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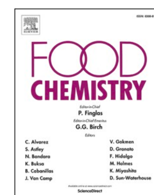

Effects of capture-related stress and pre-freezing holding in refrigerated sea water (RSW) on the muscle quality and storage stability of Atlantic mackerel (*Scomber scombrus*) during subsequent frozen storage

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A R T I C L E I N F O

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A B S T R A C T

Crowded (stressed) and unstressed Atlantic mackerel with or without pre-freezing holding in refrigerated sea water (RSW) were stored at −19 °C for ~12 months and analysed for nucleotide degradation (K value), muscle pH, water holding capacity (WHC), fillet firmness, cathepsin B/L like activity, lipid oxidation and fillet colour. The frozen storage showed the largest and most consistent direct effects on the quality metrics leading to increased lipid oxidation, discolouration (yellowing) and reduction on WHC and cathepsin activity. RSW treatment promoted nucleotide degradation and reduced WHC and fillet firmness in interaction with frozen storage and affected fillet colour lightness and saturation. Although showing only marginal main effects, crowding stress modified WHC, cathepsin activity and fillet firmness and colour through significant interactions with the frozen storage and RSW treatment. Further studies with larger sample sizes would be needed to elucidate their complex effects and interactions on the quality and storage stability of mackerel.

1. Introduction

Northeast Atlantic mackerel (*Scomber scombrus*) is one of the most economically important species targeted extensively by the pelagic purse seine fishing industry in Europe. Mackerel is highly susceptible to capture related stressors (Huse & Vold, 2010). The crowding of fish during purse seining triggers behavioural physiological responses of sufficient severity to cause mortality (Marçalo et al., 2019 and references therein). Entire schools, potentially consisting of several hundred tons of mackerel, can be crowded into extremely high densities (>200 kg/m^3) (Huse & Vold, 2010). Intense physical activity in the fish in such situations will exhaust the intracellular supply of oxygen in the muscle causing a shift from aerobic to anaerobic energy pathways, which will eventually manifest in the exhaustion of adenosine triphosphate (ATP), as well as metabolic acid and proton accumulation (Huss, 1988). The detrimental effect of such preslaughter stress has been extensively investigated with several species, demonstrating for example: lower muscle pH (Lerfall et al., 2015), faster onset and strength of *rigor mortis*, increased gaping and drip loss, discoloration (Roth, Slinde, & Arildsen, 2006) and decreased fillet firmness (Bahuaud et al., 2010) in Atlantic salmon (*Salmo salar* L.) and reduction in water holding capacity (WHC) in farmed Atlantic cod (*Gadus morhua*) (Hultmann, Phu, Tobiassen, Aas-Hansen, & Rustad, 2012). Similarly, killing methods that involve struggling (e.g. air asphyxiation) have been shown to accelerate quality loss in mackerel species, through increased nucleotides degradation, faster onset of *rigor mortis*, muscle softening and increased gaping in the struggled fish when compared to fish killed instantly by e.g. pithing (Ando et al., 2001; Mishima et al., 2005; Mochizuki & Sato, 1994; Ogata, Koike, Kimura, & Yuan, 2016; Sato et al., 2002).

Thus far, few investigations have dealt with stressors that simulate those experienced by mackerel during commercial capture, where catches are large, and fish are alive and vigorous. Recently, Anders, Eide, Lerfall, Roth, and Breen (2020) demonstrated that crowding stress during simulated purse seine capture led to faster onset and strength of *rigor mortis*, increased cathepsin B/L like activity, increased gaping and reduced fillet firmness in Atlantic mackerel when combined with ice storage. In the Norwegian mackerel fisheries, it is common to hold captured fish in refrigerated sea water (RSW) tanks for up to several days prior to landing. This is in contrast to the ice and chilled pre-freezing storage so far investigated and shown to increase rancidity, freeze

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denaturation, gaping, muscle softening and thaw drip in mackerel muscle (Aubourg, Lehmann, & Gallardo, 2002; Fukuda, Tarakita, & Arai, 1984; Hashimoto, Kawashima, Yoshino, Shirai, & Takiguchi, 2015). Moreover, while frozen storage of whole fish dominates the industrial process after landing, and frozen mackerel is highly susceptible to lipid oxidation (Sone, Skåra, & Olsen, 2019), the literature has mainly dealt with stress effects on mackerel and other fish species in combination with non-frozen storage (Anders et al., 2020; Bahuaud et al., 2010; Mishima et al., 2005). Accordingly, the objective of this study was to investigate if and how crowding stress at capture, *post mortem* RSW treatment and long-term frozen storage interact with one another to determine the final muscle quality of Atlantic mackerel.

2. Materials and methods

2.1. Experimental treatments

Using methods originally described by Anders, Roth, and Breen (2021), in July 2018 at the Austevoll Research Facility (60°N) in western Norway, schools of wild mackerel were benignly captured by attracting them into a 1728 m^3 aquaculture cage using feed pellets. In May 2019, using the same technique as described above, groups of mackerel were enticed from the aquaculture cage into two smaller net cages (5 × 5 × 5 m with an inverted 2.9-m-deep pyramidal section at the bottom; volume: 149.17 m^3) contained inside the larger cage. The pre-treatment stocking density of mackerel inside the control and treatment cages was 0.76 and 0.41 kg.m-3, respectively. They were allowed to acclimate in these cages for at least 48 h before the experiment began. The cages were mounted on a floating pontoon fixed approximately 100 m offshore. Fish foraged on natural prey items within cages with occasional supplementary feeding with aquaculture feed pellets. On the 21st of May 2019, ~75 fish were collected one-by-one from one of the cages using a handline and barbless hook to act as unstressed samples. On the 22nd of May, a crowding stress treatment was applied by manually lifting the second cage in the water column to achieve a fish density of ~180 kg/m^3. The fish were held at this density for ~60 min. This is analogous to a typical crowding density and duration that purse seine catches may experience (Huse & Vold, 2010). Once crowded, ~75 fish were removed one-by-one from this cage using a dip net. Removal of fish took place throughout the whole crowding period. Further detail of crowding methodology and associated procedures are described by Anders et al. (2021). All protocols were prospectively authorized by the local animal welfare authority (Mattilsynet, FOTS licence ID: 19238).

2.2. Blood physiology

Following capture, the first 23 (stressed group) or 21 (unstressed group) fish removed from the cages were assessed for behavioural vitality (results not reported here) and then euthanized using a percussive blow to the head. For these fish, up to ~ 3 mL of blood was collected from the caudal vasculature using 10 % EDTA treated syringes with 21G needles. The remaining fish in the sample (~50 each from the stressed and unstressed groups) were euthanised in the same way but were not assessed for vitality or blood physiology. Blood was immediately chilled on ice and processed into plasma within 4 h after collection. Resulting plasma was analysed for cortisol, sodium ions (Na^+), glucose and lactate concentrations. Analytical methodologies are described in full by Anders et al. (2020).

2.3. Pre-freezing holding in refrigerated seawater (RSW), frozen storage and sampling

After euthanisation and/or blood sampling, all fish were placed in 65L insulated plastic tubs (SÆPLAST Iceland ehf, Dalvík, Iceland) with RSW (−1.5 °C) for approximately-one hour. The mean of the initial temperature in the fish with standard deviation was 14.0 ± 1.2 °C (N = 3). The temperatures of the RSW and the fish were recorded every 30 s using a logger (Testo 176 T4, Testo SE & Co. KgaA, Titisee-Neustadt, Germany) with insertion sensors (Testo Type T (Cu-CuNI) 0628 0030) positioned close to the backbone at the centre of the fish. The fish without RSW treatment were removed from the RSW tank, placed individually in a PE-bag and frozen rapidly using dry ice reaching a temperature below −20 °C within 90 min. The remaining fish were kept immersed in the RSW tank and maintained a temperature range between −1.3 and 0 °C for three days, before being similarly frozen in the PE-bag using dry ice. In total, the experimental design consisted of four treatment groups of frozen fish representing crowded (stressed) and unstressed fish, which were either frozen immediately after slaughter or after three days of RSW treatment. All frozen samples were transferred on the following day to a freezer at −30 °C for two days, before they were further stored at −19 °C (−19.3 °C ± −1.2 °C) for either 6 or 12 months. In addition, five fish from each treatment group were kept at −80 °C to represent the storage time "0 months".

After 6 and 12 months of storage, 10 fish were randomly taken and individually weighed, and three 2.5-cm slices below the pectoral fin were collected from each using a SX350 Band Saw S (Dadaux Technologies, Bersaillin, France). These three samples and the remains of the fish were individually vacuum-packed and stored at −80 °C prior to the following analyses: the sample closest to the pectoral fin was used for fat content, fatty acid profile and determination of ATP breakdown compounds (K value) and muscle pH (at 0 and 6 months). Thiobarbituric reactive substances (TBARS) and cathepsin B/L-like activities were measured using the remaining two slices respectively. The remaining fish was thawed in water at 4 °C for four hours and filleted by hand at the core temperature between −2 and −1°C and subjected to analyses of muscle pH (at 12 months), texture (fillet firmness), colour and WHC.

2.4. Fat content and fatty acid profile

Lipids were extracted and quantified as described by Bligh and Dyer (1959). Fatty acid composition was determined as previously described (Bogevik, Nygren, Balle, Haugsgjerd, & Kousoulaki, 2018) by chromatography (GC) of fatty acid methyl esters (FAMEs). The GC analyses were conducted using a Trace GC gas chromatograph (ThermoFisher Scientific) with flame ionization detector (GC–FID), equipped with a 60 m × 0.25 mm BPX-70 cyanopropyl column with 0.25 μm film thickness (SGE, Ringwood, Victoria, Australia). Helium 4.6 was used as the carrier gas with constant flow mode at 1.2 mL/min. The injector and detector temperatures were set to 250 °C and 260 °C, respectively. The oven was programmed as follows: 60 °C for 4 min, 30 °C/min to 145 °C, then 1.2 °C/min to 217 °C, and 100 °C/min to 250 °C where the temperature was held for 7 min. The sample solution (3.0 μL) was injected in splitless mode, and the split was opened after 2 min. The identification of the FAMEs was performed by comparing the elution pattern and relative retention time against the reference FAME mixture (GLC-793, Nu-Chek Prep Inc., Elysian, MN, USA). Empirical response factors calculated from the areas of the GLC-793 mixture was used to correct chromatographic peak areas. Fatty acid composition was expressed on a sample basis as g/100 g 23:0 fatty acid methyl esters as internal standard.

2.5. Determination of ATP breakdown compounds (K value)

ATP-related breakdown compounds (adenosine-5′-diphosphate ADP, adenosine-5′-monophosphate, AMP, inosine-5′-monophosphate, IMP, inosine, HxR and hypoxhantine, Hx) in 0-month samples (i.e. stored at −80 °C) were determined based on the high-performance liquid chromatography (HPLC) procedure (Özogul, Taylor, Quantick, & Özogul, 2000) with modifications. A HPLC system (Waters Corporation, Milford, MA) was used for the analysis. Approximately-four grams of the sample was homogenized with 25 mL cold 8 % (w/w) perchloric acid solution for up to 1 min using (IKA T10 Basic, IKA, Staufen, Germany) before

being immediately placed on ice. The obtained homogenate was centrifuged for 10 min at 3000 rpm at 10–16 °C (Kubota 4200, Kubota Corporation, Tokyo, Japan). 15 mL of the supernatant was mixed with 10 mL of 2 M KOH solution and let stand for up to 30 min to reach pH ~1. The precipitated potassium perchlorate was removed through filtration (Black Ribbon, Whatman 589/1 ashless filter paper, Schleicher & Schuell, Munich, Germany) and the filtrate was diluted with the buffer solution without acetonitrile (0.21 M potassium dihydrogen phosphate, KH_2PO_4 and 0.004 M dipotassium hydrogen phosphate, K_2HPO_4, pH adjusted to 6.25 ± 0.02 with 2 M potassium hydroxide), to reach pH 5–7. The chromatographic separation of the individual nucleotide products was obtained by a 5 µm C18 column (4.6 × 150 mm mm [Sunfire, Waters, Ireland]) equilibrated at 25 °C, with continuous gradient elution with acetonitrile, the buffer solution and purified HPLC water. A 5 µL filtrate was injected into the HPLC system after filtration through a 0.45 µm filter membrane with the flow rate of 1.0 mL/min, and detection was at 254 nm. A standard curve for each nucleotide (Sigma-Aldrich) was prepared in the buffer solution at six concentrations in the range ~0.0015–0.06 mM, and peaks obtained from the sample were compared against those of the standard solutions. The K value was calculated based on the concentrations of ATP and ATP-related breakdown products using the following equation:

$$K = \frac{(H_xR + H_x)}{(ATP + ADP + AMP + IMP + H_xR + H_x)} \bullet 100(\%)$$

2.6. Muscle pH and water holding capacity (WHC)

Muscle pH was measured using 20 g of homogenized muscle mixed with 20 mL 0.15 M KCl using a benchtop pH meter at 0 month (SevenEasy pH, Mettler Toledo, Oslo, Norway), and at 6 and 12 months (EasyFive FP20, Mettler Toledo). The WHC of the muscle on the dorsal right fillet was determined as described by Skipnes, Østby, and Hendrickx (2007) using the following equation:

$$WHC = \frac{W_0 - \Delta W}{W_0} \bullet 100\%$$

where W_0 and ΔW represent the percentage of the initial water content and the exudates (the liquid separated from the sample during centrifugation) of the total sample weight, respectively. The water content of the sample was determined gravimetrically at 105 °C for 16–18 h. Two parallels were collected from each fillet for the WHC and water content analyses.

2.7. Fillet firmness

The muscle on the ventral left fillet was analyzed instrumentally using TA.XT Plus Texture Analyser (Stable Micro Systems ltd, Godalming, UK) equipped with 5 kg load cell. The cylinder probe (1/4″Ø, P/0.25) (Stable Micro Systems ltd) was pressed perpendicular to the muscle fiber at a constant speed of 1 mm/s to the end point at 90 % of the fillet height at three locations above the lateral line of each fillet. The time-force graph was recorded using the software Exponent (version 6.1.16.0). Breaking force (N) at fillet surface and force recorded at 60 and 90 % of fillet height (FH) was used to analyse the firmness of the sample.

2.8. Cathepsin B/L-like activities

Cathepsin B/L-like activity was determined according to Yang, Rustad, Xu, Jiang, and Xia (2015) with modifications. ca 2–10 g of sample in 20 mL distilled water was homogenised using T25 digital Ultra Turrax equipped with the S25 N 18G ST probe (IKA, Staufen, Germany) at 13,500 rpm in 30–40 s before being placed on ice for 30 min while stirring occasionally. The homogenised sample was centrifuged at 14 600 g for 20 min at 4 °C (Heraeus Multifuge x3 FR, ThermoFisher Scientific), and the supernatant was collected as crude enzyme extract and stored at −80 °C until analysis. 10 mM stock solution of the synthetic fluorogenic substrates *N*-carbobenzoxy-phenylalanyl- arginine-7-amido-4-methylcoumarin (Bachem Holding AG, Bubendorf, Switzerland) was prepared in dimethyl sulfoxide and stored at −20 °C until use. On the day of the analysis, the diluted substrate (12.5 µM) was prepared with distilled water. After thawing and centrifugation at 134,000 rpm for 60 s (Heraeus Fresco 17 Microcentrifuge, ThermoFisher Scientific), 15 µL of the crude enzyme extract was mixed with 135 µL activation buffer (150 mM Bis–Tris, 30 mM EDTA, 6 mM dithiothreitol, pH 6.0), vortexed and incubated at 30 °C for 10 min before 100 µL of the 12.5 µM substrate was added to activate the reaction at 30 °C for 10 min. The reaction was stopped by adding 1 mL of stopping buffer (1 % SDS 50 mM Bis-Tris, pH 7.0) into the reaction mixture before it was transferred immediately on ice for 10 min. The standard curve was constructed in the concentration range 0 to 1500 nmol using the fluorogenic product 7-amido-4-methylcoumarin (AMC) in distilled water. Amount of AMC was determined fluorimetrically at 360 nm excitation 460 nm emission wavelength using Synergy 2 Multi-Mode Microplate Reader (BioTek, VT, USA). The blank consisted of 150 µL activation buffer, 1 mL of stopping buffer and 100 µL substrate (12.5 µM). Cathepsin B/L-like activities were expressed as nmol AMC/g sample weight/min. The analyses were run in triplicate.

2.9. Fillet colour

Muscle surface colour of the right and left fillet was measured using a digital photo imaging colour-measuring system (VeriVide's DigiEye, VeriVide ltd., Leicester, UK) equipped with a DSLR camera (Nikon D90, Japan) and CIE D65 standard illuminant. Images were captured at three locations along the dorsal fillet, and the captured image was analyzed for CIELAB color scale using the DigiEye 2.9 software. The a^* and b^* values described the intensity of colour on the red–green axis ($a^* > 0$ = red, and $a^* < 0$ = green) and on the yellow–blue axis ($b^* > 0$ = yellow and $b^* < 0$ = blue) respectively, while L^* represented the sample lightness ($L^* = 100$ = white, and $L^* = 0$ = black). Furthermore, chroma (C*) represents the colour saturation calculated by $C^* = (a^{*2} + b^{*2})^{1/2}$. The hue angle ($h^*$) is the colour angle between a^* and b^*, where $h^* = 0°$ for reddish hue and $h^* = 90°$ for yellowish hue.

2.10. Lipid oxidation

Lipid oxidation measured as thiobarbituric acid-reactive substances (TBARS) values were determined as described by Dulavik, Sørensen, Barstad, Horvli, and Olsen (1998) with modifications. Approximately 7–8 g of the sample was homogenised in 30 mL of 10 % trichloroacetic acid, 0.1 % propyl gallate and 0.1 % EDTA (TCA extraction buffer) at 20 500 rpm for 30 s using T25 Ultra Turrax equipped with the S18N-10G probe (IKA, Staufen, Germany), and boiled in a water bath for 30 min. After cooling in an ice bath, the homogenised sample was centrifuged at 1500 rpm for 15 min at 4 °C (Heraeus Multifuge x3 FR, ThermoFisher Scientific). The supernatant was removed through Whatman 589/1 ashless filter paper (Schleicher & Schuell, Munich, Germany) and stored at −80 °C until further analysis. Upon analysis, 1 mL of the thawed extract was mixed with 1 mL of 0.6 % 2-thiobarbituric acid reagent (TBA) and heated at 95 °C for 30 min using a heating block (QBT4, Grant Instruments, Cambridge, UK) before cooling in ice for 10 min. The absorbance of the sample (250 µL) was read at 532 nm using Synergy 2 Multi-Mode Microplate Reader (BioTek, VT, USA). The calibration curve was constructed in the concentration range of 2–10 nmol/ using 1,1,3,3-tetraethoxypropane (Merck, Darmstadt, Germany) in the extraction buffer as malondialdehydbis (MDA) standard. The blank consisted of 1 mL TCA extraction buffer and 1 mL TBA solution. The amount of TBARS was expressed as nmol TBARS/g muscle. The analyses were run in triplicate.

2.11. Statistical analysis

The number of samples and replicates analysed per treatment group ("stressed", "unstressed", "no RSW treatment", "3 days RSW treatment") at the respective storage time (0, 6 or 12 months) is presented in Table S1. Many of the analytical methods for muscle quality involved replicate samples from the same fish. To account for such pseudo-replication, these datasets were analysed using linear mixed effects models (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). Either random intercept (the cathepsin, WHC and water content datasets), nested random intercepts (colour datasets) or random intercept plus slope structures (the texture datasets) were incorporated to account for replicates taken from the same fish, from the same fillet within the same fish, or for differences in fillet thickness within the same fish, respectively. Analysis of the lipid oxidation dataset incorporated similar nested random structures but with a gamma error structure with log link (i.e. a generalized linear mixed effect model). The pH dataset was analysed using multiple linear regression. The K value and blood physiology datasets were analysed using generalized least squares (GLS) regression. GLS modelling allows residual dependency to be incorporated into models via variance functions, thereby avoiding violations of model assumptions (Zuur et al., 2009). Variance functions were also applied to LME models where required.

Model fitting procedures were as follows: 1) removal of known erroneous datapoints; 2) a maximal model was fitted, considering (where possible) all interactions between the predictor variables of "RSW" (categorical: either "No RSW treatment" or "3 days RSW treatment"), "Storage" (categorical: either "0 months", "6 months" or "12 months") and "Stressed" (categorical: either "Stressed" or "Unstressed"); 3) model assumptions were checked using residual plots and residual dependency stabilized using variance structures, where necessary; 4) selection of the most parsimonious model, based on AIC and significance testing (models were reduced no further than main effects for the three predictor variables); and 5) the most parsimonious model was then assessed for violation of assumptions using residual plots.

Mixed and GLS models were fitted using restricted maximum likelihood (REML), with significance of predictor terms determined by Wald F testing or chi-squared testing. Multiple linear regression models were fitted using Ordinary Least Squares regression (OLS) with significance of terms determined by F testing. All statistical analysis was conducted in R (version 3.6.2), with a significance level of $p < 0.05$.

To facilitate a synthesis of the results, the effect sizes (% difference of the model coefficients) and associated *p* values in relation to the baseline of unstressed fish not subjected to RSW treatment or frozen storage were determined for the various covariates. No attempt was made to adjust for a potential increase in family-wise error rate across the multiple statistical analyses by inclusion of a Bonferroni-type correction of the p-values. The justification for this is that a Bonferroni-type correction would: i) *de facto* be testing an inappropriate hypothesis, namely that our control and treatments are not equal for all variables simultaneously; and ii) lead to an unacceptable increase in the risk of type II errors for inferences on individual variables, i.e. increasing the probability of accepting the null hypothesis when the alternative in in fact true (Nakagawa, 2004; Perneger, 1998).

3. Results and discussion

3.1. Fat and water content and fatty acid profile of raw material

The mean fat content of the mackerel was 16.5 ± 3.7 % (N = 4), corresponding to that of summer Atlantic mackerel in the Norwegian waters (Brix, Apablaza, Baker, Taxt, & Grüner, 2009). Considering that the total fat content is related to fish weight (Brix, et al., 2009), the mean weight of the mackerel in this study was high (812.6 ± 144.8 g, N = 87), suggesting that food availability and the occasional supplement of aquaculture feed pellets may have altered the muscle composition and fat deposition of the mackerel kept within the cage (Fjermestad, Hemre, Holm, Totland, & Froyland, 2000). The fatty acid profile of the mackerel was characterized by the dominant monounsaturated fatty acids (MUFA, mean 46.6 ± 2.4 g/100 g extracted lipids), followed by polyunsaturated fatty acids (PUFA, 28.0 ± 4.3 g/100 g) and saturated fatty acids (19.9 ± 1.0 g/100 g). Docosahexaenoic acid (C22:6n-3, DHA) (6.80 ± 1.8 g/100 g) and eicosapentaenoic acid (C20:5n-3, EPA) (4.0 ± 0.9 g/100 g) were among the most abundant PUFA. The mean water content of the fish was 67.3 ± 4.7 % (N = 100 fish; with 2 replicates per fish) and was not significantly affected by crowding stress ($p = 0.10$), RSW treatment ($p = 0.30$) or the frozen storage ($p = 0.79$), with no interactive effects being found either.

3.2. Blood physiology & stress responses

The crowding significantly elevated concentrations of plasma cortisol ($p < 0.001$), lactate ($p = <0.001$), glucose ($p = 0.03$) and sodium [Na^+] ($p < 0.001$) in the stressed mackerel above levels of the unstressed fish (Fig. 1). Cortisol and lactate increased substantially by 3.2 and 9.0 times, respectively. The increase due to crowding for glucose and sodium was 18 % and 14 % above control levels, respectively. These stress related responses were characterised by a high level of individual variability. Taken together, these results indicated that most individuals in the stressed group experienced a typical teleost physiological stress response (Wendelaar Bonga, 1997), characterized by the release of stress hormones (such as cortisol), which acted to increase both energy availability (such as glucose) and osmotic water loss across the gills (leading to increases in plasma ion concentrations, such as Na^+). The highly elevated levels of lactate in the crowded group suggests that anaerobic thresholds were exceeded, as has been demonstrated previously for crowded mackerel (Anders et al., 2020). This is likely explained by the tendency for crowding stress to induce increases in swimming activity in mackerel (Anders et al., 2019).

3.3. Nucleotide degradation (K value)

The extent of nucleotide degradation as affected by crowding stress and RSW treatment was assessed as K value in the sample prior to frozen storage. RSW treatment had a highly significant ($p = 0.0001$) effect on nucleotide degradation leading to a 4.7- and 5.7-fold increase in K-value for stressed and unstressed fish, respectively (**Fig. S1**). This indicated that nucleotide degradation continued in the mackerel muscle during RSW treatment, likely promoted by the low RSW temperature (Mochizuki & Sato, 1994) and higher activity of ATPase, as hypothesized by Gaarder, Bahuaud, Veiseth-Kent, Mørkøre, and Thomassen (2012).

Crowding stress was associated with a substantial increase in K values of 1.51 units, but this difference was not significant ($p = 0.1630$), most likely due to the small sample size (N = 6) and high individual variability in the RSW treated samples (**Fig. S1**). The lack of a significant stress effect contradicts the findings of Mochizuki and Sato (1994) who observed faster ATP degradation in mackerel stressed through asphyxiation compared to fish killed instantly. However, the rate of the ATP degradation is likely to vary depending on the killing method (slaughter stress), species and storage temperature. The effect of stress on the K value may also appear after a lag phase, as demonstrated by the same authors. This could account for the lack of any significant crowding stress effect on the fish in this study, which were frozen shortly after slaughter. Moreover, any stress effect on K value may have been nullified when the fish was subjected to the RSW treatment as observed for several mackerel species stored for up to 48 h *post-mortem* at non-frozen temperatures (Mishima et al., 2005; Mochizuki & Sato, 1994).

3.4. Muscle pH

Although not significant ($p = 0.1666$), crowding stress reduced muscle pH by 0.06 (equivalent to ~15 % higher acidity) (**Fig. S2**). This

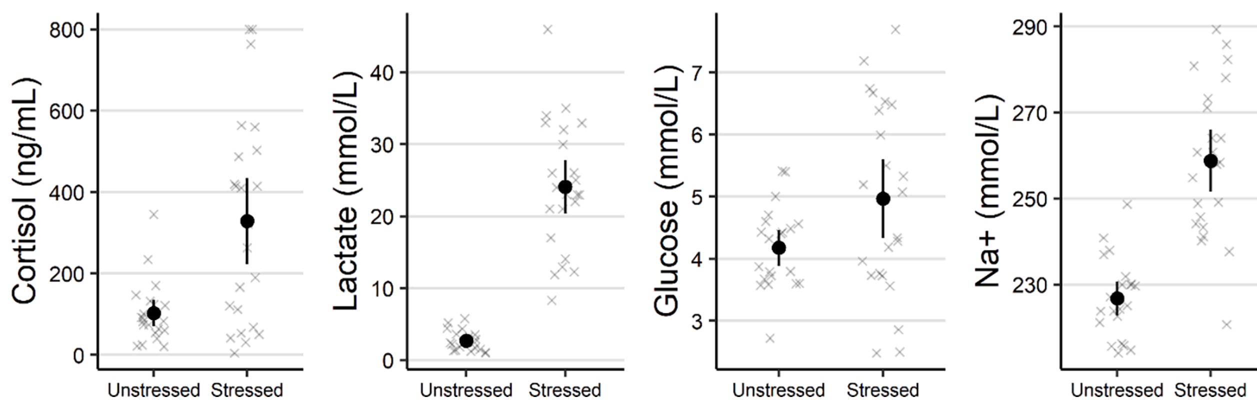

Fig. 1. Physiological responses in the blood plasma of stressed (crowded) and unstressed mackerel as measured by concentrations of sodium ions [Na^+] (mmol/L), cortisol (ng/mL), lactate (mmol/L), glucose (mmol/L). Points indicate model derived mean values with 95 % confidence intervals as whiskers. The underlying data is indicated with crosses.

agreed with stress-induced pH reduction previously reported for spotted mackerel (Ogata et al., 2016), Atlantic mackerel (Anders et al., 2020), salmon (Bahuaud et al., 2010) and cod (Hultmann et al., 2012) associated with elevated lactate production and proton accumulation through increased anaerobic metabolism in the muscle (Huss, 1988). The most parsimonious model to explain muscle pH demonstrated no significant influence of RSW treatment ($p = 0.9254$) or any interactive effects. The effect of frozen storage was, however, highly significant ($p = 0.0012$), demonstrating a slight increase from the initial pH (~pH 6.0) towards 6 months, followed by a decrease between 6 and 12 months of 0.2 (equivalent to a 58 % increase in acidity). Comparably low pH (pH < 6) has been demonstrated in *post mortem* muscle of several mackerel species (Fukuda et al., 1984; Hashimoto et al., 2015). Similar results were observed at the end of ice storage of Atlantic mackerel with and without crowding treatment (Anders et al., 2020).

3.5. Water holding capacity (WHC)

In general terms, WHC reduced throughout frozen storage, with the magnitude of this effect being modified by a highly significant interaction ($p = 0.0016$) with RSW treatment and *pre mortem* crowding stress (Fig. 2). Furthermore, WHC was significantly ($p = 0.0001$) and positively correlated with water content (Fig. 2). This apparent complexity, in which WHC is determined not only by the interactive effects of stress, RSW and frozen storage but also the properties of the raw material, implies that future experiments may need a more refined design.

As a main effect, frozen storage of mackerel is known to compromise the structural and functional properties of muscle proteins (such as muscle cell membranes and solubility of myofibrillar proteins), resulting in WHC alteration (Cropotova, Mozuraityte, Standal, Grøvlen, & Rustad, 2019; Nakazawa & Okazaki, 2020). Pre-freezing ice-cold storage also promotes freeze denaturation and the formation of larger ice crystals in the extracellular spaces of mackerel muscle during subsequent frozen

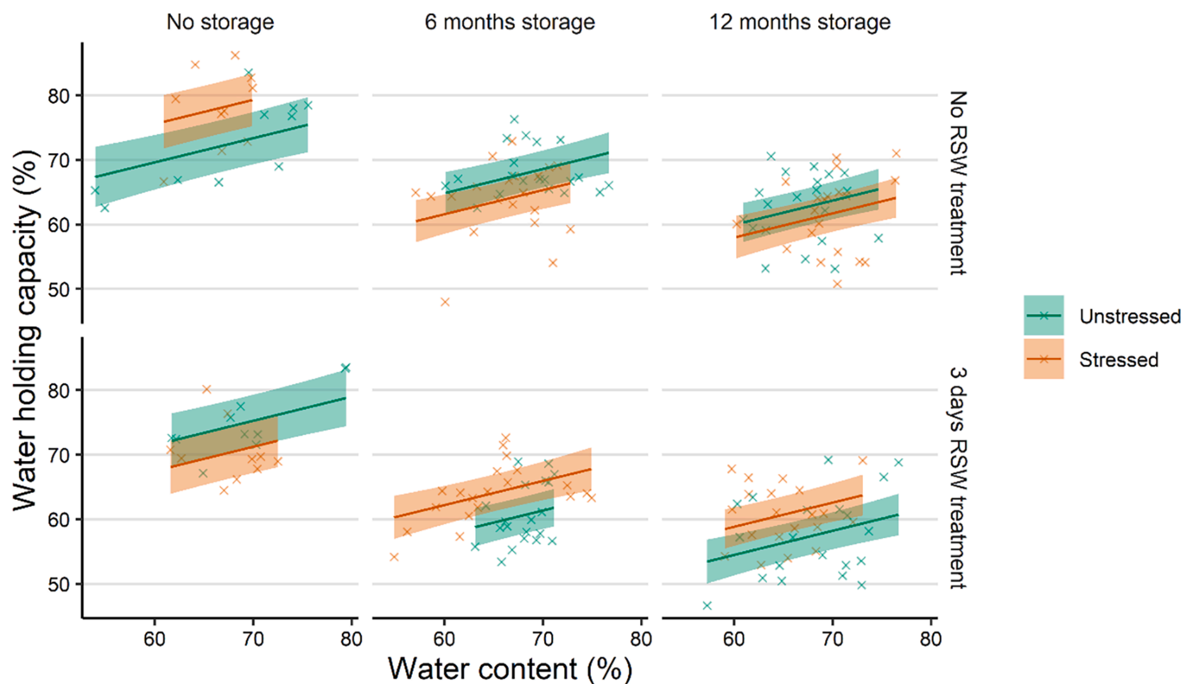

Fig. 2. The relationship between water holding capacity and water content in groups of Atlantic mackerel (*Scomber scombrus*) muscle, subjected to either crowding ("stressed", red) or no crowding ("unstressed", green) in aquaculture net cages. Post-mortem, fish were treated with or without refrigerated seawater (RSW treatment, between −1.3 and 0 °C) and then sampled after 0, 6 or 12 months of frozen storage (at −19 °C). The shaded areas indicate model derived 95 % confidence intervals. Crosses indicate the underlying dataset. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

storage (Fukuda et al., 1984; Hashimoto et al., 2015). On the other hand, brine immersion at low temperatures such as in RSW (often referred to as superchilling) has shown both positive and negative effects on various muscle quality parameters in salmon (Chan et al., 2020; Erikson, Misimi, & Gallart-Jornet, 2011). Some authors attribute the negative quality effects of superchilling to water and solute uptake and partial ice-crystalisation, causing cell rupture in the muscle and altering raw material properties (e.g. pH and solute concentration) and thereby impacting freezing dynamics and promoting protein denaturation, enzymatic reactions and autoxidation (Nakazawa & Okazaki, 2020). The water- and solute uptake and physiochemical changes in the muscle during RSW treatment can be influenced by the prior physiological state of the fish, e.g. following exhaustion, as crowding stress can increase plasma ionic concentration in mackerel (Fig. 1) (Anders et al., 2020; Anders et al., 2021). RSW treatment during commercial fishing also poses welfare and quality challenges as caught mackerel are likely killed by temperature shock. Such treatment has been shown to accelerate *post mortem* quality loss in certain mackerel species, in a manner similar to death by asphyxia (Mochizuki & Sato, 1994).

3.6. Fillet firmness

Fillet firmness measured at 90 % of the fillet height (FH) was influenced by RSW treatment through a significant interaction with frozen storage ($p = 0.0253$). This resulted in softer fillets compared to fish not treated with RSW (by 1.88 N and 0.88 N after 6 and 12 months of frozen storage, respectively) (Fig. 3B). Fillet firmness of fish without RSW treatment varied little throughout storage at 90 % FH, while the effect of crowding stress was significant ($p = 0.0127$) and caused an average decrease in fillet firmness of 10.6 %. Interestingly, RSW treatment significantly ($p < 0.0001$) increased breaking force by 0.6 N (**Fig. S3**). Significant main effects of frozen storage ($p = 0.0178$) were also found, with an increase in breaking force from 0 to 6 months followed by a decrease between 6 and 12 months. Similar fluctuations during storage occurred at 60 % FH, with marginally significant main effects of frozen storage ($p = 0.0500$) (Fig. 3A). In a similar way to 90 % FH, RSW treatment yielded softer fillets as a main effect ($p = 0.0090$) at 60 % FH. Stress and frozen storage interacted significantly ($p = 0.0036$) at 60 % FH, with stressed fish undergoing a significant reduction in firmness of 0.89 N between 6 and 12 months (Fig. 3A).

The breaking force represents the yield force of the connective tissue near the fillet surface, while force measured at 90 % FH will largely provide an indication of overall fillet firmness. Force at 60 % FH may reflect both. These results imply possible spatial differences in denaturation mechanisms which are in effect due to stress, RSW treatment and frozen storage alone and in interaction. Cropotova et al. (2019) observed hardening of superchilled, vacuum-packed mackerel fillets despite increased proteolytic activity, which the authors attributed to accelerated protein oxidation in the myofibrillar proteins of the superchilled fish. This agrees with the increased breaking force of RSW treated fish in this study, which may have been impacted by larger ice crystals and increased solute concentrations in the muscle, promoting intermolecular cross-links, protein aggregation and eventually textural hardening at the surface (Cropotova et al., 2019 and references therein). Besides the detrimental effects of pre-freezing ice- and chilled storage discussed above, the combination of RSW treatment and non-frozen storage has been shown to result in decreased fillet firmness in salmon (Chan et al., 2020; Erikson et al., 2011) corresponding to the significant interaction of RSW and frozen storage we observed at 90 % FH. This has so far attributed to the change of the chilling medium from liquid phase to air by the same authors, but the mechanisms behind it remain uninvestigated.

Corresponding to the significant effect of stress at 90 % FH, struggling at slaughter has been shown to promote collagen fibril disintegration, weaken the binding forces in the pericellular connective tissues and create larger intracellular spaces in mackerel muscle (Ando et al., 2001; Sato et al., 2002). Stress-induced reduction in muscle pH, high levels of blood lactate (Fig. 1) and mechanical damage in the muscle can further exacerbate muscle softening (Bahuaud et al., 2010; Roth et al., 2006). However, such effects of stress preslaughter and at killing on fillet firmness have only been demonstrated early *post-mortem* during non-frozen storage in mackerel (Ando et al., 2001; Sato et al., 2002), and it has been argued that extended storage will cause any treatment effects to gradually converge and result in minimal differences (Anders et al., 2020). Our study demonstrated that crowding stress influenced fillet firmness during long term frozen storage, as both a main effect (90 % FH) and interactively (60 % FH) with frozen storage and its concomitant freeze denaturation.

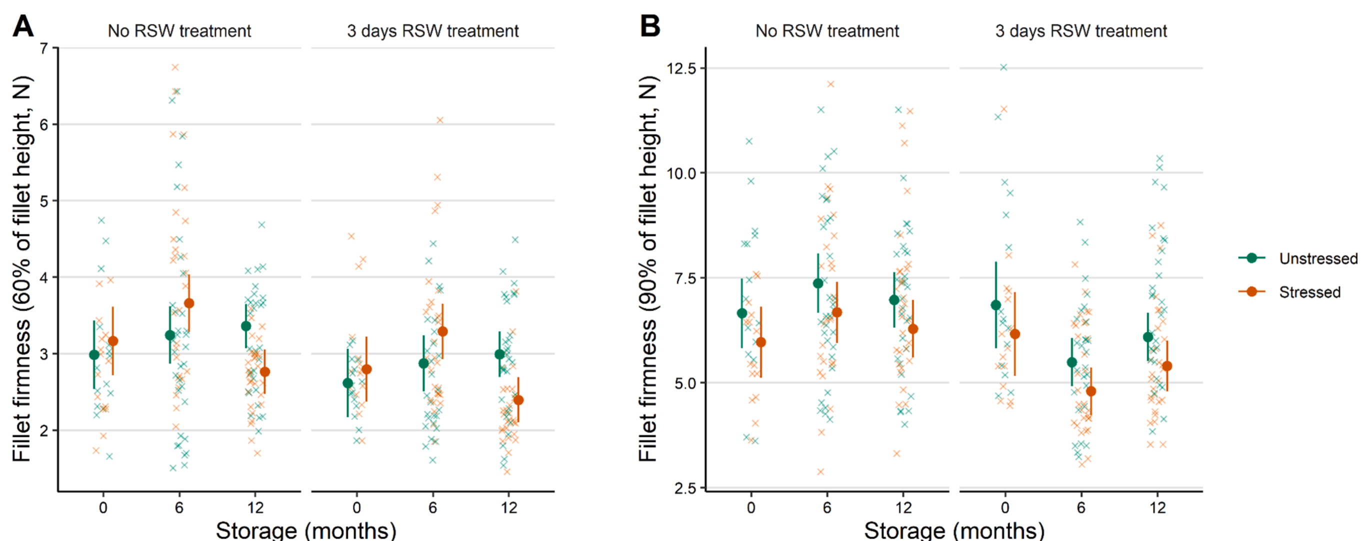

Fig. 3. A and B: The temporal effects of frozen storage (−19 °C) at 0, 6 and 12 months on Atlantic mackerel (*Scomber scombrus*) fillet firmness, expressed as compression force (N) at: (A) 60 % and (B) 90 % of fillet height. Prior to freezing, groups of mackerel were subjected to either crowding ("stressed", red) or no crowding ("unstressed", green) in aquaculture net cages, followed by either refrigerated seawater (RSW) treatment (between −1.3 and 0 °C) or no RSW treatment. Points indicate model derived mean values with 95 % confidence intervals as whiskers. The underlying data is indicated with crosses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.7. Cathepsin B/L like activity

Cathepsin B/L like activity was significantly elevated by crowding stress (p = 0.0142) at the point of death, by 39 % and 45 % for RSW treated and non-RSW treated fish, respectively (Fig. 4). There was a significant interactive effect between crowding stress and frozen storage (p = 0.0137), which effectively nullified the effect of stress by 12 months. RSW treatment also showed a tendency to increase enzyme activity up to 6 months, but to a lesser extent than the stress effect. Furthermore, there was a significant interaction between the RSW treatment and frozen storage (p = 0.0147).

Increased cathepsin activities have been reported in fish subjected to (pre-)slaughter stress, superchilling and non-frozen storage, either alone or in combination. This is presumably due to the accelerated breakage of the lysosomal membranes and increased release of proteolytic enzymes, promoted by stress-induced mechanical stress and pH change, formation of disruptive ice crystals and myocyte apoptosis (Bahuaud et al., 2010; Cropotova et al., 2019; Gaarder et al., 2012; Lerfall et al., 2015). When fish were not subjected to RSW treatment in this study, there was a significant increase in the cathepsin activity between 6 and 12 months for unstressed mackerel. This may be due to delayed leakage of enzymes by increasing freeze denaturation (Yang et al., 2019); enzymes which had initially been protected from cell lysosomes due to minimum pre-slaughter stress and rapid freezing immediately after slaughter. For stressed fish, on the other hand, crowding likely accelerated the release and activity of the enzymes prior to frozen storage (as discussed above). In addition to introducing greater variability (Fig. 1), this may have negated any freeze denaturation effect and resulted in the observed non-significant increase between 6 and 12 months for stressed fish. With RSW treatment, the cathepsin activity generally decreased during frozen storage. This could be due to the pre-freezing RSW treatment accelerating the release and activity of enzymes, resulting in higher cathepsin activity (as detected in the RSW treated fish up to 6 months) which subsequently denatured at the frozen storage temperature as hypothesised by Yang et al. (2019).

3.8. Lipid oxidation (TBARS)

TBARS concentration increased significantly (p = 0.0101) throughout frozen storage (**Fig. S4**), as the susceptibility of frozen mackerel muscle to lipid oxidation is well established (Sone et al., 2019). Although no evidence of a significant stress effect (p = 0.8616) was found on TBARS concentration, preslaughter stress or during killing has been previously shown to accelerate lipid oxidation in fish muscle (Secci & Parisi, 2016 and references therein). This has been attributed to cell rupture, generation and activation of highly reactive species such as hydroperoxides, which is also catalysed by stress-induced anaerobiosis and rapid degradation of ATP *post-mortem*. However, stress responses to lipid degradation may be species-dependent, as Duran, Erdemli, Karakaya, and Tyilmaz (2008) observed a significant stress-related increase in lipid oxidation in rainbow trout (*Oncorhynchus mykiss*) but not in carp (*Cyprinus carpio*). RSW treatment increased TBARS concentration by 17 %, although the effect was not significant (p = 0.1157). This corresponds with Aubourg et al. (2002), who observed that pre-freezing ice storage accelerated rancidity in horse mackerel. The immersion in RSW may also increase the exposure of mackerel muscle to pro-oxidative compounds such as blood and NaCl (Sone et al., 2019 and references therein).

3.9. Fillet colour

Crowding stress, RSW treatment and frozen storage all interacted to

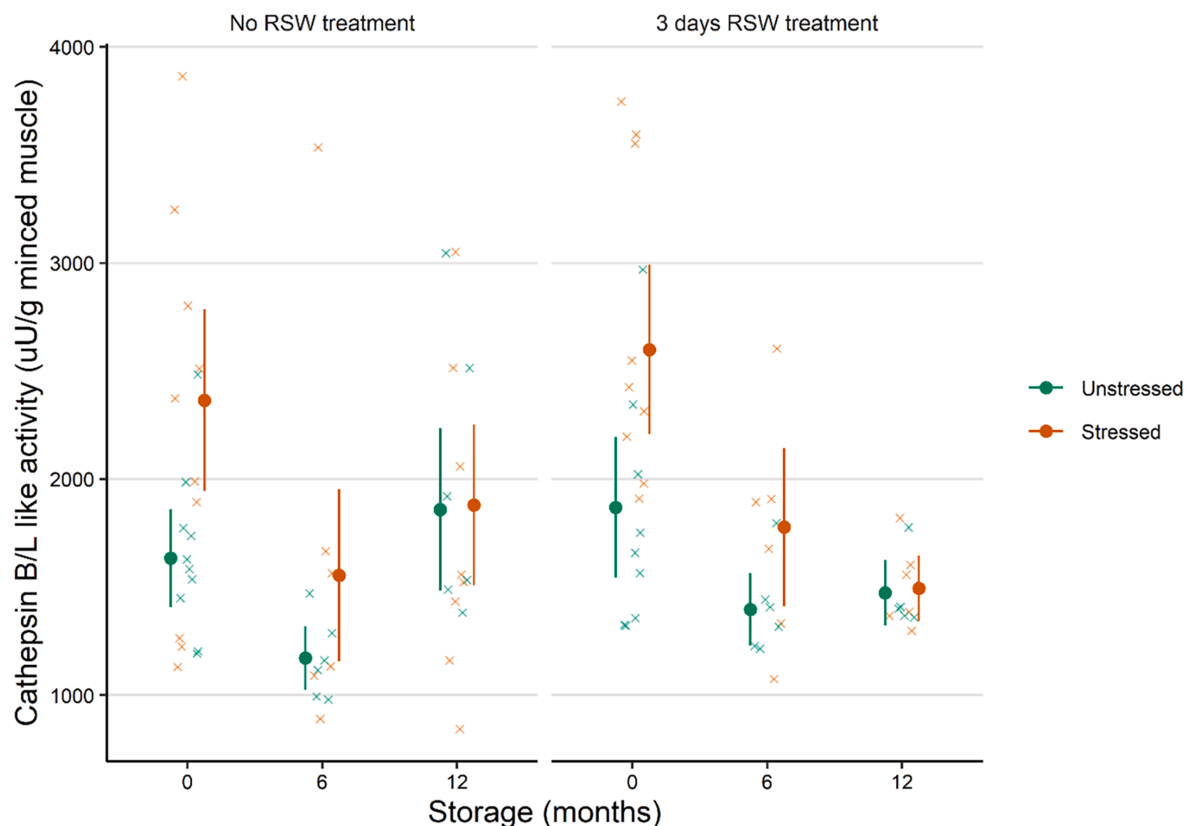

Fig. 4. The temporal effects of frozen storage (at −19 °C) on cathepsin B/L like activity in Atlantic mackerel (*Scomber scombrus*) muscle. Prior to freezing, groups of mackerel were subjected to either crowding ("stressed", red) or no crowding ("unstressed", green) in aquaculture net cages, followed by either refrigerated seawater (RSW) treatment (between −1.3 and 0 °C) or no RSW treatment. Points indicate model derived mean values with 95 % confidence intervals as whiskers. The underlying data is indicated with crosses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

determine fillet redness (a^*) (p = 0.0178) (Fig. 5A) and hue angle (h^*) (p = 0.0346) (Fig. S7). However, the directionality in the stress and RSW effect within the interaction terms was inconsistent making it difficult to explain. It is notable that as main effects, neither stress nor RSW treatment had a significant effect on a^* (p = 0.7385 and p = 0.6915, for stress and RSW treatment respectively) or h^* (p = 0.4862 and p = 0.1457, for stress and RSW treatment respectively). Prior to frozen storage, fillet colour of stressed fish increased in redness (a^*) with 3-day RSW treatment compared to unstressed fish. Although this may indicate that *post mortem* RSW treatment amplified the effect of stress on quality, the effect was marginal (17 % increase) and the confidence intervals still overlapped. Stress and RSW treatment played a significant role in determining yellowness (b^*) (p = 0.0310 and p = 0.0104, for stress and RSW treatment respectively) (Fig. 5B) and colour saturation (C^*) (Fig. S6) (p = 0.0155 and p = 0.0272, for stress and RSW treatment respectively), but the sizes of these effects were small, and no interactive effects were evident. Similarly, stress (p = 0.0131) and RSW treatment (p = 0.0341) significantly increased the fillet colour lightness (L^*) as main effects (Fig. S5), with the stressed and RSW treated fish having 2.4 % and 2.0 % lighter fillets on average than unstressed fish and fish subjected to no RSW treatment respectively. No evidence of a significant effect of the frozen storage was found on the fillet lightness (p = 0.7961), and no interactive effects between the three experimental variables were evident.

The effect of frozen storage on a^* and b^* was most evident, supported by its high significance as a main effect (p = 0.0001 and $p < 0.0001$, for a^* and b^* respectively). In general terms, fillets became less red and more yellow over time, with the most significant colour change occurring during the first six months of the frozen storage. On average, fillets become 13.6 % more yellow and 14.8 % less red between 0 and 6 months (Fig. 5A and B). Correspondingly, the effect of frozen storage was also highly significant as a main effect for h^* ($p < 0.0001$), with a mean increase of 15 % between 0 and 6 months (Fig. S7) but induced only a small and non-significant increase in C^* (mean increase of 4 %, p = 0.1284) (Fig. S6). Strong correlation between redness (a^*) loss and lipid oxidation has been established (Cavonius & Undeland, 2017 and references therein), reflecting a conversion from oxy- and met-haemoglobin (Hb) to met-Hb/myoglobin and a tentative haem-ring destruction, while the concomitant increase in yellowness (b^*) has been attributed to the formation of tertiary lipid oxidation products, e.g. Schiff bases and their further polymerization into yellow pigments. The changes in haem proteins, particularly the haem-ring destruction, have also been considered responsible for the increased fillet lightness during frozen storage of herring fillets (Cavonius & Undeland, 2017). This implies that crowding stress and RSW treatment in our mackerel led to increased Hb-mediated lipid oxidation, although this was not evident in the TBARS results.

4. Synthesis & conclusions

This study has been the first attempt to describe the collective and interactive effects of crowding stress, pre-freezing holding in RSW (3 day) and long-term (up to 12 months) frozen storage on the muscle quality of Atlantic mackerel. All three treatments affected quality parameters and muscle properties to different degrees. Table 1 shows the effect size (% difference) and associated *p* value for the various covariates with respect to the baseline (i.e., unstressed fish that were not subjected to RSW or frozen storage).

Frozen storage (6 and 12 months at −19 °C) generally showed the most consistently large and significant main effects on the fillet quality parameters, including: substantial (>10 %) increases in lipid oxidation (TBARS), breaking force at fillet surface, yellowness (b^* colour) and hue (h^*) of fillet colour; and substantial decreases in WHC (as a co-variate with water content) and fillet redness (a^* colour). However, WHC, a^* and h^* values were also significantly modulated by complex three-way interactions with crowding stress and RSW treatment. There were also effects of frozen storage on muscle pH and cathepsin B/L like activity, although these were both time dependent, and cathepsin activity was also modified through interactions with RSW treatment and crowding stress.

Pre-freezing holding in RSW for 3 days, as a main effect, substantially increased nucleotide degradation, resulting in significantly higher K values (by 473 %). It also substantially increased the breaking force at fillet surface, while reducing fillet firmness measured at 60 % as well as 90 % FH through a significant interaction with frozen storage. RSW treatment had small (<10 % effect size) but significant effects on fillet colour: increasing lightness (L^*), reducing yellowness (b^*) and colour saturation (C^*), as well as affecting redness (a^*) and hue (h^*) through complex interactions with crowding stress and frozen storage. There was no substantive evidence that *post mortem* RSW treatment mitigated any

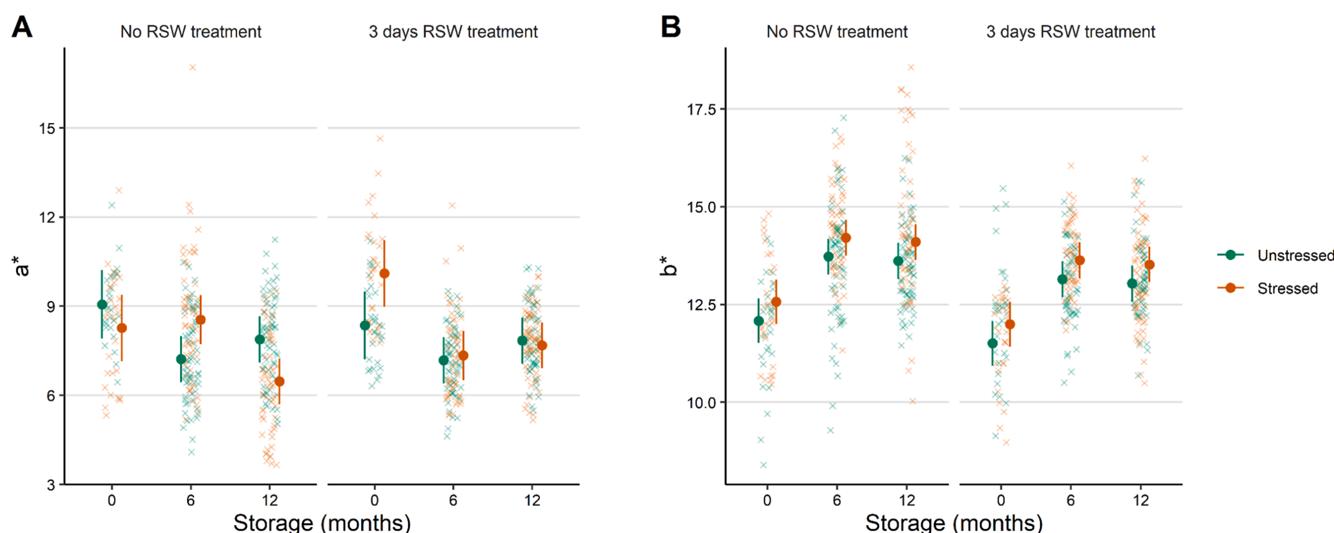

Fig. 5. A and B: The temporal effects of frozen storage (at −19 °C) on: (A) a^* (green–red component) and (B) b^* (yellow-blue component) in CIELAB colour space of Atlantic mackerel (*Scomber scombrus*) fillets. Prior to freezing, groups of mackerel were subjected to either crowding ("stressed", red) or no crowding ("unstressed", green) in aquaculture net cages, followed by either refrigerated seawater (RSW) treatment (between −1.3 and 0 °C) or no RSW treatment. Points indicate model derived mean values with 95 % confidence intervals as whiskers. The underlying data is indicated with crosses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Effect sizes (% difference of the model coefficients) and associated p values of crowding stress (Stress), pre-freezing holding in RSW (RSW) and long-term frozen storage for 6 and 12 months (M) in relation to the baseline of unstressed fish not subjected to RSW treatment or frozen storage.

	Main effects							2-way interactive effects									3-way interactive effects		
	Stress		RSW		Frozen storage (M)			Stress x RSW		Stress x Frozen storage			RSW x Frozen storage			Stress x RSW x Frozen storage		
Response	% effect	*p*	% effect	*p*	6M % effect	12M % effect	*p*	% effect	*p*	6M % effect	12M % effect	*p*	6M % effect	12M % effect	*p*	6M % effect	12M % effect	*p*
Water content	-2.10	0.0804	-1.85	0.1256	-1.03	-0.40	0.7810	-	-	-	-	-	-	-	-	-	-	-
K value	28.88	0.1630	472.78	0.0001	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pH	-0.97	0.1666	0.02	0.9254	1.50	-1.83	0.0012	-	-	-	-	-	-	-	-	-	-	-
WHC[§]	12.60	0.6826	4.02	0.0009	-10.13	-20.50	<0.0001	-21.14	0.0510	-19.52	-16.81	0.9642	-19.35	-15.64	0.8897	37.77	34.60	0.0016
Breaking force	-8.85	0.3513	25.80	<0.0001	19.90	10.33	0.0178	-	-	-	-	-	-	-	-	-	-	-
Firmness 60%	6.06	0.2289	-12.35	0.0090	8.60	12.55	0.0500	-	-	-7.87	-26.01	0.0036	-	-	-	-	-	-
Firmness 90%	-10.33	0.0127	2.94	<0.0001	10.71	4.81	0.6256	-	-	-	-	-	-31.20	-16.25	0.0253	-	-	-
Cathepsin B/L	44.71	0.0142	14.38	0.1097	-28.25	13.75	<0.0001	-	-	-21.32	-43.41	0.0137	-0.67	-37.94	0.0147	-	-	-
TBARS	1.03	0.8616	9.55	0.1157	14.11	23.42	0.0101	-	-	-	-	-	-	-	-	-	-	-
*L** colour	2.46	0.0131	2.09	0.0341	0.80	0.86	0.7961	-	-	-	-	-	-	-	-	-	-	-
*a** colour	-8.73	0.7385	-7.77	0.6915	-20.36	-13.00	0.0001	28.07	0.2854	23.44	-6.85	0.0224	7.37	7.33	0.0712	-40.99	-14.23	0.0178
*b** colour	4.01	0.0310	-4.77	0.0104	13.50	12.64	<0.0001	-	-	-	-	-	-	-	-	-	-	-
*C** colour	3.55	0.0155	-3.22	0.0272	3.95	3.04	0.1284	-	-	-	-	-	-	-	-	-	-	-
*h** colour	6.87	0.4862	5.44	0.1457	17.74	13.72	<0.0001	-18.65	0.1630	-11.66	3.82	0.1196	-6.96	-6.53	0.1829	24.81	8.94	0.0346

[§] Effect size and *p value* of water content (%) were 0.80 and *p* = 0.0001 respectively.

Colour coding:					
	p values (≤0.05)	>0.01	>0.001	>0.0001	≤0.0001
	Effect size - detrimental		>10%	>25%	>100%
	Effect size - beneficial		>10%	>25%	>100%

effects of *pre mortem* crowding stress.

Pre mortem crowding stress, at first viewing, appears only to have substantial and significant main effects on two quality parameters: increasing cathepsin activity and reducing fillet firmness at 90 % FH. Furthermore, these effects are only marginally significant and are co-dependent on interactions with frozen storage (and RSW treatment, for cathepsin activity). Like RSW treatment, crowding stress also had small and marginally significant main effects on fillet colour: increasing lightness (*L**), yellowness (*b**) and colour saturation (*C**). However, crowding stress also significantly affected a further four quality parameters through interactions with RSW treatment and/or frozen storage: WHC, fillet firmness at 60 % FH, colour *a** and *h**. In addition, K value was substantially elevated by crowding stress (by 29 %), but the effect was not statistically significant. Crowding stress responses in Atlantic mackerel have inherently high individual variability (Anders et al., 2020). This, in combination with a low sample size (N = 6), likely explain this contradictory statistical inference.

Clearly, without the comprehensive coverage of quality metrics and complex 3-way treatment design of this experiment, the potential importance of crowding stress in pre-determining the effects of RSW treatment and frozen storage on mackerel muscle quality would not have been realized. However, high individual variability in the quality and physiological stress responses has resulted in wide confidence intervals and marginally significant effects which demands cautious interpretations of the relationships between the quality parameters and crowding stress, RSW treatment and frozen storage. Moreover, the experimental unit (the cage) was replicated only once, making it difficult to characterise any variability that may exist in responses between different populations of fish. For more definitive inferences about these potential effects, further studies with larger sample sizes would be needed to better elucidate the mechanisms involved at and across these consecutive stages of the supply chain affecting fillet quality in Atlantic mackerel.

In conclusion, each of the treatments described in this study (crowding stress, RSW treatment and frozen storage at −19 °C) has the potential to substantially affect fillet quality in Atlantic mackerel, either directly or through complex interactions with the other treatments. Clearly, further work is required to better describe the natural variation in these interactive effects, as well as to better understand their underlying mechanisms, particularly for crowding stress for which there is little information. This knowledge would be invaluable for developing future best practice to promote the final muscle quality and storage stability of Atlantic mackerel.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134819.

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