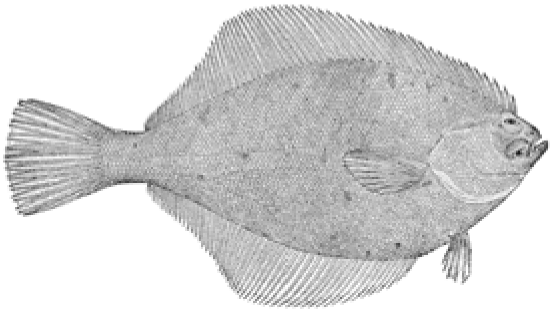
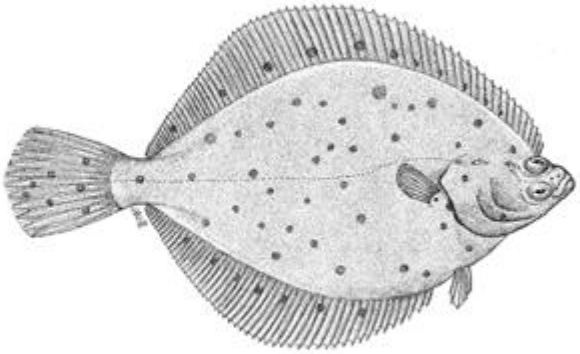
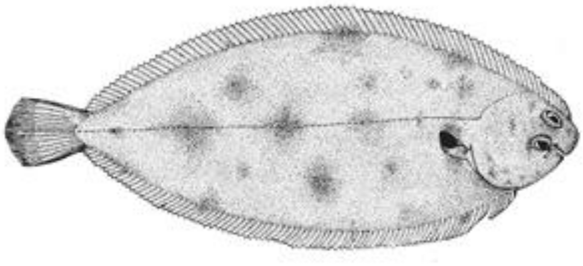
Burying behaviour and camouflage as indicators of viability in dab (*Limanda limanda*), plaice (*Pleuronectes platessa*) and sole (*Solea solea*).







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Abstract

For a long time, fish were considered to be unable to feel and experience pain and emotions. However, as recent research indicates that fish are likely to have this capacity and the public opinion is that potential animal suffering should be minimized, care should be taken to protect fish welfare. There is reason for concern regarding the welfare of wild caught fish. Catching methods may lead to substantial suffering of the fish, decreasing the viability and survival chances of those that are returned to the sea for reasons of being too small or of an unwanted species. Fisheries may lead to an allostatic overload of the caught fish and this study searches for new methods to measure the allostatic load of fish in order to assess their viability and welfare. Animal welfare consists of three main aspects, naturalness, function and affective state, of which the function based view seems especially appropriate to describe the welfare of wild caught fish. Currently used methods for assessing the welfare status of the fish are mainly based on estimating survival chance by testing for survival directly of by scoring of injuries (CDI) or testing for reflexes (RAMP). However, there are uncertainties about how to interpret the results of these tests and they do not give complete information on the welfare status of the fish. Thus, new ways to assess the welfare status of the fish are required.

In this study, burying behaviour and camouflage behaviour, which are regarded to be essential in the life of flatfish, were tested for their possibility to be used in the assessment of viability of the flatfish species sole, dab and plaice. The results were linked to the results obtained with the RAMP test performed on the same fish, to see if the different tests gave comparable results. In different treatment groups, the fish were used as control or were tested for RAMP, burying, camouflage or RAMP, burying and camouflage.

Results on burying behaviour and camouflage suggest that predictions about viability can be made based on measurements on these behaviours. For burying behaviour, the number of movements exerted during the burying action of plaice was significantly correlated with the weight difference of plaice over a 10 days period. For camouflage behaviour, the amount of camouflage reactions of dab was significantly correlated with the weight difference of dab over a 10 days survival period. Assuming growth as the reference standard, criterion (concurrent) validity is provided and the idea that burying behaviour and camouflage can be used to predict viability of flatfish is supported. Burying behaviour and camouflage are behavioural tests and it should be taken into account that their results might be influenced by individual characteristics in the same way as is the case for the reflex test. Burying and camouflage seem to involve regulation by higher cognitive processes and may be more sensitive to impairment compared to reflexes, which are typically maintained even when the animal is in a poor state of health. Therefore burying behaviour and camouflage are useful proxies for assessing viability of flatfish. Such methods for assessing welfare status of fish can be used to evaluate how (aspects of) catching methods affect wild caught fish and may be improved, for example to allow a successful return of unwanted fishes.

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1. Introduction

Welfare of fish is of increasing interest to society (Ashley, 2007; Huntingford and Kadri, 2009), especially in Europe. Following a recent focus on fish welfare in aquaculture systems, the next step is to also pay attention to the welfare in wild caught fish. There are three main aspects of animal welfare; biological functioning, naturalness and affective state (Duncan and Fraser, 1997; Fraser, 2003). Physical health, which is part of the function based view, is “the most universally accepted measure of welfare” (Ashley, 2007). The natural state view focuses on the ability of an animal to express its natural behaviour and in the affective state view, the suffering and emotional experiences determine welfare. Although it is possible to analyse the affective state of a fish in a laboratory (Hans van de Vis, pers. comm.), it is difficult to measure the affective state of fish as no instrumental methods are available to measure affective states directly. The emotional expressions of fish are difficult to recognize by humans and together with the distant evolutionary relatedness between fish and humans, this might have contributed to the fact that for a long time, fish were considered to be unable to feel and experience pain and emotions. However, recent studies indicate that this might be untrue.

There are several homologies between the brain structures of fish and mammals. This indicates a shared vertebrate brain organisation which contributes to the likeliness that fish are able to feel and experience pain and emotions as do mammals. For instance, Broglio *et al*. (2005) found that the lateral pallial region and the medial pallial region of the forebrain are homologous with the hippocampus and the amygdala in mammals, which have a function in cognition and emotions (Braithwaite and Boulcott, 2007). The cerebellum is another brain structure of fish that shows homology with the mammalian brain structure. The cerebellum is involved in the conditioning of simple motor reflexes and has clear functional similarities among mammals and fish (Rodríguez *et al*., 2005). Recent studies provide evidence that the cerebellum is also involved in processes like associative learning, emotional conditioning and spatial cognition (Rodríguez *et al.*, 2005; Broglio *et al.*, 2003). Fish also show behaviour which results from both cognition and emotion. For instance, fish can adjust their fighting behaviour when they face an opponent that they have faced, or observed, before (Chandroo *et al.*, 2004). This was shown by McGregor *et al.* (2001) for Siamese Fighting fish (*Betta splendens*). In their study, an individual fish was allowed to observe the confrontation between two other individuals. When the observing fish was later confronted with the more aggressive of the two fish it had observed, it showed more aggressive behaviour in the confrontation compared to the confrontation with the less aggressive individual. So, their behavioural response depended on the level of aggressiveness that the opponent showed in the earlier confrontation and on the outcome of that confrontation. Comparable results were found for pygmy swordtails (*Xyphophorus nigrensis* and *Xyphophorus multilineatus*; Morris *et al.*, 1995), sea trout (*Salmo trutta*; Höjesjö *et al.*, 1998) and rainbow trout (*Oncorhynchus mykiss;* Johnsson, 1997). Another example of fish showing complex behaviour that likely results from cognition and emotion is the conditioning of goldfish (*Carassius auratus*) to avoid electric shock (Ehrensing *et al.*, 1982). When the fish were given morphine, the fish could not be conditioned and the fish only responded to a high voltage of electric shock. Avoidance behaviour as a result of experience with a noxious stimulus has been shown for other fish species as well and indicates that fish indeed experience the noxious stimulus as unpleasant or painful. In angling experiments with carp (*Cyprinus carpio*) and pike (*Esox lucius*) it was shown that the catching rate decreased with time and the number of fishes caught. Also few fish were caught for a second time during the experiment (Beukema, 1970a; Beukema, 1970b).

There are arguments against higher cognitive abilities in fish as well. For instance, Rose (2007) argues that fish cannot psychologically experience pain as they lack a cerebral cortex. Also Sneddon (2004) states that the lack of a cortex-like brain structure is the main reason for discussion on the fishes’ cognitive abilities. Following the evolutionary tree of vertebrates, the most highly evolved species, like humans and primates, have the most developed (neo)cortex. Down the evolutionary tree, the cortex of vertebrate species becomes less developed. However, fish do appear to possess a rudimentary cortex area (Ashley and Sneddon, 2008) which could mean that they also possess at least some ability to consciously experience pleasure and pain. Therefore, research makes it plausible that fish are able to feel and experience pain and emotions. The public consensus is that potential animal suffering should be minimized (Ohl and van der Staay, 2012), and a precautionary principle seems appropriate here; a scientifically plausible but so far unproven risk mandates action to protect. Care should thus be taken for the protection of fish welfare (Hürlimann *et al*., 2014).

The welfare of fish has already got attention for a longer time (Ashley, 2007; Huntingford and Kadri, 2009), but this was mostly for fish in aquaculture systems. Studies on the welfare of wild caught fish are lacking. It can be imagined that fish welfare is impacted during the fishing process and that this may depend on the fishing method that is being used, e.g. a short haul with light fishing gear will have less impact compared to a haul with heavy fishing gear for a long duration. Therefore, it is necessary to investigate the welfare of fish in wild caught systems and to find practical methods or indicators to quantify the welfare of fish directly on board of fishing vessels. In the future, such indicators may also help in the development of fishing methods that account for the welfare of fish.

One way to estimate fish welfare is to use survival as a proxy for the welfare of fish. When a fish is returned to the sea and its chance to survive is reduced due to the fishing procedure, this fishing procedure apparently has a negative influence on the fish welfare. As such, survival is a measure of biological functioning or physical health. The most straightforward method to assess survival chances is to perform survival experiments in which the fish are kept in tanks and monitored for their survival rate (e.g. Depestele *et al.*, 2014b; Davis, 2007). However, in practice this method is not always possible and it is time consuming. Therefore, survival probability proxies or welfare indicators are used to assess the survival chance of the fish. These are for instance the catch damage index (CDI) and reflex test (RAMP). The CDI scores the injuries of the fish (Depestele *et al.*, 2014b) and the RAMP targets reflexes of the fish to peripheral stimuli (Davis, 2010; Depestele *et al.*, 2014a). The impact of fishing methods on caught fish can be assessed, for example, from a perspective of animal welfare, survival chances or allostatic load (Korte *et al.*, 2007). Allostatic load is part of the allostasis concept (McEwen, 1998), building on classical homeostasis (i.e. the maintenance of the internal state of an organism, Cannon, 1932), "in the context of an organism's life cycle and in relation to individual experience and how they respond to their physical and social environments" (McEwen and Wingfield, 2009). According to the allostasis concept, each organism has an allostatic state and coping abilities, which are determined by its daily activities and an accumulation of factors, such as age, size, species, season, sex, personality, physical state, time of the day and the organisms' history of environmental challenges. Changes in the condition of the animal (such as additional energy requirements for migration or breeding), changes in the environmental conditions or other disturbances may lead to an allostatic load on the organism which is comparable to a stressor (McEwen and Wingfield, 2007). The organism has to adapt to the situation in order to cope with the allostatic load and to bring its internal system and allostatic state back in balance. If the energy needed to deal with the allostatic load exceeds the available energy of the organism, this results in an allostatic overload which may impair the health of the animal as the internal system is overworked (Korte *et al.*, 2005; McEwen and Wingfield, 2007). Thus, the load on animals imposed by disturbances should be within their coping abilities in order to maintain a good health and welfare (Broom, 1986; Korte *et al.*, 2007; McEwen and Wingfield, 2007).

To assess the allostatic load of an organism, physiological parameters, such as cortisol, neurotransmitters and immune responses can be used (Korte *et al.*, 2005). Cortisol is known to be the most informative and accessible marker of stress in fish (Pottinger, 2008). Piato *et al.* (2011) showed that unpredictable chronic stress in zebrafish (*Danio rerio*) increased anxious behaviour, impaired cognitive function and increased cortisol levels. The disadvantage of the use of physiological parameters to assess the allostatic load of an organism is that it is an invasive method. Invasive methods disturb the animal and may lead to additional stress, which might bias the results. Secondly, physiological measurements often require laboratory measurements, which is a complicating factor in field studies. Therefore, non-invasive methods are preferred over invasive methods, as they are regarded to have less impact on the animal and are more practical under field conditions. Besides the currently used CDI and RAMP tests, another possibility could be to use behavioural tests to measure the viability of the fish and to assess the allostatic load. Animal behaviour tests are used typically to test the individual characteristics or traits of an animal as for instance animal personality (Gosling, 2001). Possibly, such tests can also be used to assess allostatic load by measuring viability.

When animal behaviour tests are used to measure viability, it should be made sure that the test indeed measures viability instead of individual characteristics of the animals. Therefore, behaviours or reactions of the animal should be used that the animal always shows when it is given the appropriate stimuli, independently from individual characteristics. Such reactions or behaviours are also termed fixed action patterns (FAP) (Thorpe, 1951; Schleidt, 1974). Often they contribute to the survival of the animals and therefore they are very important for the animal to express. Fixed action patterns can be very species specific and species specific characteristics should thus be taken into account to make correct predictions about the viability of an animal. The testing of fixed action patterns is comparable with the testing of reflexes (RAMP), but at a higher cognitive level.

In the case of flatfish like dab (*Limanda limanda*), plaice (*Pleuronectes platessa*) and sole (*Solea solea*), which are important target species in the Dutch fisheries (www.agrimatie.nl, 4-12-2014; www.pvis.nl, 4-12-2014), burying and camouflage behaviour are very typical in their sedentary lifestyle. Flatfish bury themselves to reduce drag forces from the water currents (Arnold, 1969; Gibson, 2005) and to reduce predation risk (Gibson, 2005; Ansell and Gibson, 1993). Burying behaviour combined with cryptic colouration is regarded to be the first line of defence in flatfish against predation (Stoner and Ottmar, 2003). Cryptic colouration, or camouflage, is a physiological response that is possibly influenced by higher cognitive processes. It is the result of changes in the distribution of pigment, skin reflectance and contrast of the patches in the skin according to visual stimuli from the background (Gibson, 2005; Saidel, 1969; Healey, 1999). As both burying and camouflage are essential for survival, flatfish are expected to show these behaviours naturally when they are given the appropriate stimuli and therefore they are regarded to be fixed action patterns that might be used to assess the viability of the fish.

So, the welfare of fish is of increasing interest to society and a precautionary principle can be supported to take care for the protection of fish welfare. To investigate the welfare of wild caught fish, it is necessary to find practical methods or indicators that can be used to quantify the welfare of fish directly on board of fishing vessels. In the future, such indicators may help in the development of fishing methods that account for fish welfare. This study investigates two possible new methods to measure the welfare of flatfish; burying behaviour and camouflage. Because little is known about the welfare of wild caught (flat)fish, this study will contribute to the understanding of how to assess the welfare of wild caught flatfish and provides the first steps in the development of new methods to do this. It will provide a basis for further research on this topic, which is necessary for the further development and validation of these and other new methods to assess the welfare of wild caught flatfish. It should be taken into account that there are many different fish species for which very different methods to assess their welfare might be required. To set the first steps in the development of new methods, sole, dab and plaice will be tested for burying behaviour and camouflage. The results will be linked to results obtained with the RAMP test performed on the same fish to see whether these new methods provide comparable results as currently used methods. This is important to evaluate the validity and usefulness of the new methods. Although the RAMP test is developed to assess the survival chances of fish, it also gives an indication of the viability and can thus be compared with the new methods. The new methods will also be evaluated on their content and construct validity to make sure that the methods indeed say something about the viability instead of individual characteristics of the fish (www.explorable.com, 15-10-2014).

2. Material and methods

This study investigates two possible new methods to measure the welfare of flatfish; burying behaviour and camouflage. To set the first steps in the development of these new methods they will be investigated for sole, dab and plaice. The same fish will also be tested for their reflexes (RAMP) in order to investigate if the new methods give comparable results with a method that is currently in use and to evaluate construct validity of the new methods.

2.1 The animals

Hatchery reared sole (*Solea solea*; 50 individuals; 45.2g ± 18.4 SD; 160.9 mm ± 19.3 SD), wild caught dab (*Limanda limanda*; 18 individuals; 105.5g ± 37.7 SD; 215.4 mm ± 23.3 SD) and wild caught plaice (*Pleuronectes platessa*; 5 individuals; 18.6g ± 9.8 SD; 121.4 mm ± 20.2 SD) were kept in a tank of 110x90x60 cm (dab and plaice were kept together) with a constant flow-through of natural seawater pumped from the Oosterschelde (120 L/hour). The tank was placed in a climate chamber which was kept at a temperature of 10 to 12 °C. The water was regularly checked for temperature (10.9 °C ± 1.9 SD) and oxygen (8.4 mg/L ± 0.7 SD). As the water was constantly refreshed, it was not necessary to monitor other parameters. The fish were fed a maintenance food (1% of their body weight) once a day. The hatchery reared sole were fed with special food for sole and the wild caught fish were fed with cut ragworms (*Nereididae*). The fish were kept at a light regime of 14L:10D (light period from 7.00 till 21.00) and exposed to a light intensity ranging from 50 lux during the survival experiments to 170 lux during the tests. The fish were caught from the holding tank with a small fishing net, which allowed precise catching of an individual fish instead of disturbing all the fish. During the experiments fish were caught by hand (slow approach, gripping the fish from the anterior side around its head while covering its eyes), as this causes less disturbance than netting the fish. At the start of the experiments, fish were measured for length and weight. To ensure that the fish could not be influenced by pheromones of the previous fish and to ensure a sufficient oxygen level and a correct temperature of the water, the water was constantly refreshed during the experiments. Fish were food-deprived 12 to 24 hours before testing and, as fish had to be used multiple times because of limited availability of fish, a recovery time of at least 24 hours was used between different tests and handling. After the experiments, the hatchery reared sole were killed with phenoxyethanol and the wild caught fish were killed by giving them a blow on the head.

2.2 Treatments and survival

Some fish functioned as a control group of which only length, weight and survival were measured. Other fish were additionally tested for RAMP, burying or camouflage or were tested for the total set of the three tests. For hatchery reared sole, 50 individuals were used during the experiments, 10 individuals per treatment (control, RAMP, burying, camouflage and total). As there were less wild caught fish available, it was not possible to test the same number of fish as with sole. For dab, 18 individuals were available, 9 of which were tested for all the tests (total) and 9 of which were used as control individuals. For wild caught plaice only 5 individuals were available and they were all tested for all three tests. Ten dab and 5 plaice were also used in an additional camouflage test at the end of the experiments.

Both, the hatchery reared sole and the wild caught dab and plaice were tested for survival for a period of ten days after a round of experiments. Their mortality was monitored during this period. At the end of the ten days they were measured for their weight, as weight gain or loss assumingly reflects the condition of the animals. For the survival experiment the animals were kept in tanks of 60x40x11.5 cm which were darkened (bottom and walls) by a covering of garbage bag plastic. A substrate of 0.2 to 0.5 mm filtered sand was placed on the bottom (2 cm height). The tanks had a constant flow of fresh seawater of 123 L/hour.

2.2.1 RAMP testing

To be able to compare the results of the new tests with the results of a test that is currently being used, a RAMP test was performed. In the RAMP tests the reflexes of the fish to several peripheral stimuli were tested. Reflex tests were performed in a white tank of 60x40x11.5 cm filled with natural seawater according to the scheme provided in table 1. The tested reflex actions were chosen on basis of their consistency across the studies done by Davis (2007), Kestin *et al.* (2002) and Depestele *et al.* (2014a), the existing protocol for the assessment of the consciousness of turbot (Hans van de Vis, pers. comm.) and on the basis of pilot experiments performed at the start of this study. A description of the reflex actions is given in table 1, as well as the protocol that should be followed when performing the test. For hatchery reared sole the reflexes were scored as present (1) or absent (0) to minimize observer subjectivity. However, as all individuals responded, this gave minimal information and a higher resolution scoring scale was used in the experiments with wild caught dab and plaice. Here, the reflexes were scored as present (2), intermediate (1) or absent (0). The RAMP score (ranging from 0 = no impairment to 1 = fully impaired) could be calculated with the formula given by Davis (2007).

(1) RAMP = 1 – (total reflex response score/total score possible)

Table 1. Description and sequence of the reflex actions used in the RAMP-test. The observation that should be seen when the fish is unimpaired is indicated as well as how the reflex has to be tested (Kestin *et al.* (2002); Depestele *et al.* (2014a); turbot protocol, Hans van de Vis, pers. comm.).

Test	**Evade**	**Breathing**	**Righting**	**Operculum closure**	**Mouth closure**	**Tail grab**
Protocol	Release the fish at the water line	Observe the fish undisturbed for at least a minute	Place the fish with its blind side facing up and watch if it returns to its natural position within 5 seconds	Open the operculum with a probe or pencil	Open the mouth with a probe or pencil	Grasp firmly the tail with two fingers and drag the fish to the water line
Location	In water	In water	In water	In water	In water	In water
Observation	Active swimming behaviour away from the tester	Movements (existence and rhythm) of the gills opercula	Ability or attempts to return to a natural position within 5 seconds	Resistance to forced opening and closure after forced opening	Closure of the mouth after opening	Attempts to escape and swim away

2.2.2 Burying

To investigate the burying behaviour of the fish, the fish were placed individually in a tank of 56.5x36.5x30.5 cm with a sandy substrate of 3 cm height on the bottom. The tank was filled to a height 17.5 cm with natural seawater. For the substrate, filtered sand with a grain size of 0.2 to 0.5 mm was used. The time to the start of burying after release in the test tank, the number of movements to bury and the percentage coverage after the burying action were monitored. The percentage coverage was scored as 0 (not buried), 1 (<50% buried), 2 (>50% buried) or 3 (completely buried). This scoring system is derived from the system used by Kristensen *et al.* (2014) to assess the burying behaviour of turbot (*Psetta maxima*) and European flounder (*Platichthys flesus*).

2.2.3 Camouflage

Camouflage, the ability of flatfish to adapt to their background by changing their body pattern, was tested by placing hatchery reared sole (N=12) in groups of three in a white tank (60x40x11.5 cm) filled with natural seawater. After 15 minutes, a photograph was taken from each individual fish (Panasonic Lumix DMC-FT3, Panasonic Corporation, 12.1 Megapixel) and the fish were transferred to a tank of the same kind, which was darkened (bottom and walls) by a covering of grey garbage bag plastic. After 15 minutes a photograph was taken and the fish were transferred back to the white tank and left for another 15 minutes after which a final photograph was taken. A 15 minutes time period is used between the photographs, as this is the time required for a flatfish to complete skin colour adaptation (Kelman *et al.*, 2006; pilot experiments). The adaptation in the body pattern was compared among the transitions by comparing the photographs for the change in expression of blotches and spots, which are two basic patterns in the skin of plaice (Kelman *et al.*, 2006). The pilot experiments showed that these can be used to describe the skin pattern of other flatfish species as well. The change in expression was scored as increase (+1), no change (0) or decrease (-1). The number of changes was also counted (min 0, max 4; spots and blotches for the transition white to dark and dark to white). Three days later the experiment was repeated, but the sequence was turned around from grey to white to grey instead of from white to grey to white, in order to determine whether the sequence influenced the reaction of the fish. To determine if the camouflage reaction differed between hatchery reared sole, wild caught dab and wild caught plaice, wild caught dab (N=10) and plaice (N=5) were tested for the transition of white to grey and back to white.

To test if the camouflage reaction of the fish was influenced by handling, a second camouflage experiment was performed in which a tank with a transparent bottom was used to be able to change the colour of the bottom without disturbing the fish by lifting it from the tank. This test was performed with the wild caught fish that had survived the 10 days survival period (10 dab and 5 plaice). Each fish was tested two times (handling and no-handling) for the colour transition white to black. The sequence was made random. The fish were placed individually in a transparant plastic tank (26x17.5x20 cm) filled with natural seawater (height 17.5 cm) with either a white plastic background (255x170 mm) in the tank (handling) or a white plastic background (225x200 mm) underneath the tank (no-handling). After 15 minutes, a photograph was taken and the white background was changed for a black one. In the handling test the fish had to be lifted out of the tank in order to replace the background, while in the no-handling test, the background could be replaced without disturbing the fish. After 15 minutes another photograph was taken. By comparing the two pictures, it could be assessed whether the fish responded to the background change or not (scored as 1 = reaction, or 0 = no reaction). Photographs were taken with a standardized distance of 20 cm and a white cover was used to standardize the light intensity of the pictures.

During the experiment, it appeared that fish did not respond well to the background that was placed underneath the plastic tank. The transparent bottom that covered the black or white background seemed to reduce the contrast in colour between the white and black background. This could be the reason why the fish did not respond to the background change. However, it could also be that the fish did not respond because of the lack of handling. Which of the two explanations was correct could not be decided on basis of the available data. Therefore, an additional test was performed to test whether handling could be the reason of the camouflage reaction. In this test, the fish were placed directly on the background and lifted out of the tank during the replacement of the background. This was done for the colour transitions white to white, black to black and black to white, as an addition to the white to black transition with handling in the original experiment.

2.3 Statistical analysis

Statistical analyses were performed with the programs IBM SPSS Statistics 20® and GenStat 17th edition ®. To investigate the weight differences before and after the 10 days survival period, a paired sample t-test was used with SPSS. As the RAMP score and burying parameters were not normally distributed, a Wilcoxon signed ranks test was used to investigate the differences before and after the 10 days survival period.

To investigate the correlations between the different burying parameters and between the results of the different tests, a principal component analysis (PCA; Jolliffe, 1986) was performed with GenStat. Procedures were as described by van Reenen *et al.*, (2004). The PCA approach involves that correlation matrices underlying sets of parameters are represented by principal components as linear combinations of parameter scores. Principal components identify parameters that co-vary (in the same or opposite direction) as indicated by relatively high absolute loadings. The importance of a component is expressed in the percentage of variation in the data set that it explains. Component scores are calculated from raw scores, using loadings as weighing factors, and thus integrate multiple parameters whilts giving most weight to those with high loadings. Loadings $>|0.4|$ are here considered significant and identify parameters that belong to a biological state or trait.

A sign test was performed with SPSS to see whether the camouflage reactions of hatchery reared sole were significantly different from zero. To test for a sequence effect of the colour transition a Restricted Maximum Likelihood (REML) test was performed with GenStat. The REML procedure (Patterson and Thompson, 1971; Harville, 1977) was used to test for the differences in reactions among the three different fish species (separately for reaction, spots and blotches), to test for the differences in reactions between handling and no handling (dab and plaice) and to test for the differences between the four different colour transitions with handling (dab and plaice). REML assumes data to have a normal distribution, which is not strictly the case for binary or count data. Therefore, the Linear Mixed Model approach was used as it takes the actual distribution of the data into account and implements REML-type analyses. Animals were fitted as random effect to account for covariance between multiple measurements. Variance components for the random animal effect and the fixed effects were estimated simultaneously in the model and means for the different levels of fixed effects were estimated while adjusting for the effects of other fixed effects. Precise statistical models used are clarified in the results section.

At last a Spearman rank correlation was used to test if the parameters from the different tests can be predictive for the weight difference of by the fish after a survival period of 10 days.

3. Results

Fish were tested for two possible new methods to measure their viability, burying behaviour and camouflage. They were also tested for their reflexes (RAMP), in order to investigate if the new methods give comparable results with a method that is currently in use and to evaluate construct validity. As there were not enough wild caught fish available for the experiments, the tests were first performed with hatchery-reared sole and later on with wild caught dab and plaice. This makes a good comparison between hatchery reared fish and wild caught fish difficult, as different species are used, but it can probably give an indication of the difference between hatchery reared and wild caught fish. Possibly, it also gives an indication of the species-specificity of the test outcomes. The tests could be taxing to the fish, affecting their survival chances. This was investigated by subjecting some fish to all the three tests, while others were only tested for one particular test. This was done with hatchery reared sole in 5 groups of 10 individuals tested for either RAMP, burying, camouflage, all the three tests or used as control group. All fish were measured for their weight (before and after a survival period of 10 days), length and survival (over a period of 10 days, as described in 'Material and methods'). In this way, it was investigated if the fishes' responses reflected viability (survival and weight) and if test outcomes were dependent on the size of the fish (length and weight), which could complicate the interpretation of the results. Weight gain or loss is assumed to reflect viability, as it gives an indication of the physiological status of the fish. The outcomes of the tests with hatchery reared sole did not show an effect of the tests on their survival chances and, as less individuals were available for wild caught dab and plaice, they were tested in different numbers. For wild caught dab, 9 individuals were tested for all the three tests (RAMP, burying and camouflage) and 9 individuals were used as control. For plaice, all individuals (N=5) were tested for all the three tests.

3.1 Survival and weight

All fish were measured for their weight (before and after a survival period of 10 days), length and survival (over a period of 10 days, as described in 'Material and methods'). For the survival experiment, the animals were kept in a grey tank of 60x40x11.5 cm with a sandy substrate on the bottom after they had been tested for either RAMP, burying, camouflage or all the three tests or were used as control. Survival and weight were measured to investigate if the fishes' responses reflected viability (weight gain or loss is assumed to reflect viability as it gives an indication of the physiological status of the fish, as does survival), while length and weight could be used to assess if the test outcomes were dependent on the size of the fish. For hatchery reared sole (N=50, 45.2 g ± 18.4 SD, 160.9 mm ±19.3 SD), no mortality occurred during the 10 days survival period. Weight loss during the survival period was only significant for the group that was tested for burying behaviour (N=10, 42.1 g ± 15.3 SD, 158.5 mm ± 17.8 SD; paired samples t-test, p=0.014). For fish that were used as control (N=10, 42.1 g ± 13.1 SD, 156.9 mm ± 22.3 SD) or that were tested for RAMP (N=10, 49.7 g ± 18.2 SD, 164.5 mm ± 22.3 SD), camouflage (N=10, 52.1 g ± 24.5 SD, 169.6 mm ± 22.6 SD) or RAMP, camouflage and burying (N=10, 40.0 g ± 19.1 SD, 154.8 mm ± 17.3 SD), no significant differences in weight over the 10 days were found (paired samples t-test, control p=0.676; RAMP p=0.262; camouflage p=0.353; RAMP+camouflage+burying p=0.120). Overall, the weight loss was not significant (paired samples t-test, N=50, p=0.069). The weight differences for the group that was tested for all three tests are presented in figure 1.

During the survival period of wild caught dab and plaice an incident occurred. The supply of fresh seawater malfunctioned and some fish died, probably because of a lack of oxygen (4.9 mg/L compared to 8.4 mg/L ±0.7 SD under normal conditions, while a minimum for survival was set at 6 mg/L). As 5 control individuals of dab and 3 test individuals of dab died, there is no reason to believe that the behaviour tests caused this mortality. For the fish that survived the incidence no significant weight differences were found between start and end of the 10-day survival period (paired samples t-test; overall p=0.069; control dab p=0.086; RAMP+camouflage+burying dab p=0.478; RAMP+camouflage+burying plaice p=1.000). The weight differences for the fish that were tested for all three tests are presented in figure 1. However, it should be taken into account that the sample sizes are very small (overall N=15, 71.4 g ± 49.2 SD, 181.5 mm ± 49.9 SD;

control dab N=4, 111.3 g ± 47.0 SD, 224.5 mm ± 31.6 SD; RAMP+camouflage+burying dab N=6, 88.6 g ± 31.1 SD, 203.0 mm ± 20.1 SD; RAMP+camouflage+burying plaice N=5, 18.6 g ± 9.8 SD, 121.4 mm ± 20.2 SD).

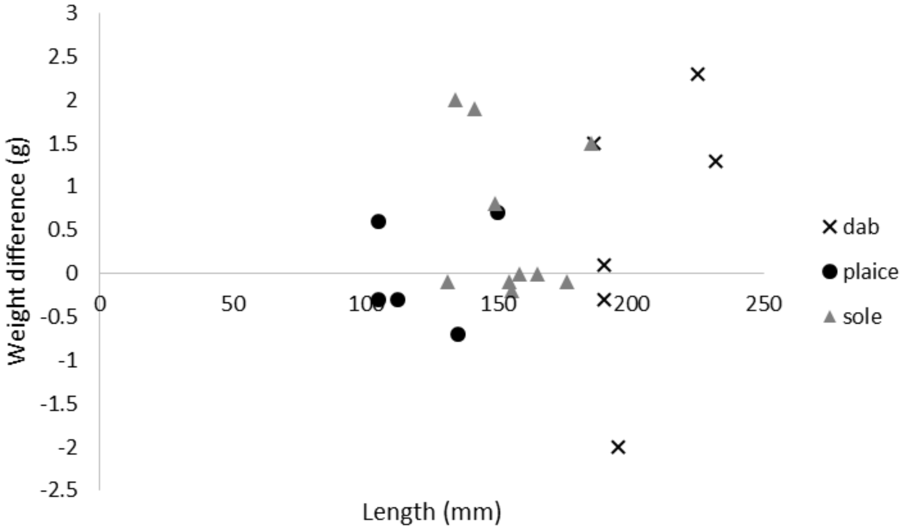

Figure 1. Weight differences over a period of 10 days for hatchery reared sole (N=10), wild caught dab (N=6) and wild caught plaice (N=5), which were subjected to tests for reflexes (RAMP), burying behaviour and camouflage at the start of the period of 10 days and were measured for their weight before and after the period of 10 days. No significant weight differences were found for these groups (paired samples t-test; sole p=0.120; dab p=0.478; plaice p=1.000).

3.2 RAMP

In the development of new methods, it is important to investigate whether they give comparable results with methods that are currently in use and which are expected to measure the same trait or state. Construct validity implies that parameters that assumingly mirror a same trait or state, associate in a logical and predictable way. Thus, the test outcomes of the fish that were tested for all of the three tests (RAMP, burying behaviour and camouflage) can be used to evaluate this. The RAMP test for reflexes may be considered a reference method with which the new methods, burying behaviour and camouflage can be compared and evaluated for criterion (concurrent) validity. Before a comparison can be made between the new methods to assess viability (burying behaviour and camouflage) and the nowadays commonly used reflex testing method, the RAMP test, basic outcomes of the latter are presented. The fish were given several stimuli and their reactions were scored as present/absent (sole) or on a 0 to 2 scale of intensity of the reaction (dab and plaice). These scores were used to calculate the RAMP score with the formula presented in the material and methods section. Hatchery reared sole responded to all stimuli given (as listed in table 1) and all individuals tested had a final RAMP-score of 0 on a scale of 0 to 1. Wild caught dab and plaice were tested for RAMP both before and after the survival period of 10 days. Because of the finer scoring system, where the reaction was scored as 2 (present), 1 (intermediate) or 0 (absent), a bit more variation in the RAMP scores was observed. However, there were no individuals that did not respond to the given stimuli. Also there was no significant difference in RAMP score observed before (dab, 0.02 ± 0.05 SD; plaice, 0 ± 0 SD) and after (dab, 0.06 ± 0.08 SD; plaice, 0.02 ± 0.04 SD) the 10 days survival period (Wilcoxon signed ranks test; dab, N=6, p=0.343; plaice, N=5, p=0.317).

3.3 Burying behaviour

Several pilot trials were done to see whether the hatchery reared fish indeed showed burying behaviour. In the first trials, the hatchery reared sole did not bury into the sand when released in the tank with a sandy substrate on the bottom. When observing the soles' burying behaviour in the tanks that were used for the survival experiment, which also contained a sandy substrate on the bottom, they buried themselves, within 3 days. Therefore a second trial for burying behaviour was done and now all the sole buried themselves into the sand when released in a tank with a sandy substrate on the bottom. Thus it seems that the hatchery reared sole were motivated to bury themselves when given the possibility to do this.

Part of (behaviour) test development is the evaluation of reliability, meaning the consistency of the outcomes. Test re-test consistency is one of the aspects of reliability and was evaluated for the burying test. In case the viability decreased over the survival period of 10 days, this would be expected to be reflected by the outcomes of the burying test, thereby providing criterion (predictive) validity. However, this could not be shown with the results obtained in this study as the viability of the fish was quite constant over the 10 days regarding the results of ‘Survival and weight’ and ‘RAMP’. For the wild caught fish, the burying before and after the survival period of 10 days was tested (Figure 2).

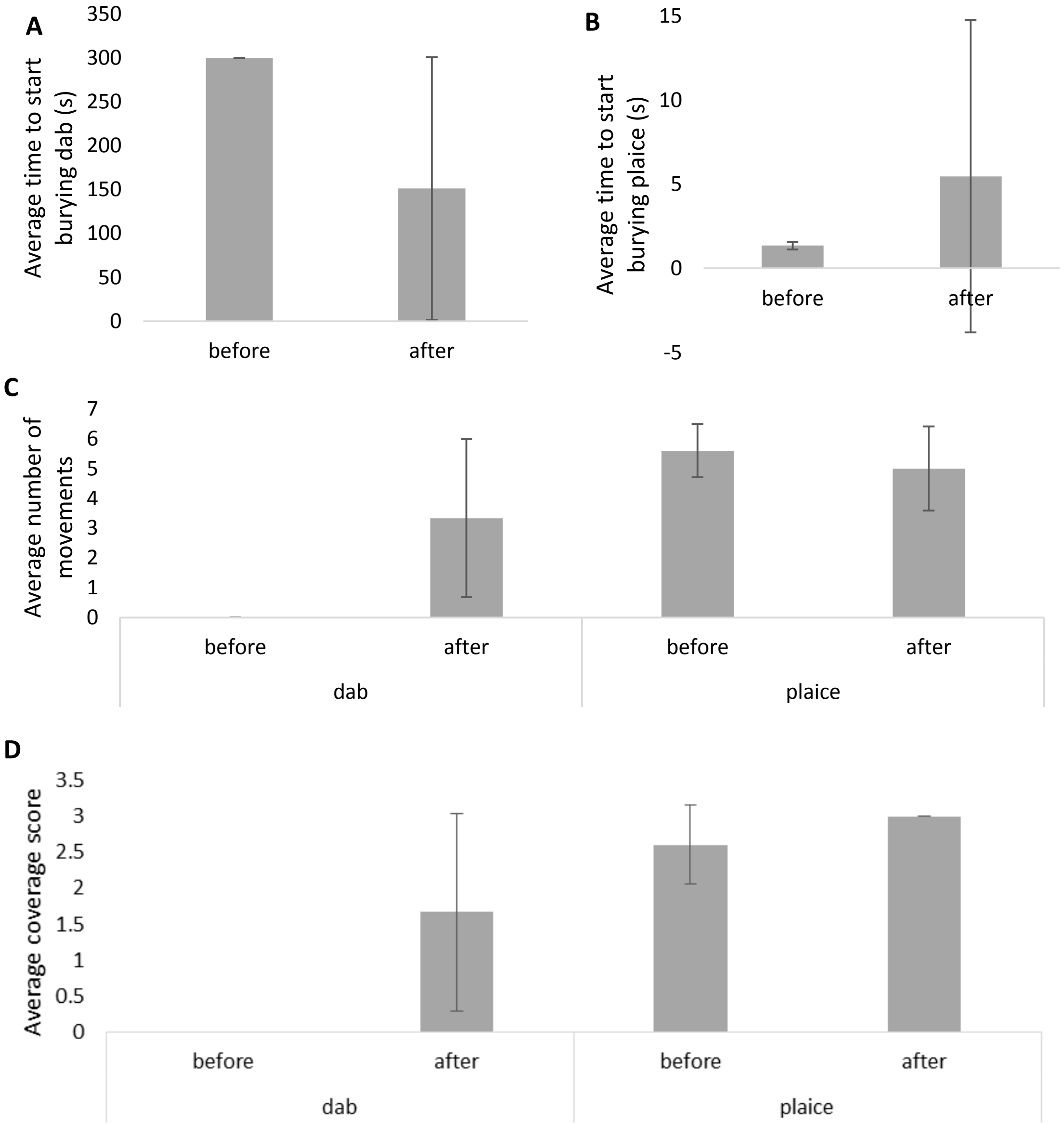

Figure 2. Wild caught dab (N=6) and plaice (N=5) were tested twice (with a 10-day interval) for burying, as scored for the time to start burying after release on a sandy substrate in the test tank (dab, A; plaice, B), the number of movements of the burying action (C) and the coverage score (from 0 to 3; 0% coverage, <50%, >50%, 100%; D). Presented are the mean scores ± standard deviations. The differences over 10 days were tested with a Wilcoxon signed ranks test, but no significant differences were found (Wilcoxon signed ranks test; dab, time to start burying p=0.068, number of movements p=0.066, coverage p=0.063; plaice, time to start burying p=0.686, number of movements p=0.257, coverage p=0.157).

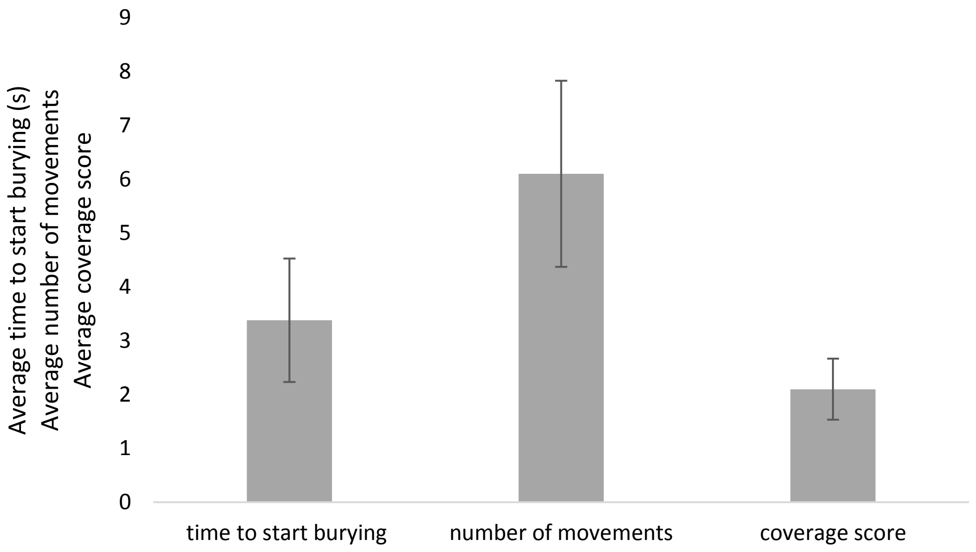

Figure 3. Hatchery reared sole (N=10) were tested for burying, as scored for the time to start burying after release on a sandy substrate in the test tank, the number of movements of the burying action and the coverage score (from 0 to 3; 0% coverage, <50%, >50%, 100%). Presented are the mean scores ± standard deviations.

To investigate the relationship between the different burying parameters and to establish construct validity, a PCA analysis was performed. At the same time, the PCA analysis indicates whether the outcome of the burying parameters is determined by the size of the fish. To perform the PCA analysis, the results of the tests before the 10 day survival period were used, as these were available for all three species used in this study. For wild caught dab and plaice these results can be found in figure 2. For hatchery reared sole, these results can be found in figure 3. The mean weights and lengths of the three species can be found in the section 'Survival and weight'. The outcomes of the PCA analysis are presented in table 2. In hatchery reared sole, time to bury and the number of movements are directly associated with weight and length. This means that larger sized sole take more time before starting to bury when they are released on a sandy substrate and that they use more movements to bury compared to smaller sized sole. This association can be explained by the age of the fish. Older fish are larger in weight and length and will thus take more time before starting to bury and will use more movements to do this. In wild caught plaice, the number of movements and coverage are directly associated with weight and length. This means that larger sized plaice use more movements to bury compared to smaller sized plaice and that the coverage of larger sized plaice after the burying action is higher compared to the coverage of smaller sized plaice. Also this association can be explained by the age of the fish, as older fish are larger in weight and length and will use more movements to bury, with a better coverage as the result of that. For wild caught dab, the PCA analysis did not reveal any correlation structure underlying the parameters but a significant correlation was found between weight and length (r=0.93; p=0.008), which is logical as larger sized fish usually also weigh more.

Table 2. Principal Component Analysis (PCA) outcomes of measures on burying parameters and size for hatchery reared sole (N=10) and wild caught plaice (N=5). Presented are loadings (significant if > |0.4|) that identify correlation structures underlying the parameters indicated in the first column. Rows 3 (latent root) and 4 (explained variance) indicate the significance of the components.

	Species	
	Hatchery reared sole	Wild caught plaice
Name	Age	Age
Latent roots	2.702	2.635
Percentage variation	54.03%	52.69%
Measures		
Weight	**0.98**	**0.96**
Length	**0.96**	**0.84**
Timetobury	**0.78**	-0.11
Movements	**0.46**	**0.91**
Coverage	-0.04	**0.42**

3.4 Camouflage

To test the ability of flatfish to adapt to their background by changing their body pattern, hatchery reared sole (N=12) were placed in a white tank and after 15 minutes a photograph was taken per individual fish and the fish were transferred to a grey tank. After 15 minutes another photograph was taken and the fish were transferred back to the white tank where after 15 minutes a final photograph was taken. In this way, the change in skin colouration of the fish could be described by comparing the photographs for the transition of white to grey and for the transition of grey to white. The skin colouration was scored, as the change in expression for spots and blotches, scored as -1 (decrease), 0 (no change) or 1 (increase), as described in the material and methods section. This experiment was also performed the other way around (grey-white-grey) to determine whether the sequence influenced the camouflage reaction. The results were tested for the presence of a reaction in spots and blotches separately (sign test) and for the effect of sequence (REML). It was shown that the fish respond to colour instead of to colour change. A transfer from a white to a grey background led to the opposite reaction of a transfer from a grey to a white background (Figure 4). Although the reactions were not so strong that it could be significantly evidenced (sign test; all transitions, blotches and spots p>0.05) the pattern shows that the reaction of the spots is stronger than the reaction of the blotches (Figure 4). Also the sequence (white to grey, grey to white or grey to white, white to grey) did not influence the strength of reactions (REML; p=0.145 for the effect of colour sequence). Thus it seems that the change in colouration of a fish's skin is determined by the present background colour and not so much by the preceding condition.

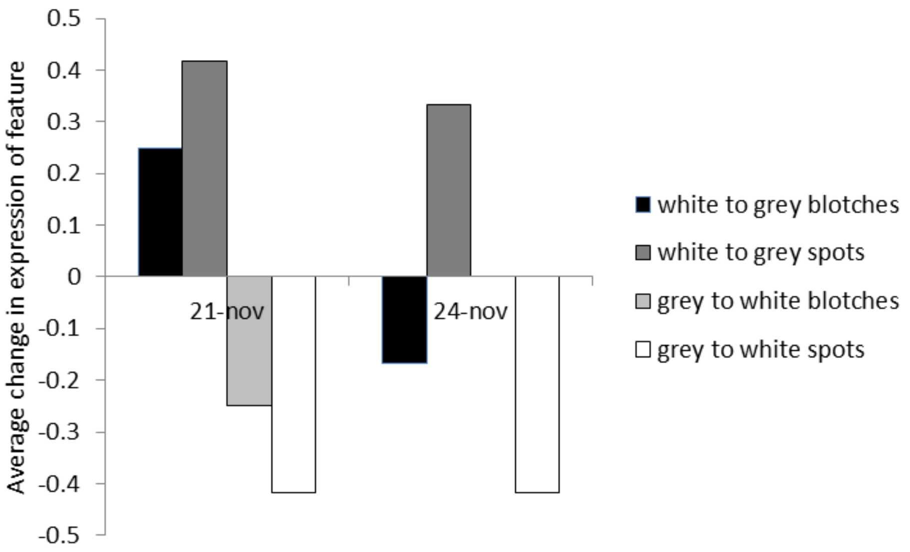

Figure 4. Hatchery reared sole (N=12) were observed for skin coloration patterns (camouflage) when on a white background and when on a grey background. Per fish, transitions from white to grey and vice versa were done. Expressed on the y-axis are the average changes in expression of the spots and blotches in the skin pattern as the result of different colour transitions. The change in expression was scored as -1 (decrease), 0 (no change) or 1 (increase). On the 21st of November, the fish were first tested for the transition from white to grey and afterwards for the transition from grey to white. On the 24th of November, this sequence was the other way around, first grey to white and then white to grey (in the graph, the order of presentation is the same).

Wild caught dab (N=10) and plaice (N=5) were also tested for the transition from white to grey to investigate if the changes in skin colouration, following a change in background colour, differed between hatchery reared sole, wild caught dab and wild caught plaice (Figure 5). The change in skin colouration was scored as the change in expression of spots and blotches in the same way as for hatchery reared sole. The predicted means for the reaction to the colour transition are comparable for both spots and blotches, but in the opposite direction (REML, predicted means; spots, grey to white=-0.35, white to grey=0.39; blotches, grey to white=-0.45, white to grey=0.53). This fits with the expectation that the expression of spots and blotches is adaptive and increases (stronger expression) when the fish is transferred to a darker background and decreased when transferred to a lighter background. In the expression of spots, sole, dab and plaice reacted in the same way to the colour transitions (REML, p=0.949 for the effect of fish species). Background colour transition did have a significant effect on the expression of spots (REML, p=<0.001 for the effect of background colour). For blotches, the interaction between fish species and colour transition was significant (REML, p=0.004). Thus, the effect of the colour transition on the expression of blotches differs between the different species.

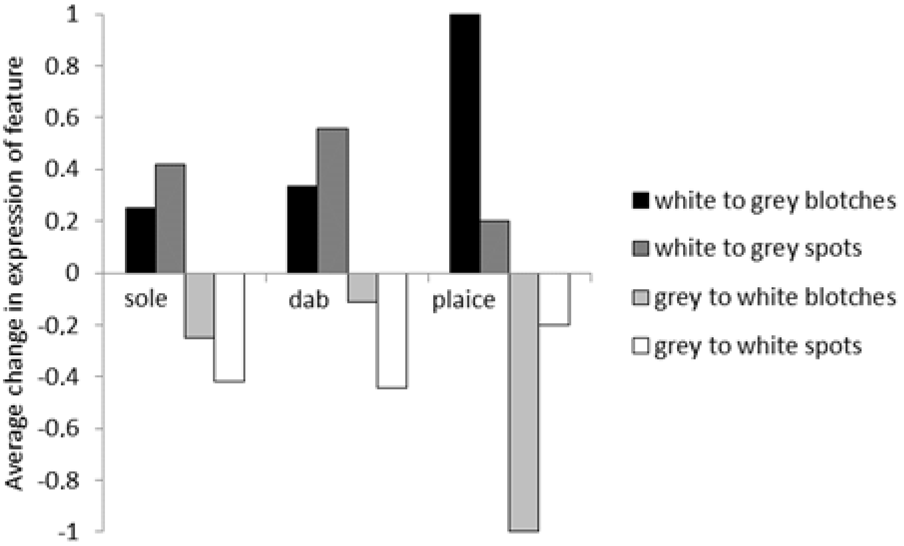

Figure 5. The average change in expression of the spots and blotches in the skin pattern of hatchery reared sole (N = 12) , wild caught dab (N=10) and wild caught plaice (N=5) as the result of different background colour transitions (i.e. from white to grey and vice versa). The change in expression of spots and blotches of the skin pattern was scored as -1 (decrease), 0 (no change) or 1 (increase).

To test if the camouflage reaction of the fish was influenced by handling, a second camouflage experiment was performed, using a tank with a transparent bottom to be able to change the background colour without disturbing the fish by lifting it from the tank. The camouflage reaction of the fish was scored by comparing the photographs taken 15 minutes after the fish was placed on the start background and 15 minutes after the background of the fish was changed. Reactions were scored as 1, reaction, or 0, no reaction. Figure 6 shows the average reaction of wild caught dab (N=10) and plaice (N=5) for the colour transition white to black with and without handling of the fish. Handling influences the reaction (REML, p<0.001 for the effect of handling). However, this is most likely due to the setup of the test, where the background is much less intense coloured in the no handling treatment compared to the handling treatment. Therefore an additional test was performed as a control, in which the four possible colour transitions (white to black, black to white, white to white and black to black) were tested with handling. In this way, it could be determined if the effect of handling differed between the different colour transitions and if handling on its own elicits a response or that it requires a change in background colour to elicit a response. The average reaction of the wild caught dab (N=10) and plaice (N=5) in this experiment is shown in figure 6. It was shown that the reaction depended on the colour transition (REML, p<0.001). The predicted means of the REML test (for the effect of handling and colour transition) show that the difference in reaction is significant between the transition from white to black (REML, predicted mean, 0.95) and the transitions from black to black (REML, predicted mean, 0.5) and white to white (REML, predicted mean, 0.25). The transition from black to white (REML, predicted mean, 0.8) does not differ from the transition from white to black, but just not enough from the transition from black to black to give a significant separation between background colour change with handling and no colour change with handling.

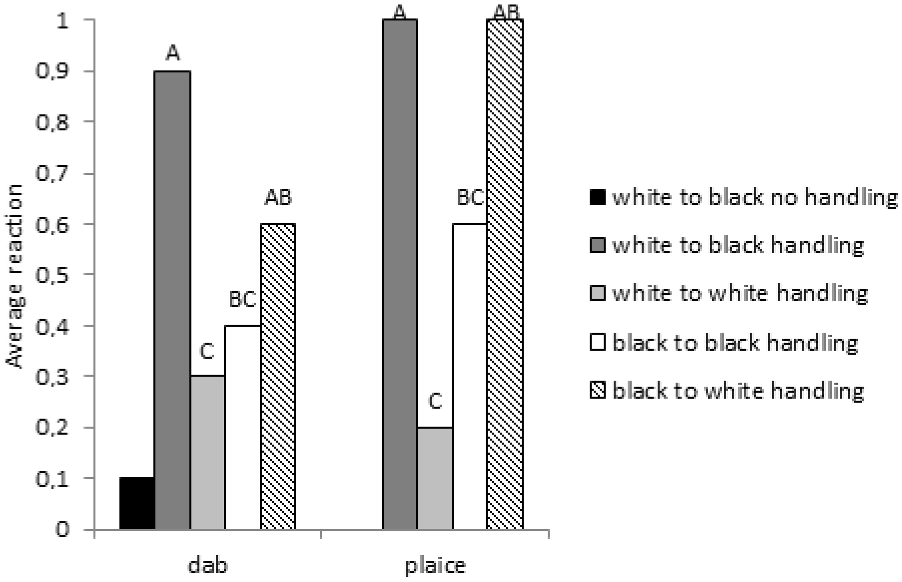

Figure 6. Wild caught dab (N=10) and plaice (N=5) were tested for their camouflage reaction on the colour transition from white to black with and without handling and the colour transitions white to white, black to black and black to white with handling, in order to determine if the effect of handling differed between the different colour transitions and if handling on its own elicits a response, or that it requires a change in background colour to elicit a response. The average reaction is shown in this graph. The reaction was scored as 1=reaction, or 0=no reaction. The letters in the graph indicate significant differences. These contrast were tested for dab and plaice combined. Only the reaction of the transition of white to black without handling was left out this analysis, as this merely belongs to another experiment. It is only presented in this graph to show the difference between handling and no handling for the same colour transition which was the reason to test the other colour transitions all with handling.

3.5 RAMP, burying behaviour and camouflage combined

During this study, different behaviour tests were performed to assess the viability of fish. A group of 6 wild caught dab and 5 wild caught plaice were subjected to all three tests (RAMP, burying behaviour and camouflage). This allowed to test for associations between test results (construct validity) and to what degree test outcome correlated with a more or less established parameter of viability (criterion validity). The reference method used in this study was the change in weight of the fishes over the 10 days survival period. To test if the parameters measured with the tests (RAMP score, time to start burying, number of movements to bury, coverage score and the amount of camouflage reactions) can be predictive for the weight difference after a 10 days survival period, a Spearman rank correlation test was performed with SPSS (Table 3). For hatchery reared sole (N=10), the camouflage experiment was not performed in the same way as for wild caught dab and plaice (see material and methods and results on camouflage) and therefore the results on camouflage of sole are left out of the Spearman rank correlation test (x in table 3) and are also left out of the PCA analysis (Table 4).

To investigate the relationship between the different test outcomes and to test for associations between test results, a PCA analysis was performed over all the test results of the fish that were tested on all three tests (hatchery reared sole, N=10; wild caught dab, N=6; wild caught plaice, N=5). The data used in this PCA analysis are the results for these fish obtained from the tests as described throughout the results section. The outcomes of the PCA analysis are presented in table 4. In hatchery reared sole, time to bury and the number of movements are directly associated with weight and length as was already shown and explained in the results on burying behaviour. No additional association with RAMP was found and the results on camouflage of sole were not included in this analysis. In wild caught dab, the RAMP score is inversely associated with weight and length. This means that the reflexes shown by larger sized dab are stronger compared to smaller sized dab, probably due to the fact that larger sized fish are more resistant for disturbances and will be impacted less compared to smaller sized fish. So, the age of the fish has an important influence on the effect that will be seen in the RAMP score. For wild caught dab, the PCA analysis also indicated a correlation between the RAMP score and number of camouflage reactions. However, this correlation was not significant (r=0.43, p=0.403). In wild caught plaice, the number of movements, coverage score and camouflage are directly associated with weight and length. This means that larger sized plaice use more movements to bury compared to smaller sized plaice, that the coverage of larger sized plaice after the burying action is higher compared to the coverage of smaller sized plaice and that the camouflage reaction of larger sized plaice is stronger compared to that of smaller sized plaice. Also this association can be explained by the age of the fish, as older fish are larger in weight and length and will use more movements to bury, with a better coverage as the result of that. The camouflage might be stronger in older fish as fish that are better camouflaged will have a higher chance of survival and thus only fish that are well able to camouflage themselves will become older and reach larger sizes.

Table 3. Results of the Spearman rank correlation to test if the parameters measured with the tests (RAMP score, time to start burying, number of movements to bury, coverage score and the amount of camouflage reactions) can be predictive for the weight difference after a 10 days survival period of wild caught dab (N=6) and wild caught plaice (N=5) and hatchery reared sole (N=10). Errors are given when the second variable was constant, because of which a correlation could not be calculated (indicated as – in table). Significant results are marked in bold. For hatchery reared sole, the camouflage experiment was not performed in the same way as for wild caught dab and plaice (see material and methods and results on camouflage) and therefore the results on camouflage of sole are left out of the Spearman rank correlation test (x).

Spearman rank correlation	**Weight difference - RAMP**	**Weight difference – time to start burying**	**Weight difference – number of movements**	**Weight difference - coverage**	**Weight difference – camouflage reactions**
Dab	r = 0.16 p=0.558	-	-	-	r = 0.89 **p=0.034**
Plaice	-	r = -0.05 p=0.493	r = 0.91 **p=0.028**	r = 0.74 p=0.152	r = -0.22 p=0.812
Sole	-	r = -0.04 p=0.428	r = -0.72 p=0.073	r = 0.31 p=0.418	X

Table 4. Principal Component Analysis (PCA) outcomes of measures on size, RAMP, burying behaviour and camouflage for hatchery reared sole (N=10), wild caught dab (N=6) and wild caught plaice (N=5). Presented are loadings (significant if > |0.4|, indicated in bold) that identify correlation structures underlying the parameters indicated in the first column. Rows 3 (latent root) and 4 (explained variance) indicate the significance of the components.

	Species		
	Hatchery reared sole	Wild caught dab	Wild caught plaice
Name	Age	Age	Age
Latent roots	2.70	2.16	2.92
Percentage variation	54.03%	54.05%	48.71%
Measures			
Weight	**0.98**	**0.97**	**0.92**
Length	**0.96**	**0.98**	**0.84**
RAMP	0.00	**-0.40**	0.00
Timetobury	**0.78**	0.00	-0.32
Movements	**0.46**	0.00	**0.78**
Coverage	-0.04	0.00	**0.45**
Camouflage	x	0.32	**0.67**

4. Discussion

For a long time, fish were considered to be unable to feel and experience pain and emotions. However, recent research indicates that fish are likely to have this capacity and the public opinion is that potential fish suffering should be minimized. Fisheries may lead to an allostatic overload of the caught fish and this study searches for new methods to measure the allostatic load of fish in order to assess their viability and welfare. Animal welfare consists of three main aspects, naturalness, function and affective state. The function based view merely assesses the viability of the fish, which gives an indication of its welfare. The non-invasive methods that are currently used to determine the state of wild caught fish focus mainly on their survival chances, which represents viability and is part of the function based view. These methods are the catch damage index (CDI) and the reflex action mortality predictor (RAMP). The CDI scores injuries of the fish and provides an objective measurement of the injuries that are relevant for the viability of the fish (Depestele *et al.*, 2014b). Wounds can lead to infections (e.g. Noble *et al.*, 2012) and loss of scales and damage to the slime layer can lead to problems with osmoregulation (Ashley, 2007; Rottman *et al.*, 1992; van de Vis, pers. comm.). However, this assessment of injuries does not show the internal damage of the fish or the stress that the fish experiences, which might also influence its viability. Therefore, besides injuries, reflex impairment is seen as a useful method to assess the viability of fish (Davis, 2007; Davis, 2010). These reflexes are stereotyped behaviours or reflex responses to peripheral stimuli (Davis, 2010; Depestele *et al.*, 2014a). They are regarded to give an indication of the viability independent from factors like age, size, sex and motivation of the fish. According to Davis (2010), 5 to 8 reflex responses should be identified to be able to calculate a composite measure of impairment. This reflex impairment score can be correlated with outcomes as mortality and can thus be used as an indicator of viability. This approach is also recommended by ICES (ICES, 2014) to assess the survival chances of fish. However, it can be questioned if the indication of the viability given by the RAMP test is really independent from individual characteristics of the fish.

A reflex is a “simple graded response to a specific stimulus” (Hill *et al.*, 2008) and is mediated by short neuronal pathways without any influence from higher brain centres. Mammalian examples that are well-studied are for instance the stretch reflex (e.g. knee-jerk response in humans) and the flexion reflex (e.g. withdraw hand when touching something hot). A reflex is minimally influenced by higher cognitive processing in the brain and is relatively independent from individual characteristics of animals. However, care should be taken when using the term ‘reflexes’, as the reflexes that are used in the RAMP test are more likely to be common reactions or a combination of behaviour with reflexes instead of true reflexes. It is likely that the reactions that are stimulated in the RAMP test can be influenced by higher brain centres of the animal and depend on individual characteristics. For instance, different fish may react differently in the tail grab test. Some species or individuals are much more active in trying to escape compared to others. This also means that this method falls under the domain of affective state, as these tests represent the affective state of the animal. The reaction of the animal can in part be explained by its coping strategy. Coping strategies are behavioural syndromes which are found in a wide range of mammalian as well as fish species (Sih *et al.*, 2004; Øverli *et al.*, 2007). Two main types of coping strategies are pro-active and reactive, with pro-active animals showing more offensive and aggressive behaviour compared to reactive animals (Koolhaas *et al.*, 2010). Therefore, the RAMP test might give different results for pro-active and reactive fishes; reactive fish might incorrectly be scored as having low viability because they do not show or only slightly show a response. Coping strategies might differ among species as well as among individuals of the same species. Another reason why the reaction of different species might differ is the period in which you test them. Sole is a nocturnal species, which is active at night and rests during the day, while plaice and dab are day-active species that rest at night (www.soortenbank.nl, 21-4-2015). The reaction of the fish might depend on whether it is tested during its’ active period or its’ resting period. It is important that these kind of individual or species differences are taken into account when interpreting the results of the RAMP test or other behavioural tests to predict viability.

Because of the uncertainties about the independency of the RAMP method of individual characteristics and because injuries alone do not give complete information on the status of the fish, the protocols for survival assessments that are used nowadays have to be improved or new non-invasive methods have to be developed. Behaviours that are regulated by higher cognitive processes may be more sensitive to impairment compared to reflexes which are typically maintained even when the animal is in a poor state of health. When the animal is in a poor state of health, behaviours that require more active input from the animals cognitive system will be shut down first, while true reflexes are usually maintained even when the animal is nearly death. In this study, burying behaviour and camouflage were tested for the possibility to be used as tests that assess the viability of the flatfish species sole, dab and plaice. These behaviours were chosen, because they are regarded to be essential in the life of flatfish. Burying behaviour combined with camouflage is regarded to be the first line of defence of flatfishes against predation (Stoner and Ottmar, 2003). It is natural avoidance behaviour of flatfish and these fish are innately motivated to perform these behaviours. As all individual flatfish will show these behaviours, it is consistent behaviour that may be used to test for viability. Burying behaviour and camouflage are sufficiently complex to involve regulation by higher cognitive processes instead of being simple reflexes and therefore they are expected to respond more sensitively to function impairments than simple reflexes do. Although they are mainly used to assess the viability of the fish, burying behaviour and camouflage combine all three domains of animal welfare. As they are behavioural tests, they represent the affective state of the animal (overtaxed animals may respond different compared to animals that are not) and as it is natural behaviour that is tested, naturalness is also involved. Thus burying behaviour and camouflage are promising possibilities to assess the welfare of wild caught fish.

Flatfishes are negatively buoyant and therefore they sink to the bottom when they do not swim actively. Although they are able to swim actively in the water column, flatfish spent most of their time on the sea bottom. To maintain their position on the bottom and to reduce the drag forces from the water currents, flatfish may bury themselves in the sediment (Arnold, 1969; Gibson, 2005). Besides avoiding water currents, burying behaviour is also important for survival by reducing predation by visual predators (Gibson, 2005; Ansell and Gibson, 1993) and by hiding the flatfish as a predator species itself. Burying behaviour is also important in energy conservation. For sole, it was shown that heart, respiratory and metabolic rate were reduced when the fish had buried themselves (Peyraud and Labat, 1962; Howell and Canario, 1987; Nasir and Poxton, 2001). So, burying behaviour has survival value in the natural life of flatfish and they are expected to show this behaviour when they are given the opportunity to do so. Gibson and Robb (1992) investigated the relationship between body size, sediment grain size and the ability to bury of juvenile plaice. They found that all fish swam to the bottom when they were released in the test tank and that almost all fish buried themselves. The results found in this study are in agreement with this. Hatchery reared sole did not bury in the first trials, but later in the experiment they did. This can be explained by the fact that the hatchery reared sole were raised on a substrate of fine gravel, which is much coarser compared to sand. It is likely that because of the difficulty of the small sole to bury in the gravel and the lack of triggers like predation or water currents, they did not bury. The relatively large grain size of the gravel might have prevented the sole from burying as there is a clear relationship between body length, sediment grain size and the extent to which individuals can bury themselves (Gibson and Robb, 1992). The reason behind this relationship is most likely the physical force that an individual can exert to bury itself. Larger fish can produce a greater forces and thus can bury in coarser sediments compared to smaller individuals which are weaker (Tanda, 1990). As larger individuals can exert a greater force to bury themselves, it can be expected that larger individuals require less movements to bury themselves compared to small individuals when they are tested on the same sediment grain size, as was done in this study. However, this could not be proved with the results obtained in this study, as the smaller individuals showed more burying activity compared to the larger ones. The dab used in this study were larger compared to the plaice (dab: 105.5g ± 37.7 SD, 215.4mm ± 23.3 SD; plaice: 18.6g ± 9.8 SD, 121.4mm ± 20.2 SD) and plaice showed more burying activity compared to dab, especially in the measurement before the survival period of 10 days. The results do show that the size is relevant for the motivation to bury. In a study by Ellis *et al.* (1997) hatchery reared sole did bury quickly when they were placed on a sandy substrate in the same way as wild caught sole did, and burying behaviour seems essential behaviour for flatfish.

To investigate the relationship between the different burying parameters, to establish construct validity and to check whether the outcome of the burying parameters was determined by the size of the fish, a PCA analysis was performed on the test outcomes of the three different species, hatchery reared sole, wild caught dab and wild caught plaice. For hatchery reared sole, a direct association was found between time to bury, number of movements, weight and length. For wild caught plaice, the number of movements to bury and the coverage score were directly associated with weight and length. Both these associations can be described by age, which has a direct connection with length and weight (size) of the individual. It should be taken into account that in the fish used in this study, size and species were very closely related. The plaice used were smaller than the dab used. Therefore, it cannot be confirmed that the results obtained are the result of size instead of a difference between species. However, many burying experiments about burying ability and sediment grain size have been performed with all kind of different flatfish species (e.g. Tanda, 1990; Gibson and Robb, 1992; Ellis *et al.*, 1997; Nasir and Poxton, 2001). As all show comparable results, namely that the flatfish intent to bury themselves when released on a sandy substrate and that the burying ability depends on the sediment grain size, it can be expected that burying behaviour is quite consistent among flatfish and thus the found results on the time to start bury and number of movements to bury for sole and the number of movements to bury and coverage score for plaice are likely to be indeed the result of size instead of a species effect.

So altogether, it is clear that burying behaviour is essential for flatfish and it is consistently shown among flatfish species, used in this study. Assumingly, flatfishes of different species and types (in terms of age etc.) are similarly motivated to perform the behaviour and this makes burying behaviour an attractive possibility to study the allostatic load of these fishes as it is a method that can be used for all flatfish. As physical force is required to bury, it can be expected that the ability to bury is impaired when a fish is injured or suffers from stress. As direct associations were found for the time to start burying with the number of movements to bury for sole and for the number of movements to bury with the coverage score for plaice, this provides construct validity and it seems that these are indeed the right parameters to measure in order to assess burying behaviour. For plaice, it was found that the weight difference of the survival period of 10 days was significantly correlated with the amount of movements exerted during the burying action, which provides criterion validity and is an indication that predictions about viability can be made based on measurements on the burying behaviour of flatfish.

A second predator defence mechanism in flatfish that we investigated for associations with viability is camouflage or cryptic colouration. Camouflage has a function in hiding from predators as well as prey, like burying behaviour. The skin of flatfish contains chromatophores, cells which contain pigment, which contribute to the cryptic colouration by changing the pigment distribution according to the visual stimuli from the background (Gibson, 2005). The camouflage is merely the result of changes in skin reflectance and contrast of the patches within the skin that are achieved by the changing pigment distribution (Saidel, 1969). In plaice, there are three types of chromatophores involved in the camouflage reactions: melanophores, lipophores and guanophores (Healey, 1999). The guanophores consist mostly of iridophores, which lay underneath the melanophores and reflect the light when they are not covered by an aggregation of melanophores. Melanophores occur dermally and epidermally and they contain dark pigment. The dermal melanophores create the background reflectance by covering the iridophores, while the epidermal melanophores create the contrast (Saidel, 1969; Healey, 1999). The lipophores consist of reddish erythrophores and yellowish xanthophores, containing carotenoid pigments (Healey, 1999). The position of the chromatophores in the skin is fixed (Lanzing, 1977) and the skin pattern is mainly created by aggregation and expansion of the melanophores which is regulated by nervous and hormonal control (Healey, 1999). Stress physiological changes can be rapid (in seconds to minutes), while metabolic and immunological changes occur more slowly (hours) (Hans van de Vis, pers. comm.). The nature of these changes is physiological and they take place at short term (seconds to hours), while flatfish may also show performance changes, which are long term (days to weeks) (Hans van de Vis, pers. comm.; Burton, 2010).

The skin pattern of flatfish consists of two major pattern components. These are the dark areas or 'blotches', which contain large numbers of epidermal melanophores, and the white 'spots', which have few or no epidermal melanophores (Kelman *et al.*, 2006; Burton, 2010). Although there are differences in the size and distribution of the white spots, both major pattern components can be found in species of the Pleuronectidae (Burton, 2010). Kelman *et al.* (2006) used these major pattern components to describe the skin pattern of juvenile plaice. Here, we used this same system to assess the camouflage reactions of hatchery reared sole, wild caught dab and wild caught plaice. Plaice and dab belong to the family of the Pleuronectidae, while sole belongs to the family of the Solidae. Although the major pattern components may differ among families, Bothidae for example are known to have more discontinuous patterns consisting of more components (Ramachandran, 1996; Kelman *et al.*, 2006; Burton, 2010), the pattern of sole, dab and plaice, could all be described by the major components of blotches and spots in this study. Comparable with the results of other studies, this study showed how the expression of the spots and blotches increased and the fish got darker when they were transferred from a white to a dark background, due to the colour of the background. The change in colouration was completed in 15 minutes and such short-term reactions are mostly under neuronal control (Burton, 2002). Hormonal control contributes to the maintenance of an equilibrium when the fish is adapted to a certain background for longer periods (Burton, 2002). In agreement with the adaptive value of the camouflage reaction, the opposite reaction was reported when the fish were transferred in the opposite direction (from a dark to a white background). In the transition from a white to a dark background, also the expression of the spots increased. This is most likely due to the aggregation of melanophores, thereby revealing the clusters of iridophores in the skin of the fish (Burton, 2010). So, the colour of the background has an effect on the expression of blotches and spots, with a higher expression of blotches and spots on a darker background.

Because the colour change has to be adaptive, you could expect that sole, dab and plaice respond in the same way to different backgrounds. Although no significant species effect on camouflage reaction was found, it cannot be concluded that sole, dab and plaice indeed respond in the same way to the background transition. However, as it was shown that the patterns of expression of spots and blotches are comparable among the three species for the background transition from white to dark and vice versa, it seems likely that sole, dab and plaice do respond in the same way to different backgrounds. For blotches a significant interaction between colour transition and species was found. Thus, the effect of the colour transition on the expression of blotches differs between the different species. Although the reactions of the species did not differ significantly from each other, the pattern observed shows that plaice responded a bit stronger in its reactions, in both spots and blotches, compared to sole and dab. It is possible that plaice in general respond stronger to changes in its background compared to sole and dab. However, as mentioned earlier for the burying experiment, size and species were very closely connected in this study. The plaice used for the experiments were smaller than the sole and dab used. As smaller fishes are more vulnerable to predation, their camouflage response might be stronger as the necessity to hide is larger. However, age effects on camouflage of flatfish have not been studied so far.

During the camouflage experiments, the fish were lifted out of the tank to transfer them to another background. Burton (1979) showed that this elicited stress-induced darkening in white-adapted winter flounders. To exclude this stress factor, the fish in this study were placed in a transparent tank with a background underneath it that could be replaced without lifting the fish or the tank. However, the response to the background transition was very low, possibly in part as the colour intensity of the background was lower compared to when the fish were placed directly on the background. Therefore as a control, the fish were tested for the four possible colour transitions (white to black, black to white, white to white and black to black) while lifting the fish out of the tank during the transition. It was shown that it was the change in background colour that determined the reaction of the fish and not the handling per se. In contrast with the results of Burton, the white-adapted fish (transition white to white) responded a bit less compared to the dark-adapted fish (transition black to black) when handled and placed back on the same background colour. Although this difference is not significant, apparently stress can have some effect on the camouflage reaction, as in both the transition of white to white and the transition of black to black the fish showed some reaction. This is very interesting, as this contributes to the possibility to use this mechanism in tests to assess the allostatic load of the fish. Fernando and Grove (1974) also showed that the stress hormones noradrenaline and adrenaline pale plaice and Burton found the same for winter flounder (Burton, 1985). Although 'the current understanding of the central processing in the chromatic regulatory system of flatfish is limited' (Burton, 2010), there are indications that stress or impairment of fish, which influences its allostatic load and viability, has an influence on the colouration of the fish and thus it can be used to assess the viability of the fish in some way.

All parameters, from burying behaviour and camouflage, were tested for their ability to predict the weight difference of the fish after the survival period of 10 days. This was done with a Spearman rank correlation and significant correlations were found between weight difference and the number of burying movements for plaice and between weight difference and amount of camouflage reactions for dab. For plaice, the weight difference became more positive with an increasing number of movement to bury. For dab, the weight difference became more positive with a stronger camouflage reaction. Thus it seems that burying behaviour and camouflage reflect the weight change of the fish, thereby providing criterion validity. As weight change is a measure for the viability of the fish, burying behaviour and camouflage could possibly be used as new methods to assess the viability of flatfish.

There were several significant relationships among the different parameters that were recorded. In hatchery reared sole, time to start burying and the number of movements were directly associated with weight and length. In wild caught dab, the RAMP score was inversely associated with weight and length and in wild caught plaice the number of movements to bury, the coverage score and camouflage were directly associated with weight and length. All three relationships can be explained mostly by age, which undermines the construct validity as age (size of the fish) has a large influence on the outcomes of the tests. This makes the interpretation of the outcomes of the tests more difficult as the age effect should be taken into account when the aim is to assess the viability of the fish.

To summarize, as all flatfish swam to the bottom when they were released on a sandy substrate and almost all fish buried themselves, it was indicated that all flatfish are naturally motivated to bury themselves when they have the opportunity to do so. Direct associations were found for the time to start burying with the number of movements to bury for sole and for the number of movements to bury with the coverage score for plaice. This provides construct validity for the burying test. For plaice, it was found that the weight difference of the survival period of 10 days was significantly correlated with the amount of movements exerted during the burying action. This provides criterion validity and is an indication that predictions about viability can be made based on measurements on the burying behaviour of flatfish. For camouflage behaviour, it was shown that the background colour has a significant effect on the expression of spots and blotches in the skin pattern of flatfish with a higher expression on a darker background, in agreement with the adaptive value of the camouflage reaction. Although not significant, this study indicated that stress, in the experiments created by handling the fish, has an influence on the colouration of the fish, which supports the idea that camouflage might be used to assess the viability of the fish. For dab, it was found that the weight difference of the survival period of 10 days was significantly correlated with the amount of camouflage reactions. This provides criterion validity and is an indication that predictions about viability can be made based on measurements on the camouflage reaction of flatfish. So there are many indications that burying behaviour and camouflage are promising new methods to predict the viability of flatfish. However, it should be taken into account that also these methods are behavioural tests which possibly might be influenced by individual characteristics, just as was mentioned for the RAMP test. This will always be the case when working with behavioural tests, but by knowing this and by searching for essential behaviours, this problem can be dealt with. Before burying behaviour and camouflage can be used in practice, more tests should be performed to validate the predictive capacity of the tests. Fish from more different length classes and species should be used to investigate whether the tests work for different sizes and different species in the same way or that adjustments should be made to the method in order to make it useful for more different sizes and species.

5. Conclusion

Burying behaviour and camouflage behaviour were tested for their possibility to be used in the assessment of viability of the flatfish species sole, dab and plaice. Although the tests on burying behaviour and camouflage behaviour were performed with only few individuals and with a small range of sizes, the results give indications that these methods can indeed be used to assess viability. Construct validity for the test on burying behaviour was provided by the test outcomes of hatchery reared sole and wild caught plaice. For sole, direct associations were found for the time to start burying with the number of movements to bury and for plaice this was the case for the number of movements to bury and the coverage score. The direct association between these parameters indicated that they measure the same state of the animal. For plaice, it was found that the weight difference of the survival period of 10 days was significantly correlated with the amount of movements exerted during the burying action. This provides criterion validity and is an indication that predictions about viability can be made based on measurements on the burying behaviour of flatfish. For the test on camouflage behaviour it was shown that the background colour has a significant effect on the expression of spots and blotches of the skin pattern of flatfish, with a higher expression on a darker background, in agreement with the adaptive value of the camouflage reaction. Although not significant, this study indicated that stress, in the experiments created by handling the fish, has an influence on the colouration of the fish, which supports the idea that camouflage might be used to assess the viability of the fish. For dab, it was found that the weight difference of the survival period of 10 days was significantly correlated with the amount of camouflage reactions. This provides criterion validity and is an indication that predictions about viability can be made based on measurements on the camouflage reaction of flatfish.

So there are many indications that burying behaviour and camouflage are promising new methods to predict the viability of flatfish. However, it should be taken into account that also these methods are behavioural tests which possibly might be influenced by individual characteristics. This was the problem with the reflex tests and therefore, new methods were required. Although the tests on burying behaviour and camouflage are also vulnerable to the influence of individual characteristics, they are sufficiently complex to involve regulation by higher cognitive processes. Behaviours that are regulated by higher cognitive processes may be more sensitive to impairment compared to reflexes which are typically maintained even when the animal is in a poor state of health. Therefore burying behaviour and camouflage are good possibilities to become new methods to assess the viability of flatfish and are worth to be investigate in more detail for their ability to do this.

6. Future perspectives

This study was performed within a project of the Wageningen UR Science Shop about the welfare of wild caught fish. In this study, the focus was chosen on flatfish, as they are important target species in the Dutch fisheries. In order to improve the welfare of wild caught fish, it is important to have validated methods that can correctly assess the current welfare state of the fish. If the current welfare status of the fish is known, possibly adjustments to the fishing methods can be applied in order to improve the welfare. The methods that are able to assess the welfare state of the fish can then be used to control if the adjustment to the fishing methods leads to the proposed improvement of welfare. Tests to predict survival chances are currently being performed in relation to discard survival. Such tests are mostly based on reflexes (RAMP) and injury scoring (CDI). Reflex tests can possibly be influenced by individual characteristics and injuries alone do not provide all information that is needed to predict the survival chances of fish. In order to test the viability of fish in more detail, this study investigated burying behaviour and camouflage as two possible behavioural tests that can be used for this purpose. It was not possible to develop the new methods in detail, but indications were found that burying behaviour and camouflage can be used to predict the viability of flatfish as can be read in the conclusion of this study. Although the tests on burying behaviour and camouflage are also vulnerable to the influence of individual characteristics, they are sufficiently complex to involve regulation by higher cognitive processes. Behaviours that are regulated by higher cognitive processes may be more sensitive to impairment compared to reflexes which are typically maintained even when the animal is in a poor state of health. Therefore burying behaviour and camouflage are good possibilities to become new methods to assess the viability, thereby giving an indication of the welfare, of flatfish and are worth to be investigate in more detail for their ability to do this.

To investigate burying behaviour and camouflage for their ability to predict the viability of flatfish, more experiments should be done with a wider range of fish sizes and more variation in actual viability of the fish. In this study, only few wild caught fish were available to be used in the experiments and they were of a limited size range. In order to investigate whether the method works for all individuals of these flatfish species, a large size range should be used in the experiments. Preferably, the fish have more variation in actual viability, so that the results obtained from the burying and camouflage tests can be linked to the data on survival and weight loss and that it can be investigated whether there is a correlation between the test outcomes and the viability. In this study, the fish all had a quite comparable viability and although significant correlations were found for the number of movements to bury for plaice and the amount of camouflage reactions for dab with weight difference, this statement would be much stronger when a wider range of viability of fish was included. If burying behaviour and camouflage could be investigated for a wider range of fish sizes and a greater variation in viability, a conclusion could be drawn about whether or not the indications that these behavioural tests might be used to assess the viability of flatfish are correct. The steps that are taken in this study to develop sound methods to measure burying behaviour and camouflage could be used in follow-up studies as well. The methods could be extended, to be able to measure in more detail. For example a more detailed measurement of the colour of the skin could be an opportunity to increase the selectivity of the camouflage test. However, it should be taken into account that the methods should be practically useful on board of fishing vessels, as that is the place where these methods ultimately are going to be used to investigate the current welfare status of fish. So far, this study provides good indications that burying behaviour and camouflage can be used to assess the viability of flatfish species. It is recommended that these methods are investigated in more detail as this is a chance to improve the methods to assess the viability and welfare status of wild caught flatfish which may ultimately contribute to the improvement of these fish.

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Appendix I: proposal

Non-invasive methods to predict the survival chances of wild caught plaice (*Pleuronectes platessa*)

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Preface

This thesis is part of my master Biology at Wageningen University and Research Centre. In my master, I have chosen the specialization Marine biology and this thesis fits very well in that specialization. I have always been fascinated by the sea and by marine life. During my bachelor Biology at the Vrije Universiteit in Amsterdam, I found out that I found every topic more interesting as long as it was related to salt water and the sea. Therefore I decided to do my bachelor internship at IMARES. During this internship I studied the diet of the grey gurnard in the North Sea. When I finished my bachelor, I wanted to specialize in marine biology and therefore I decided to do my master at Wageningen University where they offered this possibility.

During the first year of my master Biology program, I participated in an ACT (Academic Consultancy Training) project about the welfare of wild caught plaice. This ACT project was part of a larger project of the Wageningen UR Science Shop about the welfare of fish in wild caught fisheries. I enjoyed the project, as it was marine oriented and dealt with fisheries as well as with animal physiology and behaviour. The ACT project was merely an inventory of how the current fishery practices may affect the welfare of wild caught plaice and it became clear that the welfare of wild caught fish is a very new area of research with much to be investigated. This motivated me to react on the question of the Wageningen UR Science Shop for a thesis student who could do further research on this topic.

By doing this thesis, I hope to contribute to the understanding of the welfare of wild caught fish in a scientific way. There is a lot discussion about welfare and about the question if fish are able to feel and experience pain and fear. By approaching this problem scientifically and by focusing on vitality and survival rather than on welfare, I think the discussion can be avoided and the welfare of fish might be improved with the help of scientifically based knowledge about vitality and survival.

Abstract

The welfare of fish is of increasing interest to society and following a focus on fish welfare in aquaculture systems the next step is to pay also attention to welfare in wild caught fish. Research indicates that it is likely that fish are able to feel and experience pain and emotions and therefore care should be taken to ensure their welfare. Welfare may be considered to encompass the three main domains naturalness, functioning (including health) and affect (feeling). In this study, the focus will be on the functional state of wild caught fish as an assessment of welfare. The functional state is represented by the physical health of the organism and can be measured by survival as mortality implies weakness, poor health and possibly suffering. To determine the survival chances of wild caught fish, indicators are needed that predict survival chances. These indicators should be animal based and non-invasive, to avoid additional stress from the measurement.

Non-invasive methods that are already used to assess the survival chances of fish are injury scoring and measurement of reflex impairment (RAMP). The reflexes that are tested in RAMP testing are influenced by higher brain centres, which implies that individual characteristics are likely to intervene with the readout of vitality. In general, protocols for survival assessment in wild caught fish that are used nowadays could be improved and new non-invasive methods have to be developed in order to do that. In this study, such new methods will be constructed and tested for their predictive validity, by comparing the results from the new test with results from actual survival experiments. Flatfishes respond differently as round fish, or invertebrates, and methods have to be developed specifically for a certain species. In this study, plaice (*Pleuronectes platessa*) has been chosen to be the target species for the development of new non-invasive methods to assess vitality and survival chances of wild caught fish.

The methods that will be examined in this study are the commonly used reflex tests and injury scoring, the use of echo to show the internal damages of the fish, the use of a swimming flume to test the resistance of plaice to an increasing water current, the burying behaviour when released in a tank with a sandy substrate and the camouflage behaviour. The new methods are designed to measure behaviour responses that are important in the natural life of plaice. The results obtained from the new methods will be compared with the results from survival experiments to assess the methods’ predictive validity. With the development of new non-invasive methods, the survival assessment of plaice can be done more accurately in the future. Survival assessment tools will help to gain knowledge about the capability of wild caught fishes to cope with the circumstances during the catch and when it makes sense to return fish to the wild. Although this study focuses on plaice, the results will contribute to the understanding of impairment reactions in other flatfish species as well. Also, a correct vitality assessment, i.e. chance on survival, may be used as a proxy for welfare and contribute to the understanding and acceptation of the concept of fish welfare.

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Introduction

The debate on animal welfare dates back to ancient Greece, and tended to focus on husbandry animals that were kept for production by humans (Preece, 1999; Sorabji, 1993). Over the years, guidelines were developed to ensure the welfare of husbandry animals. Aquaculture has rapidly increased the last years as well as the interest in animal welfare in general. Therefore, especially in Europe, Canada, Australia and New Zealand, attention was drawn also to the welfare of fish in aquaculture systems (Ashley, 2007; Hürlimann *et al.*, 2014). The recent interest in the welfare of fish mirrors in the increase in research being done on this topic (Huntingford and Kadri, 2009). A logical next step seems to address the welfare in wild caught fish.

For a long time, fish were considered to be unable to feel and experience pain and emotions, but this seems untrue. In mammals, the hippocampus and the amygdala have a function in cognition and emotions (Braithwaite and Boulcott, 2007). Broglio *et al.* (2005) identified homologous structures in teleost fish, the lateral pallial region of the forebrain and the medial pallial region of the forebrain, respectively. Fish also show behaviour that requires both cognition and emotion. For instance, they can adjust their fighting behaviour when they face an opponent that they have faced in the past (Chandroo *et al.*, 2004), they can learn to associate a light cue to an electric shock, thereby showing avoidance behaviour (Braithwaite and Boulcott, 2007) and they can resolve the reverse reward task in which they get the larger reward when they choose the smallest reward (Braithwaite *et al.*, 2013). Not only the lateral pallial region and the medial pallial region of the forebrain show homology with mammalian structures. The cerebellum, which has a function in for example spatial cognition and conditioning of simple motor reflexes, shows also striking functional similarities among mammals and fish (Rodríguez *et al.*, 2005). The homologies between the mammalian and the fish brain indicate a shared vertebrate brain organisation and this contributes to the likeliness that fish are able to feel and experience pain and emotions as do mammals. However, there are arguments against the cognitive ability of fish and Rose (2007) for instance argues that fish cannot psychologically experience pain as they lack a cerebral cortex. Nevertheless, the majority of the research indicates that fish are likely to be able to feel and experience pain and emotions. The public opinion is that potential animal suffering should be minimized (Ohl and van der Staay, 2012), and thus a precautionary principle can be supported in which fish are considered to be able to experience pain and emotions and thus care should be taken for the insurance of their welfare (Hürlimann *et al.*, 2014).

There are three main aspects of animal welfare, naturalness, function and, affective state (Duncan and Fraser, 1997; Fraser, 2003). From a function based view, the physical health of an animal indicates its welfare, while in the affective state view, the suffering and emotional experiences of an animal are determining. The natural state view focuses on the ability of an animal to express its natural behaviour. These different views can be used to determine a complete picture of the welfare of an animal. The natural state view on the welfare of wild caught fish would suggest poor welfare given the detrimental effects of the catching process on the expression of natural behaviour. The variation across catching procedures and between individuals is similar from this perspective, making application of the naturalistic view for assessing variation in the welfare of wild caught fish less useful. The affective state is the most direct indication of welfare, as welfare is largely determined by the way the animal experiences it. However, although research indicates that it is likely that fish have the capability to feel and experience pain and emotions, as described before, it is difficult to measure the affective state of fish. Therefore the focus in this study will be on the functional state. Physical health is "the most universally accepted measure of welfare" (Ashley, 2007) and is represented by for instance survival, which can thus be used as a proxy for welfare.

The welfare of wild caught plaice was described by Hürlimann *et al.* (2014) by the allostasis concept (McEwen, 1998). The allostasis concept states that "the capacity to change is crucial to good health and welfare" (Korte *et al.*, 2007). Animals living in their natural habitat are exposed to challenges like hunger, diseases and a changing abiotic variables, and an animal has to be able to adapt to its environment in order to maintain a good health and welfare. If an animal has difficulty to adapt and cope with the changes in its environment, this results in high allostatic load, lowering survival chances and impairing welfare. Weakened fish with poor health that possibly suffer, are at risk of dying and thus survival rate is an obvious proxy for welfare. To determine the survival chances of wild caught fish, they can be kept in tanks to assess their survival rate. In practice, this method is not always possible and it is time consuming. Therefore indicators should be found which can give a prediction of the survival chances of the fish. Hürlimann *et al.* (2014) made an inventory of possible indicators for the welfare of wild caught plaice and these indicators are relevant for the survival chances as well. Indirect indicators of fish welfare are hauling duration, hauling speed, the amount of fish and debris in the net, seasonality and the way of handling. These indicators cannot be measured on the fish itself and provide an environment based welfare assessment. To determine the actual welfare of the animal, an animal based welfare assessment is preferred. Physiological indicators, as for instance brain function, haematic indicators, muscle pH and lactic-acid concentration can be measured on the fish itself and can give information on the stress experienced by the animal and can contribute to the prediction of its survival. The problem with these physiological measurements is that they are invasive and typically are performed in a laboratory setting. Invasive methods can thereby be regarded as stressful for the fish, which may bias in results. To avoid this problem, non-invasive methods should be developed which can predict the survival chances of the fish.

There are already some non-invasive methods to assess the survival chances of fish, like the number of injuries. Wounds can lead to infections (e.g. Noble *et al.*, 2012) and loss of scales and damage to the slime layer can lead to problems with osmoregulation (Ashley, 2007; Rottmann *et al.* 1992; van de Vis, pers. comm.). Injuries can be scored for instance via the Catch Damage Index (Depestele, Desender *et al.*, 2014), which provides an objective measurement of the injuries relevant for fish survival. However, injuries do not show the internal damage of the fish or the stress that the fish experiences, which might influence its survival chances. Therefore, besides injuries, reflex impairment is often used as a method to assess survival chances of fish. These reflexes are stereotyped behaviours or reflex responses to peripheral stimuli (Davis, 2010; Depestele, Buyvoets *et al.*, 2014). They are regarded to give an indication of the vitality and survival chances independent from factors like age, size, sex and motivation of the fish. According to Davis (2010), 5 to 8 reflex responses should be identified to be able to calculate a composite measure of impairment. This reflex impairment score can be correlated with outcomes as mortality and can thus be used as an indicator of survival. This approach is also recommended by ICES (ICES, 2014). In the reflex impairment test, reflexes can be scored simply as present or absent to minimize observer bias in the results. In case of doubt, a reflex is scored as absent, so impaired, thereby following a precautionary principle and preventing overestimation of the survival chances (Depestele, Buyvoets *et al.*, 2014). This method, also called reflex action mortality predictor (RAMP), is seen as a useful predictor of survival (Davis, 2007; Davis, 2010). However, if the indication of the survival chance given by this method is really independent from individual characteristics of the fish can be questioned.

The reflexes that are used in the RAMP testing can be influenced by higher brain centres, rather than being simple reflexes with short neuronal pathways only, and the result of the test may depend on individual characteristics (i.e. traits). One reason that animals, with similar welfare, may react differently to the given stimuli during the tests is that they may have different coping strategies. Coping strategies are behavioural syndromes which are found in a wide range of mammalian species as well as in fish species (Sih *et al.*, 2004; Øverli *et al.*, 2007). Coping strategy differentiates between pro-active and reactive animals, with pro-active animals showing more offensive and aggressive behaviour compared to reactive animals (Koolhaas *et al.*, 2010). Therefore, the RAMP testing might give different results for pro-active and reactive fishes; reactive fish might incorrectly be scored as having low vitality and a low chance of survival. Coping strategies might differ among species as well as among individuals of the same species. Therefore it is important that this kind of individual differences is taken into account when interpreting the results of the RAMP test or other behavioural tests to predict survival chances.

Because the results of the reflex tests are difficult to interpret correctly and injuries alone do not give complete information on the status of the fish, the protocols for survival assessments that are used nowadays have to be improved or new non-invasive methods have to be developed. In this study, plaice (*Pleuronectes platessa*) has been chosen to be the target species for the development of new non-invasive methods to assess its survival chances.

Plaice is a demersal living flatfish species that is important in the Dutch fisheries (www.visbureau.nl, 5-10-2014). It is characterized by orange spots on the body and by having both eyes on the right side of the body. Plaice occurs over a large area along the coast of Europe, from the Mediterranean Sea towards Iceland (ICES, 2005). When developing new methods for assessing survival chances, it is important that these methods are validated to give meaning to the test results. For example, criterion (concurrent) validity can be assessed by means of comparing the results from the new tests with results from actual survival experiments (i.e. the golden standard), to test whether the predictions obtained from the new test are correct. Besides criterion validity, the new developed methods should also be evaluated on content validity (including face validity) and construct validity to ensure that the method indeed represents the survival chances instead of something else (www.explorable.com, 15-10-2014). Species-specific behaviour should be taken into account when developing tools for survival assessment to provide content validity; methods have to be developed specifically for a certain species or the interpretation of test results should be adjusted to the species tested as flatfishes respond in a different way as round fish or invertebrates (Depestele, Buyvoets *et al.*, 2014).

The reflex tests and injury scoring that are commonly used to assess the survival chances of fish might be useful for plaice and other flatfish as well (Depestele, Buyvoets *et al.*, 2014). In order to make correct predictions about the survival chances of flatfish, the protocols have to be adjusted with behaviours and reflexes that are shown by flatfish as a response to a given stimulus. Thereby, the results have to be validated by linking them to actual survival experiments in which the actual survival rate is observed. This should be done for vital fish as well as for weakened fish. Thereby, an echo apparatus can be used to assess internal injuries which are not visible when observing the fish normally. This method is not commonly used and its applicability should be tested in a pilot study first, but if internal damages of the fish can be made visible, this might contribute to a better survival assessment and therefore it is worth it to explore the possibilities of the echo apparatus for survival assessment.

A test that is commonly used to test the condition of a fish is swimming performance (Hammer, 1995). In a swimming flume, two different tests are done. Either the fish is exposed to a constant water current to measure its endurance (Winger *et al.*, 1999) or it is exposed to an increasing water current and its capability to swim against this current and to maintain its position in the tube is monitored (Plaut, 2001; Tierney and Farrell, 2004). In this way the critical swimming speed can be measured. Both, critical swimming speed and endurance tell something about the condition of the fish. The condition of the fish is a measure for its physical health, which can be linked to the survival chance of the fish. However, it should be taken into account that swimming performance is not only dependent of physical condition, but also of other factors as species, size, age and temperature (Winger *et al.*, 1999). Although not all factors influencing survival might be represented by the swimming performance, it might be regarded to contribute to the survival chances of a fish as swimming is important for a fish to escape predation, find food and find good habitats for example (Plaut, 2001). Testing for swimming performance is typically done with species for which swimming is an important part of their life style, such as upstream migrating fishes and pelagic fishes (Horak, 1972; Tierney and Farrell, 2004; Plaut, 2001; Priede and Holliday, 1980). For flatfish, swimming is not that important in their life style as they spent most of their time lying on the sea bottom luring for prey. If a flatfish is brought into a swimming flume, it adheres to the bottom instead of starting to swim against the flow (Arnold 1969; Arnold and Weihs, 1978). For a flatfish to survive it is more important to maintain its position on the sea bottom than to swim actively. The behaviour of plaice in response to water currents was described by Arnold (1969). In order to maintain its position with increasing water currents, plaice heads upstream, increases its respiration, shows burying movements, clamps down to the bottom by arching its back, starts with posterior fin beating, paddles when it slips away and shows bursts of swimming when dislocation occurs. In the case of flatfish, this position holding behaviour might be monitored instead of swimming behaviour as a measure of physical health. A flatfish which has a weaker condition is expected to dislocate earlier compared to a vital flatfish. Thus the swimming flume can be used to monitor the behaviour of plaice in response to an increasing water current and thereby tell something about its vitality and survival chances. Although this method may be impractical to use onboard of commercial fishing vessels, it can identify a promising proxy for survival that can be explored further. Because this study is a search for proxies of survival chances, all kinds of tests can be done and by linking the results to each other (construct validity) and to the golden standard (criterion validity) of the actual survival rate, the most promising proxies can be identified. These promising proxies can then be used to search for more practical tests that can assess the survival chances.

In order to make correct predictions about the survival chances of plaice, it is also important to take the species specific characteristics into account. Therefore, also new methods to predict the survival chances of plaice have to be developed which are specifically oriented on the life history characteristics and natural behaviour of plaice. Flatfishes are negatively buoyant, therefore they sink to the bottom when they do not swim actively. Although plaice is able to swim actively in the water column, it spends most of its time on the sea bottom. As mentioned before, it is important for plaice to maintain its position on the bottom, to reduce the drag forces from the water currents, flatfish may bury themselves in the sediment (Arnold, 1969; Gibson, 2005). Besides avoiding water currents, burying behaviour is also important for survival by reducing predation by visual predators (Gibson, 2005; Ansell and Gibson, 1993). Burying behaviour combined with cryptic colouration is regarded to be the first line of defence of flatfishes against predation (Stoner and Ottmar, 2003). Thereby it is also important for flatfish as a predator species itself. As this behaviour is essential for survival, plaice is expected to show this behaviour when it is released in a tank with a sandy substrate on the bottom. As the time at which burying starts and the burying behaviour after release might differ depending on the coping style (Kristensen *et al.*, 2014) a pilot study is necessary to monitor the normal burying behaviour op plaice. If a plaice is impaired, its burying behaviour might become slower or absent as it requires physical force to bury itself (Gibson and Robb, 1992). So, as the burying behaviour is related to the physical status of the plaice, it may say something about its physical status and survival chances.

A way of defence against visual predators used by plaice is cryptic colouration. The skin of flatfishes contains chromatophores, cells which contain pigment, which contribute to this colouration by changing the distribution of pigment according to visual stimuli from the background (Gibson, 2005). The cryptic colouration is merely the result of changes in skin reflectance and contrast of the patches within the skin (Saidel, 1969). This is the result of three types of chromatophores which are present in the skin of plaice, melanophores, lipophores and guanophores (Healey, 1999). The guanophores consist mostly of iridophores, which lay underneath the melanophores and reflect the light when they are not covered by an aggregation of melanophores. Melanophores occur dermal and epidermal and they contain dark pigment. The dermal melanophores create the background reflectance by covering the iridophores, while the epidermal melanophores create the contrast (Saidel, 1969; Healey, 1999). The lipophores consist of reddish erythrophores and yellowish xanthophores, containing carotenoid pigments (Healey, 1999). The morphological position of the chromatophores is fixed (Lanzing, 1977). The skin pattern is mainly created by aggregation and expansion of the melanophores which is regulated by nervous and hormonal control (Healey, 1999). Nervous changes can be rapid in seconds to minutes (Ramachandran, 1996), while hormonal changes occur more slowly (hours) (Healey, 1999). Because of the high level of controllability of the colour change in plaice, this can be expected to be disturbed when the health status of the plaice is impaired. If this is indeed the case, it could be possible to use the ability to change colour as a proxy for stress, health status and survival chances. However, it should be taken into account that there might be a lot of individual variation in this characteristic (Healey, 1999). Therefore a pilot study has to show whether colour change can be used to assess the health status of the plaice.

With the development of new non-invasive methods the survival assessment of plaice can be done more precisely in the future. This may give more insight in the welfare of the fish during the fishing process. Thereby it contributes to the knowledge about the capability of fishes to cope with the circumstances during the catch and how impairment can be identified on basis of physical and behavioural characteristics. Although this study focuses on plaice, the results will contribute to the understanding of impairment reactions in other flatfish species as well. Different flatfish species are not the same, but the same general mechanisms are involved in their lifestyle and therefore results might be extrapolated when the differences are taken into account. At last, a correct assessment of the survival chances might be used as a proxy for welfare and contribute to the understanding and acceptation of the concept of fish welfare.

Material and methods

Experimental approach

The experiments will be carried out in the laboratory facilities at IMARES in Yerseke. Each fish that is tested will be measured for length, weight and sex, as these parameters might also influence the test results.

Pilot study

Before starting with the actual experiments, pilot studies have to be carried out in order to ensure that the tests are useful. As the ideas for testing methods are derived from literature and other fish species, it is necessary to check whether the tests do indeed work the way it is expected. The pilot studies will be performed with vital fish. If this does not show the reaction that is expected during a certain test, the test can be regarded to be not useful and will not be used in the actual experiments. The pilot experiments require replicates, as the results obtained from a single fish might be due to chance. In the beginning of the pilot experiments, the best way to perform a test is investigated. This will be done with 3 fish per test. After the protocol is optimized, the method will be tested on 8 individuals to see whether the test gives the results that are expected. By performing a pilot in this way, negative results can be used to show that a method does not work for testing the survival chances.

The fish used in the pilot studies can be cultured sole, which is available at IMARES or wild caught plaice or other flatfish which is kept in the laboratory for a period of 2 to 3 weeks in order to ensure their vitality. The fish that survived this period can be regarded to be vital and would survive if they would be returned into the sea. Before the fish are used in the experiments, their length and weight are measured and their sex is determined. Weight is important to measure as weight gain or loss can be used to indicate the state of a fish when all fish survive the survival experiments that are performed at the end of the experimental procedure during the actual experiments (see actual experiment). For the pilot studies, other flatfish species as plaice can be used as well as the main mechanisms in these species are quite similar. However, it should be taken into account that in some tests plaice might react differently compared to for instance sole. Therefore at least one round of pilot studies should be performed with plaice to ensure that the tests work for plaice.

During the pilot experiments, all the results that are obtained from the tests are recorded. By recording all the results, it becomes clear what things should be improved for the actual experiments and it might also be possible that some results are already useful. Therefore it is important to record everything that is done, as this might help to improve the tests and to reflect on the tests that are done, which is very important in this stage of the method development.

Actual experiment

The tests in the actual experiment will be performed with vital fish as well as with non-vital fish. Both groups are tested to be able to compare the results for vital and non-vital fish to investigate predictive validity of the tests. Vital fish are wild-caught plaice that have survived in the laboratory for a period of two weeks. Because of their survival they are regarded to be vital. Non-vital fish are wild-caught plaice that are brought to the laboratory where they are immediately tested for their survival chances and afterwards hold to observe their actual survival rate. The weight of the fish will be measured before and after the survival experiment, to indicate the state of the fish in the case that all or most fish survive. As they are wild-caught there will be natural variation among their survival chances which will offer a broad range of vitality scores, survival chances and actual survival rates in this experiment. The actual survival rates function as the golden standard during this study and by correlating the test results with the actual survival rates, it can be showed if the test truly predicts survival (criterion validity) which confirms construct (convergent) validity.

All tests will be performed on all the individual fishes in the experiment. As the vitality might be influenced by the testing procedure, the tests will be performed in the same sequence all the time. The optimal sequence will be determined with the pilot studies. It should be taken into account that the last test might appear to be not useful as a consequence of its position in the sequence of the tests rather than its actual usability. Because of the sequence effect, it cannot be tested which test is most predictive for the survival chance of the fish, but the tests can be performed in the same circumstances among all the individuals, which makes comparisons between individuals possible. However, it should be taken into account that the performance of the tests may influence the survival chances and that non-vital fish might have an interaction effect by which their vitality score decreases steeper compared to vital fish. To validate the test results, the results are linked to the actual survival rate. In this way the predictive value of the tests is indicated.

To be able to analyse the data correctly, it is recommended that at least 40 individual fishes are tested (based on the requirements for a Chi-square test with 2x4 groups). Most tests can be performed in a correct way then. However, because the fishes have to be caught from the wild, it might not be possible to test that amount of fish and in that case this should be taken into account when analysing the data.

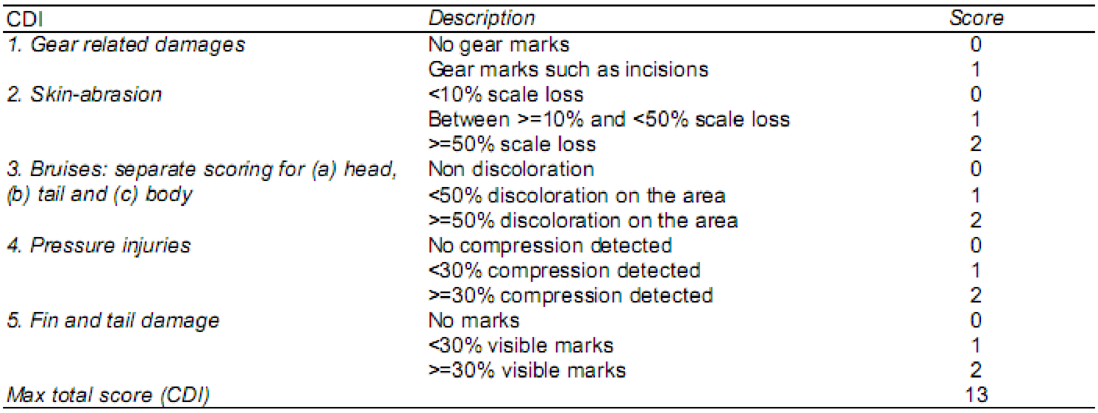
Holding of the fish

During the pilot experiments as well as during the actual experiments, the fish are housed individually in aquaria. They are not socially dependent of each other and by housing them individually social interactions which might have an effect on the test results can be avoided. Individually housing also makes identification of single individuals easily possible as marking of the fish should be avoided as this might have an effect on their survival. If there are not enough aquaria available the fish will be housed with two or three individuals per tank, so that individual identification is still possible. The tanks will have a sandy substrate on the bottom, to provide the fish with a bit of a natural habitat. The tanks in which the fish are held and the test tanks are connected to the flow-through system of IMARES which provides a constant flow-through of water from the Oosterschelde. Because of its natural origin, the temperature, salt and oxygen levels might fluctuate a bit. However, these fluctuations can be neglected as they are natural variability to which the fish would also be exposed when they would be in their natural environment. It should be checked whether the flow-through of the test tanks is sufficient, as stress of the fish during the tests might lead to increased oxygen consumption for instance. To prevent the oxygen from being depleted, extra aeration can be applied for the test tanks. The temperature, oxygen and salt levels will be monitored to check for large differences, although this is not expected to happen. Besides the water characteristics, the light intensity is also important. The light should follow the normal day/night cycle and the lamp should be located directly above the tank, the intensity should not be too high and will be determined in cooperation with the people of the laboratory facilities.

Injuries

Injuries are scored by percentage, using the Catch Damage Index (CDI) that was used by Depestele, Buyvoets *et al.* (2014). In this index the injuries are scored with a value of 0, 1 or 2 depending on the percentage damage and summarized to provide an overall catch damage score. The scoring is listed in table 1.

Table 1. Catch Damage Index (CDI) to score physical damage relevant for fish survival. Injuries are scored with a value of 0, 1, or 2 depending on the percentage damage. (Depestele, Buyvoets *et al.*, 2014)



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

RAMP

Reflex tests will be performed according to the scheme provided by table 2. The reflex actions that will be tested are chosen on basis of their consistency across the studies done by Davis (2007), Kestin *et al.* (2002) and Depestele, Buyvoets *et al.* (2014) and the existing protocol for the assessment of the consciousness of turbot (Hans van de Vis, pers. comm.). A description of the reflex actions is given in table 2, as well as the sequence that should be followed when performing the test. To prevent bias in the results, the reflexes are scored as present or absent. In case of doubt a reflex is scored as absent, thereby following a precautionary principle and preventing the vitality from being overestimated. The RAMP score can then be calculated with the formula given by Davis (2007).

(1) RAMP = 1 – (total reflex response score/total score possible)

No impairment is given by a score of 0 and maximal impairment is given by a score of 1.

Table 2. Description and sequence of the reflex actions used in the RAMP-test. The observation that should be seen when the fish is unimpaired is indicated as well as how the reflex has to be tested. Reflex actions with an asterisk (*) are prone to be influenced by individual behaviour such as coping styles.

Test	Breathing	Righting*	Vestibular-ocular response	Evade*	Operculum closure	Mouth closure	Tail grab*
Protocol	Observe the fish undisturbed for at least a minute	Place the fish with its blind side facing up and watch if it returns to its natural position within 5 seconds	Grasp firmly the fish and rotate around anterior/posterior axis. The eyes must always face the observer.	Release the fish at the water line	Open the operculum with a probe or pencil	Open the mouth with a probe or pencil	Grasp firmly the tail with two fingers and drag the fish to the water line
Location	In water	In water	In air	In water	In water	In water	In water
Observation	Movements (existence and rhythm) of the gills opercula	Ability or attempts to return to a natural position within 5 seconds	Rolling of the eyes to compensate for changes in body posture	Active swimming behaviour away from the tester	Resistance to forced opening and closure after forced opening	Closure of the mouth after opening	Attempts to escape and swim away

Burying

The fish is placed individually in a tank with a sandy substrate of 4 cm height on the bottom. Fine sand is used, to test the burying behaviour of the fish. If it appears that burying does not cost the fish energy because of the fine sand, a coarser sand type can be used. This can be tried in the pilot study. With the pilot study, several things are measured, namely the time to the start of burying after release in the test tank, the amount of movements of the fish to bury itself and the percentage coverage after 30 minutes. The percentage coverage is scored as 0 (not buried), 1 (<50% buried), 2 (>50% buried) or 3 (completely buried). This scoring system is derived from the system used by Kristensen *et al.* (2014) to assess the burying behaviour of turbot and European flounder. The measurement that is best practical and reproducible will be used in the actual experiment. If useful, a combination of the measurements is also possible.

Camouflage

An individual fish is placed in a tank with a transparent bottom which is slightly lifted from the ground so that a paper can be placed underneath. At first, a grey paper is underneath with a known reflectivity percentage of 45 (intermediate reflectivity) based on the Ostwald standards as used by Healey (1999). A photograph is taken after 15 minutes and after 30 minutes. After 30 minutes, the paper is replaced by a white paper with black dots. According to the literature, flatfish are able to adjust structure better than colour, as in nature no abrupt colour changes occur, but merely structure changes when a flatfish moves over the seabed. Therefore it would be best to test with a pattern of dots with the same reflectivity. The reflectivity of a pattern can be calculated by the formula given by Healey (1999) where R is the overall percentage reflectivity of the pattern, R_c is the reflectivity percentage of the circles and R_g is the reflectivity percentage of the background.

$$(2)\quad R = R_c\, A\, d^{-2} + R_g\, (d^2 - A)\, d^{-2}$$

For a reflectivity of 45 percent, the dots need to have a distance between the centres two circles in a straight line of d=0.37 cm and an area of A=0.06 cm^2. In the pilot study, several patterns of coarse and fine dots will be tested and the one with the clearest results will be used in the actual experiments. In the pilot study a photograph is taken every five minutes over 30 minutes in total to determine the time that is required for a clear skin pattern adaptation. The photographs are taken with an digital underwater camera to avoid reflection of light on the water which disturbs the colours of the photo. The digital photographs will be analyzed with the computer program Colour Contrast Analyser (www.paciellogroup.com, 13-10-2014). With this program you take a block of 8x8 pixels and compares that with another block of 8x8 pixels and the program gives a contrast score of maximal 21:1 (black to white) and minimal 1:1 (same colour). Besides using the program, also the number of spots can be counted and the width of the spots can be measured on basis of the photographs. Another possibility is to score camouflage as 0 (not adapted), 1 (slightly adapted) and 2 (highly adapted) on basis of visual assessment or in combination with the parameters measured. The pilot study will be used to find the best way to assess the colour change.

Swimming flume

The fish are placed individually in a transparent pipe which is located in a tank with a controllable water current. The pipe has no substrate on the bottom as that might influence the behaviour in the current. Plaice will probably bury itself to escape from the water current, but burying behaviour is already tested with the burying test and as here the physical behaviour to the water currents is to be tested, the fish will be placed on a bottom without a substrate. At the start, the speed of the water current is 0.10 m/s. After 10 minutes, the speed is increased with 0.10 m/s. This is repeated till a speed of 1 m/s or is stopped before if the fish is unable to swim against the currents. During the pilot study, it is also possible to test whether it works better when the speed of the water current is increased with 5 m/s and every time watch for 2 to 3 minutes if the behaviour changes. If the behaviour does not change, the speed can be increased further. The pilot can be used to test for the best useable speed increase and time tested per speed. During the test, the behaviour of the fish in the swimming flume is observed. It is monitored at which speeds and at which time the fish starts heading upstream, increases its respiratory movements, shows burying behaviour, clamps down to the surface by arching its back, starts fin beating, slips away, shows swimming bursts and at last is dislocated. In the pilot study it is tested which of these parameters is useful and reproducible. Those parameters will be used in the actual experiment. If more pipes are placed near each other, the number of parameters has to be reduced or cameras have to be used in order to be able to monitor the parameter changes correctly.

Echo

A pilot study has to show what the use of echo can contribute to the assessment of the survival chances of the fish. Possibly the echo apparatus is used to investigate the bone structure for fractures, to monitor the heart function (only rhythm as heart beat might be influenced by the measurement) and to monitor large parasites or large internal bleedings. If the use of the echo apparatus gives useful results, a more precise protocol can be developed for the use in the actual experiments. As in the pilot study vital fish are used, no damages will be observed then. However, it can be assessed if is likely that damages can be seen when the fish would have had them.

Survival rate

All fish, vital and non-vital will be kept for at least ten days in their holding tanks after the tests have been performed in order to monitor their actual survival rate. Every day, the mortality of the fish is monitored by scoring the death individuals. The fish that survived this experiment will be measured for their weight, as weight gain or loss is also an indication for how well the animals do. If possible, the food intake will also be monitored during the survival experiment by counting the amount of food pellets that is eaten by the fish. This also gives an indication of the state of the fish, but this is only possible when fish are housed individually.

Data analysis

For each test, the differences in the results among fish parameters as size, weight and sex will be tested. Thereby, the differences for vital and non-vital fish will be tested and the outcomes of the actual survival experiments will be tested for correlation with the results obtained from the different tests.

For the injuries and RAMP testing, the scores that are obtained from the test are continuous variables. The differences between sexes or vital and non-vital can be tested with a two sample t-test in SPSS. For differences among weight or size, first weight or size classes have to be made and the results can then be compared for the different classes with a one-way ANOVA in SPSS. If the data are not normally distributed, a Mann-Whitney U test and a Kruskal-Wallis test can be used respectively.

For the burying test, the results contain different variables. The ‘time to start burying’ is a continuous variable and can be tested with the same tests as are used for injuries and RAMP testing. The amount of movements is a discrete variable and can be tested with a Chi-square test when more than two groups are compared. The percentage of coverage is scored in four categories and can also be tested with a Chi-square test.

For the camouflage test, the pilot study has to show what kind of variables will be used. The colour contrast score is a continuous variable and can be tested with a two-sample t-test or a one-way ANOVA (or when not normally distributed, a Mann-Whitney U test and a Kruskal-Wallis test). However, if it is summarized in a categorical scoring system, the results have to be tested with a Chi-square test. So it remains open which way of data analysis will be used until the pilot study has taken place.

For the swimming test, the time and speed of the water current at which several behaviours occur is scored. Although the speed is increased in steps, it remains a continuous variable as is time as well. Therefore the results of the swimming test can be tested with a two sample t-test or a one-way ANOVA in SPSS.

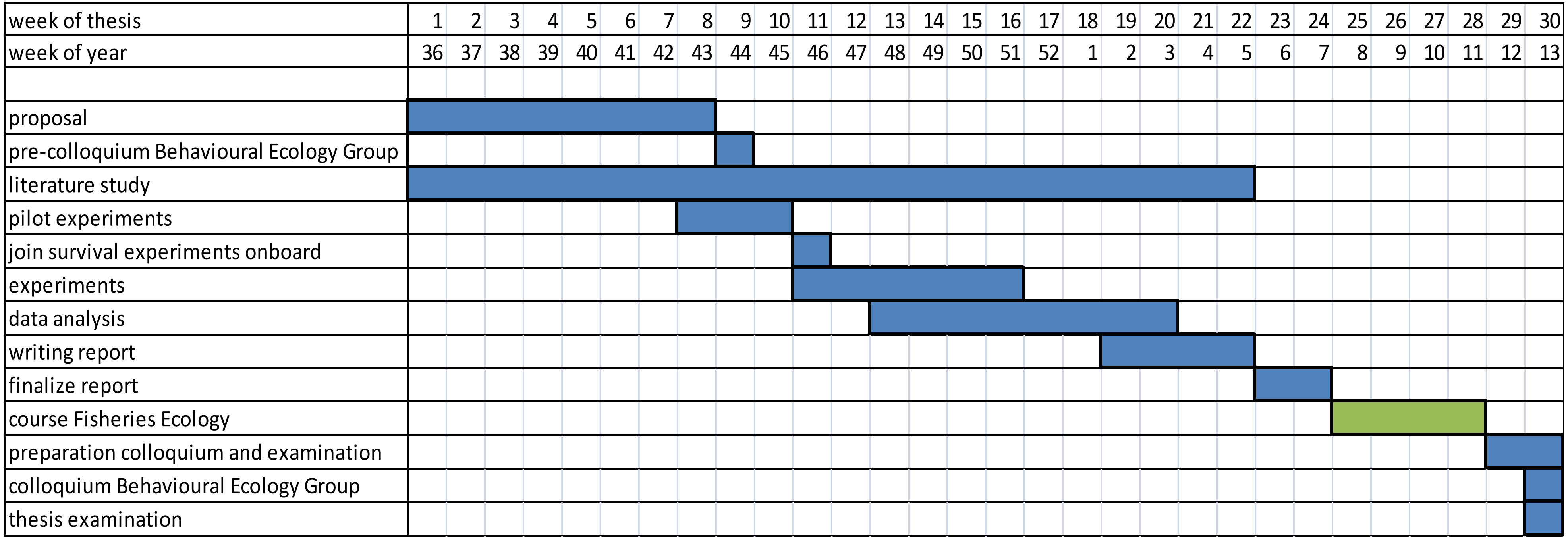
For the echo, the pilot study has to reveal how it can be used and therefore it is not yet known what kind of data will be obtained and how they can be analysed.

For the survival rate, the time until death is scored. The Kaplan Meier procedure can be used to test whether the survival time differs between different groups.

To investigate the correlations between the results of the different tests, a PCA analysis will be performed. In this way, it can be explored which tests or parameters tested correlate with each other (indicated by high PCA outcomes >0.4; <-0.4) and are important predictors for the survival chance of plaice. Depending on the number of tests that remains for the actual experiments after the pilot studies, the number of fish that has to be tested is 5 times that amount, as for a PCA analysis the number of parameters tested is 20% of the amount of individuals tested.

Project planning

In the scheme below, the planning of the thesis project is shown. Literature study is ongoing during the whole duration of the thesis. The thesis report will be finalized before the break in week 25 to 28, as then the course Fisheries Ecology has to be followed (indicated in green in the scheme below). After this course, there is another period of two weeks in which the thesis will be presented and the examination will take place.



week of thesis	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
week of year	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	1	2	3	4	5	6	7	8	9	10	11	12	13
proposal	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦																						
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literature study	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦								
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Backup strategies

It might happen that the pilot studies show that some of the tests are not useful to assess the survival chance of plaice. In that case, these tests might be left out of the analysis. If there remain too few tests to perform a Principle Component Analysis (minimum of 5 parameters tested), the correlation of the tests can be done by a normal correlation test. Another possibility is to choose one or two of the tests that seem to be most promising and investigate these tests in more detail.

It might also happen that the weather is too bad and that there is no delivery of wild-caught plaice. In that case, another fish, such as cultured sole, might be used for the experiments. To provide an impaired group, part of these fish can be impaired experimentally, for instance by simulating stressors. However, to be able to do this, a request has to be made at the Animal Experimental Committee.

If the tests do not deliver useable results it is possible to limit the thesis to a literature review without further experimentation. In this literature review the nowadays existing methods to assess fish survival chances might be investigated as well as the physiological responses of fish to stressors and the cognitive ability of stress. Thereby possibly new methods to predict the survival chances might be investigated theoretically.

Calculation of costs

Table 3. Estimated costs of thesis work.

Expenditure	**Estimated costs (€)**	**Paid by**
Fish for the experiments	500	IMARES
Medical health check field work	142.78	IMARES
Pipes for swimming flume		IMARES
Sand for burying test		
Paper prints for camouflage test		
Food for the fish		
Room in Yerseke	400	Rianne Laan

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