THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Prevention of hemolysis as a novel strategy to limit hemoglobin-mediated lipid oxidation in fish

- towards a more sustainable use of fish raw materials

SEMHAR GHIRMAI

Department of Biology and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2022

Prevention of hemolysis as a novel strategy to limit hemoglobin-mediated lipid oxidation in fish
-towards a more sustainable use of fish raw materials

SEMHAR GHIRMAI
ISBN 978-91-7905-716-9

Doktorsavhandlingar vid Chalmers tekniska högskola
Ny serie nr 5182
ISSN 0346-718X

Chalmers University of Technology
Department of Biology and Biological Engineering
Food and Nutrition Science
SE-412 96 Gothenburg
Sweden
Telephone + 46 (0)31-772 1000

Cover:
Atlantic herring (*Clupea harengus*) on an ice bed. From the fish, a zoom-in effect is made on the capillary system with a visualization of some intact and some lysed red blood cells.
Illustrated by Elsa Bairu

Printed by:
Chalmers Digitaltryck
Gothenburg, Sweden 2022

ምስጢር ናይ ዓወተይ ወለደይ እዮም

My parents are the secret to my victory

Prevention of hemolysis as a strategy to limit hemoglobin-mediated lipid oxidation in fish
- towards a more sustainable use of fish raw materials

SEMHAR GHIRMAI
Division of Food and Nutrition Science
Department of Biology and Biological Engineering
Chalmers University of Technology
Gothenburg, Sweden

ABSTRACT

Hemoglobin (Hb) has been recognized as a main pro-oxidant in fish, causing fast and intense lipid oxidation. This thesis explores the hypothesis that maintaining red blood cells (RBCs) intact for an extended time during fish processing could delay Hb-mediated lipid oxidation. The aims were to (i) unravel how selected endogenous and exogenous parameters affect fish RBC-stability, (ii) confirm the relation between hemolysis and lipid oxidation and (iii), evaluate antioxidative strategies to prevent oxidation caused by released Hb.

In **Study I**, washed and resuspended trout RBCs (wr-RBC) and whole trout blood (WB) were used to study how temperature, salinity and mechanic pressure affect hemolysis. Cold temperatures, 0-6 °C, and physiological saline were advantageous for the RBC stability while hyper- (3% NaCl) or hypotonic (tap water) conditions as well as simulated mechanic pressure hemolysis. **Study II** revealed that typical post-mortem pH's, 6.4 and 6.8, yielded a higher hemolysis rate of wr-RBCs compared to pH 7.2-8.0, and stimulated lipid oxidation of the RBC membrane. Similarly, in RBC-spiked washed cod mince (WCM), pH 6.4/6.8 stimulated both lipid oxidation and hemolysis compared to pH 7.2/7.6 **(Study III)**. Hb-analyses of the soluble phase revealed that only low levels of released Hb (7.1 µM) were needed to initiate lipid oxidation at pH 6.4-6.8, which was not the case at high pH, even when >50% of the Hb was in the metHb form. In experiments with wr-RBCs, re-addition of blood plasma (12-75% v/w) largely increased the RBC stability **(Study I-II)**. The stabilizing effect was partly ascribed to glucose, albumin, and ascorbic acid (AA). Adding blood plasma to the RBC-spiked WCM system 3% (v/w), delayed the onset of lipid oxidation at pH 6.8 with 3-4 days without delaying hemolysis; the latter most likely due to the high plasma dilution. To clarify the effect of hemolysis on WCM lipid oxidation, different ratios of intact and lysed RBCs were added to a WCM system at pH 6.8 **(Study III)**. Samples with 25-100% lysed RBCs oxidized rapidly within the first day of storage, whereas the sample with 100% intact RBCs provided a 1-day delay in the onset of lipid oxidation. Further, the sample with initially fully intact RBCs resulted in 59.5± 2.9% and 48.1± 2.9% reduced maximum peroxide value (PV) and thiobarbituric acid reactive substances (TBARS), respectively.

Short pre-incubation of herring co-products in 0.9% NaCl removed 6.6-18.0% of their Hb and slightly reduced the intensity of TBARS development during ice storage of the minced product, compared to pre-incubation in tap water or no pre-incubation **(Study IV)**. Refrigerated storage of herring co-products while submerged in tap water, 0.9% NaCl or phosphate buffered saline (PBS) (pH 6.5 or 7.5) also reduced the lipid oxidation intensity compared to air-storage; however, without any effect from pH or salinity. When adding the commercial antioxidant mixture Duralox-MANC to the pre-incubation or submerging solutions (2% vs 0.5%) the lipid oxidation lag phase was largely prolonged; e.g., from < 1 day in controls to >6 days. Direct fortification of minced herring co-products with 0.5 % Duralox-MANC confirmed its antioxidative efficiency as lipid stability was prolonged from <1 day to >8 days.

Altogether, this thesis confirmed that delaying hemolysis can be a route to delay lipid oxidation. It also revealed that current conditions used in the early handling/processing of fish, such as subjection to crowding, pumping, refrigerated sea water (RSW)-storage or tap water rinsing do not favor RBC stability and call for adjustments to limit hemolysis; and thereby Hb-mediated lipid oxidation. Intact RBCs, presence of blood plasma and elevated pH's delayed lipid oxidation in a WCM system, providing an important window of time which could allow for better utilization of blood-rich fish raw materials currently leaving the food chain. To further enhance the lipid stability of such materials, subjecting them to rosemary-derived antioxidants via pre-incubation, submerging or direct addition is recommended. Results presented can contribute to an increased robustness of our global food systems towards unprecedented challenges such as pandemics, war, and climate threats. They also contribute to the ongoing dietary protein shift in which aquatic foods as small pelagic fish and fish rest raw materials have an immense potential if correctly preserved by updated procedures.

Keywords: hemoglobin, hemolysis, erythrocyte, blood plasma, lipid oxidation, herring (*Clupea harengus)*, rainbow trout (*Oncorhynchus mykiss*), fish, antioxidant, side-stream

LIST OF PUBLICATIONS

This doctoral thesis is based on the work contained in the following papers:

I. **Ghirmai, S.**, Eriksson, L., Wu, H., Axelsson, M., and Undeland, I. (2020). "Improving the Stability of Red Blood Cells in Rainbow Trout (*Oncorhynchus mykiss*) and Herring (*Clupea harengus*): Potential Solutions for Post-mortem Fish Handling to Minimize Lipid Oxidation." Food and Bioprocess Technology **13**(8): 1344-1355.

II. **Ghirmai, S.,** Wu, H., Axelsson, M., Matsuhira, T., Sakai, H., and Undeland, I. (2022). "Exploring how plasma- and muscle-related parameters affect trout hemolysis as a route to prevent hemoglobin-mediated lipid oxidation of fish muscle." Sci Rep **12**(1): 13446.

III. **Ghirmai, S.,** Krona, A., Wu, H., Axelsson, M., and Undeland, I. (2022). "Relationship between hemolysis and lipid oxidation in washed cod mince spiked with red blood cells and its dependance on pH and blood plasma". (submitted)

IV. Wu, H., **Ghirmai, S.,** and Undeland, I. (2020). "Stabilization of herring (*Clupea harengus*) by-products against lipid oxidation by rinsing and incubation with antioxidant solutions." Food Chemistry: 126337.

Paper not included in the thesis:

Wu, H., Tullberg, C., Ghirmai, S., and Undeland, I. (2022). "Pro-oxidative activity of trout and bovine hemoglobin during digestion using a static in vitro gastrointestinal model." Food Chem **393**: 133356.

CONTRIBUTION REPORT

Paper I: The first author, Semhar Ghirmai (SG), participated in the design of the study, performed the experimental work and analyses. Parts of the statistical calculations were performed by SG. Further, interpretation of results, writing the first manuscript draft and coordination of the editing of the manuscript were also under the responsibility of SG.

Paper II: SG, the first author, co-conceived the study, planned, and performed the experimental work and analyses. Statistical analysis, primary interpretations of the results and writing the first draft of the manuscript and coordination of the editing process were also SG's responsibility.

Paper III: First author, SG, co-conceived the study, planned, and performed the experimental work and most of the analyses. Statistical analysis, primary interpretations of the results and writing the first manuscript draft and coordination of the editing were also performed by SG.

Paper IV: SG, the second author, participated in planning and performing the experimental work and analyses, interpreted the data and participated in the final manuscript writing.

ABBREVIATIONS

AA	ascorbic acid
ATP	adenosine triphosphate
BSA	bovine serum albumin
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
CO_2	carbon dioxide
COP	colloidal osmotic pressure
DEHP	di(2-ethylhexyl) phthalate
DeoxyHb	deoxyhemoglobin
DHA	docosahexaenoic acid
DHAA	dehydroascorbic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
2,3- DPG	2,3-diphosphoglycerate
EDTA	ethylenediaminetetraacetic acid
EPA	eicosapentaenoic acid
FDA	U.S. Food and Drug Administration
Fe	iron
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
ferrylHb^{+}(4+)=O	ferrylhemoglobin
ferrylHb$^{+\bullet}$(4+)=O	ferrylhemoglobin radical
FFA	free fatty acid
G6PD	glucose-6-phosphate dehydrogenase
GSH	glutathione
GSSG	oxidized glutathione
Hb	hemoglobin
[Hb]	hemoglobin concentration
HCl	hydrochloric acid
HCO_3^-	hydrogen carbonate ion
Hct	hematocrit
H&E	hematoxylin & eosin
HES	hydroxyl starch
HK	hexokinase
HMP	hexose monophosphate shunt
HMW	high molecular weight
H_2O_2	hydrogen peroxide
IMP	inosine monophosphate
$L^\bullet$	alkyl radical
LC	long chain
LH	unsaturated fatty acid
LMW	low molecular weight
$LO^\bullet$	alkoxyl radical
$LOO^\bullet$	peroxyl radical
LOOH	lipid hydroperoxide
LOX	lipoxygenase
Mb	myoglobin
MetHb	methemoglobin

NaCl	sodium chloride
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
O_2	oxygen molecule
$^1O_2$	singlet oxygen molecule
$^3O_2$	ground state oxygen molecule
$O_2^{\bullet-}$	superoxide anion
$^\bullet OH$	hydroxyl radical
$^\bullet OOH$	protonated superoxide
OxyHb	oxyhemoglobin
PEST	penicillin-streptomycin
PFK	phosphofructokinase
pI	isoelectric point
PL	phospholipid
PLA2	phospholipase A2
PUFA	polyunsaturated fatty acid
PV	peroxide value
PVC	polyvinyl chloride
RBC	red blood cell
ROS	reactive oxygen species
RSW	refrigerated sea water
RNA	ribonucleic acid
SAG-M	saline-adenosine-glucose-mannitol
SD	standard deviation
SOD	superoxidase dismutase
T_{50}	days to reach 50 % hemolysis
TAG	triacylglycerol
TBARS	thiobarbituric acid reactive substances
TBA	thiobarbituric acid
WB	whole blood
WCM	washed cod mince
wr-RBCs	washed resuspended red blood cells

CONTENT

1. INTRODUCTION

Seafood is one of the main food supplies traded in the world, with an estimated global production of 178 million tonnes aquatic animals in 2020; including both aquaculture (88 million tonnes) and wild fisheries (90 million tonnes) (5). From the wild captures, marine finfish constitute 67 million tonnes out of which 14 million tonnes are small pelagic species such as herring (*Clupea harengus*), sprat (*Sprattus sprattus*), sardine (*Sardinops asgax*), anchoveta (*Engraulis ringens*), scads nei (*Decapterus spp.*) and mackerel *(Scomber scombrus*) (5). Although decreasing, a significant amount, 16 million tonnes, marine captures were used for fishmeal and fish oil production in 2020, with bulks of whole small pelagic fish directly dedicated for this end product (5). The same destiny applies to most of the rest raw materials which emerge from filleting of fish, i.e., backbones, heads, belly flaps etc.; a biomass that can amount up to 70 % of the whole fish weight (5). It has been estimated that the total loss and waste throughout the seafood value chain is 30-35 % (53-62 million tonnes) of ingoing raw material (5).

A major hurdle which has hampered the valorization of small pelagic fish and fish rest raw materials into foods is their high susceptible to lipid oxidation, that leads to rancidity; i.e., unwanted flavor, reduced nutritional value, pigmentation, and textural changes (6-11). Reasons for this are that the mentioned raw materials are rich in both polyunsaturated fatty acids (PUFA) and heme-proteins; especially hemoglobin (Hb) which is a strong pro-oxidant found in the red blood cells (RBCs) (6, 8, 12-14). For the pelagic fish species, another reason for hampered valorization is their relatively low post-mortem muscle pH compared to e.g., cod and salmon (15), which further activates the heme-proteins as pro-oxidants. To gradually raise the food production and value of the mentioned biomasses, immense research efforts have been carried out during the last two decades to develop innovative technologies for antioxidant incorporation (16-18), separation of co-products (19) as well as production of fish minces (20-22), functional protein isolates and protein hydrolysates (23). All these efforts pave the way for blue bioeconomies with more responsible uses of aquatic biomasses.

Upon lysis of the RBCs (hemolysis), Hb is released and quickly oxidized to metHb which readily releases its hemin (24). Oxidized Hb can also be found as ferrylHb or as ferrylHb radicals. The Hb-form is an important determinant to the pathway of lipid oxidation. Initiation may be caused by ferrylHb and ferrylHb radicals, whereas metHb and hemin may propagate lipid oxidation via breakdown of pre-formed lipid hydroperoxides (LOOH) into alkoxyl radicals ($LO^{\bullet}$) and peroxyl radicals ($LOO^{\bullet}$) (25, 26). To date, most efforts to prevent the pro-oxidative action of these mechanisms has evolved around addition of various antioxidants or antioxidant-containing extracts from e.g., plants and algae (17, 27-32). Much less focus has been given to strategies for preventing the contact between Hb and PUFA. A possible strategy within this segment would be to maintain the RBCs intact as long as possible throughout the value chain. At start of this thesis, only one paper was available which reported on such an attempt. This was from Richards and Hultin (8) who reported on a small difference in the lipid oxidation lag phase when adding Hb as intact RBCs vs. as a hemolysate to washed cod *(Gadus*

morhua) mince (WCM). Later also Perez, Tatiyaborworntham (33) reported that lysed avian RBCs stimulated lipid oxidation more than intact RBCs in a turkey muscle model system. However, to the best of our knowledge, no published studies exist where different factors controlling lysis of fish RBCs are evaluated with the focus of preventing the development of Hb-mediated lipid oxidation. Our hypothesis throughout this thesis work has been that adaptation of selected process conditions used within the fish industry could maintain the Hb inside the RBCs for an extended amount of time after catch, which would preserve the fish post-mortem quality. This thesis therefore focuses firstly on the understanding and possible prevention of fish RBC lysis, and secondly, if lysis cannot be fully prevented, on the inhibition of Hb-mediated lipid oxidation via (pre-)incubation of sensitive fish raw materials in different antioxidant solutions.

2. AIMS

Although Hb is known as a strong pro-oxidant in fish, studies on preventing lysis of the RBCs and thereby release of Hb molecules as a route to avoid lipid oxidation are limited. The underlaying hypothesis throughout this thesis has been that Hb-mediated lipid oxidation can be reduced and postponed if hemolysis is limited or delayed. The specific aims of the thesis were to:

i. evaluate how selected parameters linked to early handling and processing of fish (osmotic pressure, temperature, mechanic impact, pH) affect the RBC integrity and how these parameters can be optimized to minimize hemolysis **(Study I-III)**.

ii. gain understanding on the molecules of blood plasma which explain its preventive effect on hemolysis (**Study I-II**).

iii. evaluate whether exogenously added molecules – selected with inspiration from transfusion medicine- could prevent hemolysis and thus be used in a solution to preserve the fish RBC integrity **(Study I-II)**.

iv. identify the mechanism behind an observed exponential behavior of hemolysis; also, in presence of blood plasma and in cases when process-parameters were optimized **(Study II)**.

v. confirm the link between RBC lysis and development of lipid oxidation in fish muscle (**Study III**).

vi. develop strategies to incorporate antioxidants into blood rich fish raw materials in cases when RBC lysis cannot be prevented **(Study IV)**.

Blood from rainbow trout (*Oncorhynchus mykiss*) was used as a main model, with the goal to implement the results also on other species such as herring.

3. BACKGROUND

3.1 Catch, harvest and early handling of wild-caught and farmed fish

3.1.1 Catching and harvesting

Trawling is the most common method to catch wild fish commercially. Another common fishing gear which together with trawls dominate in the Swedish fisheries is purse seine. The trawl is a funnel-shaped net that is dragged along the vessel while a purse seine is used as a vertical net curtain to surround the school of fish (34, 35).

In farmed fish the first step during harvesting is usually to separate the fish into a different holding tank, where the density of fish in the holding tank can affect the stress. Farmed fish is commonly chilled alive (36), and starved prior to slaughter, the latter to reduce feces in the intestines and feces trailing. However, starving the fish can also cause faster rigor mortis as the glycogen reserves are reduced prior to death (15). Rigor mortis makes the fish stiff, and thereby difficult to process (section 3.3.1).

Stress and other changes induced during catch and harvest
Stress is an important parameter to control prior to death as it affects blood flow to the muscles, and the efficiency of bleeding (section 3.1.3) (37). Stress also affects the accumulation of lactic acid in the muscles (38) and in blood plasma (39); causing faster rigor mortis (40). Stress can be assessed in fish through three stress responses: i) cortisol production, ii) change in behavior and physiology, such as metabolic pathways, osmoregulation, immune response, blood chemistry, and iii) immune suppression and mortality (39). To control stress and exhaustion in wild-caught fish, fishing method and time are important parameters (40). Commercial trawling results in both increased stress-levels and higher mortality rates compared to hook and line caught fish (39, 40), however it is not practical to implement hook and line catch in commercial settings. Another drawback of trawling is the 10 to 100-fold increase in microbial load compared to line-caught fish arising from contaminations on the fish from mud stirring during trawling and gut contamination (15, 41). Further, herring and sprat are particularly sensitive to scale loss; de Leitão Tomás (39) found that 98% of the scales was lost in herring after trawling and pumping the fish to the vessel. Such loss increases the risk of microbial contamination on the flesh and has osmoregulatory drawbacks.

3.1.2 On board storage in refrigerated sea water tanks

The typical procedure of a trawler in the North Atlantic is that the wild-caught fish is stored in refrigerated sea water (RSW) tanks. In RSW-tanks, the RSW is cooled to a temperature around 0±1 °C with full-strength sea water salinity (42, 43). If the fish is not dead due to time in the trawling net or pumping the fish from the net to the vessel, they will die in the RSW (40). Some of the advantages of storing fish in RSW tanks instead of in ice include: i) ensure faster cooling of the fish, ii) easier to control the temperature, iii) the fish does not get crushed due to the buoyancy of the water and injuries on the fish that can be caused by the ice are eliminated, and

iv) allows for maximum tank loading as chilled fish sediment which eliminate the risk of trapping non-chilled fish in one part of the tank (42, 43).

When storing herring in RSW-tanks for food purposes, a ratio of fish to sea water 3:1 or 4:1 is commonly used (43). To keep a high fish quality, the holding time of the fish in the RSW-tanks should be minimized. One reason for minimizing the time in the RSW tanks is that the water is quickly contaminated with blood, proteins and bacteria from the skin- (slime), gill surfaces, and viscera of the fish, which can affect the fish quality (42, 43). The other reason is that the belly-flap muscle, which is the flesh close to the visceral cavity, can become soft and even break open (belly burst), due to proteolysis, driven by endogenous or bacterial proteases. This phenomenon is especially prominent in herring during the heavy feeding period, and limits the period of time that herring can be stored in RSW to ~3 days (44).

3.1.3 Bleeding and primary processing

Bleeding of farmed fish is the final part of the slaughter process and leads to decreased blood contamination during processing, but is not performed on small pelagics, e.g., herring, due to their small size and capture in large hauls. (45). Instead, some of the blood is removed during the filleting procedure via rinsing with tap water. Further to this, some amounts of blood are leaking from the fish into the RSW water (46). As mentioned earlier, farmed fish is often chilled alive prior to stunning and bleeding to reduce pre-slaughter stress; resulting in delayed rigor mortis and reduced blood clotting (36, 47). Fish contain 1.5-6 % blood of the total body mass (48), and it has been reported that 30-40 % of the cardiac output is directed to the gastrointestinal circulation in chinook salmon (*Onchorhynchus tshawyscha*) (37, 49). During severe exercise such as stress, the blood circulation to the muscles increase and the gastrointestinal circulation can be reduced with up to 60 % (37). Thus, to reduce the blood flow to the muscles, fish should be subjected to minimal stress prior to slaughter/bleeding.

The standard procedure for blood removal from larger fish species such as salmon and cod is that the fish is first bled by cutting the gills, which are connected to the heart via the ventral aorta (50). Another technique is tail-cutting (8). For sufficient blood removal, the fish should be bled for 15-20 minutes in clean circulating seawater or in a bleeding tank (50). Harrysson, Swolin (45), showed that rainbow trout bled via gill-cut for 60 min contained 54% less total heme compared to un-bled fish. The study also comprised saline-perfused rainbow trout as a research model, from which 72% total heme was removed. Lipid oxidation in these fish developed in the order un-bled > bled > perfused (45). That traditional bleeding only removes around half of the blood has also been found by several others (51). Beyond the fact that lipid oxidation still can proceed, incomplete bleeding can result in e.g., blood spots, which is not attractive to the consumers (36, 50). For optimal blood removal it has been reported that Atlantic cod should be bled within 30 min post-mortem (52).

Gutting, washing, and filleting

There are many discrepancies about the order to perform bleeding and gutting of fish and how different bleeding methods affect blood drainage from the muscles (52). Olsen, Joensen (52) reported better blood removal by a two-stage method, in which fish is bleed for 30 min prior to gutting. Performing bleeding prior to gutting ensures that the time from death to bleeding is reduced. On the other hand, gutting prior to bleeding ensure that the time during which there is contact between the viscera and the flesh is minimized; thereby reducing the risk of bacterial and enzymatic spoilage (15, 50). However, gutting prior to bleeding would not be legally accepted in many countries, as the fish is not allowed to be killed in a painful way while it is conscious (53).

De-heading and gutting is often mechanized and is performed after landing/harvesting. It should also be mentioned that there are very large fishing vessels existing today, where whole processing lines and even freezing are installed. After gutting the fish, thorough washing of the peritoneal cavity, skin and gills is important to remove blood, bacteria, and intestinal contents (15, 40, 50). It is important to remember that any washing procedure also remove concurrent antioxidants from the surface of the flesh which can affect lipid stability (45). From studies of surimi-production (i.e., washed fish mince), it has been found that when washing is performed with limited amounts of washing solution, the net removal of antioxidants often dominates over the net pro-oxidant removal, ultimately decreasing the oxidative stability of the mince during subsequent storage (45).

Filleting and trimming of wild caught fish is often performed after rigor mortis, which result in less yield due to gaping compared to pre-rigor filleting (40). Filleting and trimming of the fillets also yield different losses depending on fish species, size, and anatomy. As an example, fish species with large head and frame such as cod naturally yield larger loss (40). Today, however, major efforts are done to also convert filleting rest raw materials into food products, thus, the filleting process should be seen more as a general cutting process, similar to the cutting of terrestrial animals, rather than simply as a fillet recovery process.

3.1.4 Typical steps in fish value chains with focus on herring

After trawling, RSW-storage and transport to the processing plant, the fish is separated into different tanks based on their size. Very small herring (< 55 g) is directed for fodder meal/oil, and the larger fish is quality inspected before continuing its process in the food value chain. After filleting, rest raw materials are directed for fodder meal/ oil, whereas the fillet is processed for food. In the Nordic countries herring is often: i) packed whole and frozen, ii) filleted and sold fresh or frozen, with or without vacuum packing with salt brines, or iii) cured in large barrels containing brines with salt, spice and vinegar or in marinades (54). **Fig. 1**, show the typical processes of a herring processing plant in Sweden.

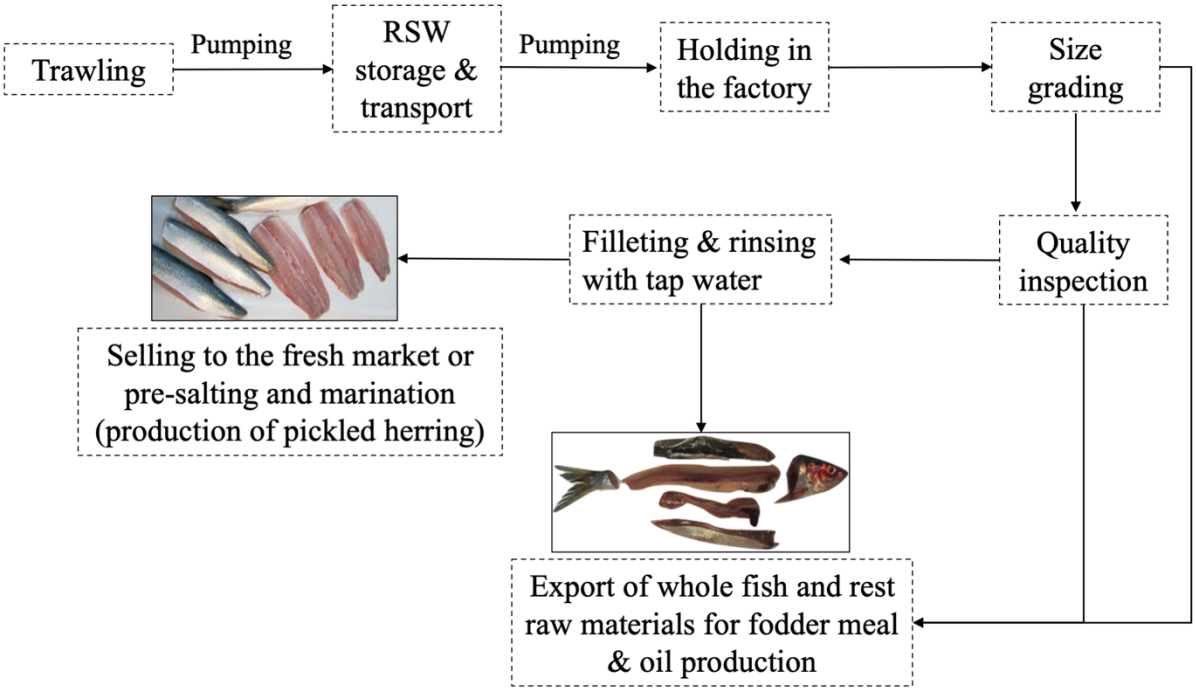

Fig. 1. A scheme over a typical herring value chain in Sweden.

It should here be highlighted that aquatic foods in general have a higher nutrient density and lower greenhouse gas emissions compared to pork and beef (55). Among fish species wild-caught small pelagics, such as herring and sprat, cause the lowest emissions, and together with its high nutrient density it calls for more attention to the 'blue protein shift'; meaning a shift in dietary proteins from terrestrial animal-based to aquatic foods, to meet the need for sustainable food production. There is a particularly large opportunity for the small pelagic species in the blue protein shift since a large percentage is currently not landed as food, but destined for production of fodder meal and oil (55, 56).

3.1.5 Possibilities with fish rest raw materials

Reduction of the large feed production and waste of e.g., small pelagic fish species, would today be possible through innovative techniques developed to produce fish minces, functional protein isolates and protein hydrolysates. However, lipid peroxidation is one of the main reactions causing a shortened shelf-life and lower overall quality of fish muscle; causing considerable economic loss in the fish industry (57-59)

In Sweden around 190,000 tonnes seafood (excluding algae) is harvested per year, from which 125,000 tonnes (66 %) are directed to biogas and production of fish meal. From the remaining 65,000 tonnes (34 %) seafood raw material, 36,000 tonnes are considered as rest material and only ~29,000 tonnes, which correspond to 15 % of the captured seafood raw material, end up for human consumption (60, 61) **(Fig. 2A)**. Herring and sprat alone constitute 70 % of the total volume caught and harvested fish in Sweden, with total catches in 2020 of 133,000 tonnes **(Fig. 2B)**. Out of this volume, only around 38,000 tonnes (29 %) are entering the food chain, but 61% (23,000 tonnes) leaves the chain again as rest raw materials from filleting production, and

are instead dedicated for feed production (60-62). Thus, altogether only 11% of the herring and sprat raw material becomes food. This is unsustainable, viewed against the growing demand of seafood as a result of a growing population and dietary recommendations (5). However, attempts to develop more holistic process lines to accelerate better valorization of aquatic resources into food products, are made. Rest raw materials which are today considered as low-value products can e.g., be transformed into high-value products such as bioactive peptides, flavor ingredients, proteins and oils which can be used for food applications or used as nutraceuticals (63, 64). Techniques used for this are for example enzymatic hydrolysis (65) and the pH-shift process (66). To maximize quality and allow tailored uses of different cuts from the fish, the mentioned processes are also combined with active cooling, pre-sorting technologies, and storage solutions for the rest raw materials (57, 64, 67, 68). It has been identified that it is often in the very early handling of the rest raw materials that further valorization is prevented, as there is commonly no active cooling in place, or the rest raw materials are stored mixed in a container allowing blood, bacterial and enzymes from one part contaminating other parts (19, 67). Thus, putting extra efforts in the immediate handling of rest raw materials open completely new doors for their maintenance in the food value chain.

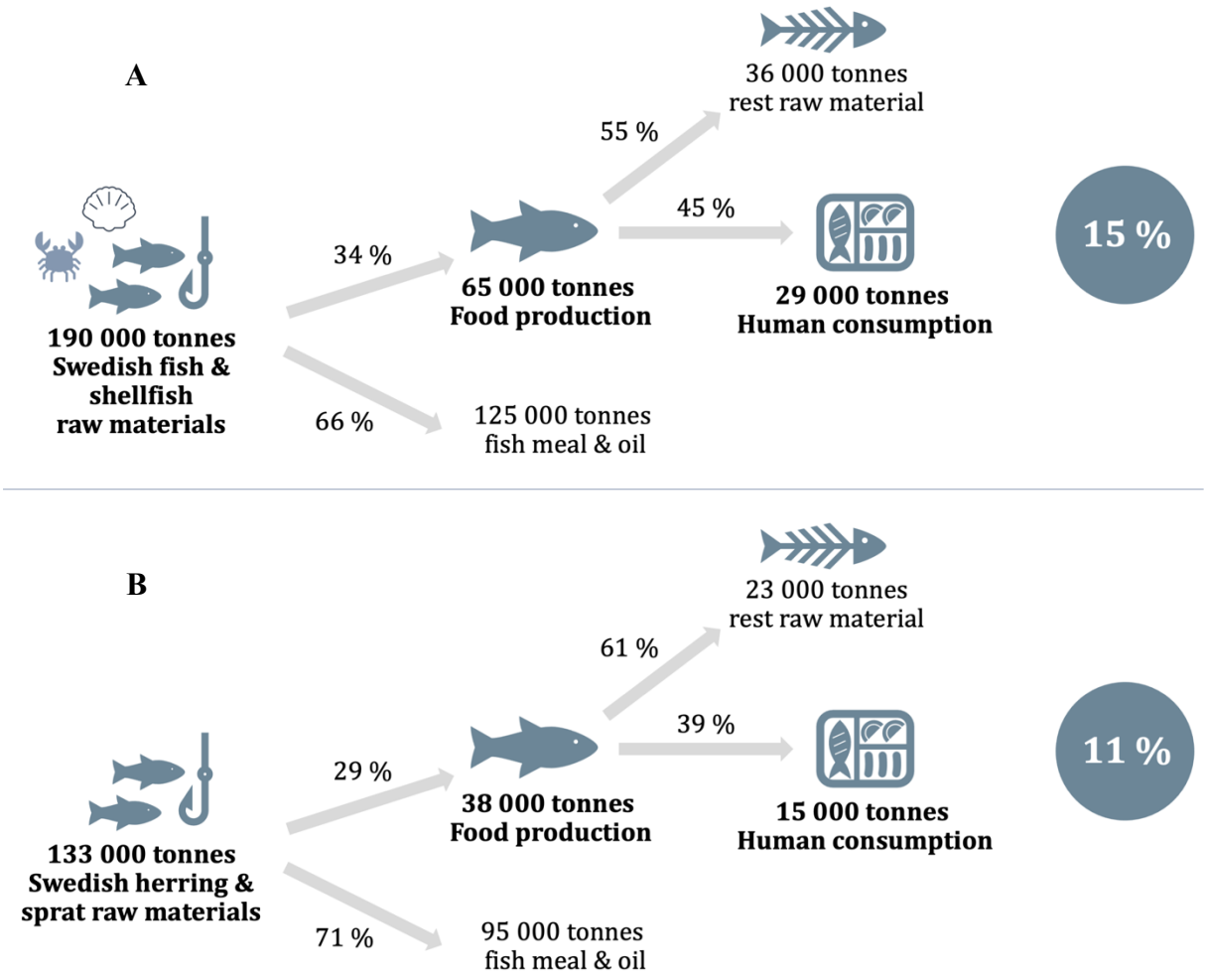

Fig. 2. Schematic pictures of (A) the annual fish and shellfish production in Sweden and the volumes directed towards animal-/aqua feed vs. food, (B) the annual catches of specifically herring and sprat in Sweden and the volumes directed towards animal-/aqua feed vs. food.

3.2 Muscle types and vascular system

3.2.1 White and dark muscle

There are two major muscle fiber types, red and white; sometimes also referred to as dark and light **(Fig. 3)**. Dark muscle is referred to as the slow muscle fibers and is used for "normal" swimming. It is dependent on aerobic metabolism and is therefore rich in capillaries that are intertwined with the muscle. It also contains high levels of heme proteins which together with mitochondria give the muscle its dark color. In teleost fish, the dark and white muscle fiber types are segregated from one another (69). Dark muscle tissue is often located along the lateral line of the fish, whereas white muscle surrounds the dark muscle (70). White muscle is used for burst-swimming and is referred to as fast fiber muscle. White muscle utilizes anaerobic metabolism and mainly breaks down glycogen to adenosine triphosphate (ATP) and lactic acid as an energy source. This gives a faster metabolism compared to using triacylglycerols (TAGs) as energy source, which is the case of dark muscle (70). Dark muscle cells are smaller in size compared to white muscle cells and contain higher levels of total lipids (70). Due to the lipid metabolism, small cell size and its abundance in mitochondria, dark muscle contains higher levels of both phospholipids (PLs) and TAGs compared to white muscle (70, 71). White muscle, on the other hand, contains more sarcoplasmic reticulum and water than dark muscle (70).

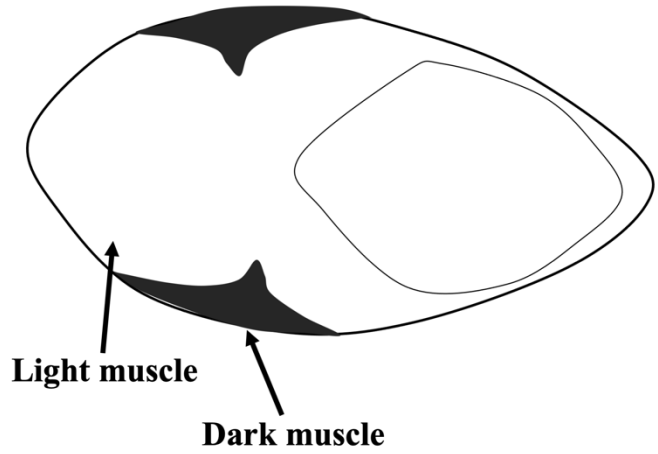

Fig.3. The location of the light- and dark muscle of herring.

3.2.2 The vascular system of fish

Fish have a single circuit circulation, meaning that blood is pumped in a single loop from the heart, through the aorta to the gills (branchial circulation) for oxygenation, and is then then distributed to the body (systemic circulation). In the systemic circulation a network of arteries supplies the muscles and organs with oxygen. Deoxygenated blood is then returned back to the heart through the venous system from the muscle, organs and gills (49, 72, 73). As mentioned in section 3.1.3, the gastrointestinal circulation receives a significant amount the cardiac output; 30-40 % (37, 49), which is significantly reduced with up to 60 % during exercise and stress (37, 73).

3.3 Post-mortem biochemical changes in fish muscle

3.3.1 Rigor mortis

After death/slaughter, ATP synthesis is maintained by glycolysis for some time. ATP synthesis fully halts when glycogen is depleted or due to accumulation of lactic acid due anaerobic respiration. The latter results in decreased pH which will inhibit glycolytic enzymes (74). The pH of unstressed Atlantic cod- or salmon muscle at the point of slaughter has been reported to be pH 7.3 but it can be as low as pH 6.8 in stressed post-mortem salmon muscle (36, 75). The ATP depletion leads to rigor mortis, a state where the muscles are rigid and stiff, as ATP is needed to allow dissociation of actin from myosin, thus to change from rigor to relaxation in the muscle contraction cycle (76). This is referred to as rigor mortis. If rigor mortis appears suddenly, it can affect fish quality through increased gaping. Gaping occurs as the connective tissue septum separating the adjacent myotomes break (myocommata), causing muscle segments (myotomes) in between to separate and create slits in the fillet. Gaping reduces the market price, and makes the fish more difficult to process as the fillet falls apart in different procedures such as skinning or slicing (40). In general, there has been a lot of research on the effects of pre- vs. post-rigor filleting and freezing of fish e.g. Esaiassen, Dahl (77), Einen, Guerin (78), this is however beyond the scope of the present thesis.

3.3.2 Autolysis and bacterial contamination

After rigor mortis one of the first factors affecting initial quality is autolysis. Nucleotides such as ATP and inosine monophosphate (IMP) are degraded by autolytic enzymes, which affect the fresh fish flavor (42) and provide catabolic activity used for bacterial growth (15). The main spoilage bacteria reported for cold-water fish species are *Photobacterium phosphoreum* (*P. phosphoreum*), *pseudomonads spp.* and *Shewanella putrefaciens* (*S. putrefaciens*) (42, 79).

Further to e.g., ATP degradation and bacterial spoilage, proteolysis is initiated during post-mortem storage of fish when endogenous proteases hydrolyze the fish proteins. Proteases such as cathepsins are present in the muscle (80), and in the gastrointestinal tract there is both peptidase and proteinase activities such as aminopeptidase, gelatinolytic proteases, trypsin and chymotrypsin, and collagenolytic proteases (81-83). Many proteases are acidic in nature, and thus, they are further activated by the natural post-mortem pH-drop, or by process-induced acidification such acid marination of herring (84). In the latter, a high degree of proteolysis is wanted as it makes the muscle soft. However, mostly endogenous proteolysis is an unwanted phenomenon as it makes the tissue “mushy” or create belly burst (see section 3.1.2).

Also, the lipids can be subjected to hydrolysis during post-mortem storage; by muscle- or gastrointestinal-derived lipases. The most common lipases are phospholipases and pancreatic lipases, acting on PLs and TAGs, respectively. Lipolysis occurs particularly extensive in the dark muscle and may cause rapid deterioration of the fish muscle (70). Lipid hydrolysis generate free fatty acids (FFAs), which are known to affect texture through interaction with proteins (85). The smaller size of FFAs is also reported make them more accessible to oxygen and pro-oxidants due to lower steric hindrance compared to TAGs and PL (86, 87). On the

other hand, FFAs also have the potential to inhibit metal- or Hb-induced lipid oxidation through chelation (88). Recent studies by Tatiyaborworntham and Richards (89) have also shown that when adding phospholipase A2 (PLA2) to Hb spiked WCM, PLA2 acts as a strong antioxidant against Hb-mediated oxidation and against heme degradation. Further description of the role of Hb in lipid oxidation can be found in section 3.5.2.

3.3.3 The lipid oxidation reaction

Lipid oxidation in post-mortem fish muscle can be stimulated when the initial balance between anti- and pro-oxidants is shifted in favor of the latter, and when then muscle is becoming disintegrated due e.g., to cutting, de-skinning or mincing operations. Such events introduce oxygen and creates contact between PUFAs and pro-oxidants which are normally compartmented. The lipid oxidation reaction is often divided into initiation, propagation, chain branching, and termination (90). During frozen storage or when pH is adjusted to prevent bacterial growth, lipid oxidation is normally the main shelf-life limiting reaction in fish. However, in some fish species and muscle types, especially heme-rich ones, lipid oxidation can limit shelf life even during refrigerated storage; thus, it has larger impact on the sensorial quality compared to bacterial spoilage (15, 91, 92).

Initiation

Initiation reactions are often divided into three different types: (A) initiation via free radicals, (B) lipoxygenase (LOX) catalyzed lipid oxidation, (C)-photo-oxidation **(Fig. 4)**. In radical-driven initiation (A), oxidation is initiated when a hydrogen atom is abstracted from an unsaturated fatty acid by a free radical, whereafter a fatty acid radical, $L^{\bullet}$, is formed. The abstraction of hydrogen atoms is most likely to take place from the methylene groups closest to the double bonds. As the hydrogen atom is abstracted the carbon is left with an unpaired electron, which can migrate, resulting in a conjugated double bond in the fatty acid (2, 74). As the general rule the $L^{\bullet}$ is short lived and highly reactive due to the unpaired electron. There are also many enzymes in the blood such as xanthine oxidase, superoxidase dismutase (SOD) and peroxidases, that can catalyze the radical driven lipid oxidation via their participation in the formation or reactive oxygen species (ROS) (26).

LOX is an enzyme found in the tissue of various animals, including fish, and could initiate lipid oxidation by catalyzing the insertion of oxygen to the unsaturated fatty acid (LH), which result in the formation of LOOH **(Fig. 4)** (19, 93).

Photo-oxidation is initiated by the formation of a singlet oxygen molecule ($^1O_2$) **(Fig. 4)**. These molecules are high energy, short-lived, highly reactive molecules. $^1O_2$ arise from normal ground state oxygen molecule ($^3O_2$) through absorption of energy from excited photosensitizers such as heme, chlorophyll, riboflavin (photo-oxidation type II) (74). Unsaturated fatty acids can then directly react with singlet oxygen though a cyclo-addition mechanism, which is different from ground state oxygen, which cannot react with a fatty acid due to the rule of spin conservation. However, in type I photooxidation, the excited photosensitizer instead gives rise to ROS, as superoxide anion ($O_2^{\bullet-}$) and the protonated superoxide radical ($^{\bullet}OOH$), which then initiate lipid oxidation (94).

Propagation and formation of primary oxidation products

The $L^{\bullet}$ very quickly reacts with oxygen forming $LOO^{\bullet}$ **(Fig. 4)**. $LOO^{\bullet}$ are high energy molecules and can therefore easily abstract hydrogens. Reaction of $LOO^{\bullet}$ with a fatty acid result in the formation of a LOOH and a new $L^{\bullet}$, thus propagating the lipid oxidation reaction (2). This step is the rate limiting one in the reaction. The ($LOO^{\bullet}$) propagation step and the subsequent breakdown of hydroperoxides result in increasing number radicals in the lipid phase, in turn reacting with large amount of oxygen.

Chain branching and formation of secondary products

$LO^{\bullet}$ are formed in the cleavage of hydroperoxides by e.g., trace elements or heme, supplying the oxidation with radicals but also resulting in formation of aldehydes, ketones and alcohols which give rise to the rancid odor and taste of oxidized lipids **(Fig. 4)**. As further discussed, in section 3.5.2, heme-driven hydroperoxide cleavage appears to be the most critical pro-oxidative mechanism for heme proteins.

Termination

The predominant product in the propagation process is the $LOO^{\bullet}$, which can react with both $L^{\bullet}$ and $LOO^{\bullet}$, in presence of oxygen, to form stable non-radical products. Under oxygen-limited conditions fatty acid dimers (L-L) rather form from the reaction of two $L^{\bullet}$ (2).

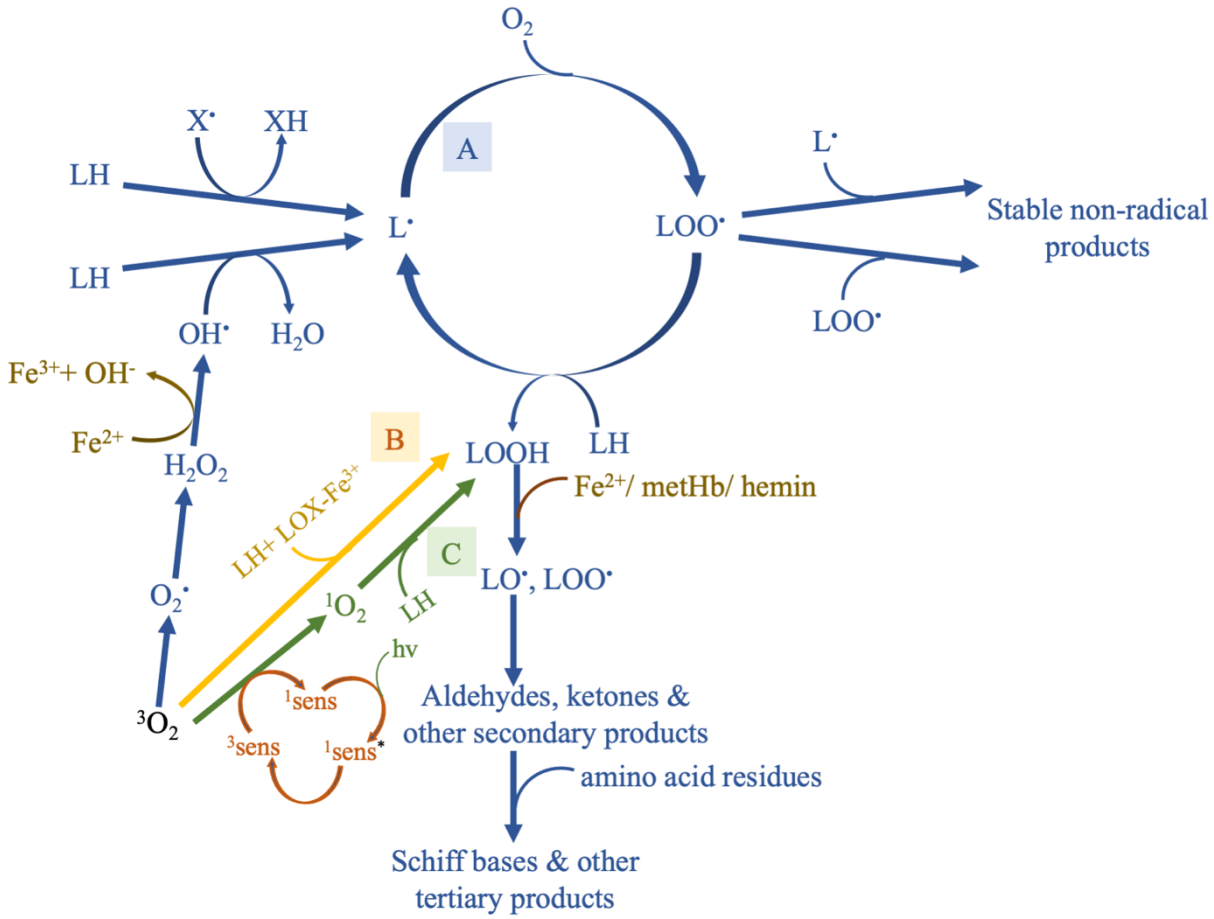

Fig. 4. The lipid oxidation reaction, (A) radical driven initiation, (B) LOX catalyzed lipid oxidation, and (C) photo-oxidation. The figure is modified after McClements (2).

Thus, the lipid oxidation reaction results in off-odors and off-flavors, but also in textural changes, color changes and nutrient loss. Texture and color can change when lipid oxidation products start acting on e.g., proteins or pigments. Nutrient losses emerge from the destruction of long chain (LC) n-3 PUFA, but also from breakdown of antioxidants, which are often vitamins. Lipid oxidation is more prominent in dark- compared to white muscle; which is related to the abundance of heme-proteins in combination with high abundance of membrane lipids (12, 42). Undeland, Ekstrand (91) reported that the rate of lipid oxidation in intact herring fillets was fastest in the skin, followed by the dark muscle and then the light muscle. However, if the dark muscle was not protected by the skin, this muscle oxidized fastest of all three tissues. Compared to e.g., cod light muscle, herring light muscle contain more heme-proteins, especially Hb, why it is more susceptible to oxidation during storage (12).

3.4 Blood, its components and changes during storage of fish

3.4.1 Blood and blood plasma components

Fish contain 1.5 to 6% blood of the total body weight, which is less compared to mammals (48). Out of the total blood volume, the RBCs make up around 39 %, in trout and herring, the rest being plasma. Plasma contains around 90% water (v/v) and approximately 10% ions, proteins and nutrients which are essential for many biological processes; the protein level being around 6-8 % (95). Some of the most important plasma proteins are albumin, which function as an osmo-regulator, has buffering capacity and act as a transport protein, fibrin- and fibrinogen proteins are involved in the coagulation and fibrinolysis cascade and, globulins are part of the immune system. Generally, the substances dissolved in plasma can be divided into: (i) mineral salts and ions, which in turn are divided into: a) sodium chloride (NaCl) which regulates osmotic pressure, b) bicarbonate which is a buffer salt regulating pH, and c) metal ions such as copper, calcium and iron which are essential for biological processes, (ii) low molecular weight (LMW) components which can be divided into: a) carbohydrates such glucose and fructose which are sources of energy. b) amino acids, c) nucleotides as ATP, d) vitamins/antioxidants, e) hormones, f) lipids g) bile acids, h) urea, and i) ammonia, (iii) high molecular weight (HMW) components divided into: a) peptides and (glycol)-proteins, b) oligosaccharides and polysaccharides, c) oligonucleotides and polynucleotides as well as d) deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), (iv) soluble gases; a) oxygen, b) carbon dioxide (CO_2) and c) nitric oxide and (v) metabolites such as lactate (96).

An essential feature of plasma is that it is isotonic, meaning that the ion concentration in plasma is very similar to the concentration inside the RBCs. If the solution surrounding the RBCs would be hypotonic, i.e., of lower ion concentration, RBCs would burst due to water entering the cell in attempt to equalize the concentration with the surrounding solution. Contrary, if the plasma would be hypertonic, i.e., of higher ion concentration, water would leave the RBCs to dilute the plasma, resulting in cell shrinkage. This is where the protein albumin plays its important role in maintaining the osmotic pressure in plasma. As the blood flows through narrow capillaries, the blood pressure (hydrostatic pressure) press plasma out of the capillaries. To avoid large loss of plasma from the capillaries, the hydrostatic pressure is balanced by a colloid osmotic pressure (COP) that is built up by a protein large enough to not leave the capillary vessels. The COP counteracts the hydrostatic pressure balancing the leak out and in the influx of the capillaries (97). Blood plasma is also known to contain several antioxidants with metHb reducing capacity. However, compared to the intracellular metHb reducing capacity, the blood plasma has limited ability, as most of the reduction in plasma is accounted by ascorbic acid (AA) (98).

AA is an important antioxidant in plasma due to its high reducing potential. AA plays a significant role as an oxygen radical scavenger and reducing agent of peroxides and superoxides (99). Most cells can regenerate oxidized AA, i.e., dehydroascorbic acid (DHAA), to reduced form by rapid transport of DHAA into the RBCs through the glucose transport protein GLUT1 (100). Inside the cell, DHAA is reduced to ascorbate, the anion of AA at

physiological pH, by glutathione (GSH). The release of ascorbate from the cells is then slow due to the negative charge, which is an advantage to the recycling and preservation of α-tocopherol in the cell membrane (100). The recycling of DHAA is dependent on GSH and nicotinamide adenine dinucleotide phosphate (NADPH) (101). GSH reduces DHAA to ascorbate and the formed oxidized glutathione (GSSG) is then recycled to GSH by glutathione reductase which is NADPH dependent. NADPH is produced through the hexose monophosphate (HMP) shunt (102). Among other important antioxidants of plasma are e.g., tocopherol, uric acid, bilirubin which can reduce free radicals (103).

3.4.2 Fish red blood cells

The main functionality of RBCs is to transport oxygen throughout the body. Fish RBCs are 8-15 µm long and have a thickness of ~2 µm (96, 104), and are therefore larger in size compared to mammalian RBCs that are ~7.2 µm long, with a similar thickness as fish RBCs (105). While human RBCs have the shape of biconcave disks and lack both nucleus and other cell organelles, fish RBCs have oval disk shapes with a compact nucleus and contain mitochondria (104). The RBC membrane is a fluid semipermeable lipid bilayer which contain ~40% lipids, whereof ~80 % are PL (104, 105). Mammalian RBCs have an approximate lifespan of 120 days whereas RBCs from teleost fish have a lifespan of 15-500 days (106).

Energy is required for the RBCs to maintain their cell shape and internal milieu by e.g., maintaining the function of transport proteins such as the sodium pumps (107) and maintaining Hb in reduced state (105). Glucose has been reported as the main exogenous substrate in the metabolism of salmonid RBCs and is mainly converted to lactate by the Embden-Meyerhof pathway (glycolysis) **(Fig. 5, black pathway)** and the HMP shunt **(Fig. 5, green pathway)** (108, 109). In the Rapoport-Luebering shunt **(Fig. 5, blue pathway),** 2,3-diphosphoglycerate (2,3-DPG) is produced; which is an important molecule for the control of Hb-oxygen affinity (105, 109). Inside the RBCs, metHb is mainly reduced by the enzymes nicotinamide adenine dinucleotide (NADH)-dependent cytochrome b5 and NADPH-dependent metHb reductase (110). These enzymes require NADH and NADPH, which are formed through the Embden-Mayerhof and HMP shunt, respectively **(Fig. 5)**. The presence of mitochondria gives fish RBCs the possibility to use lactate and pyruvate as sources for energy through further oxidation in the Krebs cycle (111).

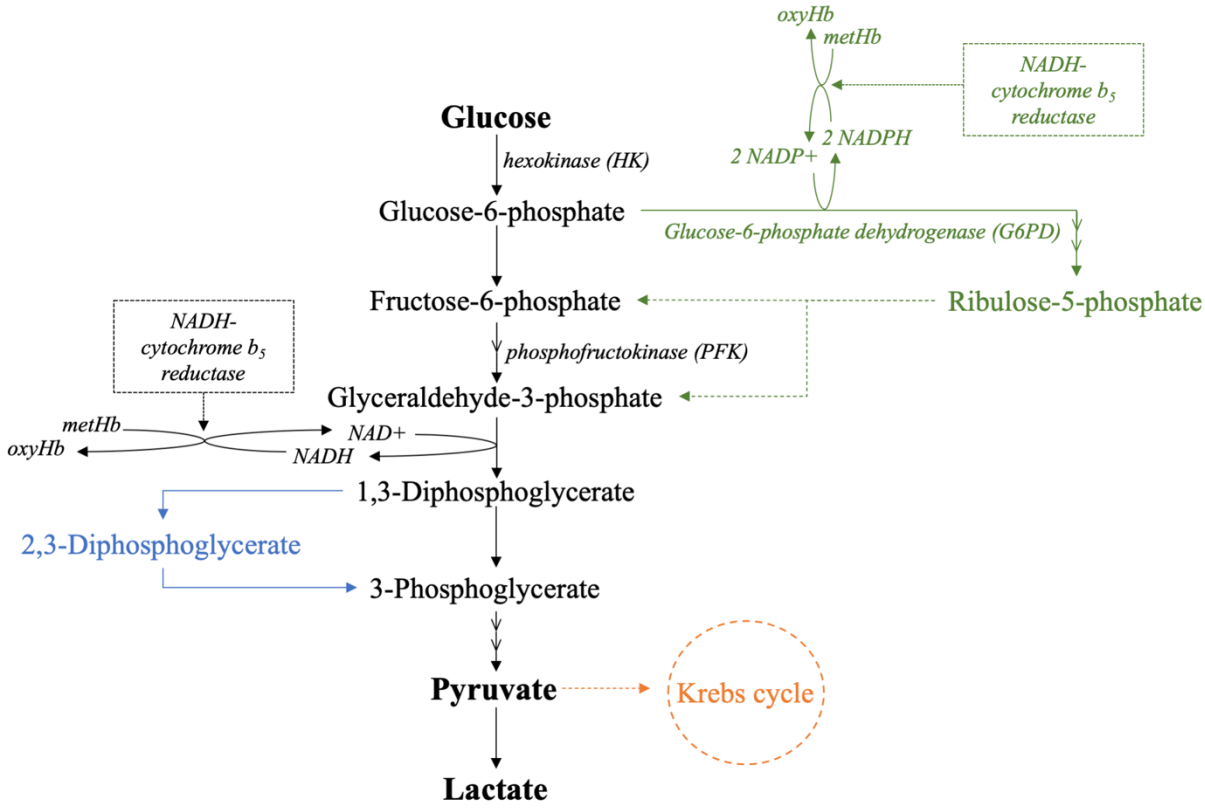

Fig. 5. Glucose metabolism of fish RBCs. Embden-Meyerhof pathway (black), HMP shunt (green) and the Rapoport-Luebering pathway (blue).

Causes of hemolysis

As stated in the introduction, studies are missing on the cause of lysis of RBCs as a function of common fish processing steps, and on the further link between lysis and lipid oxidation development. The main existing research on blood and fish quality evolve around development of blood spots e.g., in fillets of salmon (36), as this decreases their market value. However, recently, there have been some studies also of herring blood at the state of capture/pumping into RSW tanks e.g., in relation to stress during capture (39). These have indicated that a first critical point in the value chain from a lysis perspective is during capture when the fish is pressed against each other in the trawl and during pumping of the fish from the trawl to the RSW tanks on the boat; causing both stress and mechanical pressure. Based on the general knowledge on sensitivity of cells, including mammalian RBCs, to osmotic pressure (112-114), we have also hypothesized in this thesis that the subjection of fish blood to hypo- or hypertonic solutions during processing might also cause hemolysis. Another possible cause for hemolysis is oxidation of the RBC membrane, as this has been reported to induce hemolysis of rat RBCs (115).

3.5 Fish hemoglobin and its involvement in lipid oxidation

3.5.1 Hemoglobin

Hb is a globular protein with four polypeptide chains (**Fig. 6A**). Each of these polypeptide chains contain a heme group, consisting of an iron (Fe) ion within a porphyrin ring (heterocyclic ring). The porphyrin ring contains four pyrrole molecules that are linked together by methane bridges with the iron ion located at the center of the porphyrin ring (**Fig. 6B**). The iron ion has six coordination sites, four which are occupied by nitrogen atoms, one which is bound to the proximal histidine in the globin part and the sixth site being bound to ligands such as oxygen (O_2) for oxyHb and water for deoxyHb and metHb. In reduced state, oxyHb, the iron ion is in ferrous (Fe^{2+}) state and the porphyrin group is termed heme. After autoxidation, metHb is formed and the iron is in ferric state (Fe^{3+}), the porphyrin group is then termed hemin (25, 116).

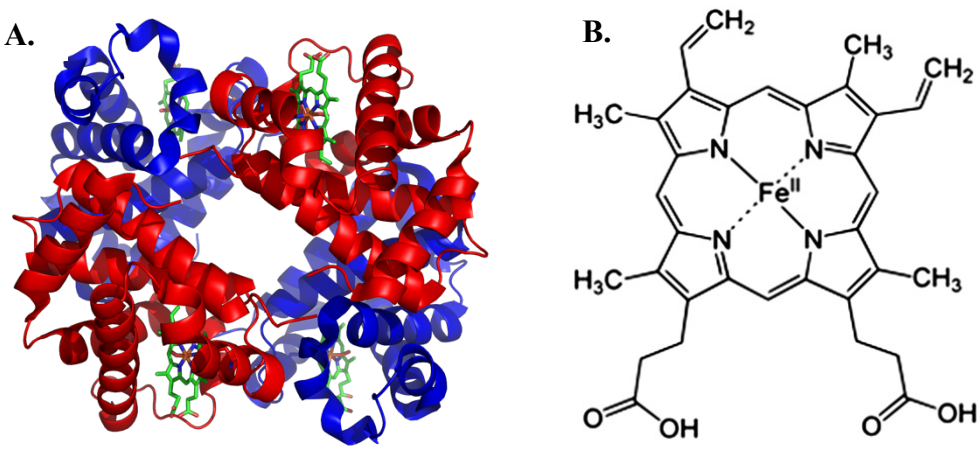

Fig. 6. The structure (A) of Hb (1) and (B) of the heme group (3) of Hb. The porphyrin ring contains four pyrrole molecules with one Fe^{2+} ion in the center of the heme group.

Although fish and mammalian Hbs have high sequence homology there are some major residue differences around the heme crevice affecting the tolerance to oxidation and sensitivity to heme release. Fish contain four types of Hb, HbI-HbIV, and according to a study performed by Aranda, Cai (24) four important amino acid differences were found between on the one hand trout and perch HbIV and on the other bovine Hb. These differences in amino acid explain the higher rate of oxidation and hemin dissociation of the trout and perch Hb which also translated into a more pro-oxidative ability towards e.g., WCM. Differences in pro-oxidative ability of Hb have also between found between fish species (Undeland et al., 2003; Richards et al) and between Hbs from the same species e.g. Sannaveerappa, Cai (117); however, in those studies, relations have mainly been done to the ability of the Hbs to become deoxygenated and oxidized, not to structural Hb-differences.

The Bohr and Root effect

The Bohr effect explains the decrease in oxygen affinity of Hb with high CO_2 concentration. CO_2 dissolved in water form hydrogen ions (H^+) and hydrogen carbonate ions (HCO_3^-); the former reducing pH. (118, 119). At lower pH, protonation of the distal histidine and other specific histidine close to the heme crevice cause structural changes that makes the heme crevice more accessible to solvent, e.g., protons and water. The accessibility of protons and water then accelerate autoxidation, as liganded O_2 in oxyHb gets protonated. The protonation of the liganded O_2 causes the release of $^{\bullet}OOH$ from the heme crevice and deoxyHb is formed. Weakly bound water then facilitates the oxidation of deoxyHb to metHb (25).

In mammalian blood, the drop in pH must be relatively large to affect the Hb oxygen affinity compared to in teleost fish where even a small reduction in pH affects the oxygen affinity to a large extent; this extreme pH sensitivity is known as the Root effect. Due to the Root effect, the fish Hb cannot be completely saturated even at high oxygen pressure, when pH is reduced. The reduced oxygen saturation of Hb enhances deoxygenation and autoxidation of Hb into deoxy- and metHb, respectively (119, 120).

Based on the described Bohr/Root effects, rapid autoxidation of Hb can take place as the pH in post-mortem fish is reduced, which in turn accelerates lipid oxidation. Low pH (5.7 and 6.3) has been proved to increase both Hb oxidation and hemin loss in perch and trout HbVI (24), two events which correlate strongly with lipid oxidation development (8, 121). Hb-autoxidation vary in different Hb-subspecies due to differences in their amino acid side chains around the porphyrin group (24). For example, HbIV was shown to be more susceptible to autoxidation and, also more pro-oxidative, than trout HbI (117). Further, oxidation of Hb and their pro-oxidative effect varies between fish species (116, 122), it is therefore important to develop antioxidative strategies that are tailored to different fish species.

3.5.2 Involvement of hemoglobin in lipid oxidation

Several studies have revealed how autoxidation and hemin-loss are critical events stimulating the pro-oxidative role of Hb against fish muscle PUFAs, e.g., Aranda, Cai (24), Undeland, Kristinsson (121), Richards, Aranda (123) Below, different mechanisms by which metHb or more oxidized species can participate in lipid oxidation are outlined.

Autoxidation reactions of Hb

Autoxidation of oxyHb to metHb generate $O_2^{\bullet-}$. These radicals are the conjugate base to the conjugate acid form $^{\bullet}OOH$. Under post-mortem pH, $^{\bullet}OOH$ is found in substantial concentrations, causing fast generation of hydrogen peroxides (H_2O_2) (25). Both $O_2^{\bullet-}$ and H_2O_2 can convert oxyHb to metHb (124). H_2O_2 can also oxidize deoxyHb to ferrylHb (ferrylHb(4+)=O), which in turn can react with another deoxyHb to produce metHb. MetHb can then further react with H_2O_2 and form ferrylHb radicals (ferrylHb$^{(\bullet+)}$(4+)=O) **Fig. 7** (25).

The link between different Hb oxidation states and lipid oxidation

FerrylHb radicals can initiate lipid oxidation by abstracting a proton from lipids (LH). The formed ferrylHb can then both initiate and propagate lipid oxidation, with metHb being formed

as a product (25). MetHb efficiently contributes to the cleavage of LOOH and hereby form ferrylHb radicals and LO• further to aldehydes and ketones **(Fig. 7)**. The LO• is efficient in abstracting a proton from lipids, and thus can re-initiate lipid oxidation. Re-initiation of lipid oxidation can also take place when the produced ferrylHb radical reacts with oxygen to form LOO•, **(Fig. 7)** (125) (25).

Hemin-propagated lipid oxidation

In metHb the porphyrin ring is loosely anchored to the globin and hemin is therefore readily released (24). This is the same phenomenon as has been found for myoglobin (Mb), where the oxidized porphyrin group is 60-fold less anchored to the globin compared to the reduced state

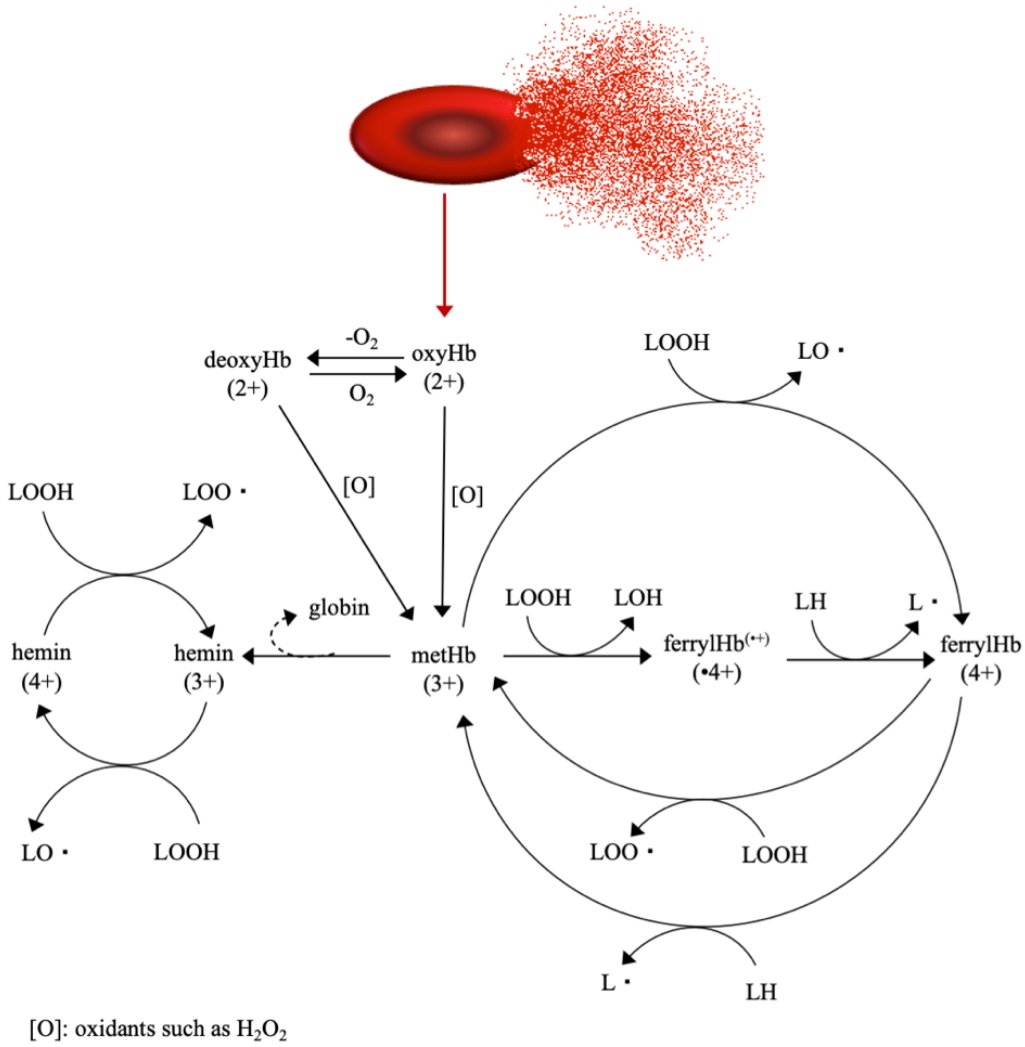

Fig. 7. Reaction pathways by which Hb can mediate lipid oxidation in muscle after release from the RBCs, and the different oxidation states where Hb is involved (4). For abbreviations, see abbreviation list.

(126). Hemin, which is lipophilic in nature, readily reacts with LOOH of e.g., cellular membranes and produce both LO$^{\bullet}$ and LOO$^{\bullet}$, see **Fig. 7** (25). Both LO$^{\bullet}$ and LOO$^{\bullet}$ are also lipophilic and can cause release of the iron from the porphyrin ring by attacking the carbon methane bonding in the tetrapyrroles of Hb (25). Thus, Hb- and hemin-mediated lipid oxidation is not possible after iron-loss. However, also free iron can mediate lipid oxidation on its own (see below), albeit of less significance in muscle compared to heme-proteins (127).

Free iron mediated lipid oxidation

In presence of activated oxygen species, LMW-iron can catalyze hydroxyl radical ($^{\bullet}$OH) formation via the reaction of Fe^{2+} with H_2O_2, i.e., the Fenton reaction (equation 3). If the LMW-Fe molecule is oxidized the $^{\bullet}$OH formation is decreased as the Fe^{3+} follows the Haber-Weiss cycle (equation 1 and 2) prior to the Fenton reaction (equation 3).

Reaction	**Description**	**Name of reaction.**	**Eq.#**
$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$	Fe^{3+} is reduced to Fe^{2+} by O_2^-	Haber-Weiss cycle	(1)
$2O_2^- + H^+ \rightarrow H_2O_2 + O_2$	Dismutation of O_2^- produce H_2O_2	Haber-Weiss cycle	(2)
$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$	H_2O_2 is decomposed to $^{\bullet}$OH + OH^-	Fenton reaction	(3)

$^{\bullet}$OH are very strong oxidants (redox potential -2.73) (99, 128) and can abstract a hydrogen atom from any molecule of lower redox potential. If it reaches to the PUFAs, it evidently can initiate the lipid peroxidation process. It has been described however, that its very high reactivity of $^{\bullet}$OH makes it likely to first react with molecules in the aqueous phase where it is formed; then the newly formed, less reactive radicals, reaches the lipid phase to initiate lipid oxidation (129).

3.5.3 Preservation of human blood within medicine to inspire fish RBC stabilization and removal

Human RBCs for transfusion are stored under hypothermic conditions (1-6 °C) to reduce the metabolic rate and the depletion of important metabolites (105, 130). Once ATP is depleted, the electrolyte balance is impaired since the sodium pumps requires energy. Also, the cell shape and membrane deformability are dependent on ATP (105), which is also true for Hb-oxygenation through depletion of 2,3-DPG (131).

Saline-adenine-glucose-mannitol (SAG-M) is an additive solution commonly used for RBC preservation in transfusion medicine. In SAG-M, saline corresponding to 0.9% NaCl is used for osmotic balance while adenine supports glycolysis by shifting the equilibrium toward ATP production (105). Glucose is added to maintain RBC metabolism and mannitol is used to stabilize the membrane (131). SAG-M is acidic (pH 5.7 at 37 °C) (132) to avoid caramelization of glucose or dextrose during sterilization of the storage solution (133). This acidification maintains ATP levels but not 2,3-DPG (105, 132). RBCs are stored in polyvinyl chloride (PVC) bags plasticized with di(2-ethylhexyl) phthalate (DEHP) (105, 133). DEHP has been reported to reduce hemolysis, shape change of RBCs and microvesicle formation (134).

3.5.4 Strategies to limit hemoglobin-mediated lipid oxidation in fish

Different modes of subjecting fish to antioxidants

One route to enhance the antioxidant level is through ingestion and regeneration of antioxidants in living organisms, which ensure that the oxidation of biological components is controlled and limited in vivo. However, regeneration of antioxidants declines in organisms after death, resulting in their depletion, which facilitates for oxidation of lipids and other biological components. On a general level, there are two mechanisms explaining how antioxidants function to reduce oxidation: i) prevention of the propagation step e.g., via radical scavenging thereby reducing the oxidation speed, ii) reaction with the highly reactive $^1O_2$ and convert it into the less reactive ground-state oxygen. It is also believed that singlet oxygen quenching antioxidants can react with ROS such as $O_2^{\bullet-}$ and $^{\bullet}OH$ (74, 135). Beside the radical scavenging antioxidants, there are many inhibitors of oxidation that act by reacting with e.g., pro-oxidants; examples here are metal chelating agents.

Antioxidants are often added into food systems to decrease the rate of oxidation. The fortification can be performed according to various approaches. Prior to death antioxidants can be added to the feed of farmed fish (136, 137) while during processing, antioxidants can be added to fillets or other pieces of whole muscle through spraying, glazing, coating, or injection. When the muscle is minced, antioxidants can be added directly via stirring or mixing (16, 138). Regarding the latter, excessive mixing is often required which can introduce oxygen, change the rheological properties (139) and increase contact between lipids and pro-oxidants; all which in turn could affect oxidation. Just as for endogenous antioxidants, protection provided by antioxidant fortification decreases as the antioxidants get depleted after reducing oxidized compounds (140). In glazing, frozen fish fillets are dipped or sprayed with water or a solution containing antioxidants which will create a protective ice layer which can decrease dehydration and oxidation (11, 141). Spraying on the surface of cooled/frozen fillets (-1 to -1.5 °C) ensures a more uniform application of antioxidants compared to dipping (142). Addition of antioxidants to the fish muscle through injection is commonly done together with a brine to also make the muscle more tender; this route is more common for meat than fish (143, 144). Injection and direct mixing of antioxidants into minces are both methods that could imply addition of excessive amounts of antioxidants into the core of the muscle matrix where oxidation is normally limited (6). Lipid oxidation is an extreme surface phenomenon and is mainly prevalent only in the outer 1-4 mm into the muscle (145).

4. METHODOLOGICAL APPROACHES

4.1 Study design

Four studies, presented in **Paper I-IV**, comprising four different models lay the ground for this Ph.D. thesis, and are designed to clarify what controls hemolysis, how Hb-mediated lipid oxidation of fish muscle can be limited by reducing hemolysis and how lipid oxidation mediated by released Hb can be prevented. An overview of the study designs can be found in **Fig. 8**, where the four different model systems are also illustrated.

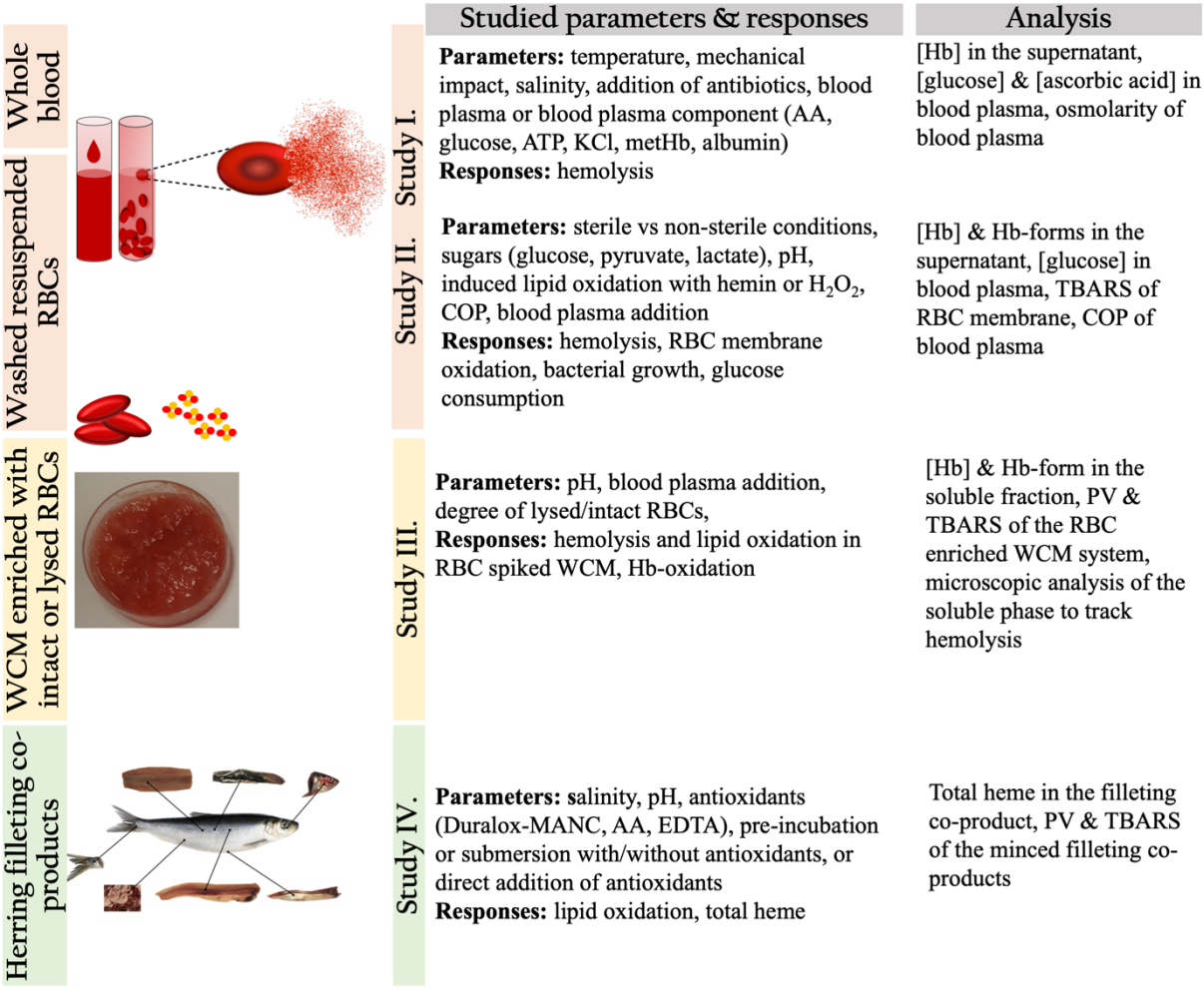

Fig. 8. Schematic overview of the four studies presented in paper I-IV. For abbreviations, see abbreviation list.

Study I-II were conducted on washed resuspended RBCs (wr-RBCs) or whole blood (WB) to understand factors affecting hemolysis. In **Study I**, temperature, mechanical impact, and osmotic stress were optimized to minimize hemolysis as these parameters were identified as critical factors affecting RBC stability and often varying during early fish handling. Since WB as more stable towards hemolysis some individual plasma components were also tested to better understand its protective effects. **Study II** was conducted to better understand how blood plasma or fish muscle related parameters can be used to prevent hemolysis. In **Study III**, the

obtained knowledge from **Study I-II** was applied in a fish model system consisting of WCM spiked with lysed or intact RBCs, to study the relationship between hemolysis and lipid oxidation, and how this relationship was affected by pH and plasma. Finally, in **Study IV**, herring filleting co-products were used to study whether Hb-mediated lipid oxidation could be limited through pre-incubation or submersion in solutions with/without antioxidants or through direct addition of antioxidants.

4.2 Sampling and handling of trout and herring blood

4.2.1 Fish supply

Atlantic herring (*Clupea harengus*) has been a target fish species in this thesis, since it represents the largest fish catch in Sweden, but due to easier maintenance of rainbow trout (*Oncorhynchus mykiss*) in tanks compared to herring, rainbow trout blood was used in most studies to investigate RBC stability. In **Study I**, it was confirmed that the trends for lysis seen in trout RBCs could be translated to herring RBCs. Rainbow trout was obtained from Antens laxodling AB, Alingsås, Sweden **(Study I)**, Vännåns fiskodling AB, Sweden **(Study II-III)** or Samegai trout farm, Shiga, Japan **(Study II)**. The fish was then maintained in tanks with aerated freshwater, ~10 °C, at Gothenburg University, Department of Biological and Environmental Sciences, Medicinaregatan 18. The fish was fed commercially available trout pellets and kept under a 12:12 photoperiod. Herring was obtained from a commercial fishing boat at the southern part of the Swedish west coast of, outside Helsingborg **(Study I)**. Further, in the development of a fish model to study the link between hemolysis and lipid oxidation **(Study IV)**, 24 h post-mortem cod (*Gadus morhua*) was obtained from a local fresh fish market, Landala Fisk AB. The choice of fish for this model is described under section 4.3.

4.2.2 Bleeding procedure

The bleeding procedure for trout and herring was approved by the regional animal ethics committee in Gothenburg, permit number 167-2013 **(Study I)**, 5.8.18-06591-2019 **(Study II-III)**. At Samegai, Shiga, Japan, the bleeding was conducted according to the purpose of the Animal Experiment Management Regulations of Nara Medical University. The fish was killed by a blow to the head and terminal bleeding was performed with heparinized syringe and needle (Heparin 5000 IU/mL; LEO Pharma AB) from the caudal vein of the fish **(Fig. 9)**.

Fig. 9. Blood withdrawn from the caudal vein of trout with heparinized needle and syringe.

An anticoagulant was used to avoid blood clotting, which is known to be more pronounced in fish compared to other vertebrates due to larger number of thrombocytes (146). Heparin was preferred over other anticoagulants such as ethylenediaminetetraacetic acid (EDTA) and citrate as it has been reported to not cause any immediate (≤ 24 h) significant changes to trout RBCs, such as swelling and hemolysis (146, 147). To avoid clotting during blood withdrawal the syringe and needle were rinsed with heparin, and an additional 50 IU heparin/ mL was added to the collected WB and gently mixed. Blood from ≥3 fish was pooled to minimize individual differences and the blood was then stored for a maximum of 24h at 3±1 °C prior to the different storage trials. Pooling the blood from several fish also gave us the opportunity to simultaneously compare several different treatments with the same batch of pooled blood since a larger volume was obtained.

4.2.3 Preparation and storage of washed resuspended red blood cells

Prior to washing of the RBCs, the hematocrit (hct) (i.e., the percentage RBC per volume WB) was measured using sodium heparinized capillary glass tubes that were centrifuged at 12 500g for 2 min at 4 °C. The remaining volume of WB was then gently centrifuged at 700g for 10 min at 4°C to collect the blood plasma. The RBCs was then washed three times according to a modified protocol by Fyhn, Fyhn (148) where the original NaCl-concentration of the washing buffer was changed from 1.7% to 0.9% NaCl to match the osmolarity of blood plasma **(Study I)**. In **Study II-III**, the final wash was performed with only 0.9% NaCl to remove residual buffer trapped between the RBCs which could have affected the final pH of the studied RBC-model system.

In **Study I**, the WB or washed RBCs was diluted 5-fold (based on WB volume) with the solution of interest and incubated on a SARMIX® blood mixer. In **Study II**, the washed RBCs were resuspended in 9-volumes of the incubation solution, and the blood mixer was not used to minimize the potential mechanical impact from constant mixing. **Fig. 10**, describe the procedure from bleeding the fish to storage of RBCs.

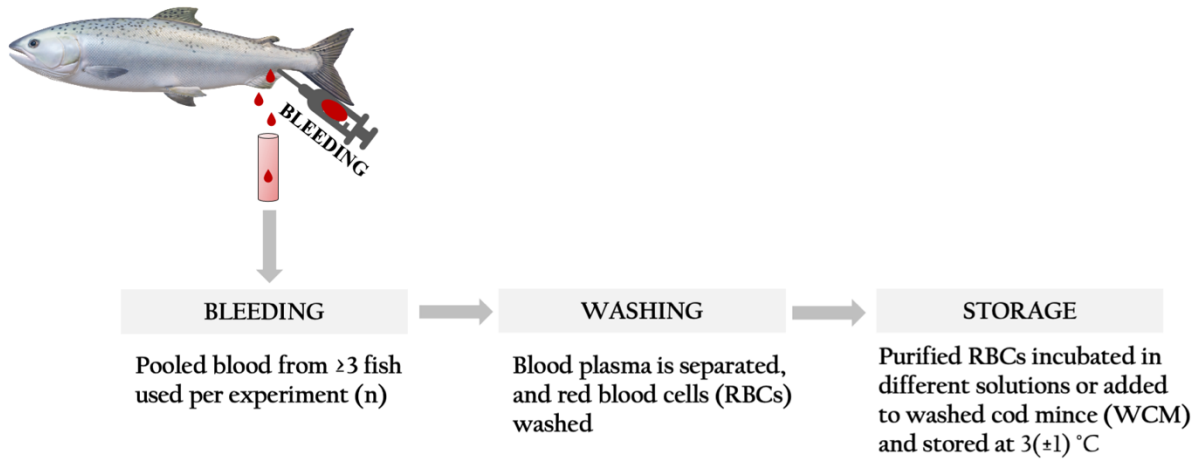

Fig. 10. A schematic overview of the bleeding, washing and storage procedure of RBCs.

4.2.4 Preparation of hemolysate

After the final wash of the RBCs, hemolysate was prepared by adding 1 mM Tris- hydrochloric acid (HCl) (pH 8.0) without any NaCl followed by incubation on ice for 1h (148). To avoid gel-formation of the DNA upon lysis, the sample was vortexed immediately after addition of the buffer. Cell-debris was then removed by centrifugation at 28 000g for 15 min at 4 °C, and the supernatant was stored at -80 °C until further use.

4.2.5 Preparation of methemoglobin

MetHb was prepared by adding 4 mole potassium ferricyanide per mole of Hb (on heme basis) to the hemolysate and incubated on ice for 1-2h. The ferricyanide was then removed with a 10 DG desalting column (Bio-Rad, Hercules) (149). The millimolar extinction coefficient 153 $mM^{-1}cm^{-1}$ was used to quantify metHb at 405 nm (on heme basis) (150).

4.3 Model systems for studies of hemolysis and lipid oxidation

Diluted WB was used as a model system to study the effect of blood plasma on the stability of RBCs towards lysis **(Study I)**. To better understand how individual endogenous plasma components, exogenously added compounds or e.g., pH, osmolarity, COP, temperature or mechanical stress affect the RBC stability, washed RBCs were resuspended in solutions with different compositions. This wr-RBC model system was used in **Study I-II**.

To study lysis of the RBCs in presence of fish muscle, the white muscle of cod was used to prepare WCM **(Study III)**. The advantage of the WCM model is that it contains intact myofibrillar proteins and cellular membranes while the sarcoplasmic proteins and other water-soluble components are removed in the washing process. Thus, the model system resembles fish muscle, but allows studies of individual pro-/antioxidants or even lipids added exogenously in controlled levels (20). The choice of cod white muscle is based on its very low levels of neutral lipids, while it still contains membrane lipids with high amounts of docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA, 20:5), similarly to other cold-water fish species such as Atlantic herring. This is important as the PUFAs such as DHA and EPA are more susceptible to lipid oxidation than less unsaturated fatty acids (25). Furthermore, cod is a white fish, thus contain low levels of endogenous pro-oxidants such as Hb and Mb, facilitating studies of the exogenously added compounds (25, 151). The high stability of WCM without addition of pro-oxidants has been confirmed in several studies (127, 152).

In **Study IV**, where prevention of Hb-mediated oxidation in fish muscle was to be studied as a function of incubation in solutions without or with antioxidants added, herring rest raw materials were used since the study was more applied in its nature. After the pre-incubation, the herring rest raw material were minced and stored in a similar manner as the WCM-samples. The antioxidants were selected based on the knowledge that many different reactants need to be efficiently prevented to reach a good lipid oxidation inhibition (153). We also took a starting

point in commercially applicable compounds and mixtures to make our methods scalable. Duralox is a commercially available mixture of rosemary extract, AA, citric acid and tocopherol which has been successfully used e.g., for meat (154). Iso-AA acid is a less expensive option than AA which has previously proven to be effective in herring muscle (155). Also, EDTA, a strong chelating agent, has previously been successfully used for herring (155).

4.4 Measurement of hemoglobin concentration

Preparation of samples for hemoglobin analysis
In storage trials of RBCs or WB in solution the sample was carefully mixed in connection to the sampling. The subsample was centrifuged at 700g for 10 min at 4 °C and Hb was measured in the supernatant using the cyanmetHb- or the azidemetHb method, described below. To measure Hb in the supernatants of the WCM system, the sample was first gently mixed with a spatula prior to subsampling. The subsample was centrifuged at 2000 g for 5 min at 4 °C, to obtain a supernatant that was collected and centrifuged at 9000g for 3 min at 4 °C to sediment fibers, membranes and other macromolecules that could hinder the Hb measurement with the cyanmetHb- or the azidemetHb method. Total heme was measured in minced herring rest raw materials by first freezing in liquid nitrogen and then grinding the sample into a fine powder to obtain a homogenous sample.

4.4.1 Cyanmethemoglobin method

The international council for standardization in hematology (ICSH) recommend using the cyanmetHb method to determine the concentration of Hb. This method was used in **Study II-III** for hemolysates and supernatants of the WB, wr-RBC or the WCM system spiked with lysed or intact RBCs. In this method all Hb-derivates (except sulph-Hb, which is converted to sulph-Hb-cyanide) are converted into metHb and the absorbance at λ=540 nm is measured. In our studies we used the Drabkin's reagent, which is an alkaline solution consisting of potassium ferricyanide, potassium cyanide, sodium bicarbonate, and a surfactant. In the first reaction Hb is oxidized to metHb by potassium ferricyanide, whereafter metHb is converted to cyanmetHb by potassium cyanide (156). The whole reaction takes 15 min at room temperature (RT) whereafter the absorbance of cyanmetHb is measured at 540 nm. If another method is used to determine [Hb] it should be adjusted to obtain comparable results with the cyanmetHb method (157).

4.4.2 Azidemethemoglobin method

A handheld HemoCue Plasma/Low Hb system was used for rapid determination of Hb concentration ([Hb]) of hemolysate or in the supernatant derived from storage solutions for RBCs, in **Study I-II**. Comparable results with the cyanmetHb method were confirmed before use. The microcuvettes compatible to the photometer contain sodium deoxycholate, sodium azide, sodium nitrate. Sodium deoxycholate is used to hemolyze RBCs, however in our experiments the cuvettes were only used for supernatants, already containing free Hb. Sodium

azide and sodium nitrate convert Hb to azidemetHb, which is then measured spectrophotometrically at 570 and 880 nm and translated to g/L Hb through a built-in microprocessor with a programmed algorithm (158).

4.4.3 Acid hematin method

In **Study IV**, heme was extracted from powdered herring rest raw material mince with the use of 80% acetone, 2% HCl and 18% water, according to (159). The sample was incubated for 1h at 8 °C in darkness, whereafter it was filtered and measured spectrophotometrically at 640 and 700 nm to estimate total heme against a standard curve. The acid hematin (Hornsey's) method was chosen as it has previously been described to have a good extractability of heme recovery ≥98% for fresh fish samples (160).

4.4.4 Analysis of hemoglobin forms

The Benesch equations were used to estimate the mixture of oxy-, deoxy- and metHb at varying pH between 6.4-7.6 **(Study III)**. The ratio between Hb-forms in the three component-system was calculated based on the absorbance at 560, 576 and 630 nm, and for each pH and wavelength, a unique extinction coefficient is used (161)**.**

4.4.5 Microscopic techniques to estimate hemolysis in a WCM system

Estimation of hemolysis by measurement of Hb in the supernatant of the RBC-spiked WCM system was not successful due to problems with decreasing Hb levels over time. To solve this issue, we aimed at tracking the RBCs instead of the Hb. The initial goal was to quantify the percentage of intact versus lysed RBCs in the WCM system as it is stored over time. The developed technique was going to be used in routine work and therefore needed to be fast and cheap to conduct, while providing a good representation of the sample.

The first technique tested was light microscopy, in which it was quickly noted that the size of the WCM muscle pieces, several millimeters, and fibers, 10-100 µm (162), created difficulties to detect the notably smaller RBCs, 8-15 µm (104). Pre-analysis processing such as filtration and centrifugation were tested to separate the RBCs from the fish tissue, however without success. Dying the RBCs prior to addition to the WCM muscle were also not a possibility due to short half-time or unspecific binding of the dyes.

Secondly, confocal laser scanning microscopy (CLSM), Zeiss LSM780, connected to an argon-laser was investigated as it provides the possibility to construct high resolution images 50-100 µm into the sample (163). With CLMS we aimed to utilize the autofluorescence of the Hb-molecules to track intact RBCs. In many studies performed on tissue the autofluorescence of RBCs is a problem as it fluoresces across multiple wavelengths, causing interferences with other signals of interest (164-166). However, in our study, Hb was the target molecule from which we wanted to obtain strong signals. Imaging of human RBCs has previously been performed by exiting the Hb-molecules with 405 nm and capturing the emission profile at 425-790 nm (167). Based on this, we assumed that similar excitation and emission ranges could be used, also for fish Hb, although small differences in the structure of Hb between mammalian

and fish Hb are known (24). Excitation at both 405 and 458 nm was tested, however, only very weak signals could be detected using the 405 nm.

The third technique tested was to cryo-section and dye the samples prior to analysis under wither light microscopy or CLSM. Thus, samples were subjected to either rapid or slow freezing in cryomolds with maximum thickness of 5 mm. Rapid freezing was performed with either liquid nitrogen or isopentane, and slow freezing was performed in a "home-made" cell freezing container with isopropanol. To protect the RBCs from cryo-damage various combinations and concentrations of the cryoprotectants dimethyl sulfoxide (DMSO), glycerol bovine serum albumin (BSA) was tested. After freezing samples were stored at -80 °C until analysis. Upon analysis samples were cryo-sectioned into ~7 µm thin slices, put on microscopy slides, dried, and then fixed with 4% paraformaldehyde for 10 min and rinsed in water before staining. Also, in this step a screening of various stains targeting different compounds of the RBC spiked WCM system had to be performed. The strongest contrast between the fish tissue and the RBCs was provided by hematoxylin and eosin (H&E), which stained acidic components (nucleic acids) in blue and the basic components (cytoplasmic proteins) in pink. However, in all samples subjected to freezing, the RBCs were cryo-damaged.

The route which was finally successful was to smear a droplet from the liquid phase of the WCM system on a microscope glass and left to dry overnight. RBCs, whereafter RBCs were fixed in 4% paraformaldehyde in PBS and stained with H&E. The stained samples were analyzed in duplicates with a light microscope. **Fig. 11A**, visualize a typical micrograph of intact RBCs with distinct and compact nucleus while **Fig. 11B** visualize a typical micrograph of a sample containing lysed RBCs, which can be identified by the nucleic content filling the RBC, thus the RBC is completely stained in blue.

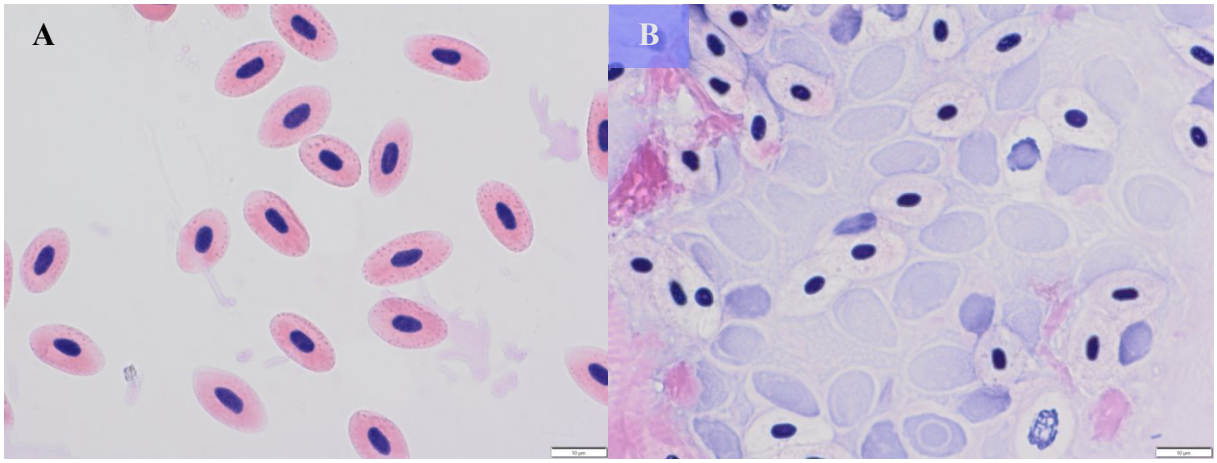

Fig. 11. Panel A shows intact RBCs with a compact dark blue nucleus and pink cytoplasm. In panel B, RBCs stored for 4 days in PBS pH 6.4 are visualized. The cytoplasm is transparent, and, in several RBCs, the nucleic content has spread out in the whole RBCs, resulting in a diffuse blue colored RBC. Images are from light microscopy analyses of H&E-stained RBCs, of the soluble phase from RBC-spiked WCM.

Summary of the various methods used to study Hb or RBCs in different model systems **(Fig. 12)**.

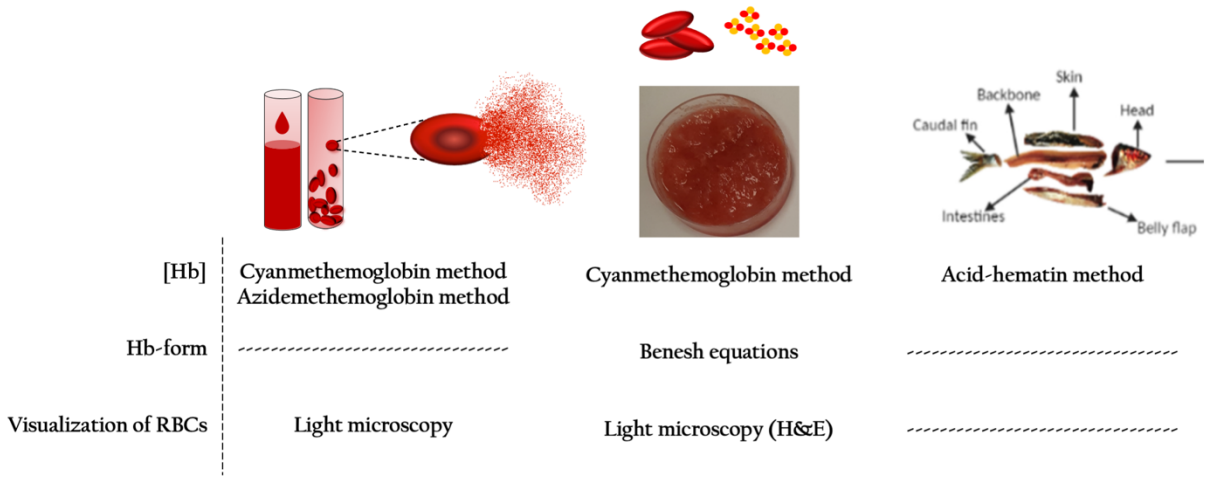

Fig. 12. The methods used to study Hb or RBCs in the various systems used.

4.5 Analysis of plasma and plasma components

4.5.1 Plasma osmolarity and colloid osmotic pressure

The osmolality in herring and trout blood plasma **(Study I)** was measured using an osmometer (Advanced® Instruments Inc., Micro-osmometer model 3320) and translated to NaCl equivalents. In trout and herring, the NaCl equivalent percentage was 0.9±0.001% (152 ± 1.2 mM) and 1.1±0.001% (195±1.3 mM), respectively. The higher osmolarity in herring could be caused by the different capture conditions. Herring was bled immediately after capture from the sea, which could have induced stress. Stress leads to an increased metabolism that increase the gas transfer and ion diffusion through the gills, this phenomenon is known as the osmo-respiratory compromise (168).

To determine the COP of blood plasma, a colloid osmometer (Osmomat 050, Gontec) with a 20 kDa cut-off membrane was used. Suspensions with COP ≥ blood plasma was then recreated using albumin, dextrin, or HMW dextran to study RBC-stability as a function of COP **(Study II)**.

4.5.2 Glucose

Glucose **(Study I-II)** was measured in trout and herring blood plasma using an UV-based glucose-hexokinase (HK) assay kit; Sigma-Aldrich), whereafter absorbance was measured at 340 nm. The kit was based on hexokinase-catalysed phosphorylation of glucose by ATP to produce glucose-6-phosphate. Oxidation of glucose-6-phosphate was then catalyzed by glucose-6-phosphate dehydrogenase (G6PD) in presence of oxidized nicotinamide adenine dinucleotide (NAD^+), which was simultaneously reduced to NADH.

4.5.3 Ascorbic acid

Plasma AA was analyzed with HPLC connected to an electrochemical detector. Fresh blood plasma was stabilized and deproteinated by 10% meta-phosphoric acid (MPA) with 2 mM EDTA. Oxidized AA, i.e., DHAA was reduced by tris[2-carboxyethyl] phosphine hydrochloride (TCEP) to AA prior to analysis at low pH (pH 2.8), to avoid irreversible conversion of DHAA to 2,3-diketogulonic acid.

4.5.4 Bacterial growth

In **Study I**, it was observed that penicillin-streptomyocin (PEST) resulted in a small reduction of the hemolysis rate. Therefore, preparation of the samples under aseptic and non-aseptic conditions was compared in **Study II**, both in terms of colony forming units (CFU/mL) and hemolysis. Plate count agar was used, and incubation of the plates was done at 4 °C.

4.6 Lipid oxidation

4.6.1 Lipid extraction

To extract fish muscle lipids or RBC membrane lipids, a solvent system consisting of varying ratios of chloroform to methanol depending on the expected lipid content**)** was used (Lee, Trevino (169). To protect the lipids from oxidation during extraction, 0.05% (w/v based on total volume) butylated hydroxytoluene BHT was added. The ratio between chloroform:methanol for fish with different fat content is suggested to be 1:2, 1:1 and 2:1 for lean, medium fat and fatty fish, respectively. For WCM which is lean, a more polar solvent system (1:2) is suggested (Lee, Trevino (169). For analysis of wr-RBCs the ratio 1:1 was selected rather than 1:2 to allow enough chloroform to be collected from the bottom phase for peroxide value (PV) measurement. For herring filleting co-products which however are fattier, 2:1 was used. A solvent-to-sample ratio of 10:1 was used in all experiments with fish tissue as this ratio had been reported to provide optimal lipid extraction for both lean- and fatty matrixes. However, for the RBC samples a sample-to-solvent ratio of 1:1 was used as the fats from the RBC membrane already were highly diluted in the storage solvent. After homogenization of the sample, 0.5% NaCl was added to create two phases (chloroform- and methanol+water phase) and to remove proteins from the chloroform phase (169).

4.6.2 Peroxide value

LOOH are primary lipid oxidation products and are commonly measured as PV, which provide a good indication of the early steps of lipid oxidation. The ferric thiocyanate method provides a simple and rapid colorimetric determination of PV by using the chloroform phase after extraction. $Fe^{2+}$are here oxidized by LOOH and form a violet/red colored ferric-thiocyanate complex which can be measured spectrophotometrically (170). A great advantage of the ferric

thiocyanate method over e.g., titration with iodine is that far less extracted lipids are required (170).

4.6.3 Thiobarbituric acid reactive substances

Carbonyls such as aldehydes, formed as secondary products from lipid oxidation, can be measured through the thiobarbituric acid reactive substances (TBARS) method. Commonly, malondialdehyde (MDA) is used as a standard, why results are expressed as MDA-equivalents. Indeed, the dialdehyde MDA is also one of the main carbonyls captured in the reaction. Schmedes and Hølmer (171) published a version of the TBARS method which comprised analysis of free carbonyls in the methanol: water phase, after lipid extraction. In the traditional method by Lemon (172) the sample is otherwise extracted with trichloroacetic acid. To use both phases obtained from the same extraction for lipid oxidation analyses allow direct comparison of PV and TBARS data and facilitates oxidation studies when sample amounts are very small. In the TBARS test, a pink colored adduct is formed after boiling, with two thiobarbituric acid (TBA) and one carbonyl molecule, which can be measured spectrophotometrically at 532 nm (171).

4.7 Statistical analysis

For experiments repeated ≥2 times, data are expressed as mean ± standard deviation (SD). To determine significant differences between sample treatments and storage points, one-way analysis of variance (ANOVA) was conducted, and a Tukey's post hoc test applied for a pair-wise comparison where significance was found ($p<0.05$). In **Study I**, a Box Behnken design was constructed to find the osmolarity where highest RBC stability was achieved. Data were evaluated with multivariate analysis to build a regression model with quadratic terms to determine the relationship between hemolysis and different process conditions. In **Study III**, Spearman's correlation coefficients and their significance was calculated to evaluate correlations between different responses, such as redness of the WCM and development of lipid oxidation.

5. RESULTS & DISCUSSION

5.1 Optimization of process parameters to limit hemolysis during early handing of pelagic fish

5.1.1 The effect of osmolarity

In **Study I**, our aim was to understand the profile of hemolysis within the range of 0-3% NaCl, with or without the presence of plasma. Two systems were therefore set up to compare the NaCl tolerance of RBCs; wr-RBCs and diluted WB. In both systems, extreme tonicity caused rapid hemolysis; 0% NaCl resulted in instant hemolysis and 3 % NaCl resulted in rapid hemolysis after 1 day of storage **(Fig. 13A)**. This indicates that in currently used fish filleting procedures, blood removal with tap water would increase the lysis rate of RBCs and thereby result in contamination of the fish flesh with Hb molecules. This is particularly critical for the filleting side-streams, such as head, backbone and caudal fin which naturally contain high levels of Hb, 39.1 to 70.9 µmol/kg (**Study IV)** (14, 173). Commonly the side streams are stored and transported for feed production at outdoor temperature, under which they will be in direct contact with a slurry of lysed RBCs. The fish filleting procedure thus calls for attention, whether it is wild-caught pelagic fish species or e.g., farmed fish.

The osmolarity in blood plasma in trout and herring corresponded to 0.9% and 1.1% NaCl, respectively (**Study I**). Based on the results above, it was of interest to study to what extent the RBCs could tolerate a deviation from this physiological salinity. In **Fig. 13B**, the results are expressed as T_{50}, meaning the days it takes for the sample to reach 50 % hemolysis. From **Fig. 13B** it can be observed that both herring and rainbow trout RBCs had a good stability in the range 0.9-1.3% NaCl, which gives somewhat larger degrees of freedom for the fish industry if they would like to adjust their handling procedures to minimize RBC-lysis. Throughout **Study**

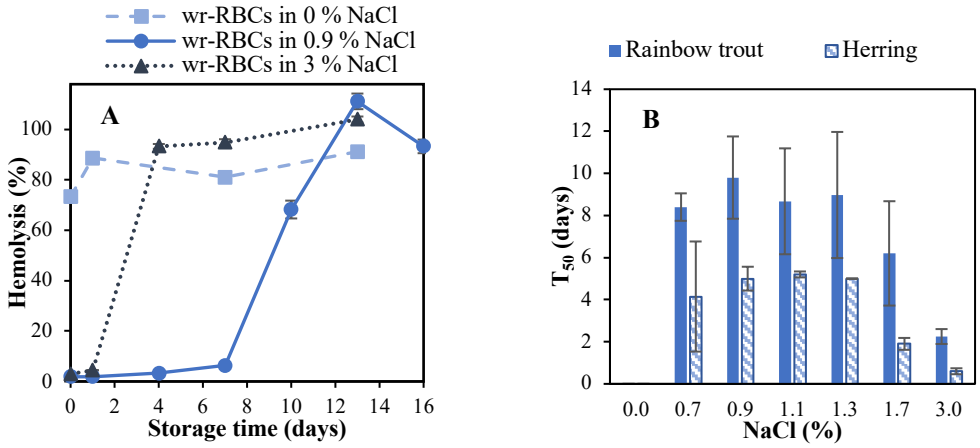

Fig. 13. The effect of salinity on hemolysis. Panel A shows how hemolysis develops in wr-RBCs during storage at 3±1 °C in 0, 0.9 and 3% NaCl. Panel B shows the differences in the time it takes for trout and herring RBCs stored under different salinities ranging from 0 to 3 % to reach 50 % hemolysis (T_{50}). Values are visualized as mean ± SD, n≥2.

I-IV, 0.9% NaCl was used as a reference when adapting the osmolarity of different RBC-incubation solutions to physiological conditions.

According to Lewis and Ferguson (113) lake trout (*Salvelinus namaycush*) RBCs are tolerant to salinities in the range 0.8-5.0 % NaCl while human RBCs are tolerant to as low as 0.4 % NaCl. However, in their osmotic fragility tests the RBCs are only subjected to the solution of interest for 30 min before measuring hemolysis. Our results therefore provide a new dimension by showing the effect of long-term storage in various salinities, on RBC stability.

5.1.2 The effect of mechanical stress

Steps such as trawling, pumping, and size grading imply mechanical impact on the fish. Loss of scales is prevalent especially in herring already after trawling and pumping, and could affect plasma osmolarity (39), which in turn affect the RBCs. Another effect that could influence the RBC stability is the actual mechanical stress induced by the three process-steps mentioned above. After size grading of the landed herring in the factories, the fish is separated into different tanks, which includes a fall from around 2-meter height. To simulate the mechanical stress induced by this procedure, a tube with wr-RBCs or diluted WB (in 0.9% NaCl) was dropped from 2-meter height, 0, 2, or 5-times, in **Study I**. A multivariate analysis was performed to investigate the time-dependent association between hemolysis and mechanical stress, from which linear relationship was found. In **Paper I, Fig. 1 e-f**, it can be observed that the time before onset of rapid hemolysis was shortened with increased mechanic stress.

5.1.3 The effect of temperature

In **Study I**, it was investigated how different temperatures (0, 4-6 and 10-12 ºC) affect the hemolysis of trout RBCs stored in 0.9% NaCl. A significant increase in hemolysis was only observed in the RBCs stored at elevated temperatures (10-12 ºC). A multivariate analysis was performed to investigate the time-dependent association between hemolysis and temperature in greater detail. It was found that the association between these two parameters grew stronger over time, from a weak linear relationship at day 7, to a strong linear and quadratic relationship at day 11 and 13. The lowest degree of hemolysis was found between 0-6 ºC with an estimated minimum hemolysis at 2.5 ºC. These results are in agreement with the U.S. Food and Drug Administration (FDA) approved span of 1-6 ºC when storing human RBCs for blood transfusion (174).

5.1.4 Bacterial contamination

Fish is operated under non-sterile conditions; thus, microbial contamination is a risk at each step of the processing chain. As mentioned in section 3.3.2, spoilage bacteria such as the Gram-negative *P. phosphoreum*, *pseudomonads* ssp. and *S. putrefaciens*, are known to be commonly present in spoiled marine fish (42, 79). Some of these spoilage bacteria, such as *pseudomonas,* are known to produce hemolysins which can destabilize and create pores through the PL-membrane of RBCs (42, 175). To study the role of bacterial growth for hemolysis under conditions of optimal salinity and temperature (**Study I**), PEST (100 ppm) was added to wr-

RBCs in 0.9% NaCl during storage at 3±1 °C. PEST is a broad-range antibiotic and is effective against both Gram- positive and negative bacteria. Addition of PEST resulted in a reduced rate of hemolysis (2.5 days delayed T_{50}). To further investigate the relationship between bacterial growth and hemolysis, total viable count was estimated in wr-RBC samples prepared under sterile and non-sterile conditions, both without the addition of an antimicrobial agent, **(Fig. 14) (Study II)**. Interestingly, the hemolysis lag-phase of both sterile and non-sterile samples was in this experiment 7 days, however, with a reduced rate of hemolysis for the sample prepared under sterile conditions, resulting in 2.5 days delayed T_{50}. Altogether, microbial growth did not seem to be the main contributor for the onset of hemolysis when other lysing conditions as osmotic or temperature stress were avoided. Instead, the relationship could even be the opposite, with hemolysis being a contributing factor to rapid microbial growth, which could stem from free Hb providing the microbes with iron which is essential for bacterial proliferation (176).

The contribution of plasma to hemolysis and microbial growth was also studied, under non-sterile conditions **(Fig. 14)**. Surprisingly, blood plasma suppressed microbial growth to 0-10 CFU/ mL during 14 days of storage compared to 2 * 10^8 CFU/mL for the sample without plasma (0.9% NaCl). Thus, these results point out the important aspect that the dilution of blood plasma in the process of rinsing after gutting or filleting, could accelerate bacterial spoilage. It has earlier been described that bleeding fish reduce bacterial growth (10), and the results seen in **Fig. 14**, clarifies that it might be that free Hb accelerate bacterial growth by making iron more accessible.

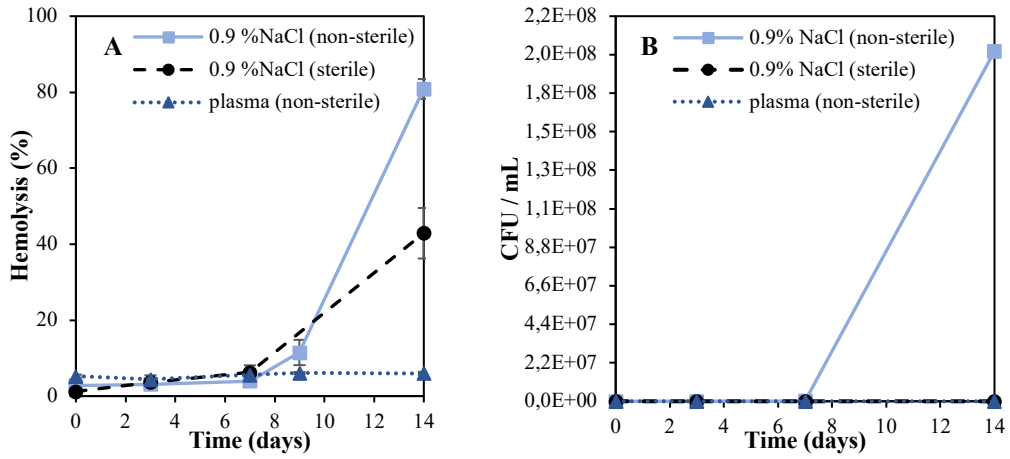

Fig. 14. The relationship between hemolysis and microbial growth under preparation and storage of wr-RBCs under sterile or non-sterile conditions at 3±1 °C. Also, the effect of plasma on both hemolysis and microbial growth is visualized. Panel A shows the development of hemolysis over time and, panel B shows total colony forming units (CFU)/ mL. Values are visualized as mean ± SD, n=2.

5.2 Role of plasma components and exogenous additives in the prevention of hemolysis

To study how different industrial conditions affect the RBCs, wr-RBCs were used as one of the model systems. However, blood plasma will always be present *in situ* in fish, undiluted or diluted. It was therefore important in this thesis to understand the role of blood plasma for the RBC stability. Throughout all studies performed on RBCs in suspension **(Study I-II)** both diluted and non-diluted plasma significantly improved the RBC stability. To better understand the nature of the protecting effects of blood plasma, different plasma-derived LMW and HMW compounds were investigated separately and later also in combination to simulate the effect of blood plasma **(Study I-II)**.

5.2.1 Low molecular weight plasma components

AA is often described as an important antioxidant and radical scavenger in human blood, as it accounts for most of the reducing capacity in plasma (98, 177). AA was measured in somewhat higher levels in trout- than herring blood plasma, 169.8 ± 19.9 µM vs. 109.6 ± 26.7 µM, respectively. Different levels of AA, ranging from 50-200 µM, were added to trout wr-RBCs to investigate their impact on hemolysis. Trout RBCs were best preserved when adding the higher level of AA (200 µM), which provided 5-11 days extra to reach 50 % hemolysis compared to the control, consisting of wr-RBCs stored in 0.9% NaCl. However, when the same level of AA was tested during incubation of herring wr-RBCs, non-systematic effects was observed **(Study I)**. Pointing at the importance of developing species-specific antioxidant solutions.

The other route to increase RBC stability with LMW plasma components was through addition of energy providing compounds. In **Study I**, various concentrations of glucose (2-20 mM) were added to 0.9% NaCl to maintain the RBC metabolism, and thereby stimulate many of the energy-dependent processes in the cell (e.g., ATP-driven volume stabilization, production of electron carriers such as NADH and NADPH); ultimately mitigating hemolysis. The addition of glucose however resulted in varying effects on RBC stability, from no effect to up to 18 days increased T_{50} **(Study I)**. No significant effects were found between the different glucose concentrations studied. Our first hypothesis was that exogenous glucose might not be consumed by the RBCs due to low permeability, which have been reported in some teleost RBCs (107, 108). However, when measuring glucose consumption of RBCs during incubation in plasma **(Study II)**, it was observed that all plasma glucose was consumed within 4 days of storage at 9 ºC, after which we expected the hemolysis to increase drastically. Surprisingly, only a slight increase in hemolysis was observed after full consumption of glucose (day 4), and as much as ~80% of the RBCs were still intact after 14 days of storage **(Fig. 15)**. This indicates that other (energy-providing) molecules might also be involved in the stabilization of RBC metabolism.

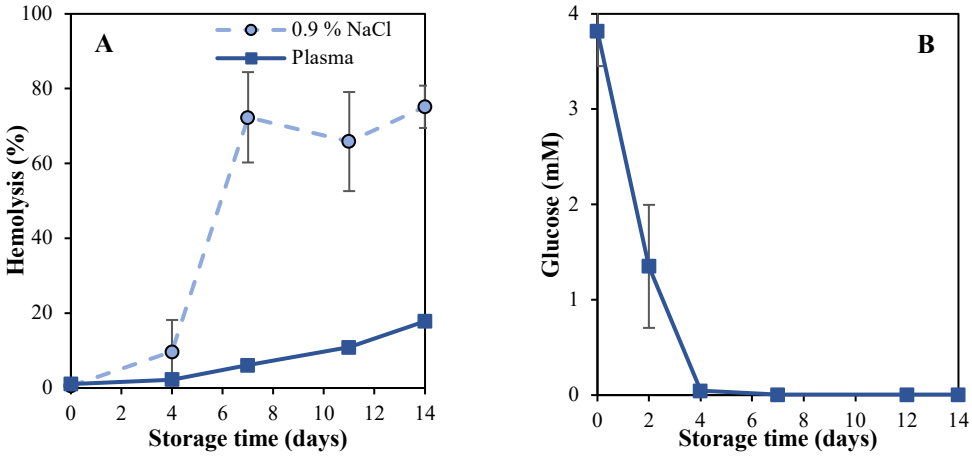

Fig. 15. Panel A: Comparison of hemolysis of wr-RBCs stored in plasma versus in saline, and panel B: glucose consumption of RBCs incubated in plasma. Both A and B are conducted at 9± 1°C, and data is visualized as mean ± SD (n=2).

Contrary to mammalian RBCs, fish RBCs contain mitochondria and are thus not only dependent on glucose as energy source (178). It was therefore of interest to understand if other molecules such as lactate and pyruvate could supplement the energy production by the RBCs, thereby preventing hemolysis **(Fig. 16) (Study II)**. However, at pH 6.8, all samples, including those with lactate (7.6 mM) and pyruvate (7.6 mM) gave rise to a rapid hemolysis that started after 5 days of storage **(Fig. 16A)**. Increasing the pH to 8.0 prolonged the RBC stability in all samples, with the lowest hemolysis rates observed in the sample with addition of glucose, followed by pyruvate and the control. The sample with lactate was least stable at pH 8.0 **(Fig. 16 B)**. It has been reported that pH 8.0 is the optimal activity for trout HK, which is also the first enzyme in the glycolysis, and phosphofructokinase (PFK) have an optimal activity pH 7.7 (110). In human blood it has been reported that the optimal activity of G6PD is at pH 8.0 (179). Thus, increased glycolysis and activity of the HMP shunt could result in increased production of electron-energy rich molecules such as NADH and NADPH. Both are important molecules for the metHb reducing enzymes in the RBC, namely NADH-cytochrome b_5 reductase and NADPH-flavin reductase (110), see **Fig. 5**. In a separate trial with trout wr-RBCs, also 30 and 100 µM ATP was tested in terms of its effect on hemolysis (**Study I**). However, no effect from ATP was found, which could be due to poor permeability through the RBC membrane (180).

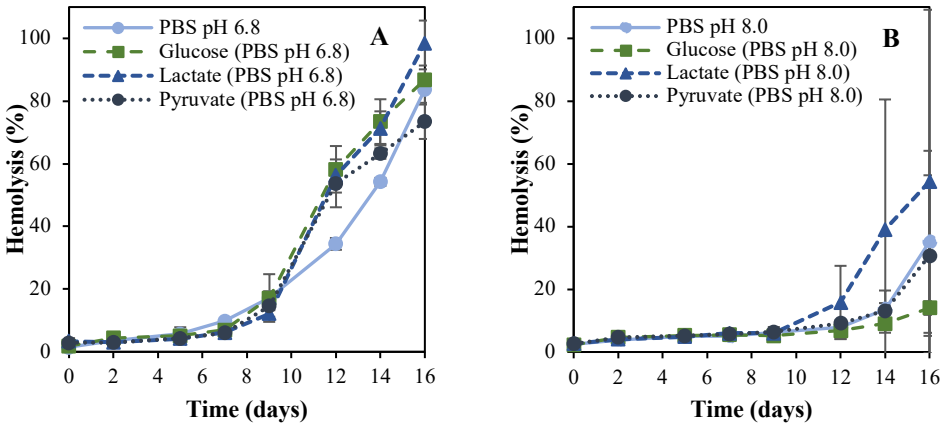

Fig. 16. Development of hemolysis in PBS solutions containing glucose (3.8 mM), lactate (7.6 mM) or pyruvate (7.6 mM). In panel A, the pH was 6.8 and in panel B, pH 8.0. Data is visualized as mean ± SD (n=2).

5.2.2 High molecular weight plasma components

In an experiment performed to understand hemolysis inducing factors under optimal storage conditions, metHb was added to wr-RBCs. The hypothesis was that extracellular metHb might cause oxidative attack on the membrane of intact RBCs, which could then weaken the membrane structure and induce hemolysis (181). A surprising finding in this trial was that trout metHb (10 g/L) increased rather than decreased T_{50}; in fact, with as much as 5 days **(Study I)**. This stabilizing effect was ascribed to COP and further exploration was therefore necessary to understand if also albumin, an important protein of blood plasma, could stabilize RBCs in a similar manner. Indeed, plasma albumin at 10 g/L provided a similar increase of T_{50} as metHb. Along the same lines, Kure and Sakai (182) encapsulated high concentrations of human serum albumins in liposomes and found that larger differences in transmembrane COP decreased the RBC membrane viscosity, which could affect RBC stability **(Fig. 17)**. Albumin, which is a negatively charged protein might also prevent water from accumulating in the RBCs, by itself attaching to the membrane and bind to sodium-ions, thus creating an osmotically active layer (183).

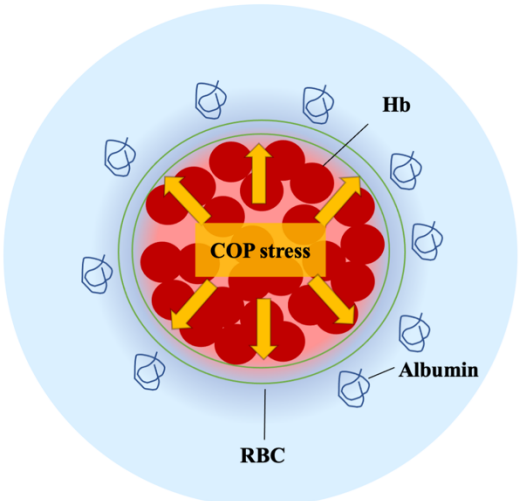

Fig. 17. Visualization of albumin attaching to the RBC membrane surface thereby stabilizing the RBC against COP stress from the high concentration of Hb inside the RBCs.

The role of COP for RBC stability was further investigated in **Study II**. To a solution consisting of PBS at pH 8.0 with 3.8 mM glucose different levels of albumin was added to reach similar or higher COP as compared to plasma. Plasma COP was measured to 1.47 kPa (11 mmHg) and correspond to 3.54 g/dL albumin, to reach an elevated COP of 4 kPa (30 mmHg), 7 g/dL albumin was added. As observed in **Fig. 18**, both concentrations of albumin resulted in significantly improved stability of the RBCs compared to the albumin-free reference, and stabilities similar to that of blood plasma were observed during 14 days of storage. It should be stressed that albumin might not only stabilize RBCs through an increased COP. Albumins also have an important role in the defense against heme, by binding free heme and limiting its damaging effects (184, 185). In addition, albumin might act as a radical scavenger and protect RBCs from oxidative damage (186).

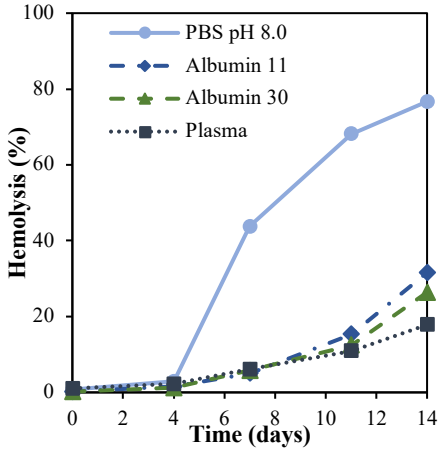

Fig. 18. Effect of COP from plasma or added albumin to reach physiological or elevated COP (11 or 30 mmHg), on hemolysis of RBCs stored at 9(±1) °C. Data is shown as mean±SD (n=2).

5.2.3 Exogenous additives used in transfusion medicine

Plasma expanders

Although it was found that albumin could stabilize the RBCs, it might be a difficult component to integrate into the fish processing-chain unless it is purified from fish plasma. Use of bovine albumin would, for example, be restrictive for pescatarians. Therefore, other potential compounds that could induce COP were investigated. In transfusion medicine, HMW-dextran and hydroxyl starch (HES), are used as a plasma replacement (187-189) and were therefore tested, although HES was replaced by dextrin, which is a similar molecule. At levels providing the COP of plasma (1.47 kPa), HMW-dextran or dextrin, did however not increase RBC stability in comparison to a reference consisting of PBS at pH 8.0 with 3.8 mM glucose. See **Paper II, Figure 7**.

SAG-M

Other additives tested to increase the RBC stability were saline-adenosine-glucose-mannitol (SAG-M) solution, which is commonly used to store RBCs of human donated blood (105). SAG-M contains nutrients; 45 mM glucose, 1.25 mM adenine and 30 mM mannitol, to provide osmotic stability, energy, and membrane stability (132). Still, when trout RBCs were incubated in SAG-M it did not provide better RBC stability compared to 0.9 % NaCl. The lack of

improvement with SAG-M might be due to its very low pH of 5.8 which e.g., may inhibit HK, PFK or G6PD in the glycolysis and HMP shunt (110, 179), which is an issue as fish RBCs in general have a higher energy requirement compared to human RBCs (133). Further pathways by which low pH may destabilize RBCs are discussed in section 5.3. It should also be mentioned that no plasma was present with the RBCs when mixed into the SAG-M, which could also influence the RBC stability.

5.3 The role of pH for hemolysis and RBC-membrane lipid oxidation

Given the profound role of pH for the pro-oxidative ability of Hb, it was of great interest to study the relation between pH, RBC membrane lipid oxidation and hemolysis. Earlier, post-mortem pH of cod and mackerel muscle was reported in the range 6.2-7.0 (15, 190) while in this thesis pH of fresh trout blood plasma was measured in the range 7.8-8.0 **(Study III)**. The pH range 6.4-8.0 was therefore selected for our studies on RBCs incubated in phosphate buffered saline. Results showed that both hemolysis and TBARS accumulation was very rapid at low pH 6.4-6.8, while higher pH strongly suppressed TBARS development **(Fig. 19) (Study II)**.

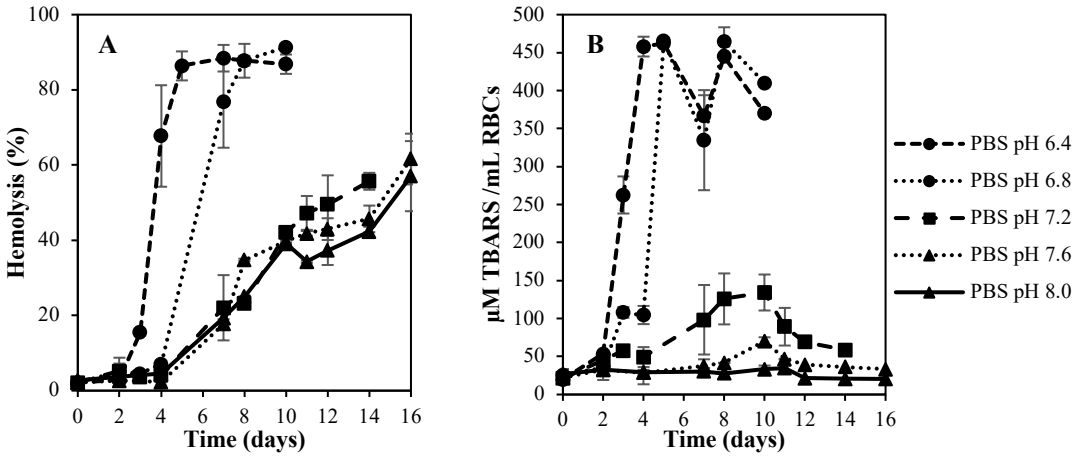

Fig. 19. Panel A show the effect of varying pH, 6.4-8.0, on hemolysis and panel B, show the effect on the development of TBARS. Samples were stored at 3±1 °C, and data is visualized as mean ± SD (n=2).

These results indicated that low pH indeed may initiate hemolysis through lipid oxidation of the RBC membrane. The oxygen affinity of fish Hbs drastically decrease even with small reductions in pH due to the Root effect, resulting in autoxidized Hb; metHb (119, 120). The 60-fold looser anchoring of the porphyrin ring to the globular part of the protein, in met-form, result in a readily released hemin-group (126). Both metHb and hemin can propagate lipid oxidation through degradation of preformed LOOH. Further, reaction of metHb with LOOH or H_2O_2 generate both ferrylHb and ferrylHb radicals, both which can initiate lipid oxidation and the former also propagating it (25). The suppressed lipid oxidation at higher pH, 7.2-8.0, could thus indicate a less pronounced hemin-loss. That hemolysis was not completely

prevented in these samples indicate that several mechanisms operate simultaneously to trigger hemolysis.

Two different oxidation mechanisms were investigated to better understand the relationship between membrane lipid oxidation and hemolysis **(Study II) (Paper II, Figure 6)**. One route was to induce lipid oxidation by addition of hemin and AA to the RBC suspension and the second route was based on addition of H_2O_2, iron and AA to stimulate the production of highly reactive $^{\bullet}OH$, through the Fenton reaction (191, 192). Surprisingly, both systems resulted in low TBARS formation, which supports the study by Yin, Lingnert (128) in which it was suggested that $^{\bullet}OH$ produced in the Fenton reaction might not be the main initiator of lipid oxidation. The reduced membrane oxidation with H_2O_2, iron and AA, could also be linked to the antioxidative effects of AA. In the experiment with hemin, lipid oxidation was almost completely suppressed, which could be explained by rapid transport of DHAA into the cell but slow release of ascorbate. Such slow release could allow for ascorbate to protect and recycle alpha tocopherol in the RBC membrane, and thereby protecting it from lipid oxidation (100). However, despite a suppressed lipid oxidation, hemin still induced hemolysis. A probable mechanism for this which has been earlier reported is that hemin can dissociate membrane skeletal proteins, thereby causing holes that can lead to hemolysis without membrane oxidation (115, 193, 194).

5.4 The contribution of hemolysis to fish muscle lipid oxidation and its dependence on pH

5.4.1 The contribution of hemolysis on WCM lipid oxidation

To investigate the effect of hemolysis for lipid oxidation in fish, different ratios of intact and lysed RBCs to a final level of 71µM Hb were added to a WCM system (pH 6.8), prior to its storage at 4 °C **(Study III) (Fig. 20)**. A control sample which had no Hb added was also included in the design and did not develop PV or TBARS over the storage period of 5 days. All samples containing lysed RBCs (25-100 % of total RBCs) resulted in rapid PV and TBARS development from day 0 and reached maximum levels after 1 day of storage. Lipid oxidation was however delayed with 1 day if RBCs were initially 100% intact. These results agree with an earlier study performed on ice at lower pH (6.3) and with lower Hb levels (5.8 µM) added as hemolysate or intact RBCs (8). The high PV/TBARS levels documented in our study are in line with the high Hb-level used (71 µM), which was chosen to simulate fish filleting rest raw materials (19, 57). Hb is however known to initiate lipid oxidation already at as low concentrations as 0.5 µM, explaining why even limited hemolysis can be detrimental for lipid oxidation. TBARS peaked at slightly lower levels when only 25% of the RBCs were lysed from start of the trial, confirming earlier observations that Hb is a reactant rather than a catalyst.

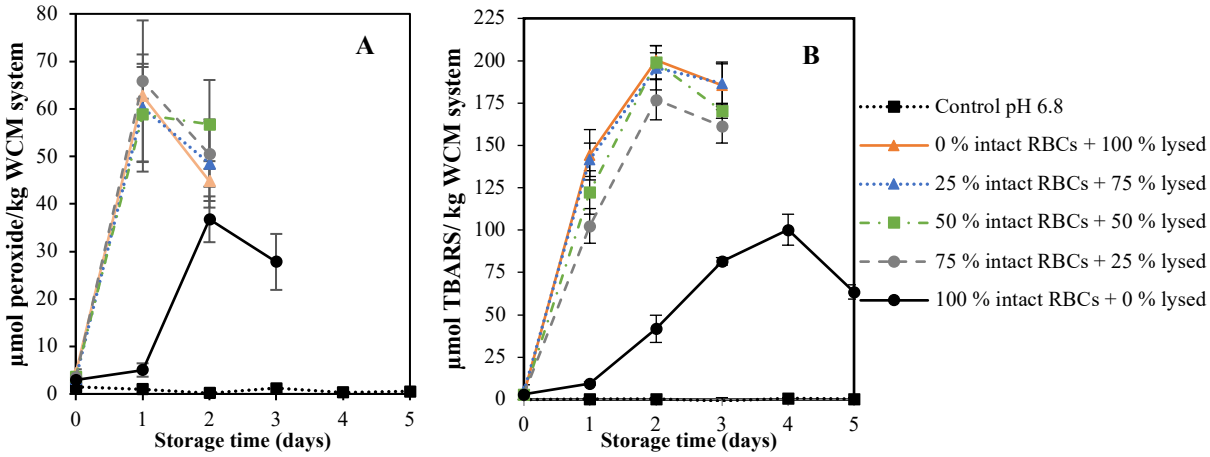

Fig. 20. Development of PV (A) and TBARS (B), with different ratios of intact and lysed RBCs in a WCM system. Samples are stored at 3±1 °C, and data is shown as mean ± SD (n=2).

One of our techniques to follow hemolysis over time in the WCM-system was to measure the concentration of Hb staying soluble in the supernatant upon centrifugation of the WCM-system. **Fig. 21** visualizes the difficulties encountered with this technique; supernatant from samples with fully lysed RBCs from start had a decreasing Hb-level over time. This decrease is hypothesized to be due to either complete degradation of the heme-group or binding of free Hb or hemin to the WCM components; the globular part of Hb interact with PLs through electrostatic forces and hemin interact through hydrophobic forces (117, 195). Due to the same phenomenon, the sample with a starting level of 50% intact and 50% lysed RBCs resulted in an almost constant line that illustrates that the net-release and net-binding/degradation of Hb or hemin is equal. For initially fully intact RBCs, Hb-levels in the supernatant were however initially low, with a rapid release observed after 3 days of storage; indicating the onset of hemolysis. That the initially added 71µM could never be recovered in the supernatant from this sample within the time frame of the trial could either be due to an incomplete hemolysis or a similar phenomenon as that described above for Hb that has been released.

Comparing **Fig. 20** and **21**, it can be observed that the limited hemolysis of the sample with 100 % intact RBCs, providing 9.8 µM Hb in the soluble phase, after 1 day of storage was enough to initiate lipid oxidation. Further, maximum PV and TBARS levels only reached 59.5 and 48.1 % of the maximum values reached for samples with 25-100% initial hemolysis. The maximum TBARS to Hb ratio in the 100% intact RBC sample (1.30 ± 0.14 µmol MDA equivalents per Hb molecule) was therefore lower than the ratio for samples with 0-75% intact RBCs; 2.72 ± 0.14 µmol MDA equivalents per Hb molecule. Across several earlier studies of Hb-mediated lipid oxidation in WCM, the ratio has been reported to be 6.7-24 µmol MDA equivalents per Hb molecule (45, 127). The lower ratio in our study was due to the very high Hb-levels used, possibly making the membrane lipid substrate limiting rather than access to Hb.

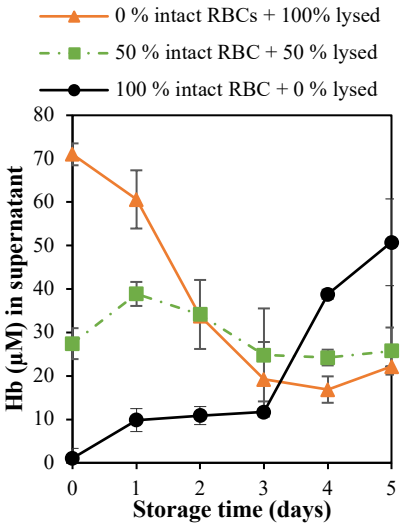

Fig. 21. Measured levels of Hb in the supernatant of WCM systems spiked with 0, 50 or 100% intact trout RBCs at a total Hb-level of 71µM.

5.4.2 The influence of pH on hemolysis and WCM lipid oxidation

Evaluating the effect of pH on hemolysis and lipid oxidation in a WCM system **(Study III)**, similar findings were obtained as the ones described in section 5.3, where a RBC-model system was used **(Study II)**. Low pH, 6.4-6.8 caused rapid hemolysis and lipid oxidation compared to pH 7.2-7.6 **(Fig. 22 A-B)**. At low pH, both PV and TBARS accumulated already after the first day of storage and reached a maximum at day 2 and day 4 respectively. Adding blood plasma to the sample with pH 6.8 delayed lipid oxidation, and accumulation of PV and TBARS was not detected until day 4 and 3, respectively. At elevated pH 7.2-7.6, lipid oxidation was suppressed during the whole storage period **(Fig. 22 A-B)**. From analyses of Hb in the supernatant of a sample after centrifugation, it was found that all samples contained low levels of Hb (7.1-17.5 µM) already after 1-day of storage and were then subjected to rapid Hb-leakage from day 3 **(Fig. 22 C)**. These data revealed that at low pH, even small amounts of Hb were

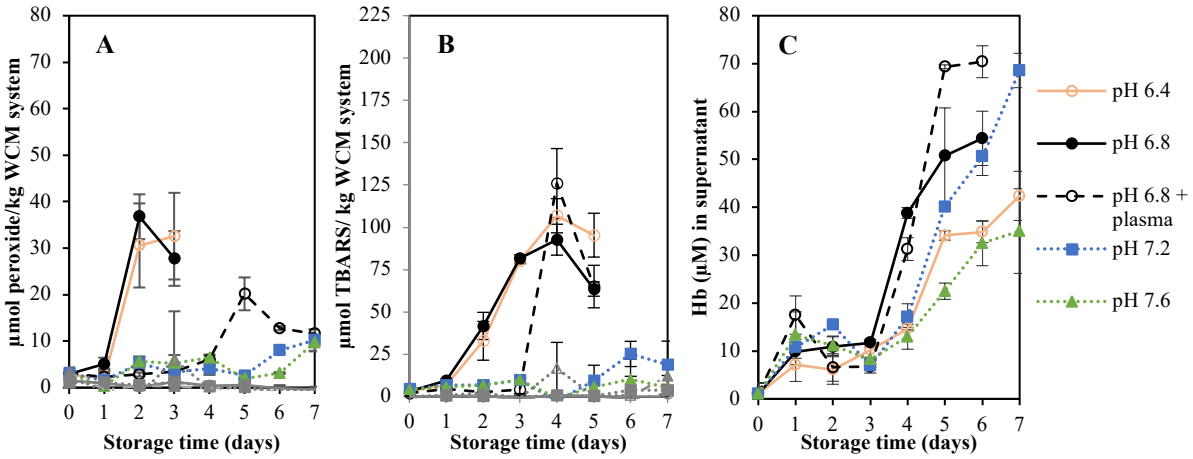

Fig. 22. The effect of varying pH, 6.4-7.6 or addition of blood plasma on the development of PV (panel A), TBARS (panel B) or on Hb-release from the RBCs (panel C). Samples were stored at 3±1 °C and data is visualized as mean ± SD (n=2).

enough to induce and propagate lipid oxidation, which was not the case in samples with plasma present or with elevated pH's.

Further to the above outlined mechanisms by which low pH can stimulate Hb-mediated oxidation, other pro-oxidative pathways stimulated by low pH are: (i) higher concentrations of $^{\bullet}OOH$ which compared to the more polar $O_2^{\bullet-}$ more easily pass into membranes (25, 196), (ii) that the H_2O_2 formed in metHb-formation can react with heme-groups according to the Fenton reaction (25, 26, 119, 120), and (iii) that HbVI can more readily interact with the negatively charged RBC membrane at pH's close to the isoelectric point (pI), causing the hemin to be readily released (117, 195). The pI of trout HbVI has been determined to 6.2-6.3 (117, 118).

The observed lower [Hb] by the end of the storage of samples at pH 6.4-6.8 can thus be explained by mechanism (iii) above, i.e., HbVI, being the most abundant Hb form in fish (118) will lose hemin from the globin via hydrophobic forces. Further, denaturation into hemichromes is more prevalent for metHb that readily release its hemin group (195). This could be another explanation to the lower detection of Hb in supernatants at reduced pH's since denatured forms more easily precipitate.

Given the obvious difficulty in following hemolysis simply via centrifugation of samples and Hb-analyses of supernatants, RBC-WCM samples at pH 6.4 and 7.6 were also subjected to H&E-staining and light microscopic analyses. As can be seen in **Fig. 23-24**, the RBC nucleus is hereby stained in blue and the cytoplasm in pink. In the pH 6.4 sample **(Fig. 23)**, the RBCs had a healthy oval disk shape, with a compact nucleus, at day 0 **(Fig. 23A)**. However, already after 1 day of storage, the cytoplasm became less colored **(Fig. 23B)** and by day 2 the cytoplasm was completely transparent **(Fig. 23C)**. At this stage, the RBCs at pH 6.4 were still intact, but the loss in cytoplasmic color could translate into the RBCs emptying its cytoplasmic content. It has earlier been reported that RBC membrane permeability is increased e.g., when Hb binds the RBC structural proteins (195). At day 3 and 4 **(Fig. 23 D-E)**, most of the RBCs were mis-shaped and had a swollen nucleus which filled the whole cell, and at the end of the storage, i.e., day 7, no RBCs with compact nucleus could be found in the sample with pH 6.4 **(Fig. 23F)**. Contrary to the rapid loss of cytoplasm color and the swelling of nucleus at low pH, the RBCs from a WCM sample at pH 7.6 resulted in better preserved cytoplasm and nucleus, until the end of the storage **(Fig. 24).** Thus, these microscopic images clearly confirm that the lower Hb-levels detected in supernatants at pH 7.6 **(Fig. 22C)** is due to less Hb release from the RBCs, while the low Hb detected in supernatants at pH 6.4 is due to that released Hb/hemin bind to the WCM structure or are subjected to porphyrin ring degradation (197). Overall, maintaining blood rich fish samples at high pH is hereby supported by several mechanisms, and should be further studied e.g., in relation to microbial growth and taste.

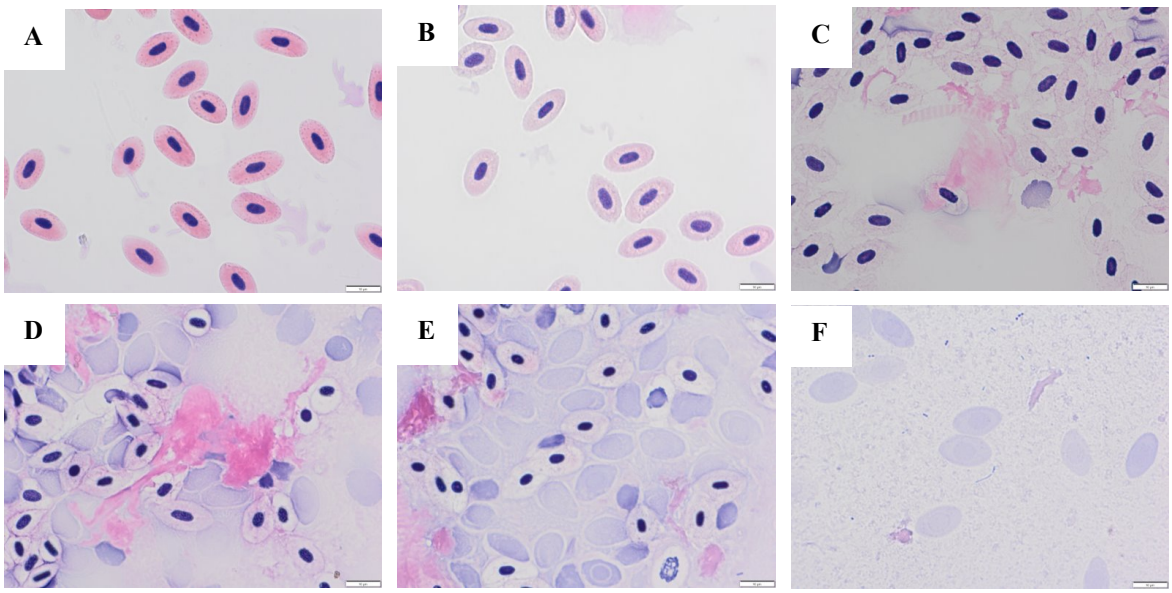

Fig. 23. Light microscopic images of RBCs from the soluble phase from the RBC-spiked WCM system at pH 6.4, following staining with H&E, day 0 (panel A), day 1 (panel B), day 2 (panel C), day 3 (panel D), day 4 (panel E), day 7 (panel F).

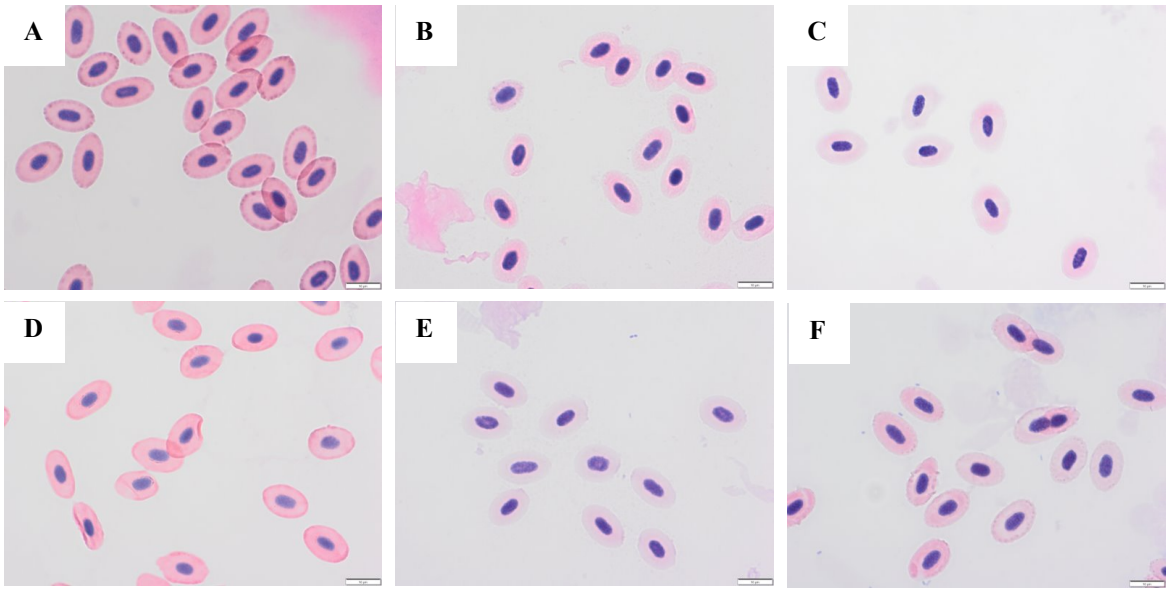

Fig. 24. Images from light microscopy of RBCs from the soluble phase of the RBC-spiked WCM system at pH 7.6, after staining with H&E, day 0 (panel A), day 1 (panel B), day 2 (panel C), day 3 (panel D), day 4 (panel E), day 7 (panel F).

5.5 Preventing hemoglobin-mediated lipid oxidation in herring byproducts

5.5.1 Pre-incubation in air-, water or physiological salt solution

As shown in the previous section, intact RBCs added at a high [Hb] (71 µM) to WCM resulted in a 1-day delay of lipid oxidation and lower maximum levels of both PV and TBARS, compared to fully or partly lysed RBCs **(Fig. 20)**. However, it was clear that once small amounts of Hb were released from the RBCs into WCM at a pH typical for post-mortem muscle (6.8), lipid oxidation developed rapidly. Thus, other strategies beyond RBC stabilization are needed to minimize the pro-oxidative power of released Hb and thereby allow valorization of Hb-rich raw materials as fish rest raw materials into ingredients **(Study IV)**.

As seen in **Tab. 1**, herring rest raw materials contain Hb-levels ranging from 39.1 to 70.9 µM, which can be compared with the herring fillet containing 5.2-14.5 µM (19) or if specified in the light and dark muscle parts; 10.7 or 28.6 µM Hb-equivalents, respectively (160). **Tab. 1**, also display the reduction in Hb which was obtained in different herring fractions after pre-incubating them in 5 volumes 0.9% NaCl for 20 min. This reduction ranged from 6.6-18.0 % of total Hb.

Tab. 1. The total amount of Hb in the different herring fractions before and after pre-incubation in 0.9% NaCl.

Treatment	Head[c]	Backbone	Caudal fin	Residuals
No pre-incubation	70.9 ± 1.1[a]	42.8 ± 0.3[a]	39.1 ± 1.8[a]	40.6 ± 1.1[a]
Pre-incubation	63.6 ± 1.5[b]	35.1 ± 0.8[b]	36.47 ± 1.3[a]	33.3 ± 0.5[b]

[a]The ratio was 5:1 for 0.9% NaCl solution/rest raw materials (volume/weight).
The process of pre-incubation was 20 minutes in 4 ° C under gentle stirring.
[b]The unit of total Hb is µmol/kg herring by-products.
[c]Data is shown as mean ± SD (n=2), mean values bearing different designations (a, b) in a column differ significantly ($P < 0.05$).

It should however be stressed that the used rest raw materials were collected after passing through a commercial filleting process. In this process, blood is removed with tap water, why some of the surface RBCs of the collected rest raw materials were most likely already defected by osmotic chock, whereupon Hb could be released. After the pre-incubation, the rest raw materials were subjected to mincing and ice storage to monitor lipid oxidation development. In this trial, also pre-incubation in tap-water was included to test the hypothesis developed from **Study I. Fig. 25** shows that both the absence of pre-incubation and pre-incubation in tap-water gave rise to significantly higher TBARS than pre-incubation in 0.9% NaCl.

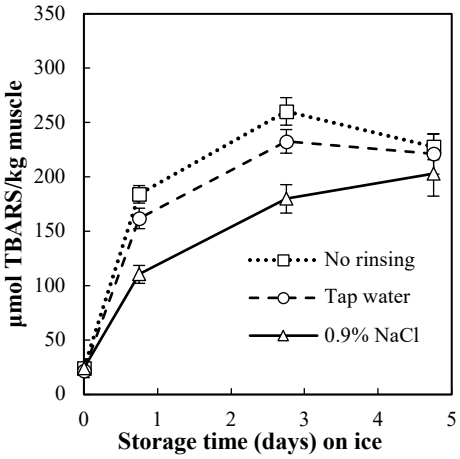

Fig. 25. The effect of pre-incubating, herring mince from rest raw materials, for 20 min in either 0% NaCl or 0.9% NaCl (1:5 byproduct to rinsing solution), prior to mincing and storage on ice. Data is shown as mean ± SD (n=2).

The small improvement from tap-water pre-incubation compared to the control is in agreement with earlier studies on mackerel fillets which showed that rinsing with 0% NaCl improved lipid stability during subsequent storage on in -20 °C compared to non-rinsed fillets cut in air (198). Our results contradict an earlier study in which 50 mM NaCl was used to wash herring fillet mince. In that study significantly reduced lipid stability was obtained during frozen storage (-18 °C) of unwashed mince compared to the washed mince (21). The contradiction may be explained by either i) that the washing concentrated the PLs of the mince (21, 199), which are more sensitive to lipid oxidation compared to TAGs (90), or ii) that the net removal of antioxidants was larger than the removal of pro-oxidants. Washing/rinsing/incubation should thus always be optimized for each specific raw material. The stabilizing effect of 0.9% could be partly ascribed to RBC-stabilization which facilitate more efficient Hb removal.

5.5.2 Pre-incubating rest raw materials in antioxidant solution prior to mincing

Since there was no major extension of the oxidation lag phase from pre-incubation in 0.9% NaCl, various antioxidant incubation solutions were developed to increase the lipid stability of the rest raw materials, as an effect of covering their surface with antioxidants **(Fig. 26)**. This strategy has earlier been used to limit lipid oxidation of mackerel fillets during frozen storage (200). A modest but significant reduction in PV and TBARS was observed the first 2.75 days of storage, when comparing pre-incubation in iso-AA plus EDTA (dissolved in either 0 or 0.9% NaCl), with the non-incubated control. Ascorbate is a well-known, water soluble, free radical scavenger and can also reduce hypervalent Hb-forms (201). EDTA on the other hand is a strong chelator of LMW-Fe. Pre-incubating in the commercial antioxidant mixture Duralox MANC significantly suppressed PV until day 7.75 and TBARS up to 12 days. Duralox-MANC is a mixture of both water and lipid soluble antioxidants. Rosemary extract which is the main component contains carnosol and carnosic acid, which are lipophilic compounds with effective radical scavenging activity (66). Duralox-MANC, also contains iso-AA which is a radical scavenger and effectively reduces LMW iron (202), citric acid and EDTA which are metal chelators, and tocopherol which is a lipophilic radical scavenger (203). Iso-AA, tocopherol, and citric acid can act in synergy with the polyphenols of rosemary extract (204). Dipping in a water-based rosemary solution has also previously been reported to delay lipid oxidation during frozen storage of mackerel (18).

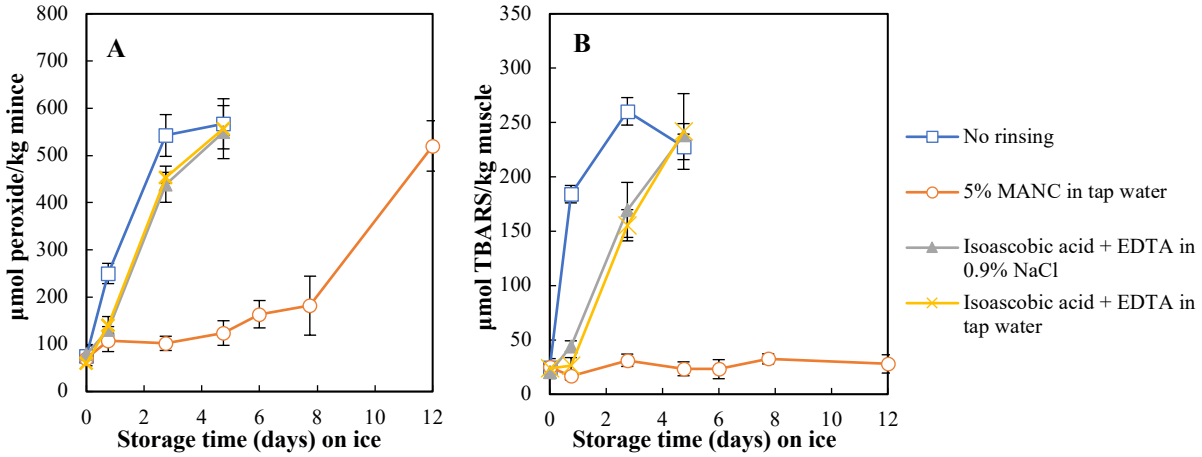

Fig. 26. The effect of antioxidant pre-incubation of herring rest raw materials. PV and TBARS are visualized in panel A and B, respectively. Samples are stored on ice and data is shown as mean ± SD (n=2).

5.5.3 Storage of intact rest raw materials in water, physiological salt, or antioxidant solution

As another strategy of stabilization, intact rest raw materials were completely submerged in 0- or 0.9% NaCl during storage at 4 °C. As control, the rest raw materials were stored in air. Rest raw materials stored in air developed significantly higher PV and TBARS values compared to submerged samples. This is line earlier studies that have shown that storage of sardines in melting ice prevent TBARS better than those stored on normal ice with exposure to air (205). Comparing storage in 0- or 0.9% NaCl provided no significant difference regarding either PV or TBARS.

Antioxidants were also added to the 0.9% NaCl to further improve the lipid stability during storage. Duralox MANC (0.5 or 1%) and iso-AA (2%) in the submerging solution both reduced PV and TBARS significantly compared to samples stored in air **(Fig. 27)**. No significant difference was however found between the two levels of Duralox MANC. To evaluate if higher pH in the submerging solution better could protect the rest raw materials, e.g., via keeping the RBCs more intact, PBS at pH 6.5 and 7.5 were compared. Surprisingly, increasing the pH from 6.5 to 7.5 did not increase lipid stability, which may be explained by that the protecting effect from reducing contact with air overruled the effect of stabilizing pro-oxidants.

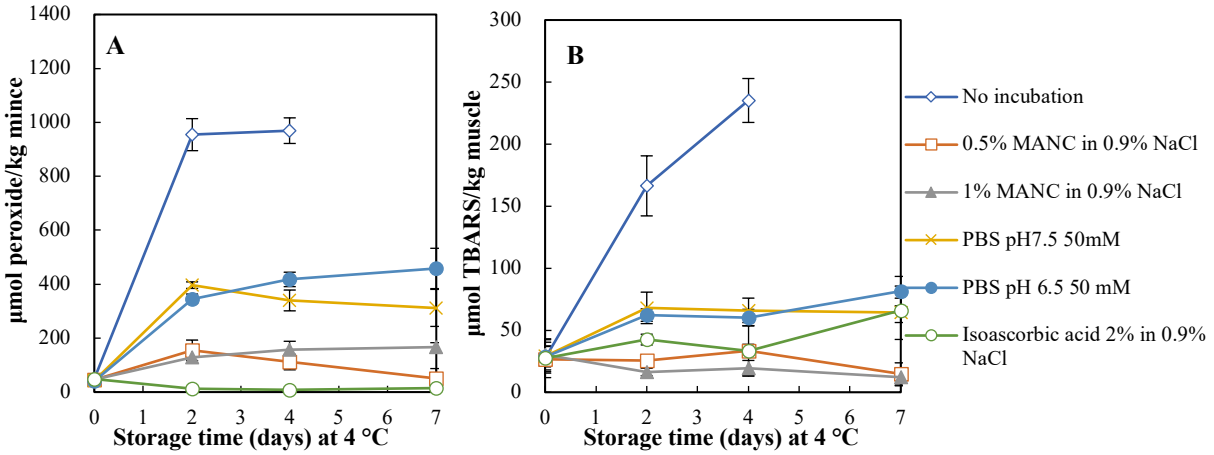

Fig. 27. The effect of storing herring rest raw materials submerged in different solutions without/with antioxidants and at different pH-values on PV (A) and TBARS (B). Samples are stored at 4 °C and data is shown as mean ± SD (n=2).

5.5.4 Direct addition of antioxidants into minced rest raw materials

Since the pre-incubation or submerging experiments did not provide direct information on the exact amounts of antioxidants being transferred over to the rest raw materials, experiments were carried out where antioxidants were directly added in controlled levels to minced herring rest raw materials. The evaluated antioxidants/antioxidant levels all provided prolonged lag phases in the order 0.50% Duralox MANC > 0.25% Duralox MANC > 0.20% iso-AA with 0.044% EDTA **(Fig. 28)**. Thus, the higher level of Duralox MANC was, just as in earlier experiments, very effective. As previously explained, the mixture of aqueous and lipophilic components in Duralox MANC, works according to different antioxidative mechanisms, and appear very powerful together in suppressing lipid oxidation in heme-rich herring rest raw materials. That iso-AA with EDTA was not so effective could reflect the low levels of LMW-metals in the system, which would otherwise be chelated by EDTA.

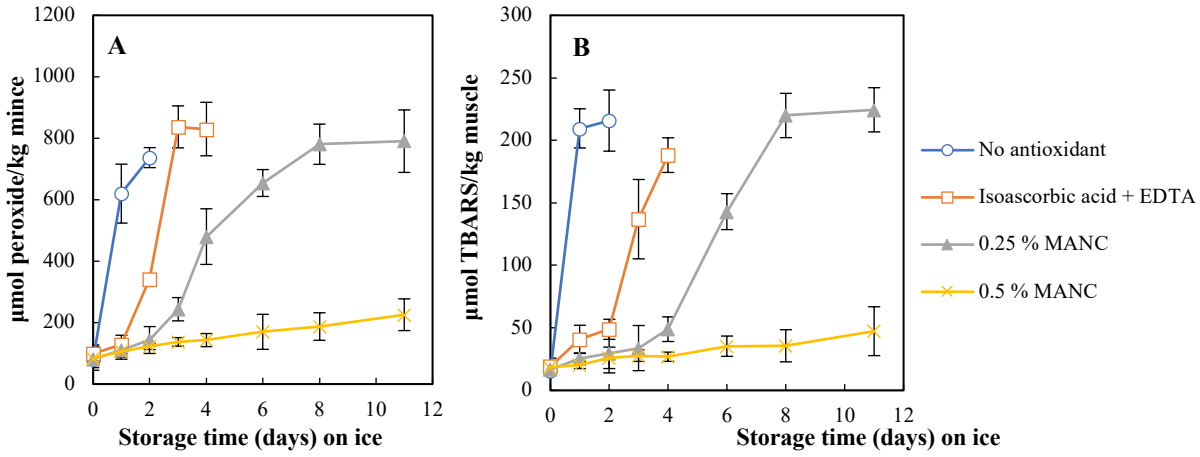

Fig. 28. Direct addition of antioxidants to minced herring rest raw materials and their effect on PV (A) and TBARS (B) during ice storage of the minces. Samples are stored at 4 °C and data is shown as mean ± SD (n=2).

6. CONCLUSIONS

The research of this thesis has generated new knowledge on measures that that can limit hemolysis and thereby delay Hb-mediated lipid oxidation, during early handling and processing of fish. All of the aims set up have been addressed and have yielded the following conclusions.

i. Several parameters linked to early handling and processing affected the RBC integrity:
   - Strong hypo- or hypertonic solutions; 0 or 3% NaCl, caused immediate hemolysis or lysis after 1 day of storage, respectively. Thus, subjecting fish to RSW (~3% salinity) or rinsing blood with tap water (0% NaCl) after filleting provides a critical environment for RBC lysis, in comparison to physiological salt concentrations. This thesis suggests that storage and rinsing solutions should rather range between 0.9-1.3 % NaCl for better conservation of the RBCs.
   - Mechanical stress stimulated hemolysis indicating that several steps e.g., crowding, pumping, and sorting the fish could compromise RBC stability, and should be re-designed, if possible.
   - Temperature fluctuations ranging between 0 and 12 °C affected the RBC stability, with temperatures > 6 °C causing significantly stronger hemolysis compared to temperatures ≤ 6 °C.
   - Low pH's (6.4 to 6.8) stimulated hemolysis while pH-values physiological to fresh blood, pH 7.6 to 8.0, reduced hemolysis and almost completely suppressed TBARS. Thus, factors affecting post-mortem acidification, such as stress, should be avoided when possible. Elevating the pH of storage solutions could be considered to reduce hemolysis but must be balanced against an increased risk for bacterial growth.

ii. Blood plasma was repeatedly the best preservation medium for RBCs. When components of plasma were tested at physiological levels, a combination of glucose, salinity albumin and pH together best simulated plasma-derived preservation of RBC. Albumin seemed to support the RBC membrane stability through COP.

iii. Exogenous additives selected with inspiration from transfusion medicine (SAG-M, HMW-dextran, and dextrin) did not prolong RBC stability.

iv. The mechanism behind rapid hemolysis during storage under optimal conditions remain unknown. However, a strong link was found between hemolysis and RBC membrane lipid oxidation.

v. Spiking a WCM model with varying ratios of intact/lysed RBCs proved the importance of maintaining RBCs intact to delay and reduce maximum levels of lipid oxidation. Less than 10 % hemolysis was needed to induce rapid lipid oxidation. In presence of blood plasma, the oxidation caused by initially intact RBCs was delayed, but not the hemolysis. Increasing pH from post-mortem to 7.2 or 7.6 suppressed lipid oxidation

during the whole 7-day storage period. It was also confirmed that hemolysis was reduced in the WCM system at pH 7.6 versus at 6.4.

vi. Different strategies to dilute or inhibit released Hb was evaluated using herring rest raw materials. Submerging intact rest raw materials in PBS pH 6.5 or 7.5 did not show significant differences in the development of lipid oxidation, most likely since submerging of samples under water *per se* limits access to air. Subjecting intact herring rest raw materials to a solution containing e.g., rosemary-derived antioxidants through pre-incubation or submerging both led to at least 7-days delay in the oxidation lag phase compared to controls. Almost complete suppression of lipid oxidation was again confirmed when the same antioxidants were added directly into minced rest raw materials.

Altogether, we foresee that a combination of RBC-stabilizing and Hb-stabilizing measures is the most powerful route to prevent lipid oxidation in fish. For each suggested strategy, careful balancing must however be made towards the cost of changed routines, and the risk of stimulating other shelf-life limiting events such as bacterial growth.

7. FUTURE PERSPECTIVES

Confirmation of results under large scale settings

All the current suggestions to improve the fish processing-routines have only been tested in lab-scale, and it would be essential to test these adjustments under large scale settings to evaluate their results in the fish industry. When it comes to temperature, this would be especially relevant to study in the handling of rest raw materials since these are often stored without temperature control under outdoor conditions. To investigate the full effect of mechanical stress and osmotic pressure on the fish RBCs, fish should be collected at each point of the process line, starting from the trawl net, after the pumping stage, after storage in RSW tanks, after sorting in the factory etc. Since coagulation of the blood and muscle stiffness (rigor mortis) might prevent blood withdrawal under these conditions, alternative blood collection strategies should be evaluated, such as more invasive techniques where the fish is cut open. Further, loss of scales from the herring during catch present additional ways via which salt could affect RBC stability also inside the *post-mortem* fish, which should be studied further.

Since new storing and rinsing solutions for fish, e.g., 0.9% NaCl or buffers with pH ≥7.2, +/- antioxidants, could bring new challenges, these should be evaluated. For example, the cooling capacity of RSW diluted to 0.9% NaCl should be tested against that of RSW with 3-3.5% NaCl, representative to most seas and oceans. Both bacterial growth and lipid oxidation could be stimulated if temperatures are not kept low enough. Also increasing the pH of various process waters might be problematic from a microbial point of view, thus microbial spoilage and oxidation should be studied together. Incorporation of antioxidants or substances to stabilize the RBCs could change the appearance or taste of the fish, therefore sensory evaluation would be important with such additives. Other antioxidants than Duralox should also be evaluated as this mixture is on the expensive side for low value materials as fish rest raw materials. To reduce costs and use of water, recycling of antioxidant solutions should finally be aimed for to ensure cost- and climate-effective production of fish products.

The stabilizing effect of blood plasma

Blood plasma has continuously shown excellent results in the preservation of RBCs. To better understand the RBC-stabilizing effect of blood plasma one route could be to fractionate it into LMW and HMW components. After identifying the fraction with the best RBC-stabilizing effect, efforts could be made to characterize and identify the main the components. If none of the fractions alone would provide an efficient RBC-stabilization, this points at synergistic effects, which are also of interest to understand.

One of the HMW plasma components which was evaluated with respect to COP was plasma albumin. However, further trials would be needed to evaluate whether COP is the main mechanism behind the stabilizing effect of plasma and pure albumin. The latter could for example also act as a radical scavenger and protect the RBC membrane from oxidative attack.

What happens to Hb under storage together with WCM?

In the current study, decreasing levels of soluble Hb were observed over time when RBCs/Hb was stored together with WCM at low pH. Identifying what happens to the Hb under such conditions could provide valuable information about Hb-mediated lipid oxidation. It would also be of interest to understand if/how plasma hinders Hb degradation or its binding to the WCM under low pH storage. Our studies revealed that relatively high levels of metHb could be present without onset of lipid oxidation as long as the pH was ≥7.2. Connected to this, further investigation of the role of pH for hemin-loss from metHb is needed.

Development of microscopic analysis to quantify RBCs in fish muscle

It was more difficult than expected to find a method to estimate hemolysis in a muscle model system. Filling this knowledge gap could be relevant not only within food science but might also have potential medical applications. It would therefore be important to expand the developed qualitative microscopic analysis, into a quantitative imaging technology.

8. ACKNOWLEDGEMENTS

This work was funded by the Swedish research council for sustainable development (FORMAS) Grant no. 2016-01181.

Thank you to all the people who has supported and believed in me throughout this journey.

Ingrid Undeland, my main supervisor, I am so grateful for your guidance and for always making time for me even during the most hectic schedule. Thank you, for giving me an opportunity to learn how to conduct research, and for introducing me to food science. I have learned a lot from our scientific discussions.

Michael Axelsson, my co-supervisor, it has been interesting to discuss science with you and thank you for always making time to helping me out with bleeding trout.

Haizhou Wu, my co-supervisor & co-vampire at the division of Food and Nutrition Science. I have enjoyed our discussions and learned a lot from you about Hb-mediated lipid oxidation.

Rikard Landberg, my examiner, thank you for your support and guidance.

Lena Ek, a true power woman, I am honored that I had you as a mentor, thank you for all the valuable advice.

Leif Bülow, thank you for making me believe in myself.

I would also like to acknowledge **Bo Ekstrand** and **Ulf Svanberg** for your constructive feedback on this thesis, and to my collaborators: **Hiromi Sakai, Takashi Matsuhira,** and **Annika Krona**, for the valuable exchange of knowledge and the novel findings that we have made together.

To the division of Food and Nutrition Science, both present and former colleagues, you have all been very important in my journey, and I hope that we will stay in touch.

Finally, thank you to my parents, siblings, partner, friends, and all my loved ones. Because of you I was able to reach my goal.

9. REFERENCES

1. Richard W. Hemoglobin. Wikipedia; 25 March 2007. p. Molecular structure.
2. McClements DJD, Eric A. Chemical deterioration of lipids: oxidative reactions. Fennema's Food Chemistry. New York: Taylor & Francis Group, LLC; 2008.
3. Yikrazuul. Heme. Wikipedia; 3 August 2010. p. Heme b.
4. Ghirmai S, Wu H, Axelsson M, Matsuhira T, Sakai H, Undeland I. Exploring how plasma- and muscle-related parameters affect trout hemolysis as a route to prevent hemoglobin-mediated lipid oxidation of fish muscle. Sci Rep. 2022;12(1):13446.
5. FAO. The State of World Fisheries and Aquaculture 2022. Toward Blue Transformation. Rome: FAO; 2022.
6. Undeland I, Lingnert H. Lipid oxidation in fillets of herring (Clupea harengus) during frozen storage. Influence of prefreezing storage. J Agric Food Chem. 1999;47(5):2075-81.
7. Undeland I, Kristinsson HG, Hultin HO. Hemoglobin-mediated oxidation of washed minced cod muscle phospholipids: Effect of pH and hemoglobin source. Journal of Agricultural and Food Chemistry. 2004;52(14):4444-51.
8. Richards MP, Hultin HO. Contributions of blood and blood components to lipid oxidation in fish muscle. J Agric Food Chem. 2002;50(3):555-64.
9. Larsson KJ, Undeland IK. Effect of caffeic acid on haemoglobin-mediated lipid and protein oxidation in washed cod mince during ice and frozen storage. J Sci Food Agr. 2010;90(14):2531-40.
10. Maqsood S, Benjakul S. Effect of bleeding on lipid oxidation and quality changes of Asian seabass (Lates calcarifer) muscle during iced storage. Food Chem. 2011;124(2):459-67.
11. Cavonius LR, Undeland I. Glazing herring (Clupea harengus) fillets with herring muscle press juice: effect on lipid oxidation development during frozen storage. Int J Food Sci Tech. 2017;52(5):1229-37.
12. Larsson K, Almgren A, Undeland I. Hemoglobin-mediated lipid oxidation and compositional characteristics of washed fish mince model systems made from cod (Gadus morhua), herring (Clupea harengus), and salmon (Salmo salar) muscle. J Agric Food Chem. 2007;55(22):9027-35.
13. Li R, Richards MP, Undeland I. Characterization of aqueous components in chicken breast muscle as inhibitors of hemoglobin-mediated lipid oxidation. J Agric Food Chem. 2005;53(3):767-75.
14. O'Brien PJ, Shen H, McCutcheon LJ, O'Grady M, Byrne PJ, Ferguson HW, et al. Rapid, simple and sensitive microassay for skeletal and cardiac muscle myoglobin and hemoglobin: use in various animals indicates functional role of myohemoproteins. Molecular and Cellular Biochemistry. 1992;112(1):45-52.
15. Huss HH. Quality and quality changes in fresh fish. Rome, Italy: FAO; 1995.
16. Wu H, Richards MP, Undeland I. Lipid oxidation and antioxidant delivery systems in muscle food. Compr Rev Food Sci F. 2022;21(2):1275-99.
17. Babakhani A, Farvin KHS, Jacobsen C. Antioxidative Effect of Seaweed Extracts in Chilled Storage of Minced Atlantic Mackerel (Scomber scombrus): Effect on Lipid and Protein Oxidation. Food and Bioprocess Technology. 2016;9(2):352-64.
18. Vareltzis K, Koufidis D, Gavriilidou E, Papavergou E, Vasiliadou S. Effectiveness of a natural Rosemary (Rosmarinus officinalis) extract on the stability of filleted and minced fish during frozen storage. Zeitschrift für Lebensmitteluntersuchung und -Forschung A. 1997;205(2):93-6.
19. Wu H, Forghani B, Abdollahi M, Undeland I. Lipid oxidation in sorted herring (Clupea harengus) filleting co-products from two seasons and its relationship to composition. Food Chem. 2022;373:131523.

20. Lin TM, Park JW. Extraction of Proteins from Pacific Whiting Mince at Various Washing Conditions. J Food Sci. 1996;61(2):432-8.
21. Undeland I, Ekstrand B, Lingnert H. Lipid oxidation in minced herring (Clupea harengus) during frozen storage. Effect of washing and precooking. Journal of Agricultural and Food Chemistry. 1998b;46(6):2319-28.
22. Wu Y-J, Atallah MT, Hultin HO. The proteins of washed, minced fish muscle have significant solubility in water. J Food Biochem. 1991;15(3):209-18.
23. Halim NRA, Yusof HM, Sarbon NM. Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. Trends in Food Science & Technology. 2016;51:24-33.
24. Aranda R, Cai H, Worley CE, Levin EJ, Li R, Olson JS, et al. Structural analysis of fish versus mammalian hemoglobins: Effect of the heme pocket environment on autooxidation and hemin loss. Proteins. 2009;75(1):217-30.
25. Richards MP. Heme proteins and oxidation in fresh and processed meats. Oxidation in Foods and Beverages and Antioxidant Applications, Volume 1 - Understanding Mechanisms of Oxidation and Antioxidant Activity: Woodhead Publishing; 2010.
26. Carlsen CU, Moller JKS, Skibsted LH. Heme-iron in lipid oxidation. Coordin Chem Rev. 2005;249(3-4):485-98.
27. Maqsood S, Benjakul S. Effect of Kiam (Cotylelobium lanceolatum Craib) Wood Extract on the Haemoglobin-Mediated Lipid Oxidation of Washed Asian Sea Bass Mince. Food and Bioprocess Technology. 2013;6(1):61-72.
28. Sánchez-Alonso I, Borderías J, Larsson K, Undeland I. Inhibition of Hemoglobin-Mediated Oxidation of Regular and Lipid-Fortified Washed Cod Mince by a White Grape Dietary Fiber. Journal of Agricultural and Food Chemistry. 2007;55(13):5299-305.
29. Wang T, Jónsdóttir R, Kristinsson HG, Thorkelsson G, Jacobsen C, Hamaguchi PY, et al. Inhibition of haemoglobin-mediated lipid oxidation in washed cod muscle and cod protein isolates by Fucus vesiculosus extract and fractions. Food Chem. 2010;123(2):321-30.
30. Li H, Zhou X, Gao P, Li Q, Li H, Huang R, et al. Inhibition of lipid oxidation in foods and feeds and hydroxyl radical-treated fish erythrocytes: A comparative study of Ginkgo biloba leaves extracts and synthetic antioxidants. Animal Nutrition. 2016;2(3):234-41.
31. Lu H, Luo Y, Feng L. Effects of Hydrolysates from Silver Carp (Hypophthalmichthys molitrix) Scales on Rancidity Stability and Gel Properties of Fish Products. Food and Bioprocess Technology. 2014;7(8):2178-88.
32. Miranda JM, Trigo M, Barros-Velazquez J, Aubourg SP. Quality Enhancement of Chilled Lean Fish by Previous Active Dipping in Bifurcaria bifurcata Alga Extract. Food and Bioprocess Technology. 2018;11(9):1662-73.
33. Perez DM, Tatiyaborworntham N, Sifri M, Richards MP. Hemolysis, tocopherol, and lipid oxidation in erythrocytes and muscle tissue in chickens, ducks, and turkeys. Poultry Science. 2019;98(1):456-63.
34. MSC. Purse seine: MSC; 2022 [Available from: https://www.msc.org/what-we-are-doing/our-approach/fishing-methods-and-gear-types/purse-seine.
35. SPF. oM FISKET: spf; 2022 [Available from: http://www.pelagic.se/fisket/.
36. Olsen SH, Sorensen NK, Stormo SK, Elvevoll EO. Effect of slaughter methods on blood spotting and residual blood in fillets of Atlantic salmon (Salmo salar). Aquaculture. 2006;258(1-4):462-9.
37. Thorarensen H, Gallaugher PE, Kiessling AK, Farrell AP. Intestinal blood flow in swiming chinook salmon Oncorhynchus tshawytscha and the effects of haematocrit on blood flow distribution. J Exp Biol. 1993;179(1):115-29.
38. Goodrick B. Postharvest Quality of Tuna Meat, a Question of Technique. Food Aust. 1987;39(7):343-5.

39. de Leitão Tomás MB, Jane; Gräns, Albin. Assessment of fish welfare in trawl cought Atlantic Herring. 2022.
40. Borderias AJ, Sanchez-Alonso I. First Processing Steps and the Quality of Wild and Farmed Fish. J Food Sci. 2011;76(1):R1-R5.
41. Shewan JM. (b) Some Bacteriological Aspects of Handling, Processing and Distribution of Fish, by J. M. SHEWAN, B.SC., PH.D., A.R.I.C., Principal Scientific Officer, Department of Scientific and Industrial Research, Torry Research Station. Journal of the Royal Sanitary Institute. 1949;69(4):394-420.
42. Lauzon lHM, Björn; Sveinsdóttir, Kolbrún; Guðjónsdóttir, María; Karlsdóttir, G. Magnea; Martinsdóttir, Emilía. Overview on fish quality research. Impact of fish handling, processing, storage and logistics on fish quality deterioration. Island: Skýrsla Matís Icelandic Food and Biotech R&D; 2010. Contract No.: ISSN 1670-7192.
43. Roach SWH, J.S.M.; , Tarr HLA. Storage and transport of fish in refridgerated sea water. Ottawa, Canada: Fisheries Research Board of Canada; 1961.
44. Barker R, D.R. Idler. Transport and storage of fish in refridgerated sea water. IV. Preliminary report on nitrogen loss, weight changes, and proteolysis (bellyburn). Fisheries Research Board of Canada; 1955.
45. Harrysson H, Swolin B, Axelsson M, Undeland I. A trout (Oncorhynchus mykiss) perfusion model approach to elucidate the role of blood removal for lipid oxidation and colour changes in ice-stored fish muscle. International Journal of Food Science & Technology. 2020;55(6):2462-71.
46. Osman A, Gringer N, Svendsen T, Yuan L, Hosseini SV, Baron CP, et al. Quantification of biomolecules in herring (Clupea harengus) industry processing waters and their recovery using electroflocculation and ultrafiltration. Food and Bioproducts Processing. 2015;96:198-210.
47. Ruis MAW, Bayne CJ. Effects of acute stress on blood clotting and yeast killing by phagocytes of rainbow trout. J Aquat Anim Health. 1997;9(3):190-5.
48. Smith LS. Blood Volumes of Three Salmonids. J Fish Res Board Can. 1966;23(9):1439-46.
49. Farrell AP. The circulatory system: an introduction. Circulation. University of Britich Columbia, Vancouver, BC, Canada: Elsevier Inc.; 2011. p. 973-6.
50. Matís. The importance of good onboard handling of fish. Matís; 2017.
51. Tretsven WI, Patten BG. Effect of Arterial Incisions on the Amount of Bleeding and Flesh Quality of Rainbow-Trout. Mar Fish Rev. 1981;43(4):16-8.
52. Olsen SH, Joensen S, Tobiassen T, Heia K, Akse L, Nilsen H. Quality consequences of bleeding fish after capture. Fish Res. 2014;153:103-7.
53. European Food Safety A. Species-specific welfare aspects of the main systems of stunning and killing of farmed fish: Rainbow Trout. EFSA Journal. 2009;7(4):1012.
54. Norway SF. Processing: SeaFood From Norway; 2022 [Available from: https://herring.fromnorway.com/value-chain/processing/.
55. Bianchi M, Hallstrom E, Parker RWR, Mifflin K, Tyedmers P, Ziegler F. Assessing seafood nutritional diversity together with climate impacts informs more comprehensive dietary advice. Commun Earth Environ. 2022;3(1).
56. Hallström E, Bergman K, Mifflin K, Parker R, Tyedmers P, Troell M, et al. Combined climate and nutritional performance of seafoods. Journal of Cleaner Production. 2019;230:402-11.
57. Wu H, Ghirmai S, Undeland I. Stabilization of herring (Clupea harengus) by-products against lipid oxidation by rinsing and incubation with antioxidant solutions. Food Chem. 2020;316:126337.

58. Kanner J. Oxidative Processes in Meat and Meat-Products - Quality Implications. Meat Sci. 1994;36(1-2):169-89.
59. Abdollahi M, Marmon S, Chaijan M, Undeland I. Tuning the pH-shift protein-isolation method for maximum hemoglobin-removal from blood rich fish muscle. Food Chem. 2016;212:213-24.
60. Sundblad E-LH, Sara; Uusitalo, Laura; Svedäng, Henrik. Svensk konsumtion av sjömat och dess påverkan på haven kring Sverige. Havsmiljöinstitutet; 2020.
61. Ericson J. Det yrkesmässiga fisket i havet, september 2022. Havs- och vattenmyndigheten; 2022.
62. Sajib M. Valorization of herring filleting co-products to silage : control of protein hydrolysis and lipid oxidation during ensilaging and possibilities for separating herring silage into multiple products: Chalmers University of Technology; 2021.
63. WaSeaBi. Optimal utilization of seafood side-streams through the design of new holistic process lines: WaSeaBi; 2019 [Available from: https://www.waseabi.eu.
64. Wu H, Forghani B, Abdollahi M, Undeland I. Five cuts from herring (Clupea harengus): comparison of nutritional and chemical composition between co-product fractions and fillets. Food Chemistry: X. 2022:100488.
65. Hemker AK, Nguyen LT, Karwe M, Salvi D. Effects of pressure-assisted enzymatic hydrolysis on functional and bioactive properties of tilapia (Oreochroniis niloticus) by-product protein hydrolysates. Lwt-Food Sci Technol. 2020;122.
66. Wu HZ, Abdollahi M, Undeland I. Effect of recovery technique, antioxidant addition and compositional features on lipid oxidation in protein enriched products from cod- salmon and herring backbones. Food Chem. 2021;360.
67. Rustad T, Storro I, Slizyte R. Possibilities for the utilisation of marine by-products. Int J Food Sci Tech. 2011;46(10):2001-14.
68. Wu H, Forghani B, Sajib M, Undeland I. A Recyclable Dipping Strategy to Stabilize Herring (Clupea harengus) Co-products During Ice Storage. Food and Bioprocess Technology. 2021;14(12):2207-18.
69. Luther PK, Munro PMG, Squire JM. Muscle ultrastructure in the teleost fish. Micron. 1995;26(5):431-59.
70. Shewfelt RL. Fish Muscle Lipolysis - a Review. J Food Biochem. 1981;5(2):79-100.
71. Bosund I, Ganrot B. Lipid Hydrolysis in Frozen Baltic Herring. J Food Sci. 1969;34(1):13-&.
72. Olson KB. Design and physiology of arteries and veins. Circulation. Indiana University School of Medicine - South Bend, IN, USA: Elsevier Inc.; 2011. p. 1085-94.
73. Ramel G. Fish circulatory system 101: how the heart and blood work 2020 [Available from: https://earthlife.net/fish/blood-2.
74. Coultate T. Food the chemistry of its components. 5th ed. Cambridge, UK: RSC Publishing; 2009.
75. Erikson U, Sigholt T, Rustad T, Einarsdottir IE, Jørgensen L. Contribution of Bleeding to Total Handling Stress During Slaughter of Atlantic Salmon. Aquaculture International. 1999;7(2):101-15.
76. Franzini-Armstrong C, Peachey LD. Striated muscle-contractile and control mechanisms. J Cell Biol. 1981;91(3 Pt 2):166s-86s.
77. Esaiassen M, Dahl R, Eilertsen G, Gundersen B, Sivertsvik M. Pre-rigor filleting and brining of farmed cod: Influence on quality and storage stability. Lwt-Food Sci Technol. 2008;41(4):724-9.
78. Einen O, Guerin T, Fjaera SO, Skjervold PO. Freezing of pre-rigor fillets of Atlantic salmon. Aquaculture. 2002;212(1-4):129-40.

79. Lunestad BT, Rosnes JT. 16 - Microbiological quality and safety of farmed fish. In: Lie Ø, editor. Improving Farmed Fish Quality and Safety: Woodhead Publishing; 2008. p. 399-427.
80. Aoki T, Yamashita T, Ueno R. Distribution of cathepsins in red and white muscles among fish species. Fisheries science. 2000;66(4):776-82.
81. Aoki H, Ahsan N, Matsuo K, Hagiwara T, Watabe S. Purification and characterization of collagenolytic proteases from the hepatopancreas of Northern shrimp (Pandalus eous). Journal of Agricultural and Food Chemistry. 2003;51(3):777-83.
82. Sriket C, Benjakul S, Visessanguan W. Post-mortem changes of muscle from fresh water prawn (Macrobrachium rosenbergii) as influenced by spawning stages. Lwt-Food Sci Technol. 2010;43(4):608-16.
83. Cao MJ, Osatomi K, Suzuki M, Hara K, Tachibana K, Ishihara T. Purification and characterization of two anionic trypsins from the hepatopancreas of carp. Fisheries Science. 2000;66(6):1172-9.
84. Fernandes P. Enzymes in Fish and Seafood Processing. Frontiers in Bioengineering and Biotechnology. 2016;4.
85. Mackie IM. The Effects of Freezing on Flesh Proteins. Food Rev Int. 1993;9(4):575-610.
86. Labuza TP, Dugan LR. Kinetics of lipid oxidation in foods. C R C Critical Reviews in Food Technology. 1971;2(3):355-405.
87. Miyashita K, Takagi T. Study on the oxidative rate and prooxidant activity of free fatty acids. Journal of the American Oil Chemists' Society. 1986;63(10):1380-4.
88. Castell CH, Moore BA, Jangaard PM, Neal WE. Oxidative Rancidity in Frozen Stored Cod Fillets. J Fish Res Board Can. 1966;23(9):1385-&.
89. Tatiyaborworntham N, Richards MP. Mechanisms involved in hemoglobin-mediated oxidation of lipids in washed fish muscle and inhibitory effects of phospholipase A2. Journal of the Science of Food and Agriculture. 2018;98(7):2816-23.
90. Srinivasan DK, L. Parkin. Fennema's Food Chemistry. 5 ed: CRC Press; 2017.
91. Undeland I, Ekstrand B, Lingnert H. Lipid oxidation in herring (Clupea harengus) light muscle, dark muscle, and skin, stored separately or as intact fillets. J Am Oil Chem Soc. 1998;75(5):581-90.
92. Undeland I, Lingnert H. Lipid oxidation in fillets of herring (Clupea harengus) during frozen storage. Influence of prefreezing storage. Journal of Agricultural and Food Chemistry. 1999;47(5):2075-81.
93. German JB, Chen SE, Kinsella JE. Lipid oxidation in fish tissue. Enzymic initiation via lipoxygenase. Journal of Agricultural and Food Chemistry. 1985;33(4):680-3.
94. Baptista MS, Cadet J, Di Mascio P, Ghogare AA, Greer A, Hamblin MR, et al. Type I and Type II Photosensitized Oxidation Reactions: Guidelines and Mechanistic Pathways. Photochemistry and Photobiology. 2017;93(4):912-9.
95. Wang CL, Liu LP, Han LH, Su Y, Qin WB, Gao LJ, et al. The effect of plasma components on hemoglobin release test in red blood cells (RBC). Toxicol Environ Chem. 2017;99(3):448-59.
96. Schaller J, Gerber S, Kaempfer U, Lejon S, Trachsel C. Human blood plasma proteins: structure and function: John Wiley & Sons; 2008.
97. Cooper C. Blood - A very short introduction. Oxford: Oxford University Press; 2016.
98. Mozzarelli A, Bettati S. Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood: Wiley; 2011.
99. Buettner GR. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. Arch Biochem Biophys. 1993;300(2):535-43.

100. May JM. Ascorbate function and metabolism in the human erythrocyte. Front Biosci. 1998;3:d1-10.
101. Rose RC, Bode AM. Biology of free radical scavengers: an evaluation of ascorbate. Faseb j. 1993;7(12):1135-42.
102. Basu S, Som S, Deb S, Mukherjee D, Chatterjee IB. Dehydroascorbic acid reduction in human erythrocytes. Biochem Biophys Res Commun. 1979;90(4):1335-40.
103. May JM, Qu Z-c, Mendiratta S. Protection and Recycling of α-Tocopherol in Human Erythrocytes by Intracellular Ascorbic Acid. Archives of Biochemistry and Biophysics. 1998;349(2):281-9.
104. Fänge R. Fish blood cells. Fish physiology, the cardiovascular system: Elsevier; 1992. p. 1-54.
105. Scott KL, Lecak J, Acker JP. Biopreservation of red blood cells: Past, present, and future. Transfus Med Rev. 2005;19(2):127-42.
106. Witeska M. Erythrocytes in teleost fishes: a review. Zoology and Ecology. 2013;23(4):275-81.
107. Nikinmaa M, Tiihonen K. Substrate Transport and Utilization in Fish Erythrocytes. Acta Physiol Scand. 1994;152(2):183-9.
108. Pesquero J, Albi JL, Gallardo MA, Planas J, Sanchez J. Glucose-Metabolism by Trout (Salmo-Trutta) Red-Blood-Cells. J Comp Physiol B. 1992;162(5):448-54.
109. Berg JM, Tymoczko, John L., Gatto Jr, Gregory J., Stryer, Lubert, editor. Biochemistry. 9th ed: W.H. Freeman and Company; 2019.
110. Saleh MC, McConkey S. NADH-dependent cytochrome b5 reductase and NADPH methemoglobin reductase activity in the erythrocytes of Oncorhynchus mykiss. Fish Physiology and Biochemistry. 2012;38(6):1807-13.
111. Tiihonen K, Nikinmaa M. Substrate Utilization by Carp (Cyprinus-Carpio) Erythrocytes. J Exp Biol. 1991;161:509-14.
112. Singh S, Ponnappan N, Verma A, Mittal A. Osmotic tolerance of avian erythrocytes to complete hemolysis in solute free water. Scientific Reports. 2019;9(1):7976.
113. Lewis JH, Ferguson EE. Osmotic fragility of premammalian erythrocytes. Comp Biochem Physiol. 1966;18(3):589-95.
114. Ezell GH, Sulya LL, Dodgen CL. Osmotic Fragility of Some Fish Erythrocytes in Hyptotonic Saline. Comp Biochem Physiol. 1969;28(1):409-&.
115. Jacob HS, Lux SEt. Degradation of membrane phospholipids and thiols in peroxide hemolysis: studies in vitamin E deficiency. Blood. 1968;32(4):549-68.
116. Maqsood S, Benjakul S, Kamal-Eldin A. Haemoglobin-mediated lipid oxidation in the fish muscle: A review. Trends in Food Science & Technology. 2012;28(1):33-43.
117. Sannaveerappa T, Cai H, Richards MP, Undeland I. Factors affecting the binding of trout HbI and HbIV to washed cod mince model system and their influence on lipid oxidation. Food Chem. 2014;143:392-7.
118. de Souza PC, Bonilla-Rodriguez GO. Fish hemoglobins. Braz J Med Biol Res. 2007;40(6):769-78.
119. Berenbrink M. Transport and exchange of respiratory gases in the blood. | Root Effect: Molecular Basis, Evolution of the Root Effect and Rete Systems. In: Farrell AP, editor. Encyclopedia of Fish Physiology. San Diego: Academic Press; 2011. p. 935-43.
120. Tsuruga MM, A.; Hachimori, A.; Sugawara, Y.; Shikama, K. The molecular mechanism of autoxidation for human oxyhemoglobin - Tilting of the distal histidine causes nonequivalent oxidation in the beta chain. J Biol Chem. 1998;273(15):8607-15.
121. Undeland I, Kristinsson HG, Hultin HO. Hemoglobin-Mediated Oxidation of Washed Minced Cod Muscle Phospholipids: Effect of pH and Hemoglobin Source. Journal of Agricultural and Food Chemistry. 2004;52(14):4444-51.

122. Richards MP, Hultin HO. Effects of added hemolysate from mackerel, herring and rainbow trout on lipid oxidation of washed cod muscle. Fisheries Science. 2003;69(6):1298-300.
123. Richards MP, Aranda R, He C, Phillips GN. Effect of pH on Structural Changes in Perch Hemoglobin that Can Alter Redox Stability and Heme Affinity. Journal of Aquatic Food Product Technology. 2009;18(4):416-23.
124. Weiss SJ. Neutrophil-mediated methemoglobin formation in the erythrocyte. The role of superoxide and hydrogen peroxide. J Biol Chem. 1982;257(6):2947-53.
125. Hrycay EG, Bandiera SM. Chapter Two - Involvement of Cytochrome P450 in Reactive Oxygen Species Formation and Cancer. In: Hardwick JP, editor. Advances in Pharmacology. 74: Academic Press; 2015. p. 35-84.
126. Tang QK, W. A.; Olson, J. S.; Bocian, D. F. Disruption of the heme iron-proximal histidine bond requires unfolding of deoxymyoglobin. Biochemistry-Us. 1998;37(19):7047-56.
127. Undeland I, Hultin HO, Richards MP. Added Triacylglycerols Do Not Hasten Hemoglobin-Mediated Lipid Oxidation in Washed Minced Cod Muscle. Journal of Agricultural and Food Chemistry. 2002;50(23):6847-53.
128. Yin D, Lingnert H, Ekstrand B, Brunk UT. Fenton reagents may not initiate lipid peroxidation in an emulsified linoleic acid model system. Free Radic Biol Med. 1992;13(5):543-56.
129. Srinivasan S, Hultin HO. HYDROXYL RADICAL MODIFICATION OF FISH MUSCLE PROTEINS. J Food Biochem. 1994;18(6):405-25.
130. Hogman CF. Preparation and preservation of red cells. Vox Sang. 1998;74 Suppl 2:177-87.
131. D'Alessandro A, Zolla L. Biochemistry of red cell aging in vivo and storage lesions. Haematologica. 2013;7:389-96.
132. Lagerberg JW, Korsten H, Van Der Meer PF, De Korte D. Prevention of red cell storage lesion: a comparison of five different additive solutions. Blood Transfus. 2017;15(5):456-62.
133. Hess JR, Greenwalt TG. Storage of red blood cells: New approaches. Transfus Med Rev. 2002;16(4):283-95.
134. Estep TN, Pedersen RA, Miller TJ, Stupar KR. Characterization of Erythrocyte Quality During the Refrigerated Storage of Whole Blood Containing Di-(2-Ethylhexyl) Phthalate. Blood. 1984;64(6):1270-6.
135. Tournaire C, Croux S, Maurette M-T, Beck I, Hocquaux M, Braun AM, et al. Antioxidant activity of flavonoids: Efficiency of singlet oxygen (1Δg) quenching. Journal of Photochemistry and Photobiology B: Biology. 1993;19(3):205-15.
136. Jiang J, Xiong YL. Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review. Meat Sci. 2016;120:107-17.
137. Farahi A, Kasiri M, Sudagar M, Soleimani Iraei M, Zorriehzahra S. Effect of dietary supplementation of Melissa officinalis and Aloe vera on hematological traits, lipid oxidation of carcass and performance in rainbow trout (Oncorhynchus mykiss). Online journal of animal and feed research. 2012;1:1-5.
138. Baptista RC, Horita CN, Sant'Ana AS. Natural products with preservative properties for enhancing the microbiological safety and extending the shelf-life of seafood: A review. Food Res Int. 2020;127.
139. Kupchak D, Lyubimova O. The study of the rheological characteristics of food systems combined minced. Science and Innovation in the Modern World: Engineering and Technology. 2017;3:102-10.

140. Gomez-Estaca J, Lopez-de-Dicastillo C, Hernandez-Munoz P, Catala R, Gavara R. Advances in antioxidant active food packaging. Trends in Food Science & Technology. 2014;35(1):42-51.
141. Taheri A. Antioxidative Effect of Rainbow Sardine (Dussumieria acuta) Protein Hydrolysate on Lipid and Protein Oxidation in Black Pomfret (Parastromateus niger) Fillet by Glazing. Journal of Aquatic Food Product Technology. 2015;24(3):241-58.
142. Sveinsdottir HI, Karlsdottir MG, Arason S, Stefansson G, Sone I, Skara T, et al. Effect of antioxidants on the sensory quality and physicochemical stability of Atlantic mackerel (Scomber scombrus) fillets during frozen storage. Food Chem. 2020;321.
143. Bosse R, Wirth M, Weiss J, Gibis M. Effect of storage temperature on volatile marker compounds in cured loins fermented with Staphylococcus carnosus by brine injection. European Food Research and Technology. 2021;247(1):233-44.
144. Jongberg S, Tørngren MA, Skibsted LH. Protein oxidation and sensory quality of brine-injected pork loins added ascorbate or extracts of green tea or mate during chill-storage in high-oxygen modified atmosphere. Medicines. 2018;5(1):7.
145. Lawrie RA. Meat Science. Oxford: Pergamon Press; 1974.
146. Maqbool A, Ahmed II, Sheikh ZA. Effects of two commonly used anticoagulants on haematology and erythrocyte morphology of rainbow trout (Oncorhynchus mykiss). International Journal of Fisheries and Aquatic Studies. 2014;2:239-43.
147. Jan K, Ahmed I, Sheikh ZA, Fazio F. Impact of three anticoagulants and their storage time on hematological parameters of snow trout, Schizothorax labiatus, habiting in river Sindh of Indian Himalayan region. Comparative Clinical Pathology. 2022.
148. Fyhn UEH, Fyhn HJ, Davis BJ, Powers DA, Fink WL, Garlick RL. Hemoglobin heterogeneity in Amazonian fishes. Comparative Biochemistry and Physiology Part A: Physiology. 1979;62(1):39-66.
149. Lee SK, Tatiyaborworntham N, Grunwald EW, Richards MP. Myoglobin and haemoglobin-mediated lipid oxidation in washed muscle: Observations on crosslinking, ferryl formation, porphyrin degradation, and haemin loss rate. Food Chem. 2015;167:258-63.
150. Benesch R, Macduff G, Benesch RE. Spectra of Deoxygenated Hemoglobin in Soret Region. Science. 1964;144(361):68-&.
151. Lochmann S, Maillet G, Frank K, Taggart C. Lipid class composition as a measure of nutritional condition in individual larval Atlantic cod (Gadus morhua). Canadian Journal of Fisheries and Aquatic Sciences. 2011;52:1294-306.
152. Undeland I, Hultin HO, Richards MP. Aqueous Extracts from Some Muscles Inhibit Hemoglobin-Mediated Oxidation of Cod Muscle Membrane Lipids. Journal of Agricultural and Food Chemistry. 2003;51(10):3111-9.
153. Kelleher SD, Silva LA, Hultin HO, Wilhelm KA. Inhibition of Lipid Oxidation during Processing of Washed, Minced Atlantic Mackerel. J Food Sci. 1992;57(5):1103-19.
154. Formanek Z, Kerry JP, Higgins FM, Buckley DJ, Morrissey PA, Farkas J. Addition of synthetic and natural antioxidants to α-tocopheryl acetate supplemented beef patties: effects of antioxidants and packaging on lipid oxidation. Meat Sci. 2001;58(4):337-41.
155. Undeland I, Hall G, Wendin K, Gangby I, Rutgersson A. Preventing lipid oxidation during recovery of functional proteins from herring (Clupea harengus) fillets by an acid solubilization process. J Agric Food Chem. 2005;53(14):5625-34.
156. Vanzetti G. An azide-methemoglobin method for hemoglobin determination in blood. J Lab Clin Med. 1966;67(1):116-26.
157. Zwart A, vanAssendelft OW, Bull BS, Lewis SM, Zijlstra WG. Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1995) and specifications for international haemiglobincyanide standard (4th edition). J Clin Pathol. 1996;49(4):271-4.

158. Lilja JN, S-E.; Svensson, J.; Eriksson, A., inventorAnalysis method and cuvette therefor. US 6,638,769 B2,2003.
159. Hornsey HC. The colour of cooked cured pork. I.—Estimation of the Nitric oxide-Haem Pigments. Journal of the Science of Food and Agriculture. 1956;7(8):534-40.
160. Chaijan M, Undeland I. Development of a new method for determination of total haem protein in fish muscle. Food Chem. 2015;173:1133-41.
161. Benesch RE, Benesch R, Yung S. Equations for Spectrophotometric Analysis of Hemoglobin Mixtures. Anal Biochem. 1973;55(1):245-8.
162. Listrat A, Lebret B, Louveau I, Astruc T, Bonnet M, Lefaucheur L, et al. How Muscle Structure and Composition Influence Meat and Flesh Quality. ScientificWorldJournal. 2016;2016:3182746-.
163. Jonkman J, Brown CM, Wright GD, Anderson KI, North AJ. Tutorial: guidance for quantitative confocal microscopy. Nat Protoc. 2020;15(5):1585-611.
164. Whittington NC, Wray S. Suppression of Red Blood Cell Autofluorescence for Immunocytochemistry on Fixed Embryonic Mouse Tissue. Curr Protoc Neurosci. 2017;81:2.28.1-2..12.
165. Alpert B, Jameson DM, Weber G. Tryptophan emission from human hemoglobin and its isolated subunits. Photochem Photobiol. 1980;31(1):1-4.
166. Hirsch RE, Zukin RS, Nagel RL. Intrinsic fluorescence emission of intact oxy hemoglobins. Biochemical and Biophysical Research Communications. 1980;93(2):432-9.
167. Rey-Barroso L, Roldán M, Burgos-Fernández FJ, Gassiot S, Ruiz Llobet A, Isola I, et al. Spectroscopic Evaluation of Red Blood Cells of Thalassemia Patients with Confocal Microscopy: A Pilot Study. Sensors (Basel). 2020;20(14).
168. Sardella BA, Brauner CJ. The Osmo-respiratory compromise in fish: the effects of physiological state and the ennvironment. In: Fernandes MN, editor. Fish respiration and environment. 1st ed. Boca Raton: Taylor & Francis Group; 2006. p. 147-65.
169. Lee CM, Trevino B, Chaiyawat M. A Simple and Rapid Solvent ExtractionMethod for Determining Total Lipids in Fish Tissue. Journal of AOAC International. 1996;79(2):487-92.
170. Shantha NC, Decker EA. Rapid, Sensitive, Iron-Based Spectrophotometric Methods for Determination of Peroxide Values of Food Lipids. Journal of Aoac International. 1994;77(2):421-4.
171. Schmedes A, Hølmer G. A new thiobarbituric acid (TBA) method for determining free malondialdehyde (MDA) and hydroperoxides selectively as a measure of lipid peroxidation. Journal of the American Oil Chemists' Society. 1989;66(6):813-7.
172. Lemon DW. An improved TBA test for rancidity. 51. Halifax, Nova Scotia: Canadian Fisheries and Marine Service; 1975.
173. Richards MP, Dettmann MA, Grunwald EW. Pro-oxidative characteristics of trout hemoglobin and myoglobin: A role for released heme in oxidation of lipids. Journal of Agricultural and Food Chemistry. 2005;53(26):10231-8.
174. FDA. Additional standards for human blood and blood products - CFR640.4. 2018.
175. Kurioka S, Liu PV. Effect of the hemolysin of Pseudomonas aeruginosa on phosphatides and on phospholipase c activity. J Bacteriol. 1967;93(2):670-4.
176. Ferguson RA, Storey KB. Glycolytic and Associated Enzymes of Rainbow Trout (Oncorhynchus Mykiss) Red Cells: In Vitro and In Vivo Studies. J Exp Biol. 1991;155(1):469-85.
177. Frei B, England L, Ames BN. Ascorbate Is an Outstanding Antioxidant in Human-Blood Plasma. P Natl Acad Sci USA. 1989;86(16):6377-81.
178. Pesquero J, Roig T, Sánchez J, Bermúdez J. Na+-K+pump and metabolic activities of trout erythrocytes during anoxia. American Journal of Physiology-Cell Physiology. 1999;277(1):C29-C34.

179. Beydemir S, Ciftçi M, Küfrevioğlu OI. Purification and characterization of glucose 6-phosphate dehydrogenase from sheep erythrocytes and inhibitory effects of some antibiotics on enzyme activity. J Enzyme Inhib Med Chem. 2002;17(4):271-7.
180. Yawata Y. Composition of Normal Red Cell Membranes. Cell Membrane2003. p. 27-46.
181. Spengler MI, Svetaz MJ, Leroux MB, Bertoluzzo SM, Parente FM, Bosch P. Lipid peroxidation affects red blood cells membrane properties in patients with systemic lupus erythematosus. Clin Hemorheol Microcirc. 2014;58(4):489-95.
182. Kure T, Sakai H. Transmembrane Difference in Colloid Osmotic Pressure Affects the Lipid Membrane Fluidity of Liposomes Encapsulating a Concentrated Protein Solution. Langmuir. 2017;33(6):1533-40.
183. Butler T, Bradley CA, Owensby JE. Plasma components protect erythrocytes against experimental haemolysis caused by mechanical trauma and by hypotonicity. Int J Exp Pathol. 1992;73(1):27-33.
184. Orf K, Cunnington AJ. Infection-related hemolysis and susceptibility to Gram-negative bacterial co-infection. Front Microbiol. 2015;6:666-.
185. Gozzelino R, Jeney V, Soares MP. Mechanisms of Cell Protection by Heme Oxygenase-1. Annual Review of Pharmacology and Toxicology. 2010;50(1):323-54.
186. Bourdon E, Loreau N, Lagrost L, Blache D. Differential effects of cysteine and methionine residues in the antioxidant activity of human serum albumin. Free Radical Research. 2005;39(1):15-20.
187. Rowe DS. Colloid Osmotic Pressures of Dextran, Serum and Dextran-Serum Mixtures. Nature. 1955;175(4456):554-5.
188. Tønnessen T, Tølløfsrud S, Kongsgaard UE, Noddeland H. Colloid osmotic pressure of plasma replacement fluids. Acta Anaesthesiol Scand. 1993;37(4):424-6.
189. Mallick A, Bodenham AR. Chapter 59 - Regulation of blood volume and electrolytes. In: Hemmings HC, Hopkins PM, editors. Foundations of Anesthesia (Second Edition). Edinburgh: Mosby; 2006. p. 709-22.
190. Kelleher SD, Feng Y, Hultin HO, Livingston MB. Role of initial muscle pH on the solubility of fish muscle proteins in water. J Food Biochem. 2004;28(4):279-92.
191. Halliwell B, Chirico S. Lipid peroxidation: its mechanism, measurement, and significance. The American Journal of Clinical Nutrition. 1993;57(5):715S-25S.
192. Sadrazadeh MHSG, Ernst; Panter, Scott S.; Hallaway, E. Philip; Eaton, W. John. Hemoglobin a biologic Fenton reagent. The Journal of Biological CHemistry. 1984;259(23):14354-6.
193. Liu SC, Zhai S, Lawler J, Palek J. Hemin-Mediated Dissociation of Erythrocyte-Membrane Skeletal Proteins. J Biol Chem. 1985;260(22):2234-9.
194. Sawas AH, Pentyala SN. Evaluation of lipid peroxidation in red blood cells by monitoring the uptake of sucrose and phenol red. J Appl Toxicol. 2004;24(3):223-9.
195. Shviro Y, Zilber I, Shaklai N. The Interaction of Hemoglobin with Phosphatidylserine Vesicles. Biochim Biophys Acta. 1982;687(1):63-70.
196. Hultin HO. Oxidation of lipids in seafoods. In: Shahidi F, Botta JR, editors. Seafoods: Chemistry, Processing Technology and Quality. Boston, MA: Springer US; 1994. p. 49-74.
197. Erdman A, Watts B. Meat pigments, spectrophotometric determination of color change in cured meat. Journal of Agricultural and Food Chemistry. 1957;5(6):453-5.
198. Richards MP, Kelleher A, Hultin HO. Effect of Washing with or without Antioxidants on Quality Retention of Mackerel Fillets during Refrigerated and Frozen Storage. Journal of Agricultural and Food Chemistry. 1998;46:4363-71.
199. Massingue AA, Torres Filho RdA, Fontes PR, Asaam S, Gomes MEdS, Ramos AdLS, et al. Effect of washing cycles and solutions on chemical composition and physicochemical

properties of the surimi-like material from mechanically deboned turkey meat. Journal of Food Processing and Preservation. 2019;43(5):e13929.
200. Pazos M, Alonso A, Fernández-Bolaños J, Torres JL, Medina I. Physicochemical Properties of Natural Phenolics from Grapes and Olive Oil Byproducts and Their Antioxidant Activity in Frozen Horse Mackerel Fillets. Journal of Agricultural and Food Chemistry. 2006;54(2):366-73.
201. Kröger-Ohlsen M, Skibsted LH. Kinetics and Mechanism of Reduction of Ferrylmyoglobin by Ascorbate and d-Isoascorbate. Journal of Agricultural and Food Chemistry. 1997;45(3):668-76.
202. Yamamoto K, Takahashi M, Niki E. Role of Iron and Ascorbic Acid in the Oxidation of Methyl Linoleate Micelles. Chemistry Letters. 1987;16(6):1149-52.
203. Hraš AR, Hadolin M, Knez Ž, Bauman D. Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. Food Chem. 2000;71(2):229-33.
204. Wada S, Fang X. The synergistic antioxidant effect of rosemary extract and α-tocopherol in sardine oil model system and frozen-crushed fish meat. Journal of Food Processing and Preservation. 1992;16(4):263-74.
205. Losada V, Barros-Velázquez J, Gallardo JM, Aubourg SP. Effect of previous slurry ice treatment on the quality of cooked sardine (Sardina pilchardus). European Food Research and Technology. 2006;224(2):193-8.