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ABSTRACT

Whole body saline-perfused rainbow trout (*Oncorhynchus mykiss*) was ice-stored for 4 weeks and compared with unwashed/washed minces from unbled and bled trout in terms of rancid odor, peroxide value (PV), thiobarbituric acid reactive substances (TBARS) and redness-loss. Muscle from saline-perfused fish, which had 72% less total heme, was deficient in rancid odour during the whole storage, while bled (54% less heme) and unbled samples developed rancid odor already after ~4 and 2 days, respectively; higher intensity without bleeding. PV/TBARS also developed in the order unbled>bled>perfused samples, however, PV/TBARS were not as completely prevented as rancid odour after perfusion. Saline-washing (3×3 volumes) of unbled mince removed 84% heme and yielded the second most stable sample, while saline-washing (1×1 volumes) destabilized unbled mince, despite 64% heme-removal. Concurrent antioxidant-removal during washing of minces obviously counteracted the effect of blood removal and washing fish mince with small volumes of solution should be used with great care.

Keywords: fish, blood, haemoglobin, heme, lipid oxidation, perfusion, bleeding, rancidity

INTRODUCTION

Haemoglobin, Hb, plays a fundamental role for the onset of lipid oxidation in fish muscle during *post mortem* handling and storage, ultimately leading to rancid odor/taste, reduced nutritional value, texture changes and colour changes (Richards & Hultin, 2002; Undeland et al., 1999; 2004; Maqsood & Benjakul, 2011; Larsson & Undeland, 2010; Cavonius & Undeland, 2017). Contamination of Hb can occur e.g. when blood vessels are damaged and erythrocytes disrupt or lyse during various process operations. There are multiple routes by which Hb then can catalyse oxidation, with a critical initiating step being the pH-drop that rapidly takes place in fish muscle *post mortem*, particularly in certain pelagic active species like herring (Cavonius & Undeland, 2017). Reduced pH will induce Hb deoxygenation, via both the Bohr and Root effect, which in turn facilitates for Hb-autoxidation and hemin loss (Aranda et al., 2009). Met-Hb can generate active oxygen species like $O_2^{\cdot -}$, $HO^{2\cdot}$, H_2O_2, and $^{\cdot}OH$, out of which H_2O_2 can react with met-Hb to form a hypervalent perferryl-Hb (Fe4+=O) radical capable of initiating lipid oxidation via hydrogen abstraction (Kanner & Harel, 1985). Lipid hydroperoxide cleavage into LOO· or LO· by met-Hb or released hemin is another very important catalytic mechanisms (Ryter & Tyrrell, 2000). A series of studies in the last 10 years (e.g. Grunwald & Richards, 2006; Cai et al., 2013) have in fact revealed that heme/hemin via this pathway may be the most critical peroxide breaking species, and that the heme/hemin-release itself, occurring as the binding to the proximate histidine gets weakened, may be one of the most critical events in fish lipid oxidation development. The low polarity of the free hemin is expected to aid dissolving it into the hydrophobic interior of membranes where lipid hydroperoxides are located (Van Der Zee et al, 1996).

The only current commercial method to limit blood contamination during fish processing is bleeding, which is applied to many commercially caught large fish species and even legislated as part of the slaughter process for aquaculture raised fish . However, it is not applied to the small pelagic species like herring, sardines, sprat etc. since there is a lack of proper techniques applicable on large volumes of very small fish. In some studies bleeding has been shown to clearly reduced lipid oxidation (e.g. Richards & Hultin. 2002; Maqsood & Benjakul, 2011) but in others not (e.g. Sohn et al,2007). Bleeding removes only ≤50% blood (Richards & Hultin, 2002; Sohn et al., 2007), mainly due to the quick drop in blood pressure as well as fast coagulation caused by extended pre-bleeding holding (Roth et al., 2005), temperature raises (Olsen et al., 2006) and stress (Roth et al., 2009).

Based on the above, it could be argued that more efficient bleeding techniques for fish should be established; and also, that severe efforts should be put on developing blood removal techniques for the small pelagic species, which indeed are highly susceptible for lipid oxidation. However, a fundamental question to put forward in this context is how dominant the role of blood/Hb is for lipid oxidation development compared to other well-known pro-oxidants of fish muscle such as myoglobin (Mb), lipoxygenases (LOX) and reduced low molecular weight (LMW) trace elements like Fe and Cu? All these compounds contribute to both formation and breakdown of lipid hydroperoxides (Qiu et al., 2013; Baron & Andersen, 2002; Schaich, 1992); for Mb according to similar mechanisms as Hb while for LMW-Fe and LOX by other mechanisms. To separate the role of Hb from other pro-oxidants under *in situ* conditions, the ultimate model would be to empty the capillaries from blood, without affecting the muscle-derived pro-oxidants. Most previous attempts to elucidate the role of pro-oxidants in fish muscle, including our own, have been to mince and then wash muscle, an approach which removes both blood and muscle-bound pro-oxidants (e.g. Richards & Hultin, 2002; Undeland et al. 2002; 2003; 2004).

During perfusion, a solution such as saline is pumped through the intact circulatory system, gradually washing out the blood. We here hypothesized that whole body perfused fish would be a strong model system to unravel the relative impact of blood/Hb for lipid oxidation in fish, in comparson to the impact from pro-oxidants of the muscle, i.e. outside the blood capillaries. To the best of our knowledge; only two studies so far have linked perfusion to lipid oxidation measurements, but then with a focus to introduce antioxidants into the capillaries, and thus, not to study the role of blood removal *per se* (Sohn et al., 2007; Tuckey et al., 2012).

The aim of this study was to investigate the development of lipid hydroperoxides, thiobarbituric acid reactive substances (TBARS), rancid odour and colour changes during ice storage of minced muscle originating from whole body saline (0.9% NaCl) perfused or intact rainbow trout *Oncorhynchus mykiss*. To obtain comparisons to more conventional methods used to reduce Hb of fish muscle; mince from standard bled fish, and minces being subjected to two versions of washing were also included in the design.

MATERIALS AND METHODS

Chemicals

Ammonium thiocyanate, barium chloride, cumene hydroperoxide, chloroform, Heparin, metaphosphoric acid, phosphoric acid, sodium nitroprusside, streptomycin, t*ris*(2-carboxyethyl)phosphine and 2-thiobarbituric acid were purchased from Sigma-Aldrich (Stockholm, Sweden), Bovine Hb, methanol and 1,1,3,3-tetraethoxypropane were obtained from Fluka (Bunchs, Switzerland). Acetone was purchased from Fischer Scientific (Gothenburg, Sweden). Iron (II) sulphate hepta hydrate was obtained from Merck (Solna, Sweden). Hydrochloric acid and trichloroacetic acid were purchased from Scharlau (Barcelona, Spain).

Fish

Rainbow trout (*Oncorhynchus mykiss*) between 0.4-0.7 kg were obtained from a local hatchery (Antens Laxodling AB, Alingsås, Sweden) and kept in 2 000-liter fiberglass tanks, supplied with recirculating water at 10°C. The fishes were subjected to a 12:12 photoperiod and fed with a maintenance diet of commercial trout pellets. Ethical permit from the regional ethical committee on animal experiments research was given for all procedures reported in this study (96/2001). The fish used was killed by a sharp blow to the head before the fish was either bled, perfused or left unbled.

Unbled fish: After the euthanization, four fishes were placed in ice-cold water for 60 min and thereafter kept in individual plastic bags separately on ice in a cooler bag (i.e. 0-3°C) until preparation of oxidation system (approximately 18-20h).

Bled fish: After the euthanization, the gills were directly cut in four fishes and they were left to bleed out in ice-cold water for 60 min. The fishes were kept in individual plastic bags separately on ice at 1-3°C until preparation of oxidation system (approximately 18-20h).

Perfused fish model: After the euthanization, 0.2 mL heparin (5000 IU/ml) was injected into the caudal vein of the four fishes before the preparation. The heart was directly exposed by a ventral midline incision. Through a cut in the exposed ventricle a P90 catheter filled with 0.9% saline solution was advanced via the bulbus cordis into the ventral aorta where it was secured using a silk suture. The catheter was connected to a peristaltic pump and the fish was perfused with 0.9 % saline solution, bubbled with air, containing 20nM sodium nitroprusside and 25 units heparin /L, for 20 minutes; the sodium nitroprusside was used for dilation of blood vessels. The fishes were thereafter perfused with 0.9 % saline solution bubbled with air for 70 minutes at a flow rate of 10 mL/min, maximum perfusion pressure was 5kPa. After the perfusion the fishes were kept in individual plastic bags separately on ice at 1-3°C until preparation of oxidation system (approximately 18-20h).

Preparation of Oxidation System

The trout was manually filleted, the skin was removed and the skinless fillets were ground in a kitchen grinder using a hole plate with a hole diameter of 5mm (Kitchen Aid, Ultra Power, Model KSM90, St Joseph, MI, USA). Minced fillets from four fishes were pooled for the three different groups (unbled, bled, perfused), respectively, by manual mixing using a stainless steel spoon. For the washed minces, a 1:1 washing was carried out by mixing 100 g minced muscle from the unbled group with 100 mL ice cold 0.9% saline and the slurry was thereafter left on ice for 20 min. The washing fluid was removed by a kitchen sieve and light manual pressure was applied towards the end to remove excess fluid. The washing fluid was then kept in -80◦C. Three 1:3 washes were also done to simulate classic surimi making; 100g mince from the same treatment group as above was mixed with 300 mL ice cold 0.9% saline where after the same procedure as above was repeated 3 times. The resulting washed minces are hereafter referred to as "single-washed" or "triple-washed", respectively. To avoid microbial growth during subsequent ice storage, 200 ppm streptomycin was added to each group of fish mince model system. Thereafter the pH of the mince was recorded (see below) and since the pH of all minces were close to 6.6; a pH typical for post mortem fish muscle, no adjustments were needed. For each mince 2×25 g was then flattened out on the bottom of two 250 mL Erlenmeyer flasks (E-flasks) yielding a layer of 5-6 mm thickness and the flasks were stored on ice in darkness at 1-3°C for up to 27 days. Samples (1g) were taken regularly throughout the storage according to the method by Larsson et al. (2007).

Measurement of pH

pH was measured regularly throughout the storage of the oxidation system. One gram minced fish was vortexed with 9 mL water and left for approximately 2 minutes before pH measurements (Radiometer analytical PHM210, Villeurbanne, France).

Sensory Analysis

Sensory analyses were carried out as described by Undeland et al. (2004) and Larsson et al. (2007). In brief, the head space of the E-flasks was sniffed regularly by a small internal panel (3 people) during ice storage of the minced trout sample. Attention was given to recognize the intensity of rancid odor development, which was marked on a scale from 0 to 100. On this scale, 0 indicated no smell, 10 slightly rancid, 50 medium rancid, and 100 maximum rancid. The internal panel was trained on both fish and fish oil samples at various stages of oxidation to learn to quantify rancid odor using the applied scale.

Colour Measurements

The colour change which was regularly measured during storage was redness (a*). A colorimeter (Minolta Chroma Meter CR-3, MinoltaCorp, Ramsey, NJ, USA) probe was pressed against the bottom of the E-flasks as described by Larsson et al. (2007). For each flask the colour was measured at five different spots.

Lipid extraction for total lipid, PV and TBARS analyses

The total fat content was extracted by the method by Lee et al. (1996). A portion of ~1g (the exact weight was recorded) minced fish was homogenized for 15 s on speed 3 using an Ultra Turrax homogenizer (model T18 basic, IKA Works, Wilmington, NC, USA) with 10 mL cold chloroform and methanol. For samples with a fat content > 5 % the chloroform: methanol ratio used was 2:1 and for samples with fat content 2-5 % the ratio used was 1:1. To obtain a two phase system 3.08 mL cold 0.5% NaCl were added and the samples were vortex for 30 s followed by centrifugation at 2600x g for 6 min at 4°C. The lower chloroform phase was further used for gravimetric determination of total lipids as well as for PV analyses according to the ferric thiocyanate method described by Undeland et al. (2002). The upper methanol/water phase was used for analyses of TBARS according to Schmedes and

Hølmer (1989). PV and TBARS results were expressed as micromole peroxides and MDA equivalents, respectively per kilogram minced fish.

Moisture content

The moisture content was determined gravimetrically by placing the minced fish (approximately 5 g) in 105°C over night. Exact weights were recorded before and after drying. The moisture content was determined on triplicates and results are expressed as weight percent.

Haemoglobin (Hb) content

Haemoglobin (Hb) was determined by a modified version of the method by Hornsey (1956) which is based on analysis of the heme group. Four grams of minced fish were vortexed for 30 s with 18 mL of freshly made acidic acetone (with final concentration 80% acetone, 2% hydrochloric acid, 18% water) in a 50 ml plastic tube followed by incubation for 60 min in darkness at 10°C and thereafter filtered through a Whatman filter no 1. In case of unclear filtrate it was filtered twice. The Hb content was determined spectrophotometrically at 640 nm. Standard curve was prepared from bovine Hb and as blank the acidic acetone was used. Hb content was determined on triplicates and results are expressed as µmole Hb-equivalents per kilogram minced fish.

Total iron

A 0.5 g portion of minced fish was mixed with 3 mL water, 150µL HCl and 750 µL HNO_3 in Teflon vials. The samples were then microwave digested (Milestone microwave laboratory system Ethos Plus Sorisole, Italy) as described earlier (Larsson et al., 2007). The analysis of iron was performed with ion chromatography as described by Fredrikson et al. (2001). The HPLC system used was from Waters (Milford, MA, USA) with a Triathlon PEEK auto injector and a Waters 626 gradient pump. Iron content was determinate in duplicates and results are expressed as micromole iron per kilogram minced fish.

Ascorbic acid

As a marker for aqueous antioxidants in the muscle, total ascorbic acid was extracted from the wash waters and analyzed by the method of Lykkesfeldt (2000). Modifications included that samples were centrifuged at 5000g and that dilutions were made in mixture of phosphate buffer (50mM, pH 2.8) and McLvaine buffer (ratio 9:1) containing 100 ppm t*ris*(2-carboxyethyl)phosphine (TCEP). Results are expressed in microgram.

Sampling and statistics

Minced fillets from four fishes were pooled for each treatment group, and during the storage trial, 2 samples of mince from each treatment group were taken out for lipid oxidation and pH analysis at each sampling point, thus n=2. Data from each analysis in each time point are presented as mean values ± (max value-min value)/2. To determine whether there were significant differences between lipid oxidation data or pH-values from the different treatment groups during storage, or to determine whether time had a significant influence on changes in lipid oxidation within a specific group, t-test was carried out using SPSS (IBM Corp., Armonk, NY, USA). Differences are described in the text as significant when $p \leq 0.05$. Data from composition analyses of the minces at time 0 are presented as mean values ±SD (n=2-3). Compositional data were subjected to ANOVA using R (R Core Team, 2019) and comparison among samples were performed with Tukey Honest Significant Differences test with $p < 0.05$ representing a significant difference. Regarding wash waters, two samples were taken for analysis from each batch of water, thus n=2. The entire experiment was carried out twice, yielding similar conclusions. However, in the first experiment, only one fish was included in each group. Here, data are presented only from the second, larger trial.

RESULTS

Composition of the minces

Table 1 summarises the composition in terms of moisture, lipids, heme (expressed as Hb-equivalents) and total iron content, of the different minces. The mean heme content in the minces decreased in the order: unbled > bled > single-washed (1x1 vol) > perfused > triple-washed (3x3 vol) with the heme content in the unbled mince being significantly higher than in the other minces. The mean total iron content ranked the samples in the same order as heme with the exception that the mince washed with one volume of 0.9% saline (i.e. 1x1 vol) had the second highest iron level. Unbled, bled and perfused trout minces had similar moisture and lipid content, whereas the group of washed minces (single- and triple washed) had significantly higher moisture content and significantly lower lipid content than the unwashed group of samples.

Rancid odour

The rancid odour scores for the different minces during storage (**Figure 1a**) shows that the

Table 1 Composition of the muscle minces from unbled, bled and perfused trout as well of trout mince washed once with one volume of 0.9% NaCl (single-washed) or three times with three volumes of 0.9% NaCl (triple-washed)

	Unbled	Bled	Perfused	Washed 1 × 1 Vol	Washed 3 × 3 Vol
Moisture (%)	75.4 ± 0.2^{c}	73.1 ± 0.2^{d}	74.7 ± 0.6^{c}	83.1 ± 0.1^{b}	86.2 ± 0.4^{a}
Lipid (%)	5.8 ± 0.6^{ab}	7.0 ± 0.2^{a}	5.7 ± 0.5^{ab}	3.7 ± 0.1^{bc}	3.3 ± 0.3^{c}
Haem (μmol Hb equiv kg^{-1})	19.6 ± 6.4^{a}	9.1 ± 2.3^{b}	5.5 ± 1.6^{b}	7.2 ± 0.2^{b}	3.2 ± 1.1^{b}
Fe (μmol kg^{-1})	50.6 ± 5.2^{a}	21.3 ± 5.3^{b}	17.6 ± 2.1^{b}	28.6 ± 5.0	6.4 ± 1.0^{b}

Data show mean values ± SD ($n = 3$ for moisture and haem; $n = 2$ for lipid and iron). Different lower-case letters denote significant differences ($P < 0.05$).

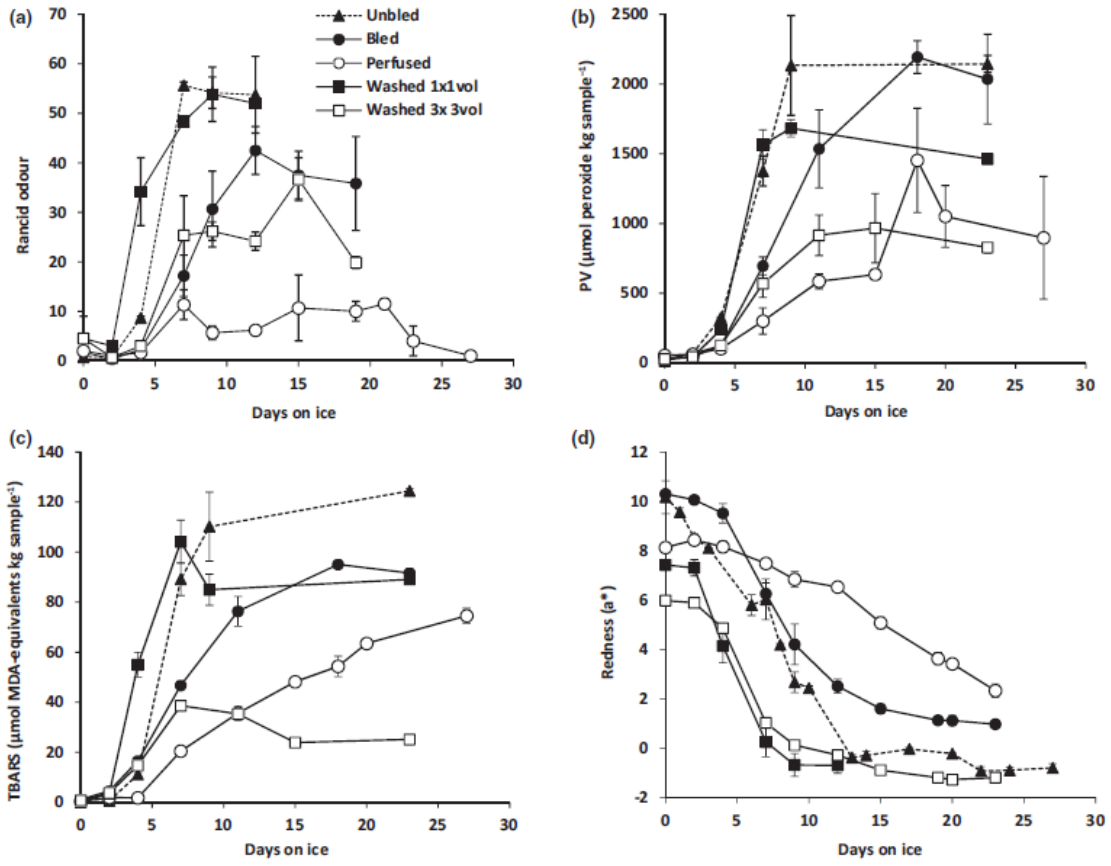

Figure 1 Changes in (a) rancid odour score, (b) PV, (c) TBARS and (d) redness during storage of minces from unbled, bled and perfused trout as well as unbled trout mince washed once with one volume of 0.9% NaCl or three times with three volumes of 0.9% NaCl. Graphs show mean values ± (max value-min value)/2 ($n = 2$).

samples could be divided into three groups. The unbled sample and the sample washed once both developed rancidity faster (lag phase ~2 days) and reached higher rancidity scores (50-55) than the other samples. For the bled sample and the triple washed mince, the lag phase lasted for ~4 days where after the rancid odour gradually increased until day 15 and 12, respectively, to finally peak at a score around 40. The decrease in rancid odour observed after this point agreed with several earlier studies (e.g. Undeland et al. 2004; Larsson et al., 2007; Sannaveerappa et al., 2007) and is most likely a result of reactions between formed carbonyls and e.g. proteins or phospholipids of the fish muscle (Dominguez et al. 2013). The mince from perfused trout did not become rancid at all during the 27 days of storage, but was in the end discarded because of microbial growth.

PV

PV-formation in all minces had a lag phase for 2 days, before the PV´s started to increase (**Figure 1b**). At day 7, samples were clearly divided in two categories, with the single washed (1x1 vol) mince and mince from unbled fish having significantly higher PV than the other samples, which were ranked according to: bled > washed (3x3vol) > saline perfused.

At day 9 the PV´s peaked for both the unbled sample and the single-washed sample, however the single-washed sample peaked and levelled out at a value that was significantly lower than for the unbled sample ≥ day 9. At day 18, the bled sample reached the same PV as the unbled sample. The triple washed and the perfused samples, which generally had the lowest PV´s during the storage, peaked at day 15 and 18, respectively.

TBARS

Formation of TBARS in the minces from the different treatments in principle followed the same pattern as the formation of PV (**Figure 1c**). However, the bled sample did not reach the same high level of TBARS as the unbled sample, and the TBARS for the saline perfused sample did not peak during the storage, which the PV did, but instead continued to increase throughout the entire storage period. In the last part of the storage (≥ 15 days), the triple-washed

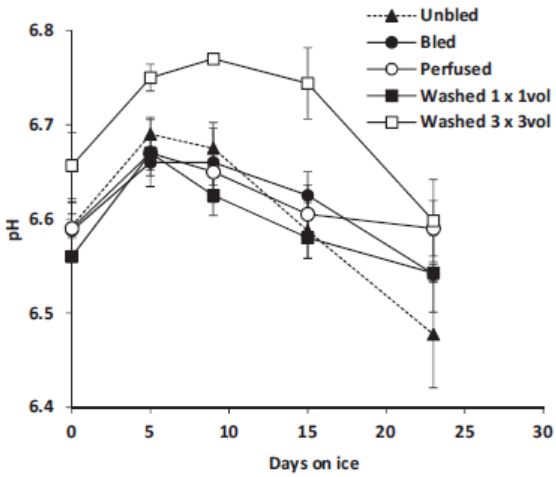

Figure 2 Changes in pH during storage of minces from unbled, bled and perfused trout as well as unbled trout mince washed once with one volume of 0.9% NaCl or three times with three volumes of 0.9% NaCl. Data are shown as mean values ± (max value-min value)/2 (n = 2).

sample had significantly lower TBARS than the other samples (≥ 18 days) and TBARS for the perfused sample was significantly lower than for the unbled and bled sample ≥ 7 days.

Changes in redness, a*

At start of the storage, the redness (a*-value) (**Figure 1d**) for the minces were divided into three significant categories according to: unbled ~ bled > saline perfused ~ single washed (1x1vol) > triple washed (3x3vol). The total decrease in a*-value during storage was largest in the unbled mince (10 units in 13 days), however, the highest rate of redness loss was recorded in the mince washed once (8.1 units in 9 days), followed by the unbled mince which dropped 7.5 units in 9 days. The third and fourth largest decreases were noted for the bled sample and the mince washed three times (6.1 and 5.9 units, respectively, during the first 9 days). The smallest and slowest relative decrease in a*-value was seen for the saline perfused sample (1.3 units in 9 days); the kinetics for this sample was also more linear than for the other ones.

pH

pH was followed in the samples as it is known to have a strong impact on Hb-mediated lipid oxidation (Undeland et al., 2004). All samples provided a similar pattern, with a slight pH-increase during the first 5-10 days of storage, followed by a decrease towards the end of the storage, see **figure 2**. All minces ended up with a pH at a lower level or at the same levels as the starting point.

Ascorbic acid content of mince and wash water

The unwashed mince contained 1092 µg ascorbic acid/100g, and based on ascorbic acid analyses of the wash water, in total, 25% and 96% of this amount was removed in the minces washed one and three times, respectively, **Table 2**.

Table 2 Amount ascorbic acid (µg) washed out from 100 g mince of the unbled trout using either one volume 0.9% NaCl or three times three volumes of 0.9% NaCl

	Total amount ascorbic acid in washing water (µg)			
	Wash 1	**Wash 2**	**Wash 3**	**Total**
Washed 1 × 1 vol	278 ± 13			278
Washed 3 × 3 vol	588 ± 33	337 ± 11	122 ± 4	1047

Total amount ascorbic acid in the 100 g unbled and unwashed mince before washing was 1092 µg (62 µmol). Data are shown as mean values ± SD (n = 2).

DISCUSSION

Although not intended as a scalable method to remove blood under industrial settings, the saline perfused fish provides an interesting research model to explore the impact of blood for storage-induced quality degradation of fish muscle according to a principle which has not been reported before. In this study, microbial growth was suppressed by addition of streptomycin to the samples right after mincing which provides an opportunity to study biochemical degradation as lipid oxidation alone (Undeland et al., 2002). Here, the focus was on primary and secondary lipid oxidation products; PV, TBARS and rancid odour. Based on our earlier reporting of strong correlation between lipid oxidation and redness loss caused by metHb formation (e.g. Larsson et al., 2007), the a*-value was also monitored throughout storage. However, different from our earlier work, redness loss in this study was most likely a combination of metHb/metMb formation and bleaching of the red astaxanthin of trout muscle as a result of co-oxidation with lipids (Haard, 2002).

The two previous studies which have used perfused fish in a lipid oxidation context applied the perfusion as a mode to introduce the antioxidants ascorbic acid, uric acid and Trolox into fish muscle via the capillary system (Sohn et al., 2007, Tuckey et al., 2012). Tuckey et al. (2012) perfused tails of Chinook Salmon (*Onchorynchus tshawytscha*), and Sohn et al. (2007) perfused whole yellowtail (*Seriola quinqueradiata*). In the former study, a significant rise in TBARS and protein carbonyls was found in dark muscle during the actual perfusion operation, which was done with oxygenated freshwater teleost saline, with and without ascorbic and uric acid. No storage was included in that study. Sohn et al. (2007) found perfusion with Trolox, but not with ascorbic acid, to reduce lipid hydroperoxides in dark muscle during 92 h storage of the minced muscle on ice. Treatments had no significant effect on metMb formation. None of the studies reported on the difference in lipid oxidation between non-perfused and perfused muscle during storage, which makes the present study novel in its kind. We here also make the comparison to conventional bleeding and to washing of fish mince; the latter being common practice in surimi making.

The bleeding in our study removed no more than about half of the total heme proteins (53%), which is in accordance with earlier studies; 34%, 44/23% and 25% Hb was removed from trout whole muscle, mackerel light/dark muscle (Richards & Hultin, 2002) and Asian seabass (*Lates calcarifer*) (Maqsood & Benjakul, 2011). Our saline perfusion removed 72% of the total heme, with the residual amount most likely

being bound in Mb and to a lesser extent in other proteins e.g. cytochromes and enzymes. Richards & Hultin reported that 35% of total heme in mackerel dark muscle was Mb while in mackerel light muscle Mb was not detectable (Richards & Hultin, 2002). However, since Hb easily dissociates upon dilution (Manning et al., 1998), it is difficult to quantitatively distinguish Hb and Mb in extractions and purifications. In trout fillets, the relative amount of dark muscle is low why it cannot be excluded that small remaining residues of Hb might have contributed to residual heme in fillets of perfused trout. Tuckey et al. (2012) found that the perfusion was much more efficient in the dark than white muscle; e.g. based on perfusion with fluorescent microspheres.

The heme content of the unbled, bled and saline perfused samples followed the same ranking order as the maximum rate and maximum level of almost all oxidation products monitored during subsequent ice storage. This relation was in accordance with previous studies done in a washed cod mince model fortified with 0.3 and 5.8 µM Hb; with and without 7.5% or 15% extra fish oil added (Undeland et al., 2002). Nearly doubling the Hb concentration here increased the maximum rancid odour intensity by ~30% and approximately doubled the maximum intensity of TBARS; regardless of lipid level. Similarly, gradually increasing Hb levels in washed cod mince between 0.06 and 5.8 µmol/kg almost proportionally increased the maximum oxidation intensities monitored as rancid odour and TBARS (Richards & Hultin, 2002). These results indicate that Hb limits the extent of the oxidation reaction and acts more as a reactant than a catalyst in lipid oxidation. Using data from several separate studies (e.g. Richards & Hultin, 200; 2002; Undeland et al., 2002), a strikingly constant ratio has in fact been obtained between maximum TBARS values and the Hb levels added to washed cod: 13.6 ± 4.8 μmol MDA equivalents/μmol Hb tetramer (range 6.7-24, n =12) (Undeland et al., 2002). In the present study; where the studied Hb was endogenous, such a ratio became surprisingly similar; 10.1 ±3.6 μmol MDA equivalents/μmol Hb tetramer (range 6.4-13.6, n=3) with Hb calculated from the analyzed amount of total heme. If also including the two washed minces, the ratio 11.36 ±3.2 (range 6.4-14.4, n=5) was obtained. This implies that Hb/heme strongly controls oxidation development also when present *in situ*, and that its quantification provides a basis for predicting the subsequent degree of oxidation in fish muscle.

The effect of removing blood via perfusion, bleeding or washing was most pronounced in terms of rancid odour development, which was nearly absent for 27 days on ice in perfused samples, although PV and TBARS still developed. It could be hypothesized that the cleavage of peroxides induced by Hb results in more short chain secondary oxidation products with lower odour thresholds than those formed by non-blood bound heme, like Mb; hereby contributing stronger to rancid odour. Thiansilakul et al. (2012) found 40% less hexanal formed in washed bighead carp in the presence of Mb compared to Hb; both added to a level of 24 µmol heme/kg mince. A contribution of blood-derived lipids or LOX to the rancid odour development in non-perfused samples can also not be excluded. Richards (2000) and Richards & Hultin (2002) found that whole blood, or plasma plus hemolysate added to hydrated filter paper developed medium to strong rancid odor during storage, suggesting that plasma lipoproteins can be a source of oxidizable lipids. Wang et al. (2012), found that LOX of grass carp gradually increased in the order skin and muscle<gill<viscera< blood. Several authors have earlier described how LOX is responsible for production of specific volatile compounds in fish tissue which are important both for the fresh and spoiled seafood odour (e.g. Lindsay, 1990; Saeed & Howell, 2001). Richards & Hultin (2002) however reported equal rancidity development in washed cod mince fortified with 5.8µM Hb in the form of hemolysate or whole blood, suggesting Hb accounted for all the lipid oxidation capacity of

blood. As LOX/LOX-products have mainly been identified in leucocytes rather than erythrocytes (Pettitt et al. 1989), presence of LOX in hemolysate is unlikely.

In agreement with our results from traditionally bled fish, Maqsood & Benjakul (2011) reported on lower levels of many volatiles in bled than non-bled Asian seabass, but particularly nonanal, 2-nonenal and heptanal and 3,5-octadien-2-one were lower. Hiratsuka (2016) found lower levels of particularly heptanal, hexanal, octanal, 1-penten-3-ol, 2-penten-1-ol and 1-octen-3-ol in bled than unbled Skipjack Tuna light muscle during 4h storage in room temperature after thawing.

Changes in TBARS can normally be detected before rancid odour is detectable. In Hb-fortified washed cod mince, rancid odour was detectable when TBARS had reached 10-12 µM (Richards et al., 2002). In our study, even a TBARS level of 70 µM did not yield rancid odour in the perfused sample, while in the 3×3-washed sample, rancid odour was detectable already at 40 µM TBARS. Beside the earlier observation that Hb and Mb yield different volatiles (Thiansilakul et al. 2012), a possibility is that the lipid level of the samples dictates the release of carbonylic volatiles; with higher lipid levels preventing a release due to a hydrophobic nature of many of the volatile compounds (Jacobsen, 1999). In the study of Richards & Hultin (2002), the washed cod mince used only had 0.1% lipid, but in this study 3×3-washed trout mince and perfused trout mince contained 3.3% and 5.7% lipids, respectively, which could explain the different correlations between TBARS and rancidity in the different samples. Another reason could be the different TBARS methods applied; here we used the one described by Schmedes and Hølmer (1989) comprising a methanol/water extract, and in the study by Richards & Hultin a TCA-extract was used as described by Lemon (1975). The former

The link between oxidation and heme-levels in the washed samples was more complex than in the three unwashed samples. Although mince washed in one volume of 0.9% saline had 63% less heme than unbled fish, it was the fastest oxidizing sample. The sample washed three times in three volumes of 0.9% saline solution, which had 84% lower heme level than unbled fish and thus the lowest heme-level of all samples, followed the oxidation of the bled sample which contained 54% less heme than the unbled fish. However, the PV and TBARS-values of the triple washed sample stabilized at lower maximum levels than bled fish samples after ~7-10 days. The results indicated a wash-out of antioxidants in parallel to the heme-removal, which was confirmed by analyzing one of the well-known endogenous aqueous antioxidants in muscle, ascorbic acid, in the wash waters. The wash-out of 25% and 96% of the total original ascorbic acid with one and three washes, respectively, lowered the final ascorbic acid concentrations of the minces by 56% and 94%, respectively, taking into account that the washing-induced weight change due to binding of water to the proteins. Interestingly, heme-concentrations were thus lowered more than ascorbic acid concentrations in the single wash, while the opposite was true in the triple wash procedure. So, even if more ascorbic acid was available per mole heme, the single washed mince was less stable than the triple washed one. Obviously, other antioxidants and/or additional factors also contribute to the net stability/instability of the washed fish mince system. It should here be stressed that ascorbic acid indeed also can act as a pro-oxidant (Ramanathan & Das, 1993).

That washing fish mince as a strategy to create stability should be used with great care is also supported by earlier studies. In herring mince stored at -18°C, oxidation proceeded faster after washing once with 5 volumes 50 mM NaCl than in the corresponding non-washed minces, even if 43% of the total iron was washed out (Undeland et al., 1998). The wash water showed a net antioxidative capacity in an iron-enriched linoleic acid emulsion. In another study (Undeland et al., 2003) PV and

rancid odour developed ~4 days faster in Hb-enriched cod mince pre-washed with 3×3 volumes water and buffer, than in Hb-enriched unwashed cod mince during ice storage. When adding back an aqueous fraction (press juice) isolated from the unwashed cod mince to the washed mince at 2−6-fold dilutions, Hb-mediated PV and rancid odor development was either delayed or completely prevented. Also double washed (3 volumes either water or 3% NaCl) minced whiting (*Merlangius merlangus euxinus*) obtained higher TBARS in the end of a refrigerated storage period than unwashed mince (Köse et al., 2006).

Since some of the depot fat was also removed in the washing (**Table 1**), washed trout minces had a higher relative ratio of membrane lipids than non-washed muscle; the latter known to be more susceptible to oxidation than neutral lipids (Undeland et al., 2003; Younathan & Watts, 1959). The heme to total lipid ratio (shown in brackets) ranked samples as: triple-washed samples ~ perfused samples (1) < bled samples (1.3) < single washed samples (1.9) < unbled samples (3.3), and thus closely followed TBARS data, but not fully the rancid odour data (**Figure 1**). This indicates that it is rather a destabilizing pro-oxidant to antioxidant balance than the pro-oxidant to lipid balance that explains the severe instability of unbled trout mince after one wash.

It should also be stressed that the high moisture content of the single-washed sample (83%) compared to unbled (75%) and bled samples (73%); the two latter which both had higher heme-levels, could also have contributed to the faster oxidation of the single washed sample. It is a known phenomenon that lipid oxidation increases at aw > 0.3 (Reid & Fennema, 2007). Our own earlier non-published work in washed cod mince, with the same Hb-to-lipid-ratio, showed oxidation to decrease in the order 90%>81%>75% moisture.

CONCLUSIONS

Altogether, our trout perfusion model revealed a profound effect of blood for rancid odour development in ice stored fish muscle under *in situ* conditions, i.e., without washing out pro-oxidants from the muscle. Saline-perfused fish was deficient in rancid odour for 27 days. The perfusion also had a relatively pronounced role for PV, TBARS and a*-value changes, but no complete inhibition of the three latter measures was obtained, indicating a selective role of Hb, or tentatively other blood components, for formation of secondary oxidation products contributing to rancidity. Fish subjected to classic bleeding was not stable towards rancidity, although the intensity became lower than in unbled fish. This implies that significant shelf life extension could be obtained upon development of new blood removal techniques for whole or processed fish that go beyond gill-/tail-cut-induced bleeding or mincing plus washing. Increased use of heme-rich fish raw materials is a reality today when by-products and small pelagic fish species are gaining increasing attention in food production. A common route is e.g. surimi-production from back-bone-derived mince generated in a meat-/bone separator, the latter which can cause high Hb-contamination due to the pressure applied. Mincing plus washing was in this study either pro-oxidative (1×1 wash), or equal to bleeding (3×3 washes) in terms of its effect on rancid odour development, pointing at the risk of removing more anti- than pro-oxidants in an insufficient washing operation and/or by increasing the Hb to lipid ratio.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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