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Reflex impairment, physiological stress, and discard mortality of European plaice *Pleuronectes platessa* in an otter trawl fishery

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The reformed European Common Fisheries Policy introduced a discard ban, with a possibility of exempting species where a high discard survival can be demonstrated. This necessitates a validation of the methods used for estimating the discard mortality of candidate species. In this study, we assess whether reflex impairment can predict short-term mortality in commercially trawled European plaice upon landing and after air exposure of up to 90 min. Sub-lethal stress was assessed by a suite of physiological variables. Over a 10-day period, mortality was monitored for a total of 199 plaice following trawl and air exposure of varying duration, and for 50 control fish scored for reflex impairment on board the vessel. Mortality was only observed in fish exposed to air for >60 min, and averaged 11.1% (95% CI = 7.1–16.3%). Reflex impairment was found to be a significant ($P < 0.001$) predictor of mortality in a generalized linear model, excluding other initially included variables by using a stepwise method. Plasma cortisol, haematocrit, and plasma osmolality all indicated a profound and increasing level of stress with air exposure, accompanied by a near depletion of muscle phosphocreatine and nucleotides. Fishing site had an unexpected, but significant ($p < 0.05$) effect on stress levels, which was also reflected in reflex impairment and mortality. Based on these findings, a possible exemption from the discard ban should include considerations on the duration of air exposure.

Keywords: adenylate energy charge, air exposure, discard survival, Pleuronectidae, RAMP, reflex action mortality predictor, stress response.

Introduction

Discard refers to the part of a catch that is not retained on board during commercial fishing operations, but instead returned to the sea (Catchpole *et al.*, 2014). Discarding occurs in most fisheries worldwide (Kelleher, 2005), including European waters, where high discard rates are observed in several important fisheries (Davis and Olla, 2011; Catchpole *et al.*, 2014; Uhlmann *et al.*, 2014). European plaice (*Pleuronectes platessa*) is one of the most important species in the North Sea, Skagerrak, and Kattegat area (Feekings *et al.*, 2012; Madsen *et al.*, 2013). The beam and otter trawl fishery for plaice in the North Sea (Aarts and Poos, 2009; Madsen et al., 2013) and Kattegat area (Feekings et al., 2012) have, until recently, been characterized by discard rates as high as 50% of the catch by weight (ICES, 2011). Discarding of plaice has occurred for a number of reasons, such as individuals being under the minimum landing size or being physically damaged.

A reform of the European Common Fisheries Policy, CFP (EEC, 2011, 2012; Sardà *et al.*, 2015) aimed to eliminate discards by landing obligations, where all individuals of certain species caught are to be landed (Sardà *et al.*, 2015) as well as count against the quota of a given vessel. Landing obligations for specific fisheries are to be gradually implemented between the years 2015 and 2019, with the possibility for either a "*de minimis*" or a "high survival" exemption (European Commission, 2013). What constitutes high survival has, however, yet to be defined by the European Commission.

While species with scientifically documented high survival rates can be exempted from the landing obligation (European

Commission, 2013), discarding is generally associated with a high mortality (Broadhurst *et al.*, 2006), albeit with some variability. For European plaice, Depestele et al. (2014a) estimated 68% survival 57 h after trawling and 48% after 77 h, Van Beek et al. (1990) estimated discard survival from a beam trawl from 2 to 48% based on an experimental observation period up to 84 h, and between 0 and 54% in an otter trawl. Finally, Kaiser and Spencer (1995) estimated a 39–40% survival in beam trawls following 120–144 h observation periods.

Estimates of discard mortality are affected by the experimental approach and available resources, and therefore carry inherent uncertainties. As such, it is relevant to consider whether additional, and less resource demanding methods, can be used as predictors of short-term mortality. Vitality assessments, based on scoring of injuries or impairment of reflexes (i.e. reflex action mortality predictor, RAMP) to predict discard mortality, are both described methods (Benoît *et al.*, 2010, 2012). Vitality assessments can be done on the fishing vessel immediately after the catch is taken on board without using extraordinary equipment. The RAMP method is well described (Davis and Ottmar, 2006; Davis, 2007, 2010) and has been tested on several species of elasmobranchs (Gallagher *et al.*, 2014) and decapods (Stoner, 2012), and a wide number of teleosts, including several species of flatfish (Davis, 2010; Humborstad *et al.*, 2016; Uhlmann *et al.*, 2016). A RAMP test consists of a suite of observations of the voluntary behaviour, stimuli response, and clinical reflexes of the fish. However, because these are species specific (Kestin *et al.*, 2002; Uhlmann *et al.*, 2016), it is necessary to conduct RAMP tests at the species level, in order to investigate how impairment of these reflexes corresponds to delayed mortality.

One possible implication of the reformed CFP is the added logistical challenge of sorting the catch. This may lead to increased handling times, exposing fish to air for prolonged periods, before they are discarded under the rule of exemption. Increased exposure to air, one of the most significant determinants of discard mortality in several species of fish (Benoît *et al.*, 2010), would decrease probability of survival. Fish react to the stress of capture, exhaustion and handling by marked physiological disturbances, that will only be aggravated by exposure to air and in severe cases lead to delayed mortalities (Wood, 1991; Wood *et al.*, 1983). Additionally, increased air exposure may lead to additional sublethal effects, e.g. inability to escape from predators because of exhaustion, or reduced spawning potential (Wilson *et al.*, 2014). The primary stress response in fish includes release of hormones (i.e. corticosteroids and catecholamines) which is followed by secondary responses including hydromineral and metabolic imbalances and changes to the cardiorespiratory system (Wendelaar Bonga, 1997). Plasma levels of cortisol, osmolality, and haematocrit are therefore often used as stress indicators in fish. How long time a fish can survive air exposure ultimately depends on its capacity for anaerobic energy production to meet metabolic demands. Once endogenous energy stores (ATP and glycogen) and anaerobic ATP production becomes exhausted, the state of metabolic stress reaches fatal levels, from which recovery is unlikely (Atkinson, 1968).

Because plaice show strong potential for survival, this species is a possible candidate for an exemption from the discard ban. In light of this, additional information of discard mortality and the development of reliable methods to estimate survival will be of value to policymakers and fisheries management. The objective of this study was therefore to assess the feasibility of using RAMP to predict short-term mortality in European plaice from a small scale otter board fishery during representative operations. Air exposure was chosen as the variable stressor, given its important influence on survival, and under the assumption of prolonged sorting times on board vessels. A second objective of this study was to characterise the levels of physiological stress in discarded plaice exposed to the same air stressors, as this will provide a better understanding of the sub-lethal effects that may have more long-term consequences for individual fitness. To assess the state of physiological stress we used haematological indicators (i.e. cortisol, osmolality, and haematocrit) and levels of high-energy phosphates (creatines and nucleotides) in white muscle to estimate energy status. This information might be of value in relation to fisheries management and to fisheries biology in general.

Material and methods

Gears and sea trials

Experimental trials were conducted at sea in the Skagerrak area (ICES Area IIIa) on an 11.8-m long-commercial trawler (RI 286) with an engine power of 92 kW. The trawler was rigged with its own single trawl for targeting flatfish, specifically plaice. Mesh size was 105 mm, except for a 2-m long top panel section behind the headline, which had 200 mm meshes to avoid bycatches of cod. Two otter boards (Type 2), were used to tow the gear. The codend was 30 meshes long and 80 meshes in circumference including 4 meshes in each selvedge, with a lifting strap 1.10 m from the codline. The codend was made from 4 mm double polyethylene (PE) netting with a nominal mesh size of 120 mm (measured to 122.4 ± 1.5 s.d., $n = 31$), which is the minimum size allowed for targeting whitefish in the Skagerrak and the northern North Sea.

A total of 5 trips, were completed between 20 November 2014 and 28 March 2015 (Table 1). The first trip had a bycatch of Atlantic cod (*Gadus morhua*) and school shark (*Galeorhinus galeus*); therefore, the following three trips were completed at a site ~20 km south. The two sites are herein referred to as "site 1" (57.3937° 9.4258°) and "site 2" (57.2351° 9.3480°). To determine if the higher delayed mortality observed at trip 1 was site related or caused by bycatch, trip 5 was completed at the first site. Towing time for all experimental hauls was fixed at 180 min, while control hauls were restricted to 15 min. Experimental hauls were conducted in the morning and control hauls as quickly as possible hereafter, and always in the same area (Table 1). The haul back (from haul stop until codend lifted out of the water) took 20–24 min. The codend was lifted out of the water at the vessel side and then emptied into a pounder with a drop height of 1.1 m. Fish were transported by a 1.6-m long conveyor belt, before dropping onto a stainless steel sorting table (68×75 cm) from a height of 0.6 m. During commercial conditions, fish are sorted manually at the sorting table and discarded fish slipped via a 1.5-m ramp to an outlet at the side of the vessel ~1 m above sea surface. Fishing area, gear configuration, tow speed, tow time, and handling as described, were all representative conditions of this fishery.

Sampling

Collection of individuals was achieved by activating the conveyer belt of the pounder until >15 individuals had landed on the sorting table. A total of 15 individuals was selected at random, and transferred to 50 l tubs filled with seawater. Of the 15 individuals,

Table 1. Summary of operational and environmental data from five trips with an otter trawl in Skagerrak, northern Denmark.

Trip	Date	Duration (min)	Speed (Kts)	Bottom temperature (°C ± s.d.)	Air temperature (°C ± s.d.)	Depth start end (m)	Sea state (m)	Total catch (kg)
1	20 November 2014	180	2.2	7.71 ± 0.0	8.11 ± 0.4	53.4–63.5	1–1.2	347
1	20 November 2014	15	2.2			45.0–43.0	1.2	97
2	02 December 2014	180	2.3	9.03 ± 0.0	6.60 ± 0.4	12.4–12.6	0.6	524
2	02 December 2014	15	2.3			19.8–18.8	0.4	60
3	03 December 2014	180	2.3	8.21 ± 0.2	7.16 ± 0.2	16.2–11.5	0.5–0.9	414
3	03 December 2014	15	2.3			12.2–14.2	0.9	41
4	18 March 2015	180	2.3	6.14 ± 0.1	6.72 ± 0.2	14.2–16.0	0.7–0.9	241
4	18 March 2015	15	2.4			13.7–13.5	1	20
5	27 March 2015	182	2.4	6.25 ± 0.1	6.10 ± 0.2	66.6–49.0	0.5	457
5	27 March 2015	15	2.4			54.8–55.3	0.9–1	30

10 were used for RAMP and injury assessments, and 5 for collection of blood and white muscle tissue. The fish selected for tissue sampling did not undergo an RAMP assessment to avoid further handling stress. The sampling procedure was repeated a total of four times at fixed time points after the codend had been lifted out of the water: Immediately, or as soon as possible (∼5 min after lift), and then at 30, 60, and 90 min after the lift. For the short control hauls, the codend was emptied directly into a 90-l tub supplied with running seawater, from where individuals were collected at random and transferred to 50 l tubs as described above.

RAMP and catch damage

Assessments of reflex impairment for RAMP (see Table 2 for description of reflexes) were performed in a separate 35 l water-filled box, immediately after individuals were scored for injuries (see Table 2 for description of injuries). The final set of reflexes was selected after an initial testing of 12 candidate reflexes (Depestele *et al.*, 2014a) on 20 completely healthy plaice, caught locally by Danish seine. All assessments were completed within 15 min after individuals had been collected from the sorting table. A score of 1 was given if a reflex was absent and a score of 0 if a reflex was present. Likewise, injuries were scored as present or absent. After assessments, total length (TL) was recorded and individuals were transferred to a 90-l box with a lid and submerged in a 310-l insulated transport container also supplied with a lid. Containers were continuously provided with running seawater and temperature and oxygen saturation was routinely monitored. From the control haul, 10 individuals were immediately transferred to the container as described above, and another 10 individuals were scored for RAMP and catch damage before being transferred to the container. An additional 5 individuals were collected for measurements of physiological variables.

Haematological and white muscle stress indicators

Blood samples were retrieved by puncture of the caudal vein using a cold 23G hypodermic needle with a 1 ml pre-heparinized syringe and immediately transferred to Eppendorf tubes and kept on ice until further processing in the laboratory. Storage time was ∼8 h, but this does not significantly affect the measured variables (Clark *et al.*, 2011). Fish were then sacrificed with a sharp blow to the head and a 1–2 g sample of white muscle was quickly excised from the anterior-ventral section, trimmed for scales and skin and frozen and stored in liquid N_2. Blood and white muscle were collected from five individuals per time point with a handling time per individual of ∼2 min. TL and wet weight (W) was recorded following sampling. In the laboratory, blood samples were mixed on a vortex mixer and a small sample of whole blood was used immediately for determination of haematocrit (see below). In the remaining sample, plasma was separated from whole blood by centrifugation (5 min, 4 °C, 10 000 *g*), aliquoted and stored at −20 °C. Muscle tissue was stored at −80 °C.

Captive observation

Arriving at the harbour after a 2–3 h return trip, the lidded 90 l boxes containing the fish and ∼45 l water were transferred, to an 1100-l insulated transport container filled with aerated seawater and transported by truck to the nearby (2.3 km) facilities of the National Institute of Aquatic Resources, Technical University of Denmark. Fish were distributed into 300 l tanks according to the time point of sampling (10 fish pr. tank). This design was chosen to avoid further stressors by tagging fish individually. To minimize possible tank effects each treatment group was randomly assigned to tanks after each trip and tanks were equipped with dividers. Tanks were supplied with running seawater (3.2 l min^{-1}, 8.5 ± 0.7 °C), and the bottom was covered by a 2-cm layer of sand. Lighting regime was 10:14 h light: dark and tanks were shielded from disturbance by room dividers. Mortality was monitored 12 h after arrival and then once daily for a total period of 10 days, during which fish were not fed. This period was judged sufficiently long to observe any capture related mortality (Depestele *et al.*, 2014b) while not imposing additional stress because of starvation. The latter assumption was based on the low standard metabolic rate of plaice at similar temperatures (Steffensen *et al.*, 1981). Any dead individuals were removed from the tank with minimal disturbance. Individuals were identified as dead when there were no visible operculum movement, the colour of the iris was opaque or when unresponsive to a gentle nudge on the caudal peduncle. At the end of the observation period, all remaining fish were euthanized in an overdose of 2-phenoxyethanol followed by a sharp blow to the head. Blood and white muscle were collected post observation period from a total of 10 individuals, to acquire unstressed reference values for physiological variables.

Haematological stress indicators

Haematocrit (Hct) values (%) were obtained by centrifugation of whole blood (5 min, 17 500 *g*) in micro capillary tubes using a Sigma 1–16 microfuge (SIGMA Laborzentrifugen GmbH, Osterode Am Harz, Germany) and determined using a micro capillary reader. Plasma osmolality was quantified using a Vapro®

Table 2. Lists and descriptions of reflexes and injuries assessed on plaice *P. platessa* caught by otter trawl in Skagerrak, northern Denmark.

Reflex	Description
Evade	Actively swims away when released into the box
Tail grab	Reacts or tries to escape when tickled/grabbed at its tale
Vestibulo-ocular response	Eyes turning around when turned around longitudinal axis
Righting	Flips back around or attempts when turned upside down within 5 s.
Operculum	Resistance to forced opening of the operculum with a probe
Mouth	Resistance to forced opening of the mouth with a probe
Injury	**Description**
Scale loss	Minor <30% scale loss
	Major >30% scale loss
Fin fraying	Fins damaged, with slight bleeding
Abrasion	Minor <30%
	Major >30%
Bleeding	Obvious bleeding from any parts on body
Wounding	Nicks or shallow cuts on body
Deep wounding	Obvious deep cuts or gashes on body
Internal organs exposed	Internal organs exposed with wounds

5600 vapour pressure osmometer (Wescor Biomedical Systems, UT, USA). Plasma cortisol titres were quantified in duplicate by ELISA using a commercially available kit (NEOGEN, Lexington, KY, USA).

Creatine compounds and nucleotides

Creatine compounds and nucleotides (ATP, ADP, AMP, and IMP) were separated by ion-par reversed phase ultra-high performance liquid chromatography and quantified by UV/VIS detection using a Flexar FX-10 system (PerkinElmer Inc., Waltham, MA, USA). Chromatography was carried out with a 5-µm (C18) 150 mm × 2.7 mm column protected with a Brownlee C18 150 mm × 2.7 mm guard column (PerkinElmer, Inc.).

Chemicals and solutions

HPLC-grade creatine compounds and nucleotides were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade Acetonitrile was purchased from VWR International (Radnor, PA, USA) and all other chemicals were purchased from Merck (Darmstadt, Germany). The mobile phase consisted of Buffer A (50 mM NaH_2PO_4, 6 mM TBAHS, adjusted to pH 5.5, filtered through a 0.2-µm membrane filter) and Buffer B (75:25 (v/V) Buffer A: Acetonitrile).

Extraction

Frozen muscle (∼100 mg) was cut on ice, quickly weighed to the nearest 0.01 mg and transferred to a 3-ml cryovial containing 1 ml ice cold 0.6 M perchloric acid. The muscle tissue was homogenized in the vial for 3 × 10 s with an ULTRA-TURRAX (IKA-WERK, Staufen, Germany) while kept on ice, then left on ice for 10 min. The extract was centrifuged at 4800 *g* for 10 min and the supernatant was transferred to a new vial, neutralized (pH 7) with 2 M K_2CO_3 (10:1.5, v/v), centrifuged at 10.000 *g* for 5 min, and supernatant frozen at −80 °C until assayed. On the day of assay samples were thawed on ice and filtered through a 0.2 µm spin filter at 10 000 *g*.

Assay procedure

Samples were diluted 1:2 with mobile phase buffer A before being injected into the column (injection volume 20 µL). A solvent gradient was run between mobile phase buffers A and B with a flow of 0.4 ml min^{-1}. The cycle began with 100% A for 2.5 min, followed by a gradient to 100% B during 7.5 min and ended with 100% B for 8 min. The column was then equilibrated with 100% A for 10 min. The wavelength for the detection of creatine compounds was 210 and 254 nm for nucleotides. Peak areas on the chromatogram was calculated post run by the Chromera™ software (PerkinElmer, Inc.) and final sample concentrations by comparing against known standards.

Data analysis

RAMP scores were combined into a single score (proportion impaired) resulting in a score between 0 (no reflexes impaired) and 1 (all reflexes impaired) for each fish. A catch damage index (CDI) was calculated for individual fish (modified from Esaiassen *et al.*, 2013), where observed injuries were scored either 1 for minor (<30% scale loss/abrasion, fin fraying), 2 for moderate (>30% scale loss/abrasion, bleeding, wounding), or 3 for major (deep wounding, internal organs exposed, intestinal prolapse). Scores were combined into a single score (proportion) by dividing by the maximum score (17) giving each fish a CDI score between 0 and 1. A generalized linear logistic regression model (GLM) was used to evaluate whether RAMP score was associated with mortality using the average RAMP score per group of 10 individuals as the predictor variable and the number of dead individuals within that group as the response variable (binomial). Air exposure time (0, 30, 60, 90 min), haul duration (180, 15 min), CDI, and length (TL) were all initially included as predictor variables in the full model. Because of the *a priori* observation of large differences in mortality between sites, this was also included as a variable. Likelihood ratio tests (ANOVA, χ^2, 1 degree of freedom) were used to compare simplified models by stepwise exclusion of variables with no explanatory power. Inference on key parameters was drawn from the final model. Linear models (LM) were used to identify the variables that had a significant ($p < 0.05$) effect on the level of physiological stress. For each variable, a full model

Table 3. Discard mortality in plaice *P. platessa* caught by otter trawl in Skagerrak, northern Denmark, and exposed to air up to 90 min.

		Mortality (min)					
Trip	**Site**	**0**	**30**	**60**	**90**	**Total mortality % (95% CI)**	**Control mortality %**
1	1	0 (10)	0 (10)	3 (10)	6 (10)	22.5 (10.8 – 38.5)	0 (20)
2	2	0 (10)	1 (10)	1 (9)	2 (10)	10.6 (2.9 – 24.2)	0 (20)
3	2	0 (10)	0 (10)	0 (10)	0 (10)	0.0 (0.0 – 8.8)	0 (20)
4	2	0 (10)	0 (10)	3 (10)	0 (10)	7.5 (1.6 – 20.4)	0 (20)
5	1	0 (10)	2 (10)	0 (10)	4 (10)	15.0 (5.7 – 29.8)	0 (20)
Total mortality %		0.0	6.0	14.5	24.0	11.1 (7.1 – 16.3)	0.0
(95% CI)		(0.0 – 7.1)	(1.3 – 16.6)	(5.9 – 27.2)	(13.1 – 38.2)		(0.0 – 3.6)

Numbers are counts of dead individuals per tank ($n = 9$–10) after 10 days observation. Total mortalities are group means (95% CI in brackets) by trip ($n = 39$–40) or by treatment ($n = 49$–50). Controls were caught in a 15 min haul immediately after main haul.

including air exposure time, haul duration, site, length, and Fulton's condition factor, K ($K = 100 \times W/TL^3$) as explanatory variables was fitted. The model was then stepwise simplified by excluding those variables that had the highest value of p (non-significant). Transformation (Log or Box-Cox) was performed on variables that did not meet the Gaussian distributional assumption. Multilinearity between variables was evaluated by calculating VIF (variance inflation factor) and excluded if VIF >5 (Zuur *et al.*, 2010). All analysis was performed using the R software (http://www.r-project.org).

All experiments were performed in accordance with European and Danish legislation on animal experimentation (Danish Animal Experiments Inspectorate, permit no. 2014-15-0201-00413).

Results

Mortality

A total of 249 plaice was collected at sea for captive observation (33.1 ± 3.3 cm TL, mean ± SD). Out of those, two individuals were confirmed dead during the RAMP and catch damage assessment while another four individuals were dead upon arrival at the holding facilities. During the 10 days observation period only a moderate delayed mortality was observed with a total of 16 deaths. There was considerable variation in mortality rates between the two fishing sites, with higher mortality occurring among fish caught at site 1 compared with no or very low mortality among fish caught at site 2 (Table 3). Mean mortality was highest in groups that had experienced prolonged air exposure. The highest mortality observed was 60% among fish exposed for 90 min (Table 3), while no mortality was observed among fish exposed to air for 30 min, nor in the control groups.

RAMP and catch damage

There was an increasing loss of reflexes in fish exposed to air and especially fish from site 1 had high RAMP scores after 90 min exposure, while the control groups consequently had the lowest RAMP scores (Figure 1). The general condition of the fish was good, i.e. the injuries sustained during the catch process were typically of a less serious nature, e.g. fin-fraying, minor scale loss and minor abrasions. The most commonly observed injury was minor dermal pinpoint bleeding (Table 4). The proportion of individuals with more severe injuries (i.e. deep wounding, internal organs exposed and intestinal prolapse) was low, ranging from 0.3 to 1.7% (Table 4). Beginning with a full model, including RAMP score, air exposure time, site, tow duration, CDI and TL as predictors of mortality, all included variables except RAMP score could be excluded stepwise, leaving a final model with RAMP score as a highly significant ($p < 0.001$) predictor of mortality: logit $(pi) = \alpha + \beta xi$, where α is the intercept and β is the coefficient for RAMP score (Table 5; Figure 2).

Haematological stress indicators

Cortisol levels significantly ($p < 0.001$) increased with increasing air exposure, peaking after 1 h of air exposure, and were furthermore influenced by fishing site, with fish from site 1 having consistently higher ($p < 0.001$) cortisol levels than fish from site 2 (Table 6; Figure 3). The other tested variables (i.e. haul duration, TL, and condition factor) did not significantly ($p > 0.05$) influence cortisol levels and were excluded from the final model. Similarly, plasma osmolality was increased with duration of air exposure, with fish from site 1 displaying significantly ($p < 0.001$) greater elevations of plasma osmolality compared with fish from site 2 (Table 6; Figure 3). Haematocrit significantly ($p < 0.001$) increased with continued exposure to air, with no significant differences in haematocrit between fish from site 1 and 2 ($p > 0.05$) (Table 6; Figure 3). Haematocrit was, however, positively ($p < 0.001$) correlated with condition factor (Table 6).

Creatine compounds and nucleotides

The pool of phosphocreatine (PCr) in white muscle was consistently low across all sampled fish regardless of air exposure duration, site, and haul duration. There was no correlation between PCr and any of the morphometric variables (Table 6). The pool of creatine (Cr) was high across all groups with no effect of air exposure time, haul duration TL, and condition factor. Fish from site 2 had marginally lower concentrations of Cr (Figure 4; Table 6). Only weak associations were found between levels of IMP, air exposure, and site ($p < 0.05$, for both), while condition factor was positively ($p < 0.001$) correlated with IMP level (Figure 4; Table 6). Air exposure did not have any significant effect on AMP and ADP levels in white muscle, but a marginally significant ($p < 0.05$) negative effect on ATP levels and the adenylate energy charge (AEC) (Figures 4 and 5; Table 6). Site on the other hand had a highly significant ($p < 0.001$) effect on both ADP and ATP levels as well as on the AEC (Figures 4 and 5; Table 6). ATP levels were also lower in fish with higher condition factors ($p < 0.05$; Table 6). Only haul duration was weakly correlated ($p < 0.05$) to AMP level being lower in the control fish (Table 6).

Discussion

The average observed total mortality (11.1%) from the five trips was lower than previously reported mortalities for similar fishing

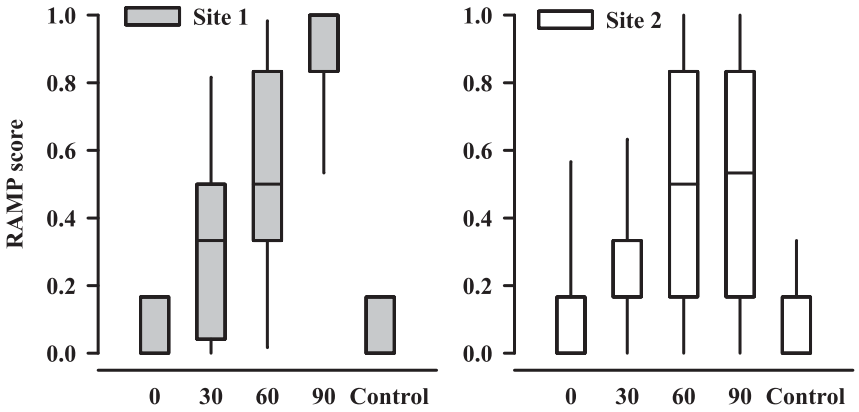

Figure 1. Reflex impairment in plaice *P. platessa* caught by otter trawl in two different sites in Skagerrak, northern Denmark and exposed to air on deck for up to 90 min. Solid line is the median, box is the 25–75th percentile and whiskers the 10–90th percentile. Grey box: site 1 ($n = 20$), white box: site 2 ($n = 30$). Control groups were caught in short hauls (15 min) immediately after main hauls.

Table 4. Catch damage and injuries in plaice *P. platessa* caught by otter trawl in Skagerrak, northern Denmark.

Type of injury	Proportion				
	0 min	30 min	60 min	90 min	Control
Scale loss <30%	0.10	0.02	0.00	0.00	0.02
>30%	0.00	0.00	0.00	0.00	0.00
Fin fraying	0.38	0.36	0.30	0.30	0.08
Abrasion <30%	0.20	0.26	0.34	0.32	0.24
>30%	0.00	0.00	0.00	0.00	0.00
Bleeding	0.72	0.88	0.80	0.90	0.34
Wounding	0.02	0.10	0.02	0.00	0.02
Deep wounding	0.00	0.00	0.02	0.02	0.00
Internal organs exposed	0.00	0.00	0.02	0.00	0.00
Intestinal prolapse	0.00	0.04	0.02	0.08	0.00
Catch damage index	0.09 ± 0.03	0.11 ± 0.01	0.10 ± 0.04	0.11 ± 0.02	0.04 ± 0.02

Values are the proportions of individuals scored for the specific injury averaged from five hauls ($n = 50$). Controls were caught in 15 min hauls after main hauls.

Table 5. Summary of logistic regression models (GLMs) fitted to short-term mortality in plaice *P. platessa* caught in otter trawl in Skagerrak, northern Denmark.

	Full model	Step 1	Step 2	Step 3	Step 4	Final model
Intercept	−57.92 (2943.00)	−31.08 (17.66)	−31.13 (16.82)	−16.31* (6.86)	−11.84* (4.93)	−4.81*** (0.64)
RAMP score	5.92 (3.04)	4.80 (2.69)	5.63*** (1.42)	4.80*** (1.00)	5.32*** (0.98)	5.16*** (0.97)
Length LT	0.99 (0.56)	0.74 (0.49)	0.74 (0.46)	0.43 . (0.20)	0.23 (0.16)	
CDI	21.01 (14.55)	18.85 (13.78)	18.82 (13.30)	9.53 (8.71)		
Site (as factor)	1.88 (1.60)	1.13 (1.34)	1.27 (1.23)			
Air exposure	0.00 (0.02)	0.01 (0.02)				
Haul duration	0.11 (16.35)					
Deviance (df)	64.6 (24)	64.6 (24)	64.6 (24)	64.6 (24)	64.6 (24)	64.6 (24)
Re. Deviance (df)	21.7 (18)	23.9 (19)	24.0 (20)	25.2 (21)	26.4 (22)	28.5 (23)
AIC	55.35	55.31	53.44	52.58	51.82	51.87

Predictor variables [RAMP score, length LT, catch damage index CDI, site (1, 2), air exposure (0, 30, 60, 90 min) and haul duration (15, 180 min)] were included in full model and then stepwise removed based on the lowest level of significance until a final model were all included variables were significant ($p < 0.05$) was found. For each included variable is stated parameter estimate, standard error (in brackets), and significance level. Deviance and residual deviance with degrees of freedom (d.f.), and the Akaike Information Criteria, AIC, are stated for each model. Significance codes: "$p < 1$, '. '$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

operations with beam and otter trawl, which ranged from 20 to 100% (van Beek *et al.*, 1990; Kaiser and Spencer, 1995; Revill *et al.*, 2013; Uhlmann *et al.*, 2016). However, comparing delayed discard mortalities between different studies is inherently difficult because of the multitude of varying factors that may affect survival, as exemplified by the marked differences between sites observed in the present study. The overall low mortality in the present study could be attributed to one of several operational,

environmental, or biological factors. For plaice, size (TL) (Revill *et al.*, 2013; Uhlmann *et al.*, 2016) as well as haul duration and temperature (van Beek *et al.*, 1990) have been reported to significantly influence survival. As sea trials were carried out during winter, water and air temperatures were low and fish were only subjected to a minimal change in temperature when brought on deck, which may have facilitated a high survival, because of lower metabolic rates. The size range in the present study was in the upper end of those earlier reports (a larger mesh size was used), offering another possible explanation for the lower observed mortality.

The higher mortality of fish from site 1 could be related to a greater fishing depth (range 49.0–66.6 and 11.5–16.2 m for sites 1 and 2, respectively). As plaice lack a swim bladder, direct barotrauma from over-inflation is not possible, but other pressure-related injuries, e.g. gas embolisms in organs or clotting of fine blood vessels cannot be excluded. Such injuries are not directly observable, but may still affect survival (Rummer and Bennett, 2005). Fish from site 1 did not have higher injury scores, but the higher mortality was, however, associated with a significantly higher degree of physiological perturbation, as discussed below.

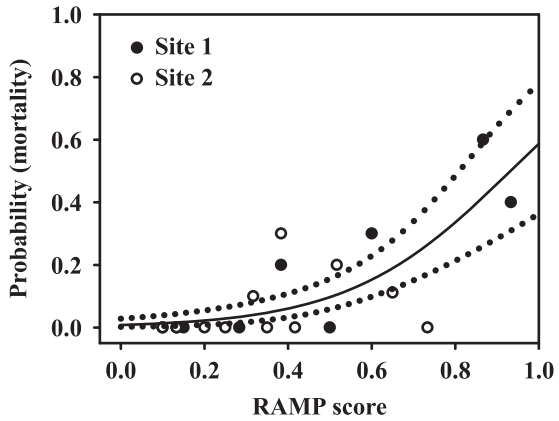

Figure 2. Regression model (GLM) of RAMP fitted to short-term mortality in plaice *P. platessa*. Solid line represents values predicted by the best model fit (see Table 5) and dotted lines the 95% CI. Data points represent proportion of dead fish per treatment group ($n = 10$) and average RAMP scores within that group. Black circles, site 1; white circles, site 2. Control groups were caught in short hauls (15 min) immediately after main haul.

Reflex impairment was sensitive to air exposure, as observed for other flatfish, e.g. Pacific halibut (*Hippoglossus stenolepis*; Davis and Olla, 2011), yellowtail flounder (*Limanda ferruginera*; Barkley and Cadrin, 2012), and in plaice (Uhlmann *et al.*, 2016), in addition to other species of fish (Humborstad *et al.*, 2007; McArley and Herbert, 2014; Nguyen *et al.*, 2014). Although delayed mortality never exceeded 60% (after 90 min of air exposure), reflex impairment could still explain the observed mortality, attesting to the use of RAMP to predict discard mortality (excluding predation) in plaice exposed to air (Raby *et al.*, 2014). To further validate RAMP as a tool for estimating discard mortality of plaice, additional field studies should be conducted applying different type of stressors relevant to the fishery (e.g. tow duration, temperature, and catch volumes) to verify that the chosen reflexes are sensitive to other type of stressors as well. The contributing effects of injuries should also be addressed, to resolve to what extent injuries may affect reflex impairments. Although integrating injury scores into RAMP for a combined vitality assessment may in some cases strengthen the mortality prediction (Campbell *et al.*, 2010; Uhlmann *et al.*, 2016), injuries that are not immediately observable (Noga and Udomkusonsri, 2002), may still contribute to mortality. Using a combined RAMP and CDI score was not attempted in the present study as CDI did not explain any of the observed mortality in the final GLM. This was likely because of the nature of the injuries, which generally could be considered to be minor and superficial. The near absence of observable compression related injuries such as bruising and protruding intestines could be because of low catch volumes (range 241–524 kg), although this relationship was not examined in the present study. Furthermore, with a mesh size of 120 mm only larger individuals were retained (i.e. sampled specimens had an average TL of 30.3 ± 3.2 cm, $N = 346$), and thus may have been less prone to pressure related injuries on internal organs given a larger muscle mass (Broadhurst *et al.*, 2006). For

Table 6. Significance of estimated (Est.) predictor variables, parameter estimates and summaries of linear models (LM) fitted to physiological variables in trawl caught plaice *P. platessa*, exposed to air up to 90 min.

Variable	Intercept		Air exposure		Haul duration		Site		Length		Condition factor		Model					
	Est.	s.e.	Est.	s.e.	Est.	s.e.	Est.	s.e.	Est.	s.e.	Est.	s.e.	Res. s.e.	Mult. R^2	Adj. R^2	*F*-statistic	DF	*p*-value
log(Cortisol)	4.23	0.13***	0.009	0.002***			−0.58	0.14***					0.73	0.26	0.25	20.36	2.117	2.59E−08
Osmolality	402.6	3.66***	0.414	0.053***			−30.19	3.80***					20.10	0.53	0.52	66.29	2.117	< 2.2e−16
log(Hct)	2.56	0.15***	0.002	0.000***							0.61	0.15***	0.17	0.25	0.24	19.80	2.116	3.99E−08
log(Cr)	3.61	0.01***					−0.03	0.02*					0.08	0.04	0.03	4.84	2.118	0.02977
log(PCr)	−1.20	0.48*							−0.028	0.016			0.54	0.03	0.02	3.35	1.118	6.99E−02
log(IMP)	0.43	0.21*	0.001	0.001*			−0.11	0.04*			0.98	0.20***	0.21	0.32	0.30	18.06	3.118	1.10E−09
log(AMP)	−0.97	0.08***			0.00	0.00*							0.34	0.05	0.04	6.24	1.118	1.39E−02
ADP.boxcox	−0.38	0.02***					0.06	0.02					0.10	0.08	0.07	9.71	1.118	2.31E−03
log(ATP)	0.57	0.93	−0.006	0.002*			1.18	0.19***			−2.01	0.88*	0.91	0.35	0.33	20.60	3.116	9.08E−11
AEC	0.43	0.02***	−0.001	0.000*			0.14	0.02***					0.13	0.25	0.24	19.88	2.117	3.69E−08

Hct, haematocrit; Cr, creatine; PCr, phosphocreatine; IMP, inosine monophosphate; AMP, ADP, ATP, adenosine mono, di and tri phosphate; AEC, adenylate energy charge. Significance codes: ''$p < 1$, '. '$p < 0.1$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

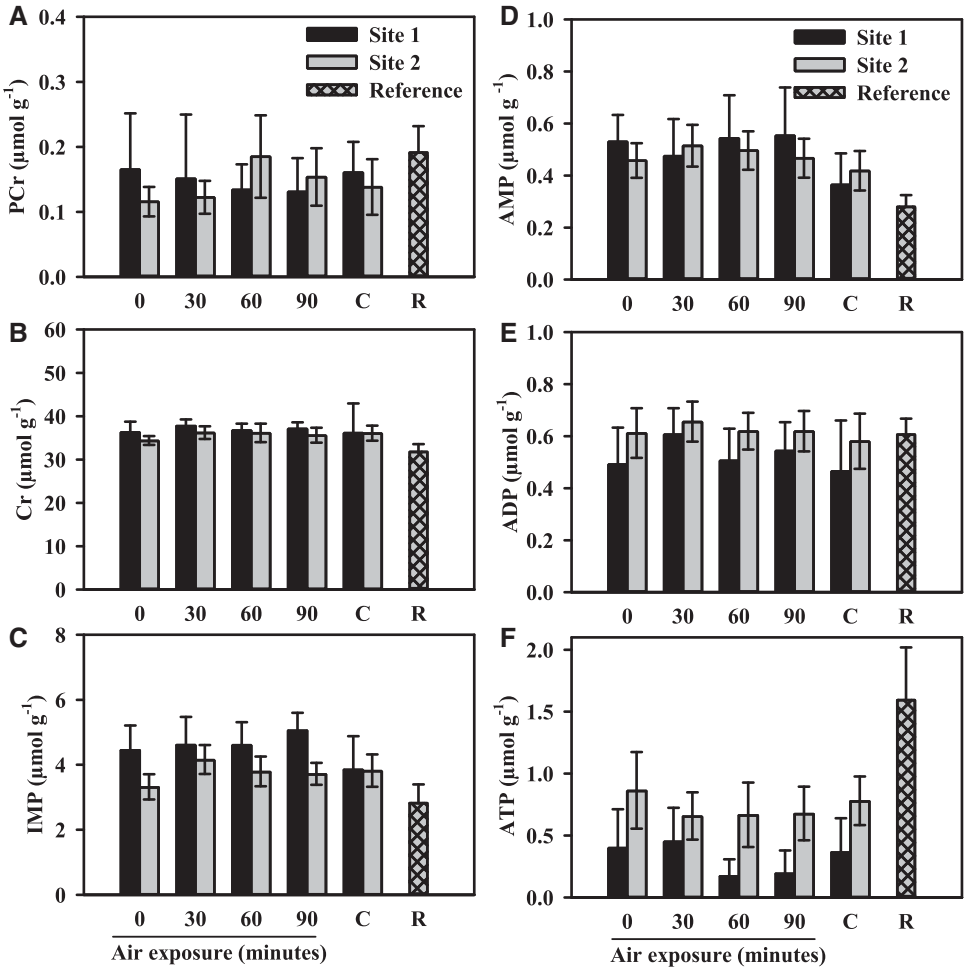

Figure 3. Haematological stress indicators in plaice *P. platessa* caught by otter trawl in two different sites in Skagerrak, northern Denmark and exposed to air on deck for up to 90 min. Controls, 'C' were caught in short hauls (15 min) immediately after main hauls. (a) Plasma cortisol (ng ml^{-1}), (b) haematocrit (%), and (c) plasma osmolality (mmol g^{-1}) (mean ± 95% CI) in fish from site 1 (dark grey, $n = 20$) and site 2 (light grey, $n = 30$). Reference values 'R' were sampled from individuals surviving 10 days captive observation ($n = 10$).

these reasons, the utility of a CDI should not be ruled out, and its capacity to be predictive of mortality should be evaluated under a broader range of fishing conditions.

The haematological stress indicators followed the pattern of reflex impairment, underpinning air exposure, and site as the major causes of physiological disturbance and stress. The peak level in plasma cortisol occurring after 1 h of air exposure follows the general dynamics of this hormone in fish, characterized by a delay in peak levels of up to 1 h following exposure to an acute stressor, and then a decrease depending on individual clearance rates (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999). To what extent the events before being hauled on board (startle, escape attempts, crowding, etc.) contributed to the overall release of cortisol is unknown. It is also uncertain if it was an initial or the continued exposure to air that resulted in the final measured levels, because of the time lag in plasma build-up of this hormone. Slightly higher peak levels (209 ± 21 ng ml^{-1}) have previously been reported for plaice after 1 h of severe hypoxia stress (White and Fletcher, 1986); however, numerous factors including feeding status, season, biogeography, and individual differences in clearance rates have been shown to affect cortisol levels (Audet *et al.*, 1993; Mommsen *et al.*, 1999). The presently observed pattern of plasma cortisol suggests that the cumulative effect of capture and air exposure elicited a maximum cortisol stress response, and that post-release, this would incur metabolic imbalances, reduced appetite, suppression of the immune system, and disruption of reproductive function (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999). A marked disturbance in the hydromineral balance, as an effect of catecholamine release increasing the permeability to water and ions in the gills, would be expected from the capture stressors and exacerbated by hyperventilation during struggling and escape attempts (Wood, 1991). As expected, a high plasma osmolality was observed in all groups including control fish (398.3 ± 9.9 mmol kg^{-1}). The progressive increase in plasma osmolality with air exposure was likely the combined effect of haemoconcentration and a gradual build-up of lactate from anaerobic metabolism (Wood, 1991), although, in plaice, release of lactate from white muscle into the circulation is limited (Wardle, 1978). Haematocrit was high in all fish sampled on board, and comparable to values (24.7–30.0%) reported for summer flounder *Paralichtys dentatus* after transport and handling stress (Sulikowski and Howell, 2003). Haematocrit was also high compared with values in the reference group (14.7 ± 0.7% s.d.) and in other flatfish, e.g. 12.8 and 16.5% in turbot *Scopthalmus*

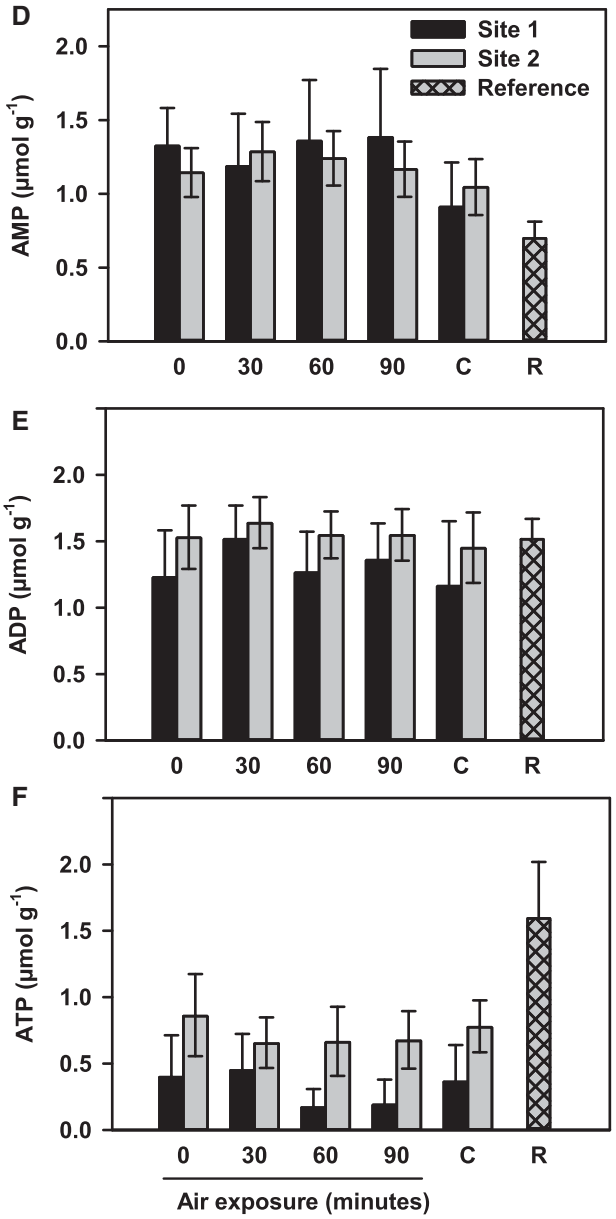

Figure 4. White muscle creatine compounds and nucleotides in plaice *P. platessa* caught by otter trawl in two different sites in Skagerrak, northern Denmark and exposed to air on deck for up to 90 min. Controls, 'C' were caught in short hauls (15 min) immediately after main hauls. (a) Phosphocreatine, (b) creatine, (c) inosine monophosphate, (d) adenosine mono-phosphate, (e) adenosine di-phosphate, and (f) adenosine tri-phosphate (mean ± 95% CI µmol per g wet mass) in fish from site 1 (dark grey bars, $n = 20$) and site 2 (light grey bars, $n = 30$). Reference values 'R' were sampled from individuals surviving 10 days captive observation ($n = 10$).

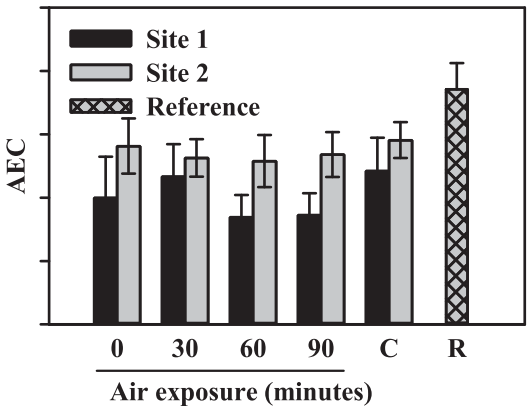

Figure 5. Adenylate energy charge (AEC) in plaice *P. platessa* caught by otter trawl in two different sites in Skagerrak, northern Denmark and exposed to air on deck for up to 90 min. Controls, 'C' were caught in short hauls (15 min) immediately after main hauls. Values are mean ± 95% CI site 1 (dark grey, $n = 20$) and site 2 (light grey, $n = 30$). Reference values 'R' were sampled from individuals surviving 10 days captive observation ($n = 10$).

maximus (Waring *et al.*, 1996; Pichavant *et al.*, 2002) and *ca.* 12–16% in American plaice *Hippoglossoides platessoides*, (Audet *et al.*, 1993). The increase in haematocrit with air exposure is likely caused by a continued release of catecholamines (Pichavant *et al.*, 2002) effecting the release of and/or a swelling of red blood cells (Nikinmaa, 1992; Reid *et al.*, 1998) and could be further exacerbated by plasma extravasation and evaporative fluid loss. Haematocrit was positively correlated with condition factor, an observation also made in *H. platessoides* (Audet *et al.*, 1993) and sardine *Sardina pilchardus* (Marçalo *et al.*, 2006) and similarly between weight and haematocrit for other species of fish (Martinez *et al.*, 1994; Nespolo and Rosenmann, 2002).

White muscle phosphocreatine, was almost entirely depleted and was coupled to increased levels of creatine in all groups regardless of sampling time and tow duration. Comparable tissue concentrations of PCr and Cr have been observed in rainbow trout *Oncorhynchus mykiss* (1.39 and 43.97 µmol g^{-1}, respectively) exercised to exhaustion (Dobson and Hochachka, 1987), and presumably high-intensity burst swimming from attempting to avoid the gear depleted PCr stores in plaice even before being brought on deck (Wardle, 1978). Similarly, ATP levels were already low when brought on deck and in the range (<1 µmol g^{-1}) of previously reported for several fish species immediately after capture by trawl (Jones and Murray, 1962; Mendes *et al.*, 2001) or in fatigued rainbow trout (Dobson and Hochachka, 1987). In several earlier studies from a range of fish species, including flatfishes, muscle ADP, and AMP levels were not found to change significantly following exhaustive exercise or hypoxia stressors, despite reductions in ATP (Jørgensen and Mustafa, 1980; Vetter and Hodson, 1982; Schulte *et al.*, 1992; Caldwell and Hinshaw, 1994; Pichavant *et al.*, 2002). Although interspecific variation exists, the levels observed in plaice fall in the range of those earlier reports (i.e. 0.44–0.97 and 0.06–0.41 µmol g^{-1} for ADP and AMP, respectively). The balance between the concentrations of ATP, and the dephosphorylated states ADP and AMP, the AEC reflects the energy status of a cell and acts as an important regulatory mechanism in energy metabolism (Atkinson, 1968). In teleost anoxic white muscle, degradation of AMP to inosine monophosphate (IMP) by AMP-deaminase occurs in response to a decrease in the ATP pool, as a mechanism to maintain a high AEC (Driedzic and Hochachka, 1976; van den Thillart *et al.*, 1980). IMP was the most abundant nucleotide in plaice white muscle, and in light of the low levels of ATP, ADP, and AMP, this suggest intense anaerobic activity during the capture process. The low AEC values currently observed in plaice reflects the depletion

of anaerobic fuel stores for generating ATP, i.e. PCr and glycogen. Although not measured in the current study, Wardle (1978) reported glycogen stores in trawl caught plaice after 30 min hauls to be depleted. It is reasonable to assume that the plaice in the current study had also exhausted their capacity for glycolytic ATP generation. AEC in teleost white muscle, during normal unstressed conditions, range from 0.89 to 0.95 (Jørgensen and Mustafa, 1980; Vetter and Hodson, 1982; Caldwell and Hinshaw, 1994; Pichavant *et al.*, 2002). An AEC below 0.5 indicates a level of stress from which the cell is unable to recover (Atkinson, 1968). The present findings imply a profound level of metabolic stress in plaice white muscle, where function is severely compromised, e.g. a loss of contractile performance, which was indeed demonstrated by a loss of reflexes coinciding with low AEC. Although not necessarily acutely fatal, this would decrease the probability of escaping predators during the initial recovery period. Previous studies have shown that energy stores in plaice white muscle took 24 h to fully recover (Wardle, 1978), meanwhile leaving them vulnerable to predation. Finally, the ∼1.5 time larger adenylate pool and ∼2.7 higher AEC in plaice sampled as reference post-observation, strongly support that the energy status was critically lowered from capture alone. Air exposure, although only marginally significant, did further aggravate the metabolic stress in terms of lower ATP, AEC and higher IMP levels, which would also be expected. Fish with higher condition factors were in a greater state of stress as reflected by lower ATP levels and higher IMP levels. Possibly, this could be related to behaviour in terms of repeated escape attempts and struggling in plaice with higher condition factors, as observed in Atlantic salmon during catch and release angling (Brobbel *et al.*, 1996).

The difference in responses from the two sites, with plaice from site 1 being in a greater state of distress, was evident not only from the creatine compounds and nucleotides, but also from the haematological variables and increased mortality. This may be an effect of size, as larger fish in general have greater anaerobic energy expenditures and greater post-exercise metabolic disturbances from greater utilization of ATP and glycogen stores (Kieffer, 2000). Fish from site 1 were on average larger than fish from site 2 (i.e. 32.0 ± 1.7 and 29.8 ± 0.2 cm TL) in addition to having higher condition factors (i.e. 1.05 ± 0.07 vs 0.96 ± 0.20). From the current observations, it can be surmized that large individuals were more vulnerable to oxygen deficiency and would have a lower chance of survival than smaller individuals if air exposure was the only stressor, which is seldom the case. In fact, size has been reported to be negatively correlated with delayed mortality in plaice (Revill *et al.*, 2013; Uhlmann *et al.*, 2016) emphasizing the point of multiple fishing related stressors adding synergistically to lethal levels of stress (ICES, 2016). For example in case of high catch volumes, smaller individuals would intuitively be more vulnerable to crushing with a thinner muscle layer or fragile skeletal structure to protect internal organs. On the other hand, smaller individuals might be able to recover more quickly from air exposure, owing to the allometric scaling of the cardiovascular system including a higher relative gill area (Hughes, 1984).

In summary, post-capture observation revealed an overall low mortality of plaice *P. platessa* caught by otter trawl. No mortality occurred when fish were exposed to air for up to 30 min, after which mortality increased with duration of air exposure. These findings add to the existing data on discard mortality of trawl-caught plaice, and may be of relevance to policymakers regarding future claims made under the "high survivability" exemptions rule. We found reflex impairment to be a valid predictor of delayed mortality in plaice exposed to air, but recommend further validation of RAMP, including testing other stressors in addition to scoring injuries to make predictions of discard mortality beyond the fishing operation currently studied. Fishing site had a marked and unexpected impact on stress levels, reflex impairment, and survival. The underlying mechanisms are obscure, but our current data suggest a relation to body size. Prolonged exposure to air resulted in increased mortalities and this coincided with severe levels of organismal stress as verified by depleted ATP and critically low energy status. The positive relationships between air exposure and mortality, reflex impairment, and physiological stress, respectively, suggests that on-board handling times are pivotal in the evaluation of possible exemptions from the discard ban.

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