REPORT ON INDICATORS (PHYSICAL, BEHAVIOURAL AND PHYSIOLOGICAL) TO ASSESS STRESS & WELFARE STATUS IN PURSE SEINE CATCHES

Deliverable 5.1 for «Fangstkontroll i notfiske etter pelagiske arter» (FHF 901350)

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RAPPORT OM INDIKATORER (FYSISK, OPPFØRSEL OG FYSIOLOGISK) FOR Å VURDERE STRESS- OG VELFERDSSTATUS I NOTFISKE FANGSTER

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Sammendrag

God fiskevelferd er avhengig av at man utvikler velferdsindikatorer som kan måles i kommersielle fangstsituasjoner (Anders et al., 2019; Breen et al., 2020). Operative velferdsindikatorer vil gi fiskerne grunnlag for å ta kunnskapsbaserte beslutninger under fisket som minimerer stress og sørger for god fiskevelferd. Imidlertid, har man så langt ikke klart å utvikle operative velferdsindikatorer til kommersielt fiske hovedsakelig på grunn av mangel på sanntids målemetoder som er praktiske og rimelige å bruke (Morgan and Iwama 1997; Huntingford et al., 2006).

I prosjekt «Fangstkontroll i notfiske etter pelagiske arter» (FHF 901350) har man undersøkt hvordan ulike fangstrelaterte stressorer påvirker fiskens stressrespons, ved hjelp av mer enn tretti forskjellige stress- og velferdsindikatorer, for å identifisere velferdsindikatorer som er brukervennlige og gir relevant og pålitelig data. Velferdsindikatorer som gir nær sanntids data og/eller data som kan tolkes intuitivt, for eksempel oksygenmetning, temperatur og atferds indikatorene avstand mellom fisk, kvalitativ score på trengingstetthet, svømmeaktivitet og vitalitet har gitt mest lovende resultat. Mange av de fysiologiske indikatorene som ble undersøkt viste signifikant korrelasjon med trengingsrelatert stress, dødelighet og filet kvalitet. Fysiologiske indikatorer ble likevel ikke inkludere blant de foreslåtte operative velferdsindikatorene. Dette på grunn av at innsamling av fysiologiske prøver krever erfaring og kan forstyrre kommersielle fiskeoperasjoner. I tillegg må prøvene ofte analyseres i laboratorie og det tar tid å få resultat. For forskningen er det likevel viktig og nyttig å måle fysiologisk stressrespons sammen med de andre indikatorene for å validere og støtte utvikling av operative velferdsindikatorer. Endring i fiskens hudfarge under stress har vist seg å være en veldig interessant mulig velferdsindikator for makrell.

Et bredt utvalg av mulige velferdsindikatorer ble undersøkt for å sikre at stress responsen til fisken blir korrekt karakterisert gjennom hele fangstprosessen, identifisere hvor og når kritiske stressnivåer oppstår, og for å kunne utvikle brukbare indikatorer som kan måle fangststatus ved disse kritiske punktene. Videre, gjennom å kombinere ulike velferdsindikatorer kan man få mer robuste og pålitelige svar på stressnivået eller velferden til fangsten.

For at velferdsindikatorene som er utviklet i dette prosjekt skal tilfredsstille kravene om relevans, brukervennlighet, pålitelighet og egnethet for godkjente operative velferdsindikatorer er det behov for videreutvikling. Det er spesielt behov for bedre forståelse av effekten høye fisketettheter og oksygenmangel har på individenes overlevelse, vitalitet og filet kvalitet og knytte disse opp mot andre potensielle velferdsindikatorene.

Kunnskapen som er generert i dette prosjekt er en begynnelse på utvikling av operative velferdsindikatorer for kommersielt fiske der det overordnede målet er å tilby målemetoder som kan brukes i ringnotfiske. Om disse blir fullt utviklet kan de bidra til å redusere dødelighet ved slipping og forbedre kvaliteten på fangsten i Norske ringnotfiskerier.

Summary

Good catch welfare depends on the development of indicators of stress that are appropriate and measurable in a real capture scenario (Anders et al., 2019; Breen et al., 2020). Establishing such operational welfare indicators will enable fishers to make informed, real-time decisions regarding fishing practices to minimize impacts upon the animals and thereby reduce the likelihood of negative outcomes. However, the measurement of aquatic animal health and welfare has been hampered by a lack of real- time field methods that are easy and inexpensive to use (Morgan and Iwama 1997; Huntingford et al., 2006).

The project “Catch control in seine fishing for pelagic species” (FHF 901350) has investigated how different catch-related stressors affect the fish's stress response, using more than thirty different metrics, in an attempt to identify practical and informative stress and/or welfare indicators (S/WIs). S/WIs that provide near real-time data and/or intuitively interpreted results, like oxygen concentration, temperature and the behavioural indicators (nearest neighbour distance (NND), crowding density score, swimming activity and vitality) show most promise as potential operational S/WIs. Many of the physiological stress indicators showed significant correlations with crowding stress, mortality and meat quality. However, they have not been identified as potential candidates as operational SWIs, because they generally require complex processing and laboratory analysis, so could be disruptive to fishing operations and would not provide information soon enough to be effective. From a research perspective, however, efforts should continue to measure physiological indicators, alongside other S/WIs, during commercial fishing operations to promote and support the development of operational S/Wis. One secondary level indicator, skin colour change, does show great potential as a S/WI for mackerel.

The rationale for this wide array of metrics has been to ensure we reliably characterise the stress responses throughout the capture process, identifying where and when critical stressors occur, as well as developing suitable indicators to monitor catch status at those critical points. By combining different stress/welfare indicators we can make more confident inferences about the stress/welfare status of the catch.

In all cases, these S/WIs will require further development to ensure they satisfy the informative (relevant, comparable, accurate & sensitive) and practical (usable, reliable & real-time) criteria for acceptable operational indicators. In particular, it will be necessary for future projects to substantiate the data on the effects of higher crowding densities and hypoxia on individual survival/vitality and quality parameters, and link these with other potential S/WIs.

The knowledge generated in this project has begun the development of pragmatic operational stress/welfare indicators, with the ultimate aim of eventually providing monitoring tools for everyday use in purse seine fisheries. If these are developed fully, they could be beneficial for promoting both the survival of the released unwanted catch and the quality of the retained catch from Norwegian purse seine fisheries.

Contents

1 Introduction

1.1 Background

The capture process in purse seines is stressful for the catch, because it crowds fish, which in turn can expose them to very low oxygen concentrations (hypoxia) and risk of traumatic injury due to contact with the net and other fish in the catch (Breen et al, 2020; Marçalo et al, 2019). We now have evidence to show that crowding stress can kill a proportion of any unwanted catch that may be released through slipping (Huse & Vold, 2010; Tenningen et al, 2012), as well as affect the quality of fillets from mackerel (Anders et al, 2019). By better understanding of how and when these stresses occur during the capture process, we hope to be able to improve both the quality of the catch retained, as well as the welfare and survival of the unwanted catch released from purse seine fisheries.

Good catch welfare depends on the development of indicators of stress that are appropriate and measurable in a real capture scenario (Anders *et al*., 2019; Breen *et al*., 2020). Establishing such operational welfare indicators will enable fishers to make informed, real-time decisions regarding fishing practices to minimize impacts upon the animals and thereby reduce the likelihood of negative outcomes.

The measurement of aquatic animal health and welfare has been hampered by a lack of real- time field methods that are easy and inexpensive to use (Morgan and Iwama 1997; Huntingford et al., 2006). The project “Catch control in pelagic purse seine fisheries” (FHF, 901350) addresses this lack of methodology in work package 5 with the main objective defined as:

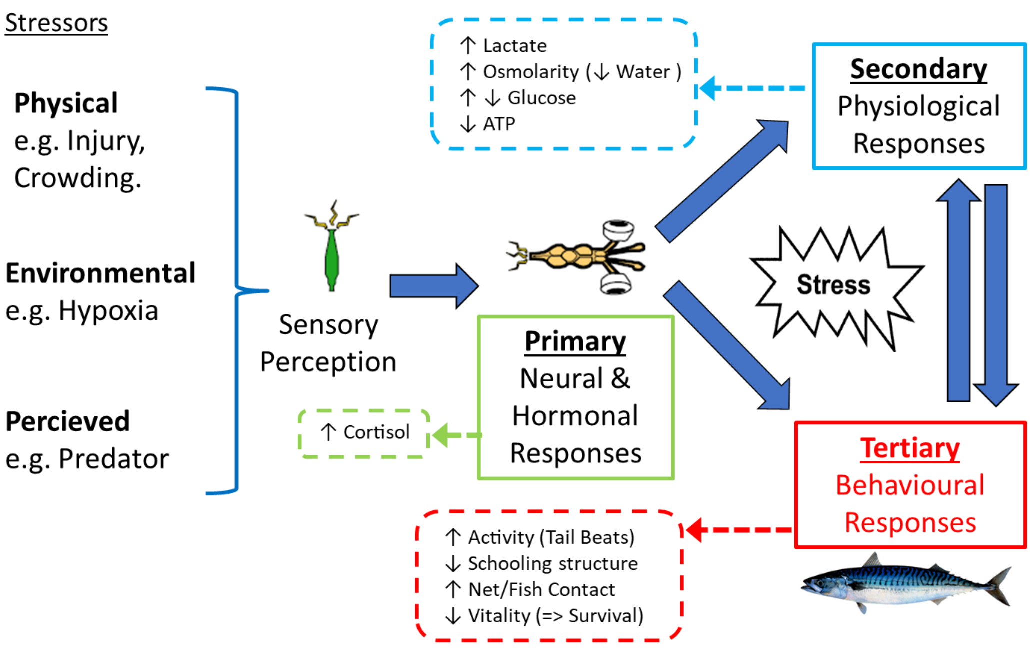
> *WP5 - Develop indicators of stress and potential survival in commercial purse seine fishing to help define safe limits for the release of unwanted catches.*

This report will describe how this work-package has tried to address this objective by systematically investigating more than thirty potential indicators of stress and/or welfare. It begins with an overview of the stress response in fish (Section 1.2). Then a functional definition of catch welfare is provided, along with our rationale for selecting potential indicators of stress/welfare status of catches in commercial purse seine fisheries (Section 1.3). An overview of our research activities is provided in section 2.0. Then section 3.0 provides a review of each candidate stress/welfare indicator, in terms of our current scientific knowledge and the evidence provided by this project. Where pertinent, this section will also discuss any practical and/or methodological challenges and limitations that were encountered during the project. Finally, an overview of the stress and welfare indicators is given in context to their potential as operational indicators, as well as how they could be combined to enable more confident inferences about the stress/welfare status of purse seine catches (Section 4.0).

1.2 Stress response in fish

Here we present only a brief summary of the key points about the stress response in fish, for more detailed explanations and background on this subject refer to Barton, 2002; Pottinger, 2007.

The stress response is the naturally occurring sum of all physiological and behavioural adaptations made in the face of a stressor (Barton, 2002; Pottinger, 2007; Wendelaar Bonga, 1997). Where a stressor is an environmental, physical, chemical or biological agent that threatens the well-being of an individual; or is perceived by that individual to be a threat. In the context of purse seine fishing several potential stressors have been identified, including: crowding, hypoxia (reduced oxygen), traumatic contact/injury, temperature change (in the retained catch), and predation (in the released catch) (Breen et al, 2020; Marçalo et al, 2019). The stress responses occur at different levels of biological organisation: Primary (neurological and hormonal; e.g. increased brain activity, adrenalin & cortisol release); Secondary (physiological & organ level; heart and breathing rate, blood chemistry) and Tertiary (whole animal level; e.g. behaviour & survival) (see figure 1). The increased energy requirements due to these responses is called the allostatic load (Ramsay & Woods, 2014). The magnitude and time-frame of the stress responses, and resultant allostatic load, vary depending on the magnitude of the response itself and the nature and duration of the stressor (Wendelaar Bonga, 1997). Stress responses, and the ability to cope with them, will also vary between individuals and within an individual, depending on their health/nutritional/sexual status, time of day, presence of other stressors, etc. (Pottinger, 2007). If the allostatic load is too high or too prolonged, the individual may not have the capacity to cope and its welfare/well-being is compromised, and ultimately it may die as a direct result.



Adapted from Barton, 2002 and Horodysky et al, 2015

Figure 1 – an overview of the multi-level general stress response in fish.

1.2.1 Physiological stress responses & indicators.

The general physiological response of fish to acute stress is well understood (and is reviewed in depth by Wendelaar Bonga, 1997). In brief, upon detection of a stressor by the central nervous system, catecholamine (mostly epinephrine/adrenaline) and corticosteroid (mostly cortisol) stress hormones are released into the blood stream. These hormones induce cardiorespiratory or metabolic alterations in their target organs to prepare the fish for a stress, i.e. flight, fight or coping, response by enhancing oxygen and energy availability. In acute scenarios, the magnitude of physiological responses can often reflect the magnitude of stress received (Wendelaar Bonga, 1997). Physiological indicators have therefore been used to inform about the welfare of a range of fish species in response to various aquaculture and fisheries procedures for several decades (e.g. Swift, 1983; Olla et al., 1998; Foss et al., 2009; Lerfall et al., 2015; McLean et al., 2020).

Physiological stress indicators are often assessed by collecting whole blood or plasma using caudal vasculature puncture followed by laboratory analysis. Sometimes, other tissues such as muscle are sampled (Sopinka et al., 2016). Recent advances in portable technology means that some indicators can be measured immediately in the field using point of care (POC) devices, providing that such instruments are calibrated and/or validated for use on the study species (Stoot et al., 2014).

Gathering an accurate picture of the full physiological response can be challenging to achieve, as not all indicators manifest and/or develop on the same timescale. Furthermore, appropriate sampling protocols are required to ensure measurements are not unduly affected by observation and sample storage effects. As our understanding of mackerel physiology grows, it may be possible to assign threshold values to welfare indicators to indicate what can be considered good or compromised welfare. However, until that understanding is achieved, the natural variability of welfare indicators in response to factors other than stress means that complimentary measurements from unstressed (control) fish are required to place any welfare inferences in better context (Pottinger, 2007).

Section 3.2 presents the main physiological indicators examined during the project and highlights those best suited to characterizing mackerel welfare.

1.2.2 Pre-mortem stress effects on post-mortem flesh quality

It is intuitive to many that the post-mortem flesh quality of animals is determined to some degree by their pre-mortem treatment. Where handling induces a physiological stress response and the time available before slaughter is inadequate to allow homeostatic recovery, undesirable flesh quality alterations may result (Poli et al., 2005). This connection has been demonstrated for a range of different fish species from several different scenarios (e.g. Hattula et al., 1995; Stien et al., 2005; Rotabakk et al., 2011; Lerfall et al., 2015). Flesh quality can be therefore be considered as a retrospective indicator of fish welfare status immediately prior to and at the point of death.

Some of the causal mechanisms behind flesh quality reduction arising from stress in fish are outlined in Figure 2. In summary, where pre-mortem stress results in intense behavioural activity, acidosis, redistribution of blood to tissues and a reduction in intracellular adenosine triphosphate (ATP) occurs. These changes then act via a variety of interacting post-mortem physical, autolytic and metabolic pathways to influence important physical attributes of fish flesh such as colour, texture and gaping (the undesirable separation of muscle myotomes).

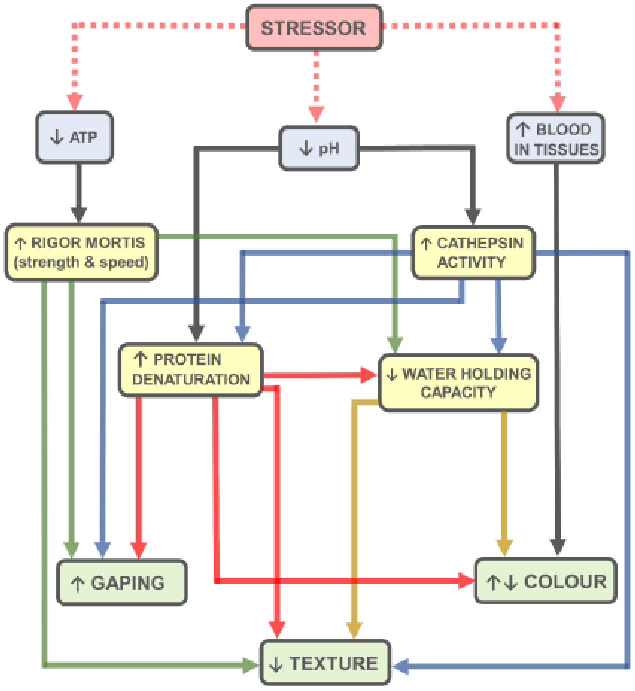


Figure 2: Physical flesh quality consequences of pre-mortem stress in fish. *Stressor/s (red box) first act to directly induce pre-mortem physiological changes (blue boxes). These stress responses then act via a variety of interacting physical, autolytic and metabolic pathways and processes (yellow boxes) to influence post-mortem physical flesh quality parameters (green boxes). Note that the figure contains just the main pathways; others exist but have relatively minor effects. Figure taken from Anders (2020).*

Previous studies conducted in Japan indicated that other members of the Scomber genius were susceptible to stress-induced reductions in quality (Mochizuki & Sato, 1996; Sato et al., 2002; Tamotsu et al., 2012; Ogata et al., 2016; Miyazaki et al., 2018), but it was unclear prior to this project as to the extent to which Atlantic mackerel were affected. Furthermore, there had been little work into the physiological basis of these quality reductions.

Section 3.2.8 presents the main flesh quality measures examined during the project, highlighting which have the greatest potential for characterizing pre-mortem mackerel welfare.

1.2.3 Behaviour as an indicator of stress.

As well as neuro-endocrine and physiological responses, animals also exhibit behavioural change in response to stressors. The purpose of such change is to mitigate an undesirable increase in allostatic load by reducing stressor exposure and intensity (Schreck *et al.*, 1997). Behavioural metrics are widely employed as acute stress indicators in fish, particularly in aquaculture scenarios (Martins *et al.*, 2012). This is because they are often the first observable expression of the stress response (Huntingford *et al.*, 2006) and can be more sensitive indicators of welfare than isolated physiological measures (Sopinka *et al.*, 2016). They are also easy to observe in a non-intrusive and non-invasive manner (Dawkins *et al.*, 2004). For pelagic species, like mackerel (*Scomber scombrus*) and herring (*Clupea harengus*), schooling behaviour is an integral part of their natural behaviour and survival strategy (Breder, 1967; Pitcher & Parrish, 1993). Therefore, it is informative to observe a suite of both collective, schooling behaviours (e.g. nearest neighbour distance and schooling order), as well as individual level behaviours (e.g. swimming activity, reflex responses and vitality), when attempting to assess the welfare of the catch during the capture process. The potential for such behavioural stress and welfare indicators will be explored in more detail in section 3.3.

Mortality, in response to a stressor, is arguably the ultimate expression of “bad welfare” for an affected animal and may indicate near-lethal conditions for the surviving individuals (Breen et al, 2020). There are many studies that have investigated the mortality different species released from commercial fishing operations, which now provide an important insight into the welfare status of animals captured using different fishing methods under a wide range of conditions (for reviews see: Broadhurst et al., 2006; Suuronen, 2005; Veldhuizen, 2017; Veldhuizen et al, 2018; Breen and Catchpole, in press). Key observations from this work include: smaller animals are typically more likely to die; and mortality generally increases with increasing exposure to the capture method (i.e. haul duration, soak times, etc) and emersion (i.e. sorting times and air exposure) (Veldhuizen, 2017; Veldhuizen et al, 2018).

Moreover, several studies have shown that crowding during simulated slipping experiments can cause mortality in herring (Tenningen et al. 2012), sardine (*Sardina pilchardus*) (Marçalo et al. 2006; Marçalo et al. 2010) and particularly mackerel ((Lockwood et al. 1983; Huse and Vold 2010). However, efforts to identify the causal mechanisms underlying these mortalities remain confounded (Anders, 2020; Marcalo et al, 2019). From a set of related studies (Pawson & Lockwood, 1980; Holeton et al., 1982; Lockwood et al., 1983; Swift, 1983) it was concluded that skin damage arising from abrasive contact with the net and other fish was likely to be the major cause of mortality in slipped mackerel. However, the validity of these conclusions, with respect to their relevance to crowding in commercial fisheries, has been questioned. Anders (2020) highlights several issues that undermine their applicability to commercial scale crowding events, including: these studies often employed relatively short post-stressor monitoring times (not more than ~5 days, Pawson & Lockwood, 1980; Lockwood et al., 1983), so likely did not observe the complete stressor related mortality (Breen & Catchpole, in press); they experimented on fish which were likely to be highly stressed as a result of capture and transfer procedures (Pawson & Lockwood, 1980; Holeton et al., 1982; Swift, 1983); and did not correlate physical damage with physiological explanations (Lockwood et al., 1983). Furthermore, they used relatively small biomasses (< 250 kg), so were unlikely to have induced any significant hypoxia in the treatments (and dissolved oxygen was not monitored); thus excluding an important likely stressor in full scale commercial crowding events (Breen et al, 2020; Marçalo et al, 2019) (see section 3.1.2 for further discussion).

Most fish in a purse seine catch are still alive when they are transferred (usually by pump) from the net into the refrigerated seawater (RSW) holding tanks (Digre et al, 2016). As such, they will experience a rapid reduction in ambient temperature, which is likely to induce “temperature shock” (e.g. Donaldson et al., 2008; Gale et al., 2013; see section 3.1.3 for further discussion). This form of slaughter method has been criticised as particularly stressful for temperate fish species, which may affect the quality of the resulting meat products (Poli et al, 2005). Furthermore, anecdotal evidence suggests that the death from “temperature shock” is not rapid (i.e. < 1 sec)(Breen, pers. obs.) and so contravenes current best practice guidelines (European Food Safety Authority, 2013) and, arguably, national animal welfare legislation (Anon, 2009).

Finally, as an informative and timely welfare indicator, mortality is not ideal because it has a low resolution (i.e. binary: alive or dead) and its appearance in a stressed population is likely to indicate that the opportunity for mitigating intervention to improve welfare is already too late. However, assessments of the impairment of reflexes/behaviours, as well as the occurrence of injuries, have been demonstrated to be good predictors of mortality (e.g. Davis, 2010, Benoît et al., 2010; Humborstad et al,. 2016b), and are now being used to systematically assess the vitality of released animals (Breen and

Catchpole, in press). As such, these vitality metrics could prove to be informative indicators of the stress and welfare status of fish, which is discussed in more detail in section 3.3.4.

1.3 Rationale for selecting stress/welfare indicators

Before discussing which potential indicators of stress and welfare should be assessed and considered for monitoring the status of the catch in commercial purse seine fisheries, it will be beneficial to define a baseline against which the status of the catch can be compared. We will refer to this as "Good catch welfare".

1.3.1 A "Functional definition of good catch welfare"

From our understanding of the stress response in fish to the capture process, we have developed a strategy for developing and promoting "responsible" (sustainable and welfare conscious) fishing practices. This is based on a "functional approach to good catch welfare" (Breen et al, 2020), from which we hypothesise that good "catch/welfare status" results from: "capture and handling practices that minimise the physical damage to, and allostatic load upon, any retained fish until the moment they are slaughtered or released, and thus promote the likelihood for post-release survival and/or good meat quality in retained fish".

1.3.2 What is a stress / welfare indicator?

All of the potential indicators discussed in this report have been selected for investigation because they describe some aspect of the stress process (i.e. the relationship between stressor and stress responses) likely experienced by fish during their capture in, and possibly release from, purse seines. This includes trying to measure some of the stressors directly (e.g. crowding density and hypoxia), as well as observing some of the responses (anatomical, physiological and behavioural) to those stressors. As such, these are "stress indicators", showing on some scale how stressed the animal is in comparison to some baseline (or control) level.

A stress indicator can be referred to as a "welfare indicator" only when it can be placed in context with the fishes' welfare status. That is, according to our functional definition of catch welfare, when a stress indicator can be directly related to either the potential survival of the animal and/or its possible effect on meat quality. To this end, we conducted a series of controlled experiments at the IMR facilities at Austevoll to investigate the effects of crowding stress on an array of stress indicators, including survival, vitality and several meat quality metrics.

1.3.3 Defining and Selecting Informative and Practical Stress / Welfare Indicators.

Finally, inspired by Noble et al (2018), we have identified several criteria for selecting appropriate indicators. For a stress or welfare indicator (S/WI) to be of benefit in assessing the stress and/or welfare status of fish in the catch it should be both "informative" and "practical".

An "Informative" indicator should be:

> **Relevant** - reflect parameters that are relevant to the capture related stress and which are likely to change during the capture process.

Comparable – quantifiable, with sufficient precision/resolution to enable reference to some baseline and/or welfare related threshold.

Accurate – consistent/repeatable and unbiased measure of the stressor and/or stress response across full range of likely values.

Sensitive – respond quickly to relatively small changes in the stressor and/or stress response to enable mitigation measures to be enacted soon enough to benefit the welfare of the catch.

A "Practical" indicator should be:

Useable in a commercial fishery setting – i.e. not so large or technically challenging that it will disrupt fishing operations.

Reliable – robust enough to be applied in the challenging operating conditions of a commercial fishery.

Real-time – provide real-time measures of the stress indicator.

For a S/WI to be of interest in this project, it must first be informative as a stress indicator, with the potential to be further tested and related to welfare related thresholds. The practicality of an informative indicator can then be developed for application in a commercial fishery context, with the ultimate aim of enabling the development of operational welfare indicators (OWIs)(c.f. Noble et al, 2018).

1.3.4 A "Toolbox" of Multiple Stress/Welfare Indicators

To address the variability associated with the stress response, it is advisable to have multiple stress metrics/indicators, selected from several levels of the stressor/response interaction (figure 1) (Wedemeyer et al, 1990). To this end we have been investigating stress indicators at all levels of the stressor/response interaction:

- Stressor level indicators
  - Crowding Density (section 3.1.1)
  - Dissolved Oxygen & Hypoxia (section 3.1.2)
  - Temperature (section 3.1.3)
- Primary & Secondary level indicators
  - Pre-mortem physiology (section 3.2.1)
  - Skin colour change (section 3.2.2)
  - Post-mortem physiology and meat quality (section 3.2.3)
- Tertiary level indicators
  - Nearest neighbour distance (NND) (section 3.3.1)
  - Schooling order (section 3.3.2)
  - Swimming activity & Tail-beat Frequency (TBF) (section 3.3.3)
  - Vitality, Injuries & Mortality (section 3.3.4)

By adopting this selection strategy, we will be less reliant on a single, potentially erroneous, indicator and therefore less likely to make false inferences about the presence or absence of a stress response and/or indication of poor welfare.

2 Overview of activities

To identify potential indicators for monitoring catch status during purse seining a series of experiments were conducted at the IMR facilities at Austevoll (section 2.1). In addition, to assess the applicability of selected indicators, and to begin to collect example data, direct observations have also been made during commercial fishing operations in both mackerel and herring fisheries (section 2.2).

2.1 Controlled experiments at Austevoll

The response of mackerel to crowding stress was determined in a series of controlled experiments, conducted in 2019 at the Austevoll Research Station of the Institute of Marine Research. These experiments built on methods developed as part of the NFR funded RedSlip project (see Handegard et al, 2017 and Anders et al, 2019 for details).

The first experiments (conducted in January/February 2019) examined the relationship between stress, physiology and flesh quality. For this, small groups of mackerel (consisting of ≤ 20 individuals) were established in one of two aquarium tanks or in an aquaculture net pen. From each group, individuals were randomly collected and sampled for blood physiology and flesh quality to provide unstressed, baseline values. The remaining fish were then subject to crowding, by placing them in a section of purse seine netting and lifting the net within the water to reduce their available swimming space. These crowded fish were then sampled to determine the effect on their physiology and flesh quality, in comparison to the individuals collected before crowding. Blood samples were collected from the caudal vasculature using heparinised syringes, and then either analysed on site or spun in a centrifuge to produce plasma for later laboratory analysis. Flesh quality sampling took place immediately for some metrics while others were determined in the laboratory after two or seven days of storage on ice. These experiments are described in detail in Anders et al. (2020) and their results reviewed in section 3.2.8.

Table 1 - Summary of crowding densities (kg/m^3 and n/m^3) for control and treatment replicates in the Austevoll crowding experiments (May-June & Aug-Sep 2019).

		Cage Volume		**Crowding Density**			
Treatment		Treatment	Total	Treatment		Total (Pre-treatment)	
Replicate Name	Date	m^3	m^3	(kg/m^3)	(n/m^3)	(kg/m^3)	(n/m^3)
Phase I							
Control 1A	21-May-19	149.17	149.17	0.76	1.00	0.76	1.00
Control 1B	28-May-19	149.17	149.17	0.59	0.78	0.61	0.81
Crowded 1A (High/prolonged)	22-May-19	NA	149.17	NA	NA	0.41	0.52
Crowded 1B (Low)	29-May-19	1.88	149.17	92.00	122.70	1.19	1.58
Crowded 1C (High/prolonged)	06-Jun-19	0.37	149.17	182.75	246.54	0.45	0.61
Phase II							
Control 2	21-Aug-19	149.17	149.17	0.76	1.17	0.78	1.21
Crowded 2A (Mod)	22-Aug-19	0.58	149.17	146.21	226.71	0.59	0.91
Crowded 2B (High)	28-Aug-19	0.51	149.17	179.87	293.59	0.64	1.04

The second set of experiments (conducted May/June and August/September 2019) investigated the relationship between stress, physiology and behaviour, and survival. Eight large groups of mackerel (each consisting of ~150 individuals) were housed in aquaculture net pens and received either crowding (by reducing the available swimming volume for 15 minutes) or no treatment (as the control), with subsequent monitoring for up to 28 days to determine survival rates. Due to methodological limitations, the induced density during crowding differed between the different trials (Table 1). Also, due to the severity of the prolonged high crowding treatments (Crowded 1A & 1C), these replicates were terminated immediately following the treatments, so no post-treatment monitoring was conducted. Video recordings of the fish schools took place prior to, during and after treatment to determine behavioural responses (in terms of swimming activity) to and recovery from the stressor (section 3.3.3). Samples of fish were removed at pre-determined intervals (pre-treatment, treatment, 2-hours and 24-hours post-treatment and pre-termination) from which vitality observations were recorded (section 3.3.4) and physiological samples taken (section 3.2) (Table 2). Sampled fish were also photographed to investigate possible stress induced changes to skin colour and had blood collected from the caudal vasculature (see section 3.2.7). These experiments are described in further detail in Anders et al. (n.d).

Table 2 - Summary of biometrics (means, 95% confidence intervals and sample sizes) for control and treatments replicates in the Austevoll crowding experiments (May-June & Aug-Sep 2019).

	Number of Fish			**Biometrics (Mean ± 95% CI)**		
	All	Treatment	Measured	Length (cm)	Weight (g)	Condition (100.g.cm^{-3})
Phase_I	**675**	**665**	**668**	**37.76 ± 0.19**	**756.02 ± 12.06**	**1.38 ± 0.01**
Control_1A	149	149	142	37.98 ± 0.39	764.68 ± 25.13	1.38 ± 0.02
Control_1B	121	116	121	37.87 ± 0.43	755.67 ± 27.70	1.38 ± 0.02
Crowded_1A	78	78	76	38.13 ± 0.63	777.03 ± 36.81	1.34 ± 0.03
Crowded_1B	236	231	236	37.63 ± 0.35	749.92 ± 21.12	1.39 ± 0.02
Crowded_1C	91	91	91	37.52 ± 0.51	741.23 ± 33.18	1.39 ± 0.03
Phase_II	**471**	**456**	**469**	**38.1 ± 0.2**	**634.55 ± 10.84**	**1.14 ± 0.01**
Control_2	180	175	179	38.25 ± 0.33	645.43 ± 18.35	1.14 ± 0.02
Crowded_2A	136	131	135	38.15 ± 0.38	643.93 ± 19.08	1.15 ± 0.02
Survivors	*133*	*128*	*132*	*38.14 ± 0.39*	*645.11 ± 19.30*	*1.16 ± 0.02*
Morts	*3*	*3*	*3*	*38.67 ± 7.59*	*591.67 ± 301.82*	*1.02 ± 0.15*
Crowded_2B	155	150	155	37.87 ± 0.35	613.81 ± 18.69	1.12 ± 0.02
Survivors	*115*	*110*	*115*	*37.94 ± 0.40*	*630.56 ± 21.58*	*1.15 ± 0.02*
Morts & Moribunds	*40*	*40*	*40*	*37.68 ± 0.72*	*565.65 ± 34.54*	*1.05 ± 0.03*
Grand Total	**1146**	**1121**	**1137**	**37.91 ± 0.14**	**705.82 ± 9.06**	**1.28 ± 0.01**

2.2 In commercial fisheries

A total of eight research cruises have been conducted during this project on which technologies and methods for monitoring stress/welfare indicators have been investigated (Table 1). These all used commercial fishing vessels in either mackerel (n = 5) or herring (NS: 3 & NVG: 1) fisheries. In total, 35 landed catches and 6 slipped catches have been monitored.

Table 3 - Summary of research cruise aboard commercial fishing vessels used to investigate technologies and methods for monitoring stress/welfare indicators.

Crusie Details				**Casts**			**CMP Deployments**				**Vitality Assessments**		
#	Vessel	Species	Date	All	Land	Slip	Net	Pump	RSW	Geil	Pump	RSW	Lactate
1	Fiskebas	Herring (NS)	Jun-17	4	2	2	2	NA	NA	2	NA	NA	NA
2	Fiskebas	Mackerel	Oct-17	4	2	1	3	NA	NA	1	NA	NA	NA
3	Eros	Herring (NS)	Jun-18	10	7	0	7	7	2	0	3	2	NA
4	Christina E	Mackerel	Sep-18	2	2	0	2	2	2	0	2	2	NA
5	Vendla	Herring (NS)	Jun-19	12	7	2	7	7	6	2	7	4	NA
6	Fiskebas	Mackerel	Sep-19	13	5	1	5	5	5	1	5	5	Y
7	Fiskebas	Mackerel	Sep-20	9	5	0	5	5	3	0	5	3	Y
8a	Fiskebas	Mackerel	Oct-20	4	3	0	4	3	2	0	3	2	Y
8b	Fiskebas	Herring (NVG)	Oct-20	2	2	0	0	2	1	0	2	1	Y

As part of WP4 in this project, we have been developing a suite of new technologies for monitoring S/WIs at different stages of the capture process (in the net, during pumping and in the RSW tank). The main tool in this suite if the Catch Monitoring Probe (CMP), incorporating a Nikon 360 Camera and RINKO ID oxygen, temperature & depth logger; in a shock proof housing (to protect, support and stabilise the instruments during deployment and operation). This has systematically been used to collect data on behavioural responses in relation to the stressors: crowding and hypoxia. When used in the net, the CMP is deployed using a pneumatic canon and comprises: a shock proof housing (to protect, support and stabilise the instruments during deployment and operation); a Sony 360 Camera (for complete contextual views around the probe); and RINKO ID oxygen, temperature & depth logger (figure 3).

Figure 3: Catch monitoring probe (CMP) in protective housing (right) prepared for deployment from a compressed air canon (left).

When used on the pump, a simpler version of the monitoring probe (containing; a RINKO ID oxygen, temperature & depth logger; and GoPro 4 camera) was attached to the vessel's catch pump, to monitor the catch during pumping; where it would be too hazardous to deploy the CMP. In the RSW tank, a RINKO ID oxygen, temperature & depth logger or a SAIV Conductivity, Temperature, Depth and Oxygen (CTDO) logger, with GoPro 4 camera attached, were lowered into the tank just prior to pumping and remained in the tank for up to 24 hours.

During slipping, two camera units (GoPro 4 with additional battery pack inside a floating housing) are also fitted to the discharge opening to observe the behaviour of any fish released through the opening (figure ##). The horizontal camera (camera #1) was positioned 5m from the "øret" looking across the opening, while the vertical camera (camera #1; looking up) was fitted in several positions. In addition, two drop cameras (vertical and horizontal) were deployed on a SAIV CTDO outside the discharge opening ("geil") to a depth of 10-25m.

As well as monitoring the behaviour of fish in the catch, on these cruises we also used a suite of behaviours/reflexes to monitor the "vitality" of individual fish sampled from the catch after pumping from the net and for sub-samples taken from the RSW tanks at various times after pumping. In addition to vitality metrics, in later cruises, a sub-sample of fish had blood samples taken (via caudal puncture), which were analysed on site for blood lactate using the Lactate Pro 2 (Arkray Inc., Kyoto, Japan) point-of-care (POC) analyser.

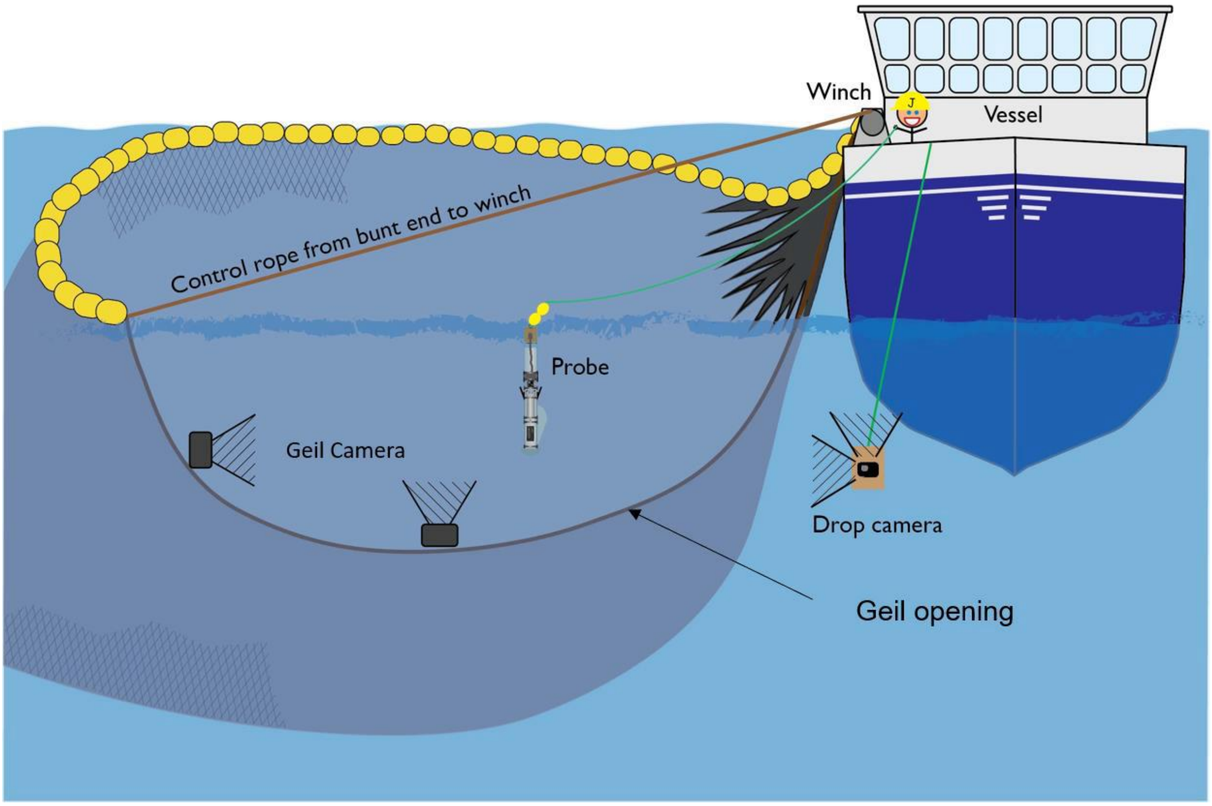


Figure 4 – an overview of the instruments used to monitor stress / welfare indicators (e.g. crowding density, dissolved oxygen, swimming activity and schooling order) during capture and slipping in purse seines.

3 Review of Potential Stress / Welfare Indicators

3.1 Stressor Level Metrics

3.1.1 Crowding Density

Excessively high crowding density is thought to be the primary stressor during purse seine fishing (Breen et al, 2020), particularly during the latter stages of the fishing operation where crowding densities of >250 kg/m3 have been estimated (Tenningen et al, 2012). Moreover, high crowding densities are likely to directly induce the potentially fatal stressors of hypoxia, exhaustion and abrasive contact/injury (see sections 3.1.1, 3.3.3 and 3.3.4 respectively, for further discussion) (Breen et al, 2020; Marçalo et al, 2019). Indeed, in experiments simulating the release of pelagic fish from purse seines, high crowding density has been shown to induce mortality in several species, including mackerel (Lockwood et al. 1983; Huse and Vold 2010), herring (Tenningen et al. 2012), sardine (Marçalo et al. 2006; Marçalo et al. 2010) and sardinops (*Sardinops sagax*) (Mitchell et al. 2002). Therefore, to be able directly or indirectly measure and control crowding density would be a major advantage in avoiding poor welfare during capture.

In principle, the concept of measuring crowding density is simple. It is the biomass (kg) of fish confined within a specified volume (m^3). Several studies, including the cage experiments at Austevol, have therefore used a simple dead-reckoning approach to estimate crowding density by recording the total number and weight of fish in each crowding treatment, and estimating the volume during treatment geometrically (Anders et al, n.d.; Ochoa, 2020, Tenningen et al, 2012). From the estimates from Austevoll, a clear relationship between crowding density during the treatments and mortality during the subsequent monitoring period (of between 5 and 14 days) could be seen (figure 5). At crowding densities less than 100 kg/m^3 no significant mortality was observed in this project (Anders et al, n.d.) and in the earlier RedSlip Project (Handegard et al, 2017).

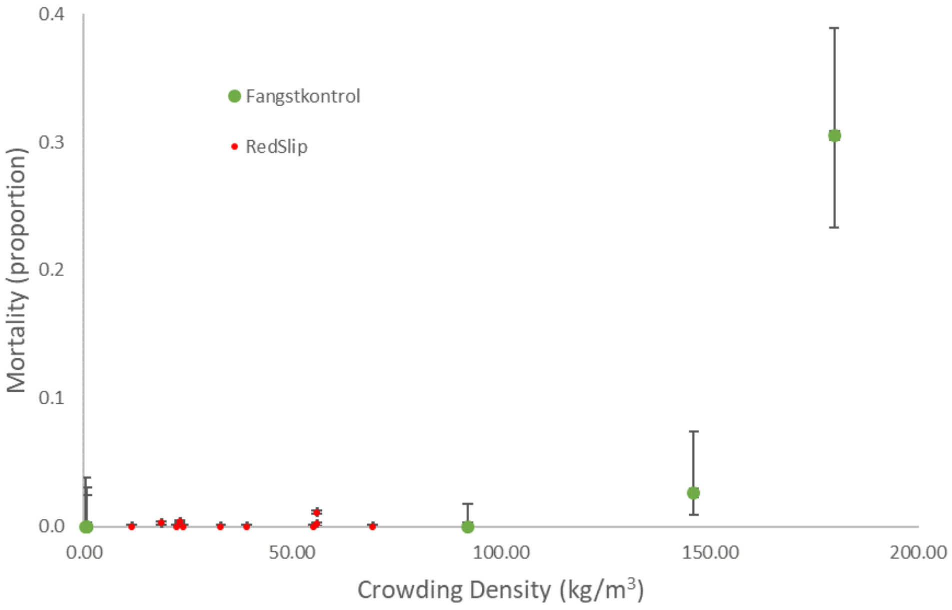


Figure 5: relationship between crowding density and subsequent mortality. *Fangstkontrol project used "Dead Reckoning" to estimate crowding density, while RedSlip estimated density during treatments indirectly using a calibrated echosounder (see main text for details).*

However, this dead-reckoning approach is impractical for obtaining estimates of crowding density in real-time during commercial fishing operations. Firstly, accurately estimating the constraining volume of the net is challenging, because its shape is constantly changing throughout the capture process, particularly during hauling (Tenningen et al, 2015, 2019). Secondly, if this S/WI is going to function in real-time, the estimate of catch biomass needs to be known during crowding, so cannot rely on estimates of catch biomass later loaded onto the vessel.

Hydro-acoustics has been proposed as a potential solution to these challenges. Tenningen et al. (2015, 2019) used sonar, in commercial fisheries, to detect the net boundary as a sequential series of cross-sections throughout the hauling process. These were used to reconstruct the three-dimensional shape and volume of the net over time, and relate these to known and theoretical catch biomasses. They concluded that, based on the assumption that fish contained within the net use the whole available volume, critically high crowding densities would only be seen in the largest of reported catches for mackerel (650-985t) when 80% of the net was hauled. However, beyond 80% hauling of the net no reliable estimates of net volume of are available, because of poor acoustic data due primarily to interference from bubble noise. Furthermore, they questioned their own assumption of volume dependent crowding densities, at least in the early stages of capture. For example, Tenningen et al (2017) observed that early in the capture process school density is independent of net volume, with schools generally occupying smaller volumes than available within the net.

Acoustic methods can be used to obtain quantitative estimates of fish density, if the acoustic equipment (echo-sounder or sonar) is calibrated and the target strength (TS) for the particular species is known (Simmonds and MacLennan, 2008). This approach was used to measure crowding density in low density crowding experiments for mackerel during the RedSlip project (Handegard et al, 2017) (figure 5). Even without TS values, relative estimates of acoustic density can be obtained using changes in volume backscattering strength (Rieucau et al., 2014). However, estimating fish density inside a purse seine is challenging because it is difficult to deploy monitoring instruments into the seine. Vessel mounted transducers are sub-optimal because of interference due to bubbles and net between instrument and fish. In addition, target strength of fish varies greatly with lateral incidence angle making sideways estimates with fishery sonars very difficult unless the orientation of the fish relative to the sonar is known. In this and previous projects several attempts have been made to overcome these challenges by deploying instruments directly into the net, e.g. flying drones with echosounder and floating acoustic probes.

In addition, density estimation by acoustic methods is confounded by the effects of acoustic attenuation and occlusion (Appenzeller & Leggett, 1992; Davies, 1973; Røttingen, 1976). While methods are available to correct for biases caused by attenuation (e.g. Foote, 1990; Zhao & Ona, 2002), these cannot address the complete occlusion of acoustic signal at the high densities (>100 kg/m^3) where the welfare of fish may be affected.

Alternative optical methods for inferring school densities from behavioural observations are available and discussed in more detail in section 3.3.1: nearest neighbour distance. However, these methods are also affected by analogous occlusion problems at high densities. Moreover, they are constrained to relatively near-field observations, depending on water turbidity and natural light levels.

In conclusion, experimental data has begun to provide some information about safe crowding thresholds for mackerel (in captive conditions), where densities less than 100kg/m^3 induce no significant mortality in the affected population. However, more information is required to better understand the effect of higher densities on mortality, and other stress/welfare metrics. Furthermore, despite the importance of crowding density as a potential S/WI, a direct method for obtaining informative and practical estimates at welfare impacting crowding densities in commercial fisheries remains elusive.

3.1.2 Dissolved Oxygen Concentration and Hypoxia

Oxygen is essential for survival in all aerobic organisms, including fish (Hughes, 1973). Hypoxia is when ambient dissolved oxygen concentrations are so low that they compromise the physiological function of a fish (Richards et al, 2009). If prolonged, hypoxia will cause critical biological systems to fail and eventually the animal's death (Hughes, 1973; Richards et al, 2009). Hypoxia is defined as oxygen concentrations below 6 mg/l (~53% saturation @ 10^{o}C) (Painting et al. 2005). During fish capture, hypoxia can result from: low oxygen (hypoxic) concentrations in the ambient water-mass; a reduced functionality in the animal's ability to breath (asphyxia) due to injury or constriction; and/or complete emersion from the animal's breathing medium (Breen et al, 2020). Hypoxic conditions have been observed in purse seine catches (Marçalo et al, 2019), and the rate at which these hypoxic conditions develop will likely depend upon the biomass of fish in the water mass (i.e. crowding density) and their activity, as well as on physical parameters (i.e. temperature, pressure, salinity and water movement).

In surface seawater, dissolved oxygen (DO) is usually in equilibrium with the overlying air mass and so will be around the maximum (100%) saturation level (Riley & Chester, 1971). However, oxygen solubility in seawater is highly dependent on temperature and pressure (both atmospheric and particularly hydrostatic) (figure 6), as well as salinity (seawater holding typically 20% less DO than freshwater) (Riley & Chester, 1971). For convenient comparison of DO concentrations from different water masses (hence different temperatures, depth and salinity) DO concentrations are often quoted as a percentage of the theoretical maximum saturation level for its source water mass. However, from a biological perspective, it is more informative to quote values in absolute units (mg/l or mg.dm^{-3}).

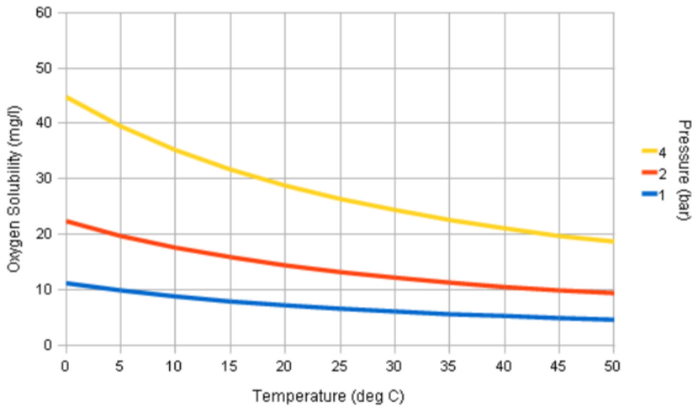


Figure 6 - Relationship between maximum oxygen solubility in seawater (salinity = 35$^{o}/_{oo}$) and temperature and hydrostatic pressure. [source: www.engineeringtoolbox.com]

Oxygen requirements and hypoxia tolerance varies between species, and is modulated by several environmental variables, in particular temperature (Domenici et al., 2013, 2017; Rogers et al., 2016). For example, Johnstone et al (1993) observed mean minimum respiration rates for captivity-acclimatised mackerel (BL = 29-38cm) swimming in 11.1° C at 0.6 B.L. s^{-1} were 1.97 mgO$_2$ kg^{-1} min^{-1}; and for herring (BL = 25.5-31cm): 1.55 mgO$_2$ kg^{-1} min^{-1}, at 9.3° C and a swimming speed of 0.3 B.L. s^{-1}. Oxygen requirements are also strongly dependent on the activity level of the fish (Claireaux et al., 2000; Hvas et al., 2017). Based on swimming metabolism data for chub mackerel (*Scomber* *japonicus*)(Guo et al, 2020), it is estimated that for mackerel in the Norwegian Sea at typical school swimming speeds of 1.0m.s^{-1} (2.9BL.s^{-1} for a 35cm fish)(Nøttestad et al, 2015) the oxygen consumption rate would be ~15 mgO_2 kg^{-1} min^{-1}. If swimming at higher speeds to escape a stressor, this rate would be exponentially higher.

Mackerel are thought to be particularly sensitive to hypoxia (Pawson et al, 1980), although recent observations have shown that mackerel can tolerate relatively low oxygen concentrations (~40% saturation) with no fatal effects (Handegard et al, 2017; Anders et al, 2019b). Hypoxia has been observed to induce increases swimming activity in mackerel at reduced oxygen saturations (40-75% saturation) (Anders et al, 2019b). Increases in swimming speed have also been seen in herring at oxygen saturations less than ~35% (Domenici et al, 2002, Herbert & Steffensen, 2006). Mackerel's apparent increased sensitivity to hypoxia is likely due to its oxyphilic nature; with a high red muscle content (Gillerman, 1980) and a highly active, fast swimming behaviour (Wardle & He, 1988, He & Wardle, 1988). Conversely, herring are known to tolerate prolonged periods of hypoxia whilst overwintering in Norwegian fjords [Dommasnes et al, 1994].

Dissolved oxygen concentrations were monitored during the crowding experiments in Austevoll (table 4) using a SAIV CTD(O), fitted with a RINKO III optical oxygen meter. Significant reductions were seen during the crowding treatments, in comparison to the controls and pre-treatment levels (not shown), but only treatments 2A and 2B could be defined as hypoxic (<6mg/l).

Table 4 - Dissolved Oxygen and Temperature (mean & 95% confidence intervals) for treatment phases of the crowding stress experiments on mackerel at Austevoll. Highlighted data were from measurements taken just after the treatment phase for Control 1B.

		CTDO Data During Treatment				
Replicate Name	**Date**	**Temperature**	**Oxygen Concentration**		**Oxygen min**	
			(mg/l)	% saturation	(mg/l)	% saturation
Phase I						
Control 1A	21-May-19	11.608 ± 0.007	10.247 ± 0.004	114.210 ± 0.045	9.26	99.83
Control 1B	28-May-19	10.991 ± 0.003	9.893 ± 0.002	110.115 ± 0.041	9.81	109.95
Control 1C (Crowd_1C PreTreat))	06-Jun-19	10.799 ± 0.001	9.616 ± 0.001	106.049 ± 0.006	9.6	105.82
Crowded 1A (High/prolonged)	22-May-19	12.257 ± 0.009	9.339 ± 0.012	86.563 ± 0.112	8.71	81.15
Crowded 1B (Low)	29-May-19	NA	NA	NA	NA	NA
Crowded 1C (High/prolonged)	06-Jun-19	13.880 ± 0.024	9.080 ± 0.016	87.409 ± 0.136	7.39	72.91
Phase II						
Control 2	21-Aug-19	16.425 ± 0.001	8.386 ± 0.001	99.553 ± 0.007	8.37	99.39
Control 2B (Crowd_2B PreTreat)	28-Aug-19	17.156 ± 0.002	8.264 ± 0.005	100.754 ± 0.030	8.19	99.72
Crowded 2A (Mod)	22-Aug-19	16.067 ± 0.002	6.182 ± 0.014	73.684 ± 0.166	5.21	65.65
Crowded 2B (High)	28-Aug-19	17.883 ± 0.002	6.781 ± 0.028	83.157 ± 0.341	5.22	64.07

Measurements made during commercial fishing operations, using the CMP with a RINKO 1D optical oxygen logger (figure 7), show DO concentrations in the net during hauling comparable to the controls at Austevoll. However, when the catches were crowded during pumping, hypoxic events (<6 mg/l) were observed in most catches. The reduction in DO was clearly related to catch size. Moreover, more hypoxic conditions were seen in herring catches compared to the mackerel catches. The much lower DO concentrations observed during the fishing operations highlights the limitations of the experiments at Austevoll for investigating the effects of hypoxia concurrently with crowding treatments. This presents us with an important knowledge gap, if the effects of crowding and the associated hypoxia are to be properly understood in terms of the welfare of the catch.

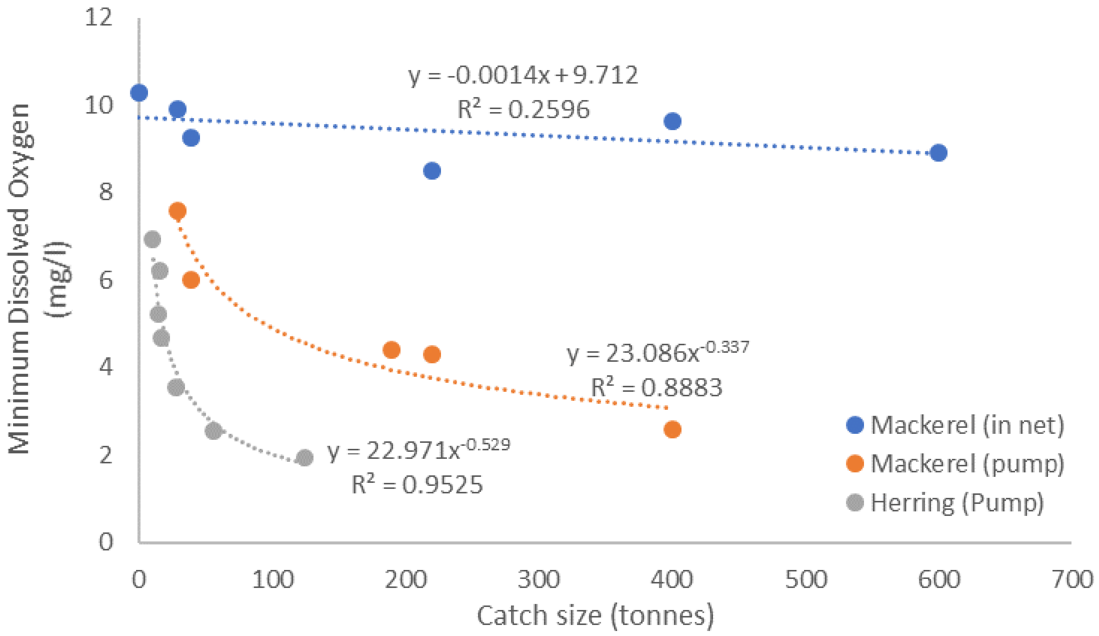


Figure 7 – minimum dissolved oxygen concentrations (mg/l) in relation to catch sizes of mackerel and herring during the hauling and pumping phases.

Dissolved oxygen is generally recognised as an important welfare indicator, which in this project has been monitored during all observations of commercial fishing operations using either RINKO 1D loggers in the CMPs or using a CTDO. A detailed description and analysis of these observations will be given in D5.2.

To be able to make inferences about the welfare status of mackerel and herring during hypoxic events, future work should investigate the maximum and standard metabolic rates (oxygen consumption) at a range of swimming activities, ambient oxygen concentrations and temperatures, as well as hypoxia tolerance limits in both species. Further controlled experiments should be conducted to investigate the compound effects of both crowding and hypoxia on vitality/mortality and the other stress/welfare indicators.

3.1.3 Temperature

Mackerel and herring are poikilotherms. That is, their body temperature varies with the ambient environmental temperature. Therefore, rapid changes in ambient temperature may cause stressful, and even fatal, disruption to their metabolism called "temperature shock" (Davis and Olla, 2001, 2002; Davis et al., 2001; Donaldson et al., 2008; Gale et al., 2013). During capture in a purse seine, fish may experience stressful temperature changes during the retrieval of the gear, if they ascend through thermoclines, and as they are transferred aboard the vessel when briefly exposed to ambient air temperatures (e.g. less than -10 ^{o}C in the Barents Sea during winter; or greater than 20 ^{o}C in the North Sea in the summer). However, the most stressful temperature change is likely be as they are transferred from the sea into the refrigerated seawater (RSW) storage tanks.

The International Mackerel Surveys (2007-2016) and the Norwegian Sea surveys (1997-2006) have recorded mackerel schools at temperatures from 4-14^{o}C (Olafsdottir et al, 2017). However, the preferred range for most mackerel schools is considered to be 7-11^{o}C (Nøttestad et al, 2015). This concurs with observations in North American waters, where the preferred temperature was above 8 °C, but Atlantic mackerel were also observed to be common in waters as cold as 7 °C and occasionally as low as 4.5 °C (Sette, 1950). A significant mortality was observed in mackerel held over winter in cages at Austervoll, when seawater temperatures dropped below 5^{o}C (Breen, pers. obs.).

Herring have a much wider tolerance for temperature: 0 to 18^{o}C (Leim et al., 1957). Moreover, herring acclimatised to summer temperatures (8.7 to 11.2^{o}C) were shown to have size related upper lethal temperatures (i.e. 50% mortality in 48 hours) of 19.5 (mean length 21.9cm) to 21.2^{o}C (mean length 11.1cm) (Brawn, 1960), During an opportunist observation, Brawn (1960) also noted that the majority (87%) of herring in a tank survived a short (overnight) exposure to water temperatures below -1^{o}C.

Seawater temperature (^{o}C) was monitored throughout our experiments at Austevoll (see table 4), as well as during all deployments of the various CMPs (in the net, on the pump and in the RSW tank) in commercial fisheries. Water temperature in Phase I generally ranged between 10 – 11 ºC, while in Phase II it was between 16 – 18 ºC (Figure #11A). Out with treatments, temperature did not show any substantial changes within the cages and stayed constant with little variation from day to day (see Ochao, 2020 for more details). There appeared to be small but significant increases in temperature during the High Prolonged crowding treatments (1A & 1C), but this may be the result of the main body of the CTDO probe being mostly out of the water for the duration of the treatment.

During observations of commercial fishing operations, no significant changes in seawater temperature have been observed during the hauling and pumping phases. However, substantial and very rapid changes in temperature have been observed as the fish are transferred into the refrigerated seawater (RSW) storage tanks, where they typically experience temperatures of around -1.5 to 2^{o}C.

Temperature change is well established as an important welfare indicator, which in this project has been monitored during all observations of commercial fishing operations using either RINKO 1D loggers in the CMPs or using a CTDO. A detailed description and analysis of these observations will be given in D5.2. To be able to make inferences about the welfare status of mackerel and herring during capture in purse seines future studies should investigate the tolerances of these species to acute temperature change, as well as the factors that may affect it.

3.2 Primary and Secondary Level (Physiological) Indicators

3.2.1 Pre-mortem physiological indicators

The following presents the main physiological indicators examined during the project and highlights those best suited to characterizing mackerel welfare.

3.2.1.1 Cortisol

Cortisol is a primary stress hormone which is released into the blood stream from inter-renal cells within the head kidney a few minutes after stressor onset. It acts to regulate hydromineral changes induced by the action of catecholamines, by stimulating water uptake in the stomach and ion extrusion from the blood at the gills, kidney and stomach. It also enhances glucose availability, mediates stress induced inflammation and suppresses non-essential immune, growth and reproductive processes during stressor exposure (Mommsen et al., 1999).

During the current project, blood plasma cortisol increased in mackerel exposed to experimental crowding compared to those which received no crowding (figure 8). The magnitude of the increase was broadly reflective of the severity and duration of the received stress (Anders et al., 2020; Anders et al., n.d). In concordance with current understanding, the peak response of cortisol was delayed, with maximal values occurring ~2 hours after stressor cessation and with a non-linear time course development during crowding itself (Anders et al., n.d). Post crowding, cortisol generally returned to baseline values within 24 hours. However, moribund fish also had slightly elevated cortisol levels (Anders et al., n.d)., but it is unknown whether these were maintained from the initial stressor treatment or whether cortisol became elevated in response to developing skin lesions (which all moribund and dead fish had)(see section 3.3.4).

These findings indicate that cortisol is a useful mackerel welfare indicator, but that care must be applied as to when it is sampled if accurate inferences are to be drawn. The considerable degree of variability in cortisol baseline values (Anders, 2020) also highlights the need for appropriate controls measurements as this metric naturally fluctuates in response to factors other than stress. Cortisol is easily analysed in the laboratory using standard assay kits on defrosted blood plasma produced from caudal vasculature puncture. Although blood plasma is easy to collect for cortisol analysis, this must currently be conducted in the laboratory due to the absence of any validated field instruments.

3.2.1.2 Ionic composition of blood

When exposed to stressors, fish release catecholamine hormones into the bloodstream which stimulate increased cardiac and operculum stroke rate and volume, as well as increased gill membrane permeability and blood flow. These alterations provide increased oxygen availability in preparation for a stress response but because the osmolality of marine fishes' body fluids is lower than the surrounding seawater, it also results in an increased rate of water loss rate across the gill membranes. An increase in the concentration of blood ions results (haemoconcentration). Osmolality also increases due to this effect and due to the presence of other solutes (e.g. lactate and glucose) diffusing into the blood. Haemoconcentration may also occur due to skin damage, when internal body fluids are exposed to hyperosmotic seawater (Wendelaar Bonga, 1997). If severe and/or unrecoverable, dehydration can contribute towards or cause of death.

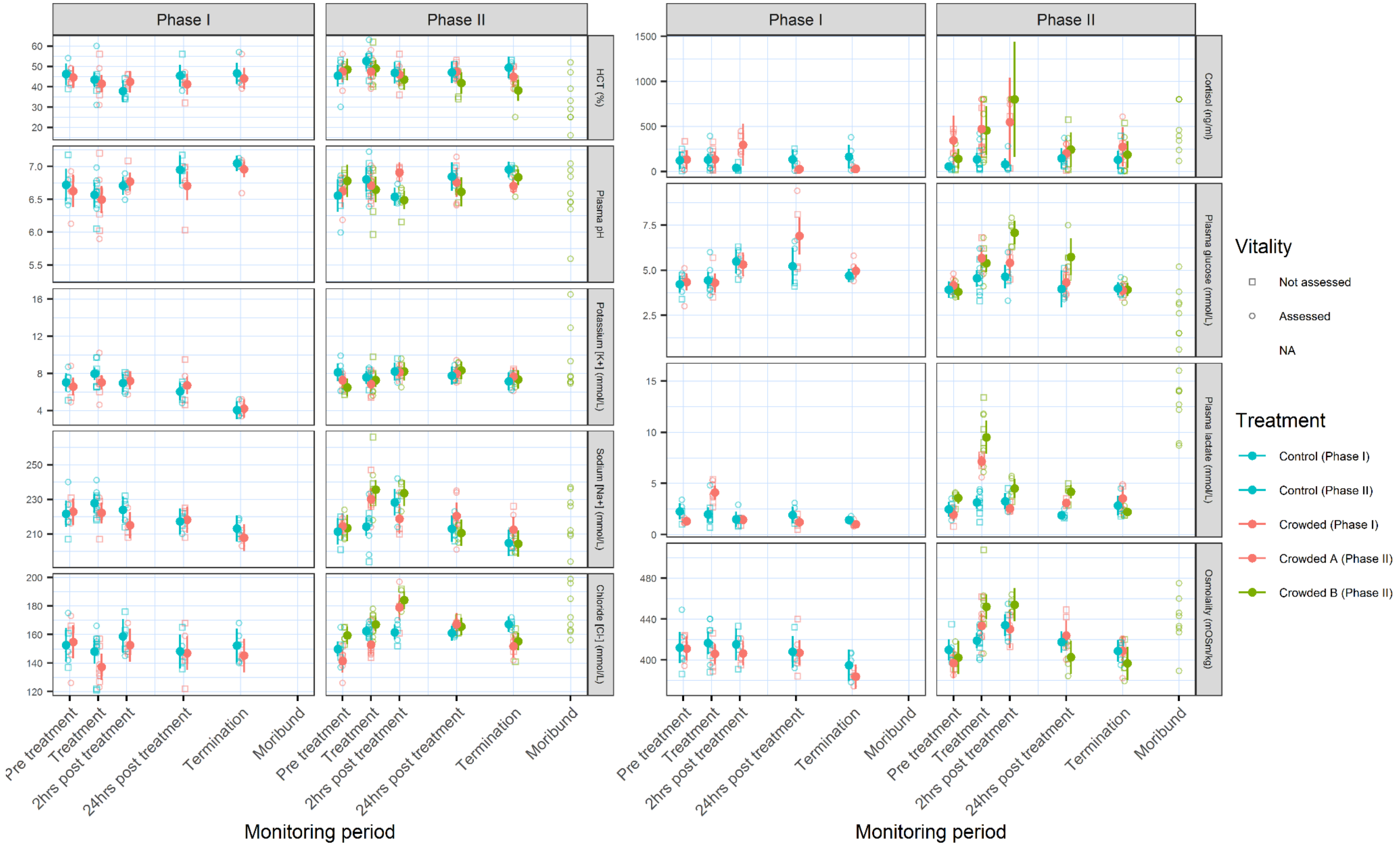

Phase I
Phase II
HCT (%)
60
50
40
30
20
Plasma pH
7.0
6.5
6.0
5.5
Potassium [K+] (mmol/L)
16
12
8
4
Sodium [Na+] (mmol/L)
250
230
210
Chloride [Cl-] (mmol/L)
200
180
160
140
120
Cortisol (ng/ml)
1500
1000
500
0
Plasma glucose (mmol/L)
7.5
5.0
2.5
Plasma lactate (mmol/L)
15
10
5
0
Osmolality (mOsm/kg)
480
440
400
Pre treatment
Treatment
2hrs post treatment
24hrs post treatment
Termination
Moribund
Monitoring period
Vitality
☐ Not assessed
○ Assessed
NA
Treatment
Control (Phase I)
Control (Phase II)
Crowded (Phase I)
Crowded A (Phase II)
Crowded B (Phase II)

Figure 8: physiological responses to crowding stress in mackerel blood. *Points indicate model derived mean estimates ± 95% confidence intervals as whiskers. The underlying dataset is indicated as points. The wide confidence intervals for cortisol in Phase II at 2 and 24 hours post-treatment reflect the lack of variability in the data for crowded fish at these monitoring periods.*

In this project, plasma osmolality, as well as the concentrations of sodium (Na^+), chloride (Cl^-) and potassium (K^+) ions, have been measured to determine the ionic composition of mackerel blood during and after crowding stress. In general, crowded mackerel showed some increases in the concentrations of Na^+, Cl^- and K^+ that were sometimes evident during the application of the stressor and up to 24 hours later (figure 8). However, these responses varied considerably in their magnitude and consistency in a way that bore little relation to stressor intensity, apart from there being no response for mildly crowded fish (Anders et al., 2020, Anders et al., n.d). Where crowding was severe enough to elicit a measurable response, overall osmolality was consistently elevated, with recovery for most fish within 24 hours and elevated values for moribund fish (Anders et al., n.d).

These findings indicate that the isolated use of blood ions as welfare indicators is questionable, as responses for these metrics were inconsistent and decoupled from the magnitude of stress. Osmolality may be a more useful indicator however, with the caveat that it appears to be unresponsive to low levels of stress in mackerel. Blood ions and osmolality are easily analysed in defrosted plasma in the laboratory using blood gas analysers. There are currently no available validated field instruments to measure blood ion indicators in the field. Portable osmometers are available for use in the field, but require the time-consuming extraction of plasma from whole blood.

3.2.1.3 Haematocrit

Stress induced release of catecholamines results in additional erythrocyte (red blood cells) release from the spleen, as well as increased erythrocyte volume and oxygen affinity (Wendelaar Bonga, 1997). These changes enhance oxygen availability in preparation for coping with the challenges of the stressor, and are reflected in the composition of the blood; increases in haematocrit (percentage volume of red blood cells in the blood) are typical for fishes exposed to stressors. Haemoconcentration (another stress induced change to blood, see previous comments) may also contribute to increased haematocrit.

Contrary to expectation, any increase in haematocrit in response to crowding during this project was non-significant (figure 8). Likewise, there was no evidence of a consistent response pattern either during or post-stressor. The lack of a clear response is difficult to account for, but it is worth noting that moribund fish did have notably reduced haematocrit levels.

It can therefore be concluded that haematocrit has limited utility as a mackerel welfare indicator, because it was generally unresponsive to crowding stress at the levels currently examined. It may, however, have some utility in identifying non-coping fish following stressor exposure; further work on various stages of moribund individuals would be required for this. Haematocrit has been quantified in this project in the field, but this is a somewhat time-consuming process that requires specialized equipment. However, faster analytical techniques are available for both the laboratory and the field (Stoot et al., 2014).

3.2.1.4 Glucose

Glucose is an energetic metabolite, used by living organisms to generate energy for maintenance and activity. In stressful scenarios, catecholamines (and later, cortisol) stimulate the breakdown of glycogen into glucose primarily within the liver, providing additional energy for a stress response

(Mommsen et al., 1999). This process is called glycogenolysis and takes time to occur. Consequently, delayed increases in plasma glucose are typically seen in fish exposed to stress. If the animal maintains a generalised stress response, any subsequent reduction in glucose may suggest an exhaustion of available energy reserves.

Mackerel exposed to crowding generally had elevated glucose levels compared to control fish, with the magnitude of response reflecting the degree of stress received (with no response evident for mildly crowed individuals, Anders et al., 2020, Anders et al., n.d). In most cases, and in accordance with other studies, glucose began to rise during the application of the stressor and continued after stressor cessation (figure 8). Return to baseline levels was achieved within 24 hours for most fish, while glucose was reduced for moribund fish. Likely due to its delayed response, there was no relationship between glucose levels and stressor duration during the time in which fish were crowded.

Taken together, these findings suggest that glucose may be a useful welfare indicator for mackerel, in that it can reflect the degree of stress received, providing that a suitable sampling time is chosen and the stressor is severe enough to elicit a response. It may also be useful for monitoring recovery from a stressor due to it taking a longer time to resolve compared to other indicators. Severely depleted levels of glucose in stressed individuals may be indicative of a non-coping fish. However, levels of circulating glucose are easily influenced by factors other than stress (such as feeding) so adequate control measures are still required for proper interpretation. Otherwise, glucose is easily measured in the laboratory using blood gas analysers on defrosted blood plasma. It has also been demonstrated that it is possible to quantify mackerel whole blood glucose in the field using "point of care" devices, providing that validation of such instruments has been undertaken (Anders et al., 2020).

3.2.1.5 Lactate

Lactate is a metabolite, produced when intracellular ATP (a high energy phosphate compound) is produced via anaerobic (without oxygen) rather than aerobic pathways. A lack of available oxygen can occur due to a reduction in availability in the environment (as can occur during high density purse seine crowding, Tenningen *et al*, 2012) and/or because of increased metabolic demands (as occurring during high intensity exercise). In such scenarios, the rate of lactate production outstrips the rate of removal. Concentrations of lactate and metabolic protons then begin to increase, first in the muscle and subsequently in the blood (Roberg et al., 2004). Lactate can therefore be considered as a marker (but not the cause) of metabolic acidosis.

Throughout the various tank and net pen experiments conducted during this project, levels of lactate were consistently, rapidly and substantially increased in mackerel exposed to crowding. Increases were also strongly correlated with stressor magnitude and exposure duration stress (Anders et al., n.d; Anders et al., 2020). However, metabolism of lactate would appear to be rapid in mackerel, with recovery to baseline levels for the majority of fish within 2 hours (figure 8). Conversely, moribund fish had notably elevated levels. A preliminary meta-analysis of lactate measures collected throughout this project (Anders, 2020; figure 9) indicated that negative welfare consequences (in terms of a rapidly increasing probability of mortality and negative flesh consequences) are associated with (but not necessarily caused by) plasma lactate levels in excess of ~6mmol/L. Further data is however needed to verify this potential reference point. Until then, adequate control sampling is necessary.

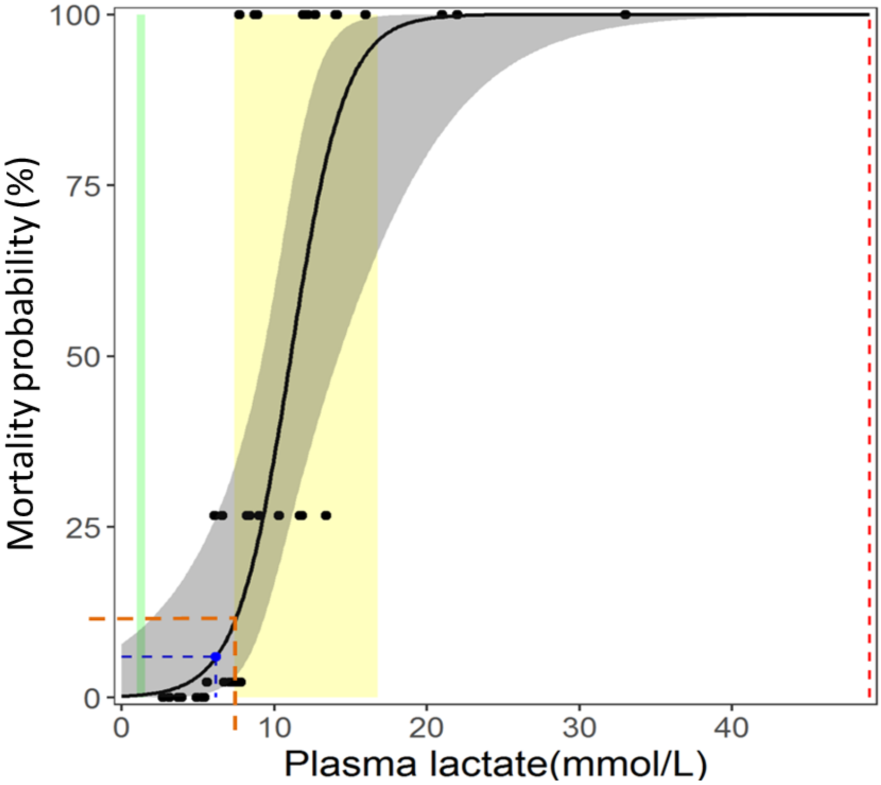


***Figure 9: The relationship between mackerel plasma lactate concentration during crowding and mortality in comparison to reference values**, based on data from the Austevoll experiments. Raw data values are shown as black dots. The black line describes a fitted Generalised Linear Mixed Model (with ± 95% confidence intervals as the grey shaded area). The green area indicates the 95% confidence interval for baseline control values (1.36 to 1.88 mmol.l-1). The yellow area indicates the 95% confidence interval for quality effects (7.4 to 13.3 mmol.l-1). The red dotted line represents the maximum lactate concentration observed during the experiments in Austevoll. Lactate concentration (6.1 mmol.l-1) at the first observed mortality is indicated by the blue point and dotted blue lines. The orange dashed line indicates the lactate concentration (7.4 mmol.l-1) at the lower 95% confidence level for quality effects, which relates to a mortality probability of 11.5% (95% CI: 3-33%). (From Anders, 2020).*

In light of these findings, lactate can be considered as perhaps the most useful physiological indicator of stress for mackerel, both during exposure to the stressor and afterwards. Chronically elevated levels may indicate a non-coping fish. The fact that lactate is such a sensitive indicator means that best-practice sampling procedures must be followed to avoid observation effects. It is important to note that lactate sampling alone does not determine whether ambient and/or functional hypoxia is occurring; additional monitoring of fish behavioural responses and environmental conditions are required to place this welfare indicator fully in context. Otherwise, lactate is also an advantageous welfare indicator because it can be easily quantified, either using colorimetric assays/blood gas analysers on plasma in the laboratory or in the field using "point of care" (POC) devices on whole blood. POC devices should be validated against laboratory measures as they can show discrepancies (Anders et al., 2020).

3.2.1.6 Blood and muscle pH

pH is a scale measuring the acidity of a substance. In non-stressed scenarios, fish's internal milieu is balanced by the exchange of acid-base elements at the gills. However, during stressor exposure, the rate of exchange can be outstripped by proton accumulation from intense exercise (metabolic acidosis) or from excess carbon dioxide (respiratory acidosis). This can lead to reductions in pH towards acidity, in both the white muscles (where metabolic protons are generated) and in the blood (as protons are transported to the gills for exchange with the external environment). Additional welfare impacts can result from acidosis, as oxygen affinity in the blood is compromised in acidic conditions due to the Root effect and haemolysis (Wendelaar Bonga, 1997). The effects of acidosis are thought to be the major factor contributing to fish death following exhaustive exercise (Wood et al., 1983).

Both blood (via plasma sampling) and muscle pH have been used as mackerel welfare indicators in the current project, with varying degrees of success. In net pen experiments, blood pH levels were generally unresponsive to crowding (both during and after the stressor) and showed variability over time (figure 8). Moribund animals did, however, show consistent evidence of plasma acidosis (Anders et al., 2020; Anders et al., n.d). Conversely, crowding in tanks did result in significantly reduced blood pH (Anders et al., 2020). This lack of concordance may have arisen due to the tank maintained mackerel being chronically stressed by their captivity (see Anders et al., 2020 for further detail); clearly more work is required to establish whether blood pH is a useful indicator. Muscle pH was found to be a more reliable indicator of acidosis, with measurements being consistently decreased for crowded mackerel (Anders et al, 2020).

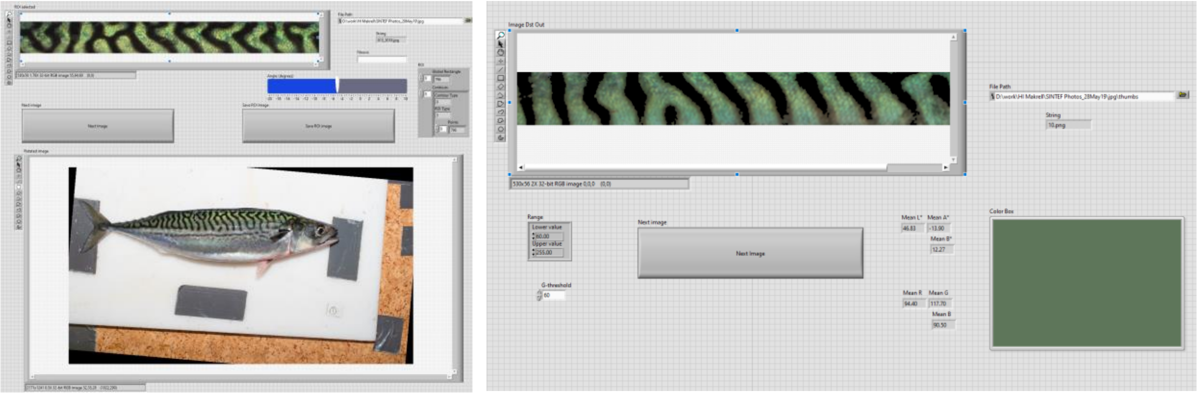
Notable and prolonged reductions in plasma pH may indicate a non-coping fish but otherwise, plasma pH cannot be reliably used to determine mackerel welfare during and post-stressor because it can be non-responsive and variable. pH is therefore best used as a welfare indicator when measuring muscle rather than blood, at least for mackerel experiencing crowding stress at the levels examined during this project. The high anaerobic capacity and presence of red, oxygenated red muscle throughout mackerel white muscle may explain why muscle pH is a more sensitive indicator for this species. Sampling defrosted plasma pH is easily achieved in the laboratory using blood gas analysers, while muscle pH can be easily recorded on site using a puncture electrode inserted into the musculature of the dorsal loin.

3.2.2 Skin colour changes

Visible indicators of stress, such as changes in skin colour, have potential as an operational stress/welfare indicator because they are relatively easily observed, and could have potential as a tool to aid fishers in determining the status of the catch during the crowding and pumping stages.

The work presented here describes the investigation of a single stress indicator (skin colour). Skin colour change in mackerel was investigated as part of the large study looking at the effects of crowding stress on multiple stress metrics (including: survival/vitality, physiological and behavioural parameters) in Atlantic mackerel described earlier. The study was conducted at the Austevoll Research Station at the Institute of Marine Research during May 2019. For the work on skin colour a total of three different treatments were conducted: one control treatment with no crowding (Control 1B), one treatment of low crowding stress (Crowding 1B) and one of high crowding stress (Crowding 1A).

The development of methodology for conducting this work, the provision of camera equipment and the subsequent image-analysis was conducted by SINTEF. During the experiments, each fish was photographed immediately following euthanisation. The camera used was a 36.3-megapixel FX-format Nikon D800E, with a Nikon 35mm f/1.8 G FX AF-S lens and a Nikon Speedlight SB900 full-spectrum flash with linear polarising filters. Images were acquired with full manual settings on the camera (1/250 sec exposure, ISO 100, F/5.6) and exported in NEF RAW format for post-processing. An X-rite ColorChecker was photographed by the same equipment and a custom colour correction profile was created and applied to the RAW-photos during post-processing in Adobe Lightroom. Each image was corrected for exposure variations, due to flash inconsistencies, by using as reference the white background the fish was photographed on. Images were then cropped, resampled and exported in JPEG-format for final processing and analysis in the LabVIEW software.



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530x56 2X 32-bit RGB image 0,0,0 (0,0)

Range
Lower value: 60.00
Upper value: 255.00

G-threshold: 60

Mean L*: 46.83
Mean A*: -13.90
Mean B*: 12.27

Mean R: 94.40
Mean G: 117.70
Mean B: 90.50

*Figure 10. Photo processing in LabVIEW. The left shows the first program selecting the range of interest (ROI). The right shows the second programme converting the ROI into CIE L*a*b* colour values.*

For final image analysis, two LabVIEW programs were developed (Figure 10). The first program was a user interface where a rectangular area on the right, dorsal-side of the fish was manually selected and exported as an RGB-thumbnail. The second program converted these thumbnails into CIE L*a*b* colour space values. A threshold was applied from the green colour plane of the RGB-thumbnail so that only the pixels of the coloured section of the fish was selected, excluding the black stripes. The mean L*, a*, b* of the subset of pixels was then calculated.

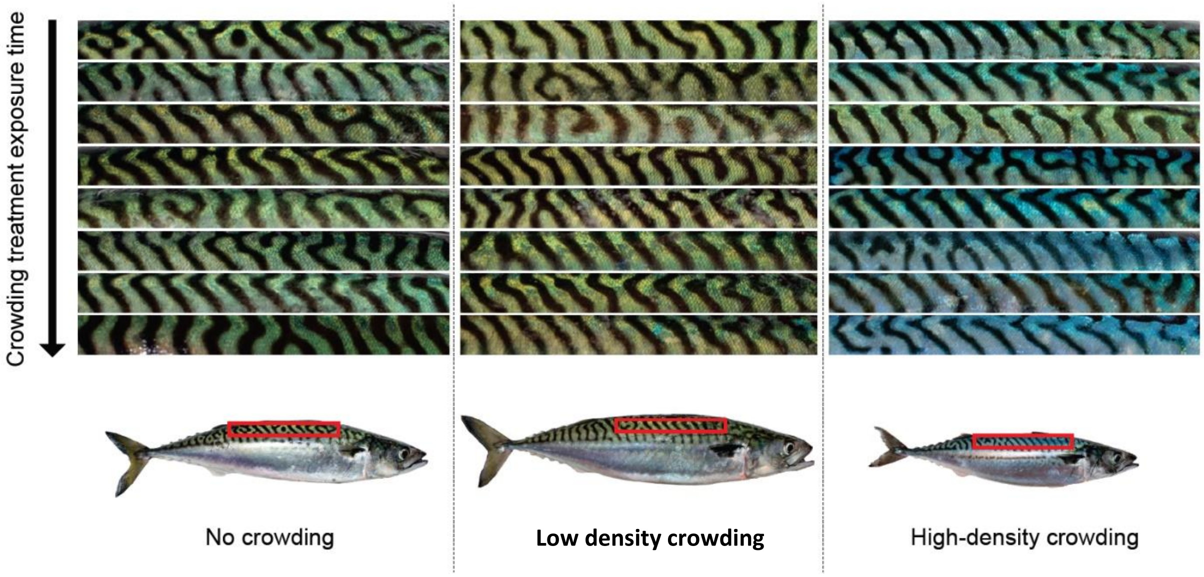


Figure 11. Cropped dorsal areas of Atlantic mackerel, under three different crowding treatments with exposure time to treatment increasing from top to bottom.

Thumbnails of fish from the control (no-crowding), moderate-density crowding and high-density crowding treatments are shown in Figure 2. The results show that the fish in the high-density crowding stress treatment have predominantly blue-coloured stripes, while in the control and moderate-density crowding stress treatments corresponding skin areas were predominantly green. The mean Yellow-blue (b*) component of the CIE L*a*b* colour space was found to be significantly different between treatments (p = <0.0001). Small significant differences were also detected between the mean Green-Red (a*) components between the three treatments (p = 0.0197). The results from the experiment suggest that there is a colour-change response in Mackerel to high-density crowding stress, and that these changes happen within the first 10 minutes of crowding stress.

Skin colour change in Atlantic mackerel (*Scomber scombrus*) shows real potential as a stress/welfare indicator. These preliminary results suggest that colour change is a responsive (after >10mins exposure to stressor) and consistent indicator of crowding stress. It is also easily seen by an observer in the field and could provide the industry with an easily applied qualitative operational indicator. Alternatively, a systematic and quantitative colour change metric requires photography under controlled lighting conditions followed by specialist image analysis.

Currently, the underlying physiological mechanism is unknown, so it is not possible to predict what other factors may influence this colour change. Therefore, further investigation is required to define underlying physiological mechanisms and potential variations in skin-colour change under different operational conditions. It would also be beneficial if this work investigated the potential for developing automated quantitative analysis in the field.

To substantiate the evidence for skin-colour change as a welfare metric, further work is currently ongoing to analyse images taken during other treatments in the Austevoll experiments. This will contribute towards manuscripts demonstrating the correlation between skin-colour change and crowding stress, as well as other potential welfare indicators, behavioural and physiological.

3.2.3 Post-mortem physiological and meat quality indicators

The following presents the main flesh quality measures examined during the project, highlighting which have the greatest potential for characterizing post-mortem effects of capture related stress in mackerel. These indicators have limited application as “informative welfare indicators”, because they monitor post-mortem effects of stress and therefore could not affect a timely migration of any poor welfare effects. However, because they are inherent in the functional definition of good welfare, as post-hoc indicators they are important for the development of informative welfare indicators, by informing when poor welfare has occurred.

3.2.3.1 Rigor mortis (rate of onset and strength)

Rigor mortis is the post-mortem stiffening of muscles. It results from a lack of residual energetic capacity in the muscle, meaning that actin and myosin fibres can no longer detach from one another. With time, proteolytic processes cause the resolution of rigor as muscles begin to break down. Fish with lower energetic muscle capacity at the point of death (such as would be found in a fish exposed to stress) would therefore be expected to take less time to enter rigor and would reach a higher degree of stiffness (Huss, 1988).

In this project, crowded mackerel stored on ice tended to be stiffer at the point of death than non-crowded individuals. Crowded fish also reached a higher degree of stiffness at a faster rate (figure 12). These effects were evident despite the use of control fish that were chronically stressed to some degree (see Anders et al., 2020 for further detail). Rigor reached maximum stiffness ~18 hours post-mortem and began to resolve ~3 hours later, with considerable overlap in stiffness values between the groups from ~10 hours post-mortem.

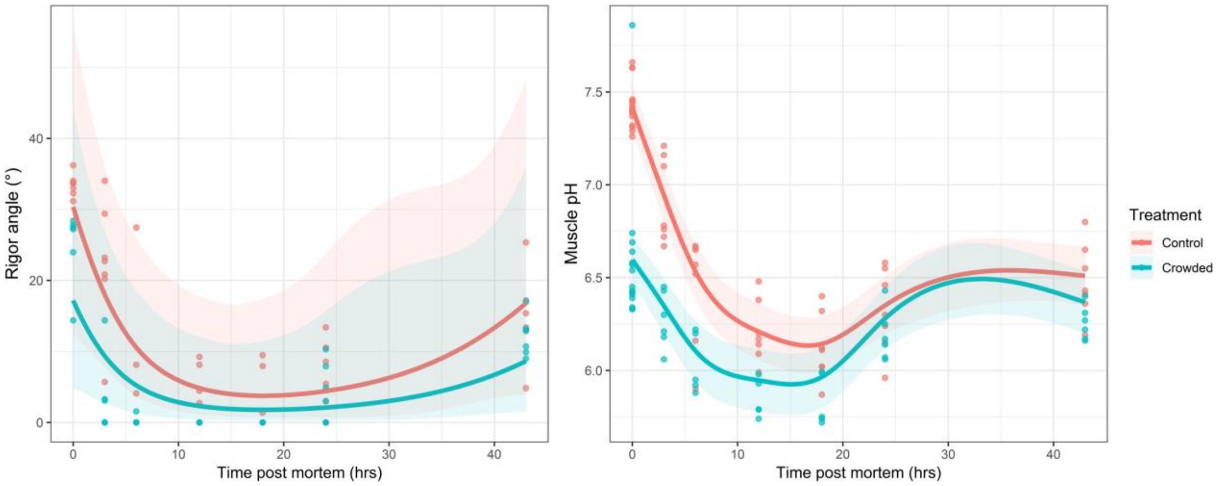


Figure 12 - Post-mortem rigor angle development (left panel) and muscle pH (right panel) response to crowding in Atlantic mackerel. The lines indicate the fitted relationship, with the 95% confidence intervals shown as shaded areas. The underlying dataset is shown as filled circles. Data points and lines are coloured according to treatment.

Overall, the evidence suggests that rigor measurement may be a robust indicator of pre-mortem welfare providing that consideration of rigor time course is given. This is because stressed individuals are most easily distinguished early on, prior to the point of maximum rigor. Additional rigor development trials using low stress controls and different storage temperatures / mediums are required to further develop the utility of this indicator. Measures of rigor are easily collected in the field using the Cutting's tail drop method (measuring the vertical drop of the tail when half of the fish’s length is placed on the edge of a table) or by measuring stiffness with handheld instruments. Experience has shown that rigor in mackerel is best expressed in absolute (e.g. angle of tail drop) rather than relative (e.g. rigor index) terms, as mackerel can display pre-mortem muscle stiffening that cannot be accounted for if relative units are applied.

3.2.3.2 Post-mortem muscle pH

Following death, intracellular energy metabolism continues but without the contribution of oxygen. Such anaerobic energy production results in an accumulation in metabolic protons, which cause the post-mortem acidification of fish flesh. When stressful conditions prior to death occurred, this effect is exacerbated by unresolved pre-mortem acidification. Stressed fish can therefore be expected to reach more acidic levels of post-mortem muscle pH at a faster rate compared to non-stressed individuals. With time, flesh becomes less acidic as proteolytic processes being to breakdown the muscle tissue (Huss, 1988).

In this project it was shown that crowded mackerel stored on ice reached more acidic post-mortem muscle values at a significantly faster rate than non-crowded fish (figure 12). pH values from both groups began to coverage at ~20 hours post-mortem, making them difficult to distinguish after that.

These findings indicate that post-mortem muscle pH is potentially a very useful indicator of pre-mortem welfare. However, as for measures of rigor, consideration of the time course must be given; stressed and non-stressed individuals are most easily distinguished earlier into the acidification process, prior to the point at which flesh acidity begins to resolve. Measuring post-mortem muscle pH is easily achieved by inserting a calibrated puncture electrode into the musculature. Further work should determine if and how post-mortem pH development is affected by different storage temperatures / mediums.

3.2.3.3 Cathepsin B & L activity

The cathepsins are an important group on intracellular proteases, responsible for breaking down waste products within the cell lysosome. At cell death, they start to become liberated from the lysosome and begin acting autolytically. Their activity rate is enhanced in acidic conditions. As metabolic acidification is a common response to stress in fish, unresolved acidosis prior to death leads to increased cathepsin activity in the post-mortem phase relative to non-stressed fish (Poli et al., 2005).

In this project, it was demonstrated that crowded mackerel stored for 7 days on ice have increased cathepsin B & L activity compared to non-crowded fish. Mean activity was 25% higher in crowded individuals, but the difference was statistically non-significant.

Elevated cathepsin activity may therefore have potential as a retrospective welfare indicator in mackerel. However, further development is needed before it can be used. It should be determined if it is possible to distinguish between stressed and non-stressed individuals when fish are stored for different lengths of time (other than the 7 days already tested), in different temperatures and in different storage mediums (other than ice). The lack of statistical significance between the groups in this project (despite the large effect size), suggests that activity is variable between individuals and that large sample sizes may be needed when using cathepsins as a welfare indicator.

3.2.3.4 *Water holding capacity (WHC) and drip loss*

The process of rigor mortis exerts physical forces on fish flesh. Furthermore, pre- and post-mortem acidification contributes towards protein denaturation and increased proteolytic enzyme activity. As a result of these processes, the water holding capacity (WHC) of fish flesh typically reduces in the post-mortem phase. The reduction in weight due to loss of water is called “drip loss” (Huss, 1988). For fish which were exposed to pre-mortem stress, these processes are accelerated and/or intensified. Flesh from stressed fish can therefore expect to have reduced WHC and increased drip loss relative to non-stressed fish.

In this project, fillets stored on ice for 5 days lost ~3% of their weight to drip loss, but the rate of loss was not different between crowded and non-crowded fish. Similarly, there was no detectable difference in WHC after 7 days of ice storage between crowded and non-crowded groups.

The lack of detectable difference in WHC and drip loss between stressed and non-stressed fish would suggest that these metrics have limited utility as indicators of mackerel welfare. Although examination of the effect of different storage times and temperatures on these parameters should be undertaken, it is likely that the naturally poor water holding capacity of mackerel flesh (due to their high fat content) will make detecting differences difficult and that large sample sizes may be required. Furthermore, although determining drip loss is easily achieved (by repeat measurement of weight), the analysis of WHC is time consuming and requires specialized laboratory equipment.

3.2.3.5 *Gaping*

Gaping is the unsightly and undesirable separation of fish muscle blocks from one another. It occurs due to the failure of the connective tissue between muscle myotomes and results in a fillet with a “split” appearance. The connective tissues fail due to the combined action of rigor mortis and muscle acidification. When greater depletion of ATP occurs prior to death (as can be expected during stressful scenarios), rigor mortis produces greater physical forces within the fillet, which may physically tear connective tissue. The higher degree of pre- and post-mortem muscle acidification in stressed fish means that proteolytic enzyme activity and protein denaturation is greater for such individuals. This also contributes to the breakdown of connective tissue and thereby the probability of gaping (Huss, 1988).

The results of this project indicate that crowded mackerel stored on ice for 5 days show significantly more gaping than non-stressed fish. However, the two groups were indistinguishable after 7 days of storage.

Taken together, these findings suggest that gaping is potentially a useful indicator of pre-mortem mackerel welfare, but that care must be taken as to when sampling is conducted. Stressed fish are indistinguishable from non-stressed individuals by gaping scores at (and potentially before) 7 days after death (likely due to an increase in autolytic processes in the control fish over time). Further research is therefore needed to determine the time course of gaping development, and how scores are affected by different storage temperatures, handling regimes and storage mediums. Otherwise, the indicator is easily recorded in either the field or the laboratory using a qualitative score to describe the gaping condition of individual fillets.

3.2.3.6 Texture

Due to the mechanical forces of rigor and protein denaturation resulting from the action of autolytic enzymes and pH reduction, changes in muscle texture occur in fish during the post-mortem phase (Poli et al., 2005). As rigor mortis, proteolytic enzyme activity and protein denaturation is stronger in fish that were stressed prior to death (see earlier notes), a greater degree of muscle softening can be expected for stressed fish compared to non-stressed individuals.

Crowded fish from this project had a ~15% less firm texture than non-crowded mackerel after 2 days of ice storage. However, there was no discernible difference in fillet texture after 7 days of storage.

As for other flesh quality metrics, these findings indicate that muscle texture may have a utility as a retrospective mackerel welfare indicator, but that the time course of texture development must be considered. Autolytic process likely explains why the two groups converged in terms of texture by day 7 after death. This indicates that texture sampling is best used as a welfare indicator prior to this point. Further work is needed to fully determine how texture develops in mackerel in the post-mortem phase, and to elucidate if and how texture is influenced by factors such as storage temperature and handling. In practical terms, the relatively small difference in texture between stressed and non-stressed individuals means that precise analytic equipment is needed for the quantification of this metric. This requires specialized laboratory equipment and is likely difficult to achieve accurately in the field.

3.2.3.7 Flesh colour

The reflective properties of fish flesh (and thereby colour) is altered in the post-mortem phase by the activity of autolytic enzymes, protein denaturation in low pH conditions and by water holding capacity changes. Intense exercise is also known to increase blood flow to muscles, which may also contribute to colour changes via the presence of residual blood. For fish stressed prior to slaughter, these processes can be expected to be accelerated and/or intensified. Hence, stressed mackerel flesh may have different colour properties than non-stressed individuals.

Using digital imaging and CIELAB colour space, this project demonstrated that crowded fish tended to be “less green” (40% mean increase in a* values [the green-red colour axis]) than their non-stressed controls. This result indicates a shift in colour towards more redness in the fillets and was evident both 2 and 7 days after death. The shift towards redness and lack of response in terms of WHC would suggest that the colour change primarily arose due to the presence of stress induced residual blood in the fillet, rather than through protein denaturation. The magnitude of the effect was however rather different between different experiments. There was no evidence of any effect of stress on flesh colour in terms of blue-yellow axis or lightness values.

Taken together, these results indicate that changes in green-red colour values in mackerel flesh may have some potential as a welfare indicator, perhaps indicative of a stress induced redistribution of blood to the fillet in the pre-mortem phase. However, additional work is required before colour can be used an indicator of welfare. The considerable differences in effect sizes between experiments in this project is difficult to explain and should therefore be the subject of further investigation to ensure the result was valid. Otherwise, the analysis of flesh colour is relatively easily achieved, providing that consistent lighting conditions are used to ensure stability between photographs.

3.3 Tertiary level (whole animal: anatomical & behavioural) indicators

3.3.1 Nearest Neighbour Distance (NND)

A social aggregation of fish is defined as a “shoal”, while a “school" is a synchronised and polarised shoal, in terms of swimming speed and direction/orientation (Pitcher, 1983). Schools tend to consist of individuals of a similar age/size, which is thought to avoid discordance with respect to hydrodynamics and relative swimming speeds (Pitcher et al, 1985). For pelagic species, like mackerel and herring, schooling behaviour is an integral part of their natural behaviour and survival strategy (Breder, 1967; Pitcher & Parrish, 1993).

A key property of this collective schooling behaviour is the distance separating individual fish: nearest neighbour distance (NND). Theoretical modelling and behavioural observation suggest there is a hierarchical order to NND, centred around an “optimal/preferred” or “neutral” NND zone (Huth & Wissel, 1994). Within this preferred or neutral zone, the fishes’ swimming behaviour (i.e. velocity: speed and direction) will remain relatively unchanged. However, when NND is too small, they are said to be in a “repulsion” or “stress” zone, and will consequently change swimming speed and/or direction to increase NND. Conversely, if NND is too large they are said to be in the “attraction” zone and will modify their swimming behaviour to reduce NND. The boundaries between NND zones are not fixed in terms of relative space (i.e. body lengths; BL), but can change depending on the biological status of the fish and/or external modifiers (e.g. stressors)(Tien et al, 2004). For example, when feeding, the NND zone boundaries may expand to reduce competition/interference between individuals. Conversely, if threatened by a predator, NND zones may contract to increase the defensive properties of the schooling behaviour (Tien et al, 2004).

NND is not only a descriptor of schooling behaviour but can also be used to infer crowding density. Stereo-photogrammetry, using calibrated stereo-cameras, can provide estimates of individual fish positions of fish in a 3D space, from which volumetric fish density can be estimated (i.e. in numbers of fish per unit volume)(Williams et al, 2018). Given information about the length-weight relationship of the population, estimates of biomass density can also be estimated (i.e. kg/m^3). As part of this project, a stereo-camera system is being developed (in collaboration with Mohn Technology) to operate in commercial fishery scenarios. It can be deployed on two different platforms, an ROV or a pneumatically deployed probe, both of which can provide live images to the fishing vessel. The primary purpose of this stereo system is to measure the length frequency distribution of fish before they are caught and/or slipped by a purse seine. However, the data gathered will also be used to estimate NND and schooling densities both before and during capture.

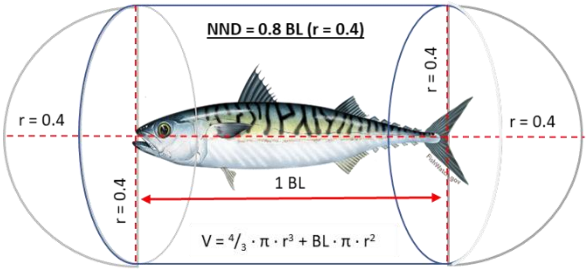


Figure 13 - by assuming the NND-space occupied by a fish approximates to a hemi-spherically terminated cylinder, and given information on mean length and weight estimates of fish in a school, the schooling or crowding density can be estimated from NND in terms of both fish/m^3 and kg/m^3. Mackerel image from NOAA

As part of the RedSlip experiments, Anders et al (2019b) used stereo-photogrammetry to estimate the neutral/preferred NND for mackerel in cages to be ~0.7 BL, which corresponded well with comparable NNDs for mackerel in captivity (0.3–0.9 BL) (Edwards et al, 1984; Glass et al, 1986). For an average sized fish in the experiments (40.5 ± 2.5cm, 887 ± 161g) this corresponds to voluntary schooling densities of ~18 fish/m^3 and ~15.8 kg/m^3 (based on the assumed volume in figure 13). Unfortunately, due to image occlusion, it was not possible for Anders et al to use stereo-photogrammetry to measure NND and densities in the more crowded treatments (i.e. > 62 fish/m^3 and >55 kg/m^3), but densities were successfully estimated using acoustic methods (see section 3.1.1). It was suggested that stereo-photogrammetric measurements in higher density schools could be made by reducing the separation between the camera lenses and/or by maintaining a greater distance between the camera and the subject fish, for example within a protective housing. But both approaches could also reduce measurement accuracy.

Table 5 - Definitions for the different levels of the Crowding Density Score, as based on estimates of Nearest Neighbour Distance (NND) in relative body lengths (BL)(from Keller, 2020). See figure 14 for example images.

Crowding score	Definition	Score
Not in view	The school is not in view. No scores applicable.	Z
Small group	Small group of fish (n<10). Always low crowding.	1
Low	The nearest neighbor distance between a fish to another of the school is larger than a fish's body length (BL). (NND > 1 BL)	2
Moderate	The distance between a fish to another of the school is approximately equal to a fish's body length. (NND ~1 BL)	3
High	The distance between a fish to another of the school is clearly less than a fish's body length. Light conditions become significantly darker and occasional fish-to-fish or fish-to-camera contact is observed. (NND < 1BL)	4
Very high	The distance between a fish to another is substantially smaller than a fish's body length. If daylight, light is shut out by high fish crowding and constant fish to fish and fish-to-camera contact is observed. (NND << 1BL)	5
Obscured View	Camera lens is covered (by something other than fish, i.e. netting) or fish are too distant to score.	X

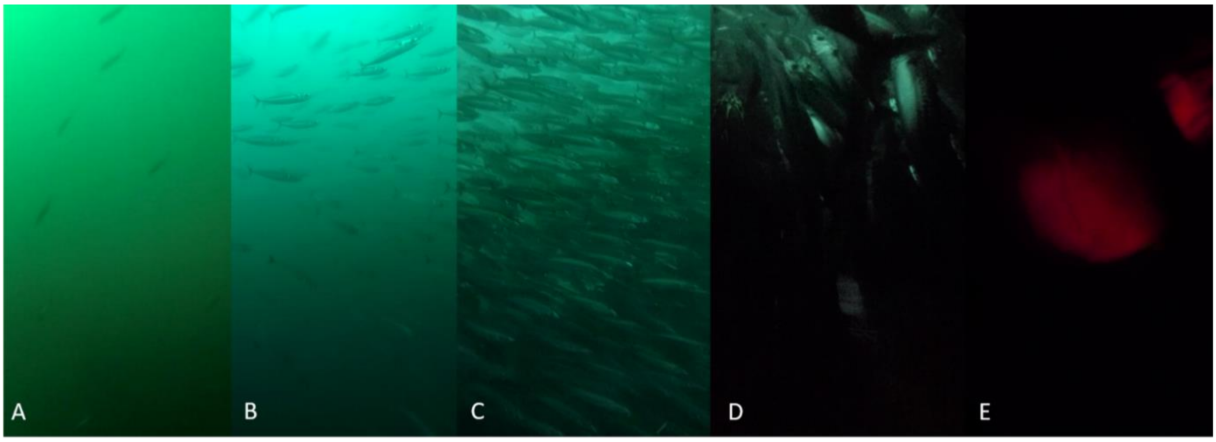


Figure 14 - Reference images of the Crowding Density Score levels A) small group B) low crowding, C) medium crowding, D) high crowding, E) very high crowding. See table 5.

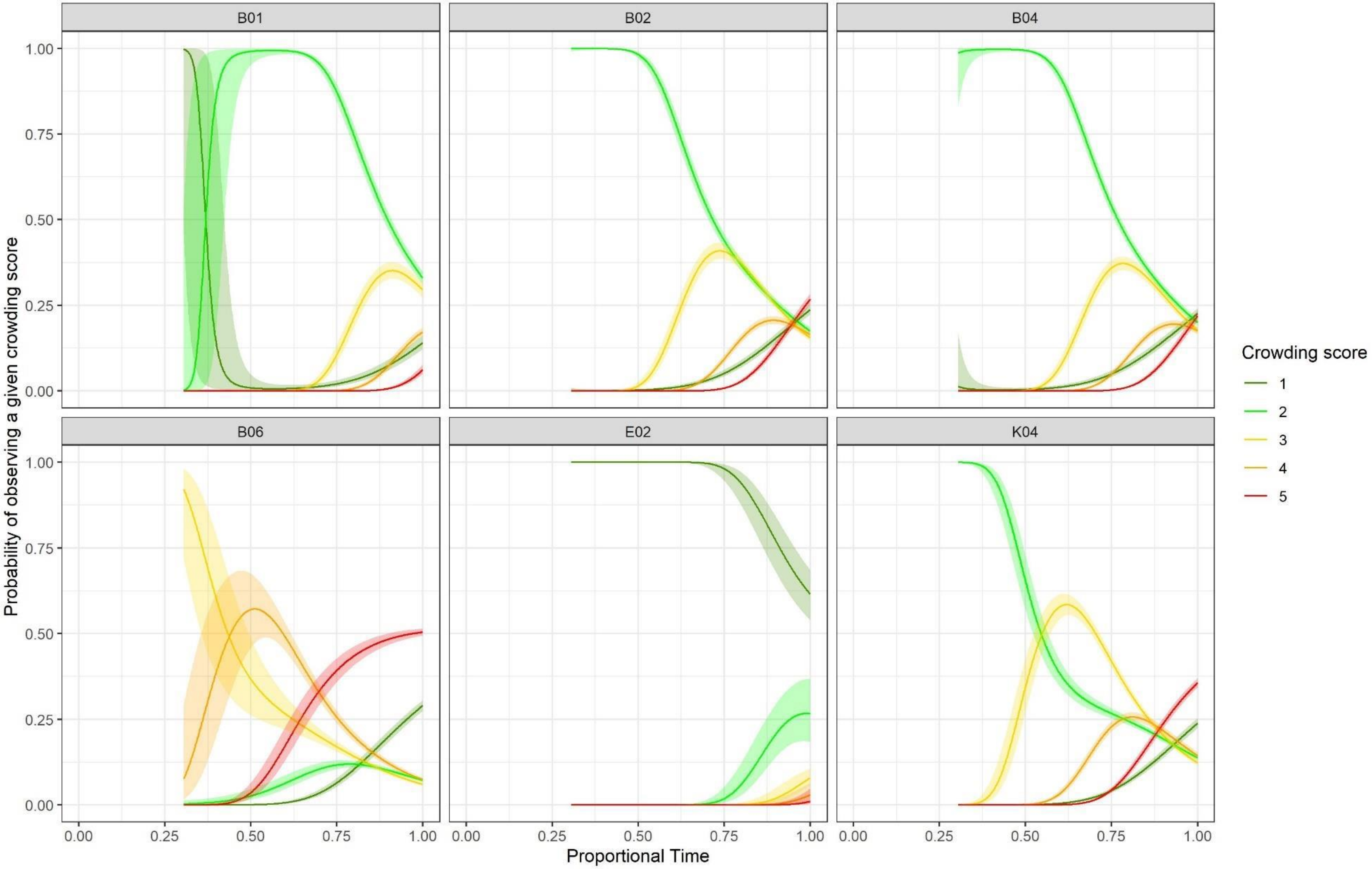

B01
B02
B04
B06
E02
K04
Probability of observing a given crowding score
1.00
0.75
0.50
0.25
0.00
Proportional Time
0.00
0.25
0.50
0.75
1.00
Crowding score
1
2
3
4
5

Figure 15: Probability of observing a given Crowding Density Score level over proportional time (from start to end of cast) in six different slipped mackerel catches: B01 (<1 t), B02 (~300 t), B04 (~1200 t), B06 (~50t slipped; 225 t landed), E02 (5-10 t) and K04 (600 t) [Keller, 2020].

In the absence of suitable methods to accurately and reliably estimate NND or crowding density (see also section 3.1.1) at high crowding densities, an ordinal scoring method was developed to estimate a Crowding Density Score (CDS) relative to NND (Table 5; figure 14)(Breen et al, n.d.; Riedinger, 2019; Keller, 2020). The method used multiple observers to provide estimates for anonymised video sequences of fish inside and/or being slipped from purse seines. These scores were compared and were shown to have consistently high comparability scores between the different observers, demonstrating the reliability and reproducibility of the method (Breen et al, n.d.; Riedinger, 2019; Keller, 2020).

In general, the probability of observing low crowding densities was highest early in the capture process and diminished over time (figure 15)(Keller, 2020). Conversely, high density scores had low probabilities of being observed early in the capture process, but these increased over time. However, these probability distributions varied from cast to cast, depending on species, catch size and whether the catch was slipped or taken aboard. Analysis of this data is ongoing and a more complete description will be provided in Deliverable 5.2.

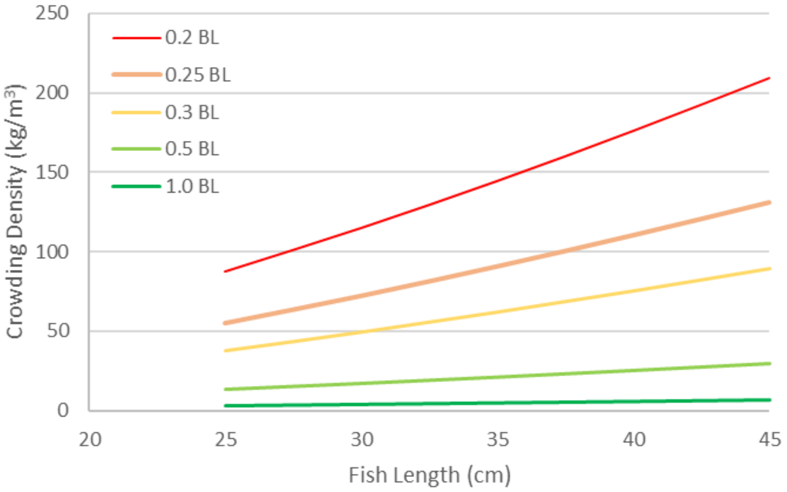


Figure 16 - estimated crowding density (kg/m^3) as defined from Nearest Neighbour Distance (NND; 0.2-1.0 BL) in relation to fish length (25-45cm); based on the assumption of the volume of NND defined space around each fish in a school described in figure 13.

Length-weight relationship parameters from Silva et al 2013.

Building on the assumption about the volume of the NND defined space around each fish in a school, described earlier, it is possible to make theoretical hypotheses about crowding density and the corresponding welfare implications of NND (figure 16). At NND of >0.3BL (CDS = "Low" and "Moderate"), crowding densities for all fish sizes are consistently less than 100 kg/m^3; which was defined in section ### as the limit below which there is consistent evidence for no significant crowding related mortality. At NND of <0.20-0.25BL (CDS = "High" and "Very High"), crowding densities begin to exceed 100 kg/m^3 and mortalities could be anticipated in at least a small proportion of the exposed population. Due to the cubic properties of 3D NND defined space (i.e. $V \sim f\{r^3\}$), crowding density increases in much larger increments as NND gets smaller, particularly at <0.3BL. So, beyond 0.20-0.25BL even small decreases in NND will rapidly results in much higher crowding densities and greater impact on the welfare and survival potential of the affected population. The relationship between NND and crowding density becomes increasingly more length dependent as NND decreases, and density increases. That is, larger fish experience greater crowding densities compared to smaller fish with the same NND. This could imply that smaller fish may experience less welfare impact. However, in general, smaller fish are more susceptible to capture related stress than larger fish, as shown by higher mortality (Veldhuizen et al, 2018). Therefore, based on the precautionary principle and in the absence of data to the contrary, it would be reasonable to assume that smaller individuals are more vulnerable to crowding stress and may die at lower crowding densities. From this we can hypothesise that when we have observations of “Very High” crowding density scores in purse seines, survival likelihood of these fish is likely to have been compromised, and therefore their welfare status is poor. The definition of “High” CDS suggests that observations of fish both below and marginally above potentially fatal crowding densities have been included in this category. Thus, it is difficult to make a definitive statement about their welfare status, other than some fish in this category may have a compromised survival likelihood, in particular if this CDS status is prolonged.

Nearest neighbour distance (NND) and Crowding Density Score (CDS) are emerging as potentially informative S/WIs, which could be central to the development of welfare monitoring in commercial catches in the future. Stereo-camera technology is enabling us to collect data to describe natural variation in preferred NND, from which natural limits of tolerance for school/crowding density in mackerel and herring could be defined. Although, stereo-photogrammetry is not practical for measuring NND at high crowding densities (because of image occlusion), an ordinal scoring method (Crowding Density Score, CDS) for estimating relative NND (in BL) has been developed and tested, and has proved to provide consistent and reproducible estimates of relative crowding density (in BL). Further development is required to refine the NND/CDS scoring method and definitively link estimates of CDS with densities indicative of poor welfare. Once properly refined, this technique could be used in combination with live video images from a catch to provide a qualitative S/WI by an observer, in real-time. However, real-time quantification and recording of the CDS would require the development of machine vision algorithms to automate the process. To this end, as part of this project, many hours of video from commercial purse seine catches have already been codified with respect to CDS (and other metrics) which could be used to expedite the development of the machine vision algorithms. Finally, these methods for estimating NND and CDS are optical and therefore dependent on good underwater visibility and sufficient natural light. To be more generally applicable in commercial fishing scenarios, particularly when fishing at night, alternative methods of visualising the target fish will need to be developed, including the application of artificial light sources that do not affect natural behaviour or alternatively high resolution “acoustic camera” technologies, as used by Handegard et al (2017).

3.3.2 Schooling Order

For mackerel and herring, an integral part of their natural behaviour and survival strategy is schooling behaviour (Breder, 1967; Pitcher & Parrish, 1993). A definitive property of a fish school is that fish within it are synchronised and polarised with respect to swimming speed and direction/orientation (e.g. Figure 17) (Pitcher, 1983). In this section we will generally refer to this synchronisation and polarisation as "schooling order". For obligate schooling fish, like mackerel and herring, if this schooling order was to breakdown (i.e. the fishes' swimming become unsynchronised and depolarised) for a prolonged period of time, in response to an external stressor, it is reasonable to assume that this may be detrimental to the fishes' well-being because the benefits of being in a cohesive school will be lost (Morgan, 2014; Handegard et al, 2017; Anders et al, 2019b).

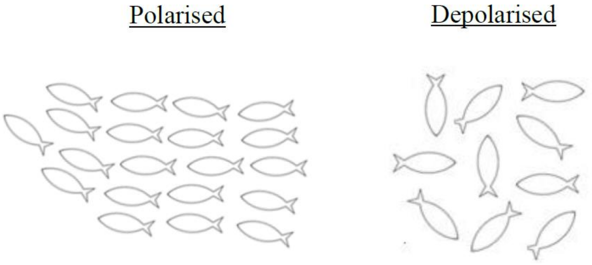


Figure 17: Polarised and depolarised fish aggregations [from Morgan, 2014]

In the RedSlip cage experiments at Austevoll, a stereo-camera was used to measure the angular deviation (AD) between individual fish and showed that unstressed mackerel were highly polarised with a mean AD of just 2^o (Anders et al, 2019b). Due to image occlusion, it was not possible for Anders et al to measure AD in crowded treatments, but unpublished anecdotal observations noted that school order broke down briefly during the onset of the crowding treatments, but then re-established after a short period of time (Breen, unpub.). Even in a seemingly ordered state, Handegard et al (2017) observed in the same experiments a reduced schooling cohesion, in the form of less ordered escape responses to a predator model, at sub-lethal crowding densities (37-78kg/m^3) compared to unstressed control groups. In the current project, loss of order was noted to be maintained throughout the potentially lethal crowding treatments (146-183 kg/m^3)(Anders et al, n.d.; Ochoa, 2020).

During commercial fishing operations schooling order has been assessed using observer-based scoring of video from the catch monitoring probe (CMP), as well as cameras at the discharge opening ("geil")(Breen et al, n.d.; Anders et al, 2019a; Riedinger, 2019; Keller 2020)(See table ## and figure ## for details). These observations have revealed that generally in the net there is a progression from order to disorder over time, as the catch becomes progressively more crowded (Breen et al, n.d.; Riedinger, 2019; Keller, 2020). At the discharge opening ("geil"), this same pattern of order to disorder is also observed over time (figure 19) (Anders et al, 2019a; Keller, 2020). However, Riedinger (2019) and Keller (2020) noted during inter-observer comparison tests that there was poor agreement between observers. This was thought to be due to inconsistent interpretation between observers of the categorical definitions for the scoring system (table 6). However, it may also be the result of biases in the test method (Krippendorf's Alpha), which has been noted to be prone to give low scores when data is predominantly grouped in a single category; which was the case with this data – with most observations being grouped in the "ordered" category. Work is ongoing to resolve this issue.

Table 6 - Definitions for the different levels of the Schooling Order score, as based on observer estimates of synchronisation and polarisation with the aggregation (from Keller, 2020). See figure 18 for example images.

Schooling Order	Definition	Score
Not in view	The school is not in view. No scores applicable.	Z
Ordered	The fish swim in an orderly fashion as one uniform school.	1
Multiple groups ordered	Multiple groups of fish are formed, sometimes swimming in opposite directions. Within sub-schools, fish swim orderly.	2
Multiple Mixed Order	Multiple groups of fish are formed, sometimes swimming in opposite directions. Within sub-schools, some fish swim disorderly	3
Disordered	The fish swim disorderly, not as a uniform school	4
Uncertain order	Camera lens is covered (by something other than fish, i.e. netting) or fish are too distant to score	X

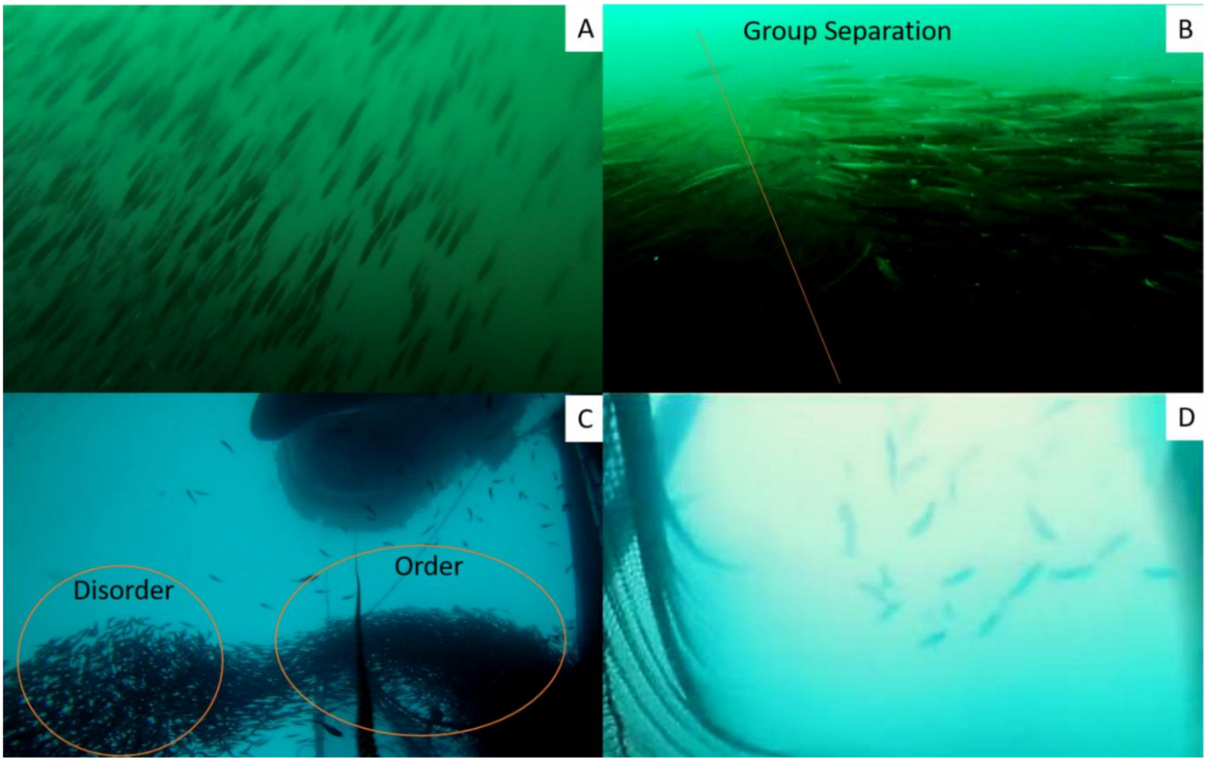


Figure 18 - example images of schooling order categories: A) ordered B) multiple ordered groups C) multiple mixed order D) disorder [from Keller, 2020].

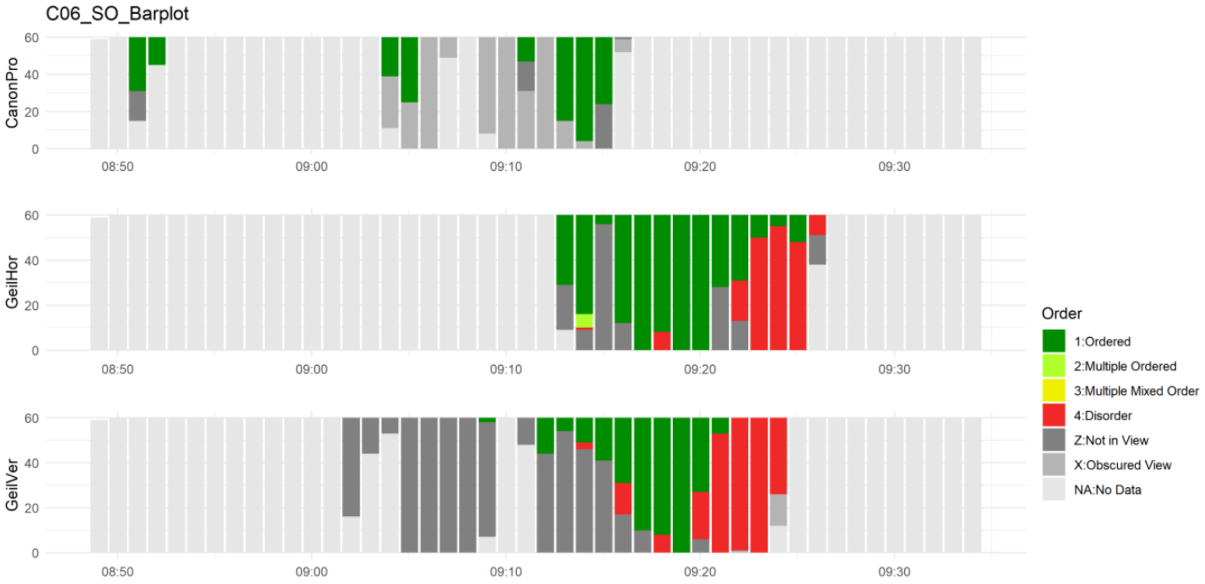


Figure 19 - An example of changes in "schooling order" from "ordered" (green) to "disordered" (red), as observed from three camera positions in the same cast: in the net and at the discharge opening ("geil", both vertically and horizontally)[from Keller, 2020].

Schooling Order appears to be affected at sub-lethal crowding densities. Therefore, it may prove to be an informative early indicator that the catch is becoming stressed. However, in isolation, it is less informative about the welfare status of the catch, because there are no definitive thresholds with which to relate this metric to poor welfare. That is, the metric is essentially binary (i.e. order vs disorder), yet states of disorder have been observed in both lethal and non-lethal treatments. If prolonged, however, it would be reasonable to assume there is an increased risk of physical contact (fish-to-fish and fish-to-net) and therefore prolonged durations of disordered behaviour could be indicative of poor welfare. Ongoing analysis will test this hypothesis by determining whether there are correlations between schooling order and net contact, as well as other candidate S/WIs. Finally, before any confidence can be placed in this metric as an informative S/WI, the issue of poor inter-observer comparability will need to be resolved.

3.3.3 Individual Swimming Activity & Tail Beat Frequency (TBF)

Increases in swimming activity and speed are a common response of pelagic schooling fish in response to stressors (Olsen, 1969; Olla et al., 1975; Misund & Aglen, 1992; Olsen et al., 2012; Rieucau et al., 2016), with the likely functional explanation being an attempt by the fish to enhance avoidance and lessen the allostatic load received from the stressor (Anders, 2020). During the current project, the behavioural response of mackerel to crowding stress has been examined in terms of changes to swimming activity. Using a combination of in-water and overhead video recordings of captive schools, our work has primarily focused upon quantifying the frequency at which individual fish beat their tails (*i.e.* tail beat frequency [TBF]). TBF is likely a better indicator of functional welfare than actual swimming speed, as it more directly reflects energy expenditure. Observations were collected prior to, during and after the application of various degrees of simulated crowding, as well as from control groups that received no treatment.

The results generated in this project (see Ochoa [2020] for further detail) indicate a consistent story that fits well with previous findings (generated during the REDSLIP project, see Anders *et al*., 2019). When exposed to crowding, mackerel TBF increases. The magnitude of the change broadly reflects the intensity of crowding stress, with larger increases in TBF for higher crowding densities (Figure 20A). Little change occurs for non-crowded, control fish (Figure 20A). Once in a crowded condition, TBF tends to decrease over time. The rate of reduction depends on the intensity of crowding, with TBF falling faster when fish are more heavily crowded (Figure 20B). Again, the rate of change is negligible for non-crowded control fish (Figure 20B). Following the cessation of crowding, TBF quickly returns to near non-stressed levels. There is some indication that this recovery takes longer from more crowded fish, but more data is required to confirm this.

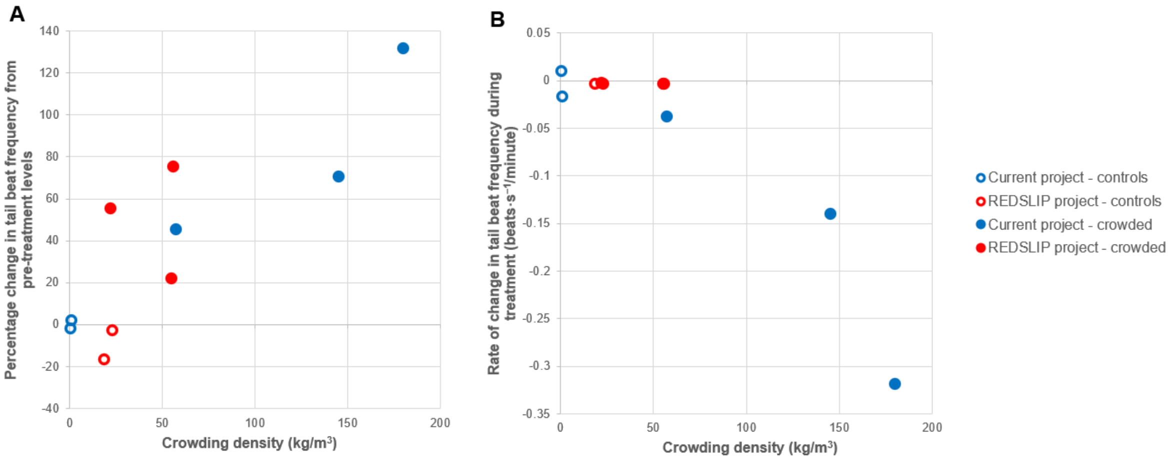


Figure 20: The effect of crowding density on tail beat frequency (TBF) in Atlantic mackerel. *Results from the current project are presented alongside previous observations of TBF during non-lethal, simulated crowding events (REDSLIP project).* ***A****: percentage change in TBF from pre-treatment to treatment start.* ***B****: rate of change in TBF during crowding.*

The maximum hypothesised tailbeat frequency for mackerel is 38 TB/sec, based on muscle twitch measurements (Wardle & He, 1988), while a maximum sustainable TBF (from tank adapted fish) was estimated to be 11 TB/sec (He & Wardle, 1988). TBFs above 11 TB/sec and approaching 20 TB/sec were observed in the crowding treatments at Austevoll. Therefore, the reduction in TBF during crowding likely reflects increasing exhaustion due to the increased activity (Ochoa, 2020). Although these treatments did exhibit a significant mortality, it is unlikely that swimming exhaustion and associated metabolic acidosis was the cause (Wood et al, 1983), because the observed mortality was delayed by at least 4 days and each dead fish had substantial skin lesions (see section 3.3.4). A more likely explanation is that excessive activity during crowding increased the risk of abrasive injury, from which the fish ultimately died (Anders et al, n.d.).

Taken together, these results demonstrate that metrics related to TBF change have high potential as informative S/WIs. This is because they are consistent, sensitive (in that they are exhibited even at sub-lethal levels of stress) and correlate well with stressor intensity. They are also present from the onset of the stressor and are relatively easy to observe. However, there is currently insufficient data to be able to set definitive thresholds for when increased TBF is likely to impact welfare.

Future work related to TBF welfare metrics should attempt to establish the relationship between TBF and stress over a wider range of stressor intensities, as swimming modes are known to alter at extremes of speed (He & Wardle, 1988). If TBF is to be developed into a metric that can used to inform real-time welfare decisions in the fishery, then it must first be confirmed that the same patterns are present in uncontrolled, field conditions. This work is currently ongoing, using footage collected from within commercial purse seine catches. From there, the challenge would be to develop methods to allow monitoring of these metrics in real-time during real capture situations. Split beam echosounders may provide such an opportunity (Handegard *et al*., 2009).

3.3.4 Vitality, Injury & Mortality

Mortality of animals due to exposure to a stressor is arguably the ultimate indicator or expression of poor welfare (Breen et al, 2020). Moreover, several studies have shown that crowding during simulated slipping experiments can cause mortality in herring (*Clupea harengus*) (Tenningen et al. 2012), sardine (*Sardina pilchardus*) (Marçalo et al. 2006; Marçalo et al. 2010) and particularly mackerel (*Scomber scombrus*) (Lockwood et al. 1983; Huse and Vold 2010). However, as an informative and timely welfare metric mortality is not ideal, because it has a low resolution (i.e. binary: alive or dead) and its appearance in a stressed population is likely to indicate that the opportunity for a mitigating intervention to improve welfare is already too late.

Mortality of individual animals is likely to be corelated with the presence of major injuries and/or the absence of characteristic behaviours or reflexes, for example: breathing, balance, locomotion and responding to intrusive stimuli (Dawkins, 2004; Davis, 2010). These physical and behaviour indicators are sometimes collectively referred to as "vitality"; an abstract property of living organisms. Collectively, vitality metrics can be used, either intuitively or systematically, to assess the well-being of an animal; where "full vitality" is the expression of a full suite of observed behaviours by a healthy animal, while "zero vitality" is the absence of any behaviours and indicates an animal is close to death. Some vitality metrics, for example orientation / equilibrium, head complex / breathing reflex, and vestibula-ocular reflex (VOR), have been used to systematically identify when a fish has lost consciousness and/or is close to death (e.g. Anders et al, 2019c; Kestin *et al.*, 2002; Lambooij *et al.*, 2012). However, when suitably correlated with survival probability, vitality metrics can be used to predict mortality in individual fish following exposure to a stressor (e.g. Reflex Action Mortality Predictor, RAMP)(e.g. Humborstad et al., 2009, 2016; Davis 2010; Campbell et al., 2010; Barkley and Cadrin, 2012; Raby et al., 2012).

As was discussed in section 3.1.1, mackerel survival in the cage experiments at Austevoll has been shown to be correlated with crowding density, with no significant mortality at densities less than 100kg.m^{-3} (table 7; also see figure 5 in section 3.1.1). Moreover, any treatment related mortality was delayed (>4 days after crowding) and all dying fish had substantial skin lesions, indicative of abrasive injury (figure 21) (Anders et al, n.d.). The proportion of fish recorded with skin injury was directly correlated with crowding density (figure 22).

Table 7 - Overview of Mortality Results and Vitality sample from Austevoll Crowding Experiments.

		Crowding Density		**No of Fish**						**Mortality**		
Replicate Name	**Date**	Treatment		Total	Removals		Dead	Moribund	Survivors	Proportion	95% Confidence Interval	
		(kg/m^3)	(n/m^3)	n	*Vitality*	*other*	d	m	s	***p***	***p** lower*	***p** upper*
Phase I												
Control 1A	21-May-19	0.76	1.00	149	21	7	0	0	121	0.000	0.000	0.031
Control 1B	28-May-19	0.59	0.78	121	$30^{\S}$		0	0	96	0.000	0.000	0.038
Crowded 1A (High/prolonged)	22-May-19	NA	NA	78	22		78	NA	NA	NA	NA	NA
Crowded 1B (Low)	29-May-19	92.00	122.70	236	$28^{\S}$		0	0	213	0.000	0.000	0.018
Crowded 1C (High/prolonged)	06-Jun-19	182.75	246.54	91	20		91	NA	NA	NA	NA	NA
Phase II												
Control 2	21-Aug-19	0.76	1.17	180	$30^{\S}$		0	0	155	0.000	0.000	0.024
Crowded 2A (Mod)	22-Aug-19	146.21	226.71	136	$26^{\S}$		3	0	112	0.026	0.009	0.074
Crowded 2B (High)	28-Aug-19	179.87	293.59	155	$37^{\S\&}$		32	8	91	0.305	0.233	0.389

High/prolonged treatments were terminated immediately following treatment, hence no survivors

Moratality = (d + m) / (d + m + s)   with Wilson Score 95% Confidence Intervals

§ - includes 5 survivors

& - includes 8 moribunds

Figure 21 - Examples of mackerel skin injuries. All fish with skin injuries, regardless of their severity or location, were classed as “injured” [from Anders et al, n.d.].

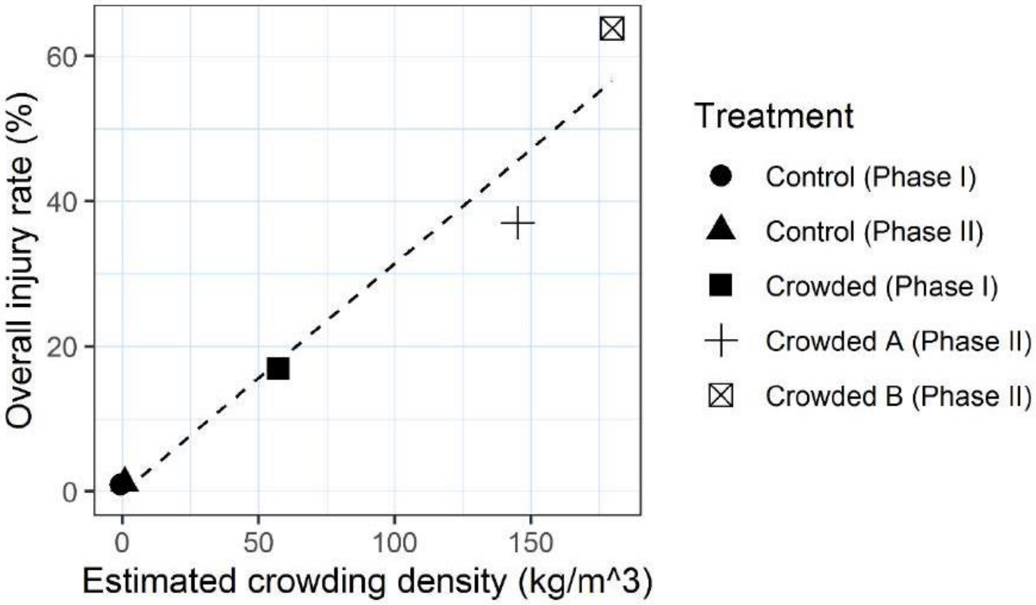


Figure 22 - The relationship between estimated crowding densities and mackerel injury rates. Mortality rates from the different treatments are shown as different points. The dotted line describes a fitted linear relationship of Injury = 0.315 × Density - 0.128 (R2 = 0.94) [from Anders et al, n.d.].

The experiments at Austevoll have demonstrated that crowding, particularly at densities >~100kg/m3, induced poor welfare in the affected fish, in the form of skin injuries and mortality. Based on the delayed mortality and the prevalence of skin injuries amongst all the dead and dying fish, it was concluded that abrasive injury, and the associated allostatic load, was the most likely cause of the mortality observed in the crowding treatments (Anders et al, n.d.). This is supported by our observations of elevated swimming activity during the crowding treatments, which likely increased the risk of the abrasive injury from which the fish ultimately died (section 3.3.3; Ochoa, 2020).

However, before extrapolating these results to a real capture scenario, it is important to recognise the limitations in the experiment set-up during these experiments. Due to the relatively small scale of the experiments, in comparison to commercial catches, the stressors and their intensities are unlikely to have been representative of real capture conditions. In particular, as discussed in section 3.1.2, although dissolved oxygen was decreased in the crowding treatments, it was not as low as seen in commercial catches. Furthermore, the modifying affects of both temperature and fish condition were not investigated (Anders et al, n.d.). This emphasises the need for additional indicators for assessing the stress and welfare of fish in commercial scale catches.

While both injuries and mortality have been very important metrics for demonstrating the effect of crowding in these experiments and for developing informative S/WIs, they themselves will not be informative or practical welfare indicators in commercial fisheries, because they simply cannot inform the fisher soon enough to improve the welfare of the catch. Therefore, further work is required to identify other potential welfare indicators that correlate well with injury prevalence and mortality in controlled experiments, but that can be interpreted during the capture process, ideally in real-time.

3.3.4.1 *Vitality Assessment*

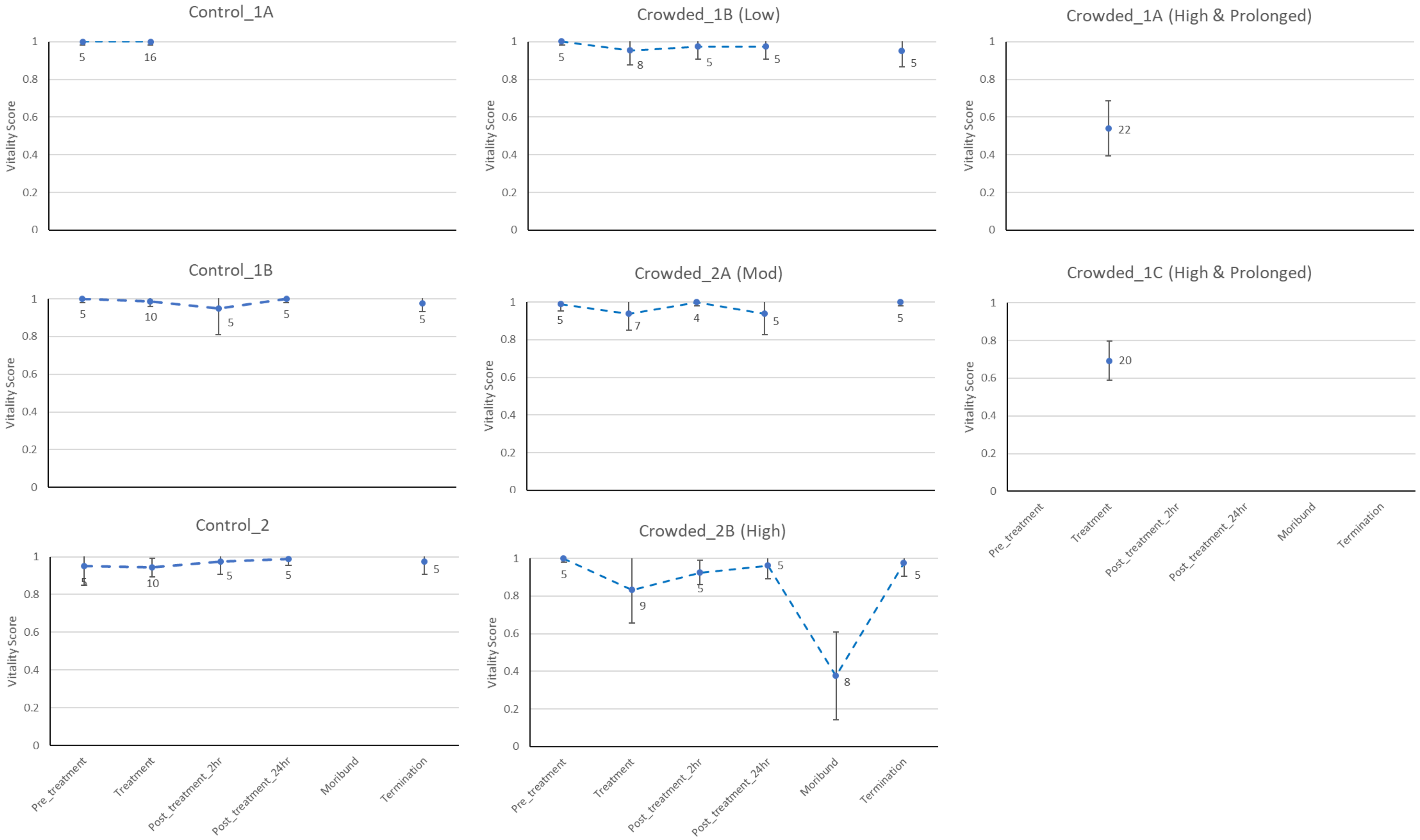
In addition to monitoring survival and the presence of injuries, vitality metrics were also monitored for each fish removed for blood sampling and as moribunds (total: 214 mackerel). Nine different vitality metrics were used in the assessment: 5 free swimming observations (in an observation tank; Evasion 1, Orientation, Head Complex, Evasion 2, Caudal Reflex) and 4 observations while handling (Body Flex 1, Vestibular-Ocular-Reflex (VOR), Mouth Reflex, Body Flex 2)(see table 8). A Total Vitality Score (VS) was then calculated for each fish, by summing the total number of positively observed vitality metrics, divided by the total number of metrics observed on that fish.

Mean total vitality scores (VS; ± 95% confidence interval, CI) for each of the three control replicates and five crowding treatment replicates are shown in figure 23, over several monitoring periods: pre-treatment, treatment, post-treatment (2 hours), post-treatment (24 hours) and termination. Also shown is the mean VS for moribund fish removed from the Crowded 2B replicate.

Table 8 – Summary of vitality metrics used for mackerel sub-sampled from the pump and RSW tanks.

	Test	**Positive Response**	**Negative implications (i.e. response absent or weak)**
Free Swimming Observations			
Evasion 1	Fish transfered from net into observation tank	A "startle" response, or swims around tank seeking "escape".	Fish lacks awareness of substantial change in environment. Or is unable to respond due to exhaustion, or physical injury.
Orientation / Self-righting	Fish transfered from net into observation tank	Can self-orientate dorsal side up within 5 seconds of transfer.	Fish has lost a basic reflex - balance. Therefore, swimming and avoidance of potential threats will be severely compromised.
Head Complex	Fish transfered from net into observation tank	A coordinated and regular use of mouth and operaculae - indicative of normal respiration (> 1 per 10 sec).	Absence - respiratory failure, fish is dead or close to death. Very strong - fish may be hypoxic or fatigued.
Evasion 2	Observer's hand, in water, approaches fish from side; in preparation for "caudal reflex test (see below).	A "startle" response, or swims around tank seeking "escape".	Fish lacks awareness of potential visible threat. Or is unable to respond due to exhaustion, or physical injury.
Caudal Reflex	Observer touches, or attempts to hold, caudal fin.	Fish immediately (<1 sec) attempts to swim away from physical contact.	Fish lacks awareness of potential physical threat. Or is unable to respond due to exhaustion, or physical injury.
Observations While Handling			
Body Flex 1 - Restrained	Observer hold fish firmly in clenched hand, with thumb and fore-finger just posterior of operculae.	Fish should flex its tail musculatur in an attempt to escape (< 3 sec). [NB - test starts in water, as observer attempts to remove fish from tank].	Fish lacks awareness of strong physical threat (i.e. restraining). Or is unable to respond due to exhaustion, or physical injury.
Vestibulo-ocular response	Observer - while holding fish as above - rotates fish on the longitudinal axis.	Fish should attempt to hold eye steady, with respect to horizonal. That is, looking from the posterior, the eye should appear to look down, as the head is rotated clockwise; and *vice versa* .	Fish has lost a basic reflex - balance. May indicate loss of functionality in brain stem.
Mouth Closure	Observer - while holding fish as above - uses finger to open open fish's mouth.	Fish should attempt to resist opening action. May also respond with a "head-complex motion" and/or "body flex" (< 3 sec).	Fish lacks awareness of an intrusive physical threat. Or is unable to respond due to exhaustion, or physical injury.
Body Flex 2 - Flat surface	Fish is laid, unrestrained, on a flat surface.	Fish should flex its tail musculatur (< 3 sec).	Fish lacks awareness of substantial change in physical status - i.e. released but emersed. Or is unable to respond due to exhaustion, or physical injury.

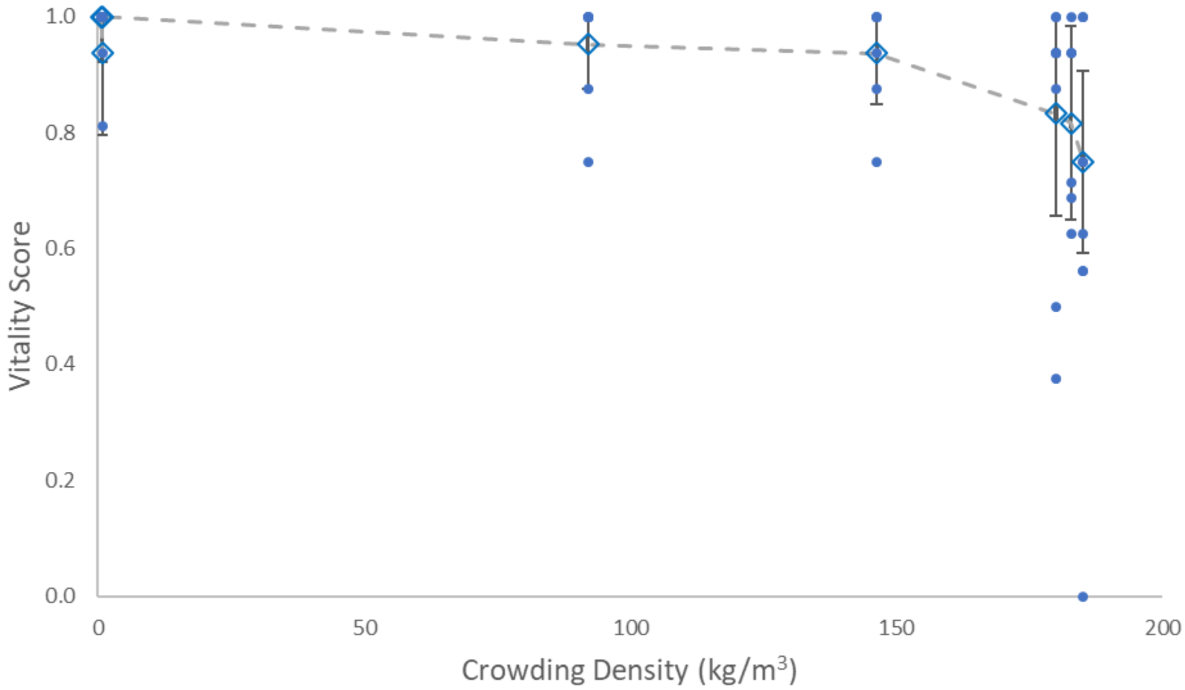
Figure 23 - Mean total vitality scores (VS; ± 95% CI) for control replicates and crowding treatment replicates over several monitoring phases.



For all control observations and all pre- and post-treatment observations in the crowded replicates mean VS was consistently high (>0.95). Moreover, these scores were generally characterised by a low level of variation, as illustrated by the relatively narrow confidence intervals (even with relatively small sample sizes; <10). Significantly low values in VS were only observed during the two prolonged, high crowding treatments (Crowded 1A & 1C) and in moribund fish removed during post-treatment monitoring from Crowded 2B, where mean values were 0.54 (± 0.15), 0.69 (± 0.10) and 0.38 (± 0.23), respectively, and showed high variability in the scores. During the crowding treatment in replicate Crowded 2B the variability was also notably high (s.d.: 0.23; range: 0.38-1.0).

Only the prolonged high crowding treatment 1A showed any significant correlation (negative) with exposure time to crowding (F = 6.2162, n = 22; p = 0.0217). However, individual variability in VS throughout the exposure was very high (s.d.: 0.33; range: 0.0-1.0; $R^2 = 0.24$). Closer examination of VS for fish exposed to 15 min (or less) crowding in each treatment (i.e. excluding the long exposures in the prolonged high crowding treatments, 1A & 1C) suggested vitality was depressed in mackerel exposed to greater than 150 kg/m^{-3} (figure 24).

Figure 24 - Vitality scores (with mean and confidence intervals) for fish exposed to 15min crowding stress, or less, decrease with increasing crowding density.



Most blood plasma stress metrics were significantly correlated to VS, including: cortisol, osmolarity, sodium ions, chloride ions, lactate and plasma pH (table 9). In particular, lactate and osmolarity (and correspondingly the blood ions sodium and chloride) were highly negatively correlated with VS. Conversely, plasma pH was significantly positively correlated with VS. However, in all cases, there was a high degree of variation associated with the relationships, as illustrated by particularly low R^2 values from the regression analysis. There was only marginal evidence that VS may have been positively related to size (i.e. length and weight) (table 9).

Table 9 – Summary of regression analysis of the relationship between Total Vitality Score (VS) and several biometric and physiological variables.

	Observations	**Coefficients**		**Significance**		
	n	Vitality	Intercept	F	p-value	R^2
Biometrics						
Length	143	1.2597	37.1638	3.0957	0.0807	0.0215
Weight	143	88.1141	639.1190	3.4276	0.0662	0.0237
Condition	143	0.0006	0.0122	1.3874	0.2408	0.0097
Physiology						
Haematocrit	143	-3.0351	48.1773	1.3315	0.2505	0.0094
Cortisol	143	-245.6659	482.2069	9.8899	0.0020	0.0655
Osmol.	142	-87.7786	505.8758	79.3056	0.0000	0.3616
Potassium	143	-1.3341	8.2329	2.7695	0.0983	0.0193
Sodium	143	-39.2120	262.9072	44.3579	0.0000	0.2393
Chloride	143	-13.6298	169.0929	7.1230	0.0085	0.0481
Plasma_pH	143	0.3964	6.2779	11.1226	0.0011	0.0731
Lactate	143	-30.7946	35.4196	167.0588	0.0000	0.5423
Glucose	143	0.0505	4.8336	0.0122	0.9123	0.0001

It was interesting to note that there appeared to be a hierarchy amongst the individual vitality metrics with respect to the order in which they were lost. That is, the swimming action metrics, evasion 1 and 2, tended to be lost first, while base reflexes like caudal reflex and head complex were the last to disappear. This concurs with vitality assessments in other species in which the head complex (ventilation) was also one of the last metrics to be lost (Kestin *et al.*, 2002; Lambooij *et al.*, 2012). Furthermore, along with high lactate levels in low vitality fish, the rapid deterioration in swimming ability supports the observation that some fish when crowded can be induced to extremely high activity levels (C.f. tail beat frequency; see section 3.3.3) leading to physiological exhaustion, particularly in high crowding treatments.

Clearly, vitality assessment has potential as an informative S/WI in mackerel during research in capture in purse seine fisheries. Low vitality (VS) was correlated with high stressor (crowding) levels, and therefore potential for mortality, as well as other stress/welfare metrics, demonstrating it is an informative indicator of stress in mackerel. It is a relatively simple technique to perform, with no requirement for specialist equipment or laboratory analysis. Indeed, trials have already been conducted on several research cruises in this project to assess the vitality of mackerel and herring pumped from purse seine catches under different crowding and hypoxic conditions (see section 4.1.2 for further discussion). However, to properly implement vitality as a true welfare metric it will be necessary to conduct further research to generate data definitively linking vitality (VS and individual metrics) with individual survival. In the meantime, work is ongoing in this project to further analyse these data (both from Austevoll and the research cruises) to investigate the correlations between individual vitality metrics and other stress/welfare metrics; with the specific aim of refining this approach to: i) select the most appropriate metrics; ii) improve resolution in the vitality scores; iii) address the high variability in the data; and iv) better understand the underlying mechanisms leading to depressed vitality and increased mortality in stressed mackerel.

Table 10 – Overview of Relevant Potential Stress and/or Welfare Indicators for monitoring stress and or welfare in commercial purse seine fishing operations.

Indicator	Type	Measures	Sampling methodology	Informative of Stress /Welfare Status (comparable, accurate & sensitive)	Practical in Commercial Fishery (useable, reliable & in real-time)	Notes & Caveats
Stressor: Direct & Indirect						
Crowding Density (Dead reckoning)	**Welfare:** survival & quality effects => needs more data	Direct measure of fish and /or biomass per unit volume; n/m^3 & kg/m^3	By dead-reckoning – small scale only	i) Comparable: Good - quantifiable by biomass & volume; with data relating to survival/vitality & quality metrics ii) Accurate: Fair - defining irregular captive volumes can be imprecise. iii)Sensitive: Poor – estimates of maximum density only available post-event.	i) Useable: Poor – impractical at fishery scale; only in experiments. ii) Reliable: Fair - but only in small/medium scale experiments. iii) Real-time: No - estimates of density only available post-event.	a) Practical method for small & medium scale experiments. b) Further data required to improve resolution of high crowding survival & quality effects
Crowding Density (Hydro-acoustics)	**Good Welfare Only**: i.e. only at low densities	Acoustic estimate of density based on target strength n/m^3 & kg/m^3	Ensonification from calibrated transducers on vessel or remotely located in catch	i) Comparable: Poor – unable to measure at high critical densities due to attenuation and occlusion. ii) Accurate: Fair – only at low densities iii) Sensitive: Fair – only at low densities	i) Useable: Good – using commercially available echosounder and sonar technology. ii) Reliable: Good – using commercially available echosounder and sonar technology. iii) Real-time: Fair – only at low densities.	a) Could be used as Good welfare indicator of low density aggregations and early warning of increasing density, before occlusion limit reached. b) could be used to further develop CDS indicator (see below)
Crowding Density (Stereo-grammetry)	**Good Welfare Only**: i.e. only at low densities	Geometrical distribution of target fish in observable volume n/m^3 & kg/m^3	Calibrated stereo-cameras inside affected fish aggregation.	i) Comparable: Poor – unable to measure at high critical densities due to occlusion. ii) Accurate: Fair – only at low densities iii) Sensitive: Poor – estimates of density only available post-event and only at low densities.	i) Useable: Poor - Not tested in fishery; requires specialist software and analysis ii) Reliable: Unknown - Not tested in fishery; but good in experiments. iii) Real-time: Poor – currently requires post-event analysis, but machine vision algorithms in development.	a) Practical method for small & medium scale experiments at low densities. b) could be used to further develop CDS indicator (see below)
Crowding Density Score (CDS)	**Welfare (possible):** survival & quality effects – after calibration with density	Observer based scoring method using perceived NND	Images from cameras (GoPro or 360) deployed on CMP inside affected fish aggregation	i) Comparable: Fair – semi-quantifiable ordinal score, theoretically relatable to crowding density. ii) Accurate: Fair – consistent inter-observer comparability; but work ongoing to calibrate with known densities. iii) Sensitive: Good – instantaneous assessment of primary stressor, if viewed in real-time.	i) Useable: Good – using commercially available cameras deployed on proto-type monitoring platform (CMP). ii) Reliable: Fair – some failed deployment, due to low battery & impact. iii) Real-time: Poor – currently requires post-processing; but direct live-feed from CMP under development.	a) further work required to calibrate with true crowding density & associated mortality. b) development of live feed from CMP with observer assessment and/or and machine vision algorisms could create a true real-time S/WI

Indicator	Type	Measures	Sampling methodology	Informative of Stress /Welfare Status (comparable, accurate & sensitive)	Practical in Commercial Fishery (Useable, reliable & repeatable)	Notes & Caveats
Stressor: Direct & Indirect (cont.)						
Hypoxia: Dissolved Oxygen Conc.	**Welfare**: survival & vitality at known safe levels. => more data needed for fatal levels	dissolved oxygen conc. via optical sensor mg/l & % sat.	Using optical dissolved oxygen sensors: RINKO ID and III	i) Comparable: Good – quantifiable oxygen concentration; related to hypoxia tolerance limits & survival, but more data needed. ii) Accurate: Good – using calibrated oxygen sensors. iii) Sensitive: Good – detects changes of <0.1 mg/l per second.	i) Useable: Good – using commercially available oxygen sensor deployed on proto-type monitoring platform (CMP). ii) Reliable: Fair – few failed deployments, mostly due to failed batteries. iii) Real-time: Fair – currently available on SAIV CTDO; but direct live-feed from CMP under development.	a) Further data need on hypoxia tolerance & survival. b) Data needed on oxygen consumption rates. c) Data during pumping may be useful for defining threshold that impact vitality.
Temperature change	**Welfare**: tolerance limits from literature => more confirma--tory data needed	Water temperature (°C)	Using SAIV CTD(O) or RINKO oxygen loggers (ID)	i) Comparable: Good – quantifiable temperature (°C); related to temperature tolerance limits from literature. ii) Accurate: Good – using calibrated temperature sensors. iii) Sensitive: Good – detects changes of <0.1 °C per second.	i) Useable: Good – using commercially available temperature sensor deployed on proto-type monitoring platform (CMP). ii) Reliable: Fair – some failed deployments, mostly due to failed batteries. iii) Real-time: Fair – currently available on SAIV CTDO; but direct live-feed from CMP under development.	a) Further data need on low temperature tolerance & survival times. b) Data needed on temperatures effects on oxygen consumption rates. c) Data in RSW may be useful for defining thresholds that impact vitality.
Primary: Pre-mortem						
Cortisol	**Stress**: more data needed for welfare inference	Elevation of hormone due to perception of a stressor m.mol/l	Blood samples then Laboratory analysis	i) Comparable: Fair - quantifiable & high res metric; but high natural variability ii) Accurate: Good - consistent response across experiments that reflects the magnitude of the stressor iii) Sensitive: Good - near-immediate response across experiments that reflects the magnitude of the stressor	i) Useable: Unknown - Not tested in fishery; but good in experiments. ii) Reliable: Unknown - Not tested in fishery; but good in experiments. iii) Real-time: No – requires post-sampling processing & laboratory analysis	Naturally variable. Requires control observations or better understanding of typical levels if welfare inferences are to be placed in context

Indicator	Type	Measures	Sampling methodology	Informative of Stress /Welfare Status (comparable, accurate & sensitive)	Practical in Commercial Fishery (Useable, reliable & repeatable)	Notes & Caveats
Secondary: Pre-mortem						
Skin Colour	**Stress**: more data needed for welfare inference	Photography under controlled lighting conditions followed by digital analysis.	Assessment of fish removed from sample population by handline, dipnet or pump.	i) Comparable: Good - consistent quantitative response across treatments that reflects the magnitude of crowding stressor. ii) Accurate: Good - systematic and quantitative metric using image analysis. iii) Sensitive: Fair – responds within ~10 mins of stressor onset; work ongoing to define sensitivity to lower crowding densities.	i) Useable: NA – not tested yet; requires dedicated camera equipment & operator in field experiments. ii) Reliable: NA – not tested yet; camera/flash occasionally failed in cage experiments. iii) Real-time: Good - systematic & quantitative method, but requires specialist image analysis.	An early stage metric which requires further investigation to define underlying physiological mechanisms and development of practical quantitative analysis in the field. Potential for developing a semi-quantitative score in association with Vitality Assessment (see below)
Ionic composition of blood (Na^+, Cl^-, K^+)	NA => not an informative indicator	Changes in blood ionic composition, changes in hydration	Laboratory analysis of blood samples	i) Comparable: Poor - non-reflective of the magnitude of stress ii) Accurate: Poor - inconsistent response across experiments. iii) Sensitive: Poor - inconsistent response across experiments.	i) Useable: Fair – specialist acquisition & processing of samples. ii) Reliable: Fair – ensuring consistent processing & storage of samples could be challenging on fishing vessel iii) Real-time: Poor – only laboratory methods available	
Osmolality	**Stress**: => use as a stress indicator in research	Changes in overall blood composition, changes in hydration	Laboratory analysis of blood samples	i) Comparable: Fair – measurable response at higher crowding densities ii) Accurate: Fair - consistent response across experiments iii) Sensitive: Poor - non-responsive at low crowding densities	i) Useable: Fair – specialist acquisition & processing of samples ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Fair – field methods available but result not immediate	Some application as a confirmatory stress indicator in research.
Haematocrit	NA => not an informative indicator	Changes in oxygen capacity, changes in hydration	Field analysis of blood samples	i) Comparable: Poor - inconsistent response across experiments. May have utility for moribund animals ii) Accurate: Poor - inconsistent response across experiments. iii) Sensitive: Poor - inconsistent response across experiments.	i) Useable: Fair – specialist acquisition & processing of samples ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Fair – field methods available but result not immediate	

Indicator	Type	Measures	Sampling methodology	Informative of Stress /Welfare Status (comparable, accurate & sensitive)	Practical in Commercial Fishery (Useable, reliable & repeatable)	Notes & Caveats
Secondary: Pre-mortem (cont.)						
Glucose	**Stress**: => use as a stress indicator in research	Changes in energetic capacity	Laboratory analysis of blood samples or field analysis using point-of-care device	i) Comparable: Fair – measurable response at higher crowding densities ii) Accurate: Fair - consistent response across experiments iii) Sensitive: Poor - non-responsive at low crowding densities. Response delayed post-stressor.	i) Useable: Fair – specialist acquisition & processing of samples ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Good – POC field methods available with near immediate results	Response delayed. Requires knowledge of the sampling time relative to the stressor and general stress response of individual if welfare inferences are to be placed in context.
Lactate	**Welfare (possible):** survival & quality effects => needs more data	Increase in anaerobic respiration rates, acidosis	Laboratory analysis of blood samples or field analysis using point-of-care device	i) Comparable: Good - measurable response across all crowding densities ii) Accurate: Good – consistent response across experiments iii) Sensitive: Good – immediate response	i) Useable: Fair – specialist acquisition & processing of samples ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Good – POC field methods available with near immediate results	a) more data needed to correlate with mortality b) Should be measured in context with environmental dissolved oxygen concentrations.
Blood pH	NA => not an informative indicator	Acidosis	Laboratory analysis of blood samples	i) Comparable: Poor - inconsistent response across experiments. May have utility for moribund animals ii) Accurate: Poor - inconsistent response across experiments. iii) Sensitive: Poor - inconsistent response across experiments.	i) Useable: Fair – specialist acquisition & processing of samples ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Fair – lab methods adapted to field work but result not immediate	Should be measured in context with blood lactate and environmental dissolved oxygen concentrations.
Muscle pH	**Stress**: => use as a stress indicator in research	Acidosis	Tissue sampling with puncture electrode in the field	i) Comparable: Poor - Unclear whether response reflects the magnitude of stress received ii) Accurate: Fair – consistent response across experiments. iii) Sensitive: Good – immediate response	i) Useable: Fair – specialist acquisition & processing of samples ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Good - easily measured in the field or laboratory with near immediate results	Should be measured in context with blood lactate and environmental dissolved oxygen concentrations.

Indicator	**Type**	**Measures**	**Sampling methodology**	**Informative of Stress /Welfare Status (comparable, accurate & sensitive)**	**Practical in Commercial Fishery (Useable, reliable & repeatable)**	**Notes & Caveats**
Secondary: Post-mortem						
Rigor mortis	**Stress effect on quality** => use as indicator in research	Changes in pre-mortem energetic capacity	Cutting's tail drop method in the field	i) Comparable: Fair – stressed animals most easily distinguished prior to maximal rigor. ii) Accurate: Fair - Unclear whether response reflects the magnitude of stress received or is consistent due to lack of replication. iii) Sensitive: Poor – Post-mortem effect	i) Useable: Fair – Lab methods adapted to field work. Easily scored in the field and validated against analytical equipment if needed ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Poor – Post-mortem effect	Effect magnitude dependent on time. Requires knowledge of the sampling time relative to the stressor if welfare inferences are to be placed in context
Post-mortem muscle pH	**Stress effect on quality** => use as indicator in research	Pre-mortem acidosis	Tissue sampling with puncture electrode in the field	i) Comparable: Fair – stressed animals most easily distinguished earlier into the acidification process. ii) Accurate: Fair - Unclear whether response reflects the magnitude of stress received or is consistent due to lack of replication. iii) Sensitive: Poor – Post-mortem effect	i) Useable: Fair – Lab methods adapted to field work. Easily measured in the field or laboratory ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Poor – Post-mortem effect	Effect magnitude dependent on time. Requires knowledge of the sampling time relative to the stressor if welfare inferences are to be placed in context
Cathepsin B & L activity	**Stress effect on quality** => use as indicator in research	Pre-mortem acidosis	Laboratory analysis of tissue samples	i) Comparable: Fair – large effect size of crowding on values. ii) Accurate: Fair - Unclear whether response reflects the magnitude of stress received or is consistent due to lack of replication. iii) Sensitive: Poor – Post-mortem effect. High variability between individuals means that large sample sizes are likely required.	i) Useable: Poor – only laboratory methods available. ii) Reliable: Fair – ensuring consistent processing & storage of samples could be challenging on fishing vessel iii) Real-time: Poor – Post-mortem effect	

Indicator	Type	Measures	Sampling methodology	Informative of Stress /Welfare Status (comparable, accurate & sensitive)	Practical in Commercial Fishery (Useable, reliable & repeatable)	Notes & Caveats
Secondary: Post-mortem (cont.)						
Water holding capacity	NA => not an informative indicator	Pre-mortem acidosis, changes in pre-mortem energetic capacity	Laboratory analysis of tissue samples	i) Comparable: Poor – no clear response evident. ii) Accurate: Poor – no clear response evident. iii) Sensitive: Poor – no clear response evident.	i) Useable: Poor – only time-consuming laboratory methods available ii) Reliable: Fair – ensuring consistent processing & storage of samples could be challenging on fishing vessel iii) Real-time: Poor – only time-consuming laboratory methods available	
Drip loss	NA => not an informative indicator	Pre-mortem acidosis, changes in pre-mortem energetic capacity	Repeat measurements of weight	i) Comparable: Poor – no clear response evident. ii) Accurate: Poor – no clear response evident. iii) Sensitive: Poor – no clear response evident.	i) Useable: Fair – Lab methods adapted to field work. Easily measured in the field or laboratory ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Poor – Post-mortem effect	
Gaping	**Stress effect on quality** => use as indicator in research	Pre-mortem acidosis, changes in pre-mortem energetic capacity	Qualitative scoring	i) Comparable: Fair – small but significant and consistent effect. ii) Accurate: Fair - Unclear whether response reflects the magnitude of stress received due to lack of replication. iii) Sensitive: Poor – Post-mortem effect	i) Useable: Fair – Lab methods adapted to field work. Easily measured in the field or laboratory ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Poor – Post-mortem effect	Effect magnitude dependent on time. Requires knowledge of the sampling time relative to the stressor if welfare inferences are to be placed in context
Texture	**Stress effect on quality** => use as indicator in research	Pre-mortem acidosis, changes in pre-mortem energetic capacity	Laboratory analysis of tissue samples	i) Comparable: Fair – small but significant effect. ii) Accurate: Fair - Unclear whether response reflects the magnitude of stress received due to lack of replication. iii) Sensitive: Poor – Post-mortem effect	i) Useable: Poor – only laboratory methods available ii) Reliable: Fair – ensuring consistent processing & storage of samples could be challenging on fishing vessel iii) Real-time: Poor – only time-consuming laboratory methods available	Effect magnitude dependent on time. Requires knowledge of the sampling time relative to the stressor if welfare inferences are to be placed in context

Indicator	Type	Measures	Sampling methodology	Informative of Stress /Welfare Status (comparable, accurate & sensitive)	Practical in Commercial Fishery (Useable, reliable & repeatable)	Notes & Caveats
Secondary: Post-mortem (cont.)						
Flesh colour	**Stress effect on quality** => use as indicator in research	Pre-mortem acidosis, changes in pre-mortem energetic capacity, pre-mortem redistribution of blood	Photography under standard lighting conditions followed by digital analysis	i) Comparable: Fair – large but inconsistent effect. ii) Accurate: Fair – inconsistent effect. Unclear whether response is reflective of the magnitude of stress received due to lack of replication. iii) Sensitive: Poor – Post-mortem effect	i) Useable: Fair - can be easily recorded in the laboratory or field, providing standardized lighting is available ii) Reliable: Fair – ensuring consistent processing of samples could be challenging on fishing vessel iii) Real-time: Poor – Post-mortem effect	
Tertiary: Pre-mortem						
Nearest Neighbour Distance (NND)	**Good Welfare Only**: i.e. only at low densities	Geometrical separation of target fish in observable volume in cm or BL	Calibrated stereo-cameras inside affected fish aggregation.	i) Comparable: Poor – unable to measure at high critical densities due to occlusion. ii) Accurate: Fair – only at low densities iii) Sensitive: Poor – estimates of NND only available post-event and only at low densities.	i) Useable: Poor - Not tested in fishery; requires specialist software and analysis ii) Reliable: Unknown - Not tested in fishery; but good in experiments. iii) Real-time: Poor – currently requires post-event analysis, but machine vision algorithms in development.	a) Practical method for small & medium scale experiments at low densities. b) could be used to further develop CDS indicator (see above)
Crowding Density Score (CDS)	**Welfare (Possible):** survival & quality effects – after calibration with density	Observer based scoring method using perceived NND	Images from cameras (GoPro or 360) deployed on CMP inside affected fish aggregation	i) Comparable: Fair – semi-quantifiable ordinal score, theoretically relatable to crowding density. ii) Accurate: Fair – consistent inter-observer comparability; but work ongoing to calibrate with known densities. iii) Sensitive: Good – instantaneous assessment of primary stressor, if viewed in real-time.	i) Useable: Good – using commercially available cameras deployed on proto-type monitoring platform (CMP). ii) Reliable: Fair – some failed CMP deployments due to low battery & impact. iii) Real-time: Poor – currently requires post-processing; but direct live-feed from CMP under development => could then be scored by observer and/or analysed using machine vision.	a) further work required to calibrate with true crowding density & associated mortality. b) development of live feed from CMP with observer assessment and/or and machine vision algorisms could create a true real-time S/WI indicator.

Indicator	**Type**	**Measures**	**Sampling methodology**	**Informative of Stress /Welfare Status (comparable, accurate & sensitive)**	**Practical in Commercial Fishery (Useable, reliable & repeatable)**	**Notes & Caveats**
Tertiary: Pre-mortem (cont.)						
Schooling Order – stereo-grammetry	**Stress**: onset of disorder indicates stress; prolonged disorder may infer poor welfare	Geometrical separation of target fish in observable volume in cm or BL	Calibrated stereo-cameras inside affected fish aggregation.	i) Comparable: Poor – unable to measure at critically high densities due to occlusion. ii) Accurate: Fair – only at low densities iii) Sensitive: Poor – estimates of NND only available post-event and only at low densities.	i) Useable: Poor - Not tested in fishery; requires specialist software and analysis ii) Reliable: Unknown - Not tested in fishery; but good in experiments. iii) Real-time: Poor – currently requires post-event analysis, but machine vision algorithms in development.	a) Practical method for small & medium scale experiments at low densities. b) could be used to further develop Schooling order score indicator (see below)
Schooling Order – score	**Stress**: onset of disorder indicates stress; prolonged disorder may infer poor welfare	Observer based scoring method using perceived NND	Images from cameras (GoPro or 360) deployed on CMP inside affected fish aggregation	i) Comparable: Poor – semi-quantifiable ordinal score, but low resolution (binary). ii) Accurate: Poor – inconsistent inter-observer comparability; but work ongoing to improve this. iii) Sensitive: Fair – disorder tends to develop before damaging crowding densities => could provide early onset indicator.	i) Useable: Good – using commercially available cameras deployed on proto-type monitoring platform (CMP). ii) Reliable: Fair – some failed CMP deployments due to low battery & impact. iii) Real-time: Poor – currently requires post-processing; but direct live-feed from CMP under development => could then be scored by observer and/or analysed using machine vision.	a) further work required to improve consistency of observer scoring b) development of live feed from CMP with observer assessment and/or and machine vision algorisms could create a true real-time S/WI indicator.
Swimming Activity & Tail Beat Frequency (TBF)	**Welfare (possible):** effects on survival => needs more data	Observer based video analysis to count TBF / sec	Images from cameras (GoPro or 360) deployed on CMP inside affected fish aggregation	i) Comparable: Good – TBF correlates well over wide range of crowding densities in cage experiments; also correlated to mortality. ii) Accurate: Good – consistent & unbiased measurement across treatments iii) Sensitive: changes in TBF seen at sublethal levels of crowding stress.	i) Useable: Untested in fishery; but good in experiments, using commercially available cameras deployed on proto-type monitoring platform (CMP). ii) Reliable: Fair – some failed deployment, due to low battery & impact. iii) Real-time: Poor – currently requires post-processing; but direct live-feed from CMP under development.	a) more data require to set definitive welfare thresholds. b) work ongoing to measure TBF from CMP in net to assess practicality. c) Real-time application would require development of live feed cameras and machine vision; or application of split-beam hydro-acoustic technology.

Indicator	Type	Measures	Sampling methodology	Informative of Stress /Welfare Status (comparable, accurate & sensitive)	Practical in Commercial Fishery (Useable, reliable & repeatable)	Notes & Caveats
Tertiary: Pre-mortem (cont.)						
Vitality Score (VS)	**Welfare (possible):** correlation with survival => needs more data	Observer based score of several vitality metrics	Assessment of fish removed from sample population by handline, dipnet or pump.	i) Comparable: Fair, show a correlation with stressors (CD & hypoxia), but more data needed to better define relationships ii) Accurate: Fair – high individual variability. iii) Sensitive: Good – some metrics (e.g. body flex 1) affected soon after exposure to stressor.	i) Useable: Good – easily applied technique, depending on ease of sampling affected population. ii) Reliable: Good – dependent on robustness of observer. iii) Real-time: Good – assessment made immediately.	a) further work required to relate VS to individual survival, and other S/WIs, and better understanding underlying mechanisms. b) work ongoing with current data to better refine technique.
Injury	**Welfare**: All dead fish had substantial skin injury => but not a real-time S/WI	Observer based assessment, with photographic records	Assessment of fish removed from crowding experiments	i) Comparable: Good – injuries definitely link with mortality in experiments. ii) Accurate: Fair – potential for injuries to develop from non-treatment abrasion. iii) Sensitive: Poor – injuries take several days to manifest.	i) Useable: Good - simple observation / photography – easily conducted in field, depending on ease of sampling affected population. ii) Reliable: Good – simple observation / photography – easily conducted in field iii) Real-time: Poor - currently no technique available for assessing undeveloped abrasive skin injury in real-time.	a) as a post-hoc indicator this is intrinsically linked with mortality and hence poor welfare. However, slow development (>4 days) precludes it as an informative welfare indicator (WI). b) development of a real-time method for assessing abrasive injury to the skin would be a major benefit.
Mortality	**Welfare**: Definitive indicator of welfare => but not a real-time S/WI	Observer based vitality assessment, with biometric records	Assessment of all fish removed from crowding experiments	i) Comparable: Good – a definitive indicator of welfare. But individual resolution poor (i.e. binary) so large sample size require to describe effect. ii) Accurate: Fair – potential for non-treatment related mortality; use of controls always recommended. iii) Sensitive: Poor – mortality can take days to manifest post-stressor. NB – vitality assessment used to define death.	i) Useable: Good – easily applied technique, depending on ease of sampling affected population. ii) Reliable: Good – dependent on robustness of observer. iii) Real-time: Good – assessment made immediately.	a) as a post-hoc metric this is a definitive indicator of poor welfare. However, slow development (>4 days) precludes it as an "informative" welfare indicator (WI). b) As S/WI, vitality assessment is better option: higher individual resolution; and used to define death anyway.

4 Discussion

To identify informative stress and/or welfare indicators (S/WIs) this project has investigated over thirty stress related metrics at all levels of the stressor/stress-response interaction:

Stressors – estimating crowding density; directly measuring dissolved oxygen concentrations (hypoxia) and water temperatures; and observing the development of injuries in crowding experiments in Austevoll.

Neurological/Hormonal – measuring plasma cortisol in in crowding experiments in Austevoll.

Physiological –In tank/cage experiments, we have measured a wide range of parameters:

Whole blood – haematocrit, lactate, glucose;
Plasma – pH, osmolarity, sodium, chloride, lactate, glucose;
Muscle – pH, rigor mortis (strength & duration), water holding capacity, etc;
Skin – colour change (green to blue, when stressed).

- in commercial fisheries - only blood lactate (& glucose) in the catch, to date.

Quality – In cage experiments, we have measured presence of injuries, gaping, texture and fillet colour.

Behaviour – from video recorded in cage experiments, as well as in the fishery in the net and during pumping, we have analysed changes in schooling behaviour and order, as well as individual activity (tailbeat frequency). Survival rates have been estimated in dedicated cage experiments in Austevoll, while "vitality" (based on nine different individual reflexes/behaviours) is assessed in individual fish removed from cage experiments and the catch.

This work has identified several potentially useful stress/welfare indicators, which were selected for further investigation in commercial purse seine fisheries – see table 10. The following text provides a brief discussion on the status of candidate stress and/or welfare indicators (S/WI), with respect to the selection criteria defined in section 1.3.3.

Stressor level indicators

Crowding density – Unfortunately no reliable direct metrics for crowding density in commercial fishing scenarios could be identified. However, an indirect measure based on observer-based scoring of NND has proven to be a reliable, repeatable and comparable indicator (see below).

Dissolved oxygen concentration & Hypoxia – is well established as an important welfare indicator. Using the different versions of the CMP, containing RINKO 1D oxygen loggers, systematic measurement of dissolved oxygen has been conducted during hauling, pumping and in RSW tanks in multiple fishing operations. Ongoing analysis will investigate the potential correlation of dissolved oxygen concentrations with crowding density (CDS), and other C/WIs, to enable us to make inferences and hypotheses about the likely importance of hypoxia related effects on slipped catches.

Temperature – is well established as an important welfare indicator, which in this project has been monitored during all observations of commercial fishing operations using either RINKO 1D loggers in the CMPs or using a CTDO. To be able to make inferences about the welfare status of purse seine catches further work should investigate the tolerances of mackerel and herring these to acute temperature change, as well as the factors that may affect it.

Physical contact and skin injury – from our observations at Austevoll, it was apparent that abrasive skin injury takes several days to develop into visible and measurable lesions. Moreover, we currently have no practical methodology for assessing the occurrence and magnitude of abrasive skin injuries on mackerel skin in the field in "real-time", at a gross or histological level. Therefore, it was considered impractical to measure skin injury during commercial fishing operations. However, observations from the CMP video have been made of fish-to-fish and fish-to-net contact. Ongoing analysis will investigate potential correlation of contact frequency with crowding density (CDS), and other C/WIs, to enable us to make inferences and hypotheses about the likely importance of abrasion related injuries amongst slipped catches.

Secondary level (Physiological) indicators

Many of the measured physiological S/WIs responded predictably to the crowding stress treatments at Austevoll, which confirmed that the mackerel were indeed stressed, from a classical physiological perspective. This provided important context for assessing the responses of other S/WIs, particularly the behavioural metrics. However, only whole blood lactate has been systematically measured during commercial fishing operations. This was because most indicators from blood and tissue samples generally require laboratory analysis, so do not meet the "real-time" criterion.

Lactate – both plasma and whole blood lactate proved to be very good S/WIs during the controlled crowding experiments. Furthermore, they were highly correlated with several behavioural S/WIs, most notably swimming activity (TBF) and vitality. It was decided to try to monitor whole blood lactate in commercial fisheries, using a POC device, in blood samples from fish taken during pumping and in RSW storage, because of lactate's role in anaerobic swimming metabolism (Wood et al, 1983). In combination with vitality assessments and dissolved oxygen, it is hoped that changes in lactate will provide more information on the respective roles of ambient and/or functional hypoxia in depressing individual and collective vitality metrics. Note – attempts were also made to measure whole blood glucose, but the glucose POC measuring devices proved unreliable in the damp conditions on deck.

Skin Colour – this novel metric has considerable potential to be an operational S/WI for mackerel in commercial fisheries. More work is required to better understand underlying mechanisms behind the colour changes, before it can be considered as a welfare indicator. Unfortunately, due to lack of resources, we have not been able to investigate the potential for this S/WI during observations in commercial fisheries. Its potential as a real-time S/WI could be further developed by incorporating it as a qualitative score in an observer-based vitality assessment, perhaps aided by the use of standard colour reference cards.

Tertiary level (Behavioural, anatomical & whole animal) indicators

Nearest Neighbour Distance (NND) and Crowding Density Score (CDS) – in the absence of reliable direct methods of measuring the primary stressor, crowding density, these related S/WIs have become an essential metric for mapping welfare status during the capture process. It is now systematically scored from all videos recorded by the CMP. Although it is a semi-quantitative observer-based score, multiple inter-observer comparison assessments have demonstrated it as a reliable, repeatable and comparable indicator (Breen et al, n.d.; Riedinger, 2019; Keller, 2020). Further work is required to determine how crowding density (fish/m^3 and/or kg/m^3) translates to the NND scale used by this metric, before it can be considered a true welfare indicator. Moreover, further work should be conducted to better understand the relationship between crowding density, mortality and meat quality, particularly at densities greater than 100 kg/m^3, to properly define thresholds for welfare status.

Schooling Order - proved to be an informative early indicator that the catch is becoming stressed. However, in isolation, it is less informative about the welfare status of the catch, because there are no definitive thresholds with which to relate this metric to poor welfare. Ongoing analysis will test the hypothesis that, if prolonged, schooling disorder may be linked with is an increased risk of physical contact (fish-to-fish and fish-to-net) by determining whether there are correlations between schooling order and net contact, as well as other candidate S/WIs. Finally, before any confidence can be placed in this metric as an informative S/WI, an ongoing issue of poor inter-observer comparability will need to be resolved.

Swimming Activity (TBF) – and linked metrics, have high potential as informative S/WIs. However, there is currently insufficient data to be able to define definitive thresholds for when increased TBF is likely to impact welfare. Future work related to TBF welfare metrics should attempt to establish the relationship between TBF and stress over a wider range of stressor intensities, as swimming modes are known to alter at extremes of speed (He & Wardle, 1988). If TBF is to be developed into a metric that can used to inform real-time welfare decisions in the fishery, then it must first be confirmed that the same patterns are present in uncontrolled, field conditions. This work is currently ongoing, using footage collected from within commercial purse seine catches.

Vitality - has strong potential as an informative S/WI in mackerel during research in capture in purse seine fisheries. It is a relatively simple technique to perform, with no requirement for specialist equipment or laboratory analysis. However, to properly implement vitality as a true welfare metric it will be necessary to conduct further research to generate data definitively linking vitality (VS and individual metrics) with individual survival. In the meantime, work is ongoing in this project to further analyse these data (both from Austevoll and the research cruises) to investigate the correlations between individual vitality metrics and other stress/welfare metrics; with the specific aim of refining this approach to: i) select the most appropriate metrics; ii) improve resolution in the vitality scores; iii) address the high variability in the data; and iv) better understand the underlying mechanisms leading to depressed vitality and increased mortality in stressed mackerel.

Clearly, not all indicators can be measured practically at all stages of the capture and handling process. For example, it has proved impractical to get vitality and physiology samples from fish in the net during hauling and/or slipping, because mackerel easily evade our sampling net. For this reason, samples were taken from the pumped catch to provide some indication of what impact crowding and hypoxia during pumping had on vitality (and physiology); working on the assumption that this would be representative of a worst-case scenario of crowding during a slipping event.

Moreover, not all indicators could be considered practical for measuring on a routine basis in a fishery context, i.e. operational stress/welfare indicators. In particular, physiological indicators from blood and tissue samples generally require laboratory analysis, so do not meet the "real-time" criterion. Even indicators, like lactate and glucose, for which there are practical point-of-care devices giving near real-time results require sampling protocols that will be practical from a research context, but likely disruptive as an operational S/WI in a commercial fishery. S/WIs that provide near real-time data and/or intuitively interpreted results, like oxygen concentration, temperature and the behavioural indicators, show more promise as potential operational S/WIs. But in all cases further work is required to make them either more "informative" and/or "practical" as indicators of welfare status of purse seine catches.

4.1 The importance of using integrated metrics

As discussed, the stress response in fish is complex and variable. It is expressed at multiple biological levels: neurologically, hormonally, physiologically and behaviourally. Moreover, it is dependent on what is stressing the animal, and to what degree, and responses can vary between, and even within, individual fish. So, to be confident about our interpretation of the stress responses during different phases of the capture process, we must investigate different stress indicators simultaneously. However, not all stress indicators are usable at all stages of the fishing operation, so it is necessary to combine and compare indicators at different stages of the capture process to better understand them and give us more confidence in our conclusions. For example, in the net we are limited to observing crowding (using CDS), behaviour and oxygen concentrations. But during pumping, which is analogous to crowding during a slipping event, we can also observe vitality and physiology indicators, which gives us a better insight into what may be happening to the catch in terms of potential survival, if released, and fillet quality, if retained.

We will now briefly discuss these two scenarios to demonstrate how the separate S/WIs can be interpreted together to make inferences about the welfare status of the catch.

4.1.1 Observations in the net during capture.

Observations from the CMP have confirmed that crowding density increases, as expected, during the hauling process (figure ##). This initiates behavioural changes in the catch, which progresses from orderly, coordinated schooling behaviours to increasingly more disordered behaviours (figure 25). The hypoxic stressor typically does not develop until after the school has become very crowded and disordered (figure 25).

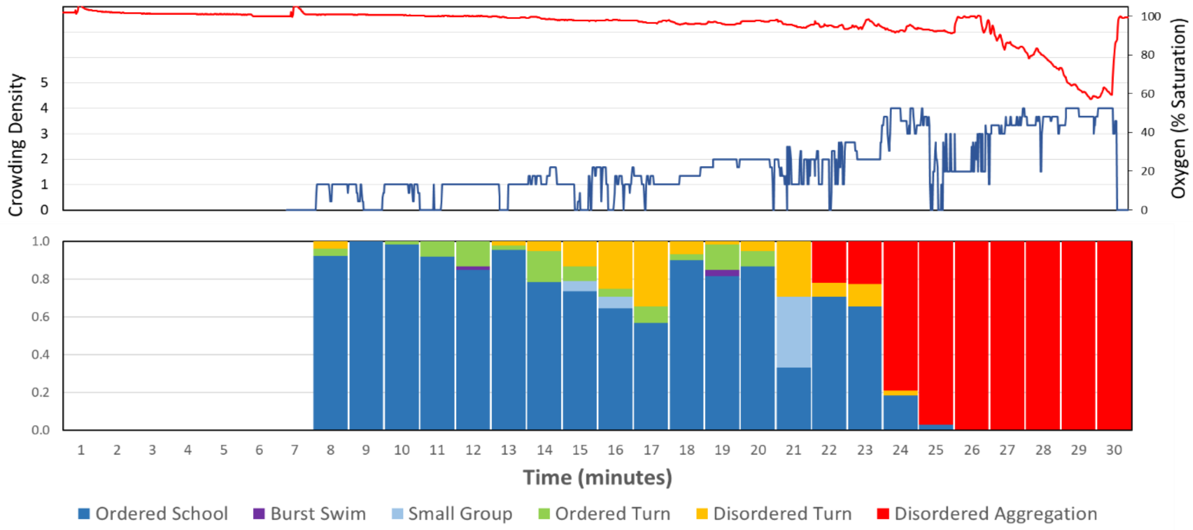


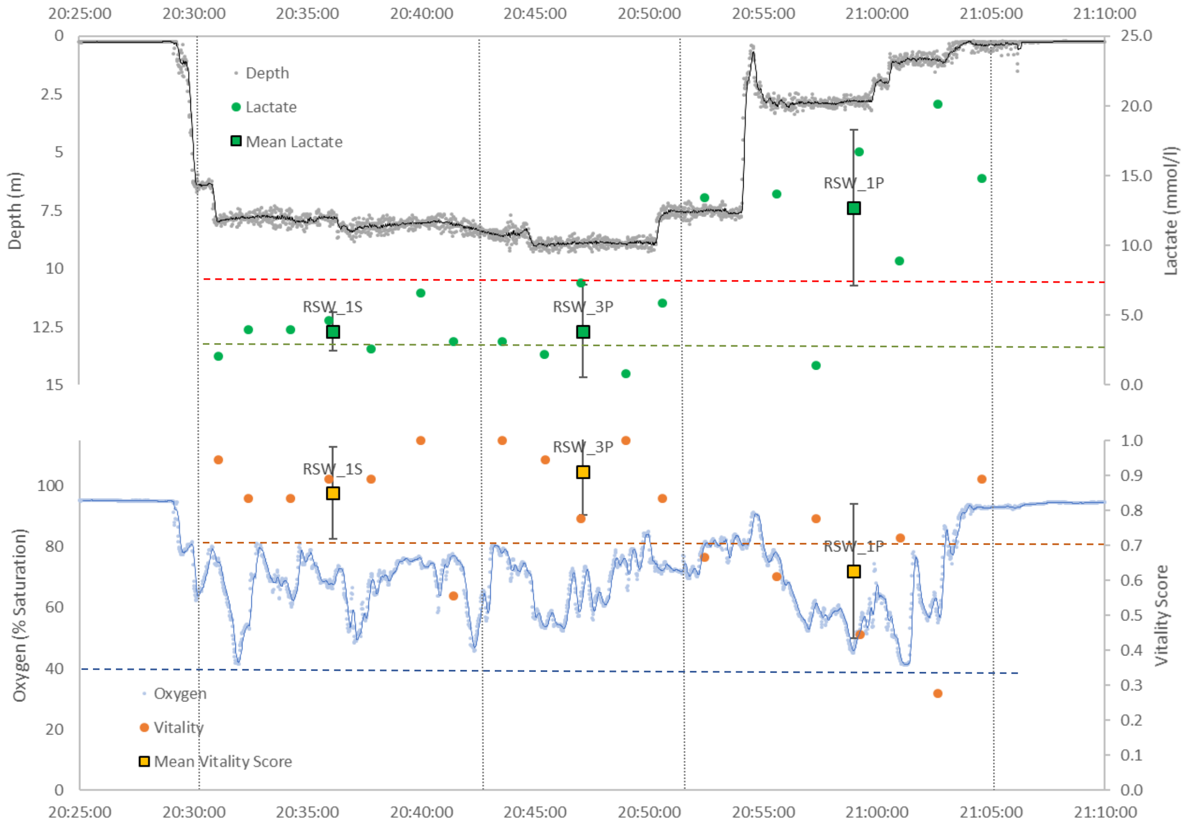
Figure 25: crowding density (score), oxygen concentration (% saturation) in relation to observed behaviours in a retained catch during hauling.

Similar observations have been made in slipped catches, using the CMP, but without the development of severe hypoxic events (i.e. <40% saturation). In addition, using cameras placed at the discharge opening, as well as outside the opening, observations have been made of the behaviour of slipped catches which demonstrate a similar progression from ordered to disordered behaviour over time (Anders et al, 2019a). This suggests a link between the disordered behaviour at the discharge opening and the state of order of the catch within the net. It has been challenging to acquire slipping observation data on this project, but we now have several observations of slipped events with simultaneous data from the CMP and cameras at the discharge opening. Work is ongoing to investigate this linkage, where we will attempt to combine data on crowding, behaviour, activity (tail beat frequency) and oxygen concentrations in the catch, with crowding, behaviour and activity data at the discharge opening.

4.1.2 Observations during pumping.

As discussed, it proved impractical to get vitality and physiology samples from fish in the net during hauling and/or slipping, because mackerel easily evade our sampling net. For this reason, samples were taken from the pumped catch to provide some indication of what impact crowding and hypoxia during pumping had on vitality and lactate; working on the assumption that this would be representative of a worst-case scenario of crowding during a slipping event. This has also given us the opportunity to investigate the effects of conditions during pumping and storage in the RSW tanks on the catch stress/welfare status.

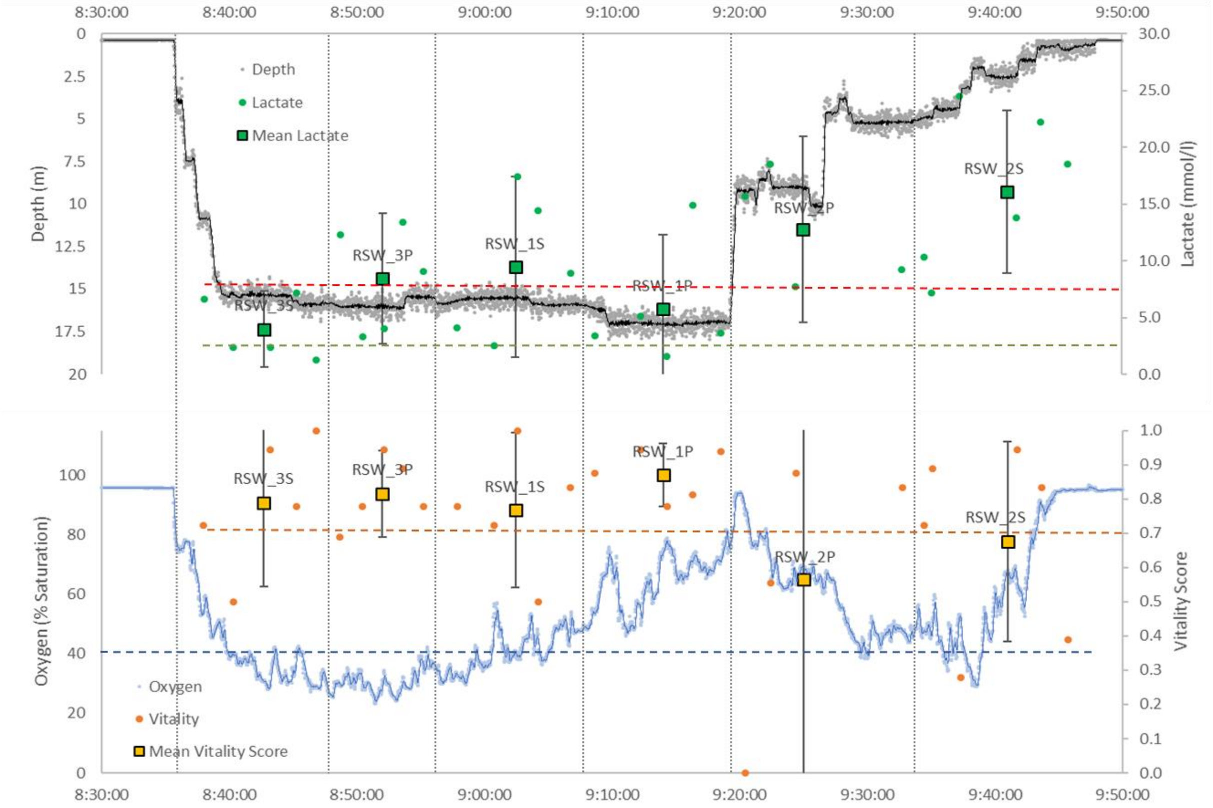
During pumping, the catch is consistently crowded (at “High” and “Very high”). Despite this, Figure 26 shows that blood lactate concentrations and vitality scores can indicate relatively low stress levels in most fish, for prolonged periods (~20 mins), provided oxygen concentrations in the water remain above the “safe threshold” of 40% saturation (as defined by Handegard et al, 2017). However, towards the end of pumping the mackerel were becoming fatigued (lactate > 7.4 mmol/l) with reduced vitality (< 0.7). This is thought to be due to excessive crowding as the net was lifted during the final stage of pumping (note reduced depth of net).



Stress/Welfare Status	Good (1.6)	Compromised? (1.4)	Compromised (1.0)	
Crowding	Compromised (1)	Bad (0)	Bad (0)	
Hypoxia	Good (2)	Good (2)	Good (2)	
Lactate	Compromised? (1.5)	Compromised? (1.5)	Bad? (0.5)	
Vitality	Good (2)	Good (2)	Compromised? (1.5)	

Figure 26 – Fiskebas, cast #13 (190 tonnes); Above: Blood lactate (mmol.l^{-1}) and CMP (pump) depth (m) over time during pumping. Vertical dotted lines: delimiting sub-sampling periods (i.e. when individual RSW tanks were being filled); Red dashed line: lactate concentrations associated with quality effects and mortality of 11.5% (95% CI: 3-33%) in Austevoll experiments (see figure 9). Green dashed line: control (non-stressed) lactate levels from Austevoll experiments. Below: Vitality scores and oxygen concentration (% saturation) over time, during pumping. Orange dashed line: nominal level for "normal" vitality (0.7), below this the fish is considered to be compromised. Blue dashed line: "safe dissolved oxygen concentration threshold" for crowded mackerel (40 % saturation) as defined by Handegard et al (2017).
Under: a summary matrix (hypothetical) of stress welfare indicators. For details of its calculation and interpretation see appendix 1.

However, figure 27 shows that during a hypoxic event (<40% saturation; probably as a result of the larger biomass in the catch) lactate concentrations begin to exceed a hypothetical "safe threshold" of 7.4mol/l, suggesting the fish are becoming physiologically exhausted (see Appendix 1 for further explanation). This is also reflected in lower vitality scores in the same period, but these do not become compromising (on average) until the end of the pumping (after >40 mins), when again the net is hauled up.



Stress/Welfare Status	Comp.? (1.3)	Bad ? (0.5)	Bad ? (0.75)	Comp.? (1.4)	Comp. (1.0)	Bad ? (0.6)	
Crowding	Comp. (1.0)	Bad (0.0)	Bad (0.0)	Bad (0.0)	Bad (0.0)	Bad (0.0)	
Hypoxia	Comp. (1.0)	Bad (0.0)	Comp. (1.0)	Good (2.0)	Good (2.0)	Comp. (1.0)	
Lactate	Comp.? (1.5)	Bad ? (0.5)	Bad ? (0.5)	Comp.? (1.5)	Bad ? (0.5)	Bad (0.0)	
Vitality	Comp.? (1.5)	Comp?(1.5)	Comp.? (1.5)	Good (2.0)	Comp.? (1.5)	Comp.? (1.5)	

Figure 27 – Fiskebas, cast #04 (400 tonnes); Above: Blood lactate (mmol.l^{-1}) and CMP (pump) depth (m) over time during pumping. Vertical dotted lines: delimiting sub-sampling periods (i.e. when individual RSW tanks were being filled); Red dashed line: lactate concentrations associated with quality effects and mortality of 11.5% (95% CI: 3-33%) in Austevoll experiments (see figure 9). Green dashed line: control (non-stressed) lactate levels from Austevoll experiments. Below: Vitality scores and oxygen concentration (% saturation) over time, during pumping. Orange dashed line: nominal level for "good" vitality (0.7), below this the fish is considered to be compromised. Blue dashed line: "safe dissolved oxygen concentration threshold" for crowded mackerel (40 % saturation) as defined by Handegard et al (2017).
Under: a summary matrix (hypothetical) of stress welfare indicators. For details of its calculation and interpretation see appendix 1.

4.2 Conclusion

To identify informative stress and/or welfare indicators (S/WIs), this project has investigated more than thirty metrics at all levels of the stressor/stress-response interaction. S/WIs that provide near real-time data and/or intuitively interpreted results, like oxygen concentration, temperature and the behavioural indicators (nearest neighbour distance (NND), crowding density score, swimming activity and vitality) show most promise as potential operational S/WIs. Many of the physiological stress indicators showed significant correlations with crowding stress, mortality and meat quality. However, they have not been identified as potential candidates as operational SWIs, because they generally require complex processing and laboratory analysis, so could be disruptive to fishing operations and would not provide information soon enough to be effective in mitigating catch welfare. From a research perspective, however, efforts should be made to continue to measure physiological indicators, alongside other S/WIs, during commercial fishing operations to promote and support the development of operational S/Wis. One secondary level indicator, skin colour change, does show great potential as a S/WI for mackerel. Work is ongoing to further substantiate the supporting evidence for this indicator.

The rationale for this wide array of metrics in this WP has been to ensure we reliably characterise the stress responses throughout the capture process, identifying where and when critical stressors occur, as well as developing suitable indicators to monitor catch status at those critical points. By combining different stress/welfare indicators, we can make more confident inferences about the stress/welfare status of the catch.

In all cases, these S/WIs will require further development to ensure they satisfy the informative (relevant, comparable, accurate & sensitive) and practical (usable, reliable & real-time) criteria for acceptable operational indicators. In particular, it will be necessary for future projects to substantiate the data on the effects of higher crowding densities and hypoxia on individual survival/vitality and quality parameters, and link these with other potential S/WIs.

Over the course of the remainder of the project, data collected on the research cruises will be used to describe, in context with the S/WIs described in the report, the behaviour and stress/welfare status of mackerel (& herring) during capture and slipping in purse seines (Deliverable 5.2; due 31-03-21).

The knowledge generated in this project has begun the development of pragmatic operational stress/welfare indicators, with the ultimate aim of eventually providing monitoring tools for everyday use in purse seine fisheries. If these are developed fully, they could be beneficial for promoting both the survival of the released unwanted catch and the quality of the retained catch from Norwegian purse seine fisheries.

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Appendix A - Hypothetical matrix of stress/welfare status indicators.

This section will introduce a hypothetical matrix of stress/welfare status indicators for mackerel during capture in purse seines. By combining and comparing different stress/welfare indicators we can develop a more complete and synergistic overview of the stress response in the affected fish. This will give us more confidence in the inferences we make about stress/welfare status of the catch, and the resultant survival of any released catch and/or fillet quality of any retained fish.

Table A1 – Hypothetical stress/welfare status indicator thresholds for mackerel during capture in purse seines.

	OWI Utility Score	**Catch/Welfare Status**		
		Good	**Compromised**	**Bad**
Mortality of UWC		Unlikely	Possible	Highly Likely
Impact on quality		Unlikely	Possible	Highly Likely
Stressors				
Crowding Density (Ordinal Score)	2	Prolonged "Low" or "Moderate" (e.g. > 5 min)	Acute "High" (e.g. < 15 min) or "Very High" (e.g. < 5 min)	Prolonged "High" (e.g. > 15 min) or "Very High" (e.g. > 5 min)
Hypoxia	2	> 40% Saturation prolonged (e.g. > 5 min)	< 40% Saturation Acute (e.g. < 5 min)	< 40% Saturation prolonged (e.g. > 5 min)
Behaviour				
Schooling Behaviour	2	Sustained order	Acute disorder (e.g. < 5 min)	Prolonged Disorder (e.g. > 5 min)
Individual activity (Tail Beat Frequency)	3	< 11 beat/second prolonged (e.g. > 5 min)	> 11 beat/second acute (e.g. < 5 min)	> 11 beat/second prolonged (e-g- > 5 min)
Vitality Score (in pumped catch)	2	>0.7	0.3 - 0.7	<0.7
Physiology / Quality				
Blood Lactate (in pumped catch)	2	< 2.5 mmol/litre	< 7.4 mmol/litre	> 7.4 mmol/litre
Skin colour	3	Green in majority of subsample	Blue in sig proportion (e.g. 25%) of subsample	Blue in majority (e.g. >50%) of subsample
Gaping §	1	No evidence in subsample from landed catch	Evidence in sig proportion (e.g. 25%) of subsample	Evidence in majority (e.g. >50%) of subsample
Texture (soft) §	3	No evidence in subsample from landed catch	Evidence in sig proportion (e.g. 25%) of subsample	Evidence in majority (e.g. >50%) of subsample
Colour §	3	No evidence in subsample from landed catch	Evidence in sig proportion (e.g. 25%) of subsample	Evidence in majority (e.g. >50%) of subsample

§ - quality metrics assumes samples held in well controlled RSW (<-1°C) for <48hours.

Operational Welfare Indicator (OWI) Utility Score

1	Can be used in fishery now with no training or specialist technology
2	Can be used in fishery now but requires training and/or specialist technology
3	Requires specialist *post-hoc* analysis and/or currently unavailable technology

Table A1 shows hypothetical stress/welfare status indicator thresholds for mackerel during capture in purse seines. While based on observations and data from this and other related projects, these remain totally hypothetical at this stage and are presented here only to illustrate the ultimate goal of this work package. Before they can be used in any meaningful way, more data and further analysis are required to better define such thresholds for a range of relevant indicators, as well as their relative importance, to enable them to be combined to give an overall indication of the stress/welfare status of the catch. These thresholds will ideally be based on empirical data demonstrating the relationship between the indicator (stressor or response) and one or both of the stress/welfare outcomes (mortality and/or fillet quality), or some corresponding physiological limits. Each threshold will incorporate an upper/lower level for the magnitude of the indicator with respect to three categories of stress/welfare: “good”, “compromised” and “bad”. Where appropriate, the threshold may also incorporate a duration defining “acute” and “prolonged” exposure. [In several example presented here we have used the arbitrary period of 5 minutes.] An example of how thresholds can be defined are illustrated for Blood lactate in figure 9.

For illustration, we will use the examples presented in figures 24 and 25 to demonstrate how the matrix could be used. Referring to figure 26, the catch was initially crowded at a “high” level, therefore in the first sampling period (20:30 to 20:43) we see from Table A1 that this relates to a status of “Compromised” and is given a score of 1.0 (table A2). Oxygen concentration in the same period remains above 40% saturation and so is defined as “Good”, scoring 2.0. The mean lactate concentration (3.86 ± 1.38 mmol.l^{-1}) is above the “safe” threshold of 2.5 mmol.l^{-1}, suggesting this indicator’s status is “Compromised”. However its confidence interval brackets the safe threshold, so there is some uncertainty on this status; therefore it is given the status “Compromised ?”, scoring 1.5. The Vitality Score and its corresponding confidence interval are clearly above the “Safe” threshold of 0.7, giving it a “Good” status which scores 2.0. These scores are arbitrary in this example, but could be refined for a real scenario by better scaling the score with respect to the magnitude of the indicator value relative to its threshold levels. Finally, for that observation period we can establish an overall score by taking the mean score of all the indicators combined. This too could be refined in the future by weighting the different indicators relative to their known effect sizes upon the stress/welfare outcomes (mortality and/or fillet quality).

Table A2: Indicator status scoring.

Stress/Welfare Status	**Indicator Score**	**Mean Score**
Good	2.0	1.6 - 2.0
Compromised ?	1.5	1.2 - <1.6
Compromised	1.0	0.8 - <1.2
Bad ?	0.5	0.4 - <0.8
Bad	0.0	0.0 - <0.4

By applying this approach to each observation period in the examples in figures 24 and 25, we see that a combination of different indicators enables us to consider the balance of evidence for the overall stress/welfare status of the catch, while accounting for the uncertainty of individual indicators. This gives us a more robust interpretation of the overall stress/welfare status of the catch.

With respect to interpreting the stress/welfare status of the catch during a slipping event, we envisage combining data on crowding, behaviour, activity (tail beat frequency) and oxygen concentrations in the catch, with crowding, behaviour and activity data at the discharge opening.