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Contributions of Blood and Blood Components to Lipid Oxidation in Fish Muscle

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There was a wide variation in the amounts of hemoglobin extracted from the muscle tissue of bled and unbled fish. Averaged values suggested that the residual blood level in the muscle of bled fish was substantial. Myoglobin content was minimal as compared to hemoglobin content in mackerel light muscle and trout whole muscle. Hemoglobin made up 65 and 56% of the total heme protein by weight in dark muscle from unbled and bled mackerel, respectively. Bleeding significantly reduced rancidity in minced trout whole muscle, minced mackerel light muscle, and intact mackerel dark muscle but not minced mackerel dark muscle stored at 2 °C. The reduction was in the number of fish that had a longer shelf life; muscle from certain bled fish had rancidity that was comparable to the rancidity in unbled controls. The soluble contents of erythrocytes accounted for all of the lipid oxidation capacity of whole blood added to washed cod muscle. Limiting lysis of erythrocytes delayed lipid oxidation, which was likely due to keeping hemoglobin inside the erythrocyte. Apparent breakdown of lipid hydroperoxides occurred only when a critical level of hemoglobin was present. Blood plasma was slightly inhibitory to oxidation of washed cod lipids. These studies suggest that blood-mediated lipid oxidation in fish muscle depends on various factors that include hemoglobin concentration, types of hemoglobin, plasma volume, and erythrocyte integrity.

KEYWORDS: Hemoglobin; myoglobin; blood; role in quality; blood plasma; erythrocytes; mackerel; trout; lipid oxidation; anodic hemoglobins; lipid hydroperoxides

INTRODUCTION

Lipid oxidation is a major cause of quality deterioration in fish muscle. Deterioration of flavor, odor, color, texture, and the production of toxic compounds can arise from lipid oxidation (*1*). Many components of blood have the potential to promote or delay lipid oxidation. Hemoglobin is highly concentrated in the erythrocyte. Various pathways by which hemoglobin can promote lipid oxidation have been described including pseudo-lipoxygenase activity (*2*). Hemoglobin can be a source of activated oxygen due to hemoglobin autoxidation, and heme or iron can be released from the protein to promote lipid oxidation. White cells can generate superoxide, hydrogen peroxide, and hydroxyl radical (*3*) and lipoxygenase products (*4*). Plasma is recognized for its many components that inhibit lipid oxidation (*5*), but fish plasma is 1.2−3% lipid, much of it in the form of lipoproteins (*6*), which could be a source of oxidizable lipid.

In trout fillets, bleeding was found to reduce lipid oxidation during frozen storage (*7*). Conversely, bleeding had no effect on lipid oxidation in frozen stored salmon muscle (*8*). More research is needed in order to have a better understanding of the role of blood in quality deterioration of fish muscle. The objectives of this research were to determine the effect of bleeding on blood levels in muscle and on storage quality. Furthermore, a model system approach was used to investigate the potential contributions that blood components could make to lipid oxidation of fish muscle.

MATERIALS AND METHODS

Materials. Sodium ascorbate, 3-aminobenzoic acid ethyl ester, tetraethoxypropane, and cumene hydroperoxide were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dithionite and chloroform with ethanol preservative were obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals were reagent grade.

Fish Supply. Rainbow trout (*Onchorhynchus mykiss*) (25−31 cm) were supplied by Mohawk Trout hatchery (Sunderland, MA). Trout were maintained in an opaque, plastic tank (Nalgene, Rochester, NY). Freshwater was used that was constantly circulated with a Whisper power filter, Turbo 5 (Willinger Bros. Inc., Oakland, NJ), equipped with disposable Bio-Bags to collect waste and ammonia. Fish were fed Silver Cup trout chow (Nelson's Sterling, Murray, UT), 1 g per fish per day. The water temperature was maintained at 4−8 °C. Atlantic mackerel (*Scomber scombrus*) were obtained at sea off the coast of Gloucester, MA.

Handling of Fish for Bleeding Studies. To tail bleed, fish were pulled from the water, the tails were immediately cut, and the fish bled

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out for 30 min in water. Fish were then gutted and put back into the water for 30 min and then held in an ice-slush for 24 h. Gill-bled fish were taken through the same procedure, but 2−4 gill plates on one side were cut to bleed the fish. Unbled fish were kept whole until after 24 h of storage in the ice-slush, at which time the fish were gutted.

Extraction of Heme Pigments from Muscle Tissue. Fillets were removed from gutted fish after 24 h post mortem storage in the ice-slush. The skins were removed from fillets. Whole muscle, light muscle, or dark muscle was minced once in a Kitchen Aid (Kitchen Aid Inc., St. Joseph, MI) mincer (diameter 4.7 mm). Whole muscle of trout was used since light and dark fibers in trout muscle are integrated and therefore difficult to separate. Four grams of minced tissue was extracted in 50 mL of distilled deionized water by homogenizing for 1 min (rheostat set at 70) using a Polytron type PT 10/35 (Brinkman Instruments, Westbury, NY). The pH was adjusted to 7.0 if necessary and centrifuged at 110 000*g* for 40 min at 4 °C. The supernatant was then filtered through Whatman 1 filter paper.

Separation of Myoglobin and Hemoglobin from Light and Whole Muscle Extracts. Anion exchange chromatography was used to isolate the hemoglobin and myoglobin in muscle. Modifications of the method of Brown (*9*) were used. Disposable chromatography columns (20 mL capacity, 1.5 cm diameter) (Bio-Rad, Hercules, CA) were loaded with degassed (diethylamino)ethyl Bio Gel A Gel (Bio-Rad, Hercules, CA). Columns were equilibrated and packed by gravity with 50 mM Tris, pH 8.6, buffer. The gel support material occupied a 12 mL volume of the column. Tissue extracts were transferred to 50 mM Tris, pH 8.6, buffer using buffer exchange columns (Bio-Rad, Hercules, CA). Then 2 mL of the extract was added to the column. The 2 mL sample was allowed to pass completely into the column. Around 30 mL of 50 mM Tris, pH 8.6, buffer was then passed through the column to elute the myoglobins. Five milliliter fractions were collected. Then, the buffer was switched to 50 mM Tris, 200 mM NaCl, pH 6.95, buffer. Around 30 mL of this buffer was passed through the column to elute the hemoglobins. Chromatography was performed using blood extracts initially to determine recoveries and the distribution of hemoglobin between the two different elution buffers over the range of hemoglobin concentrations found in tissue extracts.

Separation of Myoglobin and Hemoglobin from Dark Muscle Extracts. Ultrafiltration was used to measure hemoglobin and myoglobin content in mackerel dark muscle. Tissue extracts were diluted in nine volumes of 50 mM Tris, pH 8.6. The pH was adjusted to 8.6 if necessary. This pH has been found to reduce the electrostatic interaction of myoglobin with the filtration membrane for maximum mass flow of tuna myoglobin (*10*). Two milliliters of the extract was placed in a centricon-50 ultrafiltration tube (Amicon, Beverly, MA). The tube was centrifuged at maximum speed for 5 min in a clinical centrifuge (IEC, Needham Heights, MA). This left around 1 mL of extract in the filtrate and 1 mL in the retentate to be analyzed spectrophotometrically for myoglobin and hemoglobin content. Ultrafiltration was performed using blood extracts initially to determine recoveries and the amount of hemoglobin that passed into the filtrate.

Quantifying Hemoglobin and Myoglobin Levels. The method of Brown (*9*) was adapted. Around 1 mg of sodium dithionite was added to the extract and mixed in a cuvette. Carbon monoxide gas (Matheson Gas, Gloucester, MA) was then bubbled into the samples for 30 s. The sample was then scanned from 440 to 400 nm (Soret) against a blank that contained only buffer using a model U-3110 double-beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA). The peak at 420 nm was recorded. Standard curves were constructed using bovine hemoglobin standard.

Muscle Storage Studies at 2 °C. A separate portion of the minced tissue that was used to measure heme pigment content was used for storage studies. Minces were from individual fish. A total of 10−15 g of minced tissue was stored in 30 mL capacity brown bottles with screw-on caps for up to 15 days. On one occasion, freshly excised dark muscle from bled and unbled mackerel was stored at −80 °C for 4−5 months in an Ultralow freezer, model No. U85-18 (So-Low, Cincinnati, OH) before it was thawed and stored on ice as intact dark muscle.

Syringe Removal of Blood from Caudal Vein. Trout were anesthetized in 3-aminobenzoic acid ethyl ester (0.5 g/L) for 3 min. The fish was then held belly up, and 1 mL of blood was drawn from the caudal vein by the method of Rowley (*11*) with a single-use, 3 cm^3 syringe and 25 gauge needle (Becton Dickinson, Franklin Lakes, NJ) preloaded with 1 mL of 150 mM NaCl and sodium heparin (30 units/ml).

Erythrocytes Preparation. Red cells were isolated by repeated washings with either 150 or 290 mM NaCl according to Fyhn et al. (*12*).

Plasma Preparation. Whole blood was spun at 700*g* at 4 °C for 15 min, and the supernatant was used.

Preparation of Hemolysate. The method of Fyhn (*12*) was modified. Four volumes of ice cold 1.7% NaCl in 1 mM Tris, pH 8.0, was added to heparinized blood. Centrifugation was done at 700*g* for 10 min at 4 °C using a tabletop clinical centrifuge (IEC, Needham Heights, MA). Plasma was then removed. Red cells were washed by suspending three times in 10 volumes of the above buffer and centrifuging at 700*g*. Cells were lysed in 3 volumes of 1 mM Tris, pH 8.0, for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before centrifugation at 28 000*g* for 15 min at 4 °C in a chilled No. 40 rotor in a Beckman Ultracentrifuge model L5-65B (Beckman Instruments Inc., Palo Alto, CA).

Hydrated Filter Paper (HFP) Matrix. Two grams of Whatman 1 filter paper was cut with scissors into pieces around 3 mm × 3 mm. Ten millimolar sodium phosphate buffer (pH 6.8) was added until the moisture content was 80%.

Washed Cod Matrix. Whole cod fish were hand filleted and skinned. All dark muscle was removed. The rest of the fillets were minced in a Kitchen Aid (Kitchen Aid Inc., St. Joseph, MI) mincer (diameter 4.7 mm). The mince was washed twice in distilled deionized water at a 1:3 mince-to-water ratio (w:w) by stirring with a plastic rod for 2 min, allowing the mixture to stand for 15 min, and then dewatering on a fiberglass screen. It was then mixed with 50 mM sodium phosphate buffer (pH 6.3) at the same 1:3 ratio and homogenized using a Polytron (Brinkman Instruments, Westbury, NY) for 1 min (rheostat at 30). It was allowed to stand for 15 min and then centrifuged at 15 000*g* for 20 min at 4 °C in a Beckman Ultracentrifuge model L5-65B (Beckman Instruments Inc., Palo Alto, CA).

Hemoglobin Levels Added to the Washed Cod. A volume of hemolysate sufficient to provide hemoglobin at the stated concentration was delivered into the washed cod mince. Samples contained 200 ppm streptomycin sulfate to inhibit microbial growth. The pH of samples was checked just after adding hemolysate and at the end of storage. The final moisture content of the samples that were stored at 2 °C was adjusted to 90%.

Sensory. A total of 5−8 trained panelists (*13*) sniffed samples contained in 30 mL capacity brown bottles with caps. Panelists concentrated on detecting rancid/painty odors using a scale of 0−10 with 10 being the strongest. Samples were stored at 2 °C for up to 15 days.

TBARS. Thiobarbituric acid (TBA) reactive substances were determined according to the method of Lemon (*14*). A modification included using 1 g of sample and extracting with 6 mL of trichloroacetic acid by homogenization with a Tissue Tearor model 986-370 at high speed (Biospec Products, Racine, WI).

Determination of Lipid Hydroperoxides. One gram samples were homogenized in 11 mL of chloroform/methanol (2:1). The chloroform used must have ethanol as its preservative since other preservatives caused high blank readings (*15*). Homogenates were then filtered. Two milliliters of 0.5% NaCl was added to 7 mL of the filtrate. Samples were vortexed at a moderate speed for 30 s and then centrifuged for 3 min in a tabletop centrifuge to separate the sample into two phases. Two milliliters of ice cold chloroform/methanol (2:1) was added to 3 mL of the lower phase. Ammonium thiocyanate and iron(II)chloride were added to assay for lipid hydroperoxides (*16*). A standard curve was prepared using cumene hydroperoxide.

Statistical Evaluations. All experiments were done at least twice. An unpaired *t*-test was used to determine significant differences between samples at a single time point (*16*). A general linear model procedure of the SAS system was used to evaluate data from storage studies (*17*). With bled and unbled fish, data from the entire storage period for each treatment were grouped prior to making comparisons.

Table 1. Ratio of Heme Pigments Detected in Hemoglobin Elution Buffer (HB) and Myoglobin Elution Buffer (MB) in Blood and Tissue Extracts from Trout and Mackerel Using Anion Exchange Chromatography[a]

sample	ratio HB/MB (heme basis)	% recovery of total heme pigment
trout blood	3.71 ± 1.18	≥90
trout whole muscle	4.22 ± 0.62	≥90
mackerel blood	no ratio; all in HB	70.8 ± 1.3
mackerel light muscle	no ratio; all in HB	68.8 ± 2.1

[a] $n = 6$.

RESULTS

Bleeding Studies with Rainbow Trout. Anion exchange chromatography was used to separate hemoglobin from myoglobin in muscle tissue extracts to quantify the concentration of each heme protein present. To ensure that hemoglobin subunits were not mistakenly quantified as myoglobin, blood extracts were analyzed prior to muscle extracts. The fact that around one-fourth of the total hemoglobin from trout blood extracts eluted in the myoglobin elution buffer suggests that hemoglobin subunits may have been present (**Table 1**). Tetrameric hemoglobins in fish dissociate into monomers and dimers upon dilution (*18*). The act of preparing a tissue extract is a dilution process. Kranen et al. (*19*) showed that dilution of chicken tissue extracts caused an increase of the observed myoglobin content suggesting subunits of hemoglobin were present. Hemoglobin in our trout blood extracts that eluted in the myoglobin elution buffer may also be indicative of cathodic hemoglobins separating from anodic hemoglobins. The cathodic hemoglobin, component I, makes up around 20% of the total hemoglobin in rainbow trout; component IV, an anodic hemoglobin, represents 75% of the total hemoglobin (*20*). The conditions by which hemoglobins are separated into their multiple components have similarities to our method of separating hemoglobin from myoglobin based on the method of Brown (*9*).

When extracts from trout whole muscle were prepared, around one-fourth of the heme protein eluted in the myoglobin elution buffer, similar to extracts from blood (**Table 1**). Therefore, hemoglobin levels were estimated by measuring the total heme pigment in the extracts from whole muscle of bled and unbled trout. Hemoglobin levels in whole muscle from unbled trout were compared to trout that were bled by a tail-cut or a gill-cut. Because there was no significant difference in hemoglobin level between tail-bled and gill-bled trout ($p = 0.38$), hemoglobin data from tail- and gill-bled trout were combined. On average, there was 11.10 ± 4.59 (5.1−17.8) μmol hemoglobin/kg whole muscle from unbled trout as compared to 7.39 ± 2.93 (4.0−13.9) in bled trout ($p < 0.01$) (**Table 2**).

Rancidity scores from the minced whole muscle of tail-bled and unbled trout during storage for 15 days at 2 °C are presented (**Figure 1**). There was a wide fish-to-fish variation in rancid odor during storage in the bled and unbled groups. Analysis of variance indicated that there was significantly greater rancidity in unbled samples than bled samples ($p = 0.012$). However, throughout storage, some samples within a treatment group were rancid while other samples receiving the same treatment were not rancid (**Figure 1**). Another way to present the data is by graphing the fraction of minced whole muscle samples that had a rancid odor at a given time of storage (**Figure 2**). During storage from 1 to 15 days at 2 °C, 10−60% of the unbled samples had a rancid odor as compared to 0−30% of the bled samples. Presenting the data this way illustrates that bleeding reduced the probability of a rancid odor developing during storage but it was not entirely preventative. A large fish-to-fish variation was observed in TBARS development in both the bled and unbled groups after day 3 (**Figure 1**). From day 7 to 15, average TBARS values were greater in the unbled group than the bled group. Analysis of variance did not show a significant difference between the two groups ($p = 0.44$).

Because there was wide fish-to-fish variation in both hemoglobin content and that removed by bleeding, the relationship between hemoglobin level and lipid oxidation was investigated using regression analysis at day 9 of storage. Day 9 was chosen for two reasons. First, day 9 was the day at which the greatest number of samples was analyzed. Second, the spread in the TBARS values was wide at this period of storage within

Table 2. Hemoglobin and Myoglobin Concentrations and Percent Myoglobin Present in Trout Whole Muscle and Mackerel Light and Dark Muscle from Bled and Unbled Fish[a]

sample	treatment	μmol hemoglobin/kg tissue	μmol myglobin/kg tissue	% myoglobin of total heme protein wt.
trout whole muscle	unbled	11.10 ± 4.59	ND	ND
trout whole muscle	bled	7.39 ± 2.93[b]	ND	ND
mackerel light muscle	unbled	6.07 ± 1.02	ND	ND
mackerel light muscle	bled	3.40 ± 0.48[c]	ND	ND
mackerel dark muscle	unbled	158.8 ± 21	342.0	35
mackerel dark muscle	bled	121.8 ± 17	382.8	44

[a] The total heme pigment was measured in trout whole muscle and mackerel light muscle since preliminary studies indicated hemoglobin was the only detectable heme protein. Ultrafiltration was used to quantitate hemoglobin and myoglobin levels in muscle extracts from dark muscle. ND, not detected. [b] $p < 0.01$ between bled and unbled fish. [c] $p < 0.001$ between bled and unbled fish.

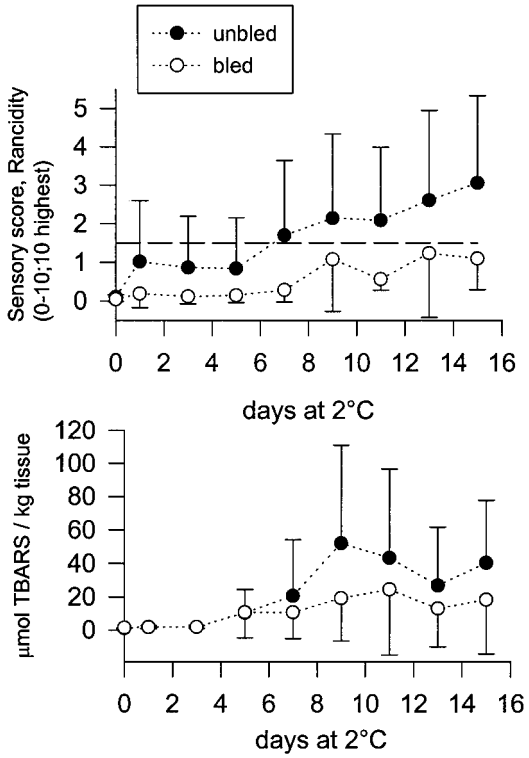

Figure 1. Sensory scores and TBARS of minced whole muscle from unbled and bled trout during storage at 2 °C. Standard deviations represent 7–11 separate minced muscle samples. Horizontal dashed line indicates slight rancidity.

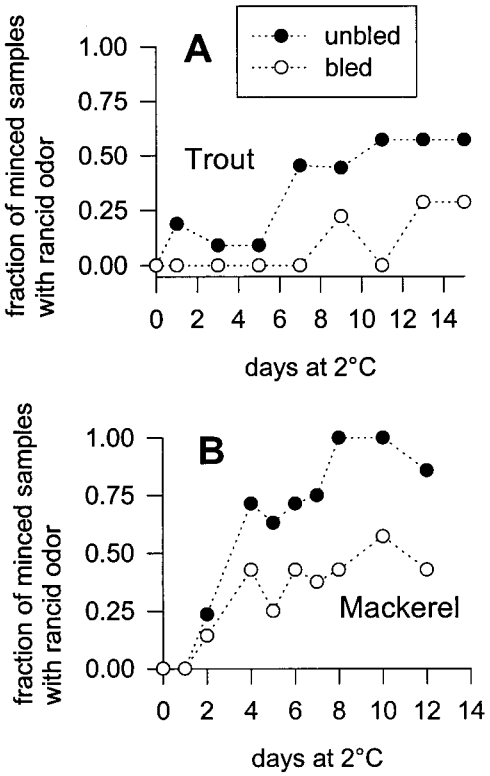

Figure 2. Fraction of minced trout whole muscle (**A**) or mackerel light muscle (**B**) samples from bled and unbled fish that had a rancid odor at different times of storage at 2 °C.

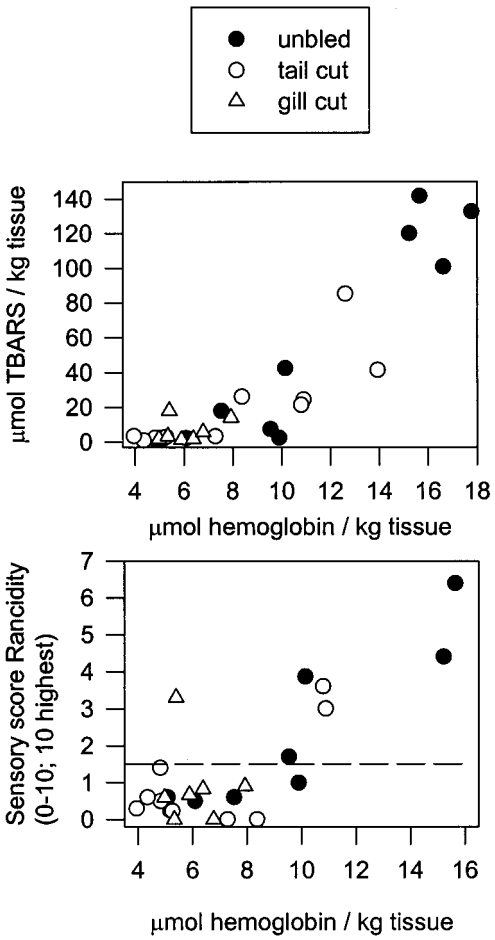

Figure 3. TBARS and rancidity scores at day 9 plotted against initial hemoglobin levels determined in minced whole muscle of unbled, tail-cut, and gill-cut trout. Horizontal dashed line indicates detectable rancidity.

treatments. TBARS values from the muscles of tail-bled, gill-bled, and unbled fish at day 9 of storage were plotted against the initial hemoglobin levels determined in those muscle tissues (**Figure 3**). A quadratic term was used to account for the flat part of the TBARS response at the lower levels of hemoglobin. The regression analysis correlating TBARS at day 9 and initial hemoglobin levels yielded an R^2 value of 0.87 ($y = 0.827x^2 - 7.62x + 19.95$). An R^2 value of 0.67 ($y = 0.039x^2 - 0.30x + 1.11$) was determined when correlating rancidity scores at day 9 against initial hemoglobin levels in the muscle (**Figure 3**).

Bleeding Studies with Atlantic Mackerel. Hemoglobin levels were determined in extracts of mackerel light muscle from bled and unbled fish. On average, there was 6.07 ± 1.02 (4.8−8.8) μmol hemoglobin/kg light muscle from unbled mackerel as compared to 3.40 ± 0.48 (2.7−4.4) in bled mackerel ($p < 0.001$).

Rancidity scores from the minced light muscle of bled and unbled mackerel during storage are presented in **Figure 4**. There was a wide fish-to-fish variation in rancid odor during storage in the bled and unbled groups. Analysis of variance indicated that there was a 90% likelihood that bleeding reduced rancidity using all of the storage data. Graphing the fraction of minced light muscle samples that had rancid odor at a given time of storage indicated that bleeding reduced the probability of a rancid odor developing during storage but it was not entirely preventative (**Figure 2**). At day 8, for example, around 40% of the bled samples had a rancid odor as compared to 100% of the unbled samples. It can also be seen in **Figure 2** that minced mackerel light muscle was more likely to develop a rancid odor than minced trout whole muscle. A large fish-to-fish variation was observed in TBARS development in both unbled and bled groups (**Figure 4**). TBARS values were on average greater in unbled samples than bled samples of mackerel light muscle after day 2 but not significantly different using all of the storage data ($p = 0.26$).

Anion exchange chromatography gave inconsistent results with mackerel dark muscle. Therefore, ultrafiltration, using a 50 kDa molecular mass cutoff membrane was used to quantitate myoglobin (~17 kDa) and hemoglobin (~68 kDa) in mackerel dark muscle. It was necessary to test if any hemoglobin subunits were present in the <50 kDa fraction. Blood extracts were subjected to ultrafiltration, and 2.2 ± 0.9% of the total heme pigment was found in the <50 kDa fraction (data not shown). After accounting for a 2.2% passage of hemoglobin into the filtrate, hemoglobin and myoglobin levels were calculated for dark muscle (**Table 2**). From unbled mackerel, there was 158.8 ± 21 μmol hemoglobin/kg dark muscle as compared to 121.8 ± 17 in bled mackerel. On average, there was 342.0 μmol myoglobin/kg dark muscle in unbled mackerel and 382.2 in bled mackerel, which indicated that myoglobin accounted for 35% of the total heme protein by weight in unbled samples and 44% in bled samples. Ultrafiltration was compatible with dark muscle because the concentration of heme pigments in dark muscle is high enough so that the extract can be greatly diluted and still have detectable hemoglobin and myoglobin. Dilution is necessary to prevent clogging of the ultrafiltration tube.

After 24 h at 2 °C, dark muscle minces from tail-cut and unbled mackerel had near maximal rancidity odors and TBARS values in excess of 350 μmol/kg tissue (**Table 3**). These data indicated a very rapid rate of lipid oxidation in both bled and

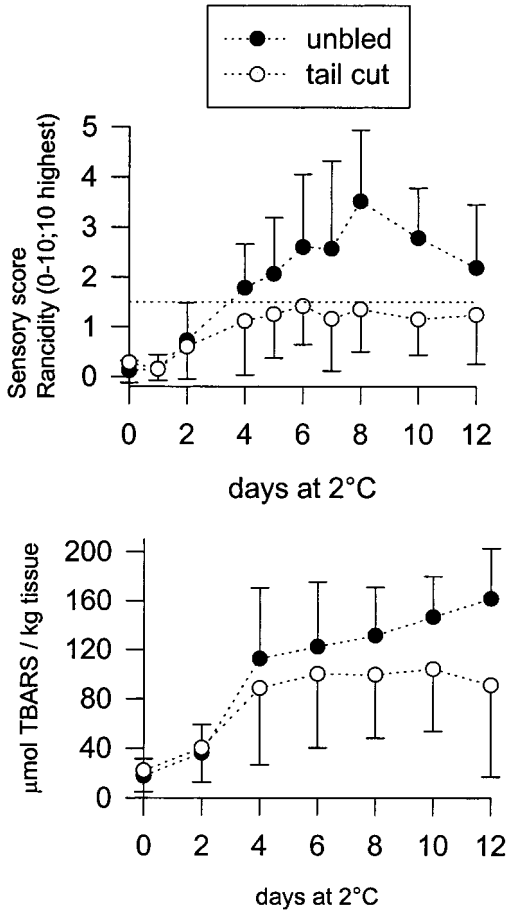

Figure 4. Sensory scores and TBARS of minced light muscle from unbled and bled mackerel during storage at 2 °C. Half of the error bar is shown to more clearly illustrate the standard deviations of each group. Standard deviations represent 6–8 separate minced muscle samples.

Table 3. Sensory Scores and TBARS Values in Intact and Minced Dark Muscle from Bled and Unbled Mackerel after 1 Day of Storage at 2 °C[a]

sample	sensory	TBARS
unbled/minced tissue	8.9 ± 0.8	360.9 ± 54
bled/minced tissue	8.5 ± 0.3 (ns)	370.2 ± 16 (ns)
unbled/intact tissue	3.2 ± 2.2	108 ± 23
bled/intact tissue	0.7 ± 0.5[b]	12.2 ± 4[c]

[a] The sensory score was a measure of rancidity (0–10; 10 highest). A score of 1.5 was detectable rancidity. TBARS units were µmol MDA/kg tissue. With intact tissue samples, bled and unbled fish were stored for 1 day on ice after capture. Dark muscle was then removed and immediately frozen at – 80 °C for 4–5 months. Samples were thawed and then stored for 1 day at 2 °C before analysis. ns, no significant difference between treatments. [b] Highly significant difference between bled and unbled fish. [c] Very high significant difference between bled and unbled fish.

unbled minced dark muscle samples as compared to minced trout whole muscle and mackerel light muscle. Rancidity and TBARS were also determined in intact dark muscle prepared from tail-bled and unbled mackerel (**Table 3**). When the dark muscle was kept intact, bleeding did significantly reduce rancidity (around 4−5-fold) and TBARS development (around 9-fold) in samples stored at 2 °C for 24 h. The intact dark muscle samples were frozen at −80 °C and then thawed prior to 2 °C storage.

Adding Blood and Blood Components to Washed Cod Muscle. Lysed and nonlysed erythrocytes were added to washed cod at a hemoglobin level of 5.8 µmol/kg washed cod. An equivalent amount of NaCl was added to lysed and nonlysed samples to ensure that NaCl was not acting as an antioxidant when used to prevent cell lysis. This experiment was done to test if limiting the rupture of erythrocyte cell membranes delayed lipid oxidation. Preventing the lysis of erythrocytes significantly reduced rancidity ($p < 0.05$) and TBARS development ($p < 0.01$) at 20 and 27 h (**Figure 5**).

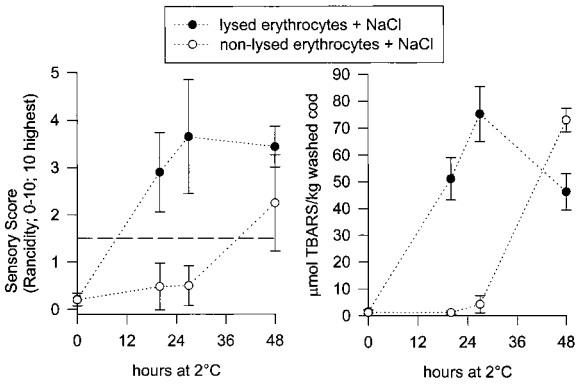

Figure 5. Sensory scores and TBARS values of lysed and nonlysed erythrocytes added to washed cod. Erythrocytes were added at 5.8 µmol hemoglobin/kg washed cod. Horizontal dashed line indicates detectable rancidity. Final pH was 6.3. An equivalent amount of NaCl (150 µmol/kg) was added to lysed and nonlysed samples to ensure NaCl was not acting as an antioxidant when used to prevent cell lysis.

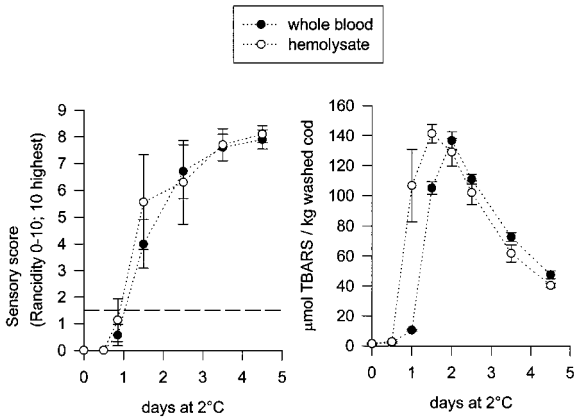

Figure 6. Sensory scores and TBARS values of trout hemolysate or whole blood added to washed cod. The hemoglobin level in each sample was 11.6 µmol/kg washed cod. Horizontal dashed line indicates detectable rancidity. Final pH was 6.3.

Hemolysate consists of the soluble components of the disrupted erythrocyte. It was suspected that the hemolysate could account for all of the lipid oxidation capacity of blood. To test this, whole trout blood and hemolysate were added separately to washed cod at equivalent hemoglobin concentrations (**Figure 6**). The maximal amount of lipid oxidation during storage based on sensory score and TBARS was nearly the same when comparing hemolysate samples to whole blood samples added to washed cod muscle. The whole blood samples developed TBARS more slowly than the hemolysate samples at day 1 ($p < 0.01$) and day 1.5 ($p < 0.01$).

Blood plasma possesses components that can both stimulate or delay the onset of lipid oxidation (*21*). Hemolysate at four different trout hemoglobin concentrations (0.06, 0.50, 1.8, and 5.8 µmol/kg) was added to washed cod in the presence or absence of plasma at 2.5% of the wet weight. This level of plasma is in the range found in trout light muscle (*22*). Plasma

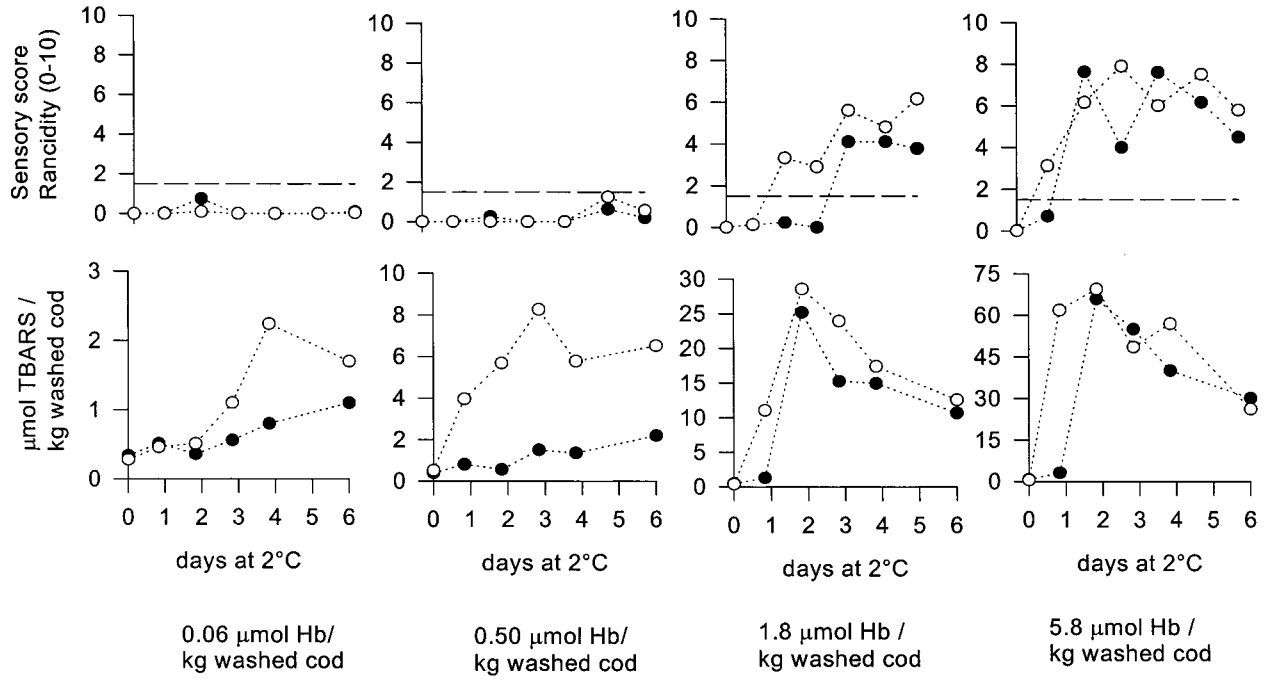

Figure 7. Sensory scores and TBARS values with different levels of hemolysate added to washed cod ± plasma (closed symbols + plasma). Plasma level added was 2.5% of the wet weight. Hemoglobin levels were 0.06, 0.50, 1.80, and 5.80 μmol/kg washed cod. Horizontal dashed line indicates detectable rancidity. Final pH was 6.3

delayed TBARS development at all four levels of hemolysate addition (**Figure 7**). Plasma delayed rancidity development as well at the two higher hemoglobin concentrations. It was suggested that vertebrates with low blood volumes are complemented with higher amounts of plasma in a secondary vascular system to aid in circulating metabolites (*20*). This may explain why the plasma-to-blood ratio in plaice muscle, a white-fleshed fish, was around 55 as compared to 0.9 in trout muscle (*22*, *23*). Muscle containing large amounts of blood, such as dark muscle, may be highly susceptible to lipid oxidation not only because of its high pro-oxidant content but also because of its low plasma volume relative to sedentary, white-fleshed species.

The highest level of TBARS determined during storage caused by the hemolysate increased with increasing hemolysate concentrations. The correlation between hemolysate level in the washed cod and maximal level of TBARS during storage was high ($R^2 = 0.99$). Hemolysate added at 0.06 and 0.50 μmol hemoglobin/kg tissue did not develop rancidity during storage, while at 1.8 and 5.8 μmol hemoglobin/kg tissue, rancidity developed in 2 and 1 day, respectively.

Oxidation of Plasma Lipids in the Presence of Hemoglobin. Plasma was added to HFP to determine if plasma lipoproteins were susceptible to lipid oxidation. Some erythrocytes ruptured during the isolation of plasma. This assessment was based on finding spectra similar to hemoglobin in the isolated plasma (data not shown). The least-contaminated plasma on the HFP sample contained around 0.02 μM hemoglobin. A cell free hemolysate prepared from erythrocytes was added to minimally contaminated plasma to increase the concentration of hemoglobin to 0.39 μM. Plasma containing either 0.02 or 0.39 μM hemoglobin was then added to the HFP. Rancidity and TBARS developed only in the samples with the higher amount of hemoglobin (**Figure 8**). Lipid hydroperoxides initially increased in the samples containing either 0.02 or 0.39 μM hemoglobin contamination during storage (**Figure 9**). Lipid hydroperoxides leveled off and decreased later in storage in the sample with the larger amount of hemoglobin, whereas lipid

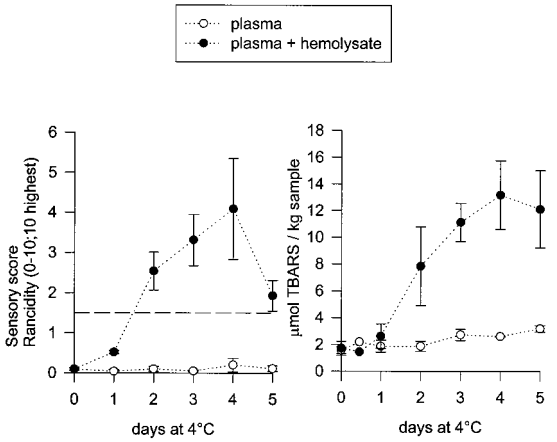

Figure 8. Sensory scores and TBARS values in plasma added to HFP with or without added hemolysate during storage. Plasma level added was 375 μL/15 g HFP. Hemolysate level was 0.02 μmol hemoglobin/kg HFP in the plasma only sample and 0.39 μmol hemoglobin/kg HFP in the plasma samples with added hemolysate. Horizontal dashed line indicates detectable rancidity. Final pH was 6.8.

hydroperoxides continued to increase during storage in the samples with the lower amount of hemoglobin. In fact, significantly greater amounts of lipid hydroperoxides were determined in the sample with lesser amounts of hemoglobin at days 4 and 5 of storage ($p < 0.05$). These data indicate that rancidity from plasma lipids occurred on HFP if adequate hemoglobin was present. The ability of different classes of blood lipids to impart rancidity has been reported (*24*).

DISCUSSION

Hemoglobin is a likely catalyst of lipid oxidation in trout whole muscle and mackerel light muscle. Myoglobin content was much lower than hemoglobin in these tissues (**Table 2**). An interesting result of these studies was that hemoglobin

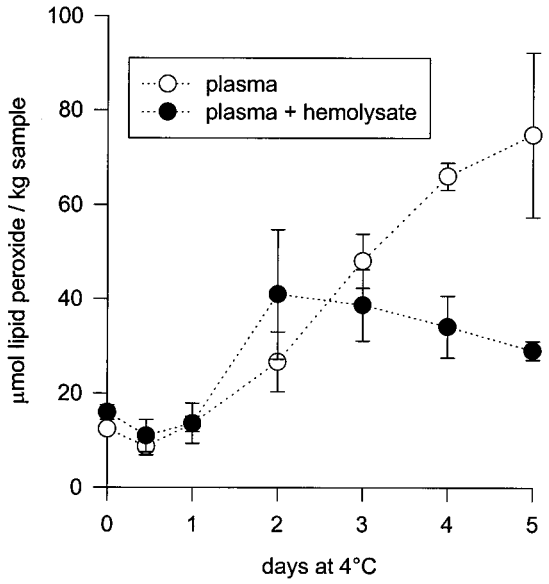

Figure 9. Lipid peroxides in plasma added to HFP with or without added hemolysate during storage. Experimental conditions were the same as in **Figure 8**.

concentration was higher in the trout whole muscle (**Table 2**); yet, mackerel light muscle was more susceptible to lipid oxidation during iced storage (**Figure 2**). To partly explain this finding, the different types of hemoglobins in fish should be considered. Anodic hemoglobins bind oxygen poorly at pH values found in post mortem muscle while cathodic hemoglobins retain high oxygen affinity (*25*). The low oxygen affinity of anodic hemoglobins is relevant because deoxyhemoglobin was found to be a more effective catalyst of lipid oxidation in liposomes as compared to oxyhemoglobin and methemoglobin (*26*). Hemoglobins from mackerel are known to have lower oxygen affinity than hemoglobins from less active fish (*27*). The potential for higher levels of deoxyhemoglobin catalyst in the highly active mackerel as compared to trout might explain the greater rate and extent of lipid oxidation in mackerel light muscle as compared to trout whole muscle despite the hemoglobin concentration being greater in trout whole muscle. Mackerel hemoglobins were found to promote lipid oxidation more rapidly than trout hemoglobins in washed cod at pH 6.3 (*28*).

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 (*29*, *30*). Although pH was not measured in the trout whole muscle and mackerel light muscle used in these studies, a decreased pH in one of the species would likely accelerate hemoglobin-mediated lipid oxidation. Decreasing the pH from 7.2 to 6.0 enhanced the ability of trout hemolysate to stimulate lipid oxidation in washed cod muscle, which was related to the higher deoxyhemoglobin content at the lower pH (*31*). Lower oxygen affinity of hemoglobin at reduced pH is known as the Bohr effect (*24*). Some fish hemoglobins exhibit an exaggerated Bohr effect called the Root effect, which is a continued decrease in oxygen affinity below pH 6.5 (*25*).

The extent of lipid oxidation in washed cod increased with increasing trout hemoglobin concentration (**Figure 7**). Similarly, Huang et al. (*32*) found that the extent of sarcoplasmic reticular lipid oxidation as indicated by TBARS was proportional to the iron levels added using Fe^{2+}-ADP as a catalyst. This indicated that the breakdown of lipid hydroperoxides was limited by the amount of iron present. In our studies, it appeared that lipid hydroperoxides were broken down if sufficient hemoglobin was present. This was based on the lower lipid hydroperoxides determined during storage in the samples with elevated hemoglobin levels (**Figure 9**). TBARS and sensory analysis support this conclusion since TBARS and rancidity, each measures of lipid hydroperoxide breakdown, were substantial only in the sample with the higher hemoglobin level (**Figure 8**). The propensity of anodic hemoglobins to be deoxygenated could promote the breakdown of lipid hydroperoxides. This is because hemoglobins with low oxygen affinity are noted for their rapid autoxidation, which produces methemoglobin (*33*). Part of the pathway that leads to heme release requires methemoglobin formation (*34*). Released heme is a likely candidate of hemoglobin-mediated lipid hydroperoxide breakdown since the reaction of heme compounds with lipid hydroperoxides is a general phenomenon (*35*) that is without the steric constraints of a lipid hydroperoxide fitting into the heme crevice of hemoglobin.

Another pathway by which hemoglobin can promote lipid oxidation is the formation of the ferryl hemoglobin radical, considered an initiator of lipid oxidation via abstraction of an H atom from a polyunsaturated fatty acid (*36*). Autoxidation of hemoglobin will form a superoxide anion radical ($O_2^{\bullet -}$) and methemoglobin (*37*). $O_2^{\bullet -}$ can be converted to H_2O_2, which will react with methemoglobin via a heterolysis reaction to form the ferryl protein radical. Lipid oxidation can also be propagated via iron released from hemoglobin. However, very high levels of hydrogen peroxide were found to be required to release the iron (*38*).

The formation of hemoglobin subunits due to hemoglobin dilution (*18*) can have a stimulatory effect on lipid oxidation. This is because hemoglobin dimers were found to undergo hemoglobin autoxidation 16 times faster than the tetramers (*39*). Dilution of hemoglobin will occur when hemoglobin leaks out of erythrocytes. pH reduction is another means to encourage subunit formation. A pH reduction from 7.5 to 6.2 accelerated dimer formation more than 10-fold using human hemoglobin (*40*). It should be noted that heme is released more readily from hemoglobin subunits than the intact tetramer (*41*), which may be related to the faster autoxidation rate of the subunits.

Bleeding fish either by a gill-cut or tail-cut had variable results in residual hemoglobin content (**Table 2**) and its ability to prevent lipid oxidation (**Figure 1**). Porter et al. (*8*) found that there was no significant difference in hemoglobin content (used to estimate blood content) between bled and unbled sockeye salmon. These authors also found that bleeding did not reduce lipid oxidation during frozen storage in the steaks. In another salmonid, rainbow trout, bleeding did reduce rancidity and TBARS during frozen storage of fillets (*7*). In skipjack mackerel, bled muscle had a less fishy odor, less heme protein autoxidation, and higher pH than unbled controls (*42*). Elevated pH lessens deoxygenation of the anodic hemoglobins. Other parameters of fish quality also show variable results from bleeding. Fish freshness as indicated by *K* value had a "tendency" to be greater in bled as compared to unbled mackerel (*43*). Bleeding delayed muscle softening in pelagic species such as mackerel but not demersal fish like red sea bream (*44*). It was reported that when Atlantic cod were bled immediately, 74% received a top grade from the inspector; whereas if a 4 h delay before bleeding was implemented, only 30% were top grade (*45*).

To thoroughly bleed, gutting and storage in an ice-slush is recommended in addition to the tail- or gill-cut (*46*). Gutting severs major blood vessels, which further facilitates blood removal. Thus, our bled fish were bled, gutted, and stored in

an ice-slush for 24 h prior to removing the muscle for analysis. This presents a dilemma as to handling of unbled control fish. Keeping unbled fish whole for the same 24 h period will prevent bleed out but will decrease exposure of the fish interior as compared to the gutted, bled group. We decided to keep unbled fish whole to allow time for blood to coagulate in an effort to minimize bleed out. Because round fish are probably more protected against lipid oxidation as compared to gutted fish, it is possible that bleeding would have inhibited lipid oxidation even more than our observations (**Figure 2**) if bled and unbled fish were gutted at the same time, but the risk was that blood would be removed from the unbled group by a rapid gutting.

Although there was a wide range of hemoglobin levels in the muscle of bled fish, generally, substantial amounts of blood remained in the muscle after bleeding based on comparison to unbled controls (**Table 2**). It has been reported that 3−4 capillaries surround each muscle cell of tuna red muscle (*47*). This author found that the capillary-to-muscle cell ratio was similar in various species and muscle types. When a major blood vessel is cut, some blood will flow out of the blood vessel at the site of the incision. At the same time, it seems reasonable that a substantial fraction of blood in the much smaller capillaries surrounding muscle cells would not empty, especially considering the drop in blood pressure due to the incision. Another factor that may have contributed to the substantial amount of blood in the muscle after bleeding is the potential for rapid blood coagulation in fish. Clotting times of blood between 5 s and 30 min have been reported in fish (*46*, *48*).

There was a good correlation between hemoglobin content and lipid oxidation in minced muscle (**Figure 3**). The mechanical action of mincing will rupture blood vessels, some erythrocytes, and muscle cells causing extracellular and intracellular components to mix. This will cause dilution of hemoglobin formerly housed inside erythrocytes. As stated, hemoglobin dilution can encourage hemoglobin subunits to form, which accelerate hemoglobin autoxidation and heme release. Releasing hemoglobin from erythrocytes was shown to accelerate lipid oxidation in the washed cod model system (**Figure 5**), and there is evidence that oxidation of erythrocyte membrane lipids can destabilize the membrane allowing additional hemoglobin to leak out (*49*). The fact that mincing will aid in releasing hemoglobin from erythrocytes and distributing hemoglobin throughout the muscle may have contributed to the good correlation between hemoglobin content and lipid oxidation in minced muscle.

The good correlation between hemoglobin content and lipid oxidation in minced muscle raises the question as to what is the probability of a similar correlation in intact fillets or gutted fish. Oxygen from the atmosphere penetrates 1−4 mm into muscle tissue (*50*). Lipid oxidation would more likely occur to a greater extent at the surface where abundant oxygen is available to oxidize lipids even if higher pro-oxidant levels were located in the interior. It was shown that the middle part of herring fillets were the least susceptible to lipid oxidation as compared to the underskin layer and inner part (*51*). At the same time, oxygen bound to hemoglobin and myoglobin should be considered as a source of dioxygen. On the basis of our results, there was around 12−28 μmol of heme iron per kg mackerel light muscle and 870−980 μmol heme iron per kg dark muscle (**Table 2**). These hemes are binding sites for dioxygen. A TBARS value of 12−20 μmol per kg tissue was the point at which rancidity was detected in surface tissue from mackerel fillets (*13*). As a first approximation, 1 mol of oxygen can add to 1 mol of fatty acid to form 1 mol of lipid hyroperoxide, which can break down to form 1 mol of malondialdehyde, a TBA reactive substance. In practice, the ratio of peroxide to TBARS was around 5−8:1 (*32*). If 7 mol of oxygen are required to produce 1 mol of TBARS, in mackerel dark muscle there is more than enough oxygen bound to heme protein to propagate lipid oxidation. Mackerel light muscle probably does not contain enough oxygen to produce a rancid odor. It should be kept in mind that some of the heme bound oxygen will form $O_2^{\bullet -}$ due to hemoglobin autoxidation, and some of the heme sites will be deoxygenated due to Bohr effects. Blood, erythrocytes, and hemoglobin will leak out of capillaries less in intact as compared to minced muscle, and hemoglobin will remain inside erythrocytes better in intact muscle. This will limit the liberation of hemoglobin catalyst and dilution of the hemoglobin. The less distributed dioxygen and hemoglobin in intact as compared to minced muscle may create a situation that reduces a notable correlation between hemoglobin concentration and lipid oxidation in the intact tissue.

Lipid oxidation was more rapid and extensive in minced dark muscle as compared to intact dark muscle (**Table 3**). The greater exposure of the minced muscle to atmospheric oxygen is probably part of the reason for the more rapid lipid oxidation. At the same time, a greater release of hemoglobin from erythrocytes in minced muscle is likely relevant. This is supported by the effect of bleeding on lipid oxidation in intact and minced dark muscle. In minced tissue, bleeding did not delay lipid oxidation while in intact muscle bleeding did delay lipid oxidation (**Table 3**). It has been documented that capillaries rupture causing "blood splash" in muscle if the animal is not bled rapidly (*52*). Apparently, without rapid bleeding, there is a failure to quickly reduce the blood pressure; hence, capillaries burst. The rupture of capillaries by the mechanical mincing of both bled and unbled samples could obscure the effect of pressure-mediated rupturing of capillaries in only unbled fish. This can explain why bleeding slowed lipid oxidation in intact but not minced mackerel dark muscle. However, the high content of hemoglobin and myoglobin in dark muscle may not serve as a good model for light muscle (**Table 2**). Future work could include examining the effect of bleeding on lipid oxidation in minced and intact light muscle. Further, a survey of the effect of bleeding on hemoglobin contents and its relation to lipid oxidation of light muscle should be investigated.

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