Full title:

Differential response to air exposure in crowded and uncrowded Atlantic cod (*Gadus morhua*): Consequences for fillet quality

Running title:

Crowding and air exposure of Atlantic cod: Consequences for fillet quality

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Declarations of interest: none

Abstract

Previous recommendations on bleeding of Atlantic cod (*Gadus morhua*) to secure proper blood drainage and good muscle quality, are based on studies done on un-stressed fish. Therefore, the aim of this study was to investigate the effects of stress by crowding in a net, followed by air exposure before and after slaughter on blood parameters and fillet quality in Atlantic cod. Live fish were either directly or after 4 h of crowding, exposed to air for 0, 15 or 30 min prior to or after killing by a blow to the head and bleeding. Blood clotting time, pH, lactate, glucose, and muscle pH were measured. Also, concentrations of haemoglobin in the muscle were measured using Vis/NIR hyperspectral imaging to indicate residual blood in muscle. Stress from crowding and air exposure before and after slaughter resulted in increased levels of muscle haemoglobin in the fillet, with a faster increase in fish crowded and slaughtered post air exposure. Blood clotting time was shorter after 15 min of air exposure, and decreased further with crowding. Blood and muscle pH, and lactate levels were mainly affected by air exposure time. Overall, air exposure had a negative effect on fillet quality, and this effect was strongest and faster if fish were crowded prior to the air exposure. However, by slaughtering the fish before air exposure, quality can be improved as this delays the increase in the amount of residual blood.

Key words: Crowding stress, blood clotting, haemoglobin in muscle, fisheries, fish physiology, bleeding, Atlantic cod, *Gadus morhua*

1. Introduction

To secure a high quality fish product, it is crucial to drain blood from the fish muscle. Residual blood in the muscle is a major quality problem aesthetically, but also because haemoglobin accelerates lipid oxidation causing an unpleasant fishy odour (Maqsood et al., 2011; Richards et al., 2002; Terayama et al., 2000). In addition, high levels of blood in fish muscle can have a negative impact on shelf life due to increased microbial growth (Maqsood & Benjakul, 2011).

On board trawlers and Danish seiners, the final phase of the fishing operations includes hauling the catch from the water and on board the fishing vessel, where the catch is commonly stored in bins without water until further processing in an onboard factory or exsanguinated and left in bins until landing. Proper exsanguination can be challenging, because catches are large and the fish are alive and vigorous. It is therefore common practice on many fishing vessels that the fish are kept a while in air prior to exsanguination, as they then become moribund and easier to handle. For this reason, bleeding of the fish is often done after a period of asphyxiation (Van De Vis et al., 2003).

It has previously been shown that the time from slaughter to bleeding is an important parameter for proper exsanguination in Atlantic cod (*Gadus morhua*), as fillet quality decreased with time due to higher levels of residual blood (Olsen et al., 2014). It was therefore concluded that the fish should be bled within 30 min after slaughter to secure a high fillet quality. However, this recommendation was based on results from unstressed fish and is therefore likely less relevant in commercial fisheries, where fish are exposed to a number of stressors, such as exhaustive swimming, crowding and barotrauma.

Capture stress has been observed in Atlantic cod by, for example, higher levels of blood lactate and lower levels of muscle and blood pH (Digre et al., 2017; Olsen et al., 2013), compared to cod that were kept rested in tanks (Svalheim et al., 2017). Furthermore, stress can have a negative impact on fillet quality, as the amount of blood in the muscle tissue tends to increase

with higher levels of stress (Botta et al., 1987; Digre et al., 2017; Esaiassen et al., 2004; Olsen et al., 2013; Rotabakk et al., 2011). In addition to stress from capture, stress from the practice of holding fish in air before exsanguination may further degrade the muscle quality of the fish. Another effect of stress is that blood-clotting time is shortened (Ruis et al., 1997; Tavares-Dias et al., 2009). This response is of paramount importance to stop the bleeding after a vascular injury and prevent blood loss in live fish, but will have an impact on quality if it affects the efficiency of bleeding. These haemodynamic and haemostatic changes may impair the bleeding process resulting in increased residual blood in the fish muscle, and thereby reduce fillet quality. The previously concluded 30 min recommendation may therefore underestimate how quickly the fish should be bled, to avoid quality defects due to residual blood in the muscle.

The aim of the present study was to investigate if stress (measured using blood lactate, glucose and pH) from crowding and air exposure for 0, 15 or 30 minutes has an effect on muscle quality in terms of residual blood as measured by muscle haemoglobin. In addition, the potential of blood clotting time as a response to stress as a contributing factor to the levels of muscle haemoglobin, was investigated.

2. Material and methods

2.1 Animals and husbandry

A total of 180 Atlantic cod (body mass 5.9 ± 2.2 kg, body length 89 ± 10 cm, and condition factor 0.81 ± 0.15 (mean ± SD); 27% females and 73% males) were used in the experiment. The fish were captured by Danish seine mid-May 2015 and kept on board in tanks supplied with running seawater. Fish were delivered to a live fish storage facility in Nordvågen, Norway for recuperation for 3 weeks followed by a 300 km transportation by boat to the Aquaculture Research Sea Facility in Tromsø, Norway. Here, the fish were held in a $5 \times 5 \times 10$ m^3 (length x width x depth) net pen until the start of the experiment in November 2015 (water temperature

7.5°C). Fish were fed three times a week with a mixture of capelin (*Mallotus villosus*) and commercial feed pellets (Skretting Amber Neptun 5 mm, Skretting ASA, Stavanger, Norway). Feeding was stopped two days prior to the experiment to ensure an empty gastrointestinal tract, as the nutritional status may influence how blood is distributed in the fish (Axelsson & Fritsche, 1991).

2.1.Experimental set up

An overview of the experimental groups is shown in Table 1. The experiment was done over the course of two days. On the first day, 40 fish were carefully collected by dip net from the net pen and immediately killed by two cranial blows, of which 10 fish were sampled for physiological measurements (control, Table 1: A1.0), and 10 fish were bled for 30 minutes in running seawater (Table 1: A1.0) and stored on ice for consecutive muscle haemoglobin analysis. The remaining 20 fish were kept in a holding bin for either 15 (N=10, Table 1: A1.15) or 30 (N=10, Table 1: A1.30) minutes prior to exsanguination and sampling. Next, 40 fish were exposed to air for either 15 (n=20, Table 1: A2.15) or 30 (n=20, Table 1: A2.30) minutes before being killed by two a cranial blows from a metal rod followed by exsanguination. Ten fish of both groups were used for physiological analyses and ten for haemoglobin measurements in muscle. On the second day, fish were first crowded for 4 hours by using a seine to reduce the volume available for ca. 100 fish to approximately 2 m^3 (fish density: ~295 kg m^{-3}). During crowding, oxygen measurements were obtained every 30 min (O_2: 66 ± 1%) using YSI ProODO handheld dissolved oxygen metre with a ProODO Optical probe (Yellow Spring Instruments, Ohio, USA). Afterwards, fish were treated following similar procedures of air exposure prior to or after slaughter as the fish on the first day. The study was done in accordance with Norwegian and European legislation related to animal research, and approved by the Norwegian Animal Research Authority (id 8222, 13.11.2015).

Table 1: Overview of experimental groups, where A = not crowded, B = crowded, 1= not euthanised, 2 = euthanised. 0 = no air exposure, 15 = 15 min of air exposure, 30 = 30 min of air exposure. All groups were sampled for haemoglobin measurements in the fillet, groups that were also sampled for physiological measurements are indicated by asterisk (*).

Group name	**Crowded**		**Euthanised**		**Air exposure (min)**		
	No	Yes	No	Yes	0	15	30
A1.0*	×			×	×		
A2.0	×		×		×		
B1.0*		×		×	×		
B2.0		×	×		×		
A1.15	×			×		×	
A2.15*	×		×			×	
B1.		×		×		×	
B2.15*		×	×			×	
A1.30	×			×			×
A2.30*	×		×				×
B1.30		×		×			×
B2.30*		×	×				×

2.2 Blood sampling

Within approximately one minute after slaughter, blood was collected from the caudal vessels using 7 ml unheparinised vacutainers with 40 × 0.9 mm needles (BD Diagnostics, Franklin Lakes, NJ, USA). Blood lactate and glucose were measured in whole blood samples, using the hand-held analysers Lactate Scout+ (SensLab GmbH, Leipzig, Germany) and FreeStyle Lite (Abbott Diabetes Care, Inc., Alameda, CA, USA), respectively.

2.3 pH measurements

Muscle pH was measured by inserting a Hamilton double pore glass electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland) of a WTW330/set-1 pH-metre (Wissenscaftliche-Technische Werkstätten, Weilheim, Germany) 15 mm into the epaxial part of the white muscle, about 30 mm ventral to the front of the first dorsal fin on the left side of the fish. Blood pH was measured in the pericardium after puncturing the *aorta ventralis*. The instrument was calibrated using pH 4.01 and 7.00 buffers at 7.5°C, and the electrode was rinsed with demineralized water between each measurement.

2.4 Blood clotting measurements

Evaluation of blood clotting time was done as previously described in Ruis and Bayne (1997). Briefly, approximately 1 mL blood was carefully decanted into 4 parallel Trombotest tubes (Trombotestrør PS 14 x 80mm, 7 ml, HEGER A.S, Rjukan, Norway,). The tubes were held in a water bath at the ambient water temperature (7.5°C). Every 30 seconds, the tubes were tilted to a ~60° angle to check for the formation of clear blood clots.

2.5 Post-mortem measurements

All fish were exsanguinated by cutting the *bulbus arteriosus* and *vena cardinalis communae*, and bled for 30 minutes in a tank supplied with running seawater (7.5°C). Afterwards, weight (kg), length (cm) and gender of each fish were obtained. Fulton's condition factor K was calculated according to Ricker (1975) (Equation 1).

$$K = \frac{W}{L^3} \quad (1)$$

Where, W=weight of fish (g), L=Length of fish (cm).

Thereafter, fish were gutted, covered with plastic film, placed with its ventral side down, covered with ice, and stored at 4ºC for maximum 72 h.

2.6 Imaging Vis/NIR Spectroscopy / Muscle haemoglobin

All the fish were manually filleted with the skin on and the black peritoneum was removed. Afterwards, hyperspectral imaging of the fillets in diffuse reflectance mode was used to assess the muscle haemoglobin concentration as an indication of residual blood in the muscle. The procedure is described in Skjelvareid et al. (2017). Birefly, a push-broom hyperspectral camera (spectral range: 430-1000 nm, spatial resolution: 0.5 mm across-track x 1.0 mm along track, model VNIR-640, Norsk Elektro Optikk, Skedsmokorset, Norway) fitted with a lens focused at 1000 mm, and mounted 1020 mm above a conveyor belt, was used. An image was generated where each image pixel contained a spectrum, which was transformed into an absorbance spectrum by characterizing the incoming light. The haemoglobin concentration was then estimated on the pixel level for each fillet.

2.7 Statistical analysis

Statistical analysis was done using the statistical software program RStudio (Version 1.0.143. Boston, MA, USA). All parameters were tested at the group level for normality using Shapiro Wilkins normality test and density plots, and further checked for heteroscedasticity by comparing the maximum and minimum group variance. Data was mostly normally distributed, but parameters showed high levels of heteroscedasticity except for blood pH. Therefore, a Welch's ANOVA (Welch, 1951) followed by a Games-Howell posthoc test (Games et al., 1976) was applied to investigate group differences. The statistical tests were done using the function "onewaytest" with var.equal = FALSE, in the package "userfriendlyscience" (Peters, 2017) and a Games-Howell test adapted from a GitHub Gist by Schlegel (2016) (R-code in supplementary materials).

3. Results

3.1 Muscle parameters

Residual blood in the fillet was estimated by measuring haemoglobin levels in muscle (Figure 1). Both stress by crowding and air exposure significantly affected muscle haemoglobin ($F_{(11, 42.4)} = 38.4$, $p < 0.001$). Crowding prior to air exposure increased levels of muscle haemoglobin. In fact, haemoglobin levels were higher in crowded air-exposed fish compared to uncrowded fish at all consecutive time points. In uncrowded fish, after 30 minutes of air exposure (Table 1: A2.20) a significant increase in muscle haemoglobin compared to 0 air exposure (Table 1: A1.0 & A2.0) was observed, independent of whether fish were killed prior to or post air exposure. In stressed fish, slaughter prior to air exposure resulted in significantly lower levels of haemoglobin in the muscle after 15 and 30 minutes of air exposure, compared to alive air-exposed fish.

Muscle pH (Figure 2A) of uncrowded fish prior to air exposure was significantly higher than all groups exposed to air ($F_{(5, 24.8)} = 10.0$, $p < 0.001$). Muscle pH was on average lower in the uncrowded fish, compared to crowded fish, however, this effect was not significant.

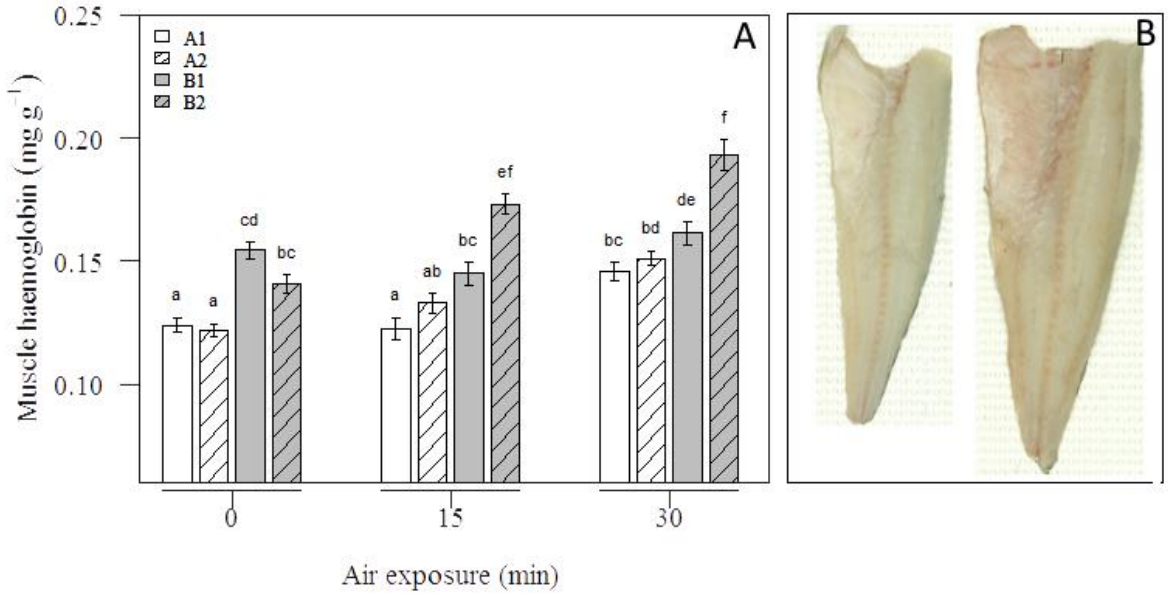

Figure 1: A) Muscle haemoglobin in cod fillets measured with VIS/NIR imaging spectroscopy. A1: Uncrowded and euthanized *prior* to air exposure. A2: Uncrowded and euthanized *post* air exposure. B1: Crowded and euthanized *prio*r to air exposure. B2: Crowded and euthanized *post* air exposure. Bars are mean values with 95 % confidence intervals. B) Cod fillets with low (left; 0.11 mg g^{-1}) and high (right; 0.19 mg g^{-1}) muscle haemoglobin levels. Different letters above bars indicate statistically significant differences.

3.2 Blood parameters

Blood clotting time (Figure 2B) was significantly different between the experimental groups ($F_{(5, 24.2)} = 11.5$, $p < 0.001$). Air exposure for 15 and 30 minutes resulted in a significant decrease in blood clotting time in both crowded and uncrowded groups. Crowding itself did not cause a significant reduction in blood clotting prior to air exposure. However, there was a significant difference in clotting time between crowded and uncrowded fish after 15 minutes of air

exposure (Figure 2B). After 30 minutes of air exposure, the difference was no longer significant, but crowded fish had on average a shorter blood clotting time than uncrowded fish.

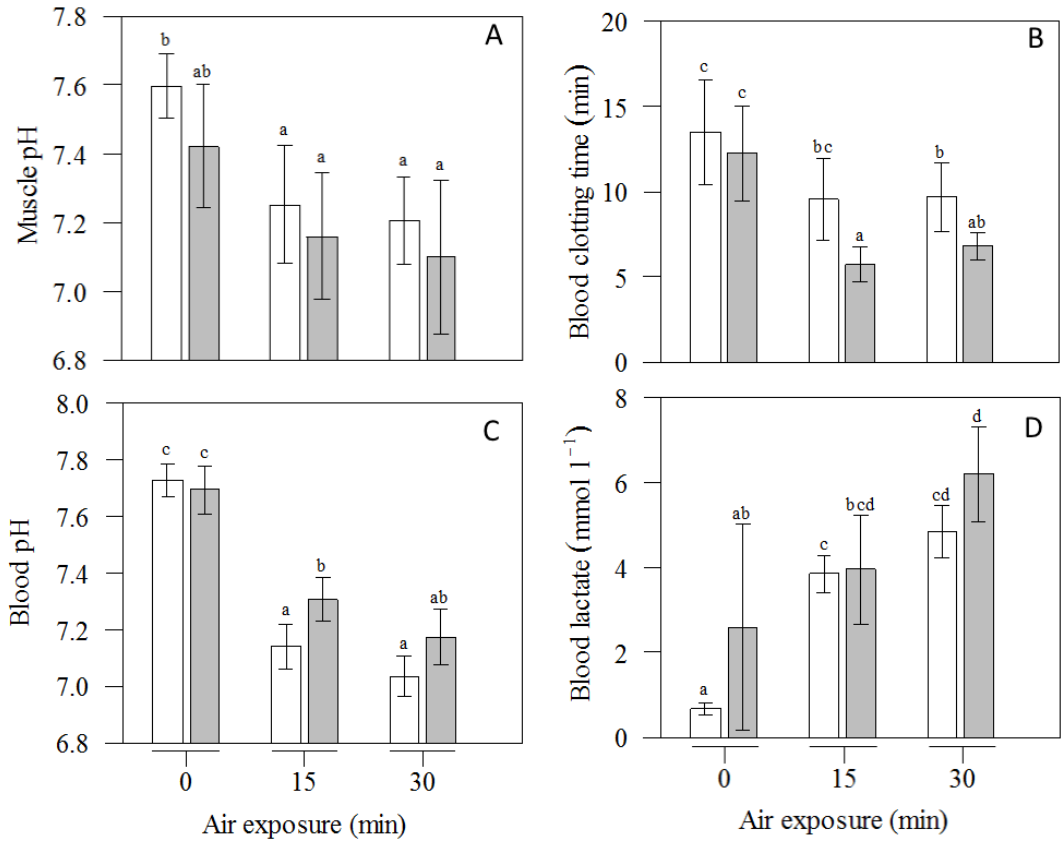

Figure 2: Muscle pH (A), blood clotting time (B), blood pH (C) and blood lactate (D) in crowded (grey bars) and uncrowded (white bars) Atlantic cod exposed to air for 0, 15 or 30 minutes. Bars are mean values with 95 % confidence intervals. Different letters above bars indicate statistically significant differences.

There was a significant effect of air exposure on blood pH ($F_{(5, 25.1)} = 82.7$, $p < 0.001$, Figure 2C), but no difference between 15 and 30 minutes of air exposure. Blood pH decreased after exposure to air, with on average a larger response in uncrowded fish. After 15 minutes of air exposure, uncrowded fish had significantly lower blood pH than crowded fish. There was no significant difference in blood pH after 30 minutes of air exposure.

Over time, air exposure significantly increased blood lactate levels ($F_{(5, 21.0)} = 103.1$, $p = 0.002$, Figure 2D), independent of the condition prior to exposure to air. However, blood lactate levels in the crowded fish was on average higher prior to air exposure, although not significantly due to a large variation in this group. Crowded fish had an overall higher variation in blood lactate levels than uncrowded fish.

Although, a significant difference in blood glucose levels (Supplement figure 1) was found between crowded fish before air exposure and uncrowded fish exposed to air for 30 minutes ($F_{(5, 21.4)} = 5.2$, $p = 0.002$), the overall glucose levels showed little response the treatment. A non-significant decrease in glucose levels was found in uncrowded fish, whereas in crowded fish glucose levels remained unchanged over time.

4. Discussion

In the present study, we assessed the effect of crowding and air exposure for 0, 15 and 30 minutes prior and post slaughter in Atlantic cod on blood parameters and fillet quality. Exposing the fish to air reduced the fillet quality, in terms of residual blood in muscle, and this effect was stronger and faster if fish are crowded. Killing the fish prior to air exposure delays the increase in the amount of residual blood in the muscle, even when fish were left for 30 minutes in air before bleeding.

Air exposure is an additive stressor to crowding and has previously been shown to have a detrimental impact on muscle quality in fish (Martine et al., 2003; Poli et al., 2005; Van De Vis et al., 2003). Our results are consistent with these studies. In addition, we found that slaughter slowed down the increase in residual blood. In cod, hypoxic conditions have been reported to increase resistance of blood vessels supplying the stomach, intestines and other digestive

organs, while somatic circulation is dilated, i.e. redistributing blood flow to the muscles (Axelsson & Fritsche, 1991). Our findings indicate that slaughter hampered the redistribution of blood to the muscle, resulting in less blood in the fillet. However, this was only the case for crowded fish, whereas the uncrowded fish did not show quality changes until 30 minutes of air exposure, which is consistent with the previous recommendation of Olsen et al. (2014) on unstressed fish. These results suggest that stressed fish have a stronger reaction towards air exposure in terms of residual muscle blood and should therefore be slaughtered within 15 minutes, or be recuperated to minimize the effect of stress (Svalheim et al., 2017). This emphasises the fact that the perimortem state of the fish is highly important to the quality of the final product.

Blood clotting is part of the physiological response to injuries to the blood vessels (Tavares-Dias et al., 2009). In the present study, there was no difference in blood clotting time between crowded and uncrowded fish before air exposure, while air exposure did reduce the blood clotting time. Intriguingly, after 15 minutes of air exposure, the blood clotting time in crowded fish was found significantly shorter than in un-crowded fish, indicating an additive effect of stress on blood clotting time. Similar results have been previously described by Ruis & Bayne (1997), showing reducing blood clotting times with increasing amount of stress.

Further, the decrease in blood clotting time appears to be reaching a plateau after 15 and 30 minutes of air exposure. It may be that the minimum blood clotting time has been reached or that the fish goes from being stressed to becoming moribund and haemostatic responses are impaired. However, this needs to be further elucidated.

Although, blood clotting time was not affected by crowding before air exposure, we did find differences in the level of residual blood in the fillets. It therefore appears that blood clotting time does not have a direct effect on residual blood. Nevertheless, because the process of

bleeding a fish involves cutting major arteries and veins, it can be hypothesised that blood clot formation may to some extent reduce the efficiency of bleeding, and thereby be a contributing factor to residual blood in the muscles.

Interestingly, after 15 minutes of air exposure, the blood pH in uncrowded fish was lower than in crowded fish. Because haemoglobin acts as a major buffer in the body (Nikinmaa, 2011), it is possible that the higher haemoglobin concentration at start of air exposure in crowded fish contributed to differential response in blood pH. Higher levels of haemoglobin is part of the general stress response in fish and results from an increased number of erythrocytes due to splenic contraction (Wendelaar Bonga, 1997). This process increases the blood oxygen transport capacity, but, as shown in the present study, had a negative effect on muscle quality, as blood is found to manifest in the muscle. Similar results regarding stress and residual blood in muscle were found in other experimental studies on crowding (Olsen et al., 2008), studies conducted on board commercial vessels (Digre et al., 2017; Olsen et al., 2013) and commercial handling of farmed cod (Jørpeland et al., 2015).

The stress inflicted by crowding in this experiment was probably not as severe as what is expected during commercial fisheries (Digre et al., 2017; Olsen et al., 2013). We found that, crowding for four hours did not cause significant differences in the measured stress parameters such as blood clotting, lactate or pH, although the lactate levels in crowded fish were on average a 2-3 fold higher. On the other hand, we did find significantly higher concentrations of muscle haemoglobin in crowded individuals. This indicates that 'mild' crowding, which leads to non-significant changes in measured physiological stress parameters, may already affect the quality of the fish based on fillet redness. Furthermore, our study was performed on fasted fish, and although wild cod have natural non-feeding periods, nutritional status of the catch will vary

with for example seasons, time of day food availability. Axelsson & Fritsche (1991) found that feeding increases the intestinal blood flow, which may in turn indicate that fed fish would have less blood distributed to the muscles during stress. This, however, remains speculative and as the fish in the present study had the same nutritional status, we interpret our result as an effect of stress inflicted by crowding and air exposure.

Conclusion

When Atlantic cod are stressed by crowding, they have a stronger reaction towards air exposure in terms of a faster increase in residual blood and decrease in blood clotting time. In order to secure best possible quality, fish should therefore be euthanised as quickly as possible after capture and should preferably not be exposed to air prior to slaughter. Future research should focus on ways to euthanise a large number of fish simultaneously without sacrificing the quality, and study methods to recuperate fish after capture to minimize the effects of stress.

Conflict of interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Acknowledgement

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Supplementary material

1. Supplementary figures

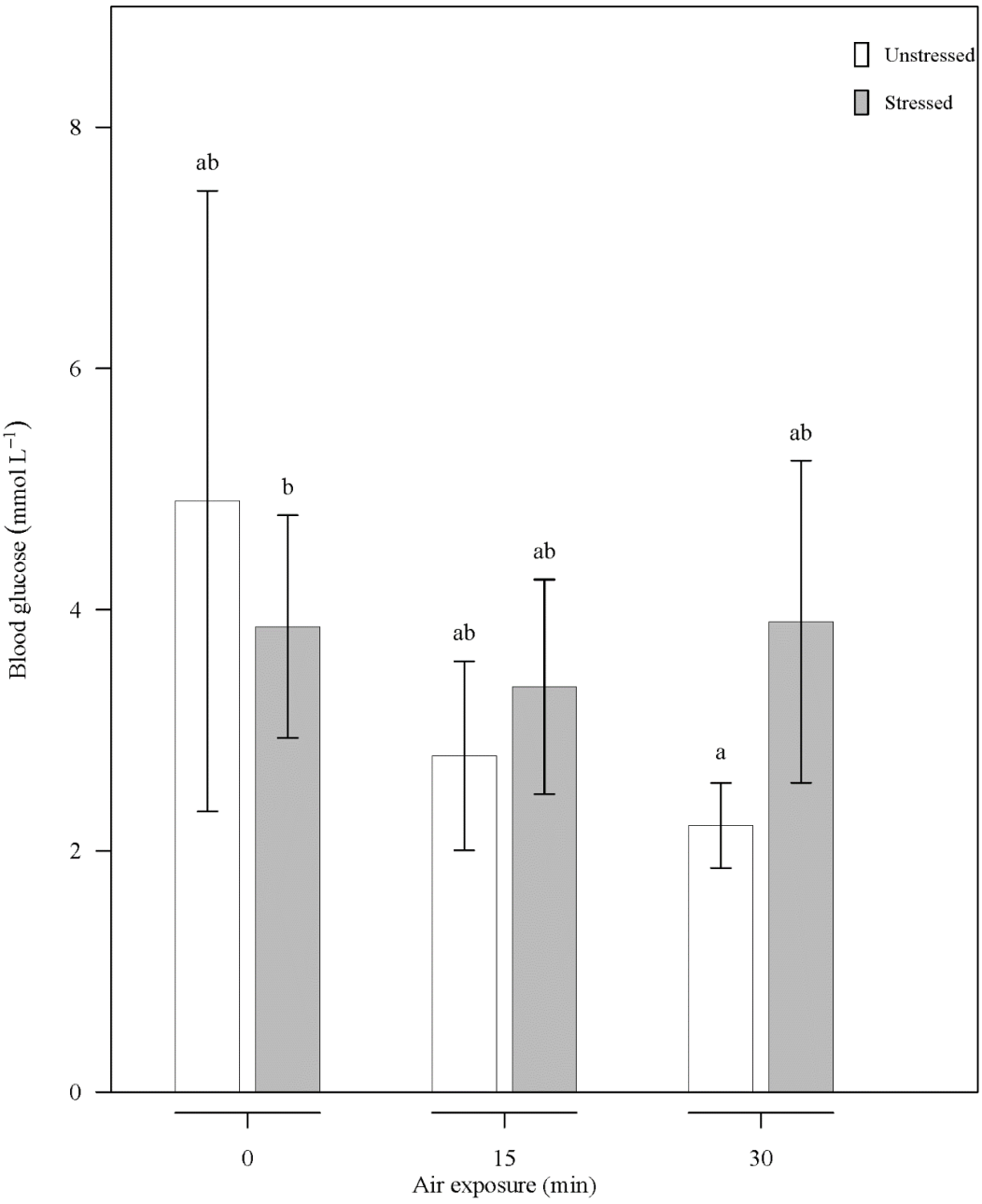

Figure 1: Change in blood glucose in response to crowding (stressed) and/or air exposure for 0, 15 or 30 minutes. Bars are mean values and arrows indicate 95% confidence intervals. Differences in letters above arrows indicate statistical differences ($p<0.05$).

2. R-CODE for Games-Howell post-hoc test

Adapted from a GitHub Gist by SCHLEGEL, A. 2016. *games_howell.R* [Online]. Available: https://gist.github.com/aschleg/ea7942efc6108aedfa9ec98aeb6c2096 [Accessed 01.01 2018]

```
games.howell <- function(grp, obs) {

  #Create combinations
  combs <- combn(unique(grp), 2)

  # Statistics that will be used throughout the calculations:
  # n = sample size of each group
  # groups = number of groups in data
  # Mean = means of each group sample
  # std = variance of each group sample
  n <- tapply(obs, grp, length)
  groups <- length(tapply(obs, grp, length))
  Mean <- tapply(obs, grp, mean,na.rm=T)
  std <- tapply(obs, grp, var,na.rm=T)

  statistics <- lapply(1:ncol(combs), function(x) {

    mean.diff <- Mean[combs[2,x]] - Mean[combs[1,x]]

    #t-values
    t <- abs(Mean[combs[1,x]] - Mean[combs[2,x]]) / sqrt((std[combs[1,x]] / n[combs[1,x]]) +
(std[combs[2,x]] / n[combs[2,x]]))

    # Degrees of Freedom
    df <- (std[combs[1,x]] / n[combs[1,x]] + std[combs[2,x]] / n[combs[2,x]])^2 / # Numerator
Degrees of Freedom
      ((std[combs[1,x]] / n[combs[1,x]])^2 / (n[combs[1,x]] - 1) + # Part 1 of Denominator
Degrees of Freedom
         (std[combs[2,x]] / n[combs[2,x]])^2 / (n[combs[2,x]] - 1)) # Part 2 of Denominator
Degrees of Freedom

    #p-values
    p <- ptukey(t * sqrt(2), groups, df, lower.tail = FALSE)

    # Sigma standard error
    se <- sqrt(0.5 * (std[combs[1,x]] / n[combs[1,x]] + std[combs[2,x]] / n[combs[2,x]]))

    # Upper Confidence Limit
    upper.conf <- lapply(1:ncol(combs), function(x) {
      mean.diff + qtukey(p = 0.95, nmeans = groups, df = df) * se
    })[[1]]

    # Lower Confidence Limit
    lower.conf <- lapply(1:ncol(combs), function(x) {
      mean.diff - qtukey(p = 0.95, nmeans = groups, df = df) * se
    })[[1]]

    # Group Combinations
    grp.comb <- paste(combs[1,x], ':', combs[2,x])

    # Collect all statistics into list
    stats <- list(grp.comb, mean.diff, se, t, df, p, upper.conf, lower.conf)
  })

  # Unlist statistics collected earlier
  stats.unlisted <- lapply(statistics, function(x) {
    unlist(x)
  })

  # Create dataframe from flattened list
  results <- data.frame(matrix(unlist(stats.unlisted), nrow = length(stats.unlisted),
byrow=TRUE))

  # Select columns set as factors that should be numeric and change with as.numeric
  results[c(2, 3:ncol(results))] <- round(as.numeric(as.matrix(results[c(2,
3:ncol(results))])), digits = 3)

  # Rename data frame columns
  colnames(results) <- c('groups', 'Mean Difference', 'Standard Error', 't', 'df', 'p', 'upper
ci', 'lower ci')

  return(results) }
```

Supplementary material

1. Supplementary figures

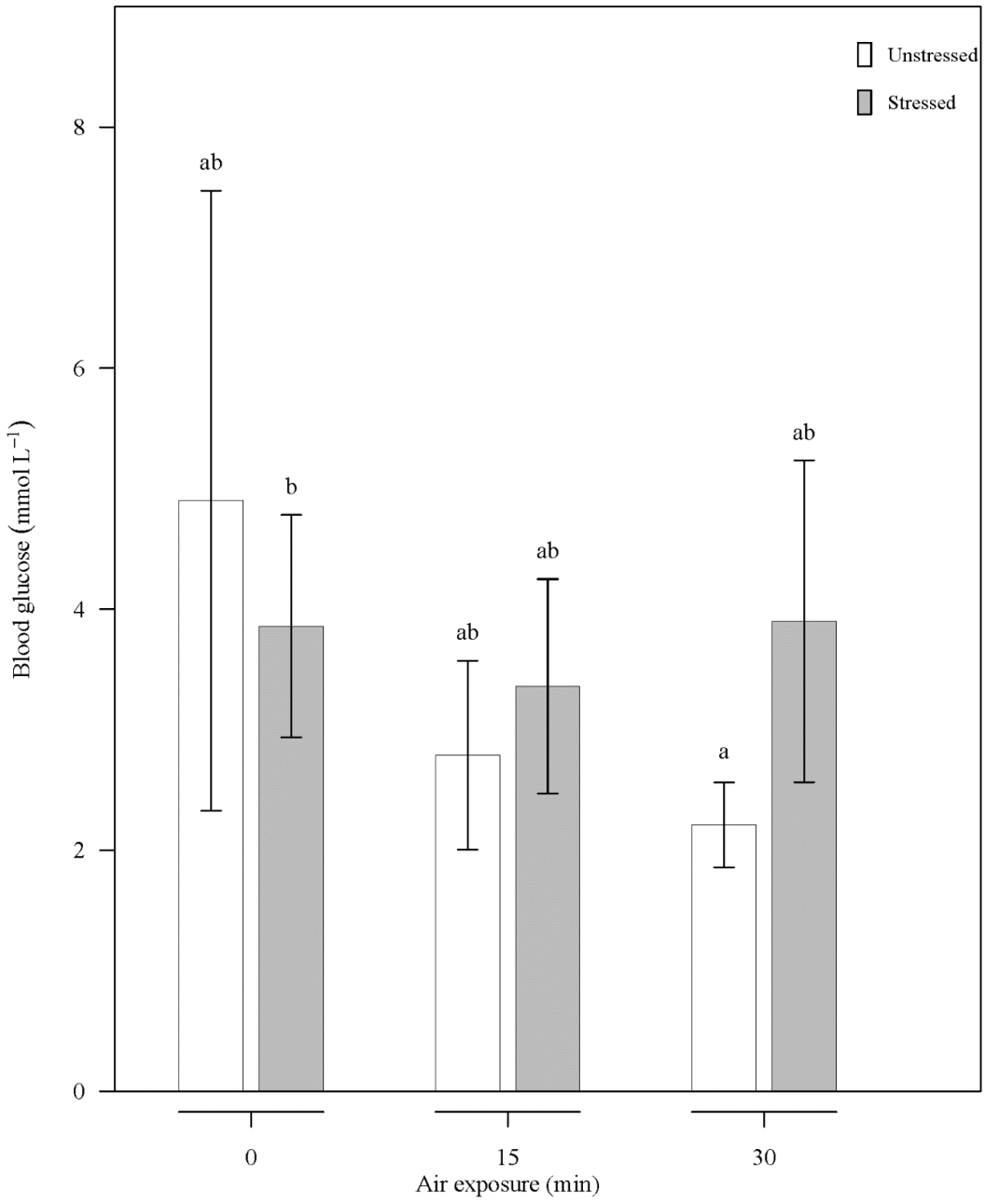

Figure 1: Change in blood glucose in response to crowding (stressed) and/or air exposure for 0, 15 or 30 minutes. Bars are mean values and arrows indicate 95% confidence intervals. Differences in letters above arrows indicate statistical differences ($p<0.05$).

2. R-CODE for Games-Howell post-hoc test

Adapted from a GitHub Gist by SCHLEGEL, A. 2016. *games_howell.R* [Online]. Available: https://gist.github.com/aschleg/ea7942efc6108aedfa9ec98aeb6c2096 [Accessed 01.01 2018]

```
games.howell <- function(grp, obs) {

  #Create combinations
  combs <- combn(unique(grp), 2)

  # Statistics that will be used throughout the calculations:
  # n = sample size of each group
  # groups = number of groups in data
  # Mean = means of each group sample
  # std = variance of each group sample
  n <- tapply(obs, grp, length)
  groups <- length(tapply(obs, grp, length))
  Mean <- tapply(obs, grp, mean,na.rm=T)
  std <- tapply(obs, grp, var,na.rm=T)

  statistics <- lapply(1:ncol(combs), function(x) {

    mean.diff <- Mean[combs[2,x]] - Mean[combs[1,x]]

    #t-values
    t <- abs(Mean[combs[1,x]] - Mean[combs[2,x]]) / sqrt((std[combs[1,x]] / n[combs[1,x]]) +
(std[combs[2,x]] / n[combs[2,x]]))

    # Degrees of Freedom
    df <- (std[combs[1,x]] / n[combs[1,x]] + std[combs[2,x]] / n[combs[2,x]])^2 / # Numerator
Degrees of Freedom
      ((std[combs[1,x]] / n[combs[1,x]])^2 / (n[combs[1,x]] - 1) + # Part 1 of Denominator
Degrees of Freedom
         (std[combs[2,x]] / n[combs[2,x]])^2 / (n[combs[2,x]] - 1)) # Part 2 of Denominator
Degrees of Freedom

    #p-values
    p <- ptukey(t * sqrt(2), groups, df, lower.tail = FALSE)

    # Sigma standard error
    se <- sqrt(0.5 * (std[combs[1,x]] / n[combs[1,x]] + std[combs[2,x]] / n[combs[2,x]]))

    # Upper Confidence Limit
    upper.conf <- lapply(1:ncol(combs), function(x) {
      mean.diff + qtukey(p = 0.95, nmeans = groups, df = df) * se
    })[[1]]

    # Lower Confidence Limit
    lower.conf <- lapply(1:ncol(combs), function(x) {
      mean.diff - qtukey(p = 0.95, nmeans = groups, df = df) * se
    })[[1]]

    # Group Combinations
    grp.comb <- paste(combs[1,x], ':', combs[2,x])

    # Collect all statistics into list
    stats <- list(grp.comb, mean.diff, se, t, df, p, upper.conf, lower.conf)
  })

  # Unlist statistics collected earlier
  stats.unlisted <- lapply(statistics, function(x) {
    unlist(x)
  })

  # Create dataframe from flattened list
  results <- data.frame(matrix(unlist(stats.unlisted), nrow = length(stats.unlisted),
byrow=TRUE))

  # Select columns set as factors that should be numeric and change with as.numeric
  results[c(2, 3:ncol(results))] <- round(as.numeric(as.matrix(results[c(2,
3:ncol(results))])), digits = 3)

  # Rename data frame columns
  colnames(results) <- c('groups', 'Mean Difference', 'Standard Error', 't', 'df', 'p', 'upper
ci', 'lower ci')

  return(results) }
```