standard deviation of a*value for oxy, CO and met-Hb; pH values 6.3, 6.8, and 7.3; and NaCl concentrations added of 0 and 450 during storage at 3.7°C for 0, 3, and 6 days are presented. Statistical comparisons were made between treatments using the General Linear Model Procedure and the mean separations were performed using Tukey's Least Square Means (SAS software 9.2). A statistical significance was reported as $p \leq 0.05$. The significance of interactions among Hb forms, Hb concentrations, pH values, and NaCl concentrations on lipid/protein oxidation is reported, Fisher's Z transformation correlation comparison and Correlated Correlation Comparison method were conducted (*119, 120*) comparing sensory scores and TBARS, sensory scores and a* value, and TBARS and a*value.

Results

Sensory Evaluation

A total of 13 trained panelists were used to evaluate the development of the painty odor in the tilapia muscle during storage at 3.7°C for 6 days as a measurement of oxidation. Using a 15 point scale, 1 being no off odor and 15 the strongest (Appendix E), the outcomes showed that the panelists detected painty/rancid odor under various treatments of the samples. When panelists were asked to evaluate samples containing the three different forms of Hb (oxy-, CO-, and met-Hb), no odor was detected on day 0 for any of the three Hb forms (Figure 7-1). However, for oxy-Hb samples, panelists were not able to detect ($p<0.05$) the painty odor by day 3. Similarly, no odor was detected for CO-Hb samples on day 3. Met-Hb samples, on the other hand, showed a significant development of the painty/rancid odor detected by the panelists on day 3. On day 6, for oxy-, and CO-Hb samples, panelists' scores showed a significant difference in the odor

compared to day 3, but met-Hb samples displayed no significant change between day 3 and 6 in the panelist's rating of the painty/rancid odor. The results of the interaction of Hb form by day (Figure 7-1) indicated that day 3 and day 6 for met-Hb samples were significantly different compared to oxy- and CO-Hb evaluated on the same days.

Figure 7-2, shows the results for the effect of the pH on the development on the oxidation assessed by the increase of the painty/rancid odor. It was observed that at pH 6.3, there was a significant difference between day 0 and day 3, but no difference in day 6 compared to day 3. The panelists were unable to detect the difference of the sample at pH 6.8 on day 0 or day 3. However, panelists' score showed a significant increase in the odor by day 6 for pH 6.8 samples. For pH 7.3, there was no significant difference in the panelists' rating between day 0, 3, or 6. The results of the interaction of panelists by day (Figure 7-2) showed that day 3 was rated significantly higher for pH 6.3 samples only. While day 6 for pH 6.8 was not different ($p<0.05$) from day 6 for pH 6.3 and 7.3, day 6 for pH 6.3 was significantly different from day 6 for pH 7.3.

Regardless of the concentration of NaCl used, panelists' scores indicated a significant development of the painty/rancid odor after storage for 3 days at 3.7°C (Figure 7-3). However, no additional odor was detected by day 6 for either samples with and without added NaCl. The results of the interaction of NaCl by day (Figure 7-3) showed there was no NaCl effect.

Lipid Oxidation Analysis

TBARS was used to measure lipid oxidation in the muscle system. The effect of different forms of Hb (oxy-, CO-, and met-Hb) is presented in Figure 7-4. Met-Hb had significantly ($p\leq0.05$) less oxidation lag time compared to oxy- and CO-Hb samples. However, the amount of TBARS developed on day 6 did not differ significantly from day

3 for met-Hb samples. Oxy-Hb was not significantly different from CO-Hb on day 0 or day 3, however, oxy-Hb showed more stability on day 6 of storage compared to CO-Hb. The interaction of day by TBARS showed that for all three forms of Hb, day 3 was significantly higher in TBARS for met-Hb samples compared to oxy-, and CO-Hb (Figure 7-4).

The oxidation lag phase was found to decrease significantly as pH level decreased (Figure 7-5). For pH 6.3, there was a significant difference between day 0 and day 3 in the amount of TBARS formation, but not between day 3 and day 6. The same pattern of oxidation was observed for pH 6.8. After 6 days of storage at pH 7.3, no significant change in the amount of TBARS was observed. The results of the interaction of day by TBARS indicated that all three pH levels for day 0 and day 6 had no significant difference in the amount of TBARS detected. However, at day 3, pH 7.3 was significantly different from pH 6.3 in the amount of TBARS, but not different from pH 6.8 which also did not also differ from pH 6.3. Thus, the interaction of day by TBARS indicates that only day 3 had significant differences for the three levels of pH (Figure 7-5).

The effect of NaCl on the development of oxidation during storage for 6 days at 3.7°C is shown in Figure 7-6. Regardless of the concentration of added NaCl (0, and 450 mM), the oxidation lag time did not differ significantly between different concentrations. However, there was a significant difference between the amounts of TBARS detected on day 3 compared to day 0 for both concentrations (Figure 7-6). Day 6 was not significantly different from day 3 for both concentrations. The interaction of

day by TBARS indicated no significant difference between both concentrations of NaCl on each day of analysis (Figure 7-6).

Correlation between Sensory Scores and TBARS

The amount of TBARS formed during storage for 6 days was highly correlated (Figure 7-7) with panelists ratings of painty/rancid odor ($R= 0.95$) at $p<0.0001$. As oxidation, measured using TBARS, increased in the muscle system, the panelists rating of painty/rancid odor increased. Hb forms, pH levels, and NaCl concentrations had no significant effect on the correlation between TBARS and sensory scores.

Color Analysis

Effect of different forms of Hb (oxy-, CO-, and met-Hb), different pH levels (6.3, 6.8, and 7.3), and effect of different concentrations of added NaCl (0, and 450 mM) on the stability of Hb color in tilapia washed system are presented in Table 7-5. Color analysis was conducted using a Color Machine Vision Systems where average a* (redness) value of the whole surface of the tilapia muscle was measured during 6 days of storage at 3.7°C (Table 7-5).

Redness of samples containing oxy-Hb decreased significantly after 3 days of storage. Further decrease in redness for oxy-Hb on day 6 was not significantly different from day 3. CO-Hb maintained its redness through storage at day 3, but had a significant decrease in color by day 6. Color of met-Hb samples did not change significantly during storage for 6 days, however, its redness was significantly less than oxy-, and CO-Hb forms for each day of analysis as indicated by the interaction of day by a*value (Table 7-5).

Redness of samples was affected by the level of pH (Table 7-5). As pH increased, the a*value (redness) for samples became more stable during storage. Low pH (6.3)

samples had significantly lower a*values for each day of storage. After 6 days of storage, pH 6.8 samples showed a significant loss of redness. High pH (7.3) maintained a stable a*value during storage (Table 7-5). The interaction of day by a*value indicated a significant difference in the redness for all pH levels on day 6 with pH 6.3 having significantly lower a*value and pH 7.3 having higher a*value.

NaCl had an effect on red color (Table 7-5). In samples with no added NaCl, a significant decrease in a*value was detected on day 6 of storage. However, addition of 450 mM NaCl/kg muscle resulted in significant loss of redness by day three and a further significant decrease was detected on day 6. The interaction of NaCl by day indicated a significant loss of redness for the concentration of 450mM on day 6 (Table 7-5).

Correlation between Sensory Scores and a*Value

As a*value decreased, the panelists' scores of painty/rancid odor increased and were highly negatively correlated ($R=-0.81$, $p<0.0001$) (Figure 7-8). For correlation between panelists' scores and color, pH levels and NaCl concentrations had no significant effect. However, different forms of Hb did (Table 7-6). Met-Hb samples did not have as strong a correlation ($R=-0.66$) as the other forms (oxy and CO had correlations of $R= -0.96$). The p value for comparing met and oxy is 0.0013 and p value for comparing met and CO was 0.0017 (Table 7-6).

Correlation between TBARS and a*Value

It was observed that there was a correlation ($R= -0.71$) between TBARS and a*value at $p<0.0001$ (Figure 7-9). The a*value (redness) decreased as oxidation proceeded over time during storage for six days at 3.7°C and TBARS value increased. For correlation between TBARS and a*value, pH levels and NaCl addition had no

significant effects. However, different forms of Hb had an effect. The met-Hb samples (R=-0.50) did not have as strong a correlation as oxy-Hb (R=-0.84, p =0.0663) and the CO-Hb samples (R=-0.88, p =0.026) as presented in Table 7-6. The correlation between TBARS and a*value was significantly different from the correlation between panelist and a*value (p =0.0001) (Figure 7-9).

Discussion

The degree of deterioration in sensory quality and freshness (odor, color, texture, and flavor) of seafood is thought to be highly dependent on the degree of lipid peroxidation, which in turn may be affected by a number of conditions, including pH, storage temperature, prevalence of certain types of Hb, extent of storage, etc (*80, 81*). Specifically, the level of rancidity of the lipids marked by painty/off odor development in various fish samples was correlated to a degree of lipid peroxidation (*23*). The findings of this study demonstrated a statistically significant correlations between trained panelists scores, the development of thiobarbituric acid reactive substances (TBARS), and deterioration in red color (a*value). Furthermore, the observed correlation was not significantly affected by the type of treatment, Hb form, pH levels, NaCl added concentrations, or extent of storage, verifying the usefulness of descriptive analysis to assess oxidation in the washed tilapia muscle system. The accuracy of the sensory evaluation as well as the strong relationship between painty odor and the degree of lipid peroxidation in tilapia muscle meat in this experiment certifies the use of painty/off odor as a valid marker of lipid oxidation.

Clear association was observed between panelists' scores and results of lipid oxidation analysis determined by the TBARS method (Figure 7-7). This is in agreement with previous reports investigating different markers of lipid oxidation in fish products.

The TBARS method has been shown to be a useful measure of rancidity development, and also correlates well with the results of sensory evaluation in many seafood products (*11, 55*). Specifically, when asked to evaluate the odor in fish samples treated with different forms of Hb, it appears that panelists' ratings were accurately corresponding to the presence and quantity of products of oxidation expressed as TBARS. The addition of met-Hb to tilapia produced a significant rancid odor by day three which did not change significantly by day 6 (Figure 7-1). In contrast, in the samples with Oxy- and CO- Hb, off odor was not detectible on day 3, and appeared only by day 6. TBARS analysis demonstrated shorter oxidation lag time and time needed to reach a plateau in samples treated with met-Hb (Figure 7-4). The met-form of Hb has been shown in numerous experiments to induce lipid oxidation in different muscle systems (*80*). It was proposed that the pro-oxidant activity of met-Hb is due to its structural characteristics promoting autoxidation, and ultimately, lipid peroxidation. Recent research has found that met-Hb is a potential pro-oxidant at the pH found in fresh meat (between 5.3 and 6.2) which emphasizes the importance of hydroperoxides in met-Hb initiated lipid oxidation (*53*). Electrostatic and hydrophobic interactions are involved when Hb binds to phospholipids. Met-Hb affects the structural and physiochemical parameters of the lipid-water interface (*54*). This results in the formation of hemichrome, a poor initiator of lipid oxidation (*41*) at physiological pH in model systems containing long-chain free fatty acids, resulting in non-catalytic activity. At lower pH values, the electrostatic and hydrophobic interactions are not involved, most likely due to the different charge distribution on both the fatty acid and the heme protein (*5*). Thus, in the presence of lipids, met-Hb at physiological pH, due to the formation of the non-catalytic hemichrome,

can undergo rapid neutralization. However, in a high lipophilic environment, denaturation of the heme protein may result in exposure of the heme group to the surrounding lipids and induce lipid peroxidation. However, at lower pH values, met-Hb is able to initiate lipid oxidation in a lipid hydroperoxide-dependent mechanism.

In contrast, oxy-Hb and CO-Hb have lesser pro-oxidant activity which was reflected by slower TBARS production as well as retarded development of rancid odor by day 6 (Figures 5-4 and 5-7). This supports results of previous investigations (*57*). Replacing oxygenated Hb and Mb with CO-Hb and CO-Mb in the dark muscle of fish was shown to offer some protection against lipid oxidation (*4, 69, 70*). High concentrations of unsaturated fatty acids (e.g. linoleic acid) in dark muscle of fish, in the presence of oxy-Hb and deoxy-Hb, may result in increased levels of lipid peroxides. This is due mainly to the autoxidation and deoxygenation of Hb, especially at reduced pH levels. CO treatment retards autoxidation of Hb and myoglobin to the ferric form, and thus plays a role in decreasing lipid oxidation, and possibly extending the shelf life of fish fillets treated with CO. Danyali (*71*) found that CO and filtered smoke (FS) treatment can be effective in retarding lipid oxidation. Filtered smoke, which contains phenolics that have the potential to serve as antioxidants, was not more effective than the other gas treatments in retarding oxidation development. After tuna steaks were treated with FS, 4%, 18% and 100% CO, Kristinsson and others (*72*) measured lipid oxidation using TBARS (thiobarbituric acid reactive substances). They found that the 4% CO treated tuna had higher TBARS than untreated tuna. The authors theorized that the higher levels of oxygen and CO_2 (which results in a drop in muscle pH) in the 4% CO may have promoted oxidation of heme protein. The 18% CO and FS (containing

approximately 18% CO) may have protected the heme molecule against the action of CO_2. Garner and others (*121*) reported that 100% CO was more effective than 100% nitrogen in retarding lipid oxidation, which suggests that the effect is not due solely to the absence of oxygen. Richards and Hultin (*4*) reported the presence of pro-oxidants of fish muscle, which may be reduced through the stabilization of the heme protein by CO (*72*). Kristinsson and others (*72*) conclude that treating yellowfish tuna with medium to high levels of CO may stabilize the heme protein molecule against lipid oxidation.

Tested pH levels (6.3, 6.8, and 7.3) were chosen to reflect the range of post mortem pH of fish products. Our findings revealed accelerated formation of TBARS at an acidic pH when compared to more basic pH levels. These findings are in agreement with previous investigations describing more rapid lipid oxidation at lower pH values. Richards and others (*80*) suggest that at low pH values (pH 6.3), tilapia Hb subunits dissociate faster and increase release of hemin. This may be due to the weakened heme-globin linkage caused by the protonation of proximal histidine. The structural change in Hb increases the proton transfer mechanism. Kristinsson and others (*69*) and Kristinsson and Hultin (*10*) reported that at lower pH, the protein is partly unfolded, giving the heme portion greater ability to participate in oxidation. At higher pH levels, oxy and CO Hb may have greater stability of the protein structure, resulting in lower lipid peroxide formation. In contrast, met-Hb, which is readily formed at acidic pH, is believed to catalyze oxidation by the breakdown of preformed lipid peroxides (*11*). A greater degree of rancid odor at lower pH values (6.3) compared to intermediate (6.8) versus highest (7.3) was also observed. This trend was not affected by the extent of storage (0, 3, or 6 days).

The degree of redness of fish muscle is known to be affected by a multitude of factors, most importantly: pH, type of Hb present, and extent and temperature of storage (*122*). In this study, samples with CO-Hb displayed the greatest stability of a*values, with significant decrease in color only by day 6 (Figure 7-1). It was previously described that CO treated samples have the ability to retard lipid oxidation, and stabilizing redness of fish muscle samples (*122*). The data from the present experiment establishes a strong link between lipid oxidation and degree of color stability/changes. Met-Hb, recognized as a strong promoter of lipid oxidation in muscle food systems, produced a considerable decrease in redness on day 0, and did not significantly change by day 6 (Table 7-5). Although the rancid odor of samples contained met-Hb was detected by panelists only by day three, no further changes in odor were detected by day 6 (Figure 7-2). Results of color analysis for oxy-Hb were somewhat intermediate between CO- and met-Hb, with decrease in redness detected by day 3 without significant further changes by day 6. Sensory evaluation of corresponding samples showed off odor by day 3 increasing in intensity by day 6.

Lastly, in the present study we observed no significant effect of addition of different concentrations of added NaCl (0, and 450 mM) on TBARS, a*value, or sensory scores. It was previously reported that NaCl may disrupt the stability of oxy-Hb, accelerating its deoxygenation and formation of met-Hb (*84*). From accumulated data it appears that the pro-oxidative activities of NaCl may be due, in part, to its ability to hasten release of iron from heme (*82*). In particular, the chloride anion was implicated in Hb autoxidation, serving as a substrate for a chloroperoxidase in the presence of preformed hydrogen

peroxide (*84, 111*). In addition, it was proposed that NaCl may have the ability to accelerate lipid peroxidation by shifting iron-oxygen to iron-hydroperoxide interactions.

Osinchak and others (*123*) used a press juice of atlantic mackerel (model system) to examine the effect of NaCl on lipid oxidation. The authors found that NaCl was clearly a potent pro oxidant of lipid oxidation which they moniterd by the development of TBARS. Harel (*85*) found NaCl to inhibit the hydroxyl radical. It was suggested that NaCl inhibits ferrous iron oxidation. Wallace and others (*84*) found that NaCl affected the stability of oxy-Hb and accelerated the oxidation of oxy-Hb to met-Hb, thus enhancing Hb autoxidation. This appeared not to be consistent with the findings of the washed tilapia model system.

Conclusion

Sensory analysis of washed mince muscle containing 12 µmol Hb/kg of CO-, oxy-, and met-Hb at pH 6.3, 6.8, and 7.3 with and without added salt revealed that panelists' detection of rancidity was highly correlated with TBARS analysis. Sensory scores correlated negatively with a* value, giving further evidence of the accuracy of sensory analysis to detect rancidity in a washed mince model system. The significant negative correlation between a*value and TBARS gives a measure of the consistency of the results presented. Panelists found no significant differences in samples with and without addition of NaCl.

Table 7-1. Treatments used in the study. Hemoglobin was added to washed tilapia muscle at a concentration of 12 µmol Hb/kg muscle and stored at 3.7ºC.

Hb-form	pH	NaCl added (mM/Kg)
Oxy-Hb	6.3	0
		450
	6.8	0
		450
	7.3	0
		450
CO-Hb	6.3	0
		450
	6.8	0
		450
	7.3	0
		450
Met-Hb	6.3	0
		450
	6.8	0
		450
	7.3	0
		450

Table 7-2. Reference samples used to train panelists for the descriptive analysis testing

Treatment	pH	Hb [µmol/kg]	Days
Oxy-Hb without added NaCl	6.3	12	0, 2, 3, 4, 6
Oxy-Hb with 450 mM NaCl	6.3	12	0, 2, 3, 4, 6

Table 7-3. Combination of treatments used by panelists to rate the formation of painty/rancid odor as an indicator of lipid oxidation

Session	Treatment	pH	NaCl added [mM/kg muscle]	Storage Days
1	Oxy-, CO-, Met-Hb	6.3	0	0, 3, 6
2	Oxy-, CO-, Met-Hb	6.3	450	0, 3, 6
3	Oxy-, CO-, Met-Hb	6.8	0	0, 3, 6
4	Oxy-, CO-, Met-Hb	6.8	450	0, 3, 6
5	Oxy-, CO-, Met-Hb	7.3	0	0, 3, 6
6	Oxy-, CO-, Met-Hb	7.3	450	0, 3, 6

Table 7-4. Nikon D200 Settings used for measurement of change in color during storage period.

Setting	Specification
Lens	VR 18-200 mm F 3.5-5.6 G
Focal length	36 mm
Sensitivity	ISO 100
Optimize image	Custom
High ISO NR	Off
Exposure mode	Manual
Metering mode	Multi-pattern
Shutter speed and aperture	1/3s-F/11
Exposure compensation (in camera)	-1.0 EV
Focus mode	Manual
Long exposure NR	Off
Exposure compensation (by capture NX)	0 EV
Sharpening	Normal
Tone compensation	Normal
Color mode	Mode I
Saturation	Normal
Hue adjustment	0°
White balance	Direct sunlight
Zoom	Manual

Table 7-5. Changes in a*value of tilapia muscle after the addition of the Hb and NaCl at three different pH levels followed by storage for 6 days at 3.7°C

Storage time (day)	Oxy-Hb	CO-Hb	Met-Hb
0	26.3±2.6^{a*}	23.3±3.1^{a*}	8.6±0.6^{a**}
3	20.0±10.8^{b*}	20.2±8.5^{a*}	6.9±3.5^{a**}
6	16.6±13.1^{b*}	14.8±11.4^{b*}	3.6±1.1^{a**}
Storage time (day)	pH 6.3	pH 6.8	pH 7.3
0	17.8±7.1^{a*}	19.5±9.0^{a*}	20.9±9.8^{a*}
3	7.3±6.5^{bc**}	18.8±9.7^{a*}	21.1±8.5^{a*}
6	3.1±1.3^{c*}	12.6±11.1^{b**}	19.3±11.7^{a***}
Storage time (day)	0 added NaCl		450 mM NaCl
0	18.4±8.0^{a*}		20.4±8.9^{a*}
3	17.3±9.2^{ab*}		14.1±11.0^{b*}
6	14.1±12.1^{b*}		9.2±10.2^{c**}

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples. Letters within the same column indicate statistically (<0.05) significant differences for storage times. Symbols * within the same rows indicate statistically (<0.05) significant differences between oxy, CO and Met-Hb.

Table 7-6. Fishers' z transformation correlation comparison (R, $p<0.05$) between sensory scores, TBARS, and a*value in tilapia washed muscle during storage for 6 days at 3.7°C

Sensory Scores and TBARS

Oxy-Hb	CO-Hb	Met-Hb	pH6.3	pH6.8	pH7.3	0.0NaCl	450NaCl
0.94	0.97	0.94	0.93	0.97	0.98	0.96	0.95

Sensory Scores and a*value

Oxy-Hb	CO-Hb	Met-Hb	pH6.3	pH6.8	pH7.3	0.0NaCl	450NaCl
-0.96	-0.96	-0.66	-0.83	-0.80	-0.76	-0.80	-0.84

TBARS and a*value

Oxy-Hb	CO-Hb	Met-Hb	pH6.3	pH6.8	pH7.3	0.0NaCl	450NaCl
-0.84	-0.88	-0.50	-0.69	-0.73	-0.69	-0.69	-.073

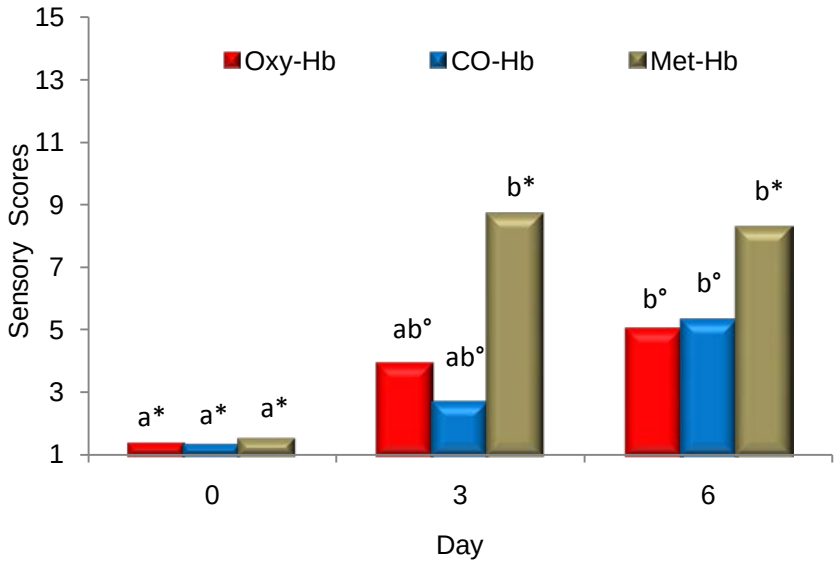


Figure 7-1. Hb form by storage interaction. Change in painty/rancid off-odor of tilapia washed muscle samples with different Hb-forms added. Samples were stored for six days at 3.7ºC. Different letters for each Hb form (Oxy, CO, Met-Hb) indicate statistically significant differences <0.05. Different symbols within each day indicate statistically significant differences <0.05. Met-Hb differed from CO and Oxy Hb on day 3 and 6.

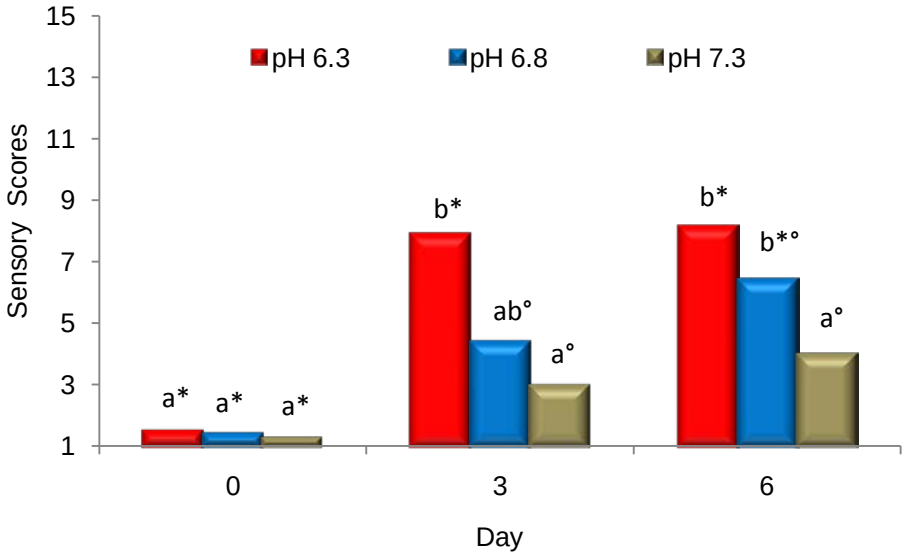


Figure 7-2. pH by storage interaction. Change in painty/rancid off-odor of tilapia washed muscle samples with different Hb-forms added. Samples were stored for six days at 3.7ºC. Different letters for each pH level indicate statistically significant differences <0.05. Different symbols within each day indicate statistically significant differences <0.05.

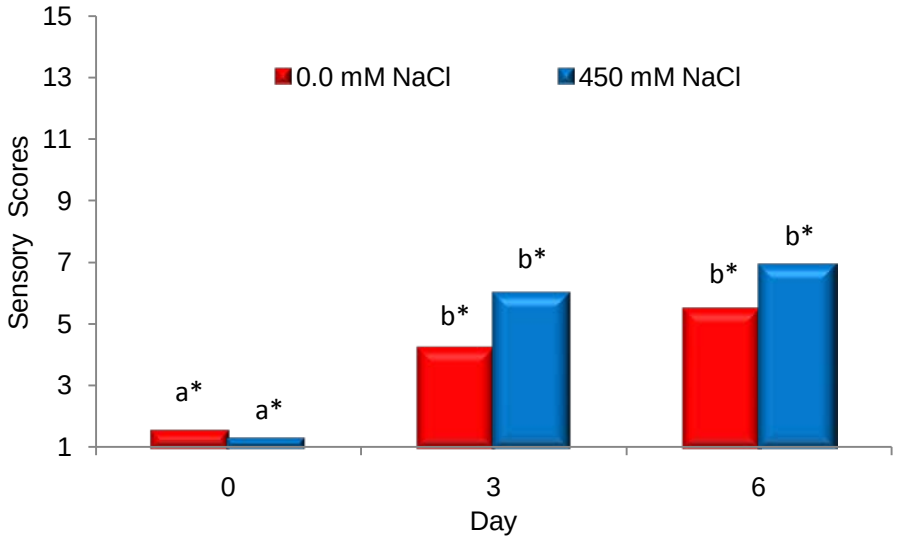


Figure 7-3. NaCl concentration by storage interaction. Change in painty/rancid off-odor at different NaCl concentrations during storage of tilapia washed muscle for six days at 3.7ºC. Different letters for each concentration indicate statistically significant differences <0.05. Different symbols within each day indicate statistically significant differences <0.05.

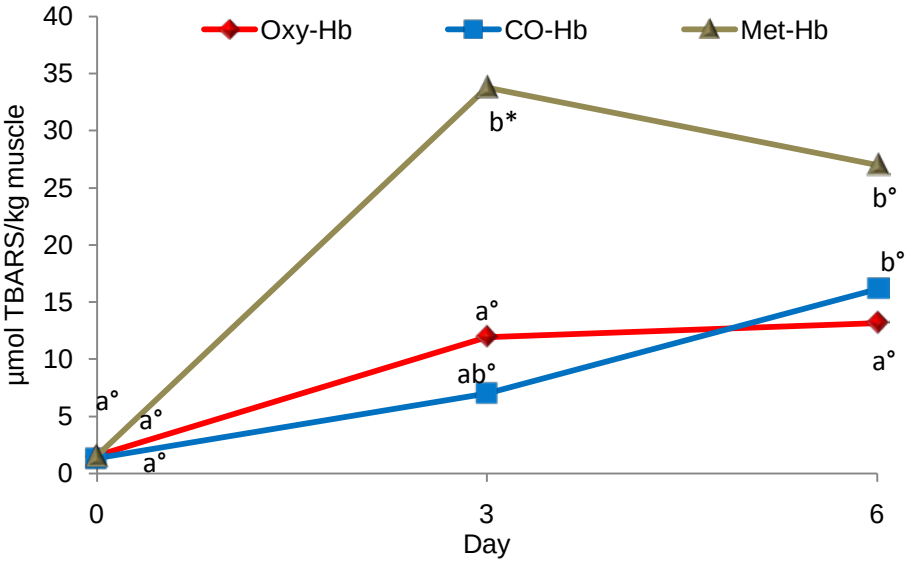


Figure 7-4. TBARS by storage interaction. Development of TBARS at different Hb-forms during storage of tilapia washed muscle for six days at 3.7ºC. Different letters for each Hb form indicate statistically significant differences <0.05. Different symbols within each day indicate statistically significant differences <0.05. Only day 3 showed a significant difference between Met-Hb versus CO and Oxy-Hb.

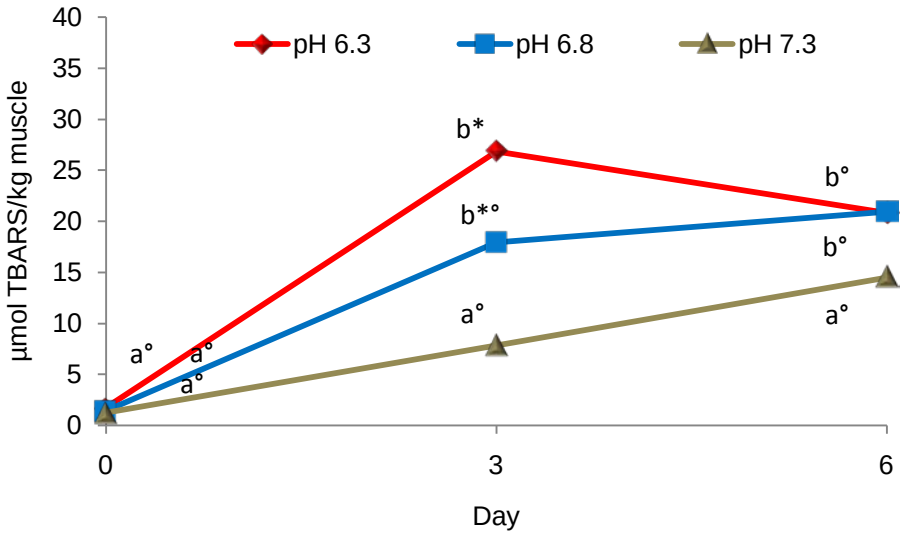


Figure 7-5. pH by storage interaction. Development of TBARS at different pH levels during storage of tilapia washed muscle for six days at 3.7ºC. Different letters for each pH level indicate statistically significant differences <0.05. Different symbols within each day indicate statistically significant differences <0.05. Only day 3 showed a significant difference between pH 6.3 versus pH 6.8 and 7.3.

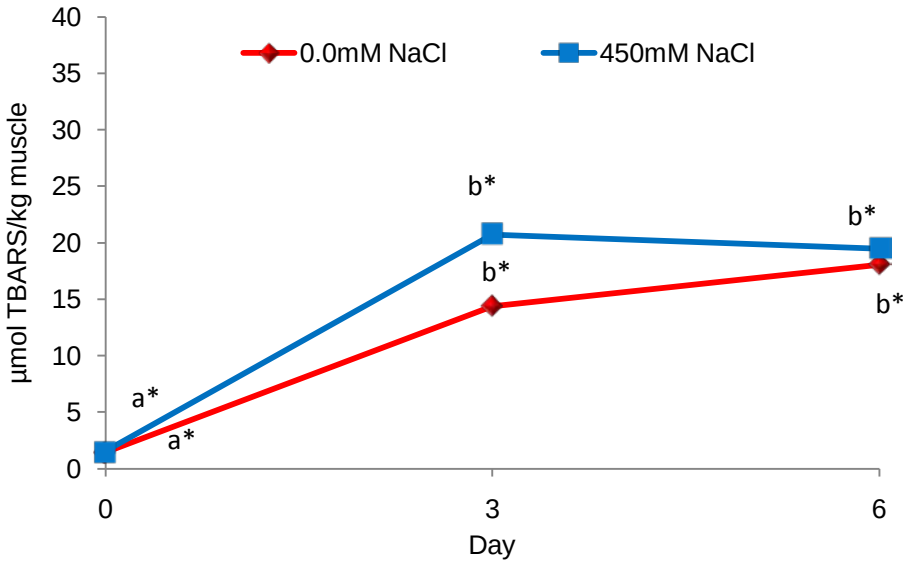


Figure 7-6. NaCl by storage interaction. Development of TBARS at different NaCl concentrations during storage of tilapia washed muscle for six days at 3.7ºC. Different letters for each concentration indicate statistically significant differences <0.05. Symbols within each day indicate there was no statistically significant difference <0.05 on each day for either concentration of NaCl.

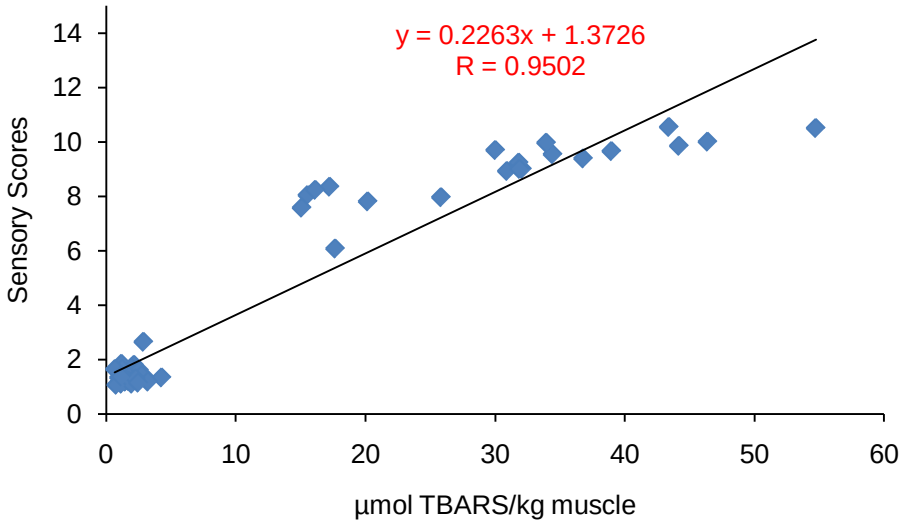


Figure 7-7. Correlation (p<0.0001) between the development of TBARS and sensory scores during storage of tilapia washed muscle for six days at 3.7ºC.

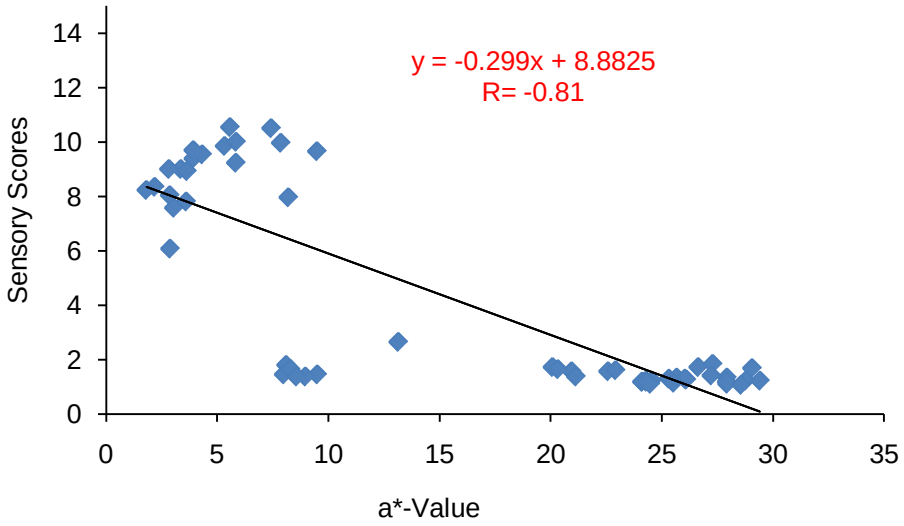


Figure 7-8. Correlation (p<0.0001) between change in a*value and sensory scores during storage of tilapia washed muscle for six days at 3.7ºC.

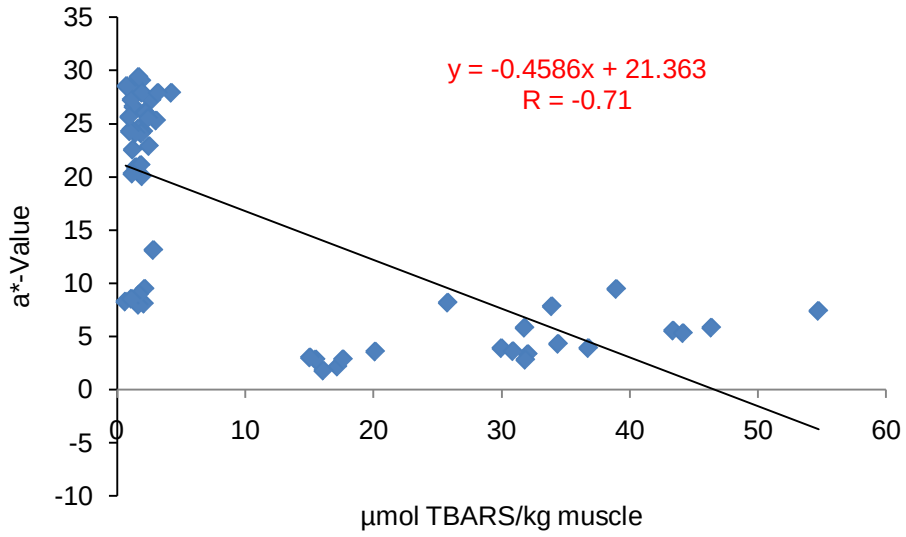


Figure 7-9. Correlation ($p<0.0001$) between the development of TBARS and change in a*value during storage of tilapia washed muscle for six days at 3.7ºC.

CHAPTER 8
SUMMARY AND CONCLUSION

Lipid and protein oxidation was measured using washed minced tilapia muscle (WMTM) adjusted to pH 6.3, 6.8 and 7.3. Oxy-Hb, CO-Hb, and met-Hb at concentrations of 6, 9, and 12 µmol/kg muscle, and NaCl at added concentrations of 0, 150 and 450 mM were added to the WMTM. Samples were stored at 3.7°C for 8 days and -25ºC for 24 weeks and assessed at intervals for oxidation. Lipid oxidation was monitored by following thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH). Protein oxidation was measured by the DNPH method. Hb oxidation state was monitored and change in color was measured using Color Vision Machine System. Formation of met-Hb was monitored spectrophotometrically. Rancidity was evaluated with a sensory panel (Appendix E).

CO-Hb was overall significantly less pro-oxidative compared to oxy- and met-Hb regardless of concentration used. The low pro-oxidative activity of CO-Hb is likely due in part to CO increasing the stability of heme protein structure, thereby reducing oxidative processes, and slowing down autoxidation. The reduced reactivity of CO-Hb with hydrogen peroxides may be due to the strong affinity of CO to Hb.

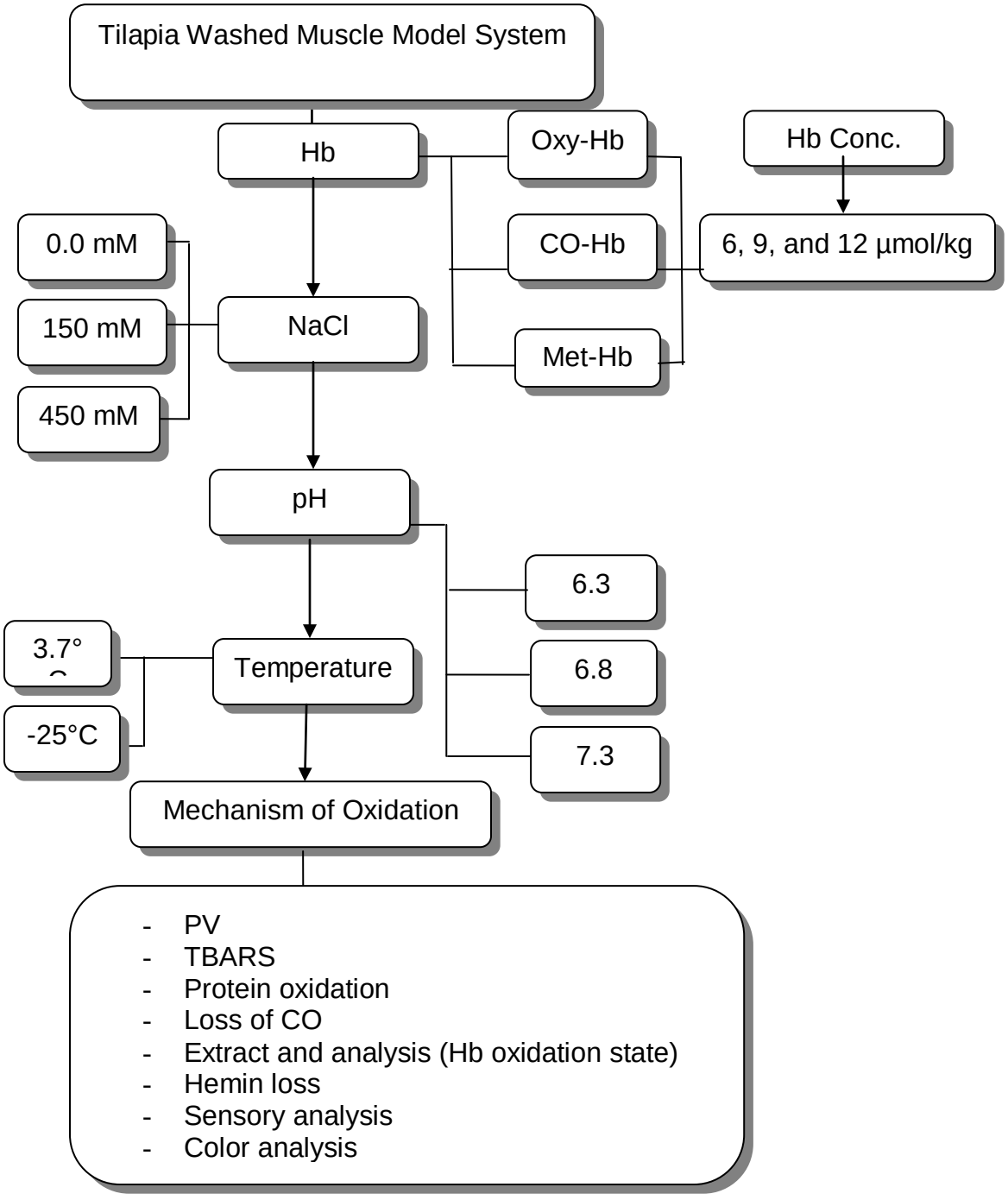
The effect of pH on oxidation superseded NaCl concentration, Hb form and concentration, or temperature in influencing oxidation and controlling for deterioration of fish muscle. Low pH increased the susceptibility of the washed minced tilapia muscle to oxidation, the lower the pH, the greater oxidation for all three forms of Hb. The color and sensory quality of the washed tilapia muscle also decreased with decreasing pH levels. Temperature significantly affected the auto-oxidation of CO-, oxy-, and met-Hb with greater % of met-Hb formed at -25°C.

NaCl significantly affected the stability of oxy-Hb and its oxidation to met-Hb. CO-Hb, on the other hand, was significantly less pro-oxidative compared to other forms of Hb, regardless of concentration used. The low pro-oxidative activity of the oxidized form (Met-Hb) could be a result of the absence of oxygen to form hydrogen peroxide required for met-Hb oxidation. The higher the concentration of NaCl, the greater the oxidation to met-Hb regardless of the concentration of Hb. Higher concentrations of NaCl also decreased the stability of color, and the sensory quality indicating NaCl has a high pro-oxidant activity in a system containing oxy-Hb and membrane lipids, thus providing insights how oxidation in seafood based products containing salt could be potentially controlled.

Sensory results are highly correlated with TBARS, indicating that Hb effectively catalyzed oxidation of the washed minced tilapia muscle system. In addition, the high correlation of sensory results with a*values further verifies the use of sensory analysis for detecting oxidation of seafood products.

In conclusion, Hb form and concentration, pH, temperature, NaCl concentration, all affected the oxidative activity of oxy-, CO-, and met-Hb. The results of this research suggest that tilapia minced muscle treated with CO could have greater stability to oxidation, thus resulting in increased color stability and sensory quality.

APPENDIX A
RESEARCH SCHEME



APPENDIX B
CHANGES IN L* VALUE AND B* VALUE

Table B-1. Changes in L*value in washed tilapia muscle containing different forms of Hb at a concentration of 6, 9, and 12µmol/kg muscle at 3.7ºC, averaged across pH level and NaCl concentration.

L* value	6µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[c]	Met-Hb[a]
0	74.8±1.5	74.4±3.3	78.1±1.8
2	75.0±1.4	74.4±3.6	77.3±1.8
4	75.9±1.9	74.3±3.6	76.8±1.7
6	76.0±2.6	74.0±4.4	77.1±1.7
8	76.0±2.9	73.0±5.1	78.0±1.9

L* value	9µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[c]	CO-Hb[b]	Met-Hb[a]
0	70.8±2.0	73.7±2.4	74.6±2.0
2	71.1±1.8	73.2±2.3	74.0±2.3
4	71.9±2.3	73.1±2.6	73.5±1.6
6	72.3±3.0	73.6±3.0	73.9±1.8
8	72.4±3.6	73.5±3.1	75.1±2.6

L* value	12µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[a]
0	68.0±2.0	71.7±2.8	71.9±1.9
2	68.2±1.9	71.1±3.1	70.6±1.6
4	69.2±2.4	71.1±2.9	70.3±1.3
6	69.4±3.1	72.1±3.0	70.7±1.6
8	69.6±3.8	71.9±3.7	72.2±2.3

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-2. Changes in L*value in washed tilapia muscle containing different forms of Hb at a concentration of 6, 9, and 12µmol/kg muscle at -25ºC, averaged across pH level and NaCl concentration.

L* value	6µmol Hb/kg muscle		
Storage time (weeks)	Oxy-Hb[b]	CO-Hb[c]	Met-Hb[a]
0	74.8±1.5	74.4±3.3	78.1±1.8
4	73.2±0.8	71.3±3.8	75.6±1.0
8	73.4±1.6	72.0±3.8	75.0±0.7
12	71.5±2.9	72.4±3.3	75.4±0.9
16	73.7±1.3	71.7±4.0	75.9±0.9
20	75.1±0.9	72.0±3.9	75.9±1.0
24	76.6±1.8	70.6±4.8	76.1±1.2

L* value	9µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	70.8±2.0	73.7±2.4	74.6±2.0
4	69.5±1.3	69.7±2.8	71.8±1.3
8	69.9±1.8	69.8±2.8	71.4±1.2
12	68.9±1.8	70.7±2.4	71.7±1.2
16	69.7±1.1	70.3±2.6	72.1±1.2
20	71.8±2.0	69.9±2.9	72.2±1.3
24	73.1±3.2	69.1±3.0	71.8±1.6

L* value	12µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[a]
0	68.0±2.0	71.7±2.8	71.9±1.9
4	65.5±1.0	68.1±2.8	68.2±0.9
8	66.0±1.2	68.1±3.3	67.6±1.0
12	66.5±3.5	69.0±2.7	67.6±1.4
16	65.8±2.0	68.3±2.7	68.3±1.3
20	67.6±3.3	68.1±2.6	68.1±1.5
24	68.8±3.5	68.6±2.6	67.8±1.5

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-3. Changes in b*value in washed tilapia muscle containing different forms of Hb at a concentration of 6, 9, and 12µmol/kg muscle at 3.7ºC, averaged across pH level and NaCl concentration.

b* value	6µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	1.8±1.4	1.8±1.5	2.6±1.0
2	2.7±1.7	3.7±2.0	4.0±0.9
4	4.4±1.8	4.2±2.2	4.4±1.0
6	4.7±1.6	4.7±2.6	5.2±1.7
8	4.4±2.6	5.1±2.2	6.8±1.8

b* value	9µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	3.4±1.8	3.1±1.5	3.8±1.2
2	4.5±2.0	4.6±1.7	5.2±1.0
4	6.5±2.2	5.2±2.6	6.2±1.6
6	7.1±2.5	6.3±2.5	7.5±2.0
8	6.4±3.2	7.2±3.2	9.3±1.6

b* value	12µmol Hb/kg muscle		
Storage time (day)	Oxy-Hb[b]	CO-Hb[c]	Met-Hb[a]
0	4.7±2.3	3.9±1.8	5.0±1.6
2	5.8±2.1	5.0±1.7	6.6±1.4
4	7.7±2.1	5.5±2.5	7.6±1.8
6	8.5±2.4	7.4±2.7	9.0±2.2
8	7.8±3.5	8.6±2.8	10.5±2.1

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-4. Changes in b*value in washed tilapia muscle containing different forms of Hb at a concentration of 6, 9, and 12µmol/kg muscle at 3.7ºC, averaged across pH level and NaCl concentration.

b* value	6µmol Hb/kg muscle		
Storage time (day)	Oxy-Hba	CO-Hbc	Met-Hbb
0	1.8±1.4	1.8±1.5	2.6±1.0
4	8.7±1.6	5.1±1.7	5.6±0.5
8	9.9±2.0	5.2±1.7	6.3±1.2
12	10.1±3.3	4.3±1.4	6.1±1.4
16	7.4±4.4	3.0±2.0	6.7±1.8
20	6.0±2.4	2.2±1.4	7.0±1.5
24	6.2±3.2	3.0±2.0	6.9±1.8

b* value	9µmol Hb/kg muscle		
Storage time (day)	Oxy-Hba	CO-Hbc	Met-Hbb
0	3.4±1.8	3.1±1.5	3.8±1.2
4	11.0±1.5	7.1±2.2	7.8±0.8
8	12.1±1.9	7.1±2.2	8.4±1.7
12	12.2±2.5	5.7±1.7	8.6±2.0
16	9.9±4.0	4.6±1.9	9.4±2.0
20	8.4±2.2	3.6±2.1	9.1±2.1
24	8.2±2.5	4.6±2.0	9.9±2.6

b* value	12µmol Hb/kg muscle		
Storage time (day)	Oxy-Hba	CO-Hbc	Met-Hbb
0	4.7±2.3	3.9±1.8	5.0±1.6
4	13.6±2.1	8.0±1.7	10.1±1.4
8	14.4±2.2	7.8±1.7	11.0±1.7
12	16.2±4.5	7.3±2.4	11.8±2.2
16	12.4±4.1	6.3±2.0	12.0±2.6
20	11.0±2.1	5.3±2.5	11.9±2.8
24	10.9±2.6	4.9±1.3	12.8±2.4

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-5. Changes in L*value in washed tilapia muscle containing different forms of Hb at pH 6.3, 6.8, and 7.3 at 3.7ºC, averaged across Hb and NaCl concentration.

L* value		pH 6.3	
Storage time (day)	Oxy-Hb[a]	CO-Hb[a]	Met-Hb[b]
0	71.9±3.8	74.3±3.2	73.9±3.0
2	72.5±3.2	73.5±3.9	73.0±2.9
4	74.9±2.9	74.6±3.0	73.4±2.3
6	76.3±2.9	76.3±2.3	74.5±2.1
8	76.8±2.9	75.9±2.8	76.2±2.3

L* value		pH 6.8	
Storage time (day)	Oxy-Hb[c]	CO-Hb[c]	Met-Hb[a]
0	71.0±3.1	72.5±3.0	73.9±2.8
2	70.9±3.3	72.8±3.2	72.7±3.0
4	71.2±3.1	71.4±3.2	72.0±2.8
6	71.3±2.8	71.7±3.0	72.0±3.0
8	71.5±3.1	71.6±3.7	72.5±2.9

L* value		pH 7.3	
Storage time (day)	Oxy-Hb[c]	CO-Hb[b]	Met-Hb[a]
0	70.7±3.2	73.0±2.8	76.9±2.9
2	70.8±3.2	72.3±2.9	76.1±3.2
4	70.8±3.1	72.5±3.0	75.2±3.3
6	70.2±3.1	71.6±3.2	75.2±3.3
8	69.6±3.3	70.9±3.6	76.6±2.9

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-6. Changes in L*value in washed tilapia muscle containing different forms of Hb at pH 6.3, 6.8, and 7.3 at -25ºC, averaged across Hb and NaCl concentration.

b* value		pH 6.3	
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	1.3±1.7	1.8±1.5	3.6±1.7
2	4.1±3.4	3.5±1.7	4.7±1.6
4	7.4±3.5	5.4±3.5	6.0±2.5
6	7.5±3.0	7.5±3.6	7.7±2.6
8	7.4±3.1	8.4±3.3	8.1±2.5

b* value		pH 6.8	
Storage time (day)	Oxy-Hb[b]	CO-Hb[c]	Met-Hb[a]
0	4.0±1.6	2.6±1.3	3.7±1.5
2	4.3±1.5	3.9±1.4	5.4±1.3
4	5.4±1.3	3.6±1.1	5.9±1.7
6	6.9±3.1	5.5±2.5	7.7±2.8
8	6.8±4.3	7.8±2.9	9.5±2.3

b* value		pH 7.3	
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	4.6±1.6	4.4±1.5	4.2±1.6
2	4.7±1.6	5.9±1.5	5.7±1.6
4	5.7±1.4	6.0±1.4	6.2±1.8
6	6.0±1.5	5.4±1.2	6.2±1.8
8	4.4±1.6	4.7±1.3	9.1±2.3

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-7. Changes in b*value in washed tilapia muscle containing different forms of Hb at pH 6.3, 6.8, and 7.3 at 3.7ºC, averaged across Hb and NaCl concentration.

b* value		pH 6.3	
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	1.3±1.7	1.8±1.5	3.6±1.7
2	4.1±3.4	3.5±1.7	4.7±1.6
4	7.4±3.5	5.4±3.5	6.0±2.5
6	7.5±3.0	7.5±3.6	7.7±2.6
8	7.4±3.1	8.4±3.3	8.1±2.5

b* value		pH 6.8	
Storage time (day)	Oxy-Hb[b]	CO-Hb[c]	Met-Hb[a]
0	4.0±1.6	2.6±1.3	3.7±1.5
2	4.3±1.5	3.9±1.4	5.4±1.3
4	5.4±1.3	3.6±1.1	5.9±1.7
6	6.9±3.1	5.5±2.5	7.7±2.8
8	6.8±4.3	7.8±2.9	9.5±2.3

b* value		pH 7.3	
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	4.6±1.6	4.4±1.5	4.2±1.6
2	4.7±1.6	5.9±1.5	5.7±1.6
4	5.7±1.4	6.0±1.4	6.2±1.8
6	6.0±1.5	5.4±1.2	6.2±1.8
8	4.4±1.6	4.7±1.3	9.1±2.3

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-8. Changes in b*value in washed tilapia muscle containing different forms of Hb at pH 6.3, 6.8, and 7.3 at -25ºC, averaged across Hb and NaCl concentration.

b* value		pH 6.3	
Storage time (day)	Oxy-Hb[a]	CO-Hb[c]	Met-Hb[b]
0	1.3±1.7	1.8±1.5	3.6±1.7
4	9.1±2.2	5.8±1.8	7.1±1.6
8	10.0±2.6	6.0±2.1	8.4±2.4
12	9.0±2.7	5.2±2.2	7.6±2.9
16	8.6±2.0	3.3±2.2	8.2±2.9
20	9.8±2.1	2.6±2.4	8.0±2.9
24	11.0±1.8	2.8±1.6	9.2±2.8

b* value		pH 6.8	
Storage time (day)	Oxy-Hb[a]	CO-Hb[c]	Met-Hb[b]
0	4.0±1.6	2.6±1.3	3.7±1.5
4	12.2±2.0	6.5±2.0	8.1±2.1
8	13.4±1.6	6.4±2.0	9.7±2.7
12	16.0±4.0	5.4±2.1	10.2±3.0
16	14.9±3.2	5.2±2.1	11.2±2.9
20	8.9±3.4	4.1±2.1	10.5±3.1
24	8.4±3.4	4.6±1.6	10.6±4.0

b* value		pH 7.3	
Storage time (day)	Oxy-Hb[a]	CO-Hb[c]	Met-Hb[a]
0	4.6±1.6	4.4±1.5	4.2±1.6
4	12.1±2.5	7.9±2.4	8.3±2.4
8	13.0±2.6	7.6±2.2	7.6±2.0
12	13.6±2.9	6.6±2.2	8.6±2.6
16	6.2±2.9	5.4±2.3	8.7±2.5
20	6.6±2.6	4.5±2.2	9.5±2.5
24	5.9±2.5	5.1±1.9	9.8±3.0

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-9. Changes in L*value in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl (0, 150, and 450mM) at 3.7ºC, averaged across pH level and Hb concentration.

L* value		0mM NaCl	
Storage time (day)	Oxy-Hb[c]	CO-Hb[a]	Met-Hb[b]
0	72.8±3.0	75.9±1.7	75.8±2.6
2	72.7±3.0	75.7±2.1	74.6±3.0
4	73.2±3.4	75.3±2.2	73.9±3.0
6	73.4±4.3	75.3±1.5	73.2±3.3
8	73.1±4.9	75.2±2.1	74.2±3.5

L* value		150.0mM NaCl	
Storage time (day)	Oxy-Hb[c]	CO-Hb[b]	Met-Hb[a]
0	71.8±2.8	73.4±2.2	75.5±3.0
2	71.9±2.8	72.9±2.5	74.5±3.4
4	72.7±3.3	72.9±2.4	74.0±3.1
6	72.8±3.8	73.5±2.7	74.6±3.1
8	72.6±4.2	72.8±3.3	75.6±2.9

L* value		450.0mM NaCl	
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	69.0±3.2	70.5±2.3	73.4±3.4
2	69.6±3.3	70.0±2.5	72.8±3.6
4	71.0±3.7	70.4±3.3	72.8±3.2
6	71.6±3.8	70.7±4.3	73.9±3.0
8	72.1±4.0	70.4±4.8	75.5±3.4

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-10. Changes in L*value in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl (0, 150, and 450mM) at -25ºC, averaged across pH level and Hb concentration.

L* value		0mM NaCl	
Storage time (weeks)	Oxy-Hb[b]	CO-Hb[a]	Met-Hb[a]
0	72.8±3.0	75.9± 1.7	75.8±2.6
4	68.5±3.5	71.9±2.9	71.8±3.2
8	69.0±3.3	72.4±3.3	71.7±3.3
12	69.8±3.4	73.3±2.0	72.1±3.4
16	69.2±3.8	72.5±2.6	72.7±3.1
20	70.4±4.0	72.7±2.2	72.6±3.1
24	72.3±4.8	71.9±2.4	72.9±3.4

L* value		150.0mM NaCl	
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	71.8±2.8	73.4±2.2	75.5±3.0
4	69.9±3.2	70.2±2.2	72.1±3.3
8	70.2±3.2	70.5±2.4	71.2±3.3
12	69.9±3.1	70.9±2.2	71.6±3.4
16	70.0±3.7	70.2±2.7	71.9±3.5
20	72.1±3.4	70.1±2.7	71.8±3.5
24	73.3±4.0	69.4±2.6	71.9±3.5

L* value		450.0mM NaCl	
Storage time (day)	Oxy-Hb[b]	CO-Hb[c]	Met-Hb[a]
0	69.0±3.2	70.5±2.3	73.4±3.4
4	69.8±3.3	67.1±3.1	71.7±3.3
8	70.2±3.8	67.0±3.1	71.2±3.1
12	67.1±3.2	68.0±2.6	70.9±3.5
16	70.0±3.4	67.5±3.0	71.6±3.6
20	72.0±4.0	67.1±3.2	71.7±4.0
24	72.9±4.4	67.1±4.1	70.8±3.9

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-11. Changes in b*value in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl (0, 150, and 450mM) at 3.7ºC, averaged across pH level and Hb concentration.

b* value		0mM NaCl	
Storage time (day)	Oxy-Hb[b]	CO-Hb[c]	Met-Hb[a]
0	2.3±2.4	1.7±1.3	2.5±1.3
2	2.8±2.3	2.7±1.2	4.2±1.5
4	4.7±1.7	2.8±1.3	4.7±1.6
6	5.1±1.5	3.9±1.5	5.3±1.5
8	4.1±1.1	5.5±2.1	6.9±2.1

b* value		150.0mM NaCl	
Storage time (day)	Oxy-Hb[b]	CO-Hb[b]	Met-Hb[a]
0	3.1±1.9	2.9±1.6	3.7±1.0
2	4.3±1.4	4.5±1.3	5.3±1.1
4	6.3±1.9	5.0±1.5	6.0±1.7
6	6.4±1.8	6.5±2.2	7.5±2.4
8	5.1±2.4	7.0±3.1	9.2±1.8

b* value		450.0mM NaCl	
Storage time (day)	Oxy-Hb[a]	CO-Hb[b]	Met-Hb[a]
0	4.5±1.7	4.1±1.8	5.1±1.3
2	6.0±2.0	6.0±1.3	6.2±1.3
4	7.5±2.8	7.2±2.1	7.4±1.7
6	8.8±3.0	8.1±2.7	8.8±2.2
8	9.4±3.3	8.5±3.3	10.6±1.7

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

Table B-12. Changes in b*value in washed tilapia muscle containing different forms of Hb at concentrations of added NaCl (0, 150, and 450mM) at -25ºC, averaged across pH level and Hb concentration.

b* value		0mM NaCl	
Storage time (day)	Oxy-Hb[a]	CO-Hb[c]	Met-Hb[b]
0	2.3±2.4	1.7±1.3	2.5±1.3
4	10.8±3.0	5.4±1.4	7.3±2.2
8	11.2±3.1	5.2±1.3	7.1±2.2
12	11.2±3.8	4.2±1.3	7.2±2.5
16	8.6±3.9	3.2±1.6	7.6±2.2
20	6.7±2.1	2.0±1.5	7.4±2.4
24	6.7±2.5	3.0±1.3	7.5±2.5

b* value		150.0mM NaCl	
Storage time (day)	Oxy-Hb[a]	CO-Hb[c]	Met-Hb[b]
0	3.1±1.9	2.9±1.6	3.7±1.0
4	11.2±2.7	6.4±1.6	8.3±2.1
8	12.1±2.6	6.1±1.5	9.3±2.4
12	12.7±4.0	5.3±1.8	9.4±2.7
16	9.6±3.8	4.0±1.7	10.5±3.1
20	8.6±2.7	3.4±1.9	10.4±2.8
24	8.9±3.4	4.2±1.3	10.5±2.6

b* value		450.0mM NaCl	
Storage time (day)	Oxy-Hb[a]	CO-Hb[c]	Met-Hb[a]
0	4.5±1.7	4.1±1.8	5.1±1.3
4	11.4±2.3	8.4±2.5	7.9±1.9
8	13.0±2.2	8.7±2.0	9.3±2.3
12	14.7±4.7	7.7±1.9	9.9±3.1
16	11.5±5.7	6.7±2.2	10.1±2.9
20	10.0±3.3	5.7±2.1	10.2±2.9
24	9.6±3.5	5.3±2.4	11.6±3.3

Values are means ± Standard Deviations for all pixels of the surface of the minced muscle samples (n=18). Letters within the same rows indicate statistically (<0.05) significant differences separated by Tukey's HSD.

APPENDIX C
PEARSON CORRELATION COEFFICIENT

Table C-1. Pearson correlation coefficient for samples stored at 3.7°C for 8 days*.

	TBARS	PV	Carbonyls	Oxy-Hb	Met-Hb	Deoxy-Hb	L*-value	a*-value	b*-value	%CO
TBARS	1	0.84*	-0.02	-0.64*	0.65*	0.48*	-0.36*	-0.25*	0.22*	0.33*
PV	0.84*	1	0.06	-0.73*	0.73*	0.60*	-0.22*	-0.37*	0.06	0.46*
Carbonyls	-0.02	0.06	1	-0.43*	0.41*	0.37*	-0.08	-0.22*	-0.20*	0.19*
Oxy-Hb	-0.64*	-0.73*	-0.43*	1	-0.98*	-0.85*	0.08	0.50*	0.27*	-0.60*
Met-Hb	0.65*	0.73*	0.41*	-0.98*	1	0.75*	-0.09	-0.48*	-0.26*	0.58*
Deoxy-Hb	0.48*	0.60*	0.37*	-0.85*	0.75*	1	0.01	-0.45*	-0.25*	0.54*
L*-value	-0.36*	-0.22*	-0.08	0.08	-0.09	0.01	1	-0.59*	-0.69*	0.10
a*-value	-0.25*	-0.37*	-0.22*	0.50*	-0.48*	-0.45*	-0.59*	1	0.50*	-0.43*
b*-value	0.22*	0.06	-0.20*	0.27*	-0.26*	-0.25*	-0.69*	0.50*	1	-0.35*
%CO	0.33*	0.46*	0.19*	-0.60*	0.58*	0.54*	0.10*	-0.43*	-0.35*	1

*Number of observations (N=810), P≤ 0.05, *indicates significant

Table C-2. Pearson correlation coefficient for samples stored at -25°C for 24 weeks*.

	TBARS	PV	Carbonyls	Oxy-Hb	Met-Hb	Deoxy-Hb	L*-value	a*-value	b*-value	%CO
TBARS	1	0.73*	0.07	-0.70*	0.71*	0.59*	-0.12	-0.62*	0.46*	0.12*
PV	0.73*	1	0.08	-0.70*	0.70*	0.63*	0.11	-0.71*	0.62*	0.15*
Carbonyls	0.07	0.08	1	-0.22*	0.19*	0.24*	0.17*	-0.12	-0.04	-0.15*
Oxy-Hb	-0.70*	-0.70*	-0.22*	1	-0.99*	-0.90*	-0.16*	0.75*	-0.36*	-0.13*
Met-Hb	0.71*	0.70*	0.19*	-0.99*	1	0.82*	0.14*	-0.74*	0.36*	0.10
Deoxy-Hb	0.59*	0.63*	0.24*	-0.90*	0.82*	1	0.17*	-0.67*	0.29*	0.21*
L*-value	-0.12	0.11	0.17*	-0.16*	0.14*	0.17*	1	-0.52*	-0.19*	0.08
a*-value	-0.62*	-0.71*	-0.12	0.75*	-0.74*	-0.67*	-0.52*	1	-0.37*	-0.19*
b*-value	0.46*	0.62*	-0.04	-0.36*	0.36*	0.29*	-0.19*	-0.37*	1	0.06
%CO	0.12*	0.15*	-0.15*	-0.13*	0.10	0.21*	0.08	-0.19*	0.06	1

*Number of observations (N=1134), P≤ 0.05, *indicates significant

APPENDIX D
TRIANGLE TEST BALLOT

Name:
Date:
Product: Tilapia washed muscle with 12µmol/kg hemoglobin

Instruction:

Please sniff the samples from left to right, in the order presented. Two samples are the same and one is different; please determine which is the odd sample and circle the code that represents it. If you cannot differentiate between the samples, please take a guess.

Which is the odd sample?

346 576 021

APPENDIX E
DESCRIPTIVE ANALYSIS BALLOT

Sample: Fish muscle Date:
Name: Panelist #:
Attribute: painty/rancid odor
Please sniff/smell samples in front of you in the order presented and rate the intensity of the painty/rancid odor using the 15 point scale presented below (none to strong)

	None	Strong
	None	Strong
	None	Strong
	None	Strong
	None	Strong
	None	Strong
	None	Strong
	None	Strong
	None	Strong

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BIOGRAPHICAL SKETCH

Sara Abdulmajeed Aldaous was born in Riyadh, Saudi Arabia, on October 31, and grew up in Jeddah, on the west coast. She graduated in 1995 from King Abdulaziz University, Jeddah, Saudi Arabia, College of Home Economics, Department of Food and Nutrition. Sara ranked in the top 5 percent of this department's graduates over the past 15 years. From 1995-2001, she joined the team of Food and Nutrition at King Abdulaziz University, teaching lab courses in Food Science and Nutrition. In 2001, she received a scholarship to study abroad to pursue her master's and PhD degrees in food science. In Fall 2003, she joined the Department of Food Science and Human Nutrition at the University of Maine, Orono, Maine. After she received her master's degree, she joined the Department of Food Science and Human Nutrition at the University of Florida, Gainesville, Florida, in Fall 2005 to pursue her PhD in Food Science. During her graduate career, her outstanding performance in academics was recognized by The University of Florida with the presentation of the Marilyn Little Altrusa Scholarship for outstanding achievement by an international female. She also presented seven posters specifying findings of her research at four different conferences, one of which received 2nd place at the Institute of Food Technologists (IFT) Aquatic Food Products Division Paper Competition in 2008.