Instituut voor Landbouw- en Visserijonderzoek

CALIBRATION TESTS FOR IDENTIFYING REFLEX ACTION MORTALITY PREDICTOR REFLEXES FOR SOLE (*SOLEA SOLEA*) AND PLAICE (*PLEURONECTES PLATESSA*): PRELIMINARY RESULTS

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Jochen Depestele
Eddy Buyvoets
Patrick Calebout
Marieke Desender
Jolien Goossens
Erik Lagast
David Vuylsteke
Christian Vanden Berghe

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Preliminary results

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Authors

Jochen Depestele, Eddy Buyvoets, Patrick Calebout, Marieke Desender, Jolien Goossens, Erik Lagast, David Vuylsteke and Christian Vanden Berghe

Contact

jochen.depestele@ilvo.vlaanderen.be

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CONTENTS

CALIBRATION TESTS FOR ESTIMATING REFLEX ACTION MORTALITY PREDICTOR (RAMP) FOR SOLE AND PLAICE: PRELIMINARY RESULTS

Authors: J. Depestele[1,2]; E. Buyvoets[1], P. Calebout[1], M. Desender[1,3], J. Goossens[2], E. Lagast[4], D. Vuylsteke[1] and C. Vanden Berghe[1]

[1] Institute for Agricultural and Fisheries Research (ILVO), Ankerstraat 1, B-8400 Oostende.

[2] Ghent University, Faculty of Sciences, Biology Department, Marine Biology Section, Krijgslaan 281-S8, B-9000 Gent.

[3] Ghent University, Faculty of Veterinary Medicine, Department of Morphology, Salisburylaan 133, B-9820 Merelbeke.

[4] skipper with >40 years of experience on-board commercial beam trawlers (e.g. Z 183).

1 INTRODUCTION

Empirical estimates of discard survival are difficult to obtain due to the complex logistics of survival studies, either by accommodating organisms in holding tanks or by tagging. There are few studies on the survival of discards in beam trawl fisheries (Table A 1 in APPENDIX for an overview until 2013), and up till today (March 2014) most of them have focused on the short-term survival using holding facilities in on-board tanks (Depestele et al., 2014; Revill et al., 2013) or underwater cages (Uhlmann et al., 2014; van Marlen et al., 2013). To our knowledge only very limited information is available on successful tagging studies in beam trawl fishery. As a consequence of the complex methodology, there are only a very limited number of species and/or individuals investigated, thus leading to the investigation of yet another modest number of influential factors. The complex logistic nature of on-board survival tank studies, especially when conducted on-board commercial vessels, resulted in a narrow focus of potential key aspects, such as haul duration, number of tickler chains (van Beek et al., 1990), catch weight (Depestele et al., 2014), or maturity (Revill et al., 2013), etc. Major influential factors could nevertheless be numerous. Beam trawl fisheries, as many other fisheries, disturb fish throughout the catching process, starting by the encounter of the chains (or pulses), followed by a stressing period (e.g. fish trying to escape the net) and culminating in a physical and internal damaging process during retention in the codend, release on deck, and subsequent sorting. The infliction of stress and damage to organisms follows the capture process as illustrated by Davis (2002) (Figure 1).

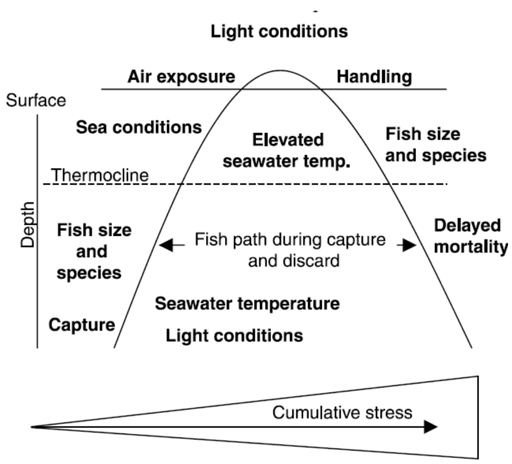

Figure 1 – The number of interacting factors that determine discard survival can be traced from the capture process. The curved line illustrates the path that fish follow (modified from ,Davis, 2002).

The range of biological (e.g. taxa, physiology, size, catch composition), technical (e.g. haul duration, gear modifications, gear weight and design) and environmental (e.g. fishing depth, sea state, visibility, and sediment type, etc.) conditions determines the stress levels that an organisms endures, and hence its internal and/or external damage. The vast range of factors, their potential antagonistic or synergistic (additive or multiplicative) effects are difficult to disentangle when only a modest number of replicates can be made available in certain circumstances. This is generally the case in holding experiments or tagging studies. Therefore, a study can opt to focus on what are presumably the main drivers for mortality, or otherwise, define a proxy for discard survival that can be easily estimated in a vast range of conditions.

The major advantage of estimating proxies for discard survival embraces a low cost, and the relative easiness and rapidity of estimation. A prerequisite is that the proxy bares a reasonable predictive power of discard survival within. The assessment of vitality was prompted in an early stage of survival studies and was based on the subjective scoring of vitality (e.g. van Beek et al., 1990). Vitality relates to the potential of an individual to survive the capture and discarding process. High vitality implies healthy, unstressed and undamaged individuals. Low vitality scores indicate individuals that have been severely injured or stressed and impaired in their functionality. Vitality has been indicated by a range of possible, but mostly subjective factors such as the movement and/or liveliness (Enever et al., 2008). Some proxies have attempted to define criteria to score vitality in a more objective manner. One of these proxies was based on the level of injuries (Catch Damage Index, CDI) and indicated considerable potential for certain taxa, such as benthic invertebrates, *Rajidae* and plaice (Depestele et al., 2014). However, injuries could not predict the mortality rate of soles and cod, and thereby highlight the need to also evaluate internal damages and/or endured stress. Time-to-mortality (TTM) is such a predictor (Benoit et al., 2013) and has shown to clearly discriminate between taxa. TTM is the time that it takes for an organism to die while being out of the water and exposed to air. This indicator is typically related to hypoxia and follows the projections that an increased handling time on deck clearly leads to reduced survival. The Reflex Action Mortality Predictor (RAMP) is another proxy for survival which can be easily measured. RAMP is user-defined and species-specific, and can include several criteria, hence embracing for instance both resistance to hypoxia and an organism's intrinsic robustness. RAMP is based on behavioural reflexes and can hence be easily applied on-board commercial fishing vessels, for instance by sea-going observers during discard monitoring programs.

A reflex in RAMP is defined as an involuntary response of the organisms to a stimulus such as being touched or stimulated by light or sound. The reflex responses are innate, fixed and present unless impaired, which implies they should not be considered as volitional behaviour that comes and goes in various degrees with motivation (e.g. fear, attraction, avoidance, hunger). They are quantified by presence or absence, and can be complemented by physical injuries. Physical injuries and reflexes are related to vitality without being confounded by factors other than health (e.g., motivation, size, and sex). Reflex actions are thus innate and fixed. Their presence should be independent of motivation, size and sex, which might important to consider during the developmental stage. If degree of impairment is scaled, then size effects for instance may appear (e.g. smaller fish have weaker reflex actions). In contrast, scale loss for instance can be scored as either present or absent in both immature and mature fish, males and females, etc. Presence-absence scoring overcomes issues with biological factors such as size, sex, maturity stage. In case of doubt about the presence of a reflex, the reflex action is considered absent and is hence scored impaired (1), while unimpaired reflexes are scored "0". A reflex action is scored not impaired (0) when strong or easily observed. Scoring injury as present-absent was also advised to improve the precision of observations and reducing observer bias in estimation of degree of injury. In this study however, injuries were nevertheless scored on an existing scaling (Depestele et al., 2014), and might need further improvement. Further details on the application of RAMP are provided elsewhere (e.g. Davis, 2007; Davis, 2010; Davis & Ottmar, 2006).

The application of RAMP on-board of commercial fishing vessels needs a preceding calibration and validation phase. Calibration of RAMP implies that species-specific reflexes are developed. Flatfishes for instance require different tests than roundfish or benthic invertebrates (Barkley & Cadrin, 2012; Hammond et al., 2013; Humborstad et al., 2009; Stoner, 2012). Calibration implies that the appropriate reflexes are determined. In a following phase, the candidate reflexes are tested are tested on a sufficient number of individuals (~20 individuals, Davis, pers. comm.). These individuals need to indicate a consistent pattern in the reflexes, given that the test organisms are in excellent condition. The latter can be traced from aquaculture-based individuals or unimpaired individuals from a catching process with minimal impact. Control fish are difficult to obtain, but short hauls of beam trawling have been illustrating to only limitedly influence fish condition and might hence provide a reasonable proxy (Depestele et al., 2014). It is advisable to also test the reflexes on fish in different conditions, e.g. several length classes, maturity stages, temperature, etc.

In this study we attempted to determine reflexes for sole (*Solea solea*) and plaice (*Pleuronectes platessa*) suitable as a proxy of discard survival and conducted a calibration test by accommodating individuals in on-board holding facilities. Individuals were first inspected to determine potential reflexes, and were subsequently tested in fish in a favourable condition, for which survival was determined.

2 PRELIMINARY CHOICE FOR TESTING REFLEX IMPAIRMENT IN RAMP

2.1 Potential reflexes

The list of potential reflex actions for scoring presence or absence of impairment and summing for RAMP calculation in fish, turtles and invertebrates is long. Not all reflexes are directly suitable for flatfish, which is the focus of this study, e.g. the orientation of flatfish is not upright, but one could assume that flatfish remain on their belly. Hence natural righting should be interpreted in this sense. The reflexes are summarized in Table 1 and in the following chapters their suitability for flatfish was investigated. A preliminary number of 10 reflexes was selected from Table 1 (based on advice by M. Davis).

Table 1 - Choices for testing reflex impairment in RAMP (based on Davis, 2010; Humborstad et al., 2009; Raby et al., 2012; Stoner, 2012, summarized online by M. Davis, website visited on 6/3/2014)[1]

Reflex	Description	Reflex	Description
Body flex 1	Place your full hand on the fish and note if it resists	Tail grab	Burst movement away from tester
Body flex 2	Body flex when placing on a flat surface	Head complex	Regular pattern of ventilation with jaw and operculum
Orientation	Normally upright, or flat for flatfish (?)	Tail flexion	Body flex when tail flanks stimulated
Righting	Returns to normal orientation when turned upside down	Evade	Active swimming away when released from testing
Startle light	Moves in response to light	Abdominal turgor	Abdomen extends horizontally or tail flip
Startle sound	Moves in response to sound	Abdominal extension	Abdomen extends outward
Startle touch	Moves in response to touch	Leg motion	Leg moves in held animal
Dorsal fin erection	Fin becomes erect when body restrained or touched	Leg retraction	Leg retracted in held animal
Operculum closure	Operculum clamps closed when lifted or opened	Leg extension / flare	Leg spread wide, extended in held animal
Mouth closure	Mouth clamps closed when lifted or opened	Pleopod motion	Pleopods retract when stimulated
Gag response	Body flexes when throat stimulated with probe	Maxilliped motion	Maxillipeds move when stimulated
Vestibular-ocular response	Eyes roll when body rotated around long axis	Maxilliped retraction	Maxillipeds retract in posterior direction
Nictitating membrane	Nictitating membrane closes on stimulation	Antenna response	Antenna moves when stimulated
Atonic immobility	Becomes immobile when stimulated	Eye turgor	Eye stalk moves when stimulated
Dorsal light reaction	Body rolls in direction of light	Eye retraction	Eye stalk retracts when stimulated
Optimotor response	Movement in response to external object motion	Chela closure	Chela closes when stimulated
Optikinetic response	Eye movement in response to external object motion	Aggressive posture	Assumes aggressive defense posture when released

1 http://yesheflowers.blogspot.be/2013/01/choices-for-testing-reflex-impairment.html

2.2 Anecdotic experiences at sea in determining reflexes

The initial set of ten reflexes (Table 2) was set up a priori based on suggestions by M. Davis and other studies (e.g. Barkley & Cadrin, 2012; Campbell et al., 2010; Davis, 2010; Raby et al., 2012). This set was complemented with a range of potential additional reflexes based on manipulations of plaice and sole from the first haul and individual experiences of the project team (Table 3). The suggestions were species specific, and tested for individuals of both species. The initial description of the reflexes, also the description in the peer-reviewed papers, was refined and discussed, as descriptions were not always straightforward to implement, i.e. open for interpretation. More details and a clear explanation of the exact meaning would eliminate differences between observers and misinterpretations.

The first haul of the campaign went smoothly (3rd March 2014), but fish were retained in the codend at the surface for a while (about 15 min). This unusual practice was due to issues preventing the net to be hauled on the deck immediately. The fish was nevertheless used for manipulation and for getting acquainted with handling. The consecutive three short hauls (<20min) were used to refine the reactions and for defining consistent reflexes. Full consistency did not seem easy to achieve, although some reflexes gave consistent correlation between observer's judgments of vitality (not *a priori* defined, hence a pure expert judgment) and the expected responses to the stimuli. There were two tests. The first test included all reflexes mentioned in Table 2 (Figure 2; Figure 3). Four individuals of plaice (haul 1) and sole (haul 2) were closely inspected from two consecutive hauls. The limited number of inspected individuals was due to the fact that manipulation took considerably long, i.e. 15 min for four individuals. The second test was limited to only 11 reflexes indicated that five reflexes were not considered at any use at all (n° and reflex name): (3) head complex, (5) body flex 1, (6) fin control, (13) headbang and (14) wave. This test aimed at the final selection of reflexes. The tested reflexes were the same for sole and plaice, but the final selection was not. The tested reflexes were (n° and reflex name): (1) righting, (2) body flex 2, (4) Vestibular-ocular response, (7) tail grab, (8) operculum, (9) mouth, (10) evade, (11) head, (12) fin lift, (15) stabilise and one additional reflex: (16) gag. A visual response of sole towards an approaching object was also noted, and at times prompted a reaction. However, this reflex was not considered. There were three plaice and six soles tested from haul 3, and four plaice and five soles from haul 4. It took about three to four minutes to test all reflexes per individual, given that all observers were relatively unexperienced, and given that only about half an hour of fish manipulations was preceding the tests (limited learning period). All tests were ended within 15 min to maintain fish in a sufficient lively condition. Stimulating the gag response, i.e. insertion of a probe to induced body movement with the tested fish, seemingly reduced the vitality of the individuals and gave contradicting results, as well as doubts between observers about its appropriateness. This reflex was therefore not retained, although consistent for plaice. The tail grab (R4) and evade (R8) in Table 5 were originally seen as rather similar. However, grabbing the tail clearly always induced a response. Releasing sole or plaice into the boxes with water did not always lead to fish that were swimming away. Grabbing or tickling the tail was later on used to evaluate whether sole and plaice in the survival tanks reacted to stimuli and hence, whether they were still alive. Also, the opening of the mouth and the gills (but especially the mouth) was used to see whether soles were still alive (without touching). Anecdotic conclusions on the different reflexes are given in Table 2 and Table 3. These conclusions are seen as valuable as the scoring in the tables, as discussion led to the final set of reflexes. The first test included a considerable range of potential reflexes, complemented with unreported manipulations of the tested fish. These manipulations led to a first set of reflexes to be tested (test 1, Table 4). A second test was performed, and led to the final set of reflexes, as detailed in Table 5 and Table 6.

Table 2 – Anecdotal description of tested reflexes for sole and plaice and preliminary conclusions from field trials. The selection of reflexes was based on suggestions by M. Davis and literature (e.g. Barkley & Cadrin, 2012; Davis, 2010; Humborstad et al., 2009)

Reflex		Description
1.	Righting	Turn fish on its back and watch if it turns back to its belly within 5 seconds. The boxes used for testing natural righting of large individuals (e.g. plaice > 27cm) most likely higher boxes (not practical), and hence the intention of righting was considered sufficient. This contradicts the rule of doubt.
2.	Body flex 2	Place a fish in a box with water and watch if it starts flapping, typically expected for plaice when placed onto a sorting table. Body flex 2 was tested on several individuals of plaice and sole. Individual fish were placed on the sorting table (in air) or in a box with water, but in both occasion the flapping did not seem a good reflex. We expected that soles and potentially also plaice, would have responded by placing them in air on a flat surface. Our experiences in measuring these individuals suggested that at times it was difficult to straighten soles or keep flapping plaice laying still for measuring. However, when testing this characteristic in a range of very vivid individuals of both species it did not appear a consistent pattern.
3.	Head complex	Look at the head of the fish: are the mouth and gills opening in a controlled manner? The mouth opens and the gills open accordingly, seemingly in all fish from the short hauls (short is <20min). The frequency of opening and closing however seemed to differ and looks as a good indicator of their vitality. However, we found this subjective and judgments across observers differed, or timing would be needed. This is rather impractical and therefore disregarded as a useful indicator for plaice and sole.
4.	Vestibular-ocular response	Are the eyes of the fish turning around when it is turned around its longitudinal axis? The eyes of plaice are rather clearly moving when turning the fish around in air. It is especially obvious that the eyes keep focusing on one element and kind of sink into the body. While the eyes do not sink deeply for plaice, this is clearer for sole. The difficulty for looking at sole in vivid individuals is that sole at times curls around and it is difficult to focus on the eyes exclusively. The eyes seem to sink into the body at times while inspecting the fish in the air. The turning of the eyes was not more than 0.5cm.
5.	Body flex 1	Place your full hand on the fish and note if it resists. Placing the hand along the longitudinal axis or in perpendicular direction to the fish did not seem to cause any clear resistance of the fish, nor any particular movement. A lack of response was apparent both in water and in air.
6.	Fin control	Does the fish react if stroked alongside of its longest fins? Response to a brushing stimulus on the fins Brushing along the fins was thought to cause any reaction, but this was not clearly apparent. However, another reaction of the fins was more clear (see below, nr. 15)
7.	Tail grab	Does the fish react if it is tickled at its tail? Tickling the tail or the after part of the main body of the fish by touching subtly with hand or pencil did not evoke a clear reaction. Therefore, we have grabbed the tail of plaice and sole with two fingers. If the fish is held like this for a while, it should swim away or at least have the intention to do so. Tickling did not work, but grabbing the tail did. It was not always easy to grab the tail of sole, which was less difficult for plaice.
8.	Operculum	Resistance to forced opening of the operculum, ideally tested under water. Opening the operculum was very easy for sole and it appeared to us that also very lively individuals did not have the force to close the operculum or try to resist the forced opening of the operculum. Opening the operculum with a probe/pencil was easy for sole and did not seem to evoke any obvious reaction which could discriminate between good or bad reflex. For sole we've concluded not to use this. Plaice on the contrary showed a clear response on the forced opening of the operculum and individuals used their muscles in order to try to close the operculum. Clear resistance against this forced opening was obvious for vivid individuals of plaice, implying that this feature could be retained for plaice. Both resistance to forced opening and re-closing the operculum after forced opening were considered as an unimpaired reflex.
9.	Mouth	Resistance to insertion of a probe into the mouth, tested under water. The mouth of soles was kept close by vivid individuals, and it was difficult by the observers to open the mouth with a pencil. This was considered a clear reaction. Opening of the mouth of plaice with a probe/pencil was easy, but plaice tried to close it. While this was not fully obvious in all individuals, this reflex was retained. Plaice did strongly close its mouth, but instead of keeping it shut like sole, plaice closed it more slowly but nevertheless clearly.
10.	Evade	Active swimming away when released from the tests. This swimming away should be clear and not just the drifting of a fish that after a while starts to stabilise itself. It should an active swimming behaviour towards for instance the corner of the aquarium/box. This seemed to work well with the tested fish.

Table 3 – Anecdotal description of tested reflexes in sole and plaice. The selected reflexes above were complemented by additions of experiences of the ILVO team. These additional tests were specifically related to handling sole and plaice.

11.	Head	When getting a fish out of the water and kept between finger and thumb, then the fish should show a reaction of the entire body, e.g. trying to erect or curl. There was a lack of any reaction in plaice individuals, but the soles curled their body upon getting them out of the water. This reaction was considered sufficient and a good reflex for sole.
12.	Fin lift	Lifting the dorsal or anal fin of a resting sole or plaice induced a reaction of the fins, i.e. the individual tried to put their fins back again on the surface and/or reacted by trying to move their fins, similar to the reaction searched for when brushing the fins. This reflex was not retained as a separate reflex, but merged with nr 15 "stabilise", because of its similarities. Simply looking at the fishes' fins gave a rapid response as there are subtle but continuous movements of the vessel.
13.	Headbang	When sole or plaice were placed on a flat surface, either in water or in air, then their heads were lifted. These movements are similar to the movement that flatfish makes when they are digging themselves into the sediment. Lifting their head was to stimulate sole and plaice to move their heads and/or tails up and down several times or at least once. These reactions showed up in some individuals, but only a minor number and therefore lifting the head was not considered sufficient for inclusion in the final set of reflexes.
14.	Wave	The pectoral fin of plaice or sole was lifted for a while. When this was initially tried with some plaice individuals, it seemed that these fish attempted to "wave" or at least moved the fin several times instead of aligning it in the longitudinal direction of their body. However, when testing on a range of fish, this reaction did not prove sufficient to be retained as a good indication of a reflex for lively fish.
15.	Stabilise	When an individual of both plaice and/or sole is placed into a box with water (and without sediment) it is clear that the fish seemingly tries to dig into the sand or stabilises itself against the movement of the vessel. It should not take longer than 5sec to look at the fish while it is moving its fins to find a good position. This reaction is what we're looking for and we are only focusing on the dorsal and anal fin, i.e. the fins along the longitudinal axis of the flatfishes' body. If this reaction does not occur, a pencil or probe can be used to go underneath the fin (while the fish is on its belly) and to see if the fish shows any reaction and tries to re-position the fins onto the bottom of the box. This reaction was very clear, although it might be sometimes subtle. Lifting the fins was not needed during the calibration tests, but it was during the investigations of which reflexes to test. Therefore, we suggest that lifting the fins should not be included in the actual, final description of this reflex. The order of testing reflexes was shown to be important. If this reflex is tested after the "evade" response, then one should allow the fish some time (<5sec) to find its position. If the fish starts floating, then the stabilise reaction can only be tested after quite some time, prolonging the investigations of the reflexes.
16.	Gag/"oesophagus"	Resistance to insertion of probe/pencil into the throat/oesophagus, i.e. after the mouth was already opened. The pencil should go at least 1.5cm deep and most likely deeper. Lively soles kept their mouth closed, and it was considered too time-consuming to insert a probe into the oesophagus of soles. Moreover, the reaction of sole after insertion was not always apparent, although most of the vivid individuals showed a full body reaction, i.e. curling of the complete body. The reactions of plaice were more obvious, but also not retained as a sufficient reflex by the ILVO team. When a pencil was inserted into the oesophagus of plaice, the gills and mouth showed a clear reaction and not the full body, looking like a panic reaction of the fish as like it was to vomit. When the fish was held under water, and the pencil was released, i.e. not continuously kept into the oesophagus by hand, then the plaice individuals attempted and succeeded to spit the probe out.
17.	Cover (not tested)	An additional reflex which was not tested during the campaign due to lack of substrate, relates to the behaviour of flatfish to dig into sediment. Sole has been observed to dig into sediment when it is placed onto sand. Therefore we suggest that sole or plaice are located on top of sediment (sand) and if they dig into the sediment within 5sec (as observed in laboratory conditions, Soetaert, pers. comm.), then the reflex action is not impaired.
18.	Tail push (not tested)	The status (alive of dead) of flatfish in the laboratory is being tested by pushing hard on the tail of sole or plaice. When an immediate reaction does not show, then fish are considered impaired.
19.	Darkness (not tested)	Place the fish in a box filled with water. Make sure that part of the box is covered in order to have a dark and lighted part. Roundfish typically move fast (e.g. within 2sec) towards the darker part. This was not tested for sole and plaice, but might be an additional reflex action to be tested.

Table 4 – Examining an extensive list of reflexes, added with manipulations of the fish which were not noted. The most apparent reflexes were noted down, although not all of them seem appropriate. Zeros indicate that a response to the proposed test was absent. This is in contrast to the zeros and ones used in Davis (2010) use of zeros and ones. The first test included a limited number of individuals, as only very vived individuals were considered and given that it took a while to evaluate the reflexes, we've ended up testing 4 individuals of sole and plaice from two different hauls on 3 March 2014. Reflexes 1-15: righting, body flex 2, head complex, vestibular-ocular response, body flex 1, fin control, tail grab, operculum, mouth, evade, head, fin lift, head bang, wave, stabilise.

Haul	Species	Length (cm)	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
1	Plaice	26	1	1	0	1	1	1	0	1	1	1	1	/	/	/	/
1	Plaice	19	0	1	0	1	1	1	0	0	0	0	1	0	/	/	/
1	Plaice	29	1	1	0	1	1	1	0	1	1	0	1	1	1	0	/
1	Plaice	29	1	1	0	0	1	1	0	1	1	0	1	0	1	1	/
2	Sole	27	0	1	0	0	1	1	1	1	0	0	0	1	1	1	0
2	Sole	19	1	0	0	0	1	1	0	0	0	0	1	1	1	1	0
2	Sole	24	0	0	0	0	1	0	0	1	1	0	0	1	1	1	0
2	Sole	39	1	1	0	0	1	1	0	0	0	1	1	0	1	1	0

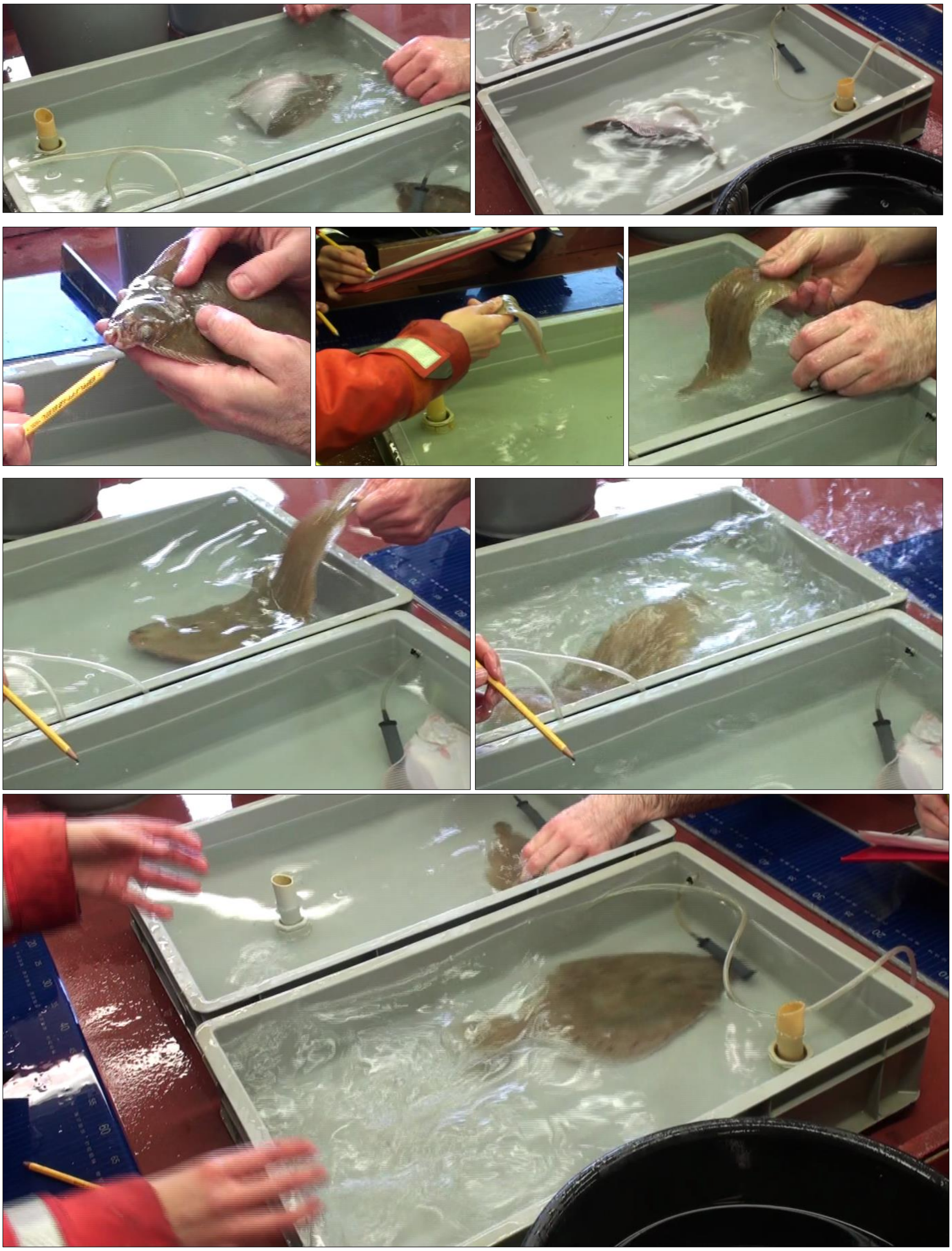

Figure 2 – Illustration of tested reflexes (from left to right and top to bottom row): righting of sole, righting of plaice, inspecting vestibular-ocular response for plaice, illustration of the absence of the "head" response for plaice, head for sole, release of sole, evade reaction of sole, evade reaction plaice

Figure 3 – illustration of retained and unretained reflexes (from left to right and top to bottom): (1) if stabilisation of the fish on the bottom is not apparent, the fins were lifted. However, this was only the case in the development of this reflex and not needed for the actual calibration test. It might therefore be eliminated from the description of this test, (2) mouth, (3) operculum (should be tested under water in contrast to what the picture shows), (4) the tail was grabbed and lifted out of the water. Generally the tail is only touched a little bit in contrast to what the picture shows, (5) brushing of the fins did not lead to a clear reflex, (6) resistance of the fish to pressure did not lead to a clear response.

Table 5 – Reflexes of sole during the second test. The tested reflexes 1-10: righting, body flex 2, vestibular-ocular response, tail grab, operculum, mouth, gag, evade, stabilise, head. H: haul, L: length (cm). A zero score indicates unimpaired reflexes. If a reflex was impaired it was scored 1.

H	L	1	2	3	4	5	6	7	8	9	10
3	27	0	0	0	0	0	0	0	0	0	0
3	20	0	1	0	0	1	0	0	0	0	0
3	24	0	1	0?	0	0?	0	0?	0	0	0
3	25	0	1	0	0	1?	0	0	0	0	0
3	18	0	0	0	0	1?	0	1	0	0	0
3	20	0	1	0	0	1	1	1	0	0	0
4	29	0	1	0	0	0	0	0	0	0	0
4	32	0	1	0	0	0	0	0	0	0	0
4	29	0	1	1	0	1	1	1	0	0	0
4	22	0	1	0	0	1	0	?	0	0	0
4	19	0	1	0	0	1	0	?	0	0	0

The reflexes from Table 3 which were not relevant have not been tested. There were a number of reflexes that consistently appeared amongst the vivid individuals: R1 (righting), R4 (tail grab), R8 (evade), R9 (stabilise) and R10 (head). Additionally R3 (vestibular-ocular response) and R6 (mouth) were considered sufficient as a consistent reflex for RAMP testing. Looking at the eyes of sole is however not always as easy, depending on how active they are during handling. The mouth is generally closed. Doubts and contradiction was found with R2 (body flex 2), R5 (operculum) and R7 (gag).

Table 6 – Reflexes of plaice during the second test. The tested reflexes 1-9: righting, body flex 2, vestibular-ocular response, tail grab, operculum, mouth, gag, evade, stabilise. H: haul, L: length (cm). Unimpaired reflexes were scored zero.

H	L	1	2	3	4	5	6	7	8	9	10
3	21	0	1	0	0	0	1	0	0	0	/
3	22	0	1	0	0	0	0	0	0	0	/
3	24	0	1	0	0	0	0?	0	0	0	/
4	36	0	1	0	0	0	0	0	0	0	/
4	23	1	1	0	1	0	0	0	0	0	/
4	45	0	1	0	0	0	0	0	0	0	/
4	38	0	1	0	0	0	0	0	0	0	/

The last reflex that was tested for soles ("head", Table 4) was not tested for plaice, because this reflex (R11 in Table 3) did formerly not result in any reaction of plaice. Reflexes that yielded consistency in all individuals were: R3 (vestibular-ocular response), R5 (operculum), R7 (gag), R8 (evade) and R9 (stabilise). R7 (gag) was however not selected, given doubt of the observers of its consequence on surviving individuals after being subjected to the "gag" test. However, plaice consistently pushed the probe outside of the gut, which was clearly visible at its mouth and gills (under water). Although not fully consistent, some reflexes were nevertheless considered relevant: R1 (righting, or serious attempts if water volume was limiting), R4 (tail grab) and R6 (mouth).

3 CALIBRATION TESTS OF RAMP AND SHORT-TERM SURVIVAL

3.1 Final set of reflexes

On Tuesday 4th of March 2014 two consecutive short hauls (<20min) were conducted (9h33 – 9h48; 10h13 – 13h28). Two observers got acquainted with handling fish during the tests above. The collected individuals of these two hauls were assessed directly after hauling, sole by Jochen and plaice by Marieke. The catch was released on the deck, fish were collected in buckets which were filled with water to minimise air exposure. They were subsequently brought to the sorting table for inspecting the RAMP-scores. Assessing the RAMP lasted max 3 minutes by individual fish and in total fish had to stay less than 20 min in the buckets with water, implying that most fish were assessed faster. The assessment of the scores was taken in the order mentioned in Table 7 for sole and Table 8 for plaice. The order is important for standardisation, as well as for a rapid possibility of assessing the reflex. Evading for instance is obviously easier to be tested after the fish have been held in the observer's hands, rather than when the stabilising reaction was tested. After assessing the scores, the fish were transferred to on-board survival tanks. Although the potential of confounding effects, one sole and one plaice were placed into a survival tank, enabling observers to assess mortality each 12 hours and identifying each individual fish without having to tag them. Fish were placed in survival tanks described in Depestele et al. (2014). Fish were monitored within 12 hours after accommodating them in the survival tanks and none of the individuals had died. All individuals survived also on Wednesday, i.e. about 30 hours after the initial tests. Only one sole died on Thursday morning (6 March 2014).

For sole it was clear that the reflexes that best described vivid individuals were "stabilise, mouth and tail grab", followed by the "vestibular-ocular response", which was however difficult to assess at times when soles curled around the hand of the observer. This implied however that the "head" reflex was present and this could therefore be an easy measure to assess. Natural righting was observed regularly, although some individuals remained at their backs for >5 sec and did not return to their natural position without stimulating them. The consistency could hence be questioned, unless there is a confounding effort of the catching process and hence mortality of these individuals. The "evade" response was not very clear as some individuals only drifted in the water. Additionally, one had to be careful to release the fish in the middle of the box as fish which were released towards the corner of the box did not need to evade further away. The use of small boxes might have been less appropriate for testing this reaction, as could have been the case for the "righting" response of larger individuals.

The most consistent reflexes of plaice were the turning of the eyes when the fish was turned around. The eyes remained focused and depending on the orientation "sank" into its body. The resistance of plaice to forced opening of the operculum was not tested for sole, but this was a clear aspect of this fish, when testing under water. Not fully consistent, but nevertheless a good indication of the reflexes was the "evade" response and the "tail grab". When the tail is touched and/or grabbed in a "good" way (which might require some practice), then the fish swim away or at least the body and/or fins make a wavy pattern and stimulates propulsion. Forced opening of the mouth of plaice was easily done, but most of the times, plaice did try to close it again or seemed to oppose against this. However, whereas the mouth of sole is difficult to open from the start, the reaction of plaice to opening of the mouth was rather after the mouth was opened by force.

The calibration tests of the reflexes were run directly after collecting fish from the codend, i.e. similarly to the discarding process. While this is appropriate for the validation tests, i.e. developing the link between discard survival and RAMP-scores, the authors suggest that it was not fully suitable for the calibration tests. The proposed reflexes of this study should therefore be seen as preliminary. The lack of full consistency was hypothesized to be due to the impairment of the tested individuals. Ideally, the reflex testing was repeated after >24hours. This would have allowed the fish to adapt and recover at least partly from the catching process. The tail grab for instance required actual grabbing of the tail such as in Figure 3, whereas controlling the fish on being alive or dead after being accommodated, revealed that touching the tails was sufficient. Similarly, the rotating of the eyes was more easily evaluated for fish that were just released from the codend, as many individuals were stunned. Fish that were lifted from the survival tanks after an accommodation period, were at times very lively which would have made it difficult to assess all reflexes for those. As a conclusion, this study suggests to test the seven detected reflexes both at the start and after the accommodation period. Testing the reflexes does last about one minute and did not seem to inflict a considerable stress on them (this was not formally tested).

3.2 Calibration tests of reflexes

There were 22 individuals of sole and plaice tested with mean (SD) lengths of 26.3 (4.6) cm for sole and 25.6 (5.1) for plaice (Table 7; Table 8). Impairment of a reflex (= no response) was scored one, and hence the presence of a reflex (response visible) scored zero. Only 6 individuals of sole showed all reflexes, while 10 plaice showed all of the investigated responses. The responses were hence not fully consistent amongst all individuals, although vitality was (subjectively) scored high for all individuals of plaice and sole. The individuals were caught in two consecutive hauls of ~15min in the morning at calm sea (<2 Bft). Only fish in a seemingly good condition were included in the tests. The tail grab was present in all sole individuals, while for plaice the vestibular-ocular response and the resistance of the operculum against opening were the only reflexes that consistently turned up. Other reflexes were <6 times absent in soles, except for evade. While this response was clearly present in preliminary tests (Table 5), it did not show up in 12 out of 22 tests. This raises doubts on the reflex. The responses of plaice showed a higher consistency and were only absent in <6 individuals, except for righting. Plaice did not turn back on its belly in 9 out of 22 cases. The mean RAMP-score of soles was 0.18 (+/- 0.16) and 0.16 (+/- 0.20) for plaice, when seven reflexes were considered.

Table 7 - The final set of reflexes was tested on 22 individuals of sole, caught in two hauls of 15 min (haul H9 and H10). Their lengths varied between 16 and 32 cm. The tested reflexes were: (1) righting, (2) vestibular-ocular response, (3) head, (4) evade, (5) stabilise, (6) mouth and (7) tail grab. Unimpaired reflexes were scored zero.

H	L	1	2	3	4	5	6	7
9	22	0	0	0	1	0	0	0
9	28	0	0	0	0	0	0	0
9	30	0	0	0	1	0	0	0
9	24	1	1	0	0	0	0	0
9	27	1	1	1	1	0	0	0
9	24	0	0	0	0	0	0	0
9	22	1	0	0	0	0	0	0
9	27	0	0	0	0		0	0
9	20	1	0	1	0	0	0	0
9	32	0	0	0	0	0	0	0
9	24	0	0	1	1	0	0	0
10	22	0	0	0	0	0	0	0
10	27	0	0	1	1	1	0	0
10	24	0	0	0	1	0	0	0
10	37	0	0	0	1	0	0	0
10	31	0	0	0	1	0	0	0
10	30	0	0	1	1	0	1	0
10	28	1	0	0	1	0	0	0
10	30	0	0	1	1	0	0	0
10	28	0	0	0	1	0	0	0
10	26	0	0	0	0	0	0	0
10	16	0	0	0	0	0	0	0

Table 8 - The final set of reflexes was tested on 22 individuals of plaice, caught in two consecutive hauls (haul H9 and H10) of 15 min. Their lengths varied between 17 and 39 cm. The seven tested reflexes were (1) righting, (2) vestibular-ocular response, (3) evade, (4) stabilise, (5) operculum, (6) mouth and (7) tail grab. Unimpaired reflexes were scored zero.

H	L	1	2	3	4	5	6	7
9	39	1	0	1	1	0	1	1
9	28	1	0	0	0	0	0	0
9	30	0	0	0	0	0	0	1
9	34	0	0	0	0	0	0	0
9	26	0	0	0	0	0	1	0
9	26	0	0	0	0	0	0	0
9	20	1	0	0	0	0	0	0
9	21	0	0	0	0	0	0	0
10	29	1	0	0	1	0	0	1
10	27	1	0	1	1	0	1	0
10	28	1	0	0	0	0	0	1
10	25	0	0	0	0	0	0	0
10	26	0	0	0	0	0	0	0
10	21	0	0	0	0	0	0	0
10	22	0	0	0	0	0	0	0
10	24	1	0	0	0	0	0	0
10	24	1	0	0	0	0	0	0
10	17	0	0	0	0	0	0	0
10	23	0	0	0	0	0	0	0
10	32	1	0	0	0	0	0	1
10	23	0	0	0	0	0	0	0
10	20	0	0	0	1	0	0	1

3.3 Physical injuries, complementing reflexes

The potential of physical injuries to predict the survival of discarded fish and benthic invertebrates was tested on-board the RV Belgica, based on a modified catch damage index (CDI) and is reported in Depestele *et al.* (2014). The potential of injuries as a complement for reflexes to predict discard survival should hence be considered. Given that injuries have the potential of predicting discard survival for plaice, we expected low CDI-scores. Mean (+-SD) CDI were indeed low for sole (0.5 +- 0.8) and plaice (1.3 +- 1.1).

Table 9 - Modified Catch Damage Index (CDI) to evaluate physical injuries for fish after catching and handling operations (modified from Esaiassen et al., 2013). Bruises are scored separately for head, body and tail.

CDI	*Description*	*Score*
1. Gear related damages	No gear marks	0
	Gear marks such as incisions	1
2. Skin-abrasion	<10% scale loss	0
	Between >=10% and <50% scale loss	1
	>=50% scale loss	2
3. Bruises: separate scoring for (a) head, (b) tail and (c) body	Non discoloration	0
	<50% discoloration on the area	1
	>=50% discoloration on the area	2
4. Pressure injuries	No compression detected	0
	<30% compression detected	1
	>=30% compression detected	2
5. Fin and tail damage	No marks	0
	<30% visible marks	1
	>=30% visible marks	2
Max total score (CDI)		13

Table 10 – Physical injury scores for sole. Column headers are explained in Table 9.

1	2	3a	3b	3c	4	5	CDI
0	1	0	0	1	0	1	3
0	0	0	0	0	0	1	1
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	1	0	0	0	0	1
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	1	0	0	0	1
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	1	0	0	0	1
1	0	0	0	1	0	0	2
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	1	0	0	0	0	1
0	1	0	0	0	0	0	1
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	1	0	0	0	0	1

Table 11 - Physical injury scores for plaice. Column headers are explained in Table 9.

1	2	3a	3b	3c	4	5	CDI
0	1	1	1	0	0	0	3
0	0	0	0	0	0	0	0
0	0	1	0	0	0	0	1
0	0	2	0	0	0	0	2
0	0	1	0	0	0	0	1
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	1	1
0	0	0	1	0	0	0	1
0	1	0	0	0	0	1	2
0	1	1	0	0	0	0	2
0	0	0	1	0	0	0	1
0	0	0	0	0	0	0	0
0	1	2	1	0	0	0	4
0	1	0	1	0	0	0	2
0	0	0	1	1	0	0	2
0	0	0	0	0	0	0	0
0	1	0	0	0	0	0	1
0	1	1	0	0	0	0	2
0	1	0	0	0	0	1	2
0	1	1	0	0	0	0	2

3.4 Short-term survival

The tested individuals of sole and plaice in the calibration tests (Table 7; Table 8) were accommodated in holding tanks (Figure 5). Hauls were similarly short (<20min) as the "reference hauls" in Depestele *et al.* (, 2014), which indicated high survival and were used as control for the holding tanks. The beam trawl used (8m beam width) had a double codend of 40mm mesh size. Fish were collected from the codend and directly transferred to buckets filled with sea water in order to minimize air exposure and stress. Reflexes were evaluated at the sorting table. Only fish that were in apparently perfect condition, based on subjective vitality scoring by the observers, were selected for the tests.

Figure 4 – Collection of fish in a favourable condition from short beam trawl hauls on-board the RV Belgica. Fish were directly transferred to buckets with sea water to minimize stress.

Figure 5 – Holding facilities were supplied with a continuous flow of sea water. Two individuals were accommodated in each of the tank: 1 plaice and 1 sole, enabling easy identification of the individuals.

All monitored individuals except one sole of 16 cm remained alive after an observation period of 70 hours. The sole individual died after on observation period of 48hours. It had a RAMP-score of zero, indicating a perfect condition. Its CDI was 0.43, a score driven by scale damage and blood mark on its body. All other individuals survived and were hence a good selection of fish in a favourable condition for determining the reflexes. The observers noted however that the fish were more vivid than they were during the testing of the reflexes. This seems to suggest that the selected reflexes are not independent of the condition of the fish. Fish that were released from the codend seemed to be impaired, although this was not formally tested. Fish that were accommodated in the holding tanks were evaluated within 12hours after being submerged, and then followed up by 24h observation period in order to minimize disturbance. Those individuals showed a clearer response to certain reflex actions. Therefore, when initially testing for consistent reflex responses, it is advisable to accommodate the fish for 24 h prior to testing of reflexes.

4 RECOMMENDATIONS FOR FOLLOW-UP

4.1 Monitoring environmental conditions during tests

Vitality is inversely related to reflex impairment. The envisaged selection of reflex actions should reflect a set of innate fixed response patterns that relate to the internal state of the fish. There cannot be any confounding factors during the calibration tests of this initial set-up. However, tests of unimpaired reflex actions were not fully the case during our experiments, although we have indications of what the unimpaired reflexes might be.

As highlighted above, the capture process (technical factors) was indicated as a confounding factor, and should be accounted for in future developments (see 4.2 Lessons learnt for the experimental design of the following-up). There are more potential confounding elements, namely environmental and biological factors. External factors can hardly be controlled for during the collection of test organisms. Therefore it is additionally important to register them. Environmental conditions such as temperature, capture depth, hypoxia, catch composition, etc. might all play a role in selecting “test” individuals. The registered parameters of the hauls are listed in Table 12. This list should be further extended in the follow-up experiment by for instance water temperature of the survival tanks, temperature on the seafloor, etc. Registrations of biological conditions of the fish were limited to length, but gender and maturity stage would provide a valuable addition, given that maturity affects survival and hence is likely to induce impairment (e.g. Revill et al., 2013).

Table 12 – Environmental conditions of experimental hauls

Haul	Depth (m)	Towing direction*	Current speed (kn)	Towing speed (kn)	Wind force (Beaufort)	Wave height (m)
1	34-37	against	1.3	3.5-4	5	1
2	26-33	against	2	3.5-4	4	0.5
3	24-25	with	1.7	3.5-4	4	0.5
4	37	with	2.1	3.5-4	4	0.5
9	45-52	against	1	3.5-4	3	0.5
10	48-51	against	2	3.5-4	3	0.2

* towing direction is either against the current, with or perpendicular to the current

4.2 Lessons learnt for the experimental design of the following-up study

The increased responsiveness of both fish species after the accommodation period indicated that they are sensitive to capture and handling stress. The capture and handling process led to sublethal effects based on subjective judgment by the observers. The effect of sublethal stressors can be objectively quantified, but to achieve this, consistent reflex actions should be tested *a priori* on “good condition” individuals, i.e. on individuals in an unstressed condition. A follow-up experiment should thus be designed to evaluate fish that has been accommodated for >24hours in the on-board holding facilities. Given the experienced vitality of fish that have been accommodated, it will be assumed that these fish are in “perfect” condition. This is currently based on subjective observations from the March 2014 trial. The subjective notion of the observers was founded in several observations, e.g. the fact that only slightly touching the tail (instead of grabbing it) resulted in a clear response for both sole and plaice. This subjective notice was also indicated by the escape reaction of both species when they were taken out of the water. The activity of some fish was pertinently high that they could not be held steady for observation. These individuals can be scored fully responsive to all reflex actions and are expected to survive the on-board holding.

The reflex actions which were tested during the campaign in March 2014 should thus be re-evaluated on individuals that have undergone an accommodation period in order to eliminate the obvious sublethal effects. Primarily an accommodation period of 24h should be tested, but also 48h could be recommended to evaluate recovery potential. The top four reflex actions are expected to be consistent across all individuals since this was almost already the case in the individuals that were impaired from the capture and handling process (Table 13). These individuals cannot show any physical injuries and should fully survive accommodation in the on-board holding facilities. Although only four out of seven reflex actions are expected to be fully consistent, the remaining three reflexes should also be tested, as they might be responsive for the induced stress of holding individuals in the tanks. Fish in unimpaired conditions can only be found in their natural habitat. Approximation can be achieved through on-board holding tanks, but this assumption is an important consideration in the developmental stage. Therefore the three reflex actions which were not consistent should still be included in the assessment. Additionally, it might well be that those reflex actions will become consistent given that responsiveness changes after holding them in the tanks. However, since the objective of the study is to correlate reflex actions with discard survival rather than an impairment assay that is most sensitive to sublethal effects, the most conservative estimates are considered as focal reflexes (M. Davis, pers. comm.). The top four reflex actions, i.e. the last and least susceptible to impairment, should receive primary attention.

Table 13 – The top four unimpaired reflexes for sole were the tail grab (7), mouth (6), stabilize (5) and the vestibular-ocular response (2). The reflexes for plaice include the vestibular-ocular response (2), the operculum (5), the evade reflex (3) and the mouth (6) as well.

Reflex*	1	2	3	4	5	6	7
# impaired (sole)	5	2	6	12	1	1	0
# impaired (plaice)	9	0	2	4	0	3	6

* The tested reflexes for sole were: (1) righting, (2) vestibular-ocular response, (3) head, (4) evade, (5) stabilise, (6) mouth and (7) tail grab. The seven tested reflexes for plaice were (1) righting, (2) vestibular-ocular response, (3) evade, (4) stabilise, (5) operculum, (6) mouth and (7) tail grab.

Depending on the outcome of the following-up tests, we expect that the RAMP will be based on at least the top four reflex actions and on the physical injuries (given the fatal event in this study, and earlier tests in Depestele *et al.*, 2014). Assessment of injuries might be improved in objectivity by scoring presence and absence instead of scaling severity. An injury symptom might be scored absent (0) when not present or when there is doubt about presence, and present (1) when easily observed. Reflex and injury scores for an individual animal are then summed and divided by the total observable impairments possible to calculate proportion impairment (RAMP score). It should be decided which injuries are primarily triggered mortality. Slight impairment might however not be fully excluded. To test this, fish should be accommodated in survival tanks until mortality stabilizes. This is difficult to test and a holding period of 60 h might be insufficient (Depestele *et al.*, 2014). Even if reflex actions are not 100% consistent and the survival curve does not level off completely, modelling might reveal that a RAMP-score which is only near the highest possible value can also be sufficient to predict 100% survival, given the limitations of the holding experiment.

The follow-up experiments will thus in a first instance focus on fish in a good condition. Once reflex actions and injuries have been found consistent, a suite of indicator for full survival potential was developed. A next step is to account for the potential impairment of fish after for instance the catching process. A full range from zero to 100% survival should be tested and reflex actions which are sensitive to impairment should be identified. Therefore the seven reflexes found should all be accounted for. Some of them might relate to the capture and handling process, or even being accommodated in holding facilities. The effect of stressors and the sensitivity of reflex actions and injuries to impairment is the validation phase. The final RAMP-curve is likely to be the relationship between discard survival and the RAMP-score. RAMP-score can either be based on both reflexes and injuries, or one can opt to create a three-dimensional graph where reflexes are assessed on an X-axis, injuries on a Y-axis and their relationship with discard survival on the Z-axis. This would require a minimum number of reflex actions for a smooth curve rather than only the four. The identification of >4 reflex actions is therefore also important in the follow-up experiment.

The process of developing a RAMP-curve can be summarized in these steps:

1. ***Collection of unimpaired individuals of a particular species, representative of the natural environment***

The collation of unimpaired individuals is an iterative process as illustrated above. One needs to identify which reflex actions are fully consistent in individuals. Several tests might be needed to get accustomed with the investigated species, and preferably in many different conditions. Wild-captured organisms have the advantage of being representative of the actual fish population, but there is a confounding element in capturing them. Cultivated organisms might resolve this, since reflex actions are genetically determined, i.e. innate and fixed. Cultivated organisms should have the same reflexes as wild ones. The differences in strength of reflexes are factored out by testing for presence-absence rather than scaling the reflex actions. Reflex action testing on “perfect condition” fish might therefore complement the suggested reflex actions found at sea.

2. ***Development of reflex action tests in unimpaired individuals: calibration***

While the iterative process of collating unimpaired individuals develops, observers interact freely with the organism. A vast range of reflexes is tested and assessed for their consistency. When utilizing wild-captured animals, the biological, environmental and technical factors that interplay during collating and accommodating should be minimized. Therefore reflex action tests should ideally be evaluated in a range of situations in order to identify when reflex consistency appears and when it leads to a minimal variation in reflex responses. While the situation with minimal variability brings about the identification of the final reflexes, the “other” situations indicate which factors lead to impairment. This is to be tested in the following step, i.e. the validation phase. Once the observers have noted when fish are in a situation which minimizes impairment, a baseline set of assumptions will undoubtedly be apparent. These assumptions should be acknowledged as the framework in which the reflexes will be related to discard survival. For instance, testing reflexes in holding facilities assumes that holding facilities do not impair them. The reflex actions which are tested in fish in the “best” condition and which consistently are scored unimpaired, are the final set of reflexes that reflect 100% discard survival. “Best condition” fish is *a priori* subjectively assessed on the basis of activity (motion) and responsiveness to reflex action tests. External physical injuries should be absent and survival in holding facilities should be 100%.

3. ***Relate reflex action tests to discard survival: validation***

The RAMP-curve relates the RAMP-score to the discard survival. The RAMP-score can be either based on a mathematical combination of injuries and reflexes or on reflexes or injuries separately. This needs to be explored, and requires hence RAMP-scores of a wide set of reflexes and injuries for many individuals in dead and alive status. In any case should 100% survival be related to unimpaired individuals and the “optimal” RAMP-score. The most conservative reflex actions should be selected to this end, and it should be tested whether these reflexes are lacking when impaired fish are tested. This implies that hauls with a longer duration are needed, and accordingly temperature, catch weight and composition and other stress-related factors should be identified to assess reflex impairment relates to some of these factors. The final goal would be to link discard survival of these individuals with the most conservative reflex actions and RAMP-score.

5 CONCLUSION

Preliminary investigations have been undertaken on-board the RV Belgica to assess the potential presence of a range of reflexes in sole and plaice. A wide range of potential reflexes was investigated prior and during the sea trial, leading to a final selection of seven reflexes with a good potential of being consistently present in fish in a favourably vivid condition. Fish in a “perfect” condition could not be retrieved, but 22 individuals of plaice and sole were selected from short hauls and their survival potential was evaluated during 70 hours in on-board holding facilities. Only one sole died, and indicated hence that the control fish for the calibration test serve purpose.

The final selected reflexes were very similar for sole and plaice, except for one. Forced opening of sole’s operculum did not reveal much resistance of the fish, while holding plaice at its head did not induce any curling of the fish. The most consistent reflexes for sole were called “stabilise, mouth and tail grab”, followed by the “vestibular-ocular response”. Vivid individuals seemingly dig into the sand or stabilise themselves onto the floor of the water-filled box. They also keep their mouth closed when trying to open it with a probe. When fish have stabilised, they respond clearly to grabbing their tale or even tickling it. The “head” reflex was easy to assess, though not always present. However, it is clear that vivid soles curled around one’s hand when they had been in the holding tanks. This was not that obvious for fish that were just released from the codend. Natural righting was observed regularly, although some individuals remained at their backs for >5 sec and did not return to their natural position at all or only after stimulating them. The consistency could thus be questioned, but good candidate reflexes were proposed for sole, and should be further evaluated. The most consistent reflexes of plaice were the turning of the eyes when the fish was turned around longitudinally. The resistance of plaice to forced opening of the operculum was a clear reaction as well. Not fully consistent, but nevertheless a good indication of the reflexes was the “evade” response and the “tail grab”. When the tail is touched and/or grabbed in a “good” way (which might require some practice), then the fish swim away, or at least the fins stimulate propulsion. The mouth of plaice was easily opened, but mostly the individuals tried to close it or seemingly opposed to the forced movement.

Our investigations confirmed that on-board holding facilities result in high survival of plaice and sole from very short hauls (<20min). Investigated individuals were non-randomly selected and thus it was not surprising that their physical injuries were limited. These individuals were suitable for developing the reflexes, although they were limited in number (22 for plaice and 22 for sole) and they also did not range over a wide variety of fish conditions (e.g. limited length variability). The seven reflexes from these preliminary investigations are therefore proposed as candidates for to the development of a RAMP score for sole and plaice.

The tests of the reflexes were run directly after releasing fish from the codend. When examining the survival from fish that were accommodated for some time (e.g. 48 hours), we noted that they reacted more strongly and had much clearer responses to the reflex tests. In particular the tail grab worked very nicely for sole when their status (alive or dead) was tested. Therefore we suggest that the proposed reflexes are tested once more on surviving individuals of short hauls after an accommodation period of >24hours. Consistency of the outcome of the reflex tests is expected to be improved when the impairment from the catching process is accounting for. Other recommendations for follow-up tests relate to the registration of potential environmental and biological confounding factors.

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7 APPENDIX

Table A 1 - Survival estimates of invertebrate discards in beam trawl fishery, based on holding tank experiments (>=48 h). The number of estimates is limited for a 4 m beam trawl with chain mat and haul duration >60 min, both for number of individuals and species. Number of individuals indicated in parentheses (Modified from Depestele et al., 2014).

Species	*Haul duration (<30 min)*			*Haul duration (60-150 min)*		
	4 m chain mat[1]	*4 m tickler chain*[2]	*12 m tickler chain*[3]	*4 m chain mat*[2]	*4 m tickler chain*[2]	*12 m tickler chain*[4]
Annelida (Polychaeta)						
Aphrodita aculeata	0.91 (125)		0.98 (653)	1 (15)		0.86 (248)
Mollusca (Bivalvia, Cephalopoda, Gastropoda)						
Aequipecten opercularis	0.97 (60)					
Arctica sp.			0.09 (130)			0.1 (1480)
Chlamys sp.						0.98 (53)
Pecten maximus	1 (65)					
Spisula elliptica						
Spisula substruncata		0.59 (439)			0.68 (360)	
Eledone cirrhosa	0.88 (25)					
Buccinum undatum	1 (37)		0.4 (96)*			0.96 (171)
Neptunia antiqua	1 (35)					
Euspira catena			1 (10)			
Echinodermata (Asteroidea, Ophiuroidea, Echinoidea)						
Asterias sp.	0.99 (126)		0.93 (414)	1 (62)	0.96 (200)	
Astropecten sp.	1 (17)		0.93 (771)		0.93 (88)	0.91 (660)
Crossaster papposus	0.92 (24)					
Luidia sarsi			0.98 (246)			
Ophiura sp.		0.91 (85)	0.6 (133)	1 (59)	0.91 (153)	0.88 (520)
Psammechinus miliaris	0.38 (100)					
Arthropoda (Crustacea)						
Cancer pagurus			0.58 (12)		0.66 (53)	0.14 (21)
Corystes cassivelaunus			0.48 (872)		0.5 (14)	0.34 (1667)
Crangon sp.		0.92 (106)				
Liocarcinus sp.	0.58 (120)	0.84 (88)	0.62 (803)		0.61 (150)	0.47 (275)
Macropodia rostrata	0.74 (23)					
Nephrops sp.			0.83 (40)			0.58 (45)
Pagurus sp.	0.92 (169)		1 (30)	1 (23)	0.93 (27)	0.8 (244)
Portunidae				0.86 (99)		

*Only survival estimate of an observation period which is much longer than the others (37 days, Mensink *et al.*, 2000).

Table A1 (continued). - Survival estimates of fish discards in different configurations of beam trawl fishery. All figures are based on survival experiment in holding tanks (>=48 h). Note that the number of estimates is limited for a 4 m beam trawl with chain mat (number of individuals) and for roundfish, elasmobranchs and non-commercial fish. No figures exist for a 12 m beam trawl with chain mat, except *Scyliorhinus canicula*. Number of individuals indicated in parentheses.

Species	*Haul duration (<30 min)*			*Haul duration (60-150 min)*				
	4 m chain mat[1]	*4 m tickler chain*[2]	*12 m tickler chain*[3]	*4 m chain mat*[2]	*4 m tickler chain*[2]	*9 m chain mat*[4]	*12 m chain mat*[4]	*12 m tickler chain*[5]
Roundfish								
Gadus morhua[a]								
Merlangius merlangus			0 (42)					
Trigla sp.			0 (18)					
Elasmobranchs								
Raja naevus	0.59 (32)							
Scyliorhinus canicula	0.93 (42)[b]			0.98 (120)				
Flatfish								
Solea solea		0.87 (272)	0.47 (1202)	0.12 (104)	0.2 (275)	0.53 (114+50)[c]	0.76 (186+40)[c]	0.16 (581)
Pleuronectes platessa	0.39 (122)	0.94 (336)	0.34 (3568)	0.31 (55)	0.07 (425)	0.37 (515+40)[c]	0.47 (207+40)[c]	0.1 (2051)
							0.80 (256+40)[c]	
Plathichthys flesus		0.79 (14)		0.25 (8)	0.06 (341)			
Limanda limanda	0.23 (22)	0.82 (350)	0.52 (1063)	0.02 (166)	0.01 (3984)			
Microstomus kitt			1 (2)					
Scophthalmus rhombus					0.19 (31)			
Scophthalmus maximus					0.58 (50)			
Non-commercial fish								
Agonus cataphractus			1 (7)					
Arnoglossus laterna			0 (33)					
Buglossidium luteum		0.16 (44)	0.75 (155)					
Callionymus lyra	0.16 (115)	0 (19)	0.52 (23)					
Pomatoschistus sp.		0.95 (19)						
Trachinus vipera			0.94 (254)					

[a] Lindeboom and de Groot (1998: 167) considered all gadoids dead within few minutes after being brought aboard
[b] Survival estimate based on 12 m beam trawling with chain mat
[c] Total mortality estimated were based on immediate and short-term mortality estimates (first and second number in parenthesis) and adjusted for control deaths. (Revill *et al.*, 2013)
[1] Kaiser and Spencer, 1995; [2] de Groot and Lindeboom, 1994; Keegan, 2002; [3] Bergman *et al.*, 1990; de Groot and Lindeboom, 1994; Fonds, 1991; Keegan, 2002; mensink *et al.*, 2000; Van Beek *et al.*, 1990; [4] Revill *et al.*, 2013; [5] de Groot and Lindeboom, 1994; Fonds, 1991; Fonds *et al.*, 1992; Van Beek *et al.*, 1990

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Contact:

Jochen Depestele,
Wetenschappelijk onderzoeker
Instituut voor Landbouw- en Visserijonderzoek ILVO
Eenheid Dier
Ankerstraat 1 - 8400 Oostende
Tel. +32 (0)59 56 98 38
jochen.depestele@ilvo.vlaanderen.be

Deze publicatie kan ook geraadpleegd worden op:
www.ilvo.vlaanderen.be

Instituut voor Landbouw- en Visserijonderzoek
Burg. Van Gansberghelaan 96
9820 Merelbeke - België
T +32 (0)9 272 25 00
F +32 (0)9 272 25 01
ilvo@ilvo.vlaanderen.be
www.ilvo.vlaanderen.be

ILVO Instituut voor Landbouw- en Visserijonderzoek

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