



# 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

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## Abstract

This study aimed at limiting hemolysis of fish red blood cells (RBCs) as a strategy to limit hemoglobin (Hb)-induced lipid oxidation during *post-mortem* handling and processing. Effects of varying temperature, salinity, and mechanical impact were studied using washed resuspended RBCs (wr-RBCs) and whole blood (WB) from rainbow trout (*Oncorhynchus mykiss*) and herring (*Clupea harengus*). The wr-RBCs were most stable avoiding mechanical stress, keeping isotonic conditions (0.9–1.3% NaCl) and low temperature 0–6 °C, with predicted minimum at 2.5 °C. When compared at the same salinity, it was found that hemolysis was more pronounced in herring than trout wr-RBCs. Furthermore, WB was more stable than wr-RBCs, showing protecting the effects of blood plasma. Studying individual plasma components, stabilizing effects were found from glucose, proteins, and ascorbic acid. This study indicates that small adjustments in the early handling and processing of fish such as changing salinity of storage and rinsing solutions could minimize Hb contamination of the fish muscle and thereby improve quality.

**Keywords** Fish · Hemoglobin · Hemolysis · Erythrocyte · Blood plasma components · Lipid oxidation

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## Abbreviations

BSA	Bovine serum albumin
Hb	Hemoglobin
Hct	Hematocrit
metHb	Methemoglobin
PEST	Penicillin-streptomycin
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RSW	Refrigerated seawater
WB	Whole blood
wr-RBC	Washed resuspended red blood cells

## Introduction

*Post-mortem*, fish muscle is highly susceptible to lipid oxidation due to its abundance of polyunsaturated fatty acids (PUFAs) and, in some cases, blood, the latter containing the strong pro-oxidant hemoglobin (Hb) (Larsson et al. 2007; Li et al. 2005; Richards and Hultin 2002; Undeland and Lingnert 1999). Lipid oxidation gives rise to unwanted flavor,

pigmentation, textural changes, and nutritional loss (Cavonius and Undeland 2017; Larsson and Undeland 2010; Maqsood and Benjakul 2011; Richards and Hultin 2002; Undeland et al. 2004; Undeland and Lingnert 1999), causing underutilization of particularly small pelagic dark muscle fish species for human consumption. Large fractions of, e.g., herring, sardines, and anchovy caught globally are instead converted into low-value products such as feed for fur animals or fish meal and fish oil for aquafeed production. Similarly, by-products from the filleting industry are highly susceptible to lipid oxidation due to richness in both blood and PUFAs (Wu et al. 2020). This renders food production from by-products difficult, despite their large amounts of high-quality muscle.

As the pro-oxidative nature of fish Hb is well established (Larsson et al. 2007; Li et al. 2005; Richards and Hultin 2002; Undeland et al. 2003), several routes have been developed in the past decades to prevent Hb-mediated lipid oxidation in fish, aiming at maintaining Hb in its reduced form or at scavenging lipid- or Hb-derived radicals by adding specific antioxidants or antioxidant containing extracts from, e.g., plants and algae (Babakhani et al. 2016; Li et al. 2016; Lu et al. 2014; Maqsood and Benjakul 2013; Miranda et al. 2018; Sánchez-Alonso et al. 2007; Wang et al. 2010). Other common strategies to reduce Hb-mediated lipid oxidation include bleeding the fish immediately after capture and applying rinsing with tap water during the filleting process to ensure maximal bleed out (Olsen et al. 2014; Roth et al. 2009). The practice of bleeding is however not applicable or economically feasible in commercial vessels targeting small pelagic fish species, as such fish is captured in large hauls, and bleeding has to be performed within minutes to ensure efficient removal of the blood (Olsen et al. 2014).

In a study conducted by Richards and Hultin (2002), preliminary evidence emerged that minimizing hemolysis of red blood cells (RBCs) could minimize lipid oxidation of washed cod muscle mince. They also found that the full lipid pro-oxidative capacity of whole blood was accounted for the soluble content of RBCs, with Hb playing a profound role. Similarly, Perez et al. (2019) reported that lysed RBCs promoted faster lipid oxidation in washed turkey muscle compared with intact RBCs. Based on these findings, we hypothesize that several process parameters typical to the fishing and fish processing industry negatively affect the fish RBC stability and would therefore stimulate lipid oxidation development. In steps with liquid involved, perhaps the most obvious process parameter expected to affect the stability of RBCs is the salinity of surrounding media (Singh et al. 2019; Waymouth 1970). Scattered studies have explored the osmotic fragility of fish RBCs (Ezell et al. 1969; Fyhn et al. 1979; Khan et al. 1972; Lewis and Ferguson 1966), but no previous publications report on how osmotic pressure in a range of relevance for the seafood industry affects the stability of fish RBCs. For example, during storage of whole fish in refrigerated seawater (RSW) or common ice on the fishing vessel, the RBCs are exposed to

hypertonic (~ 3% NaCl, 513 mM) or hypotonic (~ 0% NaCl) conditions, respectively, through the gills or wounds on the fish (Graham et al. 1992; Huss 1995). In addition, the mentioned tap water (0%NaCl) rinsing applied during the filleting would also subject residual RBCs to hypotonic conditions. Similarly, high osmotic pressure could appear, e.g., in pre-salting and marinating steps which comprise salt solutions from om 3% up to saturation. Other factors which could be critical to RBCs would be the mechanical stress which appears, e.g., during transport and sorting of fish, as well as temperature fluctuations (Blaine et al. 2019). Although the temperature is normally close to 0 °C early in the process chain (Graham et al. 1992; Huss 1995), it can raise, e.g., in filleting and washing steps and in the handling of by-products (Kuhlin 2019). The latter are often stored in outdoor temperature without any active cooling or antimicrobial protection which can induce the formation of, e.g., hemolysins (Vesper and Vesper 2004). Any step with liquid involved also implies a general dilution of the blood which can cause a shortage of nutrients and antioxidants (e.g., ascorbic acid), required for normal RBC function and integrity (B. Frei et al. (1989).

Altogether, there is thus a range of parameters which directly, or after prolonged storage, could induce lysis of fish RBCs. To the best of our knowledge, such parameters have however not before been systematically investigated. The aim of this study was to unravel how variations in salinity, mechanical impact, and temperature affect trout and herring RBC stability, thereby identifying the conditions that could maintain as much as possible of the Hb in its natural “encapsulated state” to prevent Hb contamination of the fish muscle and subsequent lipid oxidation. The results could ultimately lead to new routes to control seafood quality.

## Material and Methods

### Fish Supply

Rainbow trout (*Oncorhynchus mykiss*) was obtained from Antens laxodling AB, Alingsås, Sweden. The fish was maintained in tanks with aerated freshwater, ~ 10 °C, at Gothenburg University, Department of Biological and Environmental Sciences, Zoophysiology. The fish was kept under a 12:12 photoperiod and fed commercially available trout pellets. Live herring (*Clupea harengus*) was obtained from a commercial fishing boat at the southern west coast of Sweden, outside Helsingborg.

### Bleeding Procedure

The bleeding of trout and herring was approved by the regional animal ethics committee in Gothenburg, permit number 167-2013. Both fish species were killed by a blow to the head, and then, the blood was withdrawn from the caudal vessels with heparinized syringes (Heparin 5000 IU/mL; LEO Pharma AB,

Malmö, Sweden) and an additional 50 IU heparin/mL withdrawn blood was added. The blood was stored on an asymmetric blood mixer (Sarstedt AB, SARMIX® GM 1; Helsingborg, Sweden) in a cold room,  $3(\pm 1)^\circ\text{C}$  and used within 24 h after blood sampling. Hematocrit (hct) percentage was determined by sampling blood in sodium heparinized capillaries (Paul Marienfeld GmbH & Co.KG; Lauda-Königshofen, Germany) and centrifuged at 15,000 rpm for 2 min (Thermo Scientific™, Heraeus™ Fresco™ 17 Microcentrifuge; Massachusetts, U.S.A.). The hct-% was later used to calculate the volume of red blood cells (RBCs) to be transferred for the different storing solutions.

### Preparation of Washed Resuspended Red Blood Cells

The whole blood (WB) was centrifuged (Thermo Scientific™ Heraeus® Multifuge® 1 centrifuge; Massachusetts, U.S.A.) at 700g for 10 min at  $4^\circ\text{C}$  to collect the blood plasma. The RBCs were then washed three times according to a modified protocol by Fyhn et al. (1979) in which the 1.7% (290 mM) NaCl was replaced by 0.9% (154 mM) NaCl to further prevent the RBCs from harm due to osmotic pressure. After the last wash, the supernatant was removed and the RBCs distributed to 5-mL polypropylene tubes. The blood volume for each treatment was predetermined to 400  $\mu\text{L}$ ; thus, the amount of washed RBCs transferred was calculated based on the hct-% measured on the WB. Based on original WB volume, the washed RBCs were diluted fivefold with the specific treatment and incubated on an SARMIX® blood mixer in a cold room,  $3(\pm 1)^\circ\text{C}$ , while measuring lysis of washed resuspended RBCs (wr-RBCs) over time. To ensure that washed RBCs were not damaged by the mechanical stress applied during the actual washing procedure, washed RBCs were resuspended in plasma and the stability towards hemolysis was compared with that of WB; no differences in hemolysis rate could be detected between these two samples.

### Preparation of Methemoglobin

Methemoglobin (metHb) was prepared according to Lee et al. (2015). The millimolar extinction coefficient of  $153\text{ mM}^{-1}\text{ cm}^{-1}$  at 405 nm was used to quantify metHb on a heme basis (Benesch et al. 1964). The absorbance was analyzed using a UV-Vis spectrophotometer (Agilent Technologies Cary 60 UV-Vis, California, U.S.A.).

### Effects of Osmotic Pressure, Temperature, and Mechanical Stress RBC Stability—Univariate Approach

The osmotic fragility was investigated by diluting the washed RBCs/WB in different salinities ranging from 0% (0 mM) up to 3% (513 mM) NaCl, including salinities around the osmolality measured in trout and herring blood plasma (see

Analysis of Blood Plasma Components). Experiments conducted on trout WB consisted of pooled blood from three fish and were repeated 2–9 times. Hemolysis (see Measurement of Hemolysis) was then measured in duplicates or triplicates in each sample during storage up to 42 or 53 days for wr-RBCs and WB respectively. Experiments on herring blood were conducted on pooled blood from 11 and 17 fish ( $n = 2$ ). Hemolysis (see Measurement of Hemolysis) was then measured in replicates ( $r = 2$ ) in each sample during storage for up to 20 days. Effect of temperature was investigated by incubating trout wr-RBCs or WB in 0.9% (154 mM) NaCl at different temperatures:  $0^\circ\text{C}$  (on ice),  $4\text{--}6^\circ\text{C}$ , or  $10\text{--}12^\circ\text{C}$  (in temperature-controlled refrigerators). To study the effect of mechanical impact, wr-RBCs or WB was incubated in 0.9% (154 mM) NaCl in polyethylene tubes at  $4\text{--}6^\circ\text{C}$ ; the tubes were then dropped two or five times from 2 m high. The experiments designed to investigate temperature and mechanical stress were performed with two different batches of trout blood ( $n = 2$ ), each batch consisting of pooled blood from three fish. Hemolysis (see Measurement of Hemolysis) was then measured in triplicates ( $r = 3$ ) in each sample during storage up to 16 days for wr-RBCs and WB respectively.

### Effects of Osmotic Pressure, Temperature, and Mechanical Stress on RBC Stability—Multivariate Approach

Interaction, linear, or/and quadratic effects between the study parameters salinity, temperature, and mechanical impact were investigated in a Box-Behnken design with three center points (see Supplement Information). Three levels were used for each of the parameters: (i) salinity: 120 mM (0.7%), 145 mM (0.85%), or 171 mM (1.0%) NaCl; (ii) mechanical stress: no drop, drop from 2 m high, two times or five times; and (iii) temperature:  $0^\circ\text{C}$ ,  $5(\pm 1)^\circ\text{C}$ , or  $11(\pm 1)^\circ\text{C}$ . The experiments were performed with two different batches ( $n = 2$ ) of trout blood, each batch consisting of pooled blood from three trout. Hemolysis (see Measurement of Hemolysis) was then measured in triplicate ( $r=3$ ) in each sample at seven different time points: before mechanical stress (0 h), after mechanical stress (0.5 h), and after 3, 5, 7, 11, and 13 days of incubation, at the specific temperature and salinity. Hemolysis is log10 transformed in the graphs visualizing linear and quadratic effects of mechanical stress and temperature on hemolysis.

### Effects of Individual Plasma Components, Antimicrobial Agents, KCl, and methHb on Hemolysis

Plasma glucose concentration was measured to  $6.0 \pm 0.4$  and  $6.0 \pm 0.3$  mM ( $n = 1$ ,  $r = 3$ ), respectively, in trout and herring blood plasma (see Analysis of Blood Plasma Components). Endogenous ascorbic acid level found in trout plasma was

169.8 ± 19.9 μM ( $n = 3$ ,  $r = 3$ ) and in herring plasma 109.6 ± 26.7 μM ( $n = 3$ ,  $r = 3$ ) (see Analysis of Blood Plasma Components). To evaluate concentrations both below and above the endogenous ones, 2, 6, 10, or 20 mM glucose (Sigma-Aldrich) and 50, 100, or 200 μM ascorbic acid (Sigma-Aldrich) were therefore added to herring and/or trout wr-RBCs in 0.9% (154 mM) NaCl. MetHb (10 g/L) (see Preparation of Methemoglobin) was added based on the Hb released at the start of the exponential hemolysis phase and bovine serum albumin (BSA, 10 g/L) was added (Sigma-Aldrich) in the same concentration as metHb to compare the effect of an other globular protein. Hemolysis was analyzed as described in the “Statistical Evaluations” section. Also, 100 μg/mL streptomycin/penicillin-streptomycin (PEST) (Sigma-Aldrich) was tested for its antimicrobial effects. In all experiments, above wr-RBCs were suspended in 0.9% (154 mM) NaCl. However, also resuspension in 0.9% (154 mM) KCl and a mixture of 0.45% (77 mM) KCl and 0.45% (77 mM) NaCl was evaluated. All samples were stored at 3 ± 1 °C for up to 42 days to evaluate the ability of these compounds to increase RBC stability.

### Analysis of Blood Plasma Components

Trout and herring blood plasma osmolality was measured using an osmometer (Advanced® Instruments Inc., Micro-osmometer model 3320; Massachusetts, U.S.A.) and the results were later translated into NaCl equivalents in millimolar. The osmolality of trout blood plasma as measured in three blood batches ( $n = 3$ ) with three replicates ( $r = 3$ ) 0.9 ± 0.001% (152.0 ± 1.2 mM). Osmolality in herring plasma as measured in one batch ( $n = 1$ ) with four replicate measurements ( $r = 4$ ) was found to be 1.1 ± 0.001% (195.0 ± 1.3 mM).

The glucose concentration of herring and trout plasma was measured using an enzyme-based glucose assay (Glucose (HK) assay kit; Sigma-Aldrich) where after spectra at 340 nm was measured in a microplate reader (SpectraMax 190 microplate reader, Fisher Scientific, U.S.A.). A blood batch ( $n = 1$ ) was analyzed in triplicates ( $r = 3$ ). Plasma ascorbic acid was measured in two batches of blood with three replicates ( $n=2$ ,  $r=3$ ) using a modified version of the method described by Lykkesfeldt (Lykkesfeldt 2000). Upon use, the blood plasma sample was thawed, vortexed, sonicated 5 min × 2, and centrifuged at 16,000g, 4 °C, and 2 min. The supernatant was then centrifuged a second time with the same settings and then let to react for 15 min at room temperature with reaction buffer (1:1). The reaction buffer consisted of 0.312 mM tris(2-carboxyethyl)phosphine (TCEP) (Honeywell Fluka) in (9:1) mobile phase (50 mM phosphate buffer, pH 2.8) and McIlvaine buffer (0.46 M sodium phosphate dibasic and 0.27 M citric acid, pH 4.5). Further dilutions before measurement in the HPLC were made with dilution buffer: (9:1) mobile phase mixed with McIlvaine buffer. For

analysis, an HPLC (Jasco PU-2080Plus) coupled with an electrochemical detector (Decade II electrochemical detector, Antec Leyden, Netherlands) was used. The mobile phase consisted of 50 mM phosphate buffer pH 2.8 and the sample injection volume was 10 μL. The flow rate was set to 0.4 mL/min and the electrochemical detection in the glassy carbon flow cell was set to 0.60 V (DC mode, range 50 nA).

### Measurement of Hemolysis

The stability of the RBCs was determined through the measurement of Hb in the supernatant, following centrifugation of a sub-sample (100 μL) at 700g at 4 °C for 10 min. The supernatant was allowed to reach room temperature before measurement with a HemoCue® Plasma/Low (HemoCue®, HemoCue® Plasma/Low Hb System; Ängelholm, Sweden). The total Hb of each wr-RBC/WB batch used in the study was estimated by preparing a hemolyzed sample according to the method by Fyhn et al. (1979). Hemolysis was expressed as the amount of Hb in the supernatant divided by the total Hb in the wr-RBC/WB used.

### Statistical Evaluations

For experiments repeated two times or more (i.e.,  $n \geq 2$ ), the standard deviation (SD) is calculated and visualized as error bars and one-way analysis of variance (ANOVA) was conducted to determine statistical significance between different storage conditions. Tukey’s post hoc test was conducted for a pairwise comparison when a significant effect was found, with a threshold of  $p < 0.05$ . In cases where a representative graph from a series of experiments is visualized, SD is calculated based on the 3 repeated subsamples taken from each sample type ( $r = 3$ ). Multivariate analysis of the data from the Behnken design was performed using a regression model with quadratic terms to assess the relation of hemolysis to temperature, salinity, and mechanical stress. Two-way ANOVA was performed on these factors to study the significance of each factor, with a threshold of  $p < 0.005$ . All analysis was performed in R-studio version 3.5.1 (Team, R. C. 2019).

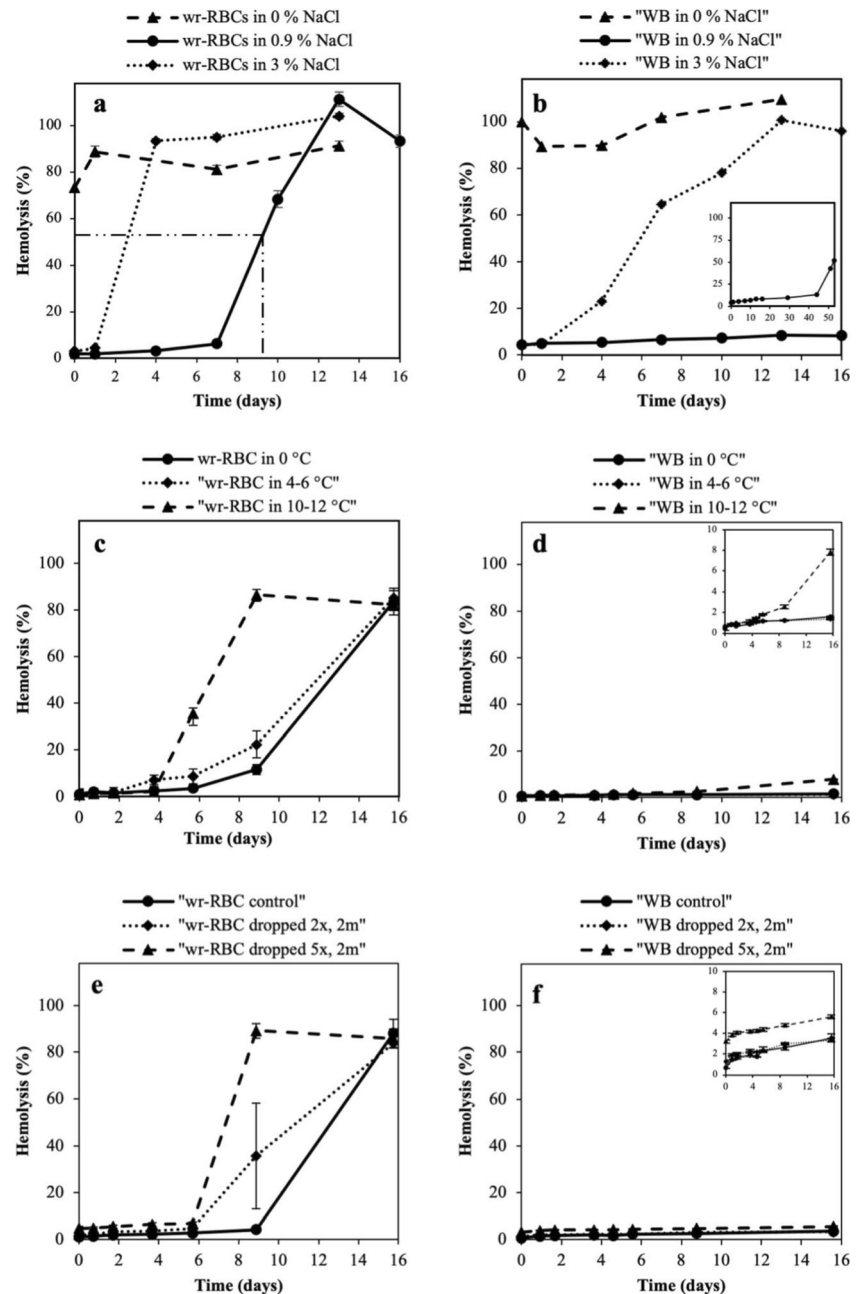
## Results

### Effect of Salinity, Temperature, and Mechanic Stress on Hemolysis

As illustrated in Fig. 1 a and b, wr-RBCs or WB in 0% NaCl resulted in instant hemolysis within 1 h of incubation. The wr-RBCs or WB in 3% (513 mM) NaCl gave the second-fastest hemolysis, with 50% hemolysis ( $T_{50}$ ) after around 3 and 6 days, for wr-RBCs and WB respectively. Both wr-RBCs and WB incubated in physiological salt concentration, 0.9% (154 mM)



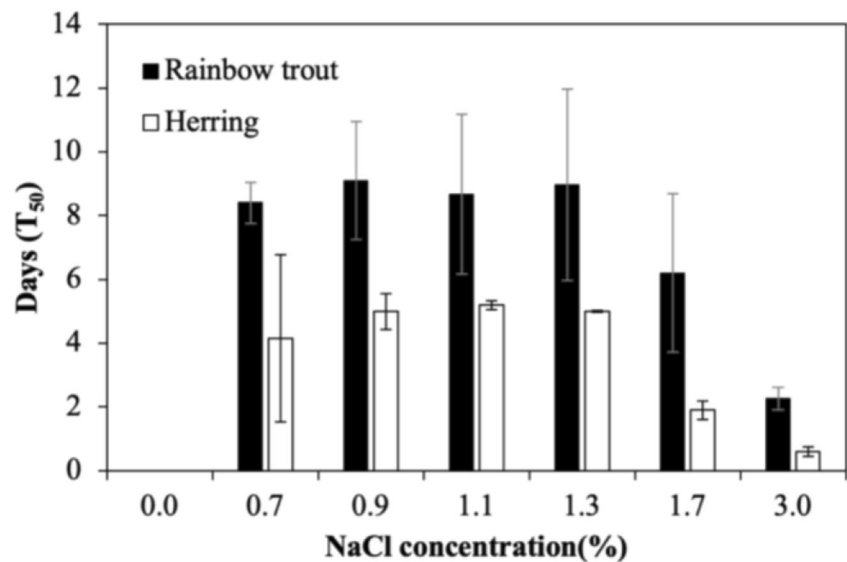
**Fig. 1** Representative graphs of hemolysis of rainbow trout RBCs over time. Each point represents mean values  $\pm$  standard deviation, SD ( $r = 3$ ) for one blood batch. In graphs **a**, **c**, and **e**, wr-RBCs are shown, whereas graphs **b**, **d**, and **f** show data from WB. Panels **a** and **b** visualize the effect of osmotic pressure on RBCs using 0% (0 mM), 0.9% (154 mM), and 3% NaCl (513 mM). The dash-double-dotted line (graph a), mark when 50% hemolysis was reached ( $T_{50}$ ). Panels **c** and **d** show the effect of temperature (0 °C, 4–6 °C and 10–12 °C) when samples are incubated in 0.9% NaCl. Panels **e** and **f** show the impact of two different levels of mechanical stress (dropped 2 times from 2 m height and dropped 5 times from 2 m height) on hemolysis in comparison to the control



NaCl, had the slowest hemolysis and reached  $T_{50}$  after 9 and 53 days, respectively. Results with 0.9% NaCl were significantly different ( $p < 0.001$ ) from those obtained in wr-RBCs with 3% NaCl at 4 and 7 days and from WB in 3% NaCl during the whole time span. There was slight variability in the time required to reach  $T_{50}$  between experimental repetitions, which was presumably due to biological variations between blood batches. However, the different salinities tested showed consistency in the order they caused hemolysis. No significant difference in hemolysis could be found between samples stored at 0 °C or 4–6 °C at the most protective salt concentration 0.9%

(154 mM) NaCl (Fig. 1 c and d). However, at the higher temperature, 10–12 °C, a significantly higher level of hemolysis was found ( $p < 0.01$ ) at days 6–9 and days 6–16 for wr-RBCs and WB respectively, compared with the lower temperature range 0–6 °C. Samples subjected to mechanical impact prior to storage in 0.9% (154 mM) NaCl (Fig. 1 e–f), resulted in significantly ( $p < 0.01$ ) faster hemolysis in both wr-RBCs and WB, only when the highest mechanical impact (5 drops from 2 m high) was applied. Furthermore, in all treatments (Fig. 1 a–f), a lower degree of hemolysis was found in WB, i.e., when plasma was present, compared with wr-RBCs.

**Fig. 2** Days until trout and herring wr-RBCs reached 50% hemolysis ( $T_{50}$ ) in samples with different salt concentrations, ranging from 0% NaCl (0 mM) to 3.0% NaCl (513 mM). Data are shown as the mean value  $\pm$  SD for all experiments. Experimental replicates for rainbow trout wr-RBCs were as follows: 0% and 3% NaCl ( $n = 2$ ); 0.7%, 1.1%, 1.3%, and 1.7% NaCl ( $n = 4$ ); 0.9% NaCl ( $n = 9$ ). All experiments for herring were repeated twice ( $n = 2$ ) and all measurements for herring and trout were done in duplicates ( $r = 2$ )

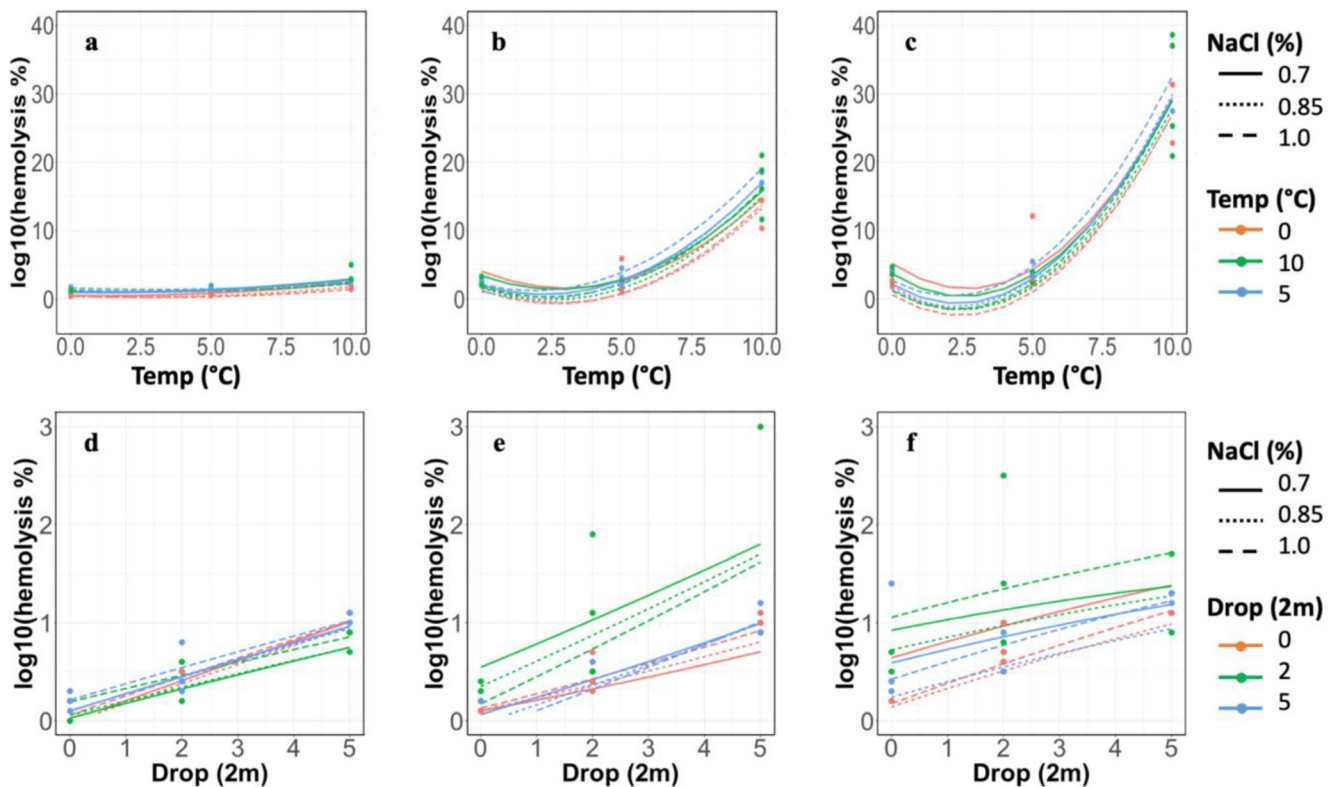


Osmotic fragility of trout and herring wr-RBCs was further investigated to specify the optimal salinity range causing minimal hemolysis. Figure 2 shows the number of days required for the sample to reach  $T_{50}$ . Herring wr-RBCs in the salinity range 0.9–1.3% (154–222 mM) NaCl have a significantly longer stability ( $p < 0.05$ ) compared with incubation in both 0% and 3% (513 mM) NaCl, whereas trout wr-RBC stability is significantly longer ( $p < 0.05$ ) when incubated within the salinity range 0.7–1.3% (120–222 mM) NaCl, compared with incubation in 0% or 3% (513 mM) NaCl. Overall, herring wr-RBCs were more fragile compared with trout, within the studied salinity range.

Multivariate analysis using data from a Box-Behnken design was performed to elucidate the nature of the relation between (i) temperature, salinity, and mechanical stress and (ii) hemolysis at different time points. The design also allowed investigation of interaction effects; however, no significant interaction effect was found. Investigating the association between temperature and hemolysis, a weak linear term was found at day 7 (Fig. 3a). The association between temperature and hemolysis increased over time and at time points 11 and 13 days (Fig 3b and c, respectively) both a strong linear and quadratic term was found, with a minimum for hemolysis at 2.5 °C. Therefore, in further experiments (see Effect of Blood Plasma Components and Potential Causes for Storage-Induced Hemolysis—metHb, KCl Leakage, and Bacterial growth), samples were kept in a cold room at  $3 \pm 1$  °C. A strong linear association was found between mechanical stress and hemolysis right after mechanical stress was applied (Fig. 3d). This association decreased with time but could also be seen at time points 3 and 5 days (Fig. 3e and f, respectively). Within the chosen range of salinity in the multivariate analysis 0.7–1.0% (120–171 mM) NaCl, no significant association with hemolysis was found.

### Effect of Blood Plasma Components

WB suspended in 0.9% (154 mM) NaCl were up to three times more stable towards hemolysis in comparison with wr-RBCs in the same salinity (Fig. 4). To better understand the protecting effect of plasma, the effect of energy-providing compounds and antioxidants found in plasma was investigated. Glucose was measured to  $6.0 \pm 0.4$  and  $6.0 \pm 0.3$  mM ( $n = 1$ ,  $r = 3$ ) in trout and herring blood plasma, respectively. Glucose (6 mM) added to wr-RBCs in 0.9% (154 mM) NaCl had a varying effect on hemolysis ranging from no effect up to 18 days prolongation of  $T_{50}$  ( $n = 6$ ,  $r = 2$ ). In herring, saline with 6 mM glucose resulted only in a slight extension of  $T_{50}$  (Fig. 4) with up to 1 day ( $n = 2$ ,  $r = 2$ ). The addition of 10 and 20 mM of glucose to the trout wr-RBCs did not provide increased stability compared with 6 mM glucose. The effect of adding adenosine triphosphate (ATP) (30 or 100  $\mu$ M) to the 0.9% (154 mM) NaCl, reduced  $T_{50}$  with 1.5 and 3 days, respectively ( $n = 2$ ,  $r = 2$ ); the stability decreased with increasing ATP concentration (data not shown). The ascorbic acid concentration in trout and herring blood plasma was measured to  $169.8 \pm 19.9$   $\mu$ M and  $109.6 \pm 26.7$   $\mu$ M ( $n=2$ ,  $r=3$ ), respectively. Adding ascorbic acid to the 0.9% (154 mM) NaCl to a final concentration of 200  $\mu$ M increased the  $T_{50}$  with 5–11 days in trout wr-RBCs ( $n = 3$ ,  $r = 3$ ). However, the effect of this concentration of ascorbic acid on herring wr-RBCs resulted in non-systematic effects, from a 2.5 days increase of  $T_{50}$  down to a decrease with 2.3 days (Fig. 4). Comparing trout and herring wr-RBCs when stored in plasma or 0.9% (154 mM) NaCl with added glucose or ascorbic acid, it was noted that herring wr-RBCs had shorter stability. Blood plasma resulted in 20.5 and 14.7 days longer  $T_{50}$  of trout and herring wr-RBCs respectively. Blood plasma was also significantly better at maintaining wr-RBC stability compared with any of the other added substances (Fig. 4).



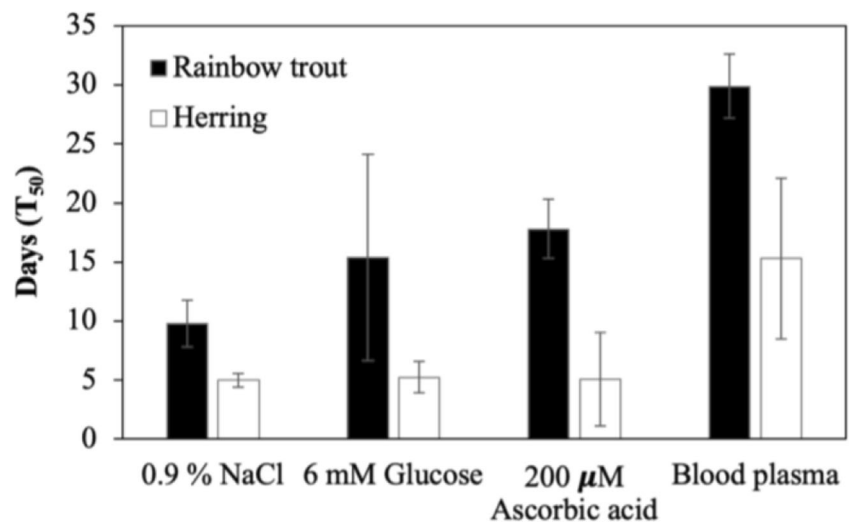
**Fig. 3** Association between temperature at time point 7, 11 and 13 days (a–c) and mechanical impact at time point 0.5h, 3 days and 5 days (d–f) with hemolysis of trout wr-RBCs, as revealed when evaluating the Box-Behnken design

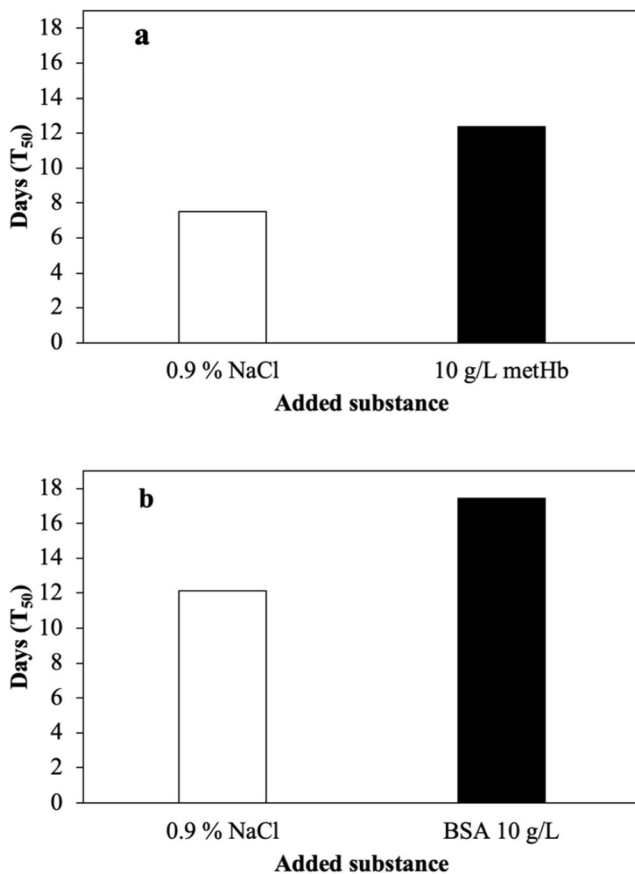
### Potential Causes for Storage-Induced Hemolysis—metHb, KCl Leakage, and Bacterial Growth

The cause of the sudden hemolysis seen during storage of wr-RBCs was investigated, with one theory being that released Hb oxidized to metHb, thus causing an external oxidative attack on the membrane of still intact wr-RBCs. Adding 10 g/L metHb to the 0.9% (154 mM) NaCl saline unexpectedly increased  $T_{50}$  with 5 days of the trout wr-RBCs (Fig. 5a). To

investigate the effect of a globular protein *per se* on the stability of RBCs, 5, 10, and 20 g/L bovine serum albumin (BSA) was added to 0.9% (154 mM) NaCl. The same stabilizing effect that was provided by metHb was found with the addition of these concentrations of BSA, i.e., an increase of  $T_{50}$  with around 5 days (Fig. 5b). Another theory was that KCl was released into the storage solution after hemolysis, which could cause further hemolysis of still intact RBCs. Therefore, 0.9% (154 mM) NaCl was replaced with 0.9% (154 mM) KCl or with 0.45% (77 mM) KCl plus 0.45% (77 mM) NaCl.

**Fig. 4** Number of days passing until trout and herring wr-RBCs in their own plasma or in 0.9% (154 mM) NaCl with added glucose or ascorbic acid reached 50% hemolysis ( $T_{50}$ ). Data represent mean values  $\pm$  SD. Experimental replicates for the trout wr-RBCs were as follows: 0.9% (154 mM) NaCl ( $n = 9$ ); 6 mM glucose ( $n = 6$ ); 30  $\mu$ M and 200  $\mu$ M ascorbic acid ( $n = 3$ ); blood plasma ( $n = 2$ ), with the measurement being replicated twice ( $r = 2$ ). For herring RBCs,  $n = 2$  and  $r = 2$





**Fig. 5** Effect of 10 g/L methHb (graph a) or BSA (graph b) in 0.9% (154 mM) NaCl on days needed to reach 50% hemolysis - ( $T_{50}$ ) in the trout wr-RBCs ( $n = 1$ ,  $r = 2$ )

However, no change in stability of trout wr-RBCs was noted from this change in salt (data not shown). Streptomycin/PEST (100  $\mu\text{g}/\text{mL}$ ) in 0.9% (154 mM) NaCl was finally tested as broad range antibiotics to investigate whether microbial growth could be the cause of the sudden hemolysis. The antibiotics resulted in a slight increase of  $T_{50}$  with 2.6 days, in trout wr-RBCs (data not shown in graph).

## Discussion

As a tentative new approach to minimize Hb-mediated lipid oxidation in herring and other fish species, this study for the first time investigated how a selection of chemical and physical parameters, typically varying during early handling of fish, affected the stability of RBCs. This knowledge can thus be used to minimize Hb contamination of the fish muscle, which would be of particular interest for small pelagic fish species such as herring which are not commercially bled after capture. However, this strategy would also be of interest in bled fish species, as less than 50% of the blood is eliminated from bleeding (Olsen et al. 2014).

## Osmotic Fragility and Species Variation

The rapid hemolysis seen when RBCs were in contact with hypo- or hypertonic solutions (Fig. 1 a and b) reveals that the conditions used in, e.g., RSW tanks and in the filleting process, respectively, are highly critical for the RBC stability. Highest stability of the RBCs of both species was found with salinities close to the physiological, 0.9–1.3% (154–222 mM) which indeed is in agreement with the general behavior of cells under varying salinity conditions (Ayik 2009; Ezell et al. 1969; Frei and Perk 1964; Khan et al. 1972; Schmidtnielsen 1974; Singh et al. 2019). Optimizing the salinity of storing and rinsing solutions used in processing could thus be a particularly beneficial strategy to avoid oxidation in muscle from small active and blood-rich fish species, such as herring, which are not commercially bled. The fact that muscles in these species are highly capillarized (Greerwalker and Pull 1975; Shewfelt 1981), and thus very rich in Hb, could explain their high susceptibility to lipid oxidation (Richards and Hultin 2002; Undeland et al. 2003).

Despite the ability of fish to regulate the osmolality of their body fluids (Schmidtnielsen 1974), some batch and species variation in osmotic fragility was noted when wr-RBCs were stored in solutions with varying salinity (Fig. 2). The batch variations could be linked to factors such as diseases, hormones, and access to feed (Fänge 1992; Kiron et al. 1994), whereas species variation could be linked to stress during capture. The blood from herring, which was bled immediately after capture from the sea, had a higher osmolality in its plasma compared with trout. The higher osmolality could be caused by stress during the capture of the herring, causing the gills to open to ensure maximal oxygen uptake. The increased diffusion area in the gills in turn causes osmotic problems, showing up as raised plasma osmolality (Sardella and Brauner 2006). Furthermore, the species variation in hemolysis could also reflect differences in the antioxidant systems of trout and herring blood, similar to the difference found in the antioxidant system between the muscle (R. Li et al. 2005; Passi et al. 2002; Undeland et al. 2003) or the blood (Perez et al. 2019; Rudneva 1997; Stagsted and Young 2002) from different animal species, including fish.

## The Effect of Temperature

In the early steps of the process chain, whole fish is stored under cold conditions either in boxes with ice (normally 1:3 fish to ice ratio) or in RSW (Stroud 2001). Generally, reduced temperature implies reduced metabolism of cells, reduced bacterial growth, and reduced enzymatic activity, thus maintaining fish quality (Huss 1995). The specific relation between process temperature and stability of fish RBCs has not previously been reported. In our study, less hemolysis was found at 0–6 °C, with a minimum at 2.5 °C (Fig. 3 a–c), which is in



agreement with the results of (Blaine et al. 2019), who found that storage at 2 °C give better viability of human RBCs which they ascribed reduced RBC metabolism, such as slower pH drop due to slower formation of lactate. Furthermore, the FDA-approved storage temperature of human RBCs is at 1–6 °C (FDA 2018).

### The Effect of Mechanical Stress

In several steps of the fish processing chain, the risk of inducing mechanical stress on the RBCs can be identified. The negative quality effect from crowding and pumping has previously been ascribed as a possible cause for, e.g., blood spotting in fish muscle (S. H. Olsen et al. 2006; Roth et al. 2005). Studying the impact of mechanical stress on trout wr-RBCs, we found a linear relation between hemolysis and mechanical stress (Fig. 3 d–f). Hemolysis was highest in close connection to the event of mechanical stress, meaning that wr-RBCs injured by the mechanic stress hemolyze in proximity to the event, whereas some wr-RBCs are completely unaffected by the stress event. In order to rule out eventual effects of damage to the wr-RBCs during the washing procedure (see Preparation of Washed Resuspended Red Blood Cells), washed RBCs was resuspended in blood plasma and stored while hemolysis was measured over time. No stability difference could be found compared with WB.

### Protecting Blood Plasma Components

Interestingly, all experiments performed on WB showed longer RBC stability compared with the same treatment on wr-RBCs. These findings suggest that there are components in blood plasma which protect the RBCs from hemolysis. Similar effects of plasma were also identified by Richards and Hultin (2002), in which they found that limiting hemolysis delayed lipid oxidation in washed cod muscle and that blood plasma delayed the lipid oxidation. Blood plasma is well known for high levels of antioxidants such as ascorbic acid, uric acid, tocopherol, and organic molecules such as various proteins (Wayner et al. 1987). Since oxidation of the cell membrane lipids can cause lysis (Fukunaga et al. 1999), antioxidants could have a preventive effect. This was partly confirmed when 50, 100, or 200 mM ascorbic acid was added to wr-RBCs. This is around the endogenous level found in trout ( $169.8 \pm 19.9 \mu\text{M}$ ) and herring ( $109.6 \pm 26.7 \mu\text{M}$ ). The antioxidative and radical scavenging activity of ascorbic acid is well known, and ascorbic acid has previously been identified as an outstanding antioxidant in human blood (Frei et al. 1989). However, in all experiments, WB was still more stable than wr-RBCs in saline with added ascorbic acid. The unattainable protection provided by blood plasma may thus be due to the cooperative activity of antioxidants and compounds protecting the RBCs according to other mechanisms (Frei

et al. 1989). One such candidate could be glucose, which is widely used in different RBC storing solutions, such as SAG-M to maintain the metabolism of the RBCs (Hess and Greenwalt 2002). The addition of glucose around and above the endogenous levels to blood plasma (2, 6, 10, or 20 mM) indeed increased the stability of wr-RBCs, which was more pronounced for trout wr-RBCs compared with herring wr-RBCs. The latter could be due to species differences in the permeability of glucose to RBCs (Fänge 1992). In a study performed on amphibian blood increased metabolism, thus higher ATP levels, decreased the osmotic fragility of RBCs (Goniakow 1974). Similarly, other studies have shown that increased metabolism and ATP production in the RBC prevent intracellular potassium loss (Whittam 1968). However, adding extracellular ATP as an additional protective candidate of plasma surprisingly decreased the wr-RBC stability, more so with increasing ATP concentration (100 vs. 30  $\mu\text{M}$  ATP). The cytotoxicity of ATP has previously been seen in human cervical cancer cells (Mello et al. 2014).

### Potential Reasons for the Exponential Lysis

Implying that oxidation of lipids in the RBC membrane is a contributing reason to hemolysis also implies that pro-oxidants could induce hemolysis. MetHb is a highly oxidative form of the Hb molecule and compared with mammalian Hb, fish Hb undergo rapid autooxidation (Aranda et al. 2009). During metHb formation, superoxide-anion radicals are formed which can induce lipid peroxidation of the fish RBC membrane (Kawatsu et al. 1991; Soldatov and Parfenova 2001). One of our initial hypotheses to explain the exponential behavior of the observed hemolysis (Fig. 1) was that it involved an oxidative attack from Hb molecules that had leaked out from the RBCs and been oxidized to metHb, thus a sort of “self-attack”. This phenomenon has been seen when exposing RBCs to ozone, causing oxidation of RBCs and the formation of metHb, due to Hb-derived reactive oxygen species (Fukunaga et al. 1999). In our experiments, an increasing metHb concentration was observed in the samples over 1-week storage time (data not shown). However, incubating wr-RBCs with metHb surprisingly increased the stability towards hemolysis (Fig. 5a), possibly due to increased colloidal osmotic pressure (COP) of the external solution, which would decrease the transmembrane difference in COP and thereby stabilize the wr-RBCs (Kure and Sakai 2017). This hypothesis was confirmed by adding another globular protein, bovine serum albumin (BSA), at the same concentration (Fig. 5b). Results in this direction have also been noted by previous researchers (Sumpelmann et al. 2000; Hughes et al. 1986). Exposing a suspension of human RBCs in a gelatin and albumin fluid, instead of 0.9% (154 mM) NaCl, to mechanical stress, showed increased stability of the RBCs (Sumpelmann et al. 2000). Another theory explored to explain the

exponential behavior of hemolysis which was noted in all trials was that extracellular KCl would gradually accumulate by hemolysis of RBCs and then stimulate further sudden lysis. However, fully or partly replacing 0.9% (154 mM) NaCl by KCl showed similar results, implying that the electrolyte can be exchanged as long as the external and internal electrolyte concentrations are in equilibrium. These results are in accordance with previous studies performed on human RBCs (Nepal and Rao 2011). This could be of importance in the development of novel storage solutions for fish, targeting maximal RBC stability.

## Conclusion

Overall, the results of this study indicate the possibility to significantly improve the stability of RBCs with few adjustments in a conventional fish processing chain. Cold temperature 0–6 °C, with a minimum at 2.5 °C, and minimal mechanical impact turned out to be highly important factors. Furthermore, the osmolarity (salinity) of the process water should be adapted to the osmolarity of the blood, rather than utilizing seawater or tap water in pure form. The highest stability of the RBCs was obtained when incubated in blood plasma or in 0.9% (154 mM) NaCl and adding components endogenous to plasma, 6 mM glucose, or 200 µM ascorbic acid. Further investigations in whole fish muscle and blood-enriched fish muscle model systems are however necessary to investigate how *post-mortem* fish quality in a broader perspective will be affected by increased RBC stability, as a result of new fish handling protocols. For example, it should be ensured that, e.g., a change in salinity of the process water or use of additives does not give negative effect on the flavor of the fish muscle or harm the cooling capacity of RSW tanks.

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