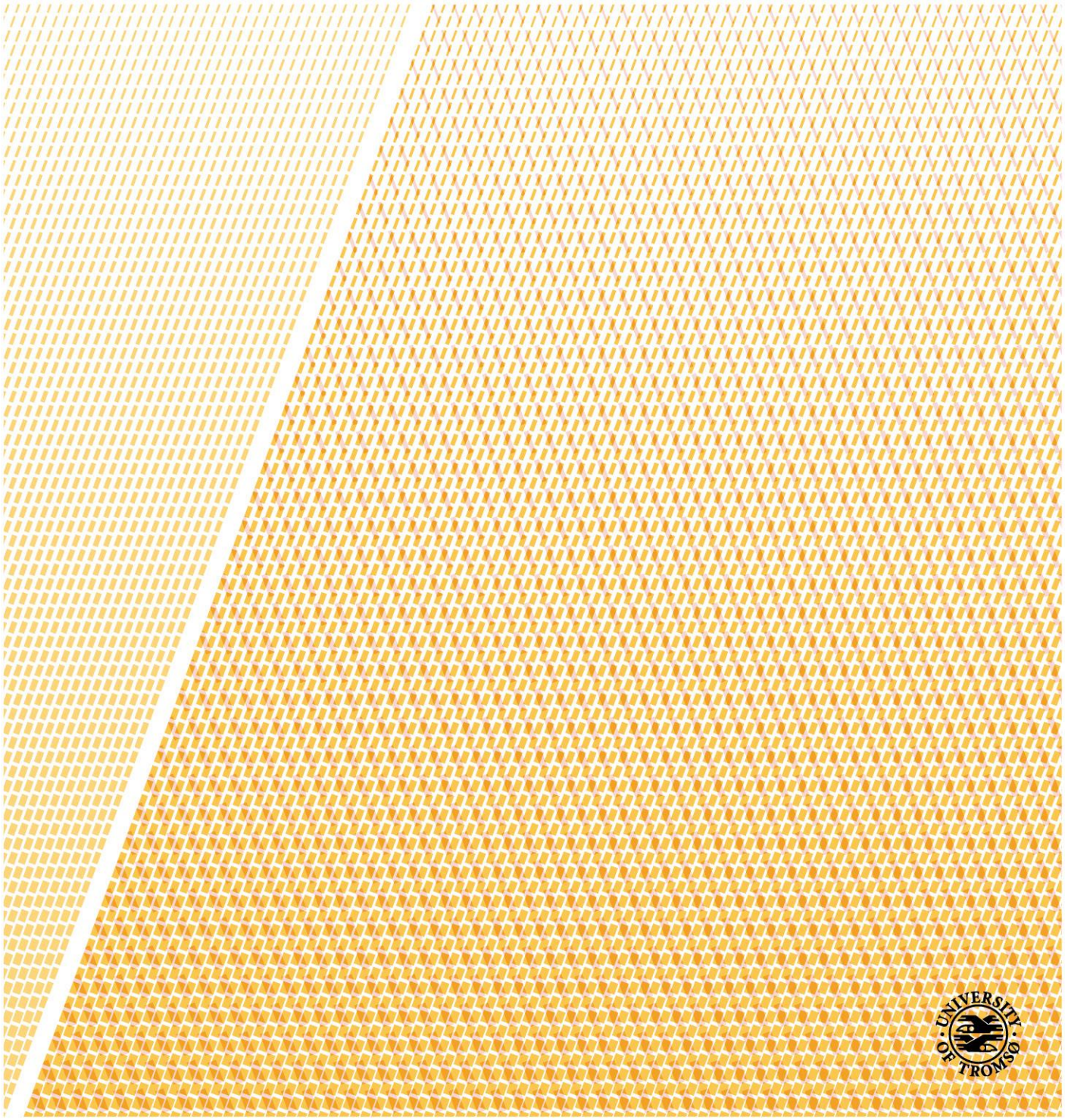
UiT
THE ARCTIC UNIVERSITY OF NORWAY

Faculty of Bioscience, Fisheries and Economics
The Norwegian College of Fishery science

Stress responses influencing fillet quality of trawled Atlantic cod and haddock

Ragnhild Aven Svalheim

A dissertation for the degree of Philosophiae Doctor – November 2018



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‘If we knew what is was we were doing, it would not be called research, would it?’

Albert Einstein

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I. Acknowledgement

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II. Thesis abstract

During trawling, fish become stressed through exercise, interactions with fishing gear and crowding in the cod-end. As catches are dropped on deck and exposed to air, light and temperature differences, fish are exposed to cumulative stress as well as potential bruising and death by asphyxiation. There is increasing evidence suggesting that pre-mortem stress is of great importance to muscle quality of fish, and this may explain why the catches from trawl fisheries have variable quality. Yet, little is known about how individual steps of the trawling process affect the muscle quality of the fish.

In this thesis, an experimental swim tunnel and cod-end was used as a model to investigate how stress during various stages of trawl capture affects fillet quality in terms of residual blood, time and hardness of post-mortem muscle stiffness and muscle colour of cod and haddock. In addition, the effect of stress on the importance of timing of euthanasia was also addressed.

The first stage of trawl capture that was chosen to study was the herding of fish in front of the trawl mouth. Two experiments were conducted to address this issue; the first involved exhaustive swimming of cod and the second focused on critical swimming speed of haddock. These studies showed that exhaustive swimming causes a moderate stress response, recovery takes longer than 6 hours and that exercise has a short-lasting effect on muscle texture, with little or no effect on muscle colouration. It was concluded that other stages of trawl capture have a higher impact on fillet quality.

The third study for this thesis aimed to investigate how extreme crowding for 1 or 3 hours in the cod-end, following exhaustive swimming, would affect the physiology and muscle quality of cod. Findings from this study showed that crowding caused a severe stress response and that fish probably suffered from hypoxia due to a significantly reduced ability to move their opercula. In addition, fillet quality was significantly reduced due to increased amount of residual blood in the muscles. Moreover, the detrimental effects of crowding are not fully reversed after 6 hours of recuperation.

In the last investigation, the final stage of trawl capture process, i.e. the effect of air exposure on deck, was studied. Fish were stressed by mild crowding and then exposed to air for 15 or 30 minutes, or directly euthanised by terminal blow to the head and then left in air for 0, 15 or 30 minutes before exsanguination. We found that stress/crowding triggered a stronger response to the air exposure by faster increase in residual blood in the muscles, resulting in lower fillet quality. However, direct euthanasia stopped blood flow to the muscle and quality was significantly improved.

Together these four studies show that there is a strong connection between the type of stress inflicted on the fish during capture and the quality of the fish product (fillets). Measures that may secure top quality fish from trawlers, include reducing crowding time in the cod-end and implementing direct euthanasia or live recuperation for more than 6 hours.

III. List of papers

Paper I

Recovery from exhaustive swimming and its effect on fillet quality in haddock (*Melanogrammus aeglefinus*)

Anders Karlsson-Drangsholt, Ragnhild Aven Svalheim, Øyvind Aas-Hansen, Stein-Harris Olsen, Kjell Midling, Michael Breen, Endre Grimsbø, Helge Kreutzer Johnsen

Fisheries Research 197, (2018), 96-104.

Paper II

Effects of exhaustive swimming and subsequent recuperation on flesh quality in Atlantic cod (*Gadus morhua*)

Ragnhild Svalheim, Anders Karlsson, Stein-Harris, Helge K. Johnsen, Øyvind Aas-Hansen,

Fisheries Research 193 (2017) 158-163.

Paper III

Simulated trawling: Exhaustive swimming followed by extreme crowding as contributing reasons to variable fillet quality in trawl-caught Atlantic cod (*Gadus morhua*)

Ragnhild Aven Svalheim, Øyvind Aas-Hansen, Karsten Heia, Anders Drangsholt-Karlsson, Stein Harris Olsen, Helge Kreutzer Johnsen

PLoS One: In review

Paper IV

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

Ragnhild Aven Svalheim, Erik Burgerhout, Karsten Heia, Sjurdur Joensen, Stein-Harris Olsen, Heidi Nilsen, Torbjørn Tobiassen

Food Bioscience: In press

Introduction

Ethical and economical aspects of quality improvements in white fish fisheries

Fish as food represents an important resource for human nutrition and health and is also appreciated for cultural and gastronomic reasons. Globally, regionally, nationally and locally, the use and importance of aquatic foods vary greatly. Production of wild captured seafood has more or less stabilized the last two-three decades (Fig 1), with most fish stocks assessed by FAO to be fully, but sustainably exploited (FAO 2018). However, the global demand for aquatic food sources are expected to increase faster than population growth, due to an increasing proportion of middle-class people with greater spending power who typically consume more animal protein than people with lower income. Therefore, it becomes more important to ensure that harvested fish are utilised in such a way that the proportion of the fish suitable for human consumption is maximized. That is, by reducing waste that is caused by quality impairment activities.

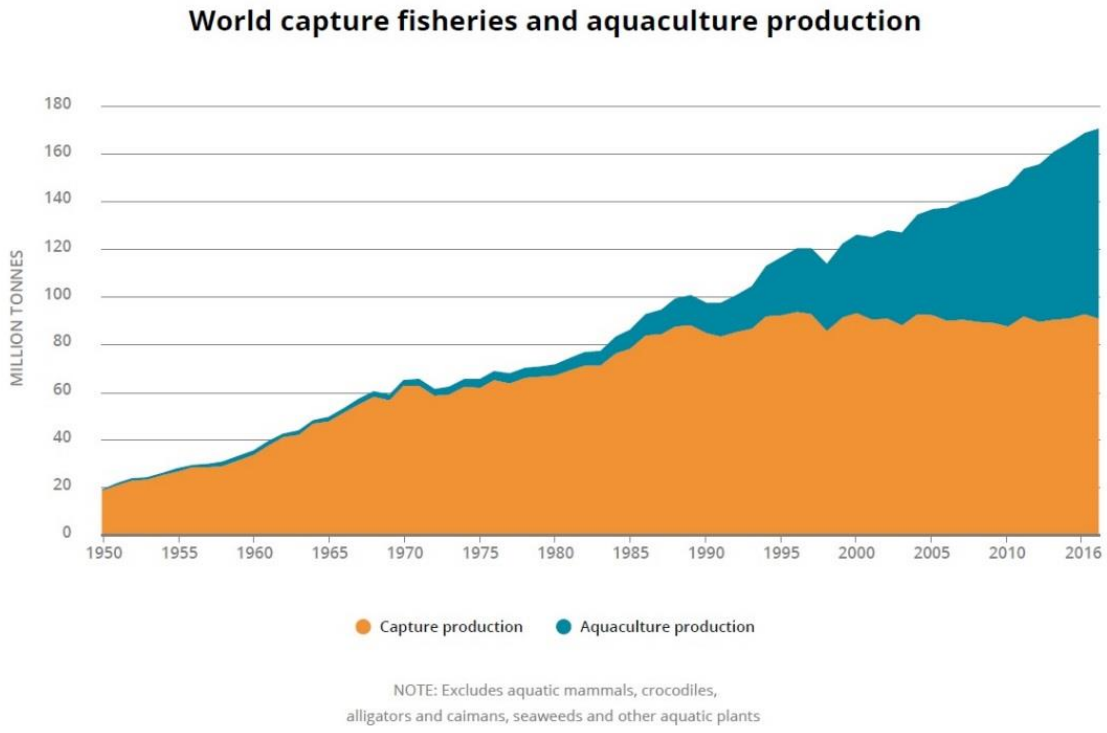

Figure 1. Trends in world capture fisheries and aquaculture production the last ~70 years. Aquaculture production is both food fish (~80 tons) and aquatic plants (~30 tons).

In Norway, governmental authorities, sales organizations, industry and researchers often emphasize the need for quality improvement of white fish. Nevertheless, the quality of landed fish from the coastal fisheries shows a negative trend (Akse et al. 2014, Akse et al. 2004). This is especially true for fish caught by gillnet and Danish sein, whereas fish caught by line are stable, and overall, have good quality. Furthermore, the difference in quality of fish caught by different gears is often not reflected in the ex-vessel price of fresh fish, where the trend is that vessels delivering the largest quanta per time unit get the highest price (The Norwegian Fishermen's Sales Organization, 2018). However, this may lead to a direct financial loss for the fish processors. A study comparing high and low quality cod and haddock showed that poor quality fish could lead to a potential value loss of 13% in the filleting industry, which corresponds to about NOK 100 million (EURO 11 million) based on the export value in 2013 (Svorken et al. 2015).

It is important to keep in mind that the gadoid fishery is diverse, extending from small coastal vessels utilizing hand-baited long-lines, gillnets, and jig machines with daily deliveries of fresh catches to local fish plants, to massive ocean going bottom trawlers and auto liners that process and freeze the catch at sea. The frozen fish is usually sold at auctions, which presents the opportunity to evaluate product quality and raise complaints when quality standards are not met. This creates a better, but not perfect, correlation between quality and price and emphasizes the fact that quality does matter. For example, fish caught by trawls and Danish seine are known to yield more variable quality than fish caught by auto line. In an ongoing study of prices for frozen cod and haddock covering a period of nine years (2009-2017), it is found that for Atlantic cod, fish caught by autoline gain 9.5% and 16.1% higher prices than trawl and Danish seine, respectively, controlled for the influence of fish size and season (Sogn-Grundvåg, unpublished data). For haddock, autoline get 22.5% higher price compared with Danish seine. Trawl, considered to be one of the most catch efficient fishing gears, lands the largest quantum of cod and haddock in Norway (Fig 2), and hence improvement to the quality of trawl-caught cod and haddock may therefore have a great ethical and financial impact on the fishery industry.

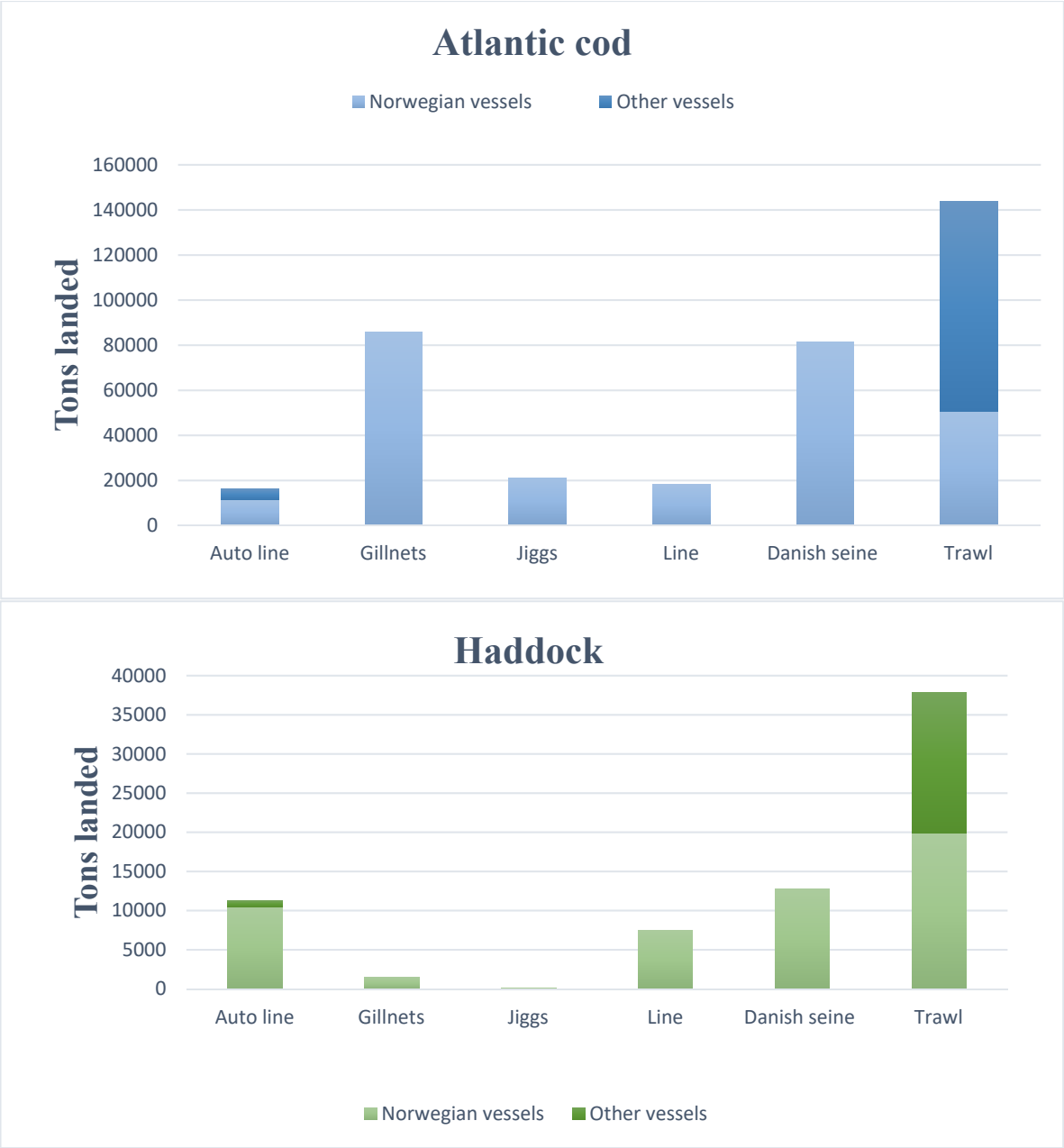

Figure 2. Metric tons landed haddock (a) and cod (b) by fishing gear. Period 01.01.2018 - 01. 10. 2018. Source: The Norwegian Fishermen's Sales Organization, 2018.

Fillet quality

There are many factors which determine the quality of a fish or a fish product (Fig 3). Fillet quality is a complex set of characters involving intrinsic factors such as chemical composition, texture, fat content and colour which in turn are influenced by extrinsic factors such as feeding regime, diet composition and pre- or post-slaughter handling procedures. The term 'quality' frequently refers to the visual appearance and freshness or degree of spoilage the fish has undergone. These features often have bearing on food safety in terms of harmful bacteria,

viruses, parasites or chemicals, that can create an off odour and bad taste, soft texture or altered muscle colour. Many quality traits of a fish product also depend on other biotic and abiotic factors such as fish species, season (Botta et al. 1987b), gender (Ageeva et al. 2017), and type of food eaten (Ageeva et al. 2018).

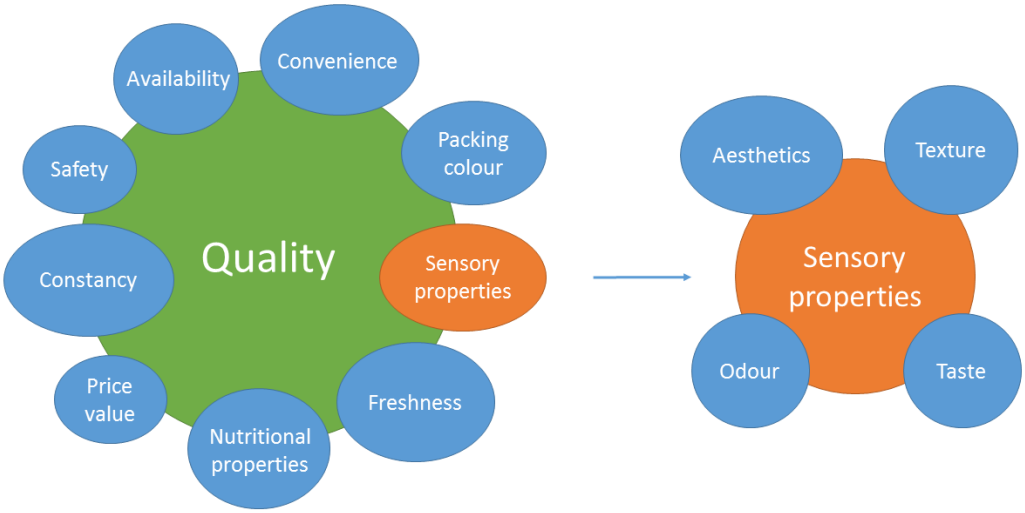

Figure 3. Overview over multiple factors influencing the quality of fish products. Modified from Olafsdóttir et al. (1997).

Texture of fish muscle is a highly important quality parameter, as softness may cause downgrading in the processing industry (Michie 2001). The texture of a fish muscle is influenced by inherent characteristics such as amount and composition of connective tissue and muscle fibres density, which in turn undergo substantial seasonal variations (Botta et al. 1987b). For the fish, white muscle with its high protein content, constitutes an important energy resource, and seasonal events like spawning, periods with starvation and prolonged stress may reduce protein content of the muscle (Ageeva et al. 2017, Ladrat 2000, Ageeva et al. 2018). These changes can alter contractile properties and metabolic characteristics of the muscles, and may ultimately influence the flesh quality (Delbarre-Ladrat et al. 2006). Post-mortem proteolysis in fishes is not considered beneficial to flesh quality, as fish meat generally does not need to be tenderized. Rather, the protease mediated muscle tissue degradation contributes to softening of the meat, increased drip loss, and increased gaping (Bahuaud et al. 2010, Mørkøre et al. 2008, Ofstad et al. 1996, Roth et al. 2006, Sigholt et al. 1997, Thomas et al. 1999).

The colour of fish muscle is another important quality parameter. When potential consumers evaluate fillets, they expect white fish to be white and may therefore reject pinkish or dark

fillets. Residual blood in the fillets is the main factor responsible for colour change in white fish (Olsen et al. 2008). The haem pigments in red blood cells contain iron molecules, which bind oxygen and makes the blood cells appear red. Hence, residual blood in the muscles makes the fillet appear pink or reddish. In addition, large amounts of haemoglobin may accelerate lipid oxidation, causing an unpleasant odour (Maqsood and Benjakul 2011, Richards and Hultin 2002, Terayama and Yamanaka 2000). Therefore, exsanguination of fish by cutting the throat or gills is decreed by Norwegian legislation (FOR-2013-06-28-844).

The type of fishing gear can also greatly influence the quality of the fish product (Botta et al. 1987a, Digre et al. 2010, Esaiassen et al. 2004, Huse et al. 2000, Larsen and Rindahl 2008, Rotabakk et al. 2011). Some quality defects are directly related to the gear, such as gaffing damages from longline (Larsen and Rindahl 2008) and bruising and net marks from trawls (Digre et al. 2010). Other quality issues may arise as an effect of the stress inflicted on to the fish by the capture process itself and are not necessarily notable until the fish is processed into fillets. These issues involve increased amount of residual blood in fillets and faster onset of *rigor mortis*, followed by textural changes such as gaping and dry flesh (Stien et al. 2005, Hultmann et al. 2016, Aursand et al. 2010, Digre et al. 2017, Olsen et al. 2008). These effects are most likely related to altered physiological characteristics of the fish, due to pre-mortem stress.

Trawls

The use of trawl to catch fish triggers a complex sequence of behavioural responses by the captured fish. A large part of the knowledge on behavioural patterns of trawl-caught fish is based on studies from the 1960s, using underwater observations of fish during trawling (Glass and Wardle 1989, Beamish 1969, Reviewd in Winger et al. 2010).

The trawl itself is a cone shaped net made from two, four or more panels, which is towed by one or two boats. The net is wide at the opening and then narrows to a bag called the cod-end, where the fish become trapped (Fig 4). The net opening is held open by beams, otter boards (doors) or distance between two towing vessels (pair trawling).

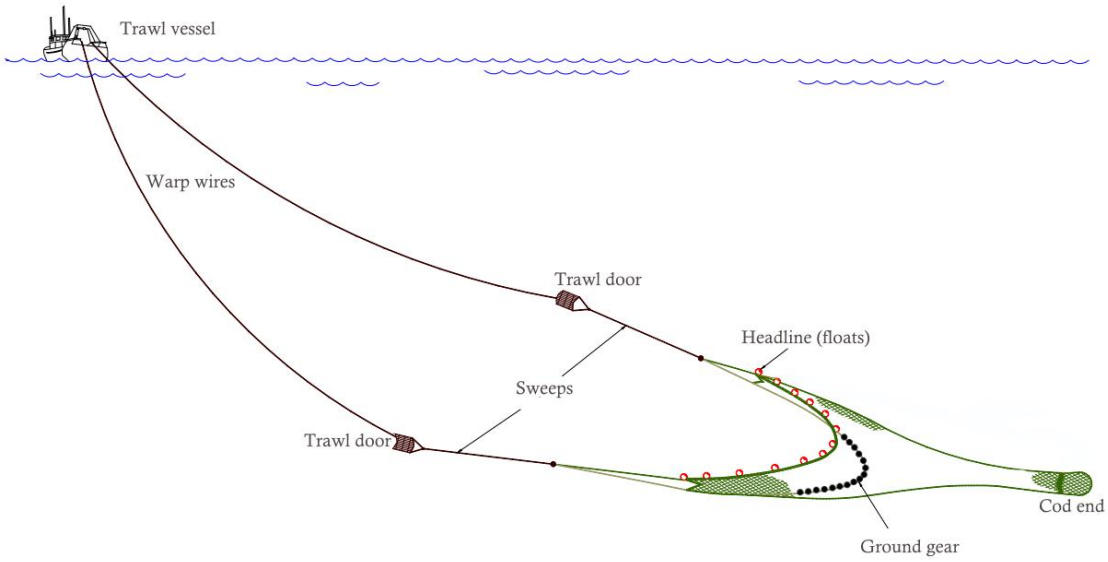

Figure 4. Schematic overview of a bottom trawl. Modified from and image by Institute of Marine research, Norway.

Otter trawl is the most commonly used type in Norway. The boards rest on the sea bottom and creates large mud clouds when they are dragged along the sea floor. These clouds are important for the catch efficiency, as they mask the visual appearance of the netting and footgear, making the fish swim towards the net instead of escaping to the side (Sistiaga et al. 2015). When these components become visible to the fish, the fish tend to alter course, turn around and if towing speed allows it, start swimming in the direction of the tow in the net opening. This behaviour occurs when light intensities are high enough for the fish to see the movement of the approaching net and is most likely an optomotor reflex (Winger et al. 2010). Normal towing speeds vary from 1-7 knots depending on target species. Common towing speeds for cod and haddock is 2-5 knots (1 to 2.5 m s^{-1}). These towing speeds exceed the sustained swimming speed of the target species (Breen et al. 2004, He 1991), suggesting that the fish may be exhausted or fatigued as they enter the cod-end.

Fish swimming in the net opening

Before ending up in the cod-end, fish engage in numerous behaviours that all involve swimming. These behaviours include reacting to and trying to avoid an approaching trawl, fleeing from the net opening and avoiding the gear or actively trying to escape once inside (Suuronen et al. 2005, Suuronen et al. 1996). In general, the swimming performance has been classified into three distinct categories: sustained, prolonged and burst swimming. Sustained swimming speed are speeds which can be maintained for more than 200 minutes, whereas

prolonged activity, as classified by Beamish (1978), can be maintained for 20 seconds – 200 minutes. Speeds which cannot be maintained for more than 20 seconds are classified as burst swimming (Beamish 1978). The endurance of cod is highly sensitive to changes in towing speed, with higher towing speeds reducing how long time the cod can swim in the trawl opening (Winger et al. 2010). By choosing a towing speed higher than the sustained swimming speed of the target species, the fish in front of the net opening will eventually drift back into the net. However, the extent to which fish are able to maintain their position in front of a trawl opening, is highly species specific and also depend the on physical (i.e. size and length) (Suuronen et al. 2005) and physiological conditions of the fish when presented with the trawl opening (Winger et al. 2010).

Video footage from a trawl opening shows that fish engage in a burst and glide behaviour before drifting into the net. This behaviour represents an intermediate mode between prolonged and burst swimming and has a predicted energy savings of about 50% (Weihs 1974). It is however, not an endless swimming mode as fish eventually terminate this swimming behaviour. Most likely, fish cease swimming due to a combination of metabolic exhaustion and accumulation of anaerobic waste products in combination with a behavioural decision of the fish to stop swimming (Tudorache et al. 2013). For example, Breen et al. (2004) found that exhausted haddock where in fact not exhausted, but were seemingly 'unwilling' to continue swimming under laboratory conditions. It has since been clearly shown that 'exhaustion' and 'fatigue' are not interchangeable descriptions and that 'exhaustion' relates to a condition in which the energy stores of the fish are fully depleted, whereas 'fatigue' is a behavioural decision of the fish that may occur before the fish has depleted its energy stores (Farrell 2007).

Crowding in the cod-end

As fish are captured they accumulate in the cod-end and eventually become the catch, and the same time, crowding pressure increases. The intensity of the crowding situation vary with the amount of fish entering and exiting the cod-end and the water flow in the cod-end. The water flow in the cod end depends on the type of twine used, mesh-type and amount of fish (Winger et al. 2010). There are few studies on the extent and exact measurements of crowding pressure inside a cod-end. However, large catch sizes (15–30 metric tons) and visual observation strongly indicate that the degree of crowding pressure is sever (Fig 5). The most dramatic crowding probably occurs during the haul back when lifting of the cod-end reduces ambient pressure, causing the swim bladder, and hence the whole fish, to expand (Tytler and Blaxter

1973, Taylor et al. 2010, Ferter et al. 2015). Swim bladder expansion occurs in cod and haddock because they have a physoclist (i.e. closed) type of swim bladder. Fish with this type of swim bladder can only adjust the gas inside by actively secreting gas from the blood to the swim bladder via the gas gland (*rete mirable*) and reabsorb gas by controlled passive diffusion over a highly vascularised area, called the oval. Both the secretion into the bladder and reabsorption of gas from the swim bladder are slow processes which take several hours to complete (Midling et al. 2012).

Figure 5. Recently captured gadoids crowded in the cod end on deck of the fishing vessel. Photo by Jesse Brinkhof.

Furthermore, because the Northeast Atlantic cod tend to be found in high abundance and in dense aggregation, massive catches can be obtained during short towing times (10-20 minutes). This has led to a practice among Norwegian trawlers, called 'buffer towing'. This practice involves deploying the trawl immediately after the catch is on board. In the case when the desired amount of fish is caught prior to completion of processing of the catch from the previous haul, the trawl is simply lifted off the seabed and towed at low speed (~1-2 knots). This tactic is employed in order to ensure a continuous supply of fish for processing, but has negative

effects on quality and will lengthen the time the fish are crowded in the cod-end (Brinkhof et al. 2018).

Post-capture air exposure

Processing of live fish to food necessarily involves a moment of slaughter. The most commonly employed slaughtering technique of wild fish involves cutting the throat followed by exsanguination. However, large catches and vigorous fish can make bleeding challenging. It is therefore common practice on many fishing vessels that the fish are exposed to air prior to exsanguination, as fish then become moribund and easier to handle. For this reason, bleeding of the fish is often performed after a period of air exposure, which lead to asphyxiation (Van De Vis et al. 2003).

Asphyxia is characterized by a prolonged period with suffocation before death. The time it takes for fish to die from asphyxiation before bleeding depends on the hypoxia resistance of the species and temperature (Poli et al. 2005). Furthermore, previous recommendations states that the fish should be bled within 30 minutes of slaughter in order to ensure proper exsanguination (Olsen et al. 2014). However, cod and haddock show brain activities up to 2 hours after being taken on board and kept in dry tanks (Lambooij et al. 2012). Hence, air exposure cannot be considered a slaughter method for these fish species, but is rather an additional stressor in the capturing process which can potentially lead to reduced quality and shorter shelf life of the fish product (Lambooij et al. 2012). In addition, the practice with air exposure is considered unacceptable in terms of animal welfare (Van De Vis et al. 2003).

Live storage of fish on board fishing vessels

Storing live fish after catch may improve the quality of the captured fish. In Norway, live cod captured by demersal seine are placed into capture based aquaculture to supply markets with fish throughout the year, and thus increase the value of the catches (Ottolenghi et al. 2004, Midling et al. 2012). During the last decade, a similar procedure has been investigated for short-term storage of trawl-captured cod and haddock (Olsen et al. 2013, Lambooij et al. 2012, Digre et al. 2017). Short-term storage involves keeping fish in water-filled tanks until the crew is prepared for slaughter. The main advantages of this practice are improved fish welfare and potentially higher product quality. Keeping fish alive for as long as possible may increase shelf-life of the product, as less time will pass from slaughter to market. Furthermore, because time from slaughter to bleeding is important for proper exsanguination, live storage could be a practical approach allowing better control of the timing of slaughter. Furthermore, the study by

Olsen et al., (2013) showed that muscle quality of cod and haddock improved after 6 hours of live storage, although Digre et al., (2017) found that quality only improved marginally with recuperation in water with variable oxygen saturation (46 - 117% dissolved oxygen).

Fish musculature and cardiovascular system

White and red muscles

The fish skeletal muscles are organized in segments (myotomes) shaped like a sideways W and arranged in series so that they stack like cones (Fig 6). The myotomes are separated by connective tissue (the *myoseptum*) and are easily visible in heat treated fish, as high temperatures breaks down the connective tissue allowing separation of the muscle blocks. Fish swim with two types of muscle fibres, the metabolically aerobic red muscles and the metabolically anaerobic white muscles. The red muscles typically represent maximum 10% of the total muscle mass and is used for slow to moderate, sustained swimming. The white muscles constitutes about 90% of the total muscle mass (and sometimes over 50% of the total body mass) and is used for brief burst and high speed swimming, such as predator-pray chase (Nelson 2011).

The red muscles are located directly under the skin, parallel to the length of the fish. They get their colour from a high myoglobin content, which functions as an internal oxygen transport system. The red muscles are made up primarily from slow-twitched oxidative fibres generating their ATP by mitochondrial oxidative phosphorylation, which produces 36 ATP for each glucose equivalent. This process require fuel in forms of substrates (lipids, carbohydrates or protein) and oxygen as terminal electron acceptor. Oxygen and substrates are provided via rich blood supply (Wang and Richards 2011).

White muscles are composed of fast glycolytic fibres that produce most power at high contraction frequencies and which rely almost exclusively on intracellular fuel stores to generate ATP anaerobically via substrate-level phosphorylation (predominantly glycolysis). Glycolysis produces two ATP in the conversion of glucose to pyruvate and then lactate. White muscles are poorly vascularised and contain no or little myoglobin. Although oxidation of glucose in the mitochondria yields more ATP then fermentation (30-36 vs 2 ATP), there are at least two physiological conditions where anaerobic pathways are preferred over aerobic, both of which occurs when mitochondria meets their limit to generate ATP.

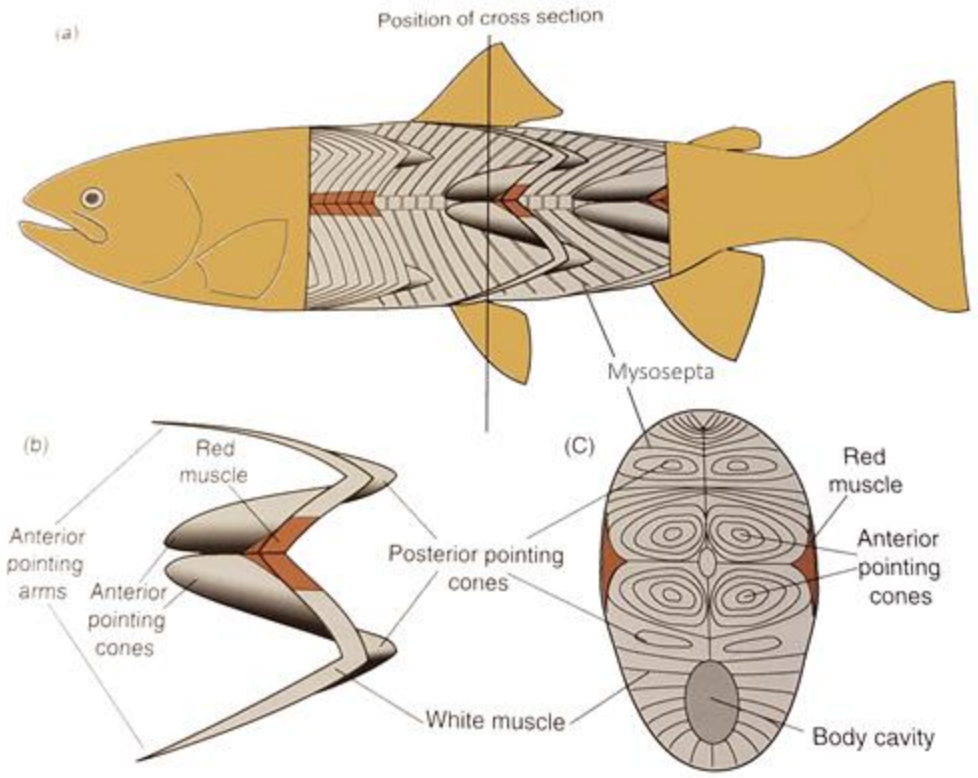

Figure 6. Myotomal muscle anatomy. (a) Lateral view of typical teleost. One myotome of the body axis is shown in situ with adjacent tissue removed to illustrate its three-dimensional structure. (b) Component part of the myotome. (c) Body cross section revealing muscle fibre types and myosepta. Modified from Nelson (2011).

The first involves lack of oxygen, for example due to exposure to a hypoxic environment. The second situation is where there is the requirement for a high generation rate of ATP in order to support intensive muscle contractions, for example during periods of intensive exercise. This is because ATP can be produced about twice as fast by anaerobic metabolism compared to aerobic metabolism (Wang and Richards 2011). Anaerobic metabolism leads to a rise in lactate levels, which can more than double in situations that cause the body to shift from aerobic metabolism to anaerobic metabolism. Therefore, lactate is frequently used as a stress indicator in fish. The rate of production and disposal of lactate is dependent on the stressor itself. For example, exercise may cause a more rapid formation and disposal of lactate than hypoxia does (Weber et al. 2016). This can be explained by differences in blood flow and in metabolic rate of the tissues that metabolize lactate.

Muscle contraction and development of *rigor mortis*

Although the anatomical structure of fish muscles is different from those of mammals, the process of muscle contraction by the striated muscles is the same. Production of force from shortening of the skeletal muscles is caused by myosin cross-bridge cycling, which involves a sequence of molecular events that underlie the sliding filament theory (Huxley and Hanson

1954, Huxley and Niedergerke 1954). In short, the myosin (thick) filaments of muscle fibres slide past the actin (thin) filaments during muscle contraction, while the two groups of filaments remain at relatively constant length. This process requires ATP energy and the binding and release of Ca^{2+} from troponin C, which is a protein component of thick myosin filaments. Crossbridge cycling can continue as long as there are sufficient amounts of ATP and Ca^{2+} in the cytoplasm

In the first hours to days following slaughter, the texture of fish muscle is particularly influenced by the process of *rigor mortis*. After death, fish cease respiration and aerobic production of ATP is no longer possible. However, the tissue will continue to produce ATP via anaerobic glycolysis until the glycogen stores are depleted. Following glycogen depletion, the ATP concentration declines and the body enters *rigor mortis* because there is no ATP available to break the crossbridges. Additionally, Ca^{2+} enters the intracellular fluids after death, due to the deterioration of the sarcoplasmic reticulum. Ca^{2+} allow the myosin heads to bind to the active sites of actin proteins and the muscle is unable to relax until further enzyme activity degrades the complex. Rigor completion has been achieved when cross-bridge affinity and tension are at their maximum. During rigor, nearly 100% of all possible binding sites form cross-bridges, as opposed to about 20% during normal muscle contraction. Muscle tension will decrease as a result of proteolytic degradation.

The process of *rigor mortis* has consequences for the processing of the fish as traditionally captured fish are often delivered in rigor. Mechanical handling of such fish by gutting or filleting machines can cause severe quality defects such as gaping and reduced fillet yield (Love 1988, Stroud 1969). It is therefore common practice to halt production until rigor is completed. This evidently has consequences for freshness and shelf life of the fish product as rigor may last for several days. Prolonging the time before onset of rigor opens up for pre-rigor filleting and production of exceptionally fresh fish that can have longer marketing time and may serve additional benefits in terms of less weight and thus energy for transport (fillet vs. whole fish). With that said, there are some issues related to pre-rigor filleting such as lower water holding capacity and increased drip loss, strong fillet contraction and fillet shrinkage (Kristoffersen et al. 2006, Kristoffersen et al. 2007).

Circulation

The fish circulatory system consists of two major vascular beds, the respiratory gill and the systemic circulation. The heart of the fish is situated in the *pericardium*, which is a membranous sac. Within the *pericardium*, there are four distinct chambers that constitute the fish heart: the *Sinus venous*, the atrium, the ventricle, and an outflow tract (*Bulbus arteriosus*) (Farrell and Pieperhoff 2011). The heart pumps deoxygenated blood through a short arterial network into the gills via *Aorta ventralis*. After passing through the capillary network of the gills, oxygenated blood is collected into *A. dorsalis* and distributed to the peripheral tissue via the systemic vessels. The venous system returns deoxygenated blood from the peripheral tissues to the heart (Olson 2011a).

White and red muscles are supplied with oxygenated blood by the segmental arteries. These arteries branch off *A. dorsalis* at each vertebra, usually in alternating myosepta. There are three groups of segmental arteries: dorsal, lateral and ventral (Fig 7). Dorsal and lateral arteries travel in the septa between the myotomes and branches leave to enter myotomes and perfuse the capillaries of muscle fibres. Circulation of the peripheral tissue is regulated by vessel diameter, which affects the resistance and hence the rate of blood flow through the tissue. The vessel diameter in turn is regulated by layers of smooth muscle cells wrapped around the arteries. Large arteries can be heavily innervated, but the degree of innervation becomes progressively less in smaller vessels (Olson 2011a).

The resistance is under the influence of two control systems; the remote system and the local system. The remote system includes the autonomic part (both the sympathetic and parasympathetic division) of the central nervous system and circulating catecholamines (CA; noradrenalin and adrenalin) released primarily from the chromaffin cells in the head kidney. Both components operate throughout the entire circulatory system and may affect blood flow through the heart (cardiac output), the gills as well as the arteries and some veins. In general, increased sympathetic activity will lead to increased blood flow and redistribution of blood from the intestinal tract to oxygen consuming tissues, including the red swimming muscles. This system is usually activated both during exercise and during hypoxia.

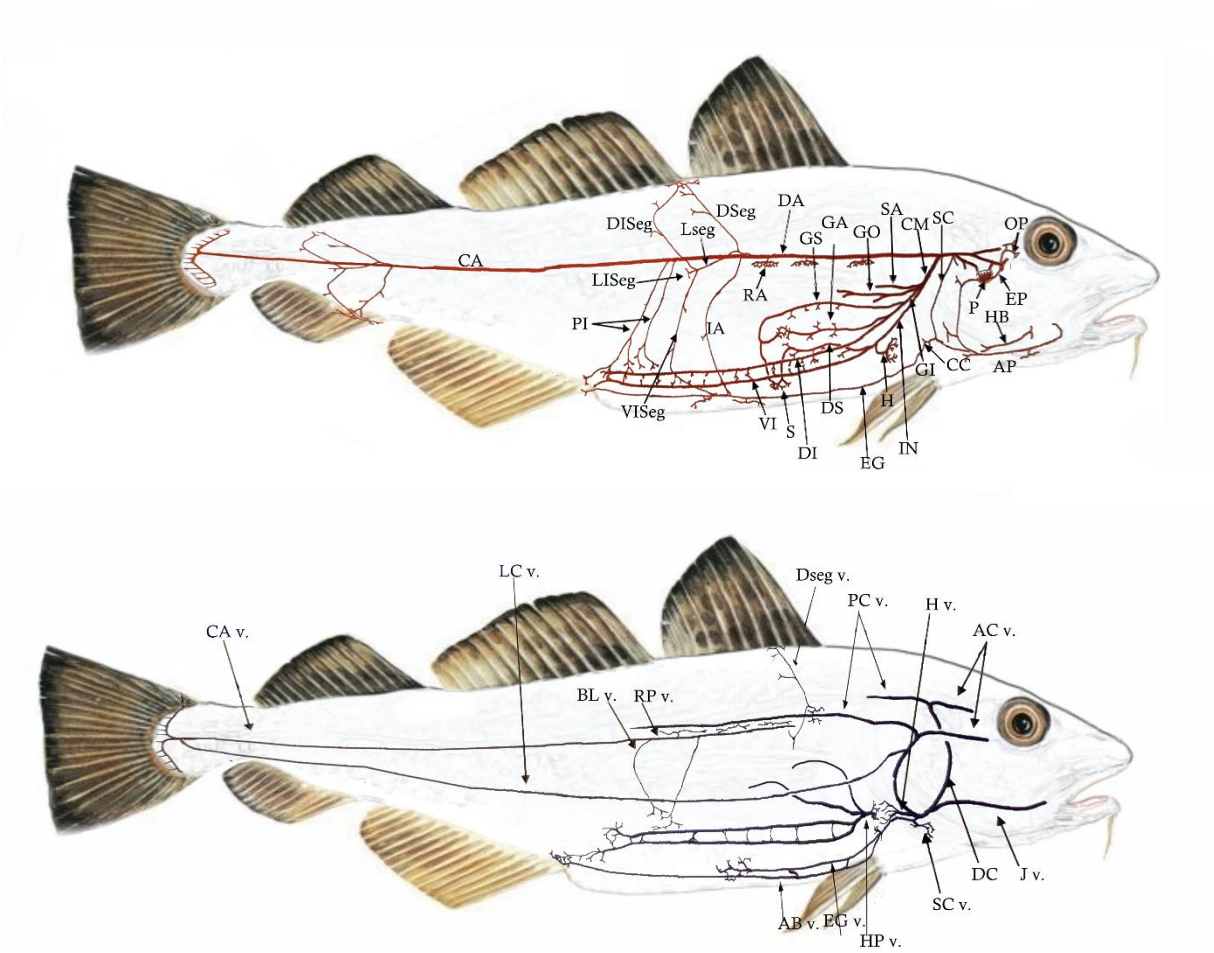

Figure 7. Major systemic arteries (top) and veins (bottom) in fish. AB v., abdominal vein; AC v., anterior cardinal vein; AP, afferent pseudobranch; CA, caudal artery; CA v., caudal vein (central vein); CM, celiacomesenteric artery; DA, dorsal aorta; DC, ductus cuvieri; DI, dorsal intestinal artery; DISeg, dorsal intersegmental; DS, duodenosplenic artery; Dseg v., dorsal segmental vein; EG v., epigastric vein; EG, epigastric artery; EP, efferent pseudbranch artery; GA, gastric artery; GI, gastrointestinal artery; GS, astrosplenic artery; H, hepatic artery; H v., hepatic vein, HB, hypobranchial artery; HP v., hepatic portal vein; IA, intercostal artery; IN, intestinal artery; J v., jugular vein; LC v., lateral cutaneous vein; LISeg, lateral intersegmental; Lseg, lateral segmental,; OP, ophthalmic artery; P, pseudobranch; PC v., posterior cardinal vein; RA, renal artery; RP v., renal portal vein; S, spleen; SA, swim bladder artery; SC, subclavian artery; SC v., subclavian vein; VI, ventral intestinal artery; VISeg, ventral intersegmental artery. Modified from Olson (2011a).

The local system, on the other hand, regulate blood flow locally in the peripheral tissue in response to production of metabolic waste products, which acts directly on smooth muscle cells in precapillary sphincters, causing vasodilation and increased blood flow in the tissue. This mechanism is referred to as local hyperemia and is important to ensure adequate oxygen supply locally in response to increased metabolism (e.g. muscle activity). Some of the putative vasodilatory agents include, but are not restricted to, H^+, CO_2, ATP, ADP, AMP and nitric oxide (NO) (Satchell 1991).

During sustained swimming, blood flow to the skeletal muscle of fish can increase four-fold due to local regulatory mechanisms, and blood is directed to the red muscles at the expense of

the white muscles (Satchell 1991). At higher swimming speeds, blood flow to the gastrointestinal tract is reduced due to increased vascular resistance triggered by sympathetic activity (Axelsson and Fritsche 1991). The redistribution of blood from the gastrointestinal tract to the red muscles ensures that the heart can maintain supply of oxygen to the working muscles. Temperature and hypoxia will also affect vascular resistance in fish through local mechanisms. For example, a decrease in temperature will lower metabolism, thereby increasing resistance. Furthermore, stimulation of vascular smooth muscle cells by circulating catecholamines may be blocked locally by NO, which is synthesized by the endothelium and the perivascular nerves and is a potent dilator of fish blood vessels (Olson 2011b).

The stress response in fish

Capture and transport are acknowledged causes of acute stress in fish (Sampaio and Freire 2016). All stages of trawl capture, including exhaustive swimming during the initial stage of trawling, crowding in the cod-end during trawling and air exposure after the fish are landed on deck, have the potential to induce stress in fish. Hence, each stage of the trawling operation may be considered a stressor, which can potentially affect the physiology of the fish and eventually also the quality of the flesh.

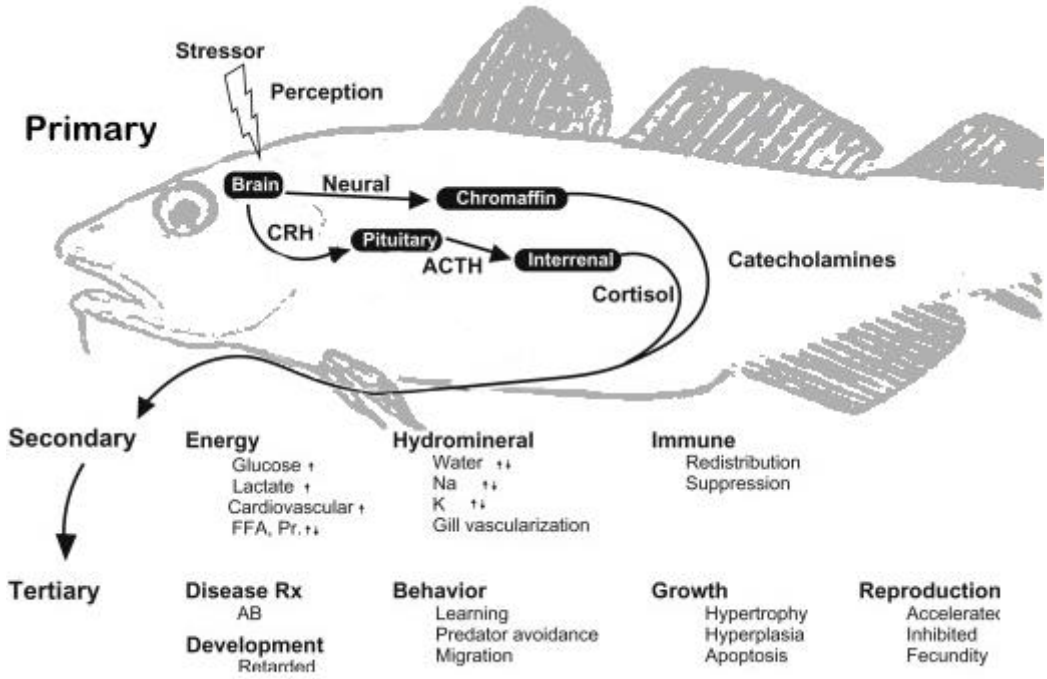

Figure 8. Overview over the primary, secondary and tertiary stress responses. CRH, Corticotropin releasing hormone, ACTH; Ardenocorticotropic hormone. Modified from Schreck and Tort (2016).

The term ‘stress’ is often a loosely used expression without consensus on its definition. Originally, stress was defined as the non-specific response of the body to any noxious stimuli (Selye 1950a). Later, this concept was revised and a distinction was made between a ‘stressor’ and a ‘stress response’. The stressor is the stimulus that jeopardize homeostasis and the stress response is how the organism copes with the stressor to regain or defend homeostasis (Chrousos 2009). The concept of homeostasis was first used to describe the physiological inner equilibrium (Cannon 1932) in terms of maintaining e.g. blood pressure, fluid volume, pH, salt concentrations etc. However, as nearly all activities of an organism relate directly or indirectly to the defence of homeostasis, the definition of stress as a threat to homeostasis seem illogical. For example, feeding will increase blood sugar and the mere action of waking up will cause a rise in level of the ‘stress hormone’ cortisol, but neither of these actions are considered stressors. Activities such as feeding and waking up are more or less predictable actions, and a more recent suggestion is that physiological stress is either the absence of an anticipatory response or a reduced recovery of the neuroendocrine reaction (Koolhaas et al. 2011).

Selye (1950b) made the distinction between primary, secondary and tertiary responses, collectively known as the General Adaptation Syndrome (GAS). The concept of GAS describes the overall stress response as a cascade of responses consisting of a primary response (alarm stage) which includes neural and endocrine responses, a secondary response (resistance stage) which covers changes in metabolic, respiratory, osmoregulatory, haematological and immunological responses, and a tertiary response in which the animal can no longer maintain homeostasis (Fig 8). The primary and secondary responses are considered adaptive, enabling the organism to mobilize sufficient energy to cope with the stressor, while the tertiary response is considered maladaptive leading to long-term detrimental effects. Behaviourally, the stress response triggers the organism to either move away from the stressor or to stay and fight the stressor, known as the ‘fight-or-flight’ reaction. Energy is attained via a set of catabolic reactions that brake down organic compounds such as carbohydrates, fats or protein in order to generate adenosine triphosphate (ATP) (Nelson 2011). ATP is a universal metabolic carrier of chemical bond potential energy and can be produced aerobically by mitochondrial oxidative phosphorylation and anaerobically via anaerobic glycolysis. To match the energy demand associated with stress, physiological mechanisms must be activated to ensure the availability of sufficient energy substrates. This is accomplished by a two-way communication between the central nervous system (CNS), the cardiovascular system, the immune system and other

systems via neural and endocrine mechanisms that are responsible for increasing concentrations of circulating glucose and lipids.

Primary stress response

Briefly, the CNS perceives the stressor and CAs are secreted from chromaffin cells, which are located in the walls of the posterior cardinal vein with the highest concentration of cells found in the rostral region of the vein in the head kidney (Reid et al. 1998). Circulating CAs rises rapidly and act on the heart to increase both heart rate and stroke volume, as well as on blood vessels to alter resistance and blood flow. In the gills, CAs enhance O_2 uptake. Furthermore, CAs increases the blood oxygen transport capacity and causes the spleen to contract, thereby releasing more erythrocytes into the blood stream. This together with erythrocyte swelling increases the haematocrit in stressed fish (Reid and Perry 2003). CAs also have a direct effect on the swimming performance (Moon 2011), although the exact underlying mechanism for how CAs are involved in muscle contractions is unclear. In the liver, CAs stimulate gluconeogenesis and glycogenolysis, while in adipose tissue they stimulate lipolysis. Gluconeogenesis is the metabolic pathway responsible for glucose production from non-carbohydrate sources such as amino acids, whereas glycogenolysis is the production of glucose from glycogen.

Cortisol has a broad range of functions in fish and important target tissues are gills, intestine and liver. These organs reflect some of its major purposes, namely regulation of hydromineral balance and energy metabolism. In addition, cortisol has immune suppressive effects. Furthermore, cortisol may stimulate a proteolytic function in white muscle cells, and possibly also in the liver, which can fuel gluconeogenesis (Moon 2011). Cortisol is produced in the head kidney, more specifically in interrenal cells. The production and secretion cortisol is regulated via the brain-pituitary-interrenal axis (Fig 8). During acute stress, the concentration of plasma cortisol tends to increase rapidly, within a minute to an hour, followed by a gradual decrease to pre-stress levels within a day. Cortisol is perhaps the most commonly used indicator for stress in fish because basal levels are low (<5 ngL-1) for most fish species and usually increases by 10-100 folds during stressful situations, depending on the stressor and the species (Sopinka et al. 2016). Some of the critiques on using only cortisol as a stress indicator comes from the fact that circulating glucocorticoids respond rapidly (i.e. often within 3–5 min) to capture and handling (Romero and Reed 2005), and so it is often difficult to obtain baseline levels in wild animals.

Secondary stress response

The secondary stress response in fish involves metabolic changes such as increase in circulating glucose and lactate and decrease in tissue glycogen (Barton 2002), as well as osmoregulatory disturbances by altering levels of plasma chloride, plasma sodium and water balance. These changes are caused by the release of hormones during the primary stress response.

Glucocorticoids (primarily cortisol) also increases circulating levels of glucose by stimulating gluconeogenesis and glycogenolysis (Moon 2011). Glucose is an important oxidative substrate to many cells and tissues in fishes. In fishes, glucose is most likely subordinate to lipids and protein and blood glucose levels varies tremendously between fish species and even within species. The variations depend on life stage, temperature, feeding regimes etc., and baseline values of glucose can therefore be difficult to interpret. The role of glucose increases with stressful situations, where energy requirements are high and urgent, because glucose have the potential to create energy in the form of ATP quickly and in the absence of oxygen. Therefore, glucose can be useful for assessing the acute stress response to specific stressors (Sopinka et al. 2016).

There are also studies reporting that both cortisol and CAs may affect glycogenesis in fish skeletal muscle (Pagnotta and Milligan 1991, Girard et al. 1992, Milligan and Girard 1993). Glycogenesis is the process of glycogen synthesis, and production of glycogen from lactate is one is the main end-points of lactate in fish. The white muscles of rainbow trout can retain as much as 80-85% of the lactate produced during exercise (Milligan and Girard 1993) and this retention of lactate is stimulated by catecholamines (Wardle 1978). Lactate is an important metabolite in fish that serves as an oxidative fuel, a glycolytic end-product, a gluconeogenic precursor, and an intracellular signalling molecule, and can also be a useful stress indicator because the rate of appearance is faster than the rate of disposal during situations where ATP supply is limited, such as during stress (Sopinka et al. 2016).

Acute stress also has a pronounced effect on cardiovascular function and tends to cause an increase in heart rate and stroke volume. In addition CAs lead to a rise in blood haematocrit by causing erythrocytes to swell and increasing the number of red blood cells and reducing blood clotting time due to higher levels of circulating thrombocytes (Tavares-Dias et al. 2009). The circulating CAs also stimulate branchial blood flow and oxygen diffusing capacity and increased oxygen transport capacity of the blood. Furthermore, vascular resistance of the systemic blood vessels are affected by high levels of circulating CAs (Wendelaar Bonga 1997).

The primary and secondary stress responses are highly adaptive in fish, in terms of mobilizing and distributing energy, thus preparing the fish for fight or flight. However, it may influence the quality of final fish product because it changes the chemistry of the muscle tissue and may influence the efficiency of bleeding fish (Jørpeland et al. 2015, Olsen et al. 2013, Olsen et al. 2008, Digre et al. 2017).

Aims of the study

During trawl capture, fish are exposed to a number of stressors which may reduce the quality of the final product. These stressors involve swimming to exhaustion, crowding in the cod end, severe barotrauma, and lack of controlled killing and bleeding. Identifying or singling out the most important factors is very challenging on board trawlers, due to weather conditions, differences in haul size and duration. Therefore, the overall aim of this thesis is to isolate and experimentally test how exhaustive swimming, exhaustive swimming followed by crowding and traditional slaughtering techniques (asphyxiation) effects the physiology and the fillet quality of haddock and Atlantic cod, and to see how short-term recuperation affect these parameters.

General results and discussion

Trawlers land the largest quantum of cod and haddock in Norway. Understanding why quality from trawl-caught fish varies will have great ethical and financial impact on the fishery industry, as it sets the base for development of technologies to prevent or reverse impairment of fish quality.

Swimming

Capture of cod and haddock by trawl involves an initial phase where the fish swim in the net opening for some time before capture. The duration of this phase is dependent on towing speed, water temperature and condition of the fish (Winger et al. 2010). At some stage, the fish usually change its swimming behaviour from sustained to burst-and-glide swimming, which is an indication of a switch from the use of aerobic red muscles to anaerobic white muscles. During swimming, blood flow to the red muscles may increase four-fold (Satchell 1991). In Atlantic cod, exercise is reported to induce a decrease in the total vascular resistance and redistribution of blood from the intestinal tract to the other parts of the systemic vasculature (Axelsson and Fritsche 1991). This can be explained by hyperaemia in the working muscles, due to release of metabolites, which leads to increased blood flow to the muscles. We therefore hypothesized that an increase in blood flow to the working muscles could lead to deposition of residual blood and hence an increase in fillet redness after exhaustive exercise. Furthermore, Olsen et al., (2013) observed an increase in fillet redness after 3 hours of recuperation from trawl capture. Blood flow to both white and red muscles have been documented to increase after muscular activity (Neumann et al. 1983), and therefore we speculated whether short-term recuperation would reduce residual blood in fillets. We addressed these issues by using a large-scale swim tunnel to physically exhaust haddock (Paper I) and Atlantic cod (Paper II and III), followed by recuperation for 0, 3 or 6 hours (paper I) or 0, 2, 4, 6 or 10 hours (paper II).

When comparing the swimming experiments of haddock (paper I) and cod (paper II) to studies done on commercial trawlers (Digre et al. 2010, Olsen et al. 2013) it was clear that the changes we found in muscle pH, blood lactate and fillet redness were less pronounced than had been observed previously. This indicates that the exhaustive swimming procedure we used should probably be considered a moderate stressor. Yet, the observed swimming behaviour in the experimental set-up with kick and glide just before the fish ceased swimming, were similar to that observed in the net opening of a trawl (Beamish 1969).

We further concluded that exhausted swimming is not the main cause of reduced quality in terms of fillet colour. However, exhaustive swimming did have a significant effect on time and strength of post-mortem muscle stiffness. We found the time it took for haddock to reach maximum muscle stiffness was significantly reduced (paper I) and that swum cod had a higher maximum muscle stiffness during *rigor mortis* than rested cod (paper II). This indicates that, although swimming is not a severe stressor, it reduces the white muscle stores of glycogen, thereby causing a faster and stronger onset of *rigor mortis* (Stroud 1969). This effect was reversed sometime between 0 and 3 hours of recuperation for haddock (paper I) and 0 and 2 hours for cod (paper II), indicating that muscle energy stores are beginning to recover. However, a full recovery may take up to 12 hours (Kieffer 2000), as indicated by elevated blood glucose levels throughout the whole recovery period (6 hours for haddock, paper I) and (10 hours for cod, paper II).

Although exercise-induced hyperaemia may result in increased blood flow to the working muscles, we did not detect any increase in fillet redness for either haddock (paper I) or cod (paper II and III). It seems therefore that the amount of blood entering the working muscles during exercise is removed at the same rate as it appears. We concluded that exhaustive exercise probably has an effect on the textural quality of the fillets of both cod and haddock, but no effect on visual residual blood in the muscle. Hence, swimming in front of the net is not the major source of the quality impairment frequently seen in trawl-captured cod and haddock.

Crowding

In paper I and II, we concluded that the stress associated with exhaustive swimming had a low overall impact on fillet quality. Several studies have reported a relationship between the duration of trawl hauls and the frequency of fillet quality defects, and literature suggests that crowding may trigger a severe stress response in fish (Bahuaud et al. 2010, Lerfall et al. 2015, Montero et al. 1999, Ortuno et al. 2001, Pickering and Stewart 1984, Skjervold et al. 2001, Tort et al. 1996, Wedemeyer 1976). There is, however, little information on the timing of the stress response associated with the 'crowding' stage of the trawling operation, and we therefore addressed this issue by swimming commercially sized cod to exhaustion followed by extreme crowding (736 ± 50 kg m^3) of the fish for 0, 1 or 3 hours in an experimental cod-end. The fish were then allowed to recuperate for 0, 3 or 6 hours in a net pen prior to slaughter, in order to assess if any potential impairment of fillet quality could be reversed within a reasonable timeframe (Olsen et al. 2013).

We found that exhaustive swimming combined with crowding was associated with a marked metabolic stress response, as indicated by high levels of plasma cortisol, blood lactate and blood haematocrit levels, as well as a reduction of fillet quality in terms of increased visual redness and a drop in muscle pH. The severity of the metabolic stress response, as judged from the metabolic markers, was comparable to that reported for lengthy (>5 hrs) and large (>20 tons) trawl hauls (Olsen et al. 2013) . Furthermore, the evaluation of fillet redness presented in paper III indicated that the fillets of our fish were assessed as having even more red colouration than fillets of commercially caught fish (Fig 9).

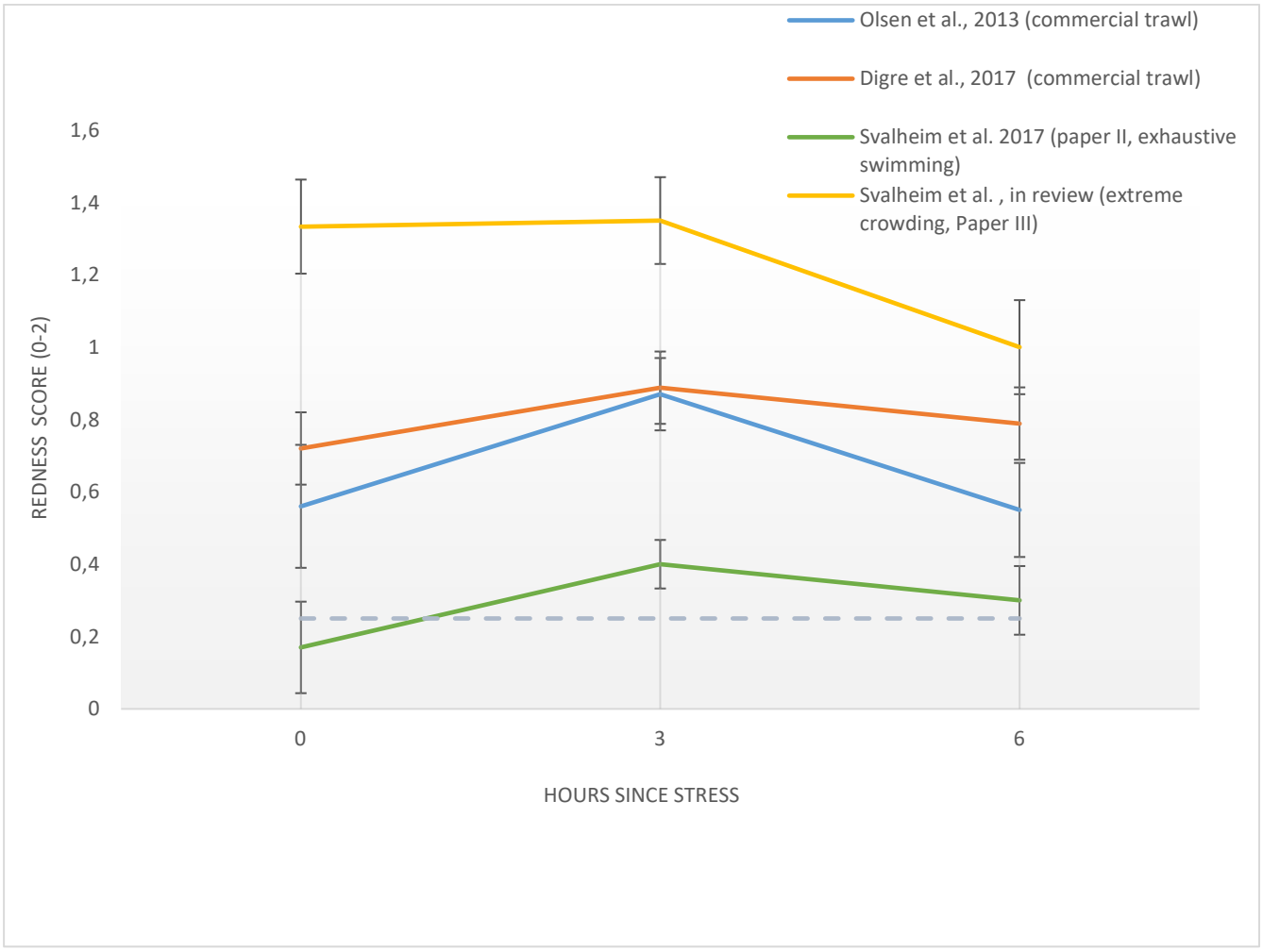

Figure 9. Comparison of five different experiments with sensory evaluation of fillet redness. All numbers have been transformed to the same scale (0-2), 0 being perfectly white, 2 being very red and blood filled. Stress in this setting is defined as either exhaustive exercise, exhaustive exercise followed by crowding or commercial trawl capture.

However, this result must be interpreted with caution, as the sensory evaluation of redness may be better suited for evaluating relative differences within the same experiment, rather than making comparisons across experiments.

Short-term recuperation only had positive effects on quality in terms of time to maximum muscle stiffness (paper I), the level of maximum muscle stiffness (paper II) in the process of *rigor mortis* and increase in muscle pH (paper III), but did not have a large impact on quality in terms of fillet redness (paper III). Studies conducted on board commercial trawlers, found that fillet redness tended to increase during the first 3 hours of recuperation after capture and then decreased to at-capture levels after 6 hours (Olsen et al. 2013, Digre et al. 2017) (Fig 9). Notably, no such increase was found in experimental setting (paper III), where redness remained unchanged throughout the recuperation period. Based on these findings, recuperation for 6 hours in our experiments had little effect on quality in terms of fillet redness.

Air exposure

On board trawlers, the final phase of the trawling operation include hauling the catch from the water and on-board the fishing vessel, where the catch is usually stored dry in bins until further processing or exsanguination. During this stage, the fish is normally exposed to air for some time prior to exsanguination, so they become asphyxiated and easier to handle in terms of bleeding (Van De Vis et al., 2003). In paper IV we investigated the effects of asphyxiation on stress parameters (muscle and blood pH, lactate and glucose) and fillet quality in terms of amount of haemoglobin in muscle of cod.

As clearly stated in paper III, and previously shown in studies on board commercial trawlers, the trawling operation represents a strong overall stressor that affects the fillet quality of cod. We therefore hypothesized that the accumulation of stress throughout the trawl capture, from swimming to crowding to air exposure, is the major cause of variable quality of trawl captured gadoids. The last paper of this thesis (paper IV) investigated how pre-mortem stress by mild crowding followed by air exposure for 0, 15 or 30 minutes effected metabolic stress parameters, blood coagulation time and muscle haemoglobin concentrations (as a measure of residual blood in the fillet).

As in paper III, paper IV also showed that stress from crowding resulted in increased levels of muscle haemoglobin in the fillets. Furthermore, we found that air exposure had an additional negative effect on fillet quality, which was stronger when the fish were crowded prior to air

exposure. However, by euthanising the fish before air exposure, the accumulation of residual blood was delayed. Interestingly, crowding for four hours did not cause a significant increase in lactate or pH. Nevertheless, we did find significantly higher concentrations of haemoglobin in muscles of crowded individuals, indicating that 'mild' crowding may already affect the quality of the fish in terms of residual blood in the fillet.

Previous recommendation states that fish should be bled within 30 minutes of slaughter. However, the study asserting that recommendation is done on unstressed fish. In our study, we found that the effect of crowding on haemoglobin concentration in the muscles masked the effect of bleeding fish. This suggests that the practice of leaving fish in air may be detrimental to the fillet quality and should be avoided.

Why is crowding important for fillet redness?

It was surprising to us that mild to moderate stress from exercise (paper I and II) did not cause any increase in muscles redness, whereas stress from mild crowding (paper IV) did. It appears as if the amount residual blood in fillets is dependent on whether stress is induced from exercise or crowding. It should be noted that the evaluation of residual blood in these three papers is not the same; sensory evaluation in paper I and II versus VIS/NIR spectroscopy in paper and IV. In paper III, we tested the correlation between spectroscopy and sensory evaluation of fillet redness and found a significant positive correlation between fillet redness and muscle haemoglobin (Fig 10). Furthermore, both the spectroscopic and the sensory evaluation in paper III indicated that swimming did not cause an increase in redness, whereas crowding did. It is therefore worth speculating on why crowding appears to have the highest impact on fillet redness, even when it is done in such a way that it does not induce a severe stress response.

It is tempting to assume that the fillet redness is directly linked to the degree of stress. In paper III we found a positive correlation between cortisol and fillet redness. However, a correlation is not the same as causality and, in paper II the increase in cortisol was not linked to the increase in redness. Furthermore, metabolic stress parameters such as glucose, lactate and pH measured in crowded fish in paper IV indicated that the fish were only mildly stressed, yet haemoglobin concentration in the muscles was significantly increased.

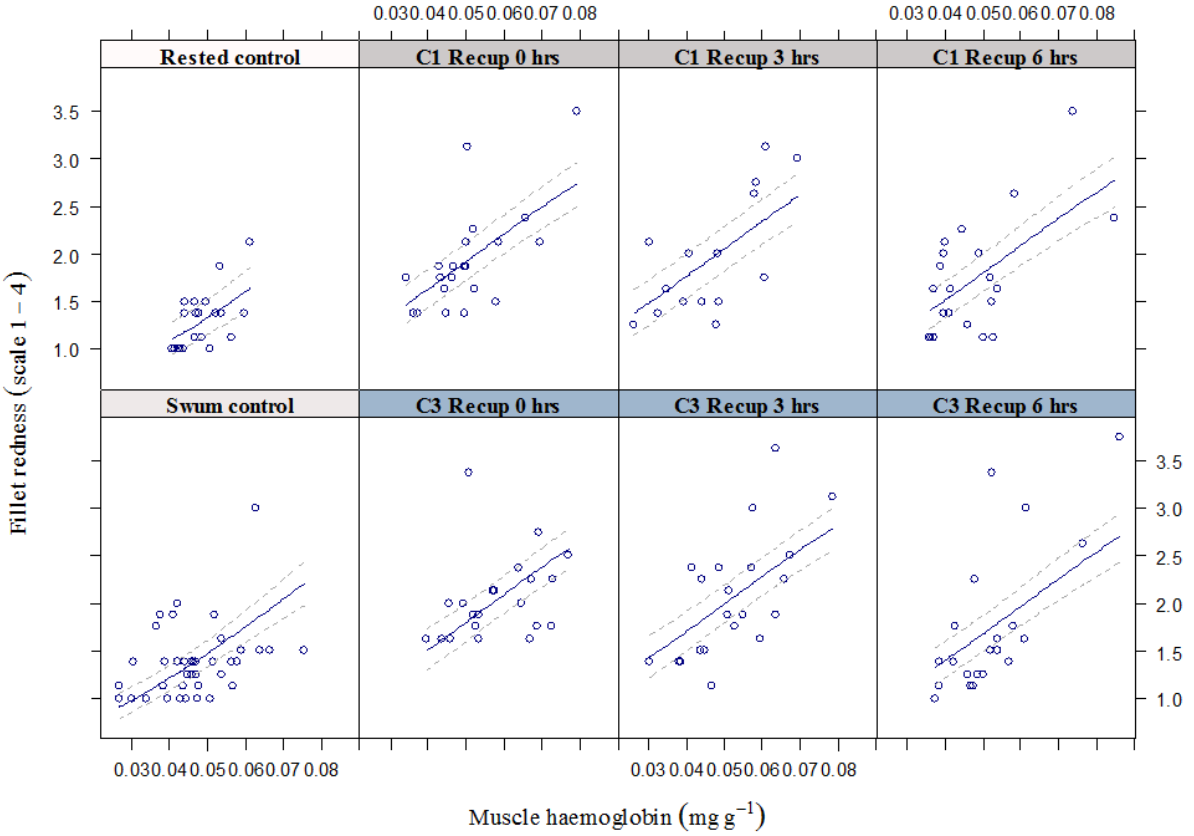

Figure 10. Correlation between filet redness and muscle haemoglobin. Continuous lines are trend lines estimated from the GLM and dotted lines indicate 95% confidence intervals (Paper III).

Previous studies have shown that blood is redistributed to white muscles during swimming (Neumann et al. 1983). However, as discussed in paper I and II, no increase in visual redness was found in fillets of cod or haddock during exercise, whereas crowded fish (paper III and paper IV) were evaluated as having a greater red colouration than swum or rested fish. It has been suggested that fillet redness is due to increased blood flow to the muscles to flush out waste products and protect against acidosis (Olsen et al. 2013). But, this does not explain why there is a difference between exhaustive exercise and crowding in terms of redness. It is interesting to speculate on some possible contributing reasons for increased fillet redness and why it occurs in crowded, but not exercising fish.

As previously mentioned, hyperaemia is the process by which the body adjusts blood flow to meet the metabolic needs of its different tissues (Satchell 1991). Functional, or active, hyperaemia leading to increased blood flow, is mediated by a rise in vasodilatory agents during

periods of increased cellular metabolism. A possible explanation for the difference in residual blood content of white muscle in exercised, compared to exercised and crowded fish, is that accumulation of residual blood in the crowded fish is caused by insufficient emptying/return of venous blood from the segmental veins, due to impaired movement of the swimming muscles. The venous blood from the swimming muscles of fish is passed to the central veins via the segmental veins and depends on the alternating movement of the lateral muscles, which squeezes the blood out of the segmental veins and into the central veins when the ipsilateral muscles contract. The segmental veins, in turn, are guarded by ostial valves (Fig 11), which prevents backflow of blood when the ipsilateral muscles relax. Hence, return of venous blood from the swimming muscles is dependent on the continuous, alternating sideways muscular movement of the body of the fish. This mechanism is similar to the muscular pump of the lower limb in humans (Ludbrook 1966), which also depend on muscle contraction to facilitate the return of venous blood to the heart. It is possible therefore that in situations where the alternating contraction/relaxation of the swimming muscles is blocked, e.g. when the fish is tightly packed in the cod end (paper III), that the return of the venous blood from the swimming muscles is impaired, resulting in accumulation of blood in the muscles. This may also be the case during recuperation in the crowding experiment (paper III), where the fish laid still on the bottom of the cage during the first few hours of hours of recuperation (unpublished observations). Interestingly, the fish in this experiment began to swim slowly sometime between 3 and 6 hours of recuperation, corresponding to the time when fillet redness began to decrease.

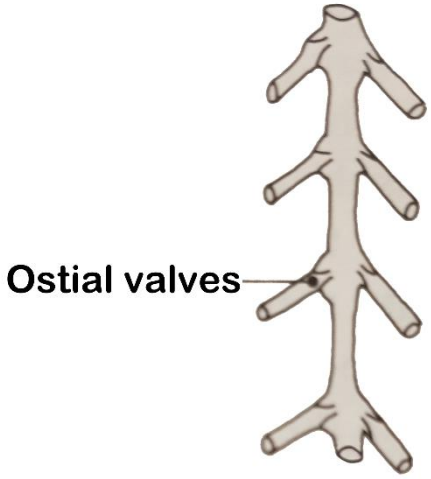

Figure 11. Location of the ostial valve on a large longitudinal (central) vein.

Conclusions

Together these four studies show that there is a connection between type of stressor affecting the fish during capture and the quality of the fish product (fillets) in terms of colour. Crowding and air-exposure cause an increase in fillet redness and concentration of haemoglobin in the muscle that was not seen for exercised fish. It is suggested that the accumulation of residual blood in the white muscles of crowded fish may be the result of insufficient emptying of segmental veins due to the static condition of the muscles during crowding. Measures that may secure top quality fish from trawlers, include reducing crowding in the cod-end and implementing direct slaughter. Recuperation may have beneficial effects on fillet quality by reducing air exposure time and gaining control over slaughter time. However, in terms of fillet colour, recuperation must be longer than 6 hours.

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Paper I

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Research paper

Recovery from exhaustive swimming and its effect on fillet quality in haddock (*Melanogrammus aeglefinus*)

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ABSTRACT

Wild haddock (*Melanogrammus aeglefinus*) of commercial size (0.8–2.49 kg, 45–60 cm) were swum to exhaustion in a large swim tunnel and then allowed to recuperate for 0, 3 or 6 h, to investigate the effects of exhaustive swimming on blood glucose, blood lactate and post mortem development of fillet quality. There was a positive linear relationship between critical (aerobic) swimming speed (U_{crit}) and body length (BL). The average U_{crit} was 1.25 ± 0.29 (SD) BL s^{-1}, which is close to that reported by others for haddock. Swimming to exhaustion resulted in reduced time to reach maximum muscle stiffness of the fillet (no recuperation vs unswum control), but the effect was remedied by recuperation for 3 h or more. Blood glucose and blood lactate increased during exercise and remained elevated throughout the entire 6-h resting period, indicating that complete recovery of these parameters may take more than 6 h. There was no significant effect of exhaustive swimming on muscle pH or colouration of the fillet. Taken together, the data suggest that swimming to exhaustion may have moderate and reversible negative effects on fillet quality in haddock. The effects observed in the present study are consistent with a recent study on exhaustive swimming in Atlantic cod, but less severe than that reported for haddock caught by trawl. This suggests that other factors (e.g. crowding/packing in the codend, barotrauma or suffocation) are contributing to the deterioration of fillet quality seen frequently in haddock caught by trawl.

1. Introduction

Trawl fishing accounted for 55% of the average yearly landings of haddock (*Melanogrammus aeglefinus*) in Norway from 2005 to 2013 (Norwegian fish landing statistics, 2014). Trawling of both haddock (Digre et al., 2017) and Atlantic cod (*Gadus morhua*) (Olsen et al., 2013) has been shown to induce poorer fillet quality than longlining (Roth and Rotabakk, 2012), depending on duration and size of the haul (Margeirsson et al., 2007; Olsen et al., 2013). Pre-slaughter fatigue is a major cause of reduced post mortem quality and shelf-life of fish (Cole et al., 2003) and line-caught fish is generally considered to be of better quality than fish caught by active gears such as trawl and Danish seine. For instance, cod and haddock caught by line may give a price premium of 18 and 10%, respectively, in the UK (Sogn-Grundvåg et al., 2013).

Discolouration of the fillet and low muscle pH are two important causes of reduced quality in white fish. Residual blood in the white muscle may give the fillet a reddish or dark appearance, which is an aesthetic problem and a quality defect as the consumer expects white fish to be white. A low muscle pH, on the other hand, affects the quality of the fillet indirectly by shortening the time to full muscle stiffness during *rigor mortis*. Before filleting, the fish has to be straightened (unfolded), which could cause muscle tears followed by gaping and dryness of the fillet if this occurs during full muscle rigor (and not during pre-rigor) (Borderías and Sánchez-Alonso, 2011; Olsson et al., 2007). Discolouration of the fillet and muscle gaping may both lead to downgrading of the fish and financial loss for the producer (Michie, 2001; Robb and Whittington, 2004).

The exact cause of the poor fillet quality frequently seen in trawled fish is unknown, but studies on farmed fish have shown that different types of handling stress prior to slaughter may increase the amount of residual blood in the fillet (Skjervold et al., 1999; Robb et al., 2003; Roth et al., 2005; Olsen et al., 2006; Olsen et al., 2008; Roth et al.,

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Table 1
Median values and 95% confidence intervals of condition factor (CF), hepatosomatic index (HSI), gonadosomatic index (GSI), muscle pH measured immediately after death (pH_{start}), pH measured at the end of the 60–80 h storage period (pH_{end}), fillet colouration (Hunter's Whiteness Index) and maximum muscle stiffness in controls (Rested control) and recovery groups (0 h, 3 h and 6 h).

	Rested control	Recovery		
		0h	3h	6h
CF	1.07 (0.84, 1.22)	1.12 (1.02, 1.29)	1.15 (1.07, 1.21)	1.13 (0.80, 1.35)
HSI	0.092 (0.04, 0.14)	0.13 (0.097, 0.15)	0.12 (0.11, 0.12)	0.12 (0.07, 0.21)
GSI	0.03 (0.00, 0.13)	0.01 (0.00, 0.082)	0.07 (0.02, 0.12)	0.07 (0.00, 0.14)
pH_{start}	7.45 (7.30, 7.60)	7.29 (7.04, 7.41)	7.36 (7.21, 7.57)	7.35 (7.19, 7.50)
pH_{end}	6.22 (6.09, 6.32)	6.10 (6.05, 6.23)	6.13 (6.037 6.227	6.11 (6.02, 6.17)
Hunter Whiteness Index	68.13 (61.96, 74.51)	63.19 (61.28, 68.94)	64.99 (63.56, 66.89)	64.14 (61.38, 69.40)
Max. muscle stiffness (kPa)	1013 (987, 1071)	1000 (985, 1058)	1000 (814, 1099)	913 (802, 1056)

2009). Thus, it is reasonable to assume that the various events of the trawl fishing operation the fish have to go through, such as swimming to exhaustion, crowding in the codend, decompression and subsequent barotrauma when lifting the trawl, may represent potential stressors that could contribute to deterioration of fillet quality (Svalheim et al., 2017). To prevent the poor fillet quality observed in trawled fish, it is therefore important to identify which stage(s) are most detrimental to quality and where efforts should be directed to improve the process.

The normal towing speed of a bottom trawl (1–2 m s^{-1}) exceeds the maximum sustainable swimming speed of haddock ($\leq$0.65 m s^{-1}; Breen et al., 2004), which have been reported to swim at the same speed and direction as the trawl with a 'kick and glide' style until they stop swimming (Main and Sangster, 1981). This suggests that most haddock will be exhausted once they enter the codend. The primary cause of exhaustion during swimming is considered to be due to insufficient supply of oxygen to fuel the aerobic red swimming muscles (Reidy et al., 2000), but swimming may also reduce white (anaerobic) muscle energy stores (Lurman et al., 2007). The white muscle mass makes up the largest part of the fillet in haddock and the pH of the white muscle at slaughter is important for the fillet quality later on (Borderías and Sánchez-Alonso, 2011).

During normal recovery from exhaustion (capture in trawl), blood lactate of haddock has been shown to return to pre-exercise levels after 12 h (Beamish, 1966). The recovery of white muscle metabolic status (i.e. white muscle pH and lactate) may be expected to follow a similar pattern, as suggested by data from other species (Beamish, 1968; Wood, 1991). The white muscle in rainbow trout (*Oncorhynchus mykiss*) receives up to 80% of the cardiac output during recovery from exhaustive swimming (Neumann et al., 1983). A similar response may take place in gadoids and could explain, at least in part, the increase in fillet redness observed in haddock (Digre et al., 2017) and cod (Olsen et al., 2013) caught by trawl. However, it remains uncertain if a full recovery of the white muscle metabolic status is necessary to return fillet quality parameters to pre-exercise levels, as fillet quality in Atlantic cod caught by trawl may improve substantially after only 6 h of live storage in tanks (Olsen et al., 2013). A recent study on Atlantic cod further suggests that white muscle pH is restored more quickly than the stress induced increase in blood glucose after exhaustive swimming (Svalheim et al., 2017). In the current study, we aim to investigate if swimming to exhaustion may contribute to the poor fillet quality reported in haddock caught by trawl, and if post exercise recuperation for 3 or 6 h can normalize fillet quality. We have addressed these questions by measurements of muscle pH, time to full muscle stiffness (rigor), muscle redness, as well as blood glucose and lactate concentrations in a group of haddock swum to exhaustion in a novel experimental swim tunnel.

2. Materials and methods

2.1. Fish and pre-swimming experimental conditions

Wild haddock were caught by a Norwegian purse seine vessel (Korsnesfisk) in Sandfjorden (Sørøya, Norway) in early November 2012 at 30–40 m depths. The haddock (n = 50) were collected randomly from the purse seine, placed in an on-board 800 L container supplied with fresh seawater (50 L min^{-1}), and subsequently transported to the Aquaculture Research Station in Tromsø, Norway, where the experiment took place. The fish were kept indoors under natural photoperiod (transparent roof, 69°N) and water temperature (maximum 8.5 °C in October, minimum 2.6 °C in mid-March) in a 2 m diameter, 4 m^3 volume circular tank for 6 months before the experiment commenced in late April 2013 (water temperature 3.5 °C). The water supply was taken at a depth of 50 m and it is assumed that the temperature of the water was close to that experienced by wild haddock in their natural habitat. The fish were inspected twice daily, and moribund fish (suffering from skin damage or barotrauma after catch) were immediately removed and euthanized by a blow to the head. The fish were fed daily to satiation, using a mixture of capelin (*Mallotus villosus*) and commercial feed (Skretting Amber 5 mm, Skretting ASA, Norway). Feed was removed 48 h prior to sampling of controls and transfer of fish into the swim tunnel (see section 2.2). A total of 26 fish (13 males and 13 females) with an average body weight (BW) of 1.57 ± 0.46 (mean ± SD) kg (range 0.80–2.49), an average body length (BL) of 51.9 ± 3.7 (mean ± SD) cm (range 45.0–59.7), an average condition factor of 1.10 ± 0.17 (mean ± SD) (range 0.60–1.34), an average gonadosomatic index (GSI) of 0.05 ± 0.06) (mean ± SD) (range 0.01–0.18) and an average hepatosomatic index (HSI) of 0.12 ± 0.04 (mean ± SD) (range 0.03–0.27) were used in the experiment (Table 1), divided between a control group of non-exercised fish (n = 6) and the experimental group subjected to swimming in the tunnel (n = 20). Prior to the start of the experiment, the control fish were netted randomly from the tank, euthanized and sampled according to the same procedure as the other fish (see Section 2.4). The remaining fish (n = 20) were then transferred to the swim tunnel and allowed to acclimate for 24 h at a low water speed of 0.1 m s^{-1} (~ 0.2 BL s^{-1}).

2.2. Swim tunnel

The swim tunnel was custom made to provide an experimental setup for swimming trials with groups of large fish. It consisted of a collection of 0.8 m inner diameter black, high density polyethylene pipes (BorsafeTM HE3490-LS, Borealis AG, Vienna, Austria), a 3-blade propeller with an integrated 5.5 kW electrical motor (Flygt PP4650.410 with 11° blade angles, Xylem Water Solutions, Charlotte, NC, USA) secured in a 0.8 m inner diameter aluminum tube housing and a 1400 L octagonal aluminum swim chamber (2.8 m in length and 0.8 m in inner height and inner width; Fig. 1). The swim chamber was equipped with an acrylic glass top hatch for loading of fish and a transparent side window for underwater camera observation of the fish. The tunnel was submerged in an 11 m inner diameter circular tank supplied with filtered seawater of natural temperature. To minimize turbulence, a flow director made from square pipes (5 × 5 cm) of 1 m length, was placed in front of the swim chamber. Grids, preventing fish from escaping the

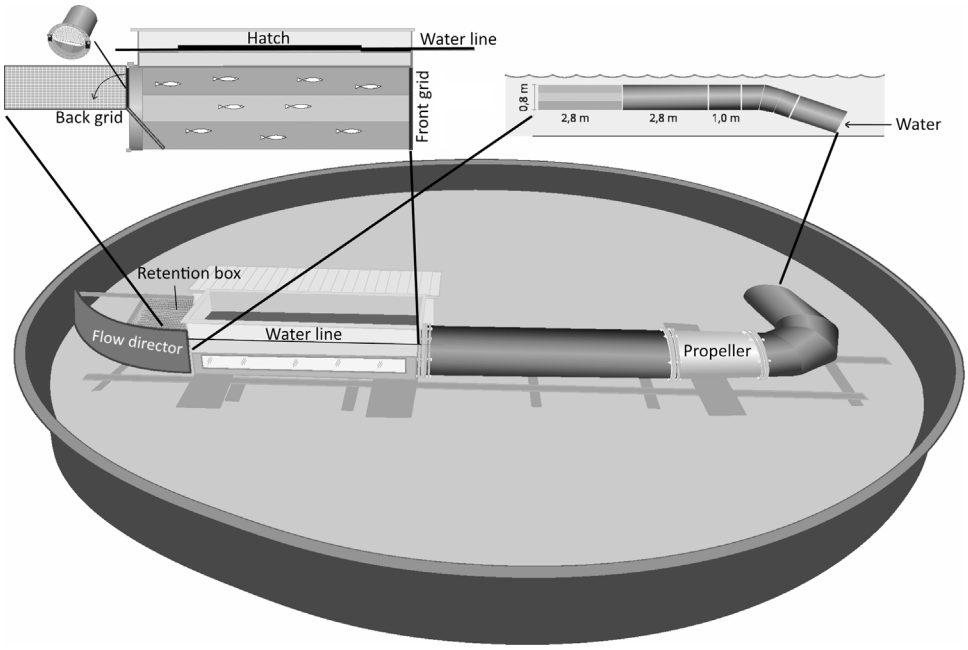

Fig. 1. Schematic drawing of the experimental swim tunnel. The construction was submerged in an 11 m diameter outdoor tank circulated with ambient seawater via an inlet pipe placed close to the edge of the tank (not shown) and a water outlet in the centre of the tank (not shown). The swim chamber could be accessed via a wooden platform (not shown) connecting the tunnel system with the area outside the tank.

swim chamber, were made from plastic coated stainless steel strands (SS grid) placed in the front and rear ends of the chamber. The lower half of the rear grid slanted, so that its lowest point protruded ca 0.4 m into the swim chamber. Thus, it directed exhausted fish to the upper half of the rear grid, which was attached to the lower half with hinges and could be opened to remove exhausted fish individually. A retaining box made from SS was placed behind the chamber to prevent fish from escaping further into the tank when the rear grid was opened (Fig. 1).

Water velocity in the tunnel was controlled by changing the electrical motor revolutions per minute (RPM) and thereby the propeller speed. RPM was controlled by sending a 4–20 mA signal on the motors frequency converters analog input (11 bit resolution). The 4–20 mA signal was generated by a NI 9265 USB measurement bundle from National Instruments, Austin, TX, USA (NI) controlled by a specially developed software application designed in LabVIEW (NI). The software application was designed as a feed forward control, where the algorithm gave an analog output 4–20 mA signal by using a transfer function calculated from the swim tunnel's flow calibration results. It was then possible to set the swim tunnel's flow velocity from the interface of the control system.

2.3. Experimental protocol and conditions

A method based on Brett (1964) was used to calculate critical swimming speed (U_{crit}) for all individuals:

$$U_{\text{crit}} = V_{n-1} + \left(\frac{t_n}{t_i}\right) \cdot V_i$$

where:

V_{n-1} = the highest water speed (m s^{-1}) the fish could successfully swim at for 30 min

V_i = the water speed increment (m s^{-1})

t_n = the time in minutes the fish swam at the speed it became exhausted

t_i = the duration in minutes of each water speed step.

After 24 h acclimation in the swim tunnel at 0.1 m s^{-1}, the water speed was increased in steps of 0.00133 m s^{-1}over 5 min, until a speed of 0.5 m s^{-1} was reached. This speed was maintained for 30 min, and after that the water speed was increased with 0.05 m s^{-1} increments every 30 min until all fish stopped swimming. Exhaustion was defined as the speed at which a fish would no longer swim, despite encouraging the fish to move away from the grid by pinching (with fingers) the tail gently for 10 s. Fish that did not respond to pinching was either transferred to separate floating cages (SS grid; 60 × 30 × 20 cm, length x width x depth) for recovery (3 or 6 h) or euthanized immediately and sampled. Water temperature during acclimation, swimming and recovery ranged from 3.5 to 4.5 °C and oxygen saturation was always between 97 and 100%.

2.4. Sampling and on-site fillet quality measurements

Blood was sampled from the caudal vessels, using 6 mL heparinized vacuum tubes (Vacutainer®) and 40 × 0.9 mm needles (BD Diagnostics, Franklin Lakes, NJ). The blood samples were analyzed immediately for lactate (Lactate Scout, EKF Diagnostics, Cardiff, UK) and glucose (FreeStyle Lite, Abbott Diabetes Care Inc., California, USA). To measure initial pH in the white muscle (pH_{start}), a small incision (ca 1 cm) was made with a scalpel approximately 3 cm ventral to the posterior attachment of the first dorsal fin (Fig. 2A). The pH was measured with a hand held WTW330/set-1 pH-meter (Wissenscaftliche-Technische-Werkstätten, Weilheim, Germany) equipped with a Hamilton double pore glass electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland). The instrument was calibrated frequently using pH 4.01 and pH 7.00 buffers, and the electrode was cleaned regularly to obtain consistent results. The fish was then bled out by cutting the *Bulbus arteriosus* and *Vena cardinalis communis* while left in cold (4 °C), running seawater for 30 min. The fish were then weighed, length measured, gutted and sex determined before the organs were weighed. Each fish was then placed in standard plastic fish boxes with the belly down, covered with ice and stored for 60 h or more to track development of muscle stiffness (as an indicator of *rigor mortis*) measured by use of a hand held Effegi penetrometer (FT 02 Penetrometer, QA Supplies LLC, Virginia, USA), according to Berg et al. (1997). Briefly, the maximum force (kPa) required to push a 8 mm diameter stainless steel plunger 5 mm into the fillet was recorded. Measurements were made repeatedly on 4 different locations (spaced approximately 50 mm apart) along the loin (Fig. 2B). Muscle stiffness was recorded approximately every 4 h the first 12 h, then approximately every 8 h for the remainder of the storage period, until the peak in muscle stiffness was passed. After the recording of muscle stiffness was completed, a final measurement of muscle pH (pH_{end}) was obtained at the same place as the initial pH_{start}. Muscle samples and measurements of pH were taken from the left loin, whereas

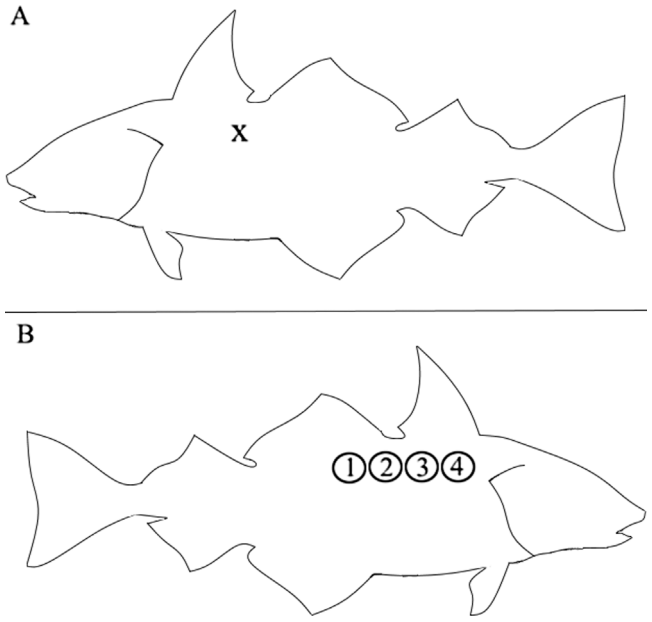

Fig. 2. Schematic diagram of the locations on the fish where measurements of muscle pH (A) and muscle stiffness (B) were made. Fillet colouration (Hunter's Whiteness Index) was recorded on the internal side of the right loin (after the fish was filleted) in the same area as measurements of muscle stiffness where obtained (B).

measurements of muscle stiffness were obtained repeatedly on the same surface area on the right loin. The fish was then filleted and colouration (given as fillet whiteness; Hunter's Whiteness Index) was recorded on three locations along the right loin (approximately 20 mm apart) in approximately the same area where measurements of muscle stiffness were obtained (see Fig. 2B), using a hand-held Minolta CR-200 Chroma Meter colorimeter (Minolta, Japan).

2.5. Statistical analyses and data management

The data were analysed with the statistical software R, version 3.2.0 (R core team, 2015). The relationships between different quality metrics [i.e. Hunter Whiteness Index, muscle pH_{start}, muscle pH_{end}, maximum rigor (kPa) and time to maximum rigor (hours)] and corresponding potential explanatory variables [i.e. recovery time (as factor; groups: control, 0, 3 & 6 h), sex (as factor), fish length (cm), U_{crit} (m s^{-1}), blood glucose (mM L^{-1}), blood lactate (mM L^{-1}), hepatosomatic index (HSI), gonadosomatic index (GSI) and Fulton's condition factor (100 g cm^{-3})] were investigated using Generalised Linear Modelling (GLM) (McCullagh and Nelder, 1999; Buckley, 2015). Before proceeding with the GLM analysis, the data were checked and prepared for modelling following the procedures described by Zuur et al. (2009). Each GLM was constructed from a linear predictor (η_i), an error distribution (e.g. Normal/Gaussian or Gamma) and a link function (g_i) (e.g. identity, inverse or log).

The linear predictor (η_i) is the systematic part of the model which describes how covariates and factors ultimately contribute to the expected value (prediction) for an observation.

$$\eta_i = b_0 + b_1.\, x_{1i} + ... + b_h.\, x_{hi}$$

The most parsimonious GLM was identified for each response variable, from all relevant explanatory variables, using a backwards selection procedure. However, because of the small sample size (n = 26), it was necessary to conduct the selection process with caution to avoid over-parameterisation of the initial full and any subsequent models. Thus, first, each response variable was modelled individually with each relevant explanatory variable, with recovery group or sex as co-variates where relevant. Then the initial full model was limited to only biologically relevant and significant ($p < 0.1$) explanatory variables. Subsequent models were constructed by sequentially removing the variables that contributed least to the model deviance, until only significant variables ($p \leq 0.05$) remained in the final model (as determined by log-likelihood ratio test and AIC) (Akaike, 1974; Burnham and Anderson, 2004). Finally, the validity of each model selection was further tested by removing any highly influential data points to assess their importance for the model coefficients and inferences.

The error distribution allows the model to estimate the random error associated with each predicted value ($\overline{y}_i$) based upon a known distribution.

$$\text{Gaussian}(\textit{Normal})\ \overline{y}_i \sim N(\mu_i, \sigma_i^2)$$
$$E(Y_i) = \mu_i \quad \text{and} \quad \text{var}(Y_i) = \sigma_i^2$$

$$\text{Gamma} \quad \overline{y}_i \sim \Gamma(\alpha_i, \beta_i)$$
$$E(Y_i) = \alpha_i.\, \beta_i \quad \text{and} \quad \text{var}(Y_i) = \alpha_i.\, \beta_i^2$$

Most of the response variables had positive values (i.e. not less than zero). Such data are best modelled using a Gamma distribution, which accounts for the skewed distribution of the model errors and prevents nonsensical negative predications. A further property of the Gamma distribution is that it assumes a constant ratio between the mean and variance (i.e. it will increase in proportion with the mean), which can help address heterogeneity in the data. Hunter's Whiteness Index, pH_{start} and pH_{end} were all initially modelled using Gaussian (Normal) error distributions. However, due to skewness in the data, Hunter's Whiteness Index and pH_{start} had better fits using Gamma error distributions.

The link function (g) relates the expected (typically mean) value (μ) of the observed values (y) to its linear predictor (η).

$$\text{identitylink}: \ \eta_i = g(\mu_i) = \mu_i \rightarrow \mu_i = g(\eta_i)^{-1} = \eta_i$$
$$\text{inverselink}: \ \eta_i = g(\mu_i) = {}^{1}/_{\mu_i} \rightarrow \mu_i = g(\eta_i)^{-1} = {}^{1}/_{\eta_i}$$
$$\text{loglink}: \ \eta_i = g(\mu_i) = \log_e(\mu_i) \rightarrow \mu_i = g(\eta_i)^{-1} = e^{\eta_i}$$

For clarity, the final parsimonious model for each response variable is presented in the results as the inverse link function form (i.e. $g(\eta_i)^{-1}$). All GLM formulae, coefficients and analytical output are presented in the supplementary materials.

Critical swimming speed (U_{crit}) was used as a potential explanatory variable in the GLMs to account for the varying levels of enforced swimming activity observed in different fish before the physiological samples were taken. Control group fish were assigned an arbitrarily low U_{crit} value (0.001), to represent the absence of enforced activity prior to sampling. In addition, five fish did not complete the first speed increment of the U_{crit} procedure (0.5 m s^{-1}) and were only fatigued to some degree and clearly not exhausted. Indeed, two fish stopped swimming only 2 min into the first speed increment and struggled vigorously after removal from the swim chamber. This complicated the calculation of U_{crit} as the fish had not completed a full speed increment. We chose to assign the fish the average water speed in the ramping from overnight to first speed increment as their fastest completed speed (V_{n-1}) in the U_{crit} calculations, as this produces U_{crit} values that are more likely than using the overnight acclimation speed (see Section 2.3). The relationships between U_{crit} and the other explanatory variables (see above) were also investigated using GLM (with a Gamma error distribution and an inverse link function).

3. Results

Critical swimming speed (U_{crit}) of haddock (0.66 ± 0.18 m s^{-1}; 1.25 ± 0.29 BL s^{-1}; average ± SD) was positively correlated with body length (p = 0.015, Fig. 3). In general, most fish swam for a considerable distance (typically greater than 1250 m) and are likely to be exhausted.

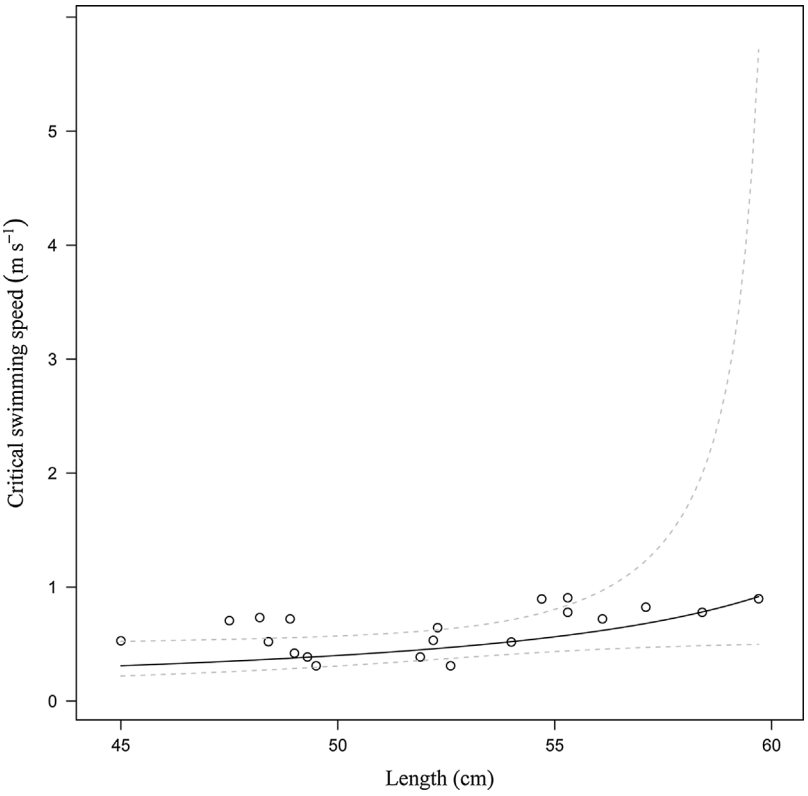

Fig. 3. Relationship between critical swimming speed and total length of individual fish in the enforced swimming trial. Data are presented as open circles; fitted values from the GLM are shown as a solid black line and the corresponding 95% confidence interval as dashed grey lines. See supplementary materials for model details.

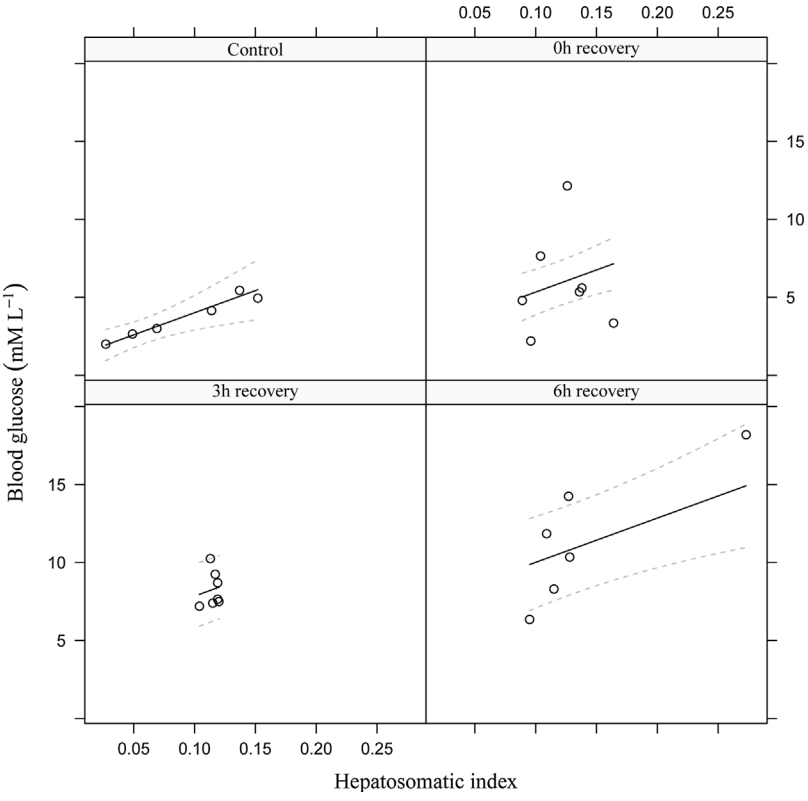

Fig. 4. Relationship between blood glucose concentration, recovery time and hepatosomatic index of individual fish in the enforced swimming trial. Each panel represents data from separate recovery groups: controls (no enforced swimming), 0 h, 3 h and 6 h recovery. Data are presented as open circles; fitted values from the GLM are shown as a solid black line and the corresponding 95% confidence interval as dashed grey lines. See supplementary materials for model details.

Blood glucose was clearly affected by the swimming regime and remained elevated ($p < 0.0001$) throughout the entire recuperation period (Fig. 4). This was an effect of both recovery ($p < 0.0001$) and a positive correlation with HSI ($p = 0.0064$). The relationship with HSI is clearly apparent in the control group animals, but was more variable in the exhausted animals. Furthermore, the range of HSI was limited in some groups, particularly the 3-h recovery group.

The swimming regime also had a transient effect on the time to reach maximum muscle stiffness (Fig. 5), which was influenced mainly by recovery ($p < 0.001$) and a positive correlation with muscle pH_{start} ($p < 0.05$). Maximum muscle stiffness (Table 1), on the other hand, was not affected by the recovery or muscle pH_{start} (see supplementary

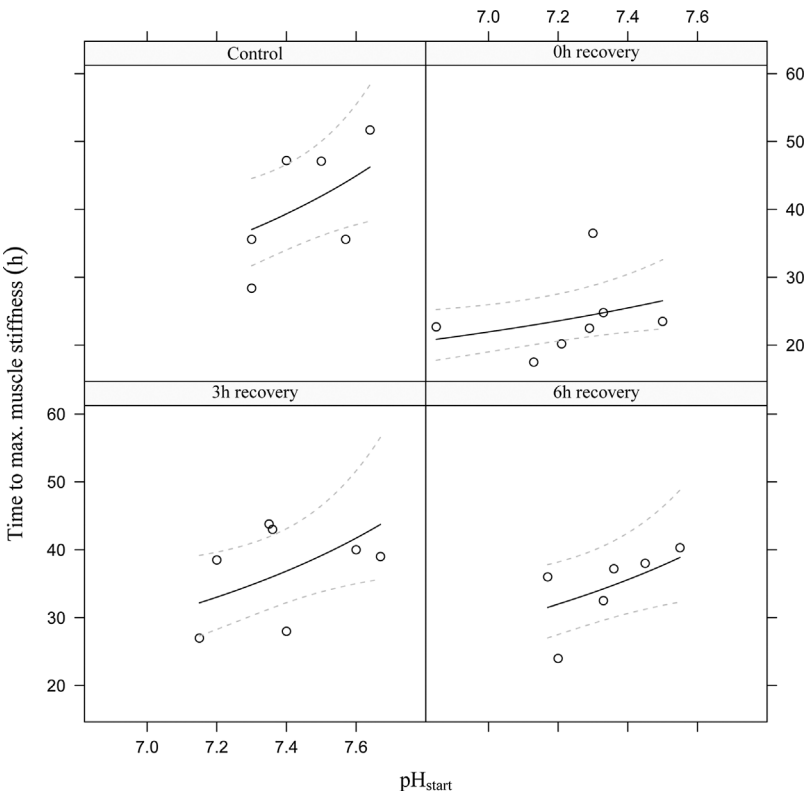

Fig. 5. relationship between time to maximum muscle stiffness, recovery time and muscle pH immediately after death (pH_{start}) of individual fish in the enforced swimming trial. Each panel represents data from separate recovery groups: controls (no enforced swimming), 0 h, 3 h and 6 h recovery. Data are presented as open circles; fitted values from the GLM are shown as a solid black line and the corresponding 95% confidence interval as dashed grey lines. See supplementary materials for model details.

materials).

The relationship between blood lactate and its potential explanatory variables was complex. When modelled individually, the effect of swimming activity was expressed by the clear effect that recovery had on lactate concentrations ($p < 0.0001$), which were elevated in all exercised groups, but particularly the 6-h recovery group (Fig. 6). In addition, sex was also significant when modelled individually ($p = 0.0029$), but this was highly dependent on just 3 females in the 6-h recovery group that had particularly high blood lactate concentrations. However, neither of these parameters were retained in the most parsimonious (final) model, selected from all relevant parameters. In this final model, the importance of swimming activity on blood lactate was expressed by the highly significant positive correlation with U_{crit} ($p = 0.0003$) (Fig. 7). In addition, in the same model, blood lactate was also positively correlated with blood glucose ($p = 0.0033$) and GSI ($p = 0.0025$) and highly negatively correlated with pH_{start} ($p < 0.0001$).

Swimming activity had no apparent effect on muscle pH_{start} or pH_{end}

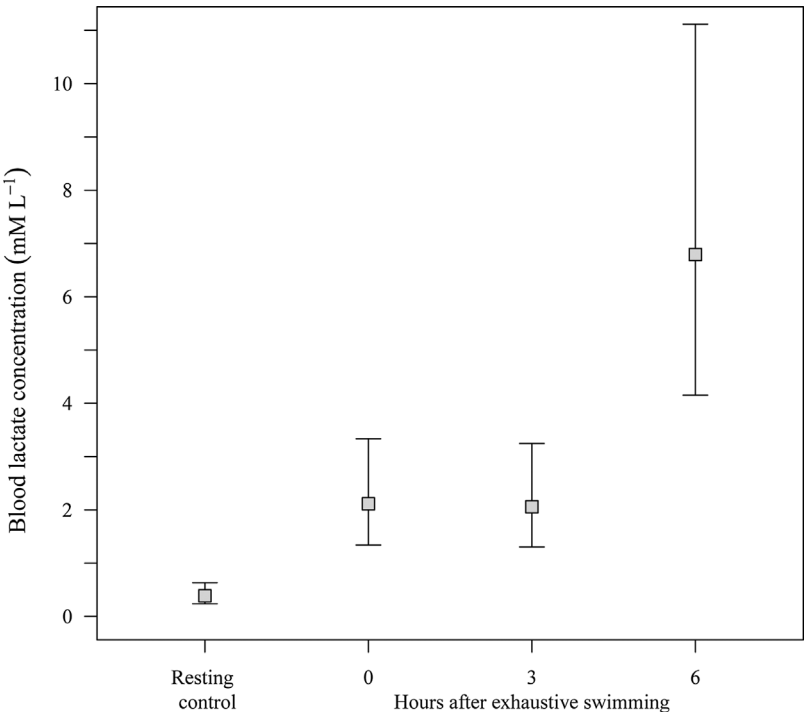

Fig. 6. Relationship between blood lactate concentration of individual fish in the enforced swimming trial and recovery time; controls (no enforced swimming), 0 h, 3 h and 6 h recovery. Fitted values from the GLM are shown as open squares and the corresponding 95% confidence interval as "T" bars. See supplementary materials for model details.

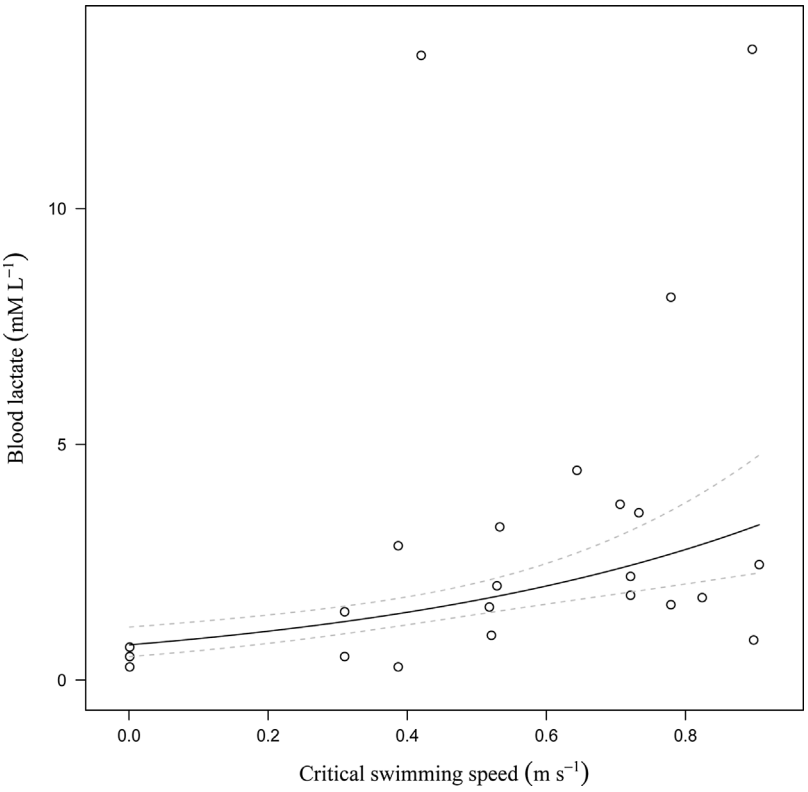

Fig. 7. Relationship between blood lactate and critical swimming speed of individual fish in the enforced swimming trial. Data are presented as open circles; fitted values from the GLM are shown as a solid black line and the corresponding 95% confidence interval as dashed grey lines. See supplementary materials for model details.

(Table 1). The only significant explanatory variable for pH_{start} was blood lactate, which had a negative correlation ($p = 0.015$), while pH_{end} was positively correlated with HSI ($p = 0.0092$), although the significance of this relationship was dependent on only two influential data points.

The whiteness of the fillet (Table 1) was also not affected by the swimming activity and only correlated positively with GSI ($p = 0.0008$) and negatively with HSI ($p = 0.0022$). The relationship with HSI was highly dependent on just four influential data points, so its significance should be viewed with caution.

4. Discussion

4.1. Swimming performance and level of exhaustion

The average U_{crit} of haddock was 0.66 ± 0.18 m s^{-1} or 1.25 ± 0.29 BL s^{-1} ($\pm$ SD) and is the first account of aerobic swimming performance in haddock larger than 45 cm BL. Interestingly, this U_{crit} corresponds well with the maximum sustainable swimming speed (U_{ms}) predicted by Breen et al. (2004) for the size of our fish (0.63 m s^{-1}), although U_{crit} and U_{ms} are different measures of aerobic swimming performance. Maximum sustainable swimming speed (U_{ms}) is a measure of the maximum speed at which a fish can maintain aerobic swimming activity, beyond which the fish will begin to utilise anaerobic swimming, start accumulating lactate in the white swimming muscles, and eventually become exhausted, if the swimming speed was maintained (Videler and Wardle, 1991). However, U_{ms} is difficult to estimate because it requires multiple replicate swimming trials on the same fish over a wide range of swimming speeds (e.g. Breen et al., 2004). Conversely, U_{crit} is a convenient method for obtaining a standardised and repeatable measure of swimming performance, although it has been criticised for its lack of ecological and eco-physiological relevance (Plaut, 2001). Furthermore, to date, no direct correlation between U_{crit} and U_{ms} has been established, although some correlations with routine activity metrics and metabolism have been demonstrated (e.g. Brett and Glass, 1973; Plaut, 2000).

In this study, we observed a positive, linear relationship between BL and U_{crit}, which is a well-established relationship observed in various species (e.g. Brett and Glass, 1973; Plaut, 2001; Videler, 1993). The critical swimming speed (U_{crit}) was based on data from individual fish swimming together in a group, while many previous studies on swimming performance in fish relied on swimming one individual at a time (Brett, 1964; Martinez et al., 2004; Sabate et al., 2013). Breen et al. (2004) also assessed swimming performance (U_{crit}) of fish in groups and demonstrated a substantial reduction in performance (> 50%) in most fish when they swam alone, compared to when swimming in a group. This difference was much greater than could be explained by any reduction in drag benefited by swimming in a school, and was thus attributed to the effect inter-conspecific competition on performance behaviour. Therefore, it is likely a combination of behavioural and physical factors (e.g. BL, temperature, fish condition) that determine the endurance of a fish swimming in the mouth of a trawl (Breen et al., 2004). Thus, the degree of exhaustion is likely to vary accordingly, including between individual fish.

4.2. Recovery from exhaustion

Blood glucose did not change significantly during swimming, but increased steadily during the entire 6-h period of recovery (Fig. 4). A similar pattern was observed for blood lactate (Fig. 6). Together, this indicates that physiological recovery after exhaustion takes more than 6 h in haddock. This is in line with the findings of Beamish (1966), who observed a return to baseline blood lactate levels after 12 h post exhaustion. However, elevated blood glucose and lactate levels could also have been caused by stress following enforced swimming, as increased cortisol levels post-exercise can prolong lactate and glycogen recovery metabolism associated with increased hepatic glucose mobilization (Milligan, 1996; Mommsen et al., 1999). Although there was a significant effect of sex on blood lactate, these results should be treated with caution due to a low sample size in the sex divided groups (n = 2–4) and large individual variation. This is especially evident for the female 6-h recovery group median value, which had a wide 95% CI

of 3.7–13.4 mM L^{-1} (Fig. 6). The pattern observed in males represents a typical response to recovery after exhaustive swimming, although it is less pronounced than one could expect if all fish had been fully exhausted (Wood, 1991) or had been caught by trawl (Beamish, 1966; Olsen et al., 2013).

4.3. Fillet quality and recovery from exhaustive swimming

The haddock that were euthanized and sampled immediately after enforced swimming reached maximum muscle stiffness faster than the control and the 3- and 6-h recovery groups. Thus, there is a similar positive effect of recovery in our study, compared to that reported previously in both haddock (Digre et al., 2017) and Atlantic cod (Olsen et al., 2013) following trawling. Our results are also consistent with a recent study on Atlantic cod, which revealed only transient effects of exhaustive swimming on muscle stiffness (i.e. maximum muscle stiffness and time to reach maximum muscle stiffness) during recovery (Svalheim et al., 2017). A slowly increasing and/or low maximum muscle stiffness is beneficial in terms of fillet quality as it reduces the chance of muscle segment gaping (Borderías and Sánchez-Alonso, 2011). In general, pre-slaughter stress accelerates myofibre-myofibre detachment, which may cause muscle segment gaping. This in turn results in lower water content with a dryer and softer texture of the fillet, and also has a negative impact on the appearance of the fillet (Sigholt et al., 1997; Skjervold et al., 2001; Erikson and Misimi, 2008; Bahuaud et al., 2010; Borderías et al., 2011; Sánches-Alonzo, 2011). Although exhaustive swimming appeared to have some effects on fillet quality, the levels of most quality parameters examined here were similar to that reported by Svalheim et al. (2017) on exhaustive exercise in Atlantic cod and not as severe as observed for fish caught by trawl (Olsen et al., 2013; Digre et al., 2017). Muscle pH recorded immediately after exhaustion in our study was 7.23 ± 0.08, compared to 6.93 ± 0.03 and 7.01 ± 0.11, respectively, in haddock (Digre et al., 2017) and Atlantic cod (Olsen et al., 2013) caught by trawl. Also, there was no significant effect of exhaustive swimming on fillet colouration, as opposed to that reported for Atlantic cod caught by trawl (Olsen et al., 2013). However, enforced swimming is only one of several events during trawling that are potentially stressful and detrimental to fillet quality in fish (Svalheim et al., 2017). Therefore, it seems likely that other factors such as injury, barotrauma, crowding or asphyxiation in the codend, may contribute to the overall deterioration of fillet quality occasionally observed in haddock caught by trawl.

4.4. Conclusions

The observations of swimming performance in large haddock obtained in the present study is in accordance with previous data from smaller congeners, but appears to be dependent on body length. A complete recovery of blood lactate and glucose after swimming exhaustion likely requires more than 6 h. All fillet quality parameters were unaffected by recovery time or had returned to control levels after 3 h of recovery, and were generally not severe. Thus, the poor fillet quality observed occasionally in haddock caught by trawl is probably not caused by exhaustive swimming alone, but is more likely the result of several factors operating during the trawling process. Further research into these factors is needed to identify the poor-quality culprit in trawled haddock and other gadoids.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fishres.2017.09.006.

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Paper II

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Full length article

Effects of exhaustive swimming and subsequent recuperation on flesh quality in unstressed Atlantic cod (*Gadus morhua*)

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ABSTRACT

Wild Atlantic cod of commercial size (1.9 ± 0.5 kg) were swum to exhaustion in a large swim tunnel in an attempt to mimic the initial stage of trawling, when fish swim at the trawl mouth until they fatigue or lose interest and subsequently drop into the trawl. The objective of the study was to investigate if exhaustive swimming in unstressed cod had any negative effects on fillet quality, and if post-exercise recuperation for 2, 4, 6, or 10 h could reverse such potential effects. Exhaustive swimming increased muscle stiffness during *rigor mortis*, but did not change time to maximum stiffness or cause any marked increase in fillet redness. Plasma lactate and cortisol were elevated after exercise, but recovered steadily during the recuperation period. Swimming to exhaustion also caused a drop in muscle pH, which returned to pre-exercise level within four hours of recuperation. We conclude that exhaustive swimming in unstressed Atlantic cod only has a moderate and reversible short lasting negative effect on flesh quality, which is less severe than the poor quality frequently observed in commercially caught cod.

1. Introduction

In the Northeast Atlantic, the majority of Atlantic cod (*Gadus morhua*) is caught by demersal trawls (ICES, 2015). Fishing by trawling is highly efficient with the potential of large catches. However stress associated with large sized catches and lengthy hauls can give poor or variable quality of the fillet, including reduction in muscle pH, fillet gaping and presence of residual blood in the white muscle (Margeirsson et al., 2007; Olsen et al., 2013). A recent study suggest that the reduction in fillet quality associated with trawling can be reversed if the fish are allowed to recover in holding tanks for a minimum of six hours after they are taken on board (Olsen et al., 2013).

Fishing by trawling is an active process in which shoals of fish are herded in front of an approaching net (Winger et al., 2010). Typically, the fish swim at the trawl mouth with the same speed as the moving net until they fatigue or lose interest and subsequently drop into the trawl and get retained in the cod end (Maine and Sangster, 1981; Wardle, 1993).

Exhaustive swimming is expected to be the first of several potential causes of poor flesh quality of trawled fish, because it depletes the energy stores of the red and white muscle of fish (Lurman et al., 2007). The energy status of the white muscle, which constitutes the majority of the muscles in fish, is of great importance to the quality, as low energy following pre-mortem activity is associated with a reduction of muscle pH, fillet gaping and a faster onset of *rigor mortis* (Borderías and Sánchez-Alonso, 2011). Oher factors, such as rapid thermal changes, confinement in the codend, barotrauma (Rummer and Bennett, 2005) and burst swimming (Maine and Sangster, 1981; Wardle, 1993), also represent potential stressors during trawling operations, which could contribute to the deterioration of the fillet quality.

The physiological responses to exhaustive exercise have been well documented (reviewed by Kieffer, 2000) and several studies suggests that strenuous activity induced experimentally in tank systems (Erikson et al., 2011; Donaldson et al., 2014) or associated with commercial capture by trawl (Olsen et al., 2013; Digre et al., 2017) or gillnet (Farrell et al., 2001), may have severe negative effects on fillet (i.e. muscle) quality parameters. However, most of these studies include elements of “fright” or “stress” in addition to muscle activity, and there

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is little information about exercise-induced effects on fillet quality in unstressed fish. Here we examine if exhaustive swimming in unstressed Atlantic cod has any negative effects on fillet quality, and if post swimming recuperation can reverse such potential effects. An important part of the objective was to mimic the initial "swimming stage" during trawling operations in an attempt to compare potential negative effects of exhaustive swimming *per se* with the overall fillet quality seen after commercial capture of cod by trawl. We have addressed these questions by measurements of muscle pH, time to full muscle stiffness and fillet redness during recuperation in cod subjected to an experimental swimming regime, which ultimately led to exhaustion. In addition, blood lactate, plasma glucose and plasma cortisol were measured to provide some insight into the metabolic perturbations associated with recovery after swimming to exhaustion.

2. Materials and methods

2.1. Animals and husbandry

A total of 110 wild Atlantic cod (body mass: 1.9 ± 0.5 kg, body length: 64 ± 6 cm, mean ± SD) were captured by Danish seine in May and June 2013 on the fishing ground "Furholmen" outside Ingøy in Finnmark, Norway. Immediately after capture, the fish were placed in a holding tank supplied with running seawater on board the fishing vessel and transported approximately 290 km to the Aquaculture research station in Tromsø, Norway. At the research station, the fish were held under natural photoperiod (69°N) in an outdoor tank (3 m diameter, 7000 l) supplied with filtered seawater, until the start of the experiment in mid-September 2013. The fish were fed to satiation three times a week with thawed capelin (*Mallotus villosus*), until 48 h prior to transfer of fish into a large-scale outdoor swim tunnel (see Section 2.2). There were no differences in sex distribution for the whole batch (N = 58 females and N = 52 males), but there was a significant difference in gender distribution for the 10-h recuperation group (13 females and 5 males).

2.2. Swim tunnel

The experiment took place in a large swim tunnel submerged in an 11 m diameter circular tank. The volume of the swim chamber was ca 1400 l and measured ca 2.8 m in length and 0.8 m in height and width. A flow director consisting of 5 × 5 cm tubes of 1 m length was placed in front of the swim chamber to minimize turbulence. Grids of plastic coated stainless steel mesh were placed in the front and rear ends of the fish chamber to prevent fish from escaping the tunnel. The rear grid could be opened to remove exhausted fish. A three-bladed propeller with an integrated 5.5 kW electrical motor (Flygt PP4650.410 with 11° blade angles, Xylem Water Solutions, Charlotte, NC, USA) mounted in a 0.8 m diameter aluminum tube housing propelled the water flow to a maximum speed of 1.2 m s^{-1}.

2.3. Experimental set-up

2.3.1. Control fish

Three days before the swimming trial (see Section 2.3.3), 15 fish were collected randomly from the holding tank and sampled (see Section 2.4) to establish baseline levels of the measured parameters in rested unstressed fish (control). The procedure was completed in less than 10 min. Two days later, the remaining 95 fish were transferred to the swim tunnel and acclimated for 24 h at a water speed of 0.12 m s^{-1} (water temperature: 10.3 ± 0.2° C) prior to the swimming trial.

2.3.2. Exclusion of non-swimmers

Previous pilot studies have shown that 5–10% of gadoid fish will cease swimming in the swim tunnel before they are physically exhausted (Svalheim and Karlsson-Drangsholt, unpublished data). Therefore, non-swimmers were excluded from the trial, as the aim of the present study was to investigate effects of exhaustive swimming on flesh quality and recuperation. Non-swimmers were identified by initially increasing the water velocity from 0.12 to 0.24 m s^{-1} in two increments of 0.06 m s^{-1} during 10 min, whereafter velocity was maintained at 0.24 m s^{-1} for 30 min. Fish that refused to swim at this speed were pinched gently in the tail three times with use of fingers. The ones that remained unwilling to swim (8 fish) were subsequently taken out and excluded from the experiment. Velocity was then reduced to 0.12 m s^{-1} and the remaining fish were left in the chamber for another 24 h, until the swimming trial commenced.

2.3.3. Swimming trial

Following the exclusion of non-swimmers, the fish density in the swim tunnel was 118 kg m^{-3}. All the remaining 87 fish were swum together in the swimming trial. During the trial, the water velocity was increased by 0.001 m s^{-1} every second for 20 min, until maximum speed of 1.2 m s^{-1} was reached. This water velocity corresponds to a swimming velocity of about 2 body lengths per second, which is twice the critical velocity for sustained aerobic swimming (U_{crit}) in Atlantic cod (~1.0 body lengths per second; Lurman et al., 2007). Hence, swimming at velocities above U_{crit} should therefore induce a switch from aerobic to anaerobic swimming and ultimately result in exhaustion (see Reidy et al., 2000).

When fish ceased swimming and laid back on the grid, they were pinched gently in the tail up to three times (with use of fingers) to check if they were willing to swim further. If they did not respond to the third pinch, they were subsequently taken out of the tunnel by a dip net and either sampled directly (0 h recuperation) or assigned randomly to one of the other recuperation groups (2, 4, 6, and 10 h).

2.3.4. Recuperation

The recuperation groups (2, 4, 6, and 10 h) were kept in lid-covered steel mesh (4 × 4 cm) cages (1 × 1 x 1 m) placed in a large fibreglass tank (11 m diameter) supplied with running seawater to ensure flow-through of oxygen saturated water.

2.4. Sampling procedure

All fish were euthanized by a blow to the head and blood was collected from the caudal vessels within 1 min, using 6 ml heparinized vacutainers with 4 × 0.9 mm needles (BD Diagnostics, Franklin Lakes, NJ, USA). Measurements of pH were then obtained from the epaxial part of the white muscle tissue, rostrally to the dorsal fin on the left side of the fish, using a WTW330/set-1 pH-metre (Wissenscaftliche-Technische Werkstätten, Weilheim, Germany) equipped with a Hamilton double pore glass electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland). The instrument was calibrated frequently using pH 4.01 and 7.00 buffers at 10° C, and the electrode was cleaned with demineralized water between each measurement. Concentrations of lactate and glucose were obtained from samples of whole blood, using the hand-held meters Lactate Scout+ (SensLab GmbH, Germany) and FreeStyle Lite (Abbott Diabetes Care, Inc., Alameda, CA), respectively. The remaining blood was then centrifuged at 2700 × *g* for 5 min at 4 °C, and plasma was transferred to cryo tubes, frozen in liquid nitrogen and stored at −80° C until further analysis of plasma cortisol was undertaken (see Section 2.7).

Immediately after blood collection and pH-measurements, all the fish were exsanguinated by cutting the *Bulbus arteriosus* and *Vena cardinalis communis* on both sides. The fish were then bled for 30 min in a tank supplied with running seawater. Afterwards, weight (g), length (cm) and gender of each fish were registered. The liver and gonads were then taken out and weighed (g) to determine hepatosomatic (HSI) and gonadosomatic indices (GSI) by tissue weight x 100/total weight. The fish were then gutted, covered with plastic film and placed on ice in standard plastic fish boxes.

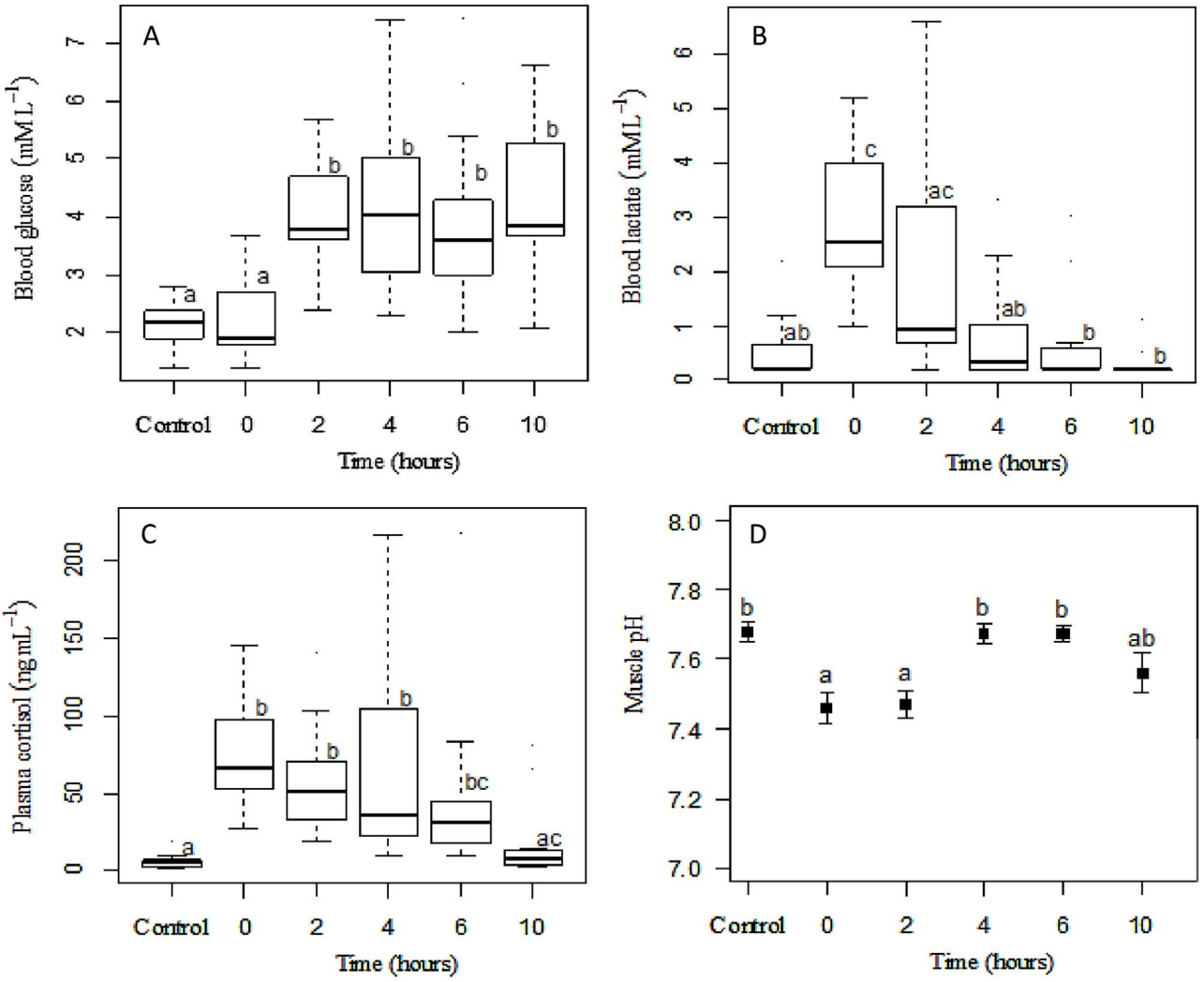

Fig. 1. Blood glucose (A), blood lactate (B), plasma cortisol (C) and muscle pH (D) in Atlantic cod during recuperation (0, 2, 4, 6, and 10 h), following swimming to exhaustion in an experimental swim tunnel. Control; non-exercised fish.

2.5. Evaluation of muscle stiffness

Muscle stiffness, used as an indirect measure of *rigor mortis*, was measured by use of an Effegi penetrometer (FT 02 Penetrometer, QA Supplies LLC, Virginia, USA), according to Berg et al. (1997). Briefly, the maximum force (kPa) required to push a 8 mm diameter stainless steel plunger 5 mm into the flesh was recorded. Measurements were made on 4 different locations (spaced approximately 50 mm apart) along the loin. Over the first 12 h, muscle stiffness was recorded approximately every 4 h. Thereafter, measurements were made, approximately every 8 h, up to 72 h *post mortem*.

2.6. Evaluation of fillet redness

All fish were filleted 72 h *post mortem* and the fillet opposite to that used for measurements of pH and sampling of muscle tissue was used for evaluation of fillet redness. The fillets were not skinned, but the black lining of the peritoneum was removed in order to evaluate the belly flap. Each fillet was evaluated by a sensory panel of 5 trained/experienced persons, according to Olsen et al. (2013), and the fillets were given a score from 0 to 2, where 0 was a white fillet, 1 was a pinkish fillet and 2 was a clearly red fillet.

2.7. Cortisol analysis

Plasma concentrations of cortisol were analyzed by use of radioimmunoassay (RIA), according to Schulz (1985) and Tveiten et al. (2010). Briefly, cortisol was extracted from 300 µl plasma with 4 ml diethyl ether under vigorous shaking for 4 min. The aqueous phase was frozen in liquid nitrogen, and the organic phase was transferred to a glass tube, evaporated in a water bath at 45 °C and then reconstituted by addition of 600 µl assay buffer prior to assaying by RIA. The antiserum used was raised in New Zealand white (NZW) rabbits and the detection limit for the assay was 0.6 ng ml^{-1} (Tveiten et al., 2010).

2.8. Data analysis

Statistical analysis was performed using the statistical software R, version 3.1.3 (R Core Team, 2013), with the packages LearnBayes (Albert, 2014) and pgirmess (Giraudoux, 2014). For each treatment group, all response variables were evaluated for normality and equal variance, using Shapiro Wilkins normality test and density plots. For normally distributed data, one-way ANOVA with type III sum of squares (F-test) was used for testing the effect of recuperation on the various parameters in question. Multiple comparisons were made using a post hoc Tukey Honest Significant Differences Test (Tukey HSD). These data are presented as means ± standard errors (SE). For the non-normally distributed data, a Kruskal Wallis rank-sum test was used to test for the

effect of recovery on non-parametric response variables, followed by a Multiple-comparison Kruskal-Wallis "kruskalmc" post-hoc test in the package pgirmess. These data are presented as medians in the text and graphically with Tukey box-plot. Outliers are defined as any value lower than 1.5 x Interquartile range (IQR) than the lower quartile and 1.5xIQR higher than the upper quartile of the boxplot. Outliers are not shown graphically.

Some fish had blood lactate levels below the detection limit (0.4 mM) of the instrument. These observations were replaced with a set value of 0.2 mM under the assumption that the unknown values is somewhere between 0 and 0.4 mM. Spearman rank-order correlation analysis was used to examine possible significant correlations between blood glucose, blood lactate, fillet redness, maximum muscle stiffness, time to maximum muscle stiffness, cortisol, condition factor (C), HSI, GSI, and muscle pH. Correlations are presented as Spearmans rank correlation coefficient rho (ρ).

3. Results

3.1. Morphological and physiological assessments

There were no significant differences in HSI (2.99 ± 1,53), GSI (0.57 ± 0.44) or C (0.72 ± 0.11) between the recuperation groups, and there were no correlations between HSI, GSI or C and the other response variables, except for a possible effect of HSI and C on the time to reach maximum muscle stiffness.

Blood glucose did not change during exercise, but increased significantly ($p < 0.001$) within two hours of recuperation from about 2–4 mmol L^{-1} and remained at this level throughout the 10-h recuperation period (Fig. 1A). There were significant correlations between blood glucose and fillet redness ($p = 0.043$), maximum muscle stiffnes ($p = 0.035$) and muscle pH ($p = 0.034$) (Table 1).

Blood lactate, unlike blood glucose, increased during exercise from about 0.2 mmol L^{-1} in the control group to 2.55 mmol L^{-1} in the group sampled immediately after exercise ($p < 0.001$) (Fig. 1B). Blood lactate then decreased gradually and returned to control level within the first 4 h of recuperation. There were significant correlations between blood lactate and maximum muscle stiffness ($p = 0.030$), cortisol ($p < 0.001$) and muscle pH ($p < 0.001$) (Table 1).

Plasma cortisol rose significantly during exercise from 4 ng ml^{-1} in the control fish to 66 ng ml^{-1} in the fish sampled immediately after exercise. Plasma cortisol then decreased gradually during recuperation to reach pre-exercise level by the end of the recuperation period (Fig. 1C). There was a significant ($p < 0.001$) correlation between plasma cortisol and blood lactate (Table 1).

Muscle pH dropped significantly ($p < 0.001$) during exercise from 7.68 ± 0.11 (control) to 7.46 ± 0.19 immediately after exercise and 7.47 ± 0.18 after 2 h of recuperation. Muscle pH then returned to pre-exercise level during the next 2 h of recuperation and remained at this level during the remainder of the recuperation period (Fig. 1D). There was a significant correlation ($p = 0.005$) between muscle pH and maximum muscle stiffness.

3.2. Fillet redness and post-mortem development of muscle stiffness

The post mortem muscle stiffness was significantly ($p < 0.001$) higher (884 ± 63 kPa) for the fish sampled immediately after exercise than both the controls (665 ± 113 kPa) and the recuperated fish (Fig. 2A). The 6- hour recuperation group took significantly longer time to reach maximum muscle stiffness than both the control group and the 10-h recuperation group, but not the other recuperation groups (Fig. 2B). There was a significant correlation between time to maximum rigor and HSI ($p = 0.002$) and C ($p < 0.001$) (Table 1).

There was a trend, although not significant, towards increased redness of the fillet among the fish recuperated for 2 and 6 h (Fig. 2C), but the variation in fillet redness within the groups were substantially. There was a significant correlation between blood glucose and fillet redness ($p = 0.043$) (Table 1).

4. Discussion

The present study suggests that swimming to exhaustion may deteriorate post mortem flesh quality of Atlantic cod by decreasing muscle pH and increasing muscle stiffness during *rigor mortis*. These effects were temporary and muscle pH and stiffness returned to pre-exercise levels when the fish were allowed to recuperate for at least 4 h

Table 1
Spearman rank correlation (ρ) and p-values for pooled cortisol, lactate, muscle pH, glucose, hepatosomatic index (HSI), gonadosomatic index (GSI), condition factor (C), fillet redness, time to maximum muscle stiffness and maximum muscle stiffness.

		Cortisol	Lactate	Muscle pH	Glucose	HSI	GSI	C	Fillet redness	Time to maximum muscle stiffness	Maximum muscle stiffness
Cortisol	ρ	1	0.46	−0.17	0.13	−0.11	0	−0.12	0.08	0.17	0.26
	p-value		> 0.001	0.091	0.197	0.285	0.976	0.239	0.435	0.098	0.009
Lactate	ρ		1	−0.35	−0.08	−0.09	−0.17	0.06	0.02	0.04	0.21
	p-value			> 0.001	0.407	0.354	0.095	0.573	0.867	0.701	0.030
Muscle pH	ρ			1	0.08	−0.14	0.11	−0.17	0.08	0.14	−0.27
	p-value				0.430	0.158	0.263	0.080	0.397	0.175	0.005
Glucose	ρ				1	0.21	0.18	0.02	0.2	0.03	−0.21
	p-value					0.036	0.076	0.853	0.043	0.741	0.035
HSI	ρ					1	0.13	0.55	0	0.32	0.12
	p-value						0.196	> 0.001	0.963	> 0.001	0.244
GSI	ρ						1	0.05	0.08	−0.03	0.01
	p-value							0.634	0.396	0.764	0.894
C	ρ							1	−0.11	0.35	0.35
	p-value								0.290	> 0.001	> 0.001
Fillet redness	ρ								1	0.02	−0.09
	p-value									0.822	0.387
Time to maximum muscle stiffness	ρ									1	0.11
	p-value										0.269
Maximum muscle stiffness	ρ										1
	p-value										

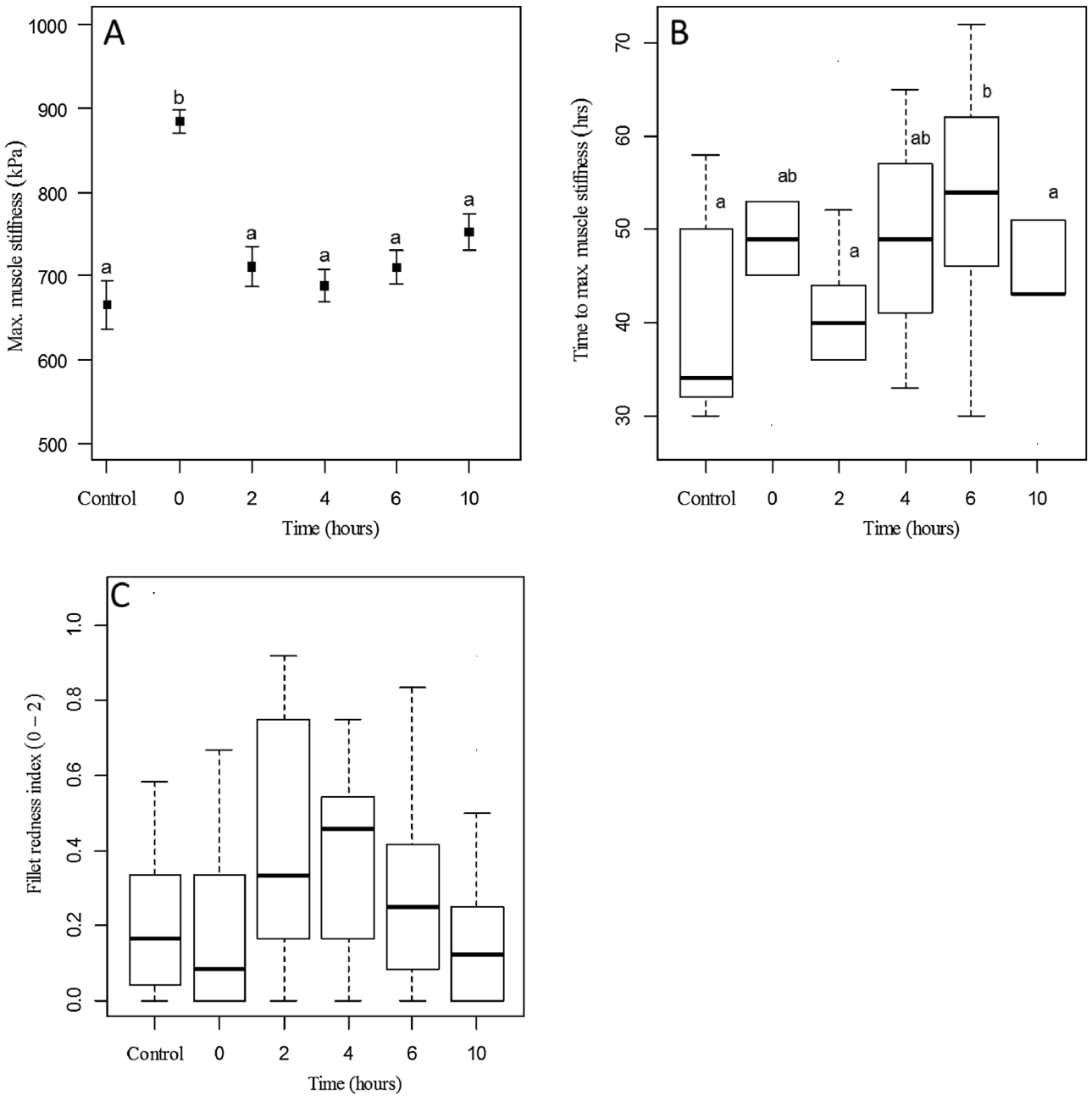

Fig. 2. Maximum muscle stiffness (A), time to maximum muscle stiffness (B) and fillet redness (C) of the white muscle of Atlantic cod slaughtered during recuperation (0, 2, 4, 6, and 10 h) after swimming to exhaustion in an experimental swim tunnel. Control; non-exercised fish.

after the swimming trial. Our results partly corroborate previous studies on Atlantic cod caught by trawl (Olsen et al., 2013; Digre et al., 2017), suggesting that recuperation for 6 h or more after catch may improve the fillet quality substantially. However, unlike the latter studies, which revealed substantial changes in muscle pH, blood lactate and fillet redness, only minor changes in these quality parameters were observed in the present study. Also, the increase in plasma cortisol observed during exercise in our study (from 4 to 66 ng ml-1) was modest and the level observed 2 h into the recuperation period (~50 ng ml^{-1}) was considerably lower than that seen during recovery in both cod caught by trawl (~120 ng ml^{-1} 3 h after catch; Digre et al., 2017) and pacific salmon caught by gillnet (~1200 ng ml^{-1} 2 h after catch; Farrell et al., 2001). It seems therefore that exhaustive swimming in unstressed Atlantic cod only has a moderate effect on metabolic stress parameters and fillet quality, and that other stress factors (i.e. crowding in the codend, barotrauma, etc.) probably contribute to the poor fillet quality frequently observed in cod caught by trawl.

Presence of residual blood in the white muscle of cod caught by trawl (Olsen et al., 2013), causing the flesh to appear red or brown, is considered a quality defect (Margeirsson et al., 2007). This could be due to insufficient exsanguination after catch or increased blood flow to the white muscle during the first few hours after exercise, as suggested by Olsen et al. (2013). Studies on rainbow trout (*Oncorhynchus mykiss*) suggest that up to 80% of the cardiac output is routed to the white muscle during recovery from exhaustive swimming (Neumann et al., 1983). A transient increase in blood flow to the white muscle is probably therefore the most likely explanation for the increase in fillet redness observed during recovery of cod caught by trawl (Olsen et al., 2013).

The increase in blood glucose (0–2 h after exercise) and circulating cortisol is most likely part of the recovery process to restore metabolic status after exhaustion. However, the exact role of cortisol in post-

exercise recovery is not clear. Some evidence suggest that cortisol is primarily associated with mobilization of energy (Gamperl et al., 1994), while other studies indicate that cortisol may be involved in the glycogen recovery metabolism (Eros and Milligan, 1996; Milligan, 2003; Mommsen et al., 1999). There is also some evidence suggesting that cortisol may even prolong the metabolic recovery following exercise, although the mechanisms of its negative effect is not clear (Gamperl et al., 1994). Nevertheless, the plasma values of cortisol observed in the present study were moderate (Gamperl et al., 1994), indicating that the level of exhaustion was not severe.

Swimming to exhaustion increased both maximum muscle stiffness of and the time to reach maximum muscle stiffness, and both parameters returned to pre-exercise levels during the recuperation period. There were however no clear pattern with respect to the time to reach maximum muscle stiffness, due to large individual variations within the recuperation groups. Onset and peak level of muscle stiffness are important indicators of fillet quality in fish. A rapidly increasing, and/or high peak of muscle stiffness, indicates low muscle energy status at slaughter and increases the potential for muscle segment gaping and dryness, flesh softening, and colorimetric changes, resulting in reduced shelf-life of the fillets (Bahuaud et al., 2010; Borderías and Sánchez-Alonso, 2011; Erikson and Misimi, 2008; Sigholt et al., 1997; Skjervold et al., 2001).

Swimming to exhaustion resulted in a significant drop in muscle pH, which recovered 2–4 h into the recuperation period. The drop in pH is likely the result of anaerobic activity and production of lactic acid during exercise, as indicated by the elevation of blood lactate during the same period. Most of the lactate produced in the white muscle is resynthesized to glycogen within the muscle itself, but 10–20% of it is released slowly into the blood (Milligan, 1996) with a peak level occurring 2–4 h post-exercise. This pattern has been observed for rainbow trout chased around in a tank (Milligan and Girard, 1993), haddock swum in a large swim tunnel (A. Karlsson-Drangsholt unpublished) and in cod caught by trawl (Olsen et al., 2013). We observed a slightly different pattern, with a peak level of blood lactate occurring immediately after exercise followed by a gradual decrease to almost full recovery 4 h into the recuperation period. It should be noted though that the removal of muscle lactate and resynthesis of glycogen is usually slower, and may require up to 12 h in some species (Kieffer, 2000). Nevertheless, all these studies, including our study, resulted in similar types of metabolic and acid/base disturbances.

We conclude that exhaustive swimming in cod only has a moderate and reversible short lasting negative effect on flesh quality, which is less severe than the poor quality frequently observed in commercially caught cod.

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Paper III

Simulated trawling: Exhaustive swimming followed by extreme crowding may be a significant contributor to variable fillet quality in trawl-caught Atlantic cod (*Gadus morhua*)

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Abstract

Fillet quality can vary tremendously in trawl-caught Atlantic cod (*Gadus morhua*). Poor quality may be caused by capture stress, crowding or exhaustion. To investigate mechanisms involved in causing variable quality, commercial-sized (size 3.5±0.9 kg) Atlantic cod were swum to exhaustion in a large swim tunnel and exposed to extreme crowding (736±50 kg m^3) for 0, 1 or 3 hours in an experimental cod-end. Further, fish were recuperated for 0, 3 or 6 hours in a net pen prior to slaughter to assess the possibility to quickly reverse the reduced quality. We found that exhaustive swimming and crowding were associated with increased metabolic stress, as indicated by increased plasma cortisol, blood lactate and blood haematocrit levels, and a reduced quality of the fillets in terms of increased visual redness and a drop in muscle pH. The observed negative effects of exhaustive swimming and crowding were only to a small degree reversed within 6 hours of recuperation. The results from this study suggest that exhaustive swimming followed by extreme crowding is a likely significant contributor to the variable fillet quality seen in trawl-caught Atlantic cod, and that recuperation for more than six hours may be required to reverse these effects.

Introduction

Fish captured in a trawl encounter a number of strenuous and stressful events such as forced swimming, crowding, confinement, crushing and barotrauma [1]. Because a trawl is an active fishing gear that involves herding the fish into the mouth of the trawl, fish will swim until exhaustion in an attempt to avoid capture. Fatiguing/fatigued fish drift back into the cod-end, where they are retained. With the increasing number of fish in the cod-end, animals will be compressed resulting in an extreme crowding situation.

Physiological measurements of trawl-captured cod, show fish in near homeostatic crisis that are highly variable in quality [2]. This indicates that the stressors to which the fish are exposed, plays a role in the degradation of quality. An increasing number of studies suggest that pre-mortem stress can strongly influence the quality of the final fish product [2-6]. Stress causes an elevation of circulating catecholamines and corticosteroids (e.g. cortisol), which in turn will alter metabolism, hydro-mineral balance and increase heart- and ventilation rate [7]. An ultimate function of the short-term stress response is mobilization of stored fuels for the physiological reactions known as "fight or flight" [8]. This pre-slaughter stress is known to cause textural changes of fish meat by altering the rate and extent of pH decline, and inducing a more rapid onset of rigor mortis [9, 10]. Furthermore, pre-mortem stress is associated with a change in muscle colour, which is considered an aesthetic quality defect in white fish [11]. Both discolouration of the fillet and textural changes play a role in downgrading of the fish and economic loss for the producer. Therefore, finding ways to reduce or reverse detrimental effects of capture stress will be of economic interest for both fishermen and producers.

During commercial trawling, it is challenging to separate the various parameters that could have an effect on quality. This also includes a variable size and length of the hauls, which is of great importance to both quality and survival of the catch [2]. Investigating trawl related stress in an

experimental setting may give a better understanding on how fillet quality parameters are influenced by different pre-mortem stressors. Previously, we have shown that neither the poor physiological state or negative fillet quality features of trawled cod could be reproduced by exhaustive swimming alone, and argue that variable fillet quality more likely is the result of several factors operating during the trawling process [12, 13]. In addition, studies performed on board commercial trawlers, have shown that it is possible to improve the quality of cod by keeping them alive in holding tanks for a few hours prior to slaughter [2].

In the current study, our aim was to experimentally simulate some aspects of a trawl capture, namely exhaustive swimming followed by extreme crowding, and investigate how this affects some key metabolic stress parameters and subsequent fillet quality in Atlantic cod. A second aim of the study was to investigate if post-stress recuperation for 0, 3 or 6 hours could reverse potential negative effects on fillet quality. We have addressed these issues by measurements of blood glucose, blood lactate, plasma cortisol, haematocrit, muscle pH, and fillet redness in cod swum to exhaustion in a swim tunnel and subsequently crowded (retained) in an experimental cod-end attached to the tunnel.

Materials and Methods

Animals and husbandry

A total of 197 wild Atlantic cod (body mass 3.5 ± 0.9 kg, body length 75 ± 7 cm, mean ± SD) (group means in Table 1, trial means in S1 Table) were captured by Danish seine in mid May 2014 outside the coast of Finnmark, Norway. The fish were kept live on board in tanks

supplied with running seawater and delivered to a live fish storage facility in Nordvågen, Norway, for recuperation for three weeks. From here, the fish were transported in a wellboat approximately 300 km to the Tromsø Aquaculture Research Station in, Norway. At the research station, the fish were held in two outdoor tanks (4 m diameter, 10 m^3) supplied with filtered seawater at natural water temperature and day-length (69ºN), until the start of the experiment in February 2015. The fish were fed three times a week, using a mixture of capelin (*Mallotus villosus*) and commercial feed (Skretting Amber 5 mm, Skretting ASA, Norway), until 48 hours before transfer of fish into an outdoor swimming tunnel (1400 L swim chamber, maximum speed 1.2 m^{-1}, we have previously described tunnel in detail [12]). There were no differences in gender distribution (N= 107 females and N = 90 males).

Table 1. Overview of biological parameters per treatment group

Group	N	Weight (g)	Length (cm)	CF	GSI	HSI
Rested ctrl	21	3477 ± 1035	74 ± 6.61	0.83 ± 0.1	4.33 ± 6.04	4.41 ± 1.21
Swum ctrl	42	3336 ± 895	73 ± 6.44	0.84 ± 0.15	4.95 ± 4.92	4.29 ± 1.39
C1.0	21	3487 ± 1015	74 ± 7.51	0.86 ± 0.13	6.57 ± 6.05	4.32 ± 1.45
C1.3	21	3761 ± 874	77 ± 4.85	0.81 ± 0.11	5.02 ± 4.96	4.2 ± 1.43
C1.6	21	3498 ± 821	74 ± 7.41	0.87 ± 0.22	3.68 ± 4.07	4.85 ± 1.41
C3.0	21	3729 ± 774	76 ± 7.21	0.84 ± 0.14	6.72 ± 6.12	4.58 ± 1.4
C3.3	21	3358 ± 922	75 ± 7.96	0.77 ± 0.12	5.03 ± 6.21	4.2 ± 1.8
C3.6	22	3497 ± 744	74 ± 5.76	0.87 ± 0.13	6.13 ± 6.52	4.75 ± 1.3

Overview of group distribution of number of fish (N), weight, length, condition factor (CF), gonadosomatic index (GSI) and hepatosomatic index (HSI). Each row show data from separate recovery groups; rested control (sampled from the holding tanks), swum control (sampled immediately after exercise), crowded for 1 hour and recuperated for 0 (C1.0), 3 (C1.3) and 6 hours (C1.6) respectively, and crowded for 3 hours and recuperated for 0 (C3.0), 3 (C3.3) and 6 hours (C3.6), respectively.

Experimental set-up

The experiment was conducted in three replicates over 26 days. There were 7 fish in each crowding group in each replica, adding up to a total of 21 individuals in each group by the end of the experiment. Three crowding durations of 1, 3 and 5 hours were selected in the original set-up to represent short, medium and long trawl hauls based reports from commercial trawl hauls [2]. However, mortality of the 5 hour crowding group reached over 80 % in the first trial and this group was therefore omitted in subsequent trials.

Control fish

Two days before each swimming trial, 7 fish were randomly dip-netted from the two holding tanks. In each trial, 3 fish were taken from one tank and 4 from the other. These fish were used to establish baseline levels for measured parameters for rested, unstressed fish (rested control). The fish were taken out and sampled within 1 min.

Swimming trial

Immediately after sampling of the control, 28 fish were transferred to a large swim tunnel housed in an 11 m tank and designed for swimming groups of large fish (sette inn referense). The fish were acclimated to the swim tunnel for 36 hours at a water speed of 0.15 m s^{-1} prior to the swimming trial. The fish density in the tunnel was on average 54 kg m^{-3}. The swimming trial started with a water velocity of 0.15 m s^{-1} and increased to 1.2 m s^{-1} in 1200 steps in 20 minutes (1 step s^{-1}). As fish ceased swimming and rested on the grid in the back of the tunnel (Fig 1), they were pinched in the tail with use of fingers to see if they would continue swimming. Non-responsive fish were considered exhausted [13] and subsequently released into the retention chamber, where water flow kept them on the grid (Fig 1). When all 28 fish in each

trial were in the retention chamber, 7 were randomly selected and sampled as swum control fish.

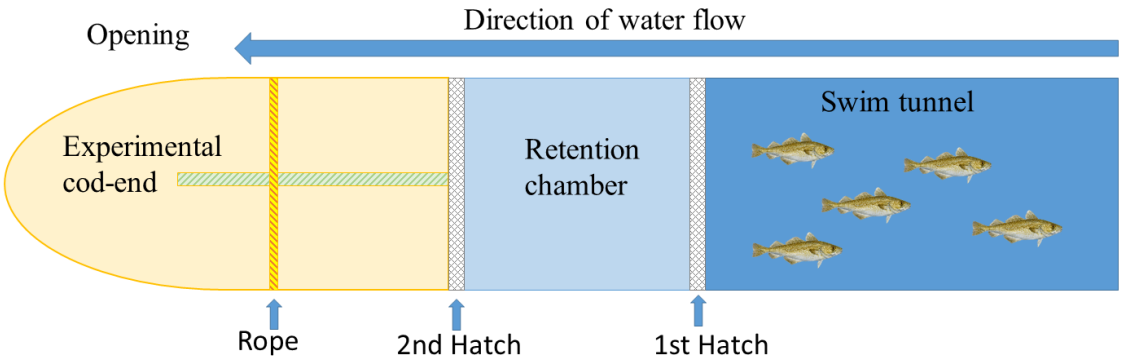


Fig 1. Schematic overview of the swim tunnel/trawl simulator. Graphic illustration of the swim tunnel and fish chamber, retention chamber and the experimental cod-end.

Crowding in the experimental cod-end.

Following removal of the 7 swum control fish, the remaining 21 fish were released from the retention chamber and into an experimental cod-end (Fig 1). The experimental cod-end was constructed as a four-panel cylindrical bag (length 200 cm height 58 cm with tension) using the same material as in a commercial cod-end (8 cm diamond cod-end mesh, 0.3 cm twine). The cod-end could be opened via a joint at the top (Fig 1). A rope was placed at a fixed position to close the cod-end, and tightened to ensure the fish were crowded. (Fig 1). When the cod-end was closed it was sphere shaped with a diameter of about 58 cm (S2 Fig) yielding a volume of about 100 L. For each trial, fish density was estimated based on the average weight of total individuals in the cod-end (S1 Table). Oxygen inside the cod-end was continuously monitored using an YSI ProODO handheld dissolved oxygen metre with a ProODO Optical probe (Yellow Spring Instruments, Ohio, USA). The fish were crowded for 1 or 3 hours. Afterwards, the fish

were taken out of the bag and randomly assigned to recuperation cages, where they were allowed to rest for 0, 3 or 6 hours.

Recuperation

The recuperation groups (0, 3 or 6 hours) were kept in 1×1×1 m lid-covered, floating steel mesh (4×4 cm) cages placed in the same tank as the swim tunnel. They fish were supplied with seawater at natural water temperature to ensure oxygen-saturated water.

Sampling procedure

All fish were euthanized by a blow to the head and blood was collected from the caudal vessels within 1 min, using 4 ml heparinized vacutainers with 4×0.9 mm needles (BD Diagnostics, Franklin Lakes, NJ, USA). Measurements of pH were then obtained by inserting a Hamilton double pore glass electrode (WTW330/set-1 pH-metre,Wissenscaftliche-Technische Werkstätten, Weilheim, Germany. Electrode: Hamilton Bonaduz AG, Bonaduz, Switzerland) via an incision (1 cm×2 cm) in the epaxial part of the white muscle tissue, rostrally to the dorsal fin on the left side of the fish. During the post-mortem pH measurements, a new incision was made 1 cm caudal to the previous incision for each measurement. pH was measured immediately after euthanasia, then there was a 20 hour period without measurements followed by measurements approximately every 8-15 hour. The instrument was calibrated frequently using pH 4.01 and 7.00 buffers at 2°C, and the electrode was cleaned with demineralized water between each measurement.

Concentrations of blood lactate and glucose were obtained from samples of whole blood, using the hand-held meters Lactate Scout+ (SensLab GmbH, Germany) and FreeStyle Lite (Abbott Diabetes Care, Inc., Alameda, CA), respectively. To calculate haematocrit, whole blood was spun using a microhaematocrit capillary tube centrifuge (Critocaps; Oxford Lab, Baxter,

Deerfield, IL) and the resulting red blood cell and total fraction measured using a millimeter ruler. The remaining blood was then centrifuged at 2700 × *g* for 5 minutes at 4°C, and plasma was transferred to cryo tubes, frozen in liquid nitrogen and stored at −80° C for later analysis of plasma cortisol. Immediately after blood collection and peri-mortem pH-measurements, all fish were exsanguinated by cutting the *Bulbus arteriosus* and *Vena cardinalis communis* on both sides. The fish were then bled for 30 min in a tank supplied with running seawater. Afterwards, weight (g), length (cm) and gender of each fish was registered. The liver and gonads were then taken out and weighed (g) to determine hepatosomatic (HSI) and gonadosomatic indices (GSI) by tissue weight x 100/total weight. The fish were then gutted, covered with plastic film and placed on ice in standard plastic fish boxes and stored at 4ºC.

Fillet redness

After approximately 72 hours storage all fish were filleted by trained personnel. The fillets were not de-skinned, but the black lining of the peritoneum was removed. Each fillet was evaluated by a sensory panel of three trained and experienced persons. To avoid expectation bias, the sensory panel was unaware of which group of fish they were evaluating. The fillets were given a score from 0 to 2, where 0 was a white fillet, 1 was a pinkish fillet and 2 was a clearly red fillet.

Imaging VIS/NIR Spectroscopy

After filleting, the muscle haemoglobin was evaluated by hyperspectral imaging of the fillets in diffuse reflectance mode. Imaging was performed with a push-broom hyperspectral camera with a spectral range of 430-1000 nm and spatial resolution of 0.5 mm across-track by 1.0 mm along track (Norsk Elektro Optikk, model VNIR-640). The camera was fitted with a

lens focused at 1000 mm, and mounted 1020 mm above a conveyor belt. By characterizing the incoming light, those spectra were transformed into absorbance spectra. Following the procedure outlined in Skjelvareid, Heia (14) the haemoglobin concentration was then estimated, on pixel level, for each fillet.

Cortisol analysis

Plasma concentrations of cortisol were analysed by use of radioimmunoassay (RIA), in accordance with previously described methods [15, 16]. In short, cortisol was extracted from 300 μL plasma with 4 mL diethyl ether under shaking for four min. The aqueous phase was frozen in liquid nitrogen and the organic phase was decanted to tubes and evaporated in a water bath at 45°C for ca 20 min and reconstituted by addition of 900 μL assay buffer before assaying by RIA. The antibody used was obtained from New Zealand white (NZW) rabbits and the detection limit for the assay was 0.6 ng mL^{-1} [15].

Statistical analysis and data management

The data was analysed with the statistical software R, version 3.4.0 [17]. The relationships between response variables (plasma cortisol (ng L^{-1}), lactate (mM L^{-1}), glucose (mM L^{-1}), pH, fillet redness, muscle pH) and corresponding potential explanatory variables (as factor; groups: crowding 1 or 3 hours, recuperated 0, 3 o 6 hours, rested control and swum control), sex (as factor), plasma cortisol, blood glucose, blood lactate, muscle haemoglobin (mg g^{-1}), hepatosomatic index (HSI), gonadosomatic index (GSI) and Fulton's condition factor (100 g cm^{-3})), were investigated using Generalised Linear Modelling (GLM) [18, 19]. Muscle pH was modelled with time post-mortem and groups: crowding 1 or 3 hours, recuperated 0, 3 o 6 hours, rested control and swum control) and curvature were checked by testing with different polynomials and interactions to determine significant differences between slopes. Note that

some variables are both response and explanatory, depending on which response is under investigation. Before proceeding with the GLM analysis, the data were checked and prepared for modelling following procedures previously described [20].

Briefly, most of the response variables had only positive values and were therefore best modelled using Gamma distribution, which accounts for skewed distribution of model errors and prevents negative predictions. In those cases where distribution was normal and there was no risk of predicting negative values, data was modelled using Gaussian (Normal) error distribution. In the case for sensory evaluation of redness, data were strictly bound between 1 and 4 and therefore fitted to a quasi-binomial distribution to make sure that predicted values also falls within this range. Link function (identity, log, inverse or logit) was chosen based on which link gave the best fit to data in terms of lowest Akaike information criterion (AIC) and by visual evaluation of the graphics. All model details are available in S3 Model details.

Results

Fish density in the cod-end varied between trials from 672 to 803 kg (S1 Table) and the oxygen saturation of the water in the cod-end always remained above 95% at any position. There were no mortalities during the swim-trial (i.e. swim tunnel and retention chamber) or following crowding for one hour, but for the group crowded for 3 hours 18 % of the fish where considered dead or moribund. The first run with 3 hours crowding had 48 % mortality, whereas the last two runs had 5 and 0 % mortality, respectively (S1 Table).

The plasma level of cortisol was clearly affected by swimming, crowding and recuperation ($p < 0.001$), but was also correlated with GSI ($p < 0.001$) (S4 Fig 1). The fish that were only swum (and not crowded) experienced a slight increase in plasma cortisol compared to the resting control. The highest levels of cortisol were found after 0 hours recuperation in the 3 hours

crowding group and after 3 hours recuperation for the 1 hour crowding group. After 6 hours of recuperation, the cortisol levels were still elevated (Fig 2A).

Blood glucose was affected by crowding and recuperation ($p<0.001$) and was positively correlated with HSI ($p < 0.001$) (S4 Fig 2). Blood glucose was higher after crowding for 1 and 3 hours compared to both resting and swum controls and remained elevated throughout the recuperation period (Fig 2B).

Blood lactate was clearly affected by swimming ($p<0.001$) and duration of crowding ($p<0.001$) (Fig 2C). Fish crowded for 1 hour had significantly higher lactate levels compared to resting and swum control ($p<0.001$), the levels remained elevated throughout the recuperation period. The animals crowded for 3 hours showed an almost 2-fold increase in lactate levels compared to 1 hour ($p<0.001$). The lactate stayed elevated throughout the recuperation period. Blood lactate levels were also negatively correlated to muscle pH ($p<0.001$) (S4 Fig3), this correlation was strongest for the 3 hours crowding group.

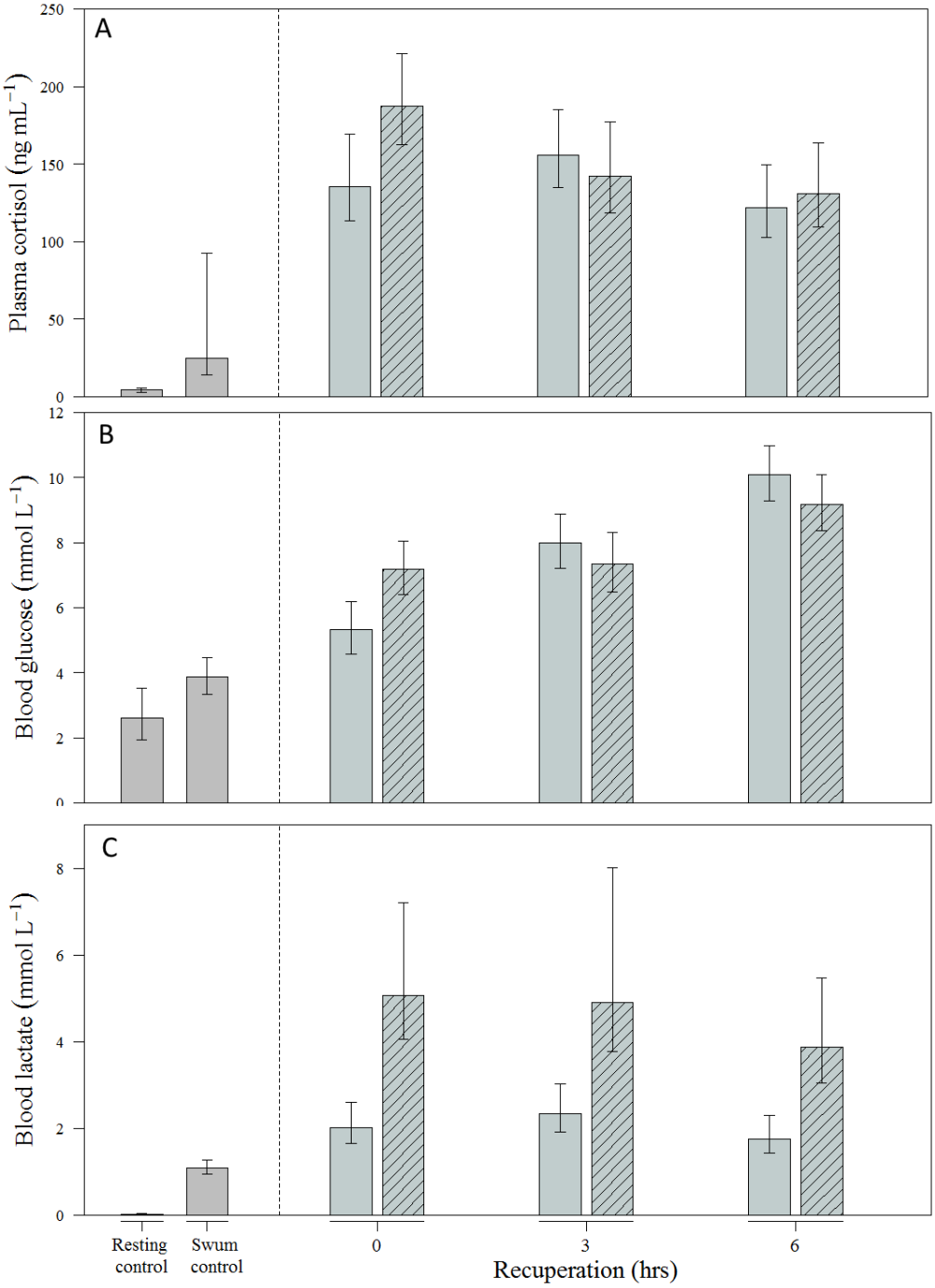
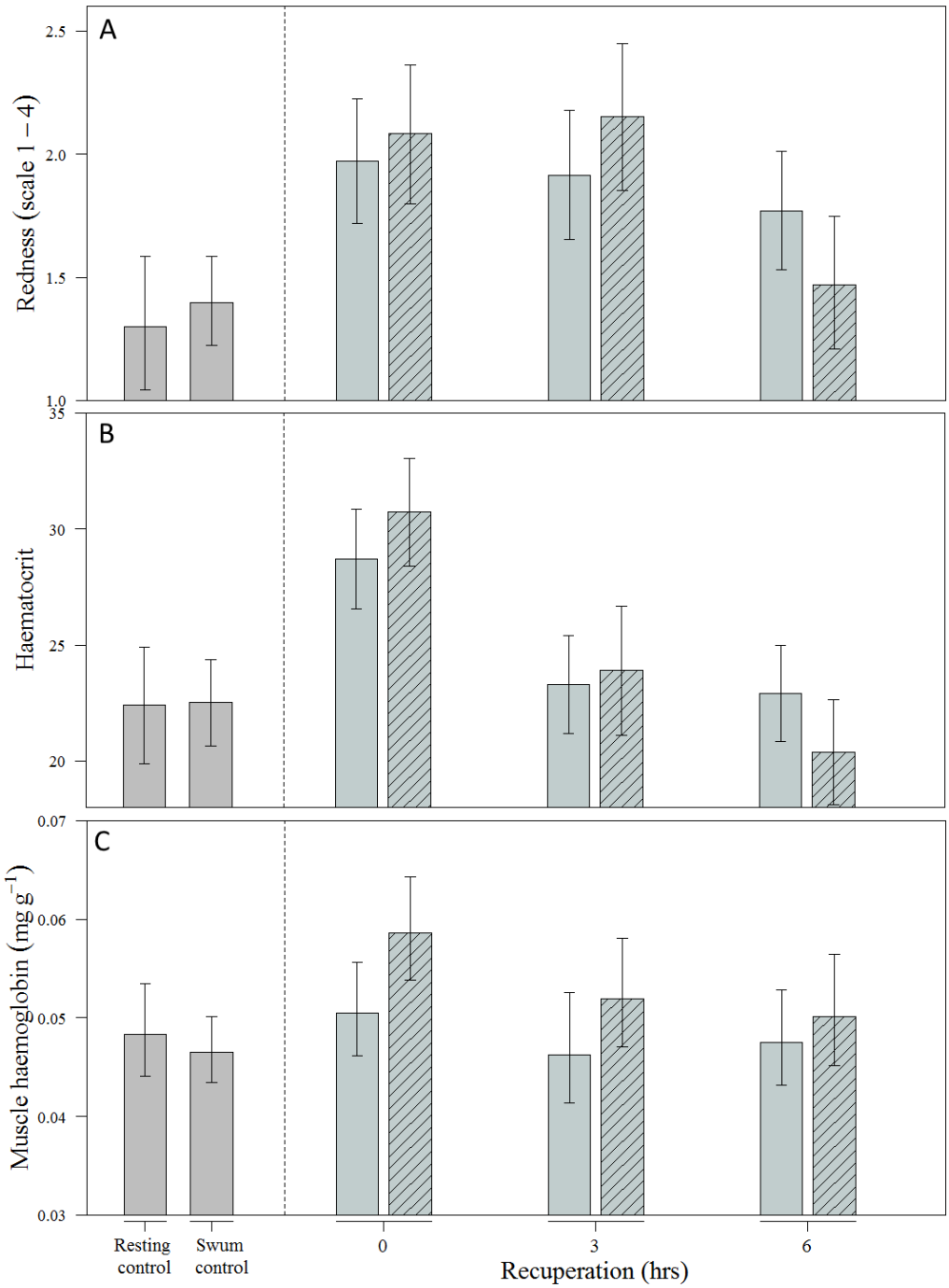


Fig 2. Physiological stress response to crowding and recuperation. Plasma cortisol (A), blood glucose (B) and blood lactate (C) in Atlantic cod during recuperation following exhaustive exercise and severe crowding for 1 hour (open bars) or 3 hours (dashed bars). Resting control are sampled from tank and swum controls are sampled immediately following exhaustive swimming exercise. Data are presented as estimated mean and errors indicate 95% confidence intervals fitted from GLM. See S3 for model details

Fillet redness was affected by swimming, crowding and recuperation and was positively correlated with muscle haemoglobin levels (S4 Fig 3). There were no major differences between fillets of fish crowded for 1 hour versus those crowded for 3 hours. After 6 hours of recuperation, the level of redness was still higher than for resting and swum control, but lower than after 0 and 3 hours of recuperation (Fig 3A). In the GLM without haemoglobin as explanatory variable, swimming, crowding and recuperation remained significant explanatory variables ($p<0.001$). In addition, a positive correlation between cortisol level and redness was found ($p=0.043$) (S4 Fig 5).

Crowding and recuperation affected muscle haemoglobin ($p=0.007$), but only the fish crowded for 3 hours without recuperation had increased muscle haemoglobin compared to the swum and rested control (Fig 3B). When modelled together with haematocrit, this effect disappeared and only haematocrit remained a significant explanatory variable ($p=0.02$) (S4 Fig 6). Because it can be argued that haemoglobin and haematocrit are dependant, a second GLM without haematocrit was run. In the second run, a positive correlation between cortisol level and muscle haemoglobin was found ($p=0.012$), also the swimming, crowding and recuperation was significant when modelled together with cortisol ($p=0.008$) (S4 Fig 7).

Swimming, crowding and recuperation affected haematocrit ($p < 0.001$) and was positively correlated to plasma cortisol levels ($p = 0.038$) (S4 Fig 8). The haematocrit increased during crowding, was highest immediately after crowding and had decreased to control levels after 3 hours of recuperation (Fig 3B).



296

297 **Fig 3. Redness, haematocrit and muscle haemoglobin**. Sensory evaluation of redness (A),
298 haemotocrit (B) and muscle haemoglobin in the surface area of fillets measured by spectroscopy (C) in
299 Atlantic cod during recuperation following exhaustive exercise and severe crowding for 1 hour (open
300 bars) or 3 hours (dashed bars). Resting control are sampled from tank and swum controls are sampled
301 immediately following exhaustive swimming excercise. Data are presented as estimated mean and errors
302 indicate 95% confidence intervals fitted from GLM. See S3 for model details

Muscle pH was affected by swimming, crowding and recuperation (Fig 4). The peri-mortem pH was lowest in un-recuperated, crowded fish, but there were no differences between groups crowded for 1 and 3 hours. However, the fish crowded for 1 hour recovered faster than fish crowded for 3 hours. The rate and shape of the slope of the post-mortem muscle pH drop was significantly affected by crowding and recuperation ($p<0.001$, Fig 4). The muscle pH drop rate was highest in control fish and fish recuperating from 1 hours crowding. Furthermore, there were significant differences in the shape of pH drop slopes that were dependant on crowding time. Fish crowded for 3 hour appeared to level at minimum pH ca 48 hours post-mortem, whereas the other groups seemed to continue the drop beyond measured time.

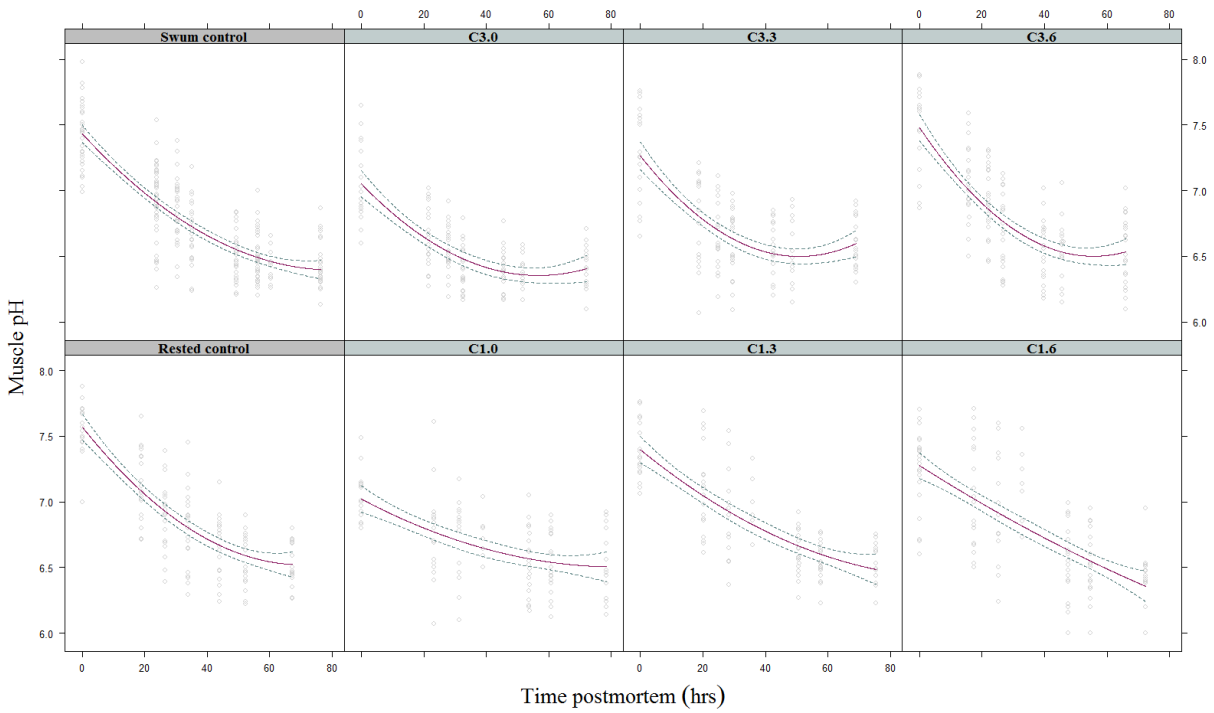


Fig 4. Postmortem change in muscle pH. Relationship between muscle pH and time postmortem. Each panel represents data from separate recovery groups: rested controls (sampled from tank), swum control (sampled immediately after swimming exercise), crowded for 1 hour and recuperated for 0 h (C1.0), 3 h (C1.3) and 6 h (C1.6), crowded for 3 hours and recuperated for 0 h (C3.0), 3 h (C3.3) and 6 h (C3.6). Data are presented as open circles; fitted values from the GLM are shown as a solid red line and the corresponding 95% confidence interval as dashed grey lines. See S3 for model details.

Discussion

There is growing interest in the fishing industry to improve the quality of fish caught by commercial trawlers. Large catches and lengthy hauls often result in lower muscle pH, muscle segment gaping and a reddish coloration of the fillet, all of which are considered quality defects that may lead to downgrading of the fish and financial loss for the producer [21, 22]. One way to circumvent this problem is to temporarily store the fish live in tanks supplied with running seawater to let the fish recover from the capture process. This procedure has successfully improved fillet quality in Atlantic cod caught by trawl [2].

We have previously demonstrated that exhaustive swimming alone does not cause the variable or reduced fillet quality frequently seen in Atlantic cod caught by trawl and suggested that crowding in the cod-end may be an important factor causing reduced fillet quality in trawl-caught fish [13]. Hence, the purpose of this study was to experimentally study the effects of exhaustive swimming and crowding in the cod-end on physiological stress parameters and fillet quality traits in Atlantic cod. We found that exhaustive swimming followed by crowding caused a severe metabolic stress response, as demonstrated by high plasma cortisol levels and elevated blood lactate and glucose levels. The metabolic stress was accompanied by a reduction in muscle pH and increased fillet redness, similar to that reported for cod caught by trawl [2, 6]. The direct cause of the stress induced by crowding is not clear, but a gradual build-up of blood lactate, which correlated with the duration of the crowding, is an indication of insufficient oxygen uptake and prolonged anaerobic metabolism during the period of confinement. Our initial expectation was that there would be less oxygen available inside the cod-end during crowding which could affect the oxygen uptake of the fish, but oxygen saturation of the water always remained above 95% at any position inside the experimental cod-end. It seems more likely, therefore, that our cod may have experienced hypoxia as a consequence of impaired

opercular movement and thus insufficient ventilation due to the very high fish density inside the cod-end.

In the present experiment, post-exercise crowding for 1 and 3 hours, were associated with 0 and 18% mortality after 6 hours of recovery, respectively. This suggests that the majority of Atlantic cod can handle extreme crowding (about 700 kg m^{-3}) for 3 hours. However, the mortality in the 3 hour crowding group varied greatly between the three trials (48, 5 and 0 %, S1 Table). The first trial of fish crowded for 3 hours had higher fish density (*i.e.* about 800 kg m^{-3}) than the last two trials. The density was similar to that in the first trial with 1 hour crowding. This indicates that crowding time is particularly important when the fish density is high and that there may be a threshold for tolerable crowding between 700 and 800 kg m^{-3}. A study from commercial trawlers found that hauls longer than 5 hours led to up to 27 % mortality [2]. This is in contrast to the initial trial in our experiment where confinement in the cod-end for 5 hours resulted in over 80% mortality. We speculate that the discrepancy between our experiment and the observations from commercial trawls, may be due to the gradual filling of the trawl under natural conditions, in which case the fish would not experience extreme crowding until the cod-end is filled up to some degree. For example, another large scale trawl study found a less severe cortisol response (~ 60 ng mL^{-1}) in cod after hauls lasting 15-55 min [6], compared to the fish in our study that were confined in the experimental cod-end for 1 hour (~ 200 ng mL-1).

During hypoxia, the metabolic fuel preference is thought to shift from mainly lipids and proteins to carbohydrates [23]. We found a marked elevation in blood glucose after crowding, which continued to increase throughout the recuperation period. This is most likely due to catecholamine and cortisol-mediated stimulation of glycogenolysis and gluconeogenesis, respectively, which is not met by a comparable increase in glucose utilisation [24, 25]. We also found that fillet redness increased as a response to crowding, and that it correlated with elevated plasma cortisol levels and muscle haemoglobin. This suggests that the sensory evaluation of

redness is a valid method for assessing amount of blood in cod fillets. In addition, the haemoglobin measurement was positively correlated with haematocrit, indicating that the amount of red blood cells also have a contributing effect to observed increase in fillet redness. In Atlantic cod, hypoxic conditions are reported to increase resistance of vessels supplying the stomach, intestines and other digestive organs, while somatic circulation is dilated [26], thereby redistributing blood flow to the muscle. Furthermore, in rainbow trout 80 % of cardiac output is found to be routed to the white muscle of during recovery from strenuous exercise [27]. It seems likely, therefore, that the increase in haematocrit, together with a presumed increased blood perfusion of the white muscle during recovery may be the most important factors causing increased redness of the fillet during recovery.

In the present study, the strong lactate response in crowded fish was negatively correlated to muscle pH. High peri-mortem lactate levels may have consequences for shelf-life of the fillets because lactate, as a carbohydrate, can be a substrate for microbial growth and production of volatiles [28]. It is frequently claimed that the formation of lactic acid causes the post-mortem decrease in muscle pH. However, the concept of lactic acidosis has been questioned [29-33]. It is now more accepted that the major source of protons is hydrolysis of ATP and formation of reduced nicotinamide adenine dinucleotide during glycolysis, with lactate production being a proton-consuming process that retards, not causes, acidosis [34].

In accordance with other studies [2, 35-38] we found that the stress associated with crowding lead to a low peri-mortem muscle pH that continued to decline post-mortem. A rapid decline in post-mortem muscle pH has been associated with softening of the muscle in cod [39]. We found that fish crowded for 3 hours reached minimum pH faster than the other groups and appeared to level out or even increase muscle pH after approximately 48 hours storage on ice. A previous study on meagre (*Argyrosomus regius*) found that a late post-mortem increase in pH was associated with decomposition of nitrogenated compounds, caused primarily by

393 microbial activity [40]. This means that an early increase in post-mortem muscle pH as
394 observed in the current study, may influence shelf-life of the final product. Interestingly, the
395 tendency of pH to increase 60-80 hours post-mortem occurred for all fish crowded for 3 hours,
396 even after 6 hours of recuperation when there were no differences in the peri-mortem muscle
397 pH. This suggests that the severity of stress fish are exposed to pre-mortem affects how muscle
398 pH changes post-mortem, and thereby may influence final quality

399 Conclusion

400 In this study, we found that exhaustive swimming together with crowding for 3 hrs cause
401 physiological responses comparable to what is seen in trawl-captured cod. The same responses
402 were not seen in fish subjected only to exhaustive swimming. This indicates that the additional
403 physiological stress caused by crowding in the cod-end is an important contributor to the often-
404 observed reduction in fillet quality of cod caught by trawl. A complete recovery from
405 exhaustive exercise and extreme crowding, most likely requires more than 6 hours.

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Supporting information

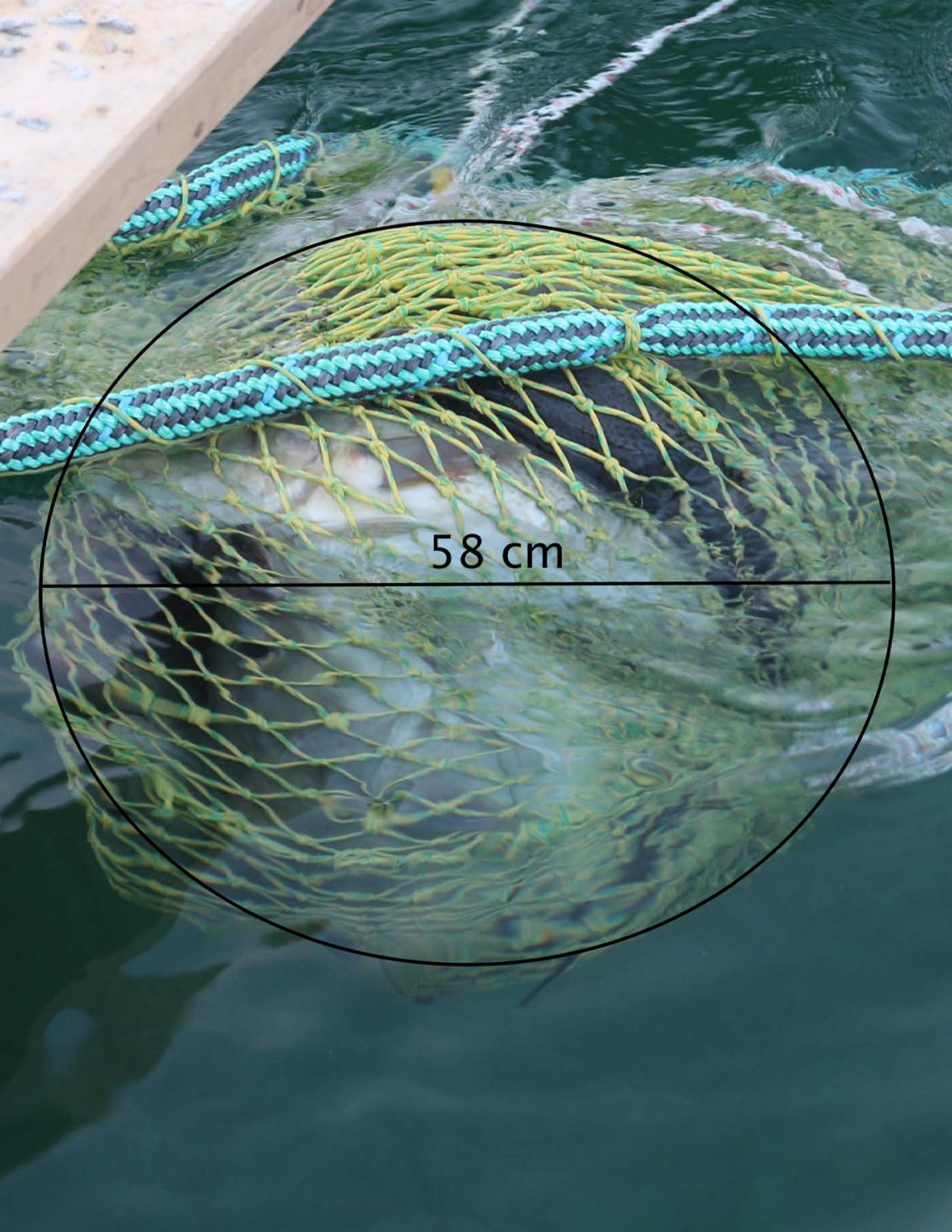
S1 Table. Overview and summary information of each trial. Trial number, dates, air temperature, biological information, fish density and mortality for each trial.

S2 Fig. Extreme crowding of Atlantic cod. Image showing the extreme crowding of cod in the experimental cod-end. The shape of the closed cod-end resembled a sphere with diameter 58 cm.

S3 Model detail. Model parameters and ANOVA output from the generalized linear models.

S4 Figures. GLM correlation plots.

Trial no.	Treatment	Recuperation time (hrs)	Date	♂	♀	N	Weight	Length	CF	GSI	HSI	Fish density in cod-end (kg m-3)	Mortality (%)	Air temp. °C
1	Rested control	Na	01.02.2015	2	5	7	3771 ± 1330	75 ± 7	0.85 ± 0.07	6.23 ± 9.17	4.80 ± 1.09	Na	0	-5±0.4
	Swum control	0	03.02.2015	3	4	7	3939 ± 616	77 ± 5	0.88 ± 0.15	7.64 ± 6.33	4.38 ± 1.39	Na		
	1 hr crowding	0	03.02.2015	3	4	7	4211 ± 1186	78 ± 10	0.89 ± 0.19	9.57 ± 6.15	4.76 ± 1.62		0	-7.8±0.4
	1 hr crowding	3	03.02.2015	2	5	7	4171 ± 1124	81 ± 6	0.78 ± 0.12	4.87 ± 4.21	4.41 ± 1.77	803		
	1 hr crowding	6	03.02.2015	6	1	7	3343 ± 839	73 ± 10	0.92 ± 0.38	5.69 ± 5.31	4.48 ± 1.76			
1	Swum control	0	10.02.2015	2	5	7	3397 ± 1220	75 ± 8	0.78 ± 0.08	3.12 ± 2.99	3.98 ± 0.90	Na	0	-0.9±1.1
	3 hrs crowding	0	10.02.2015	2	5	7	3603 ± 804	77 ± 6	0.78 ± 0.14	7.03 ± 6.81	3.93 ± 1.09			
	3 hrs crowding	3	10.02.2015	5	2	7	3934 ± 248	80 ± 5	0.78 ± 0.13	6.19 ± 4.46	3.83 ± 1.02	802	48	
	3 hrs crowding	6	10.02.2015	3	5	8	3645 ± 597	77 ± 6	0.79 ± 0.13	6.08 ± 5.74	4.34 ± 0.95			
2	Rested control	Na	08.02.2015	1	6	7	3626 ± 911	76 ± 6	0.81 ± 0.11	3.48 ± 4.34	4.34 ± 1.30	Na	0	-6±0.4
	Swum control	0	12.02.2015	2	5	7	3293 ± 889	75 ± 6	0.77 ± 0.15	4.79 ± 5.62	4.69 ± 1.30	Na	0	
	1 hr crowding	0	12.02.2015	2	5	7	2683 ± 601	68 ± 5	0.83 ± 0.12	5.79 ± 6.98	3.56 ± 1.34			-4.9±0.8
	1 hr crowding	3	12.02.2015	4	3	7	3423 ± 852	75 ± 4	0.79 ± 0.11	4.73 ± 4.86	3.40 ± 1.44	672	0	
	1 hr crowding	6	12.02.2015	4	3	7	3706 ± 889	76 ± 7	0.84 ± 0.06	1.74 ± 2.19	5.28 ± 1.15			
2	Swum control	0	17.02.2015	2	5	7	3418 ± 706	73 ± 5	0.87 ± 0.18	5.75 ± 5.96	4.72 ± 2.14	Na	0	
	3 hrs crowding	0	17.02.2015	4	3	7	3776 ± 975	76 ± 10	0.88 ± 0.17	7.37 ± 7.11	5.30 ± 1.47			1.3±0.54
	3 hrs crowding	3	17.02.2015	2	5	7	3304 ± 1104	74 ± 10	0.79 ± 0.12	8.49 ± 8.28	4.22 ± 1.74	706	5	
	3 hrs crowding	6	17.02.2015	4	3	7	3222 ± 454	71 ± 4	0.91 ± 0.11	9.29 ± 8.78	4.25 ± 1.22			
3	Rested control	Na	22.02.2015	4	3	7	3034 ± 784	72 ± 7	0.82 ± 0.12	3.27 ± 3.52	4.07 ± 1.28	Na	0	-1.3±2.27
	Swum control	0	24.02.2015	3	4	7	3364 ± 898	72 ± 5	0.90 ± 0.11	5.11 ± 4.71	4.51 ± 0.85	Na	0	
	1 hr crowding	0	24.02.2015	4	3	7	3567 ± 539	74 ± 4	0.87 ± 0.07	4.34 ± 4.31	4.65 ± 1.23			0.9±1.0
	1 hr crowding	3	24.02.2015	3	4	7	3690 ± 472	75 ± 3	0.86 ± 0.10	5.48 ± 6.35	4.78 ± 0.66	733	0	
	1 hr crowding	6	24.02.2015	2	5	7	3446 ± 818	73 ± 5	0.86 ± 0.10	3.62 ± 3.63	4.78 ± 1.36			
3	Swum control	0	26.02.2015	5	2	7	2608 ± 676	69 ± 8	0.81 ± 0.17	3.26 ± 3.29	3.44 ± 1.40	Na	0	
	3 hrs crowding	0	26.02.2015	5	2	7	3808 ± 609	76 ± 6	0.86 ± 0.09	5.78 ± 5.11	4.52 ± 1.46			0.1±1.1
	3 hrs crowding	3	26.02.2015	4	3	7	2836 ± 921	72 ± 8	0.74 ± 0.11	0.43 ± 0.22	4.56 ± 2.55	702	0	
	3 hrs crowding	6	26.02.2015	4	3	7	3604 ± 1089	73 ± 5	0.92 ± 0.11	3.04 ± 3.23	5.71 ± 1.35			


58 cm

S3 Model details

Output from Generalized linear models

Cortisol

```
Call:
glm(formula = cort ~ treatment + gsi, family = gaussian(inverse),
    data = df)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-165.60   -23.93    -3.34    30.73   163.00

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)         2.237e-01  7.357e-01   0.304    0.762
treatmentpack.1.0  -2.184e-01  7.357e-01  -0.297    0.767
treatmentpack.1.3  -2.189e-01  7.357e-01  -0.298    0.766
treatmentpack.1.6  -2.168e-01  7.357e-01  -0.295    0.769
treatmentpack.3.0  -2.203e-01  7.357e-01  -0.299    0.765
treatmentpack.3.3  -2.182e-01  7.357e-01  -0.297    0.767
treatmentpack.3.6  -2.180e-01  7.357e-01  -0.296    0.767
treatments.control -1.855e-01  7.359e-01  -0.252    0.801
gsi                 4.471e-04  7.317e-05   6.111 9.17e-09 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 2737.255)

    Null deviance: 1112242  on 149  degrees of freedom
Residual deviance:  385946  on 141  degrees of freedom
  (40 observations deleted due to missingness)
AIC: 1623.6

Number of Fisher Scoring iterations: 9
```

Analysis of Deviance Table

```
Model: gaussian, link: inverse

Response: cort

Terms added sequentially (first to last)


          Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                        149    1112242
treatment  7   554006       142     558235 28.913 < 2.2e-16 ***
gsi        1   172290       141     385946 62.943 5.956e-13 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


>
```

Glucose

```
Call:
glm(formula = glu ~ treatment + hsi, family = gaussian(log),
    data = df)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-4.9957  -1.1436  -0.0911   0.9309   6.4114
```

```
Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)        0.62007    0.16346   3.793 0.000205 ***
treatmentpack.1.0  0.71497    0.16867   4.239 3.63e-05 ***
treatmentpack.1.3  1.13090    0.15933   7.098 3.04e-11 ***
treatmentpack.1.6  1.31248    0.15660   8.381 1.66e-14 ***
treatmentpack.3.0  0.99200    0.16123   6.153 5.04e-09 ***
treatmentpack.3.3  1.04070    0.16217   6.417 1.26e-09 ***
treatmentpack.3.6  1.22771    0.15805   7.768 6.47e-13 ***
treatments.control 0.39689    0.16786   2.364 0.019157 *
hsi                0.07691    0.01334   5.764 3.64e-08 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 3.330925)

    Null deviance: 1809.80  on 183  degrees of freedom
Residual deviance:  582.91  on 175  degrees of freedom
  (6 observations deleted due to missingness)
AIC: 754.34

Number of Fisher Scoring iterations: 5
```

Analysis of Deviance Table

```
Model: gaussian, link: log

Response: glu

Terms added sequentially (first to last)


          Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                        183    1809.80
treatment  7   1111.8       176     698.01 47.682 < 2.2e-16 ***
hsi        1    115.1       175     582.91 34.555  2.05e-08 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Lactate

```
Call:
glm(formula = lac ~ treatment + mpH, family = Gamma(inverse),
    data = df)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-2.58366  -0.33619  -0.00047   0.27781   0.99015

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)        36.53743    4.37497   8.351 1.99e-14 ***
treatmentpack.1.0 -39.24542    4.35518  -9.011 3.46e-16 ***
treatmentpack.1.3 -39.46750    4.35499  -9.063 2.51e-16 ***
treatmentpack.1.6 -39.24458    4.35523  -9.011 3.47e-16 ***
treatmentpack.3.0 -39.48641    4.35498  -9.067 2.44e-16 ***
treatmentpack.3.3 -39.51815    4.35497  -9.074 2.34e-16 ***
treatmentpack.3.6 -39.55227    4.35491  -9.082 2.22e-16 ***
treatments.control -38.97400    4.35532  -8.949 5.11e-16 ***
mpH                 0.45794    0.05559   8.238 3.96e-14 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for Gamma family taken to be 0.2488991)

    Null deviance: 318.337  on 183  degrees of freedom
Residual deviance:  95.924  on 175  degrees of freedom
```

```
  (6 observations deleted due to missingness)
AIC: 442.75

Number of Fisher Scoring iterations: 5
```

Sensory evaluation of redness with muscle haemoglobin

```
Call:
glm(formula = (sens + 1)/4 ~ treatment + mbr, family = quasibinomial(),
    data = df)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-0.39348  -0.15090  -0.03632   0.10519   0.84174

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)         -2.1613     0.1912 -11.302  < 2e-16 ***
treatmentpack.1.0    0.6153     0.1465   4.200 4.25e-05 ***
treatmentpack.1.3    0.7535     0.1601   4.708 5.10e-06 ***
treatmentpack.1.6    0.4951     0.1472   3.363 0.000948 ***
treatmentpack.3.0    0.4948     0.1497   3.306 0.001151 **
treatmentpack.3.3    0.6906     0.1469   4.701 5.25e-06 ***
treatmentpack.3.6    0.3723     0.1459   2.552 0.011566 *
treatments.control   0.1552     0.1307   1.188 0.236567
mbr                 29.2960     3.2643   8.975 4.49e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.0516939)

    Null deviance: 17.5746  on 182  degrees of freedom
Residual deviance:  9.3824  on 174  degrees of freedom
  (7 observations deleted due to missingness)
AIC: NA

Number of Fisher Scoring iterations: 4
```

Analysis of Deviance Table

```
Model: quasibinomial, link: logit

Response: (sens + 1)/4

Terms added sequentially (first to last)


          Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                        182    17.5746
treatment  7   3.8829       175    13.6917 10.731 3.288e-11 ***
mbr        1   4.3093       174     9.3824 83.362 < 2.2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Sensory evaluation of redness with plasma cortisol

```
Call:
glm(formula = (sens + 1)/4 ~ treatment + cort, family = quasibinomial,
    data = df)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-0.47022  -0.16657  -0.03464   0.09097   1.17908
```

```
Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)        -0.7397988  0.1585117  -4.667 7.07e-06 ***
treatmentpack.1.0   0.5154926  0.2228334   2.313  0.02216 *
treatmentpack.1.3   0.4308118  0.2269462   1.898  0.05971 .
treatmentpack.1.6   0.3279003  0.2163676   1.515  0.13191
treatmentpack.3.0   0.5452524  0.2421873   2.251  0.02592 *
treatmentpack.3.3   0.6802684  0.2398435   2.836  0.00524 **
treatmentpack.3.6   0.0074835  0.2289634   0.033  0.97397
treatments.control  0.0812914  0.1875940   0.433  0.66544
cort                0.0014818  0.0007247   2.045  0.04275 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.07156997)

    Null deviance: 14.116  on 148  degrees of freedom
Residual deviance: 10.392  on 140  degrees of freedom
  (41 observations deleted due to missingness)
AIC: NA

Number of Fisher Scoring iterations: 3
```

Analysis of Deviance Table

```
Model: quasibinomial, link: logit

Response: (sens + 1)/4

Terms added sequentially (first to last)


          Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                        148     14.116
treatment  7   3.4236       141     10.692 6.8338 5.525e-07 ***
cort       1   0.2999       140     10.392 4.1901   0.04253 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Muscle haemoglobin modelled with haematocrit

```
Call:  glm(formula = (mbr) ~ hct, family = gaussian(), data = df)


Deviance Residuals:
      Min         1Q     Median         3Q        Max
-0.022056  -0.007248  -0.000999   0.006001   0.038645

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0372313  0.0050855   7.321 7.22e-11 ***
hct         0.0005040  0.0002062   2.444   0.0163 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 0.0001254236)

    Null deviance: 0.012915  on 98  degrees of freedom
Residual deviance: 0.012166  on 97  degrees of freedom
  (91 observations deleted due to missingness)
AIC: -604.47

Number of Fisher Scoring iterations: 2
```

Analysis of Deviance Table

```
Model: gaussian, link: identity
```

```
Response: (mbr)

Terms added sequentially (first to last)


     Df   Deviance Resid. Df Resid. Dev      F  Pr(>F)
NULL                     98   0.012915
hct   1 0.00074912        97   0.012166 5.9727 0.01634 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Muscle haemoglobin without haematocrit

```
Call:
glm(formula = mbr ~ treatment + cort, family = gaussian(), data = df)

Deviance Residuals:
      Min         1Q     Median         3Q        Max
-0.021899  -0.007157  -0.001759   0.005926   0.036879

Coefficients:
                    Estimate Std. Error t value Pr(>|t|)
(Intercept)         4.977e-02  2.970e-03  16.761   <2e-16 ***
treatmentpack.1.0  -2.771e-03  4.329e-03  -0.640   0.5231
treatmentpack.1.3  -7.339e-03  4.591e-03  -1.598   0.1123
treatmentpack.1.6  -6.657e-03  4.182e-03  -1.592   0.1138
treatmentpack.3.0   2.019e-03  4.741e-03   0.426   0.6709
treatmentpack.3.3  -3.577e-04  4.677e-03  -0.076   0.9391
treatmentpack.3.6  -4.212e-03  4.378e-03  -0.962   0.3377
treatments.control -4.062e-03  3.518e-03  -1.155   0.2503
cort                3.767e-05  1.473e-05   2.557   0.0117 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 0.0001145962)

    Null deviance: 0.018644  on 144  degrees of freedom
Residual deviance: 0.015585  on 136  degrees of freedom
  (45 observations deleted due to missingness)
AIC: -893.54

Number of Fisher Scoring iterations: 2
```

Analysis of Deviance Table

```
Model: gaussian, link: identity

Response: mbr

Terms added sequentially (first to last)


          Df   Deviance Resid. Df Resid. Dev      F   Pr(>F)
NULL                         144   0.018644
treatment  7 0.00230938       137   0.016334 2.8789 0.007785 **
cort       1 0.00074931       136   0.015585 6.5387 0.011652 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Haemotocrit

```
Call:
glm(formula = (hct) ~ treatment + cort, family = gaussian(),
    data = df)

Deviance Residuals:
```

```
     Min        1Q    Median        3Q       Max
-17.0103   -1.6971    0.3383    2.3208    7.3038

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)       22.326168   1.511959  14.766   <2e-16 ***
treatmentpack.1.0  4.194346   2.165753   1.937   0.0568 .
treatmentpack.1.3 -1.464930   2.119666  -0.691   0.4917
treatmentpack.1.6 -1.446498   2.112939  -0.685   0.4958
treatmentpack.3.0  5.400214   2.233996   2.417   0.0182 *
treatmentpack.3.3 -0.838822   2.452710  -0.342   0.7334
treatmentpack.3.6 -5.592258   2.114374  -2.645   0.0101 *
treatments.control -0.234210   1.882553  -0.124   0.9013
cort               0.016383   0.006802   2.409   0.0186 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 15.99685)

    Null deviance: 2265.3  on 79  degrees of freedom
Residual deviance: 1135.8  on 71  degrees of freedom
  (110 observations deleted due to missingness)
AIC: 459.27

Number of Fisher Scoring iterations: 2
```

Analysis of Deviance Table

```
Model: gaussian, link: identity

Response: (hct)

Terms added sequentially (first to last)


          Df Deviance Resid. Df Resid. Dev     F    Pr(>F)
NULL                        79     2265.3
treatment  7  1036.69        72     1228.6 9.258 4.331e-08 ***
cort       1    92.81        71     1135.8 5.802   0.01861 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Muscle pH

```
Call:
glm(formula = pH ~ adj.hrs * treatment + I(adj.hrs^2) * treatment,
    family = gaussian(), data = rigor)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-0.73550  -0.15952  -0.01063   0.16668   0.84081

Coefficients:
                                Estimate Std. Error t value Pr(>|t|)
(Intercept)                    7.571e+00  5.091e-02 148.715  < 2e-16 ***
adj.hrs                       -2.988e-02  3.150e-03  -9.486  < 2e-16 ***
treatmentpack.1.0             -5.497e-01  7.239e-02  -7.594 6.32e-14 ***
treatmentpack.1.3             -1.744e-01  7.192e-02  -2.425  0.01548 *
treatmentpack.1.6             -2.957e-01  7.130e-02  -4.147 3.61e-05 ***
treatmentpack.3.0             -5.178e-01  7.236e-02  -7.155 1.47e-12 ***
treatmentpack.3.3             -3.065e-01  7.441e-02  -4.120 4.06e-05 ***
treatmentpack.3.6             -9.082e-02  7.252e-02  -1.252  0.21073
treatments.control            -1.370e-01  6.119e-02  -2.239  0.02534 *
I(adj.hrs^2)                   2.126e-04  4.425e-05   4.804 1.75e-06 ***
adj.hrs:treatmentpack.1.0      1.731e-02  4.334e-03   3.993 6.93e-05 ***
adj.hrs:treatmentpack.1.3      1.052e-02  4.435e-03   2.371  0.01788 *
adj.hrs:treatmentpack.1.6      1.479e-02  4.547e-03   3.252  0.00118 **
```

```
adj.hrs:treatmentpack.3.0        5.229e-03  4.310e-03   1.213  0.22530
adj.hrs:treatmentpack.3.3       -2.206e-04  4.533e-03  -0.049  0.96120
adj.hrs:treatmentpack.3.6       -5.302e-03  4.606e-03  -1.151  0.24994
adj.hrs:treatments.control       4.093e-03  3.700e-03   1.106  0.26884
treatmentpack.1.0:I(adj.hrs^2)  -1.362e-04  5.827e-05  -2.337  0.01960 *
treatmentpack.1.3:I(adj.hrs^2)  -1.161e-04  6.060e-05  -1.915  0.05568 .
treatmentpack.1.6:I(adj.hrs^2)  -1.795e-04  6.343e-05  -2.830  0.00473 **
treatmentpack.3.0:I(adj.hrs^2)   4.963e-06  5.844e-05   0.085  0.93234
treatmentpack.3.3:I(adj.hrs^2)   8.367e-05  6.195e-05   1.350  0.17714
treatmentpack.3.6:I(adj.hrs^2)   1.035e-04  6.448e-05   1.606  0.10865
treatments.control:I(adj.hrs^2) -5.230e-05  5.072e-05  -1.031  0.30274
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 0.06071145)

    Null deviance: 185.904  on 1198  degrees of freedom
Residual deviance:  71.336  on 1175  degrees of freedom
  (265 observations deleted due to missingness)
AIC: 69.225

Number of Fisher Scoring iterations: 2
```

Analysis of Deviance Table

```
Model: gaussian, link: identity

Response: pH

Terms added sequentially (first to last)


                        Df Deviance Resid. Df Resid. Dev         F    Pr(>F)
NULL                                    1198    185.904
adj.hrs                  1   86.570      1197     99.334 1425.9288 < 2.2e-16
***
treatment                7   11.190      1190     88.144   26.3295 < 2.2e-16
***
I(adj.hrs^2)             1   10.265      1189     77.880  169.0742 < 2.2e-16
***
adj.hrs:treatment        7    4.168      1182     73.712    9.8070 6.355e-12
***
treatment:I(adj.hrs^2)   7    2.376      1175     71.336    5.5904 2.353e-06
***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Fig 1. Correlation between plasma cortisol and GSI

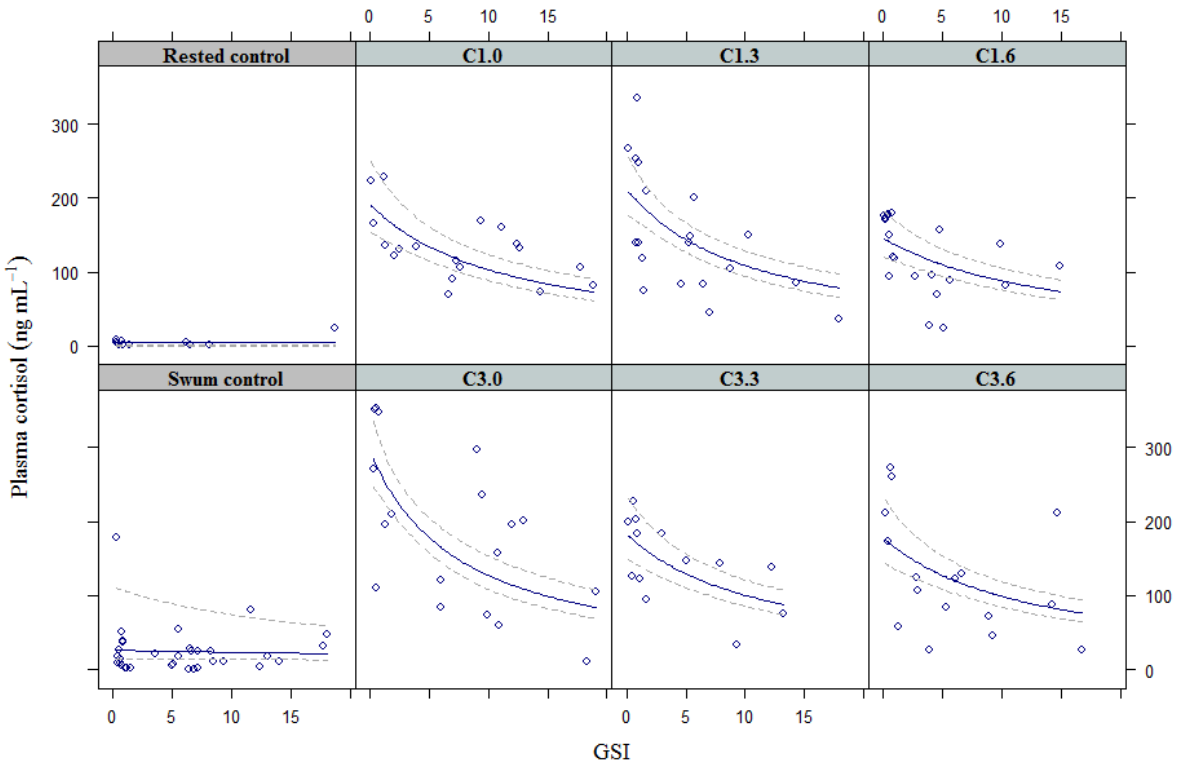


Fig 2. Correlation between blood glucose and HSI

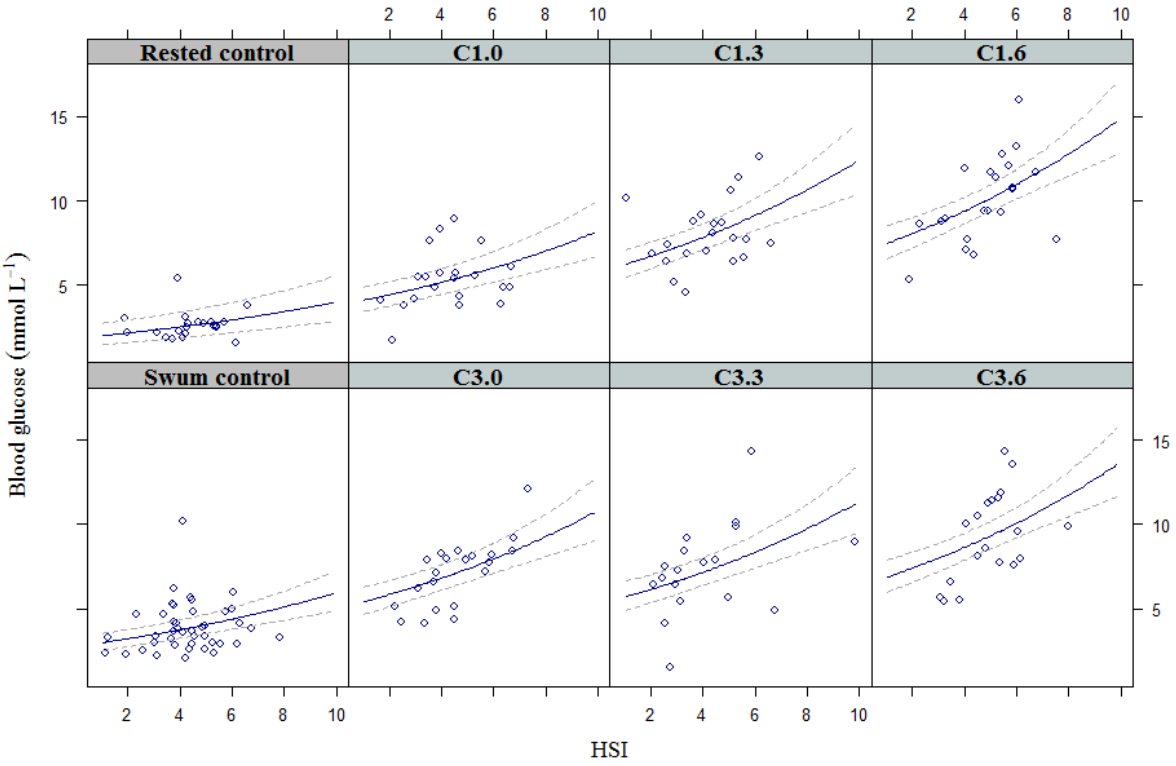


Fig 3. Correlation between blood lactate and muscle pH.

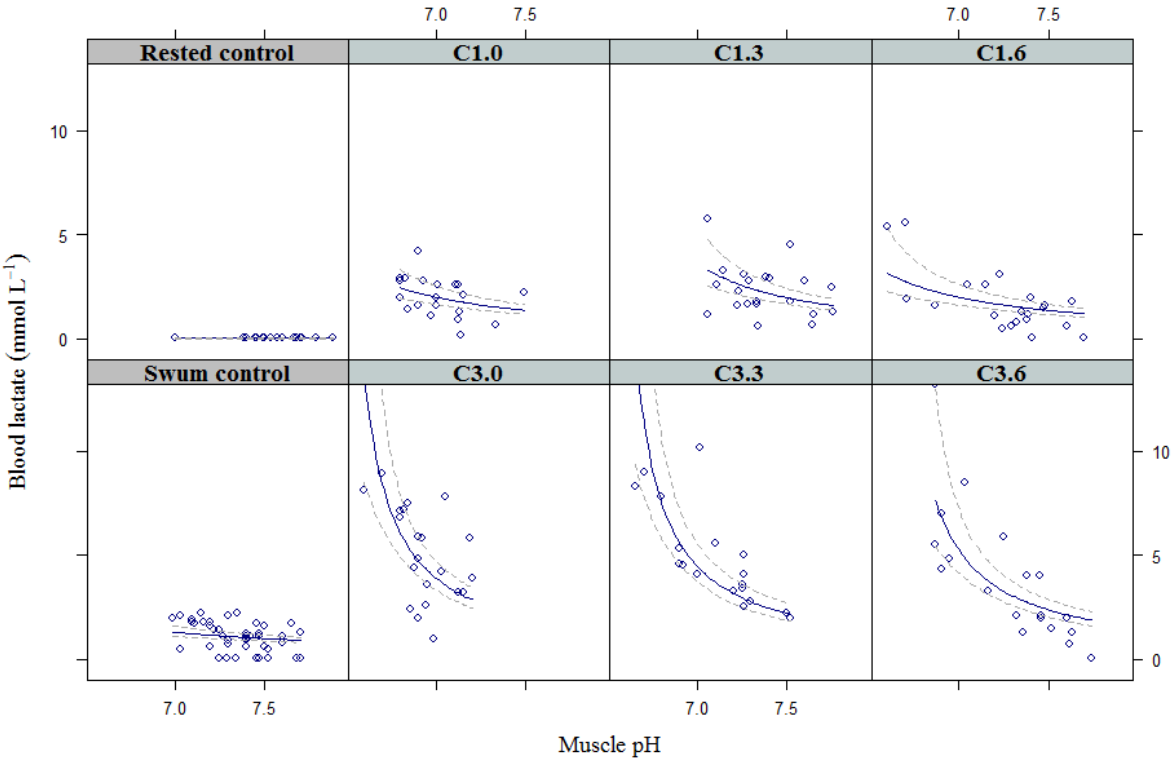


Fig 4. Correlation between fillet redness and muscle haemoglobin

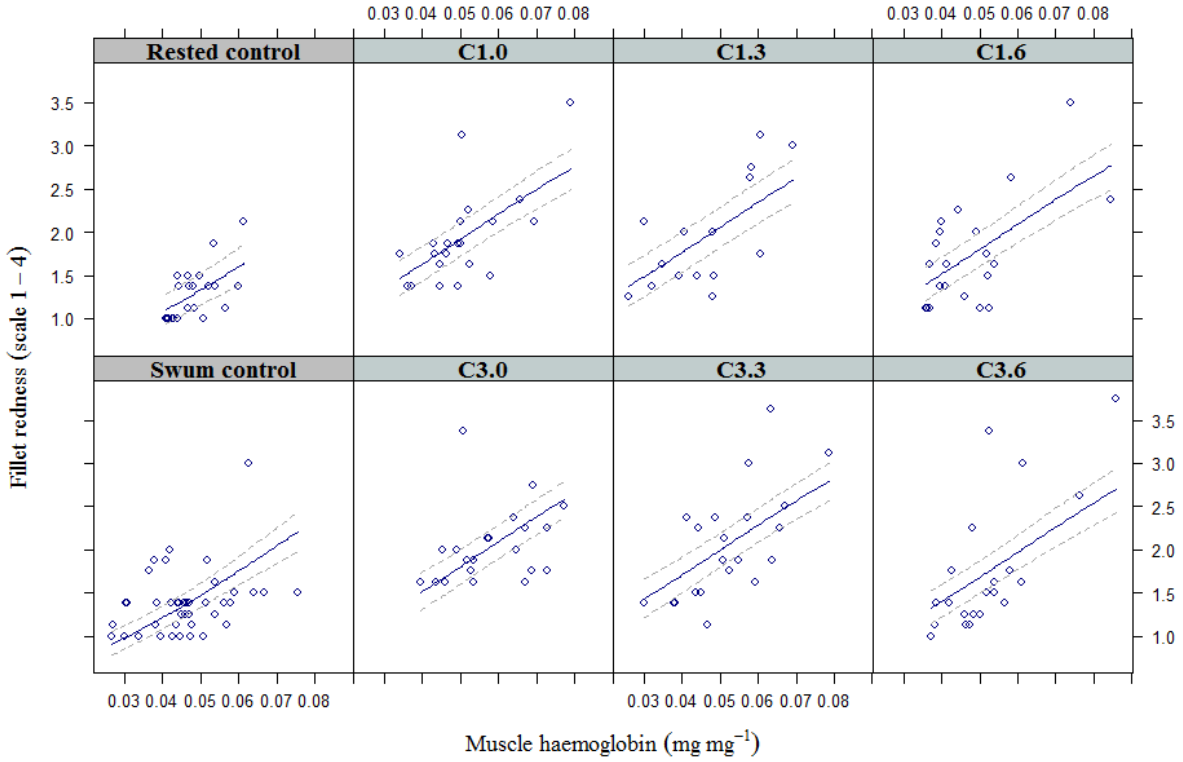


Fig 5. Correlation between fillet redness and plasma cortisol

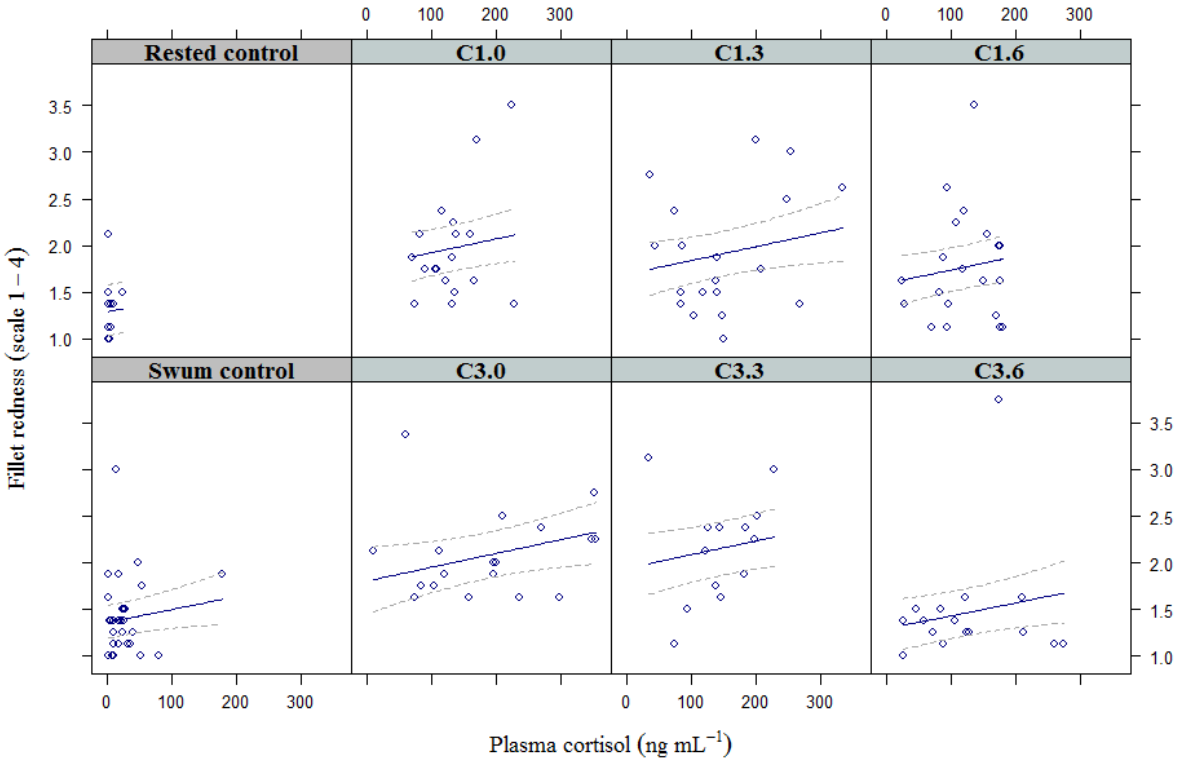


Fig 6. Correlation between muscle haemoglobin and haematocrit

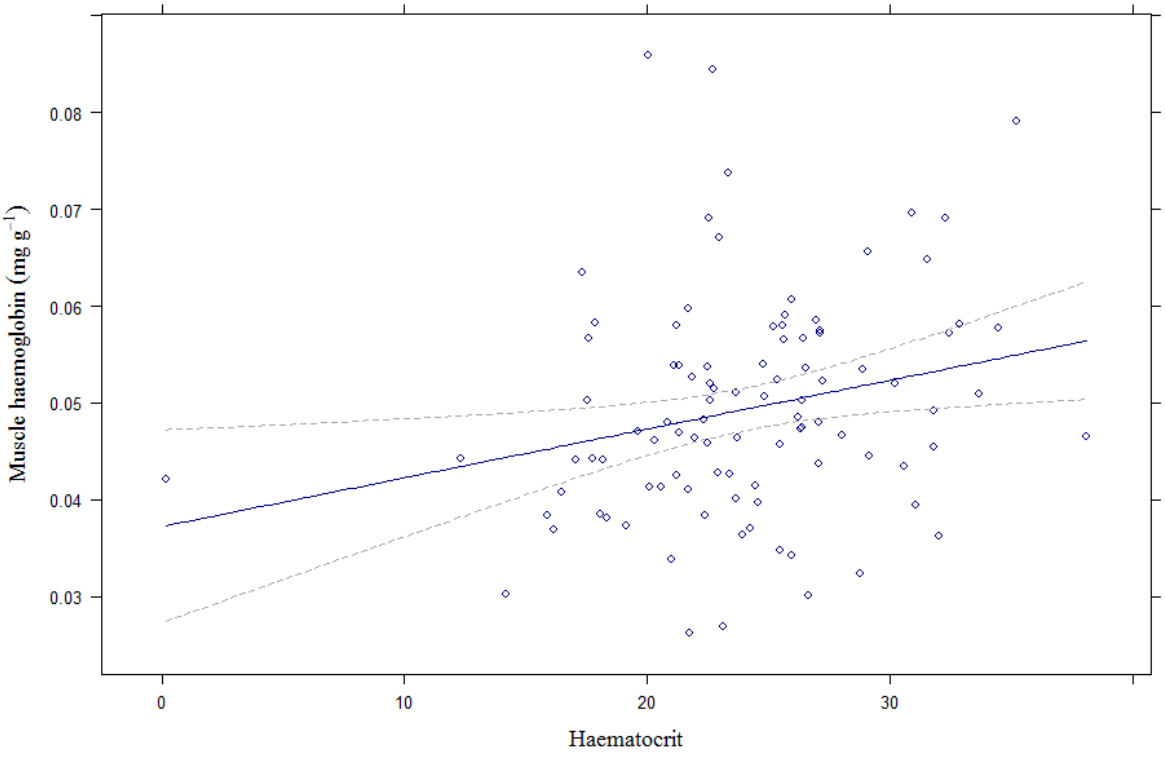


Fig 7. Correlation between muscle haemoglobin and cortisol

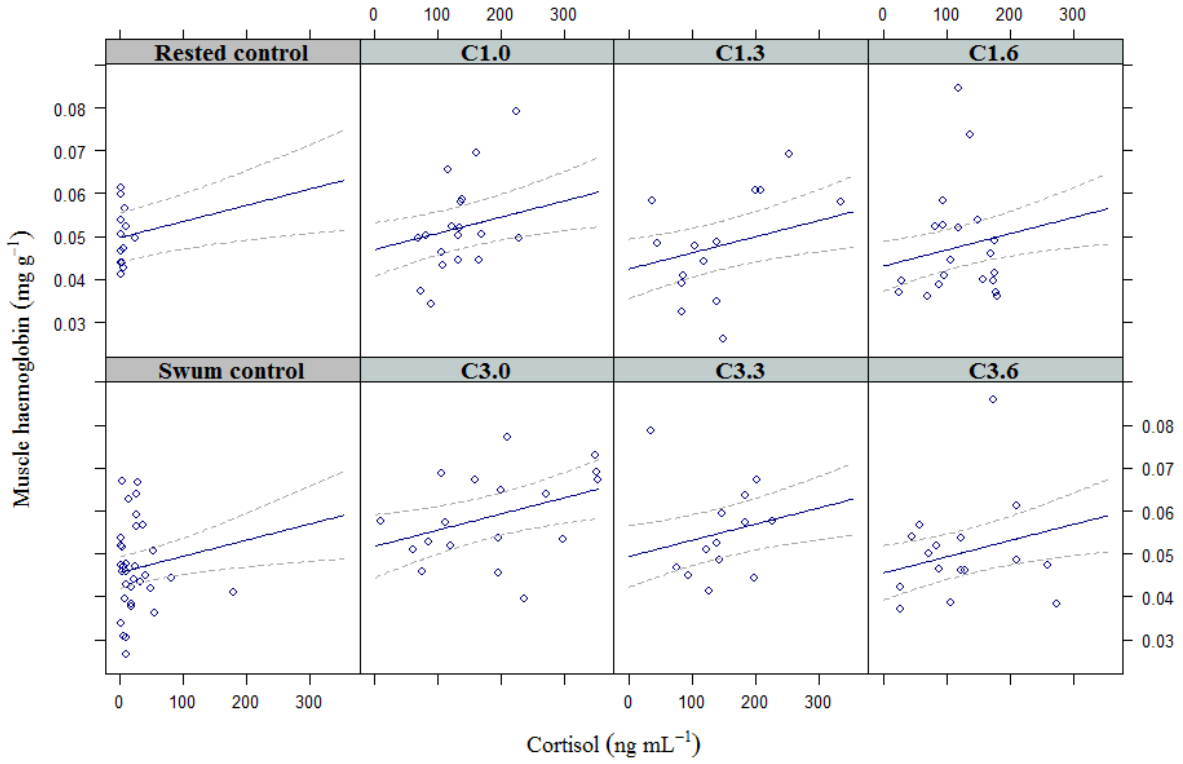
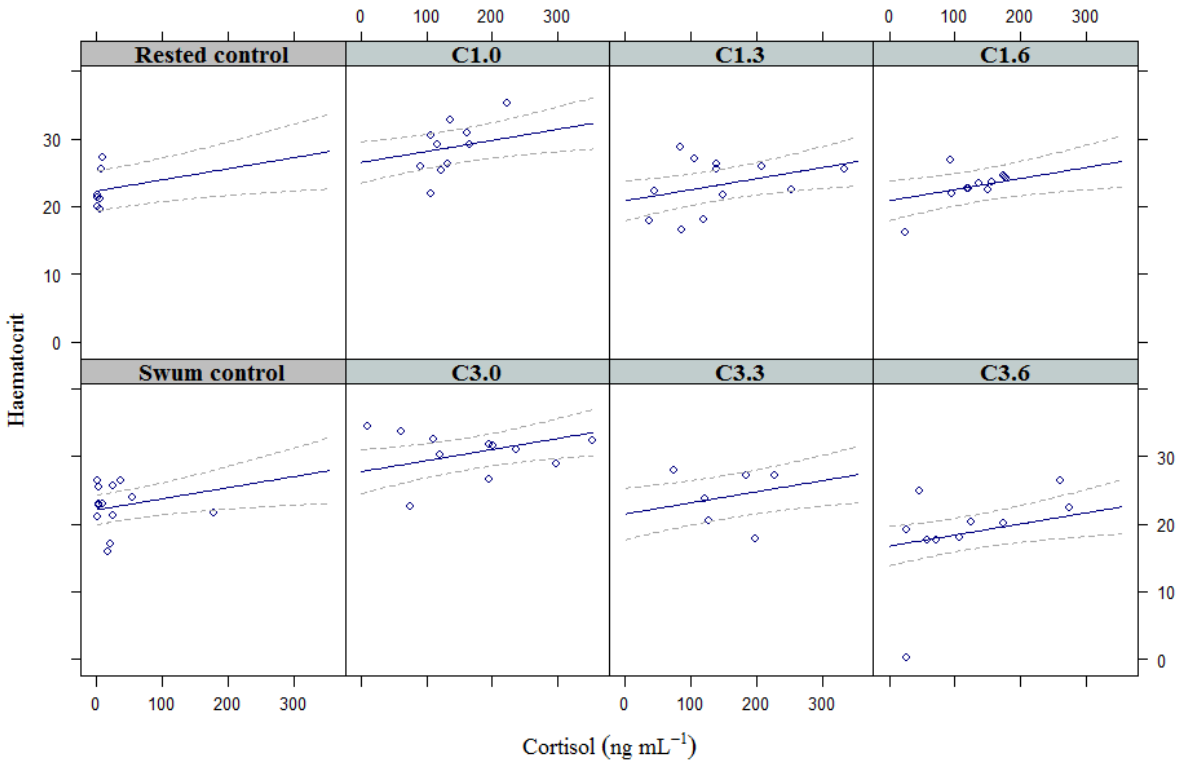


Fig 8. Correlation between haematocrit and plasma cortisol



Paper IV

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.

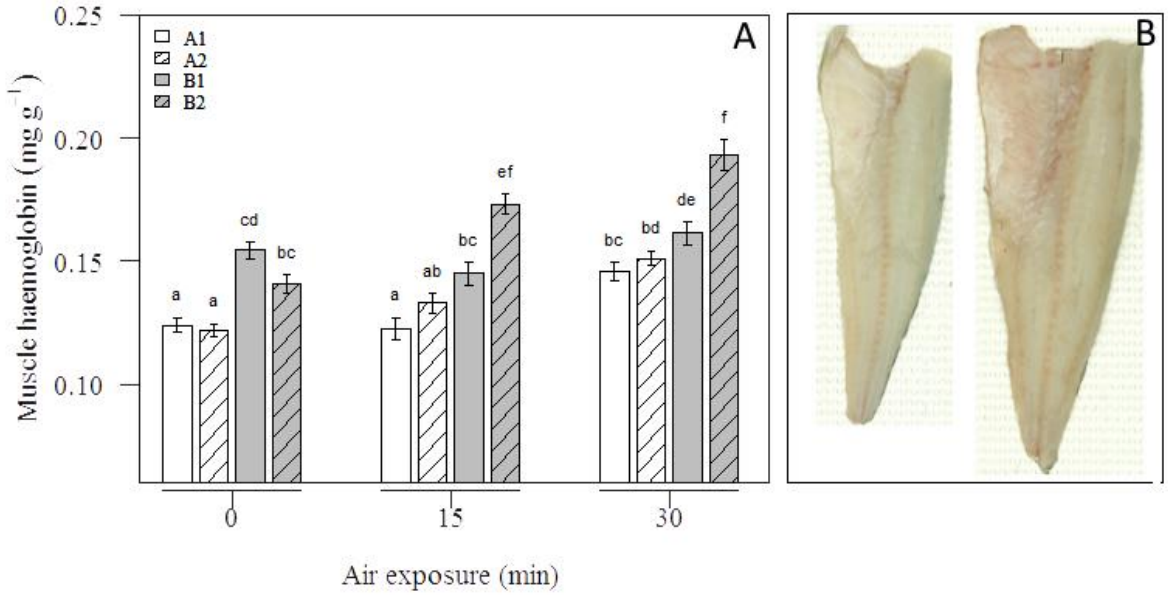

Figure 1: A) Muscle haemoglobin in cod fillets measured with VIS/NIR imaging spectroscopy. A1: Uncrowded and euthanized *prior* to air exposure. A2: Uncrowded and euthanized *post* air exposure. B1: Crowded and euthanized *prio*r to air exposure. B2: Crowded and euthanized *post* air exposure. Bars are 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.

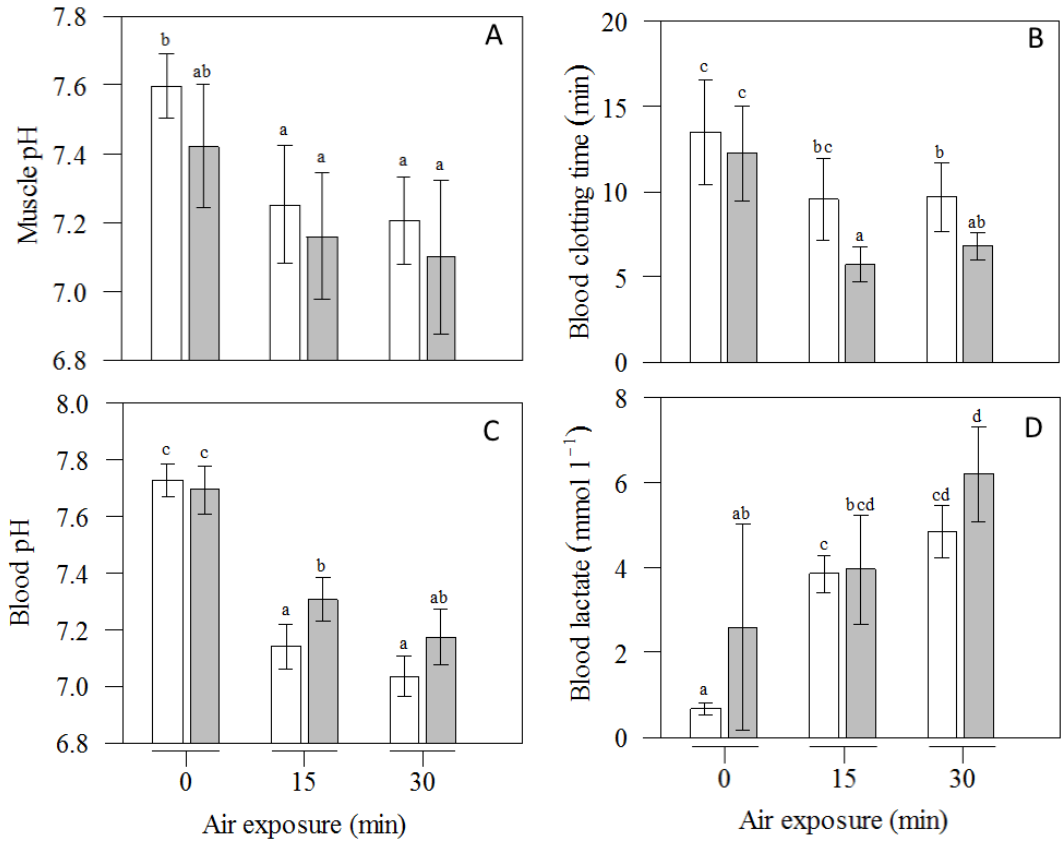

Figure 2: Muscle pH (A), blood clotting time (B), blood pH (C) and blood lactate (D) in crowded (grey bars) and uncrowded (white bars) Atlantic cod exposed to air for 0, 15 or 30 minutes. Bars are mean values with 95 % confidence intervals. Different letters above bars indicate statistically significant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

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Supplementary material

1. Supplementary figures

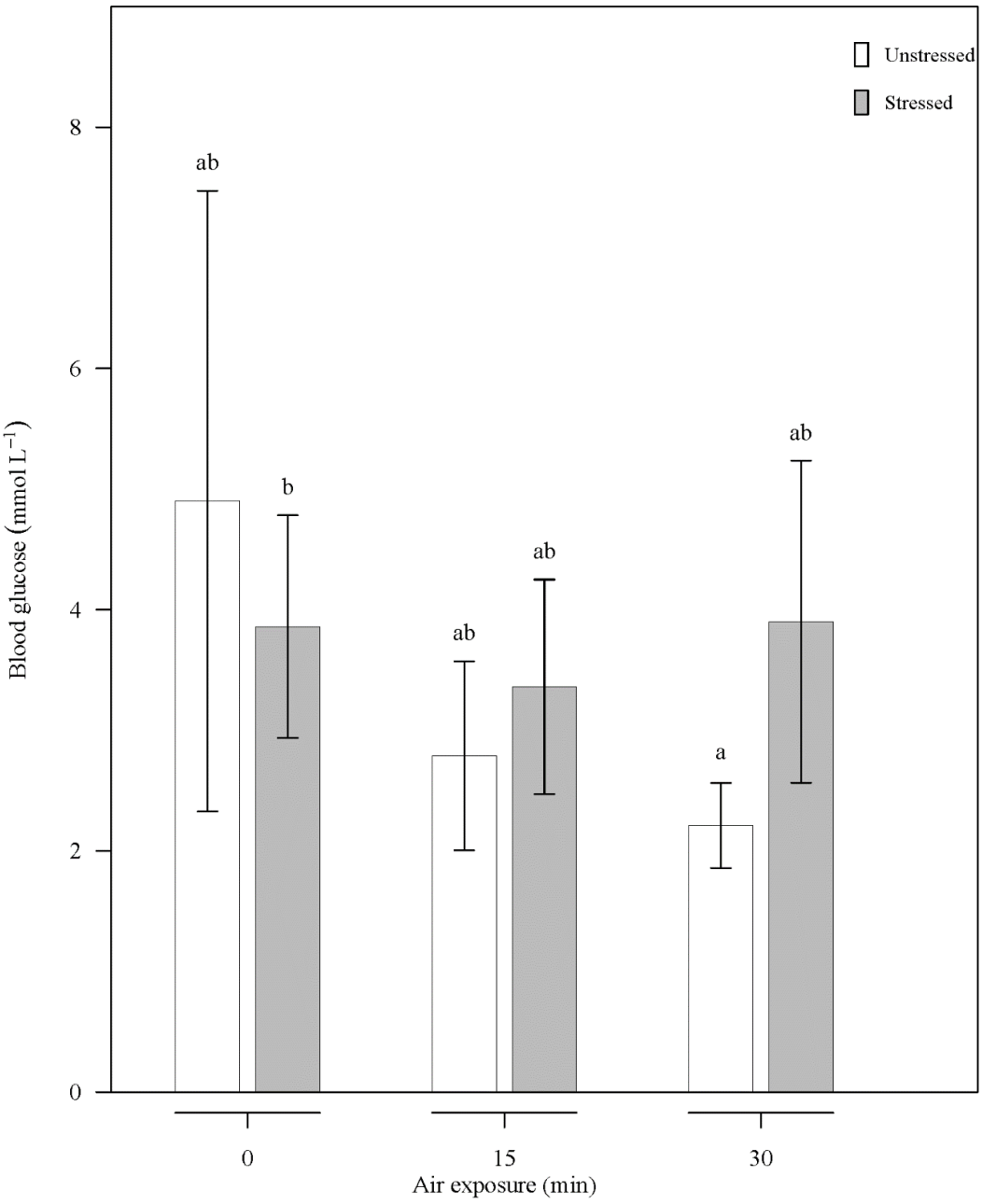


Figure 1: Change in blood glucose in response to crowding (stressed) and/or air exposure for 0, 15 or 30 minutes. Bars are mean values and arrows indicate 95% confidence intervals. Differences in letters above arrows indicate statistical differences ($p<0.05$).

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))

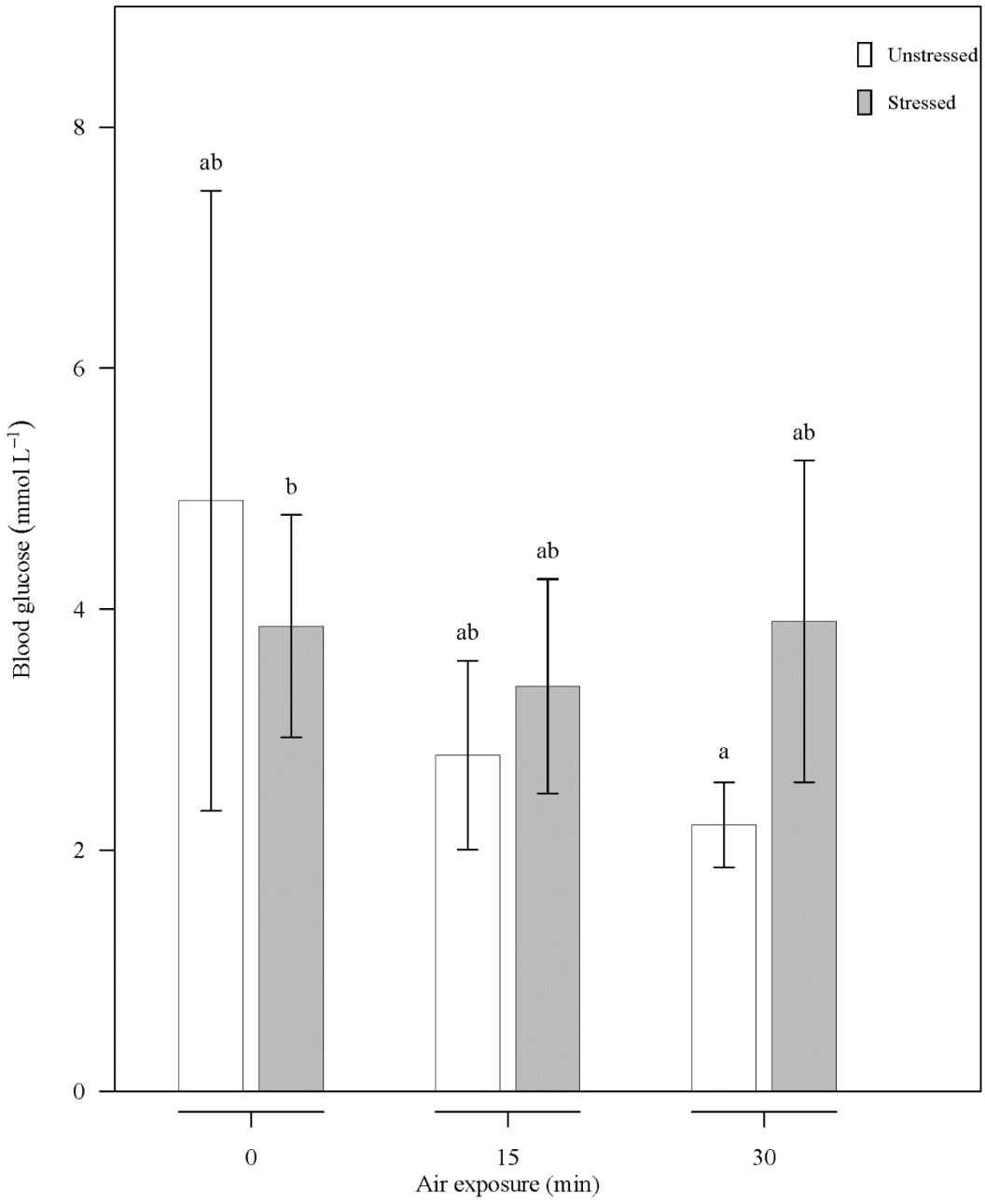
  # Select columns set as factors that should be numeric and change with as.numeric
  results[c(2, 3:ncol(results))] <- round(as.numeric(as.matrix(results[c(2,
3:ncol(results))])), digits = 3)

  # 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



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  # Mean = means of each group sample
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  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) {

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    #t-values
    t <- abs(Mean[combs[1,x]] - Mean[combs[2,x]]) / sqrt((std[combs[1,x]] / n[combs[1,x]]) +
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    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
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  # Rename data frame columns
  colnames(results) <- c('groups', 'Mean Difference', 'Standard Error', 't', 'df', 'p', 'upper
ci', 'lower ci')

  return(results) }
```