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Florian Mauduit

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présentée par

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Préparée au Laboratoire PFOM-ARN du  
centre Ifremer de Brest

**Assessing fish health:  
the missing cornerstone in ecological  
impact assessment and management.**

**Thèse soutenue le 31 Mars 2017**

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## ***List of abbreviations***

CT<sub>max</sub>: critical thermal maximum

CT<sub>min</sub>: critical thermal minimum

ILOL: incipient lethal oxygen level

ILOS: incipient lethal oxygen saturation

NEBA: net environmental benefit analysis

O<sub>2crit</sub>: critical oxygen level

PAH: polycyclic aromatic hydrocarbons

PCB: polychlorinated biphenyl

T<sub>opt</sub>: optimum temperature

U<sub>crit</sub>: critical swimming speed

## *Introduction*



With nearly 25% of humans living within 100 km of the coast, population densities in coastal regions are about three times higher than global average (Small and Nicholls, 2003). Marine coastal areas provide a wide range of goods and services, including energy production, transportation routes, tourism and recreational activities, pharmaceuticals, nutrient cycling and climate moderation (UNEP, 2006). Among the most crucial and influential utilities provided by coastal ecosystems throughout human evolution has certainly been food supply (Crawford *et al.*, 1999; Broadhurst *et al.*, 2002). Nowadays, fish still accounts for 17% of animal protein intake and 6.5% of all protein consumed by human populations (FAO, 2014).

The demographic concentration and associated intensification of human activities along the coast line is putting considerable pressure on marine ecosystems, as revealed by ecological imbalances such as eutrophication, pollution, increased number and severity of hypoxic events, loss of habitat and loss of biodiversity (UNEP, 2006). As a result, enhancing the preservation, and management of marine biological resources is a crucial matter to secure human population's wealth and health.

To assess and predict anthropogenic impacts on marine ecosystems, substantial effort has been directed to the development of biomarkers. A biomarker is a substance, a structure or a process which can be measured accurately and reproducibly, and which reflects the interactions between a biological system and its environment (WHO, 1993). Within the terms of this definition, genes or groups of genes, biochemical pathways, organisms, populations or ecosystems can be considered as biological systems and the chemical, physical, or biological features of

the interactions between these systems and their surroundings may be used as biomarkers. However, despite potential for broad range applications, most commonly used biomarkers target effects occurring at sub-organismal organizational level (molecules, cells or tissues) with very few of them liable to indicate impacts at organism or higher organizational levels. As a consequence, whereas low-level interactions between a biological system and its environment can be easily established and used as early warning signals of environmental stress, the propagation of effects to higher organization levels remains poorly understood. Unfortunately, without such knowledge, establishing reliable projections about potential outcomes at ecologically and economically relevant levels remains very difficult (Depledge *et al.*, 1993; van der Oost *et al.*, 2003).

Even though high level biomarkers are too far removed from potential causative events to be used as early warning signals of environmental stress, they are of considerable relevance to human activities, economy and well-being. In fish, common and long-standing knowledge indicates that performance traits such as hypoxia tolerance, thermal susceptibility and swimming capacity can be used as biomarkers of functional integrity and can be considered as high-level markers of fish health (Tierney and Farrell 2004; McKenzie *et al.*, 2007, Roze *et al.*, 2012, Claireaux *et al.*, 2013). These traits are indeed the cumulative outcome of an organism's environmental history and, at the same time, they reflect their vulnerability and resilience to changes in life conditions (Deem *et al.*, 2001; Hanisch *et al.*, 2012; Stephen, 2014). Such characteristics fit with the definition of animal health which has been defined as the organism's capacity to adapt to, respond to, or control life's challenges and changes (Frankish *et al.*, 1996).

Using complex, integrative physiological traits as indicators of fish health is, however, difficult and currently of limited use. High level biomarkers indeed integrate the functioning of a large number of interacting and redundant, lower-level processes. While this diversity, redundancy and compensatory interactivity in physiological, biochemical and molecular processes contributes to make biological systems more resilient, it also makes it much harder to identify the chains of causalities linking an alteration in one of these high-level markers to a change in environmental conditions.

In that context, to better inform decisions regarding environment management and use, my thesis aimed at:

- 1) developing a methodology to evaluate fish health by combining laboratory and field experiments;
- 2) examining the underlying mechanisms and identifying the confounding factors influencing the performances measured and its interpretation as a biomarker;
- 3) validating that methodology through two case studies i.e., the treatment of an oil spill with dispersant in coastal area and the effect of domestication upon the health of farmed fish.

This dissertation is structured into four chapters of which the first (Chapter I) provides an overview on key topics and introduces current scientific knowledge on the subject. Chapter II presents the development of an ecologically relevant methodology for fish health assessment. The methodology developed here is based on high-throughput measurements of individuals' hypoxia tolerance, temperature susceptibility and swimming performance. The temporal stability of the performances measured was

assessed and their ecological relevance was demonstrated by transferring fish into semi-natural mesocosms. Also, one subset of the experimental population tested in this study was exposed to chemicals (dispersant alone, oil alone, oil + dispersant) to verify that the performances traits chosen as biomarkers were sensitive to pollutant exposure. Chapter III focuses on the identification of the possible confounding factors of biomarkers responses as well as on the examination of the physiological underlying mechanisms to strengthen biomarkers responses interpretation. This chapter is divided into three sub-chapters. Chapter III.1 presents the work on the influence of environmental history upon fish hypoxia tolerance and temperature susceptibility. Chapter III.2 exposes the results of the examination of the metabolic basis of hypoxia tolerance. To identify the mechanisms by which an oil exposure can affect hypoxia tolerance, several respiratory indexes were compared between a control group and an oil-exposed group. Chapters III.3 and III.4 aimed at exploring the contribution of the cardiac performance in determining the performances measured at challenge tests. Chapter III.3 specifically examines the underlying role of the heart in determining hypoxia tolerance. Chapter III.4 aims at verifying the effect of an oil exposure upon cardiac function. This study sought to validate the results obtained at the animal level and to verify that compensatory and regulatory mechanisms do not blur results of temperature challenge tests. Finally, Chapter IV presents two case studies in which fish health assessment shed new light on long-standing issues. Chapter IV.1 aims at settling the controversy regarding the impact of treating oil spills with dispersant on juvenile fish. For this, laboratory and field experiments were combined, representing an innovative approach in relation to traditional practices in dispersant-treated oil effect assessment. Chapter IV.2 presents a contribution to improving aquaculture practices by investigating the

consequences of domestication and selection on salmon's athleticism and ability to cope with farming conditions.

## Chapter I: State of the art



## **1. General context**

### **1.1. Ecological importance of coastal areas**

Coastal regions are transitional areas between the land and the seas. They are characterized by a very high biodiversity and they include some of the richest and most productive and fragile ecosystems on earth, like mangroves and coral reefs. These areas provide a wide range of services to human societies, including ecological support mechanisms (soil formation, photosynthesis, nutrient cycling), environmental regulation (regulation of climate, floods, disease, wastes and water quality), cultural resources (recreational, aesthetic, spiritual benefits) and provisioning services (food, water, timber). These services influence human wealth and welfare both directly, through human use, and indirectly, via impacts on supporting and regulating services on other ecosystems.

### **1.2. Degradation of coastal areas**

The size and rate of growth of human populations have been and continue to be important contributing factors in the generation of environmental disruptions (Holdren and Ehrlich, 1974). The human global population has indeed grown from a few million people 10,000 years ago, to an estimated 7 billion today (Cohen, 1995; Roberts, 2011) (Figure 1). Also, more than half of today's world population lives in coastal areas (within 60 km from the sea) and population densities on the coasts are nearly three times that of inland areas (UNEP, 2006). Therefore, human pressure upon coastal ecosystems is higher than anywhere else on the planet.

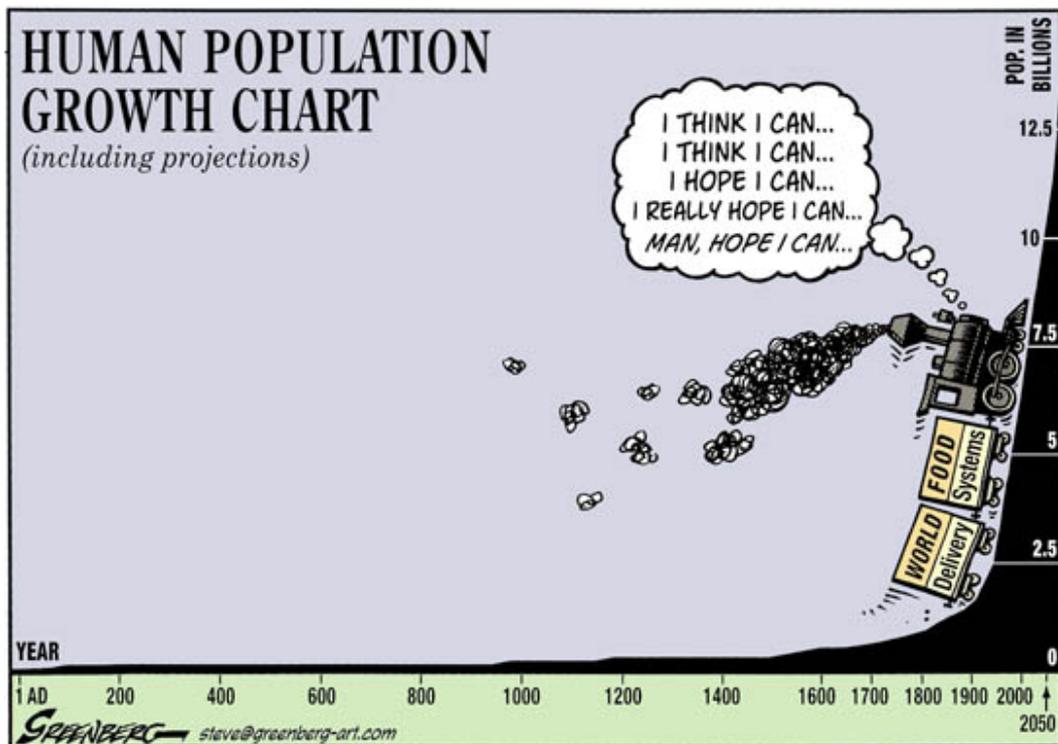


Figure 1: Human population growth (including projections to 2050). Credits: Greenberg, 2010.

Human population growth is indelibly tied together with increased use of natural and man-made resources, energy, land for growing food and for living, and waste by-products that are disposed of, to decompose, pollute or be recycled. Eighty percent of marine pollution comes from land-based sources (Brown *et al.*, 1993). Contaminants and activities that affect coastal habitats and ecosystems also contribute to the loss of marine fauna on which many people rely for food and income. Therefore, measuring the environmental impact of human activities is essential to move toward a sustainable development.

## **2. Assessment of human activities' impact upon fish**

### **2.1. The importance of fish**

Fish are a vital source of food for people and man's most important single source of high-quality protein, providing about 17% of all animal protein consumed by the world's population in 2013. This contribution of fish to human diet is even more important in developing countries, especially small island states, and in coastal regions, where over 50% of the population's animal protein can come from fish (FAO, 2016). Beyond its nutritional importance, fish also have substantial social and economic significance. The value of fish traded internationally in 2013 was around US\$ 148 billion and 54% of fish exports came from developing countries. Moreover, at least 200 million people derive direct and indirect income from fish (FAO, 2016). Besides these direct benefits for humans, fish play an important role in their ecosystem (Allgeier, 2016). Among these roles, fish transfer energy up and down the food chain. For instance, menhaden (*Brevoortia tyrannus*) is a filter feeder eating phytoplankton and thus transferring energy from the sun to its predators. Modification in one species abundance could therefore have consequences on numerous linked-species.

### **2.2. Ecological risk assessment**

Since the early sixties, mankind has become aware of the potential long-term adverse effects of its activities in general and in particular of the risks resulting from the release of chemicals in aquatic and terrestrial ecosystems. To assess these risks, ecologists developed, in the early 80', risk assessment procedures which were then put to use by the industry and insurance companies (Barnthouse *et al.*, 1982;

Barnthouse and Suter, 1986). Nowadays, ecological risk assessment protocols are used in a number of sectors such as industry, government agencies, policy makers and legislators, to determine the nature and likelihood of the effects of anthropogenic activities on animals, plants, and the environment at large. One approach to estimate ecological risks is to conduct a net environmental benefit analysis (NEBA). A net environmental benefit analysis evaluates the gain in value of an environmental service or other ecological property attained by remediation or ecological restoration, minus the value of the adverse environmental effects caused by those actions. Net environmental benefit analysis is therefore a methodology for comparing and ranking net environmental benefits associated with multiple management alternatives (Efroymsen *et al.*, 2004). Through this methodology, environmental managers strive to uphold community values (sensitive ecosystems, local businesses, health and safety, tourism/recreation, regional industries) and protect community assets with every operational decision.

Conducting a NEBA consists of four steps. These steps are illustrated below in the context of the accidental spillage of a chemical compound:

1. **Compile and evaluate data** on pollutant properties, spill trajectory and sensitivity of resources which may be affected by the spill trajectory.
2. **Predict outcomes** of a no action scenario.
3. **Reach consensus** among stakeholders on the relative priority of environmental sensitivities and understand, balance and accept the trade-offs inherent to the available response techniques.
4. **Select the best response strategy** for the planning scenario and prevailing incident conditions.

Although NEBA has a broad range of applications, it is most commonly associated with the assessment of response strategies following an accidental spill (Lunel *et al.*, 1997; Fiocco and Lewis, 1999). However, Lunel and Baker (1999) highlighted that most spills are inadequately documented for NEBA and they pointed out the lack of data about the relationship between response methods and organisms' outcomes. To bridge this gap, substantial efforts have been devoted to developing and applying biomarkers capable of informing that organisms have been or are being exposed to certain chemicals or that organisms are suffering or likely will suffer future impairments of ecological relevance as result of exposition to those chemicals (Forbes *et al.*, 2006).

### 2.3. Biomarkers

#### 2.3.1. *Definition*

Biomarkers have their origins in human toxicology, in which they have proved to be useful as measures of human exposure to specific chemicals or as early warning indicators of specific diseases or syndromes (Timbrell, 1998). The World Health Organization (WHO) defines a biomarker as a substance, a structure or a process that can be measured accurately and reproducibly, and which reflects the interactions between a biological system and its environment (WHO, 1993, 2001). Thus, biomarkers are also used as indicators of disease prognosis or a predictor of clinical response to an intervention. (Biomarkers Definitions Working Group, 2001). For instance, biomarkers can be measured to detect tumor shrinkage or regression in certain cancers (Everson *et al.*, 2014; Okegawa *et al.*, 2014) or to determine the risk

of heart disease (Eapen *et al.*, 2014; Ligthart *et al.*, 2014). According to the Canadian National Research Council (NRC, 1987), biomarkers can be classified into 3 groups:

- Biomarker of exposure has been defined as an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism. Biomarkers of exposure can be used to confirm and assess the exposure of individuals or populations to a particular substance, providing a link between external exposures and internal dosimetry.
- Biomarker of effect is any measurable biochemical, physiological or other alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease. Biomarkers of effect can be used to document adverse health effects elicited by external exposure and absorption of a chemical.
- Biomarker of susceptibility is defined as an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance. They aim to consider inter-individual differences in response to toxicant exposure.

Pollutant exposure generally triggers a cascade of biological responses, each of which may be used as a biomarker (McCarthy *et al.*, 1991). Effects at higher hierarchical levels are always preceded by changes in underlying biological processes occurring at lower organizational levels. For that reason, lower-level changes can serve as early warning signals of higher-level effects (Bayne, 1985). In

an environmental context, biomarkers offer promises as sensitive indicators demonstrating that toxicants have entered an organism, have been distributed between tissues, and are eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990).

Van der Oost *et al.*, (2003) proposed a set of 6 criteria to evaluate the strengths and weaknesses of biomarkers:

1. the assay to quantify the biomarker should be reliable (with quality assurance), relatively cheap and easy to perform;
2. the biomarker response should be sensitive to pollutant exposure and/or effects in order to serve as an early warning signal;
3. data about baseline fluctuations in the biomarker value should be available in order to distinguish natural variability (noise) from contaminant-induced stress (signal);
4. confounding factors to the biomarker response should be identified;
5. the mechanistic basis of the relationships between biomarker response and pollutant exposure (dosage and time) should be established;
6. the ecological significance of the biomarker, e.g., the relationships between its response and the (long-term) impact to the organism, should be established

### *2.3.2. Sensitivity vs ecological relevance*

According to Adams *et al.* (1989), there is a difference in sensitivity, specificity and relevance of biomarker responses depending on the biological level at which they occur. It is admitted that low-organizational level biomarkers (molecular or biochemical) are more sensitive and specific to pollutant exposure than high-

organizational level biomarkers (population or community) but their ecological relevance is poor. On the other hand, high level biomarkers provide ecologically relevant information, although causal links with pollutant exposure then become difficult to establish. Between these two opposing points, organism-level responses provide a pivotal point which allows both the establishing of causality links and the projection of the ecological consequences of the stressor of interest (Figure 2).

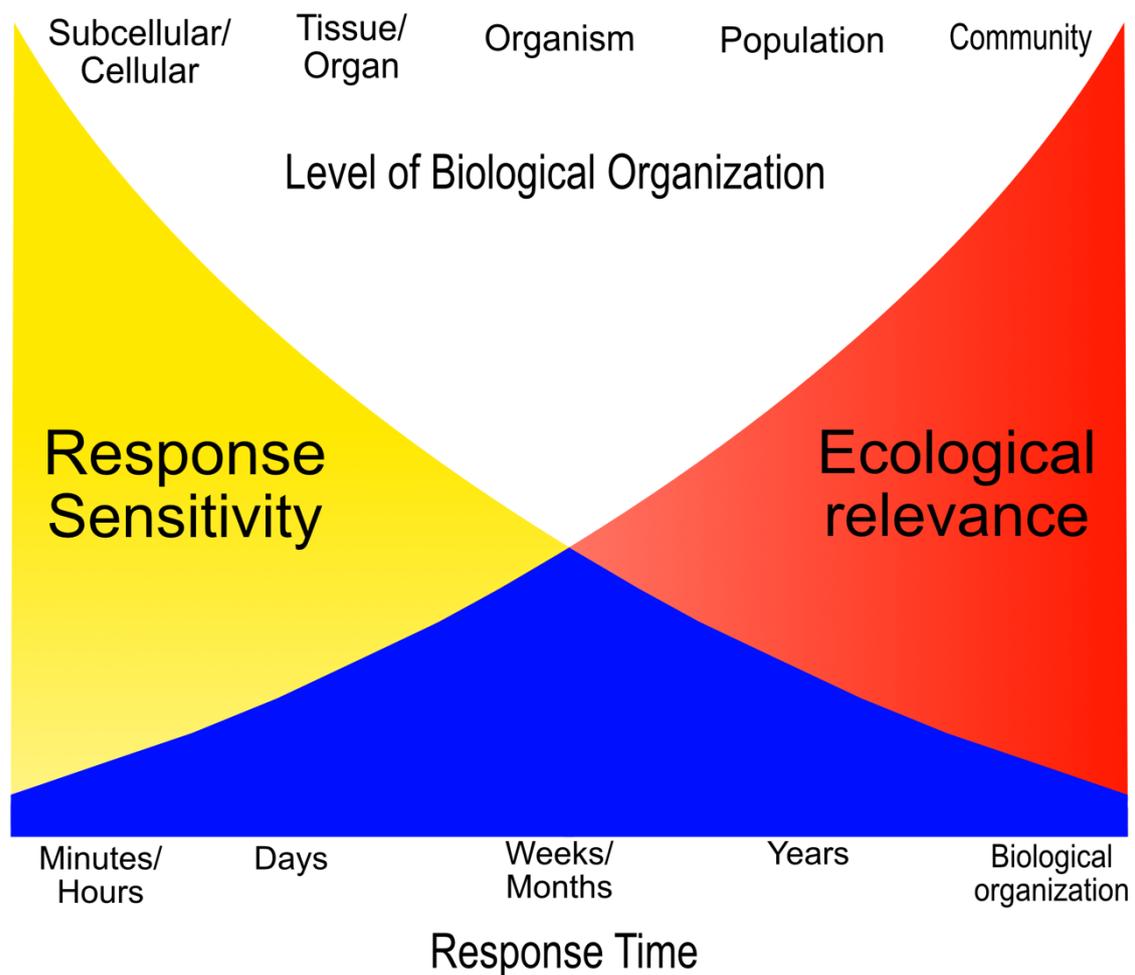


Figure 2: Illustration of the trade-off between response sensitivity and ecological relevance of biomarkers related to their level of biological organization. Adapted from Adams, 2002.

2.3.3. *The failure of biomarkers in ecological risk assessment*

Biomarkers were initially designed to provide predictions about the consequences of environmental stressors on ecologically relevant processes or functions such as ecosystem/organism integrity and health (McCarthy and Shugart, 1990; Peakall, 1994). Unfortunately, this initial goal has not been reached. Forbes *et al.*, (2006) put forward four reasons for this failure:

- 1) **Lack of integrated mechanistic models.** Most biomarker studies are based on empirical statistical models, such as correlation and simple or multiple regression, which are unable to fully describe the complexity of the processes involved. Example of hidden factors not taken into account in such studies, although crucial include differences in genetics and environmental history as well as seasonal variation in environmental conditions, nutritional status and physiological state.
  
- 2) **Complicated time- or dose-dependent responses.** Following an exposure to pollutant, changes in EROD activity, for instance, are transient (from hours to months). For example, Gagnon and Holdway (2000) demonstrated that a six days' exposure to dispersant-treated oil lead to an increased hepatic EROD activity in Atlantic salmon until four days post-exposure before retuning back to control levels. Furthermore, a study from Jimenez *et al.*, (1990) highlighted how difficult it can be to interpret biomarker responses from a fish population in the field. They investigated the responses of biochemical biomarkers in fish collected from clean and contaminated streams. They found variations in biomarkers responses over the year, with different levels between male and reproductively active

females. Also, fish from the most severely impacted site did not display the highest biomarkers levels. Their conclusion was that if biomarkers testify from an exposure, a proper interpretation of the response requires full understanding of environmental, physiological, and toxicological factors involved.

- 3) **Unclear or undetermined link to fitness.** While in theory biomarkers are classified in three groups (biomarker of (i) exposure, (ii) effect or (iii) susceptibility, see above for further details), in practice it is difficult to determine whether a given biomarker response is indicating an impairment or is part of the homeostatic response, indicating that an organism is successfully dealing with the exposure (Calow and Forbes, 1998).
  
- 4) **Confounding influences.** In the field, organisms are often exposed to mixture of chemicals. Such a complexity may lead to very different biomarker responses than those predicted from single-chemical exposures. For example, Willett *et al.*, (2001) showed that while one PAH (benzo(a)pyrene) induced EROD activity, another PAH (fluoranthene), significantly inhibited this same biomarker in the fish *Fundulus heteroclitus*.

## 2.4. Tracks of solution

### 2.4.1. *The notion of health*

During the first half of the 20<sup>th</sup> century, the definition of human health progressively evolved from “*the absence of disease*” to “*complete physical, mental and social well-being and not merely the absence of disease or infirmity*” (World Health Organization, 1948). More recently this definition was extended even further to include the capacity to adapt to, respond to, or control life’s challenges and changes (Frankish *et al.*, 1996). Unfortunately, these advance in the definition of human’s health did not translate into an upgrade of the concept of animal health and contemporary definitions, regulations and research on animal health still largely lag behind by largely focusing on the presence/absence of disease (Stephen, 2014).

The lack of a proper definition of animal health has consequences that extend far beyond the concerns of academics and into the political, social and professional fields. For instance, the Cohen Commission investigating the disappearance of the sockeye salmon from the Fraser River recognized that it was the responsibility of the Canadian Government to preserve the health of wild fish stocks. However, the Commission also pointed out that the lack of fish health standards prevented scientists and regulators from properly assessing risks to wild salmon and from taking informed preventive actions (Cohen, 2012). Yet, propositions about the defining features and forms of measurement of wildlife health recently emerged. Deem *et al.* (2008) proposed a definition focusing on the ability to efficiently respond to challenges (diseases) and to effectively restore and sustain a “state of balance”. Also, Nordenfelt (2011) argued that modern health concepts should emphasize the fact that healthy individuals / populations must have access to a minimum set of resources, functions and capacities in an environment which should allow these

individuals / populations to cope with changes and everyday life challenges. Furthermore, to improve animal health assessment, Stephen (2014) proposed to move from a measure of solely what is absent (i.e., lack of disease or hazards) to characteristics of the animals and their ecosystem that affect their vulnerability and resilience to interacting social and environmental harm.

#### *2.4.2. Complex-physiological traits as biomarkers of fish health*

In aquatic environments, the temperature and oxygenation of the water can undergo wide and rapid fluctuations and any alteration in the ability of organisms to cope with these fluctuations is likely to affect their fitness (Somero, 2005; Mandic *et al.*, 2009). For instance, Claireaux *et al.* (2013) reported that hypoxia tolerance (incipient lethal oxygen level) and thermal susceptibility (upper incipient lethal temperature) display broad inter individual variations. These authors also found that these individual performances are temporally stable and are determinants of survival and growth under field conditions. These traits can, therefore be considered as potential, ecologically relevant, biomarkers of organisms' functional integrity (Beitinger and McCauley, 1990; Davoodi and Claireaux, 2007; Pörtner and Knust, 2007; Wang and Overgaard, 2007; Claireaux and Davoodi, 2010). Similarly, swimming performance is considered as a major character for survival in many species of fish (Jones *et al.*, 1974; Taylor et McPhail, 1986; Graham *et al.*, 1990). Swimming capacity is indeed a determinant of predator-prey relationship, foraging, unfavorable condition avoidance and mate search (Drucker, 1996; Domenici *et al.*, 2012). Measurement of critical swimming speed has been demonstrated as repeatable, indicating that these traits can be considered as reliable indicators of fish coping ability and fitness.

#### 2.4.2.a Hypoxia tolerance

Because environmental hypoxia is a well-studied phenomenon, one would expect that it is well defined. Surprisingly, this is not the case and the definition of environmental hypoxia remains relative. In the literature, the transition from a normoxic to a hypoxic situation depends on the physical, chemical or biological process investigated and although the transition from one situation to the other is progressive, it is generally pinned with a threshold value. Among the most common thresholds for the transition between normoxia and hypoxia  $2 \text{ mg O}_2\cdot\text{L}^{-1}$  is often used (Diaz, 2001; Rabalais *et al.*, 2002; Rabalais and Turner, 2006). Defining such thresholds suggests, however, that hypoxia is a binary variable (i.e. water is hypoxic or it is not) and, quite clearly, this is an oversimplification. Hypoxia indeed means different things to different organisms. For instance, at one extreme of the scale crucian carp and goldfish can tolerate complete anoxia for months by preventing the accumulation of acidic end products in glycolytic energy production (Van den Thillart 1982). At the other extreme, salmonids can hardly tolerate any decrease in environmental oxygen tension (Nikinmaa, 2013). Therefore, hypoxia in the broadest possible context, can be defined as the water oxygen concentration when physiological function is first compromised (Farrell and Richards, 2009).

Fish can show various behavioral and physiological responses when facing a hypoxic environment (Chapman and McKenzie, 2009; Urbina *et al.*, 2011; Domenici *et al.*, 2012). The first line of defense of a fish against hypoxia is to avoid it. When avoidance is not possible, then regulatory physiological processes are mobilized sequentially as hypoxia develops. At an initial stage, this sequence of regulatory responses contributes to preserve a fish's routine aerobic activities while, at a later stage, it mostly defends life-sustaining ATP production. Initial responses correspond

notably to the capacity to increase gill ventilation (Stephensen *et al.*, 1992), reducing heart rate, cardiac output, ATP demand and metabolic rate (Hochachka *et al.*, 1996, Speers-Roesch *et al.*, 2010). When these responses are not sufficient to maintain a life-sustaining ATP production, other compensatory mechanisms are mobilized such as an increased gill perfusion (Russell *et al.*, 2008; Smith *et al.*, 2001), gill surface area (Sollid *et al.*, 2005; Sollid and Nilsson, 2006) and blood hemoglobin concentration or hemoglobin–O<sub>2</sub> binding affinity (Wells, 2009).

Exploring and comparing within and among populations' diversity in hypoxia tolerance requires that it is measured in a large number of individuals. For these populations, classical screening indices produced through nearly 150 years of research in animal respiratory physiology are unsuited. One example of such indices is the critical oxygen level (O<sub>2crit</sub>) which measures the minimum oxygen level required to sustain one individual standard metabolic rate. The determination of O<sub>2crit</sub> is tedious and, at very best, a dozen values are obtained every 2-3 days. To this objective, Roze *et al.*, (2012) proposed a new high-throughput and non-lethal methodology to determine fish hypoxia tolerance. This approach is based on the determination of individual's incipient lethal oxygen level (ILOL) (Figure 3) which corresponds to the oxygen level at which fish cannot maintain its equilibrium. At that point, ATP requirement to maintain life-sustaining activities are not covered and end products from anaerobic metabolism accumulate at a rate which is oxygen- and time-dependent. If the hypoxic conditions worsen and persist, fish lose their ability to maintain an upright position and death generally follows shortly. As consequence, ILOL integrates a temporal dimension (Claireaux and Chabot, 2016). The value of ILOL depends on the experimental protocol used and, particularly, on the time course

of the development of hypoxia. It is also influenced by past exposure history (Nelson and Lipkey, 2015).

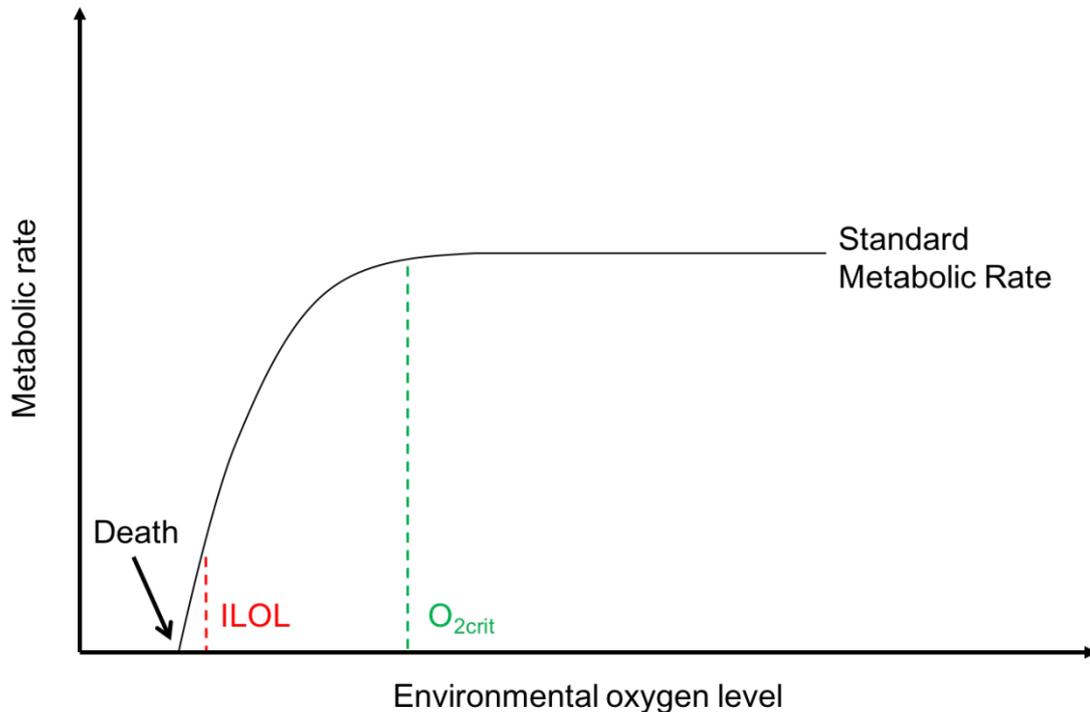


Figure 3: Schematic representation of the influence of environmental oxygen level upon the aerobic metabolic capacity of fish. The black line corresponds to the fish metabolic rate. Critical O<sub>2</sub> level (O<sub>2crit</sub>) is the minimum oxygen level required to sustain an individual's standard metabolic rate. Incipient lethal oxygenation level (ILOL) corresponds to the oxygen level at which fish cannot maintain its equilibrium. Adapted from Richards (2011).

#### 2.4.2.b Temperature susceptibility

The thermal sensitivity of ectotherms is often described by a thermal performance curve (Figure 4). This curve tends to be unimodal, left skewed and can be divided into three distinct regions. First, a rising phase as temperature increases; secondly, a plateau phase that encompasses the thermal optimum (T<sub>opt</sub>) for the trait considered; and third, a falling phase at higher temperatures (Schulte *et al.*, 2015). Low and high

temperatures associated with zero performance on either side of  $T_{opt}$  are usually defined as the minimal and maximal critical temperatures, ( $CT_{min}$  and  $CT_{max}$ , respectively). When these limits are reached, only short-term survival is possible (Fry and Hart, 1948; Brett, 1971; Pörtner and Knust 2007; Pörtner 2010; Sokolova *et al.*, 2012).

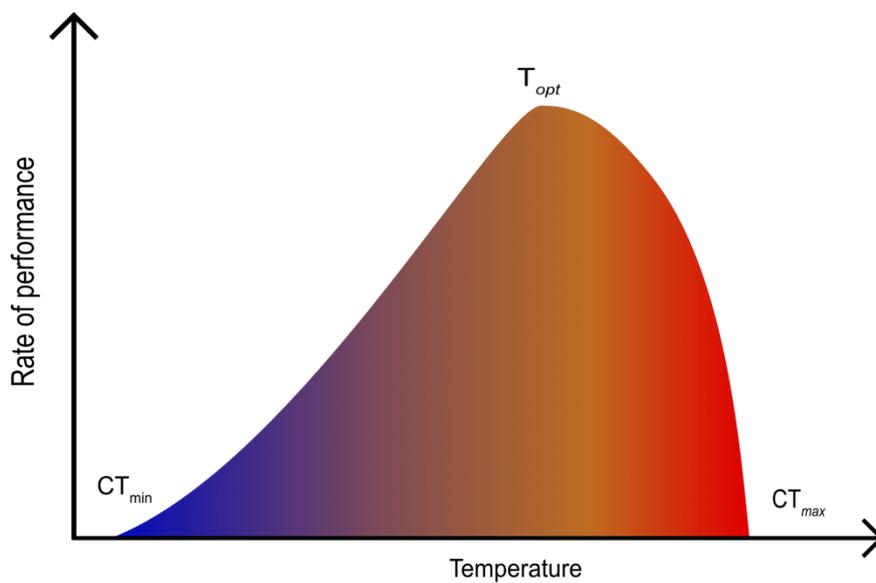


Figure 4: A thermal performance curve

Although the influence of temperature on the biology of fish has been studied for well over a century, the underlying mechanisms determining acute thermal tolerance ( $CT_{max}$ ) are still not fully understood (Beitinger and Lutterschmidt, 2011; Ekström *et al.*, 2016). Even if still debated, heart failure and maintenance of  $O_2$  delivery to the tissues are often put forward as possible factors governing thermal tolerance in fish and other ectotherms (Farrell, 2002, 2009; Lannig *et al.*, 2004; Clark *et al.*, 2008; Pörtner and Farrell, 2008).

The measure of the  $CT_{max}$  was introduced by Cowles and Bogert in 1944. It involves a period of rapid temperature increase (from acclimation to 27°C in about 2.5 H) followed by a slower increase (approximately 0.5°C / H) until a loss of equilibrium occurs (Becker and Genoway 1979). This methodology has been used for several decades to determine fish thermal tolerance (Cocking 1959; McFarlane *et al.*, 1976; Lutterschmidt and Hutchison 1997; Beitinger *et al.*, 2000; Somero 2005). Furthermore,  $CT_{max}$  has been widely used in ecotoxicology as a biomarker of fish functional integrity, notably to assess the effects of exposure to copper, zinc, nickel, and arsenic, (Burton *et al.*, 1972; Becker and Wolford, 1980; Lydy and Wissing; 1988). Furthermore, Claireaux *et al.*, (2013) argued that temperature susceptibility could represent a reliable and ecologically relevant biomarker of fish health. This statement is based on the significant relationship found between individual's  $CT_{max}$  and a fish's ability to survive in the field which indicates that this trait is a determinant of fish ecological performance and that it is under severe selection. This statement is strengthened by the fact that individuals'  $CT_{max}$  was reported temporally stable over a 2-month period. This temporal stability is one prerequisite for this trait to be targets for natural selection and evolution.

#### 2.4.2.c *Swimming performance*

As in many species, locomotory capacities are considered as a determining factor for fish survival. Swimming performance is crucial for searching for food, for predator avoidance and for migration and reproduction (Videler, 1993; Drucker and Jensen, 1996; Watkins, 1996; Walker *et al.*, 2005). Fish have different types of swimming modes which can be classified into 3 categories depending on the duration of the

effort, the muscles involved and the energy substrates used (Beamish, 1978, Webb, 1993; Shadwick *et al.*, 1998):

1. **Sustained aerobic swimming:** sustained performances correspond to those speeds that fish can maintain for long periods (> 200 minutes) without muscular fatigue (Beamish, 1978). At these speeds, energy is supplied to slow oxidative (red) muscle fibers through aerobic processes. These fibers do not fatigue and do not have high power output (Webb, 1994). Metabolic demand is matched by its supply and waste production is matched by its removal (Jones, 1982).
2. **Burst swimming speed:** burst performances are the highest speeds attainable by fish and can be maintained for only short periods of time (< 20 seconds) (Beamish, 1978). At burst speeds, energy is primarily supplied to white muscle through anaerobic processes (Webb, 1994). The conclusion of short periods of burst swimming occurs as a result of the exhaustion of extracellular energy supplies or accumulation of waste products (Colavecchia *et al.*, 1998). Fish often use burst speeds to pass through short high velocity areas, such as the falls and rapids of a river or the inlet or outlet of a culvert. Median and paired fins tend to power slow swimming, and when swimming velocity increases they are supplemented and even replaced with body and caudal (tail) fin undulation (Webb, 1994). At burst speeds, the caudal fin is expanded and made as rigid as possible (Nursall, 1962). When fish swim at low speed they modulate the frequency and amplitude of their body and caudal fin undulation whereas at high speed tail beat frequency is modulated (Bainbridge, 1958, Webb, 1971).

3. **Prolonged swimming speed:** prolonged performances correspond to those speeds that fish can maintain for 20 seconds to 200 minutes and which ends in fatigue (Beamish, 1978). The prolonged category spans the swimming speeds between sustained and burst. At prolonged speeds, energy is supplied to slow (red) and / or fast oxidative glycolytic (pink), and / or fast glycolytic (white) fibers through aerobic and anaerobic processes, respectively. Indeed, when the aerobic metabolic demand for red muscles exceeds the oxygen and nutrient capacity of respiratory and cardiovascular systems, white muscles are involved and anaerobic metabolism triggered. Therefore, as speed increases so does anaerobic metabolism (Peake and Farrell, 2004). Fish capacity to maintain swimming performances can be assessed notably through the determination of critical swimming speed (Brett, 1964; Beamish, 1978; Hammer, 1995; Plaut, 2001).

Critical swimming speed ( $U_{crit}$ ) is measured through a standardized swimming challenge during which the water velocity is increased in a stepwise fashion until the fish is no longer able to maintain position against the water current (Brett, 1964; Hammer, 1995). Such a test requires a swimming tunnel in which water velocity can be controlled (Figure 5). Fish are placed individually or in group in a swimming chamber and encouraged to swim against the current. Individual performances measured during such tests have been found repeatable over periods of up to two months (Kolok, 1992; Jain *et al.*, 1998; McKenzie *et al.*, 2007; Claireaux *et al.*, 2007, 2013).

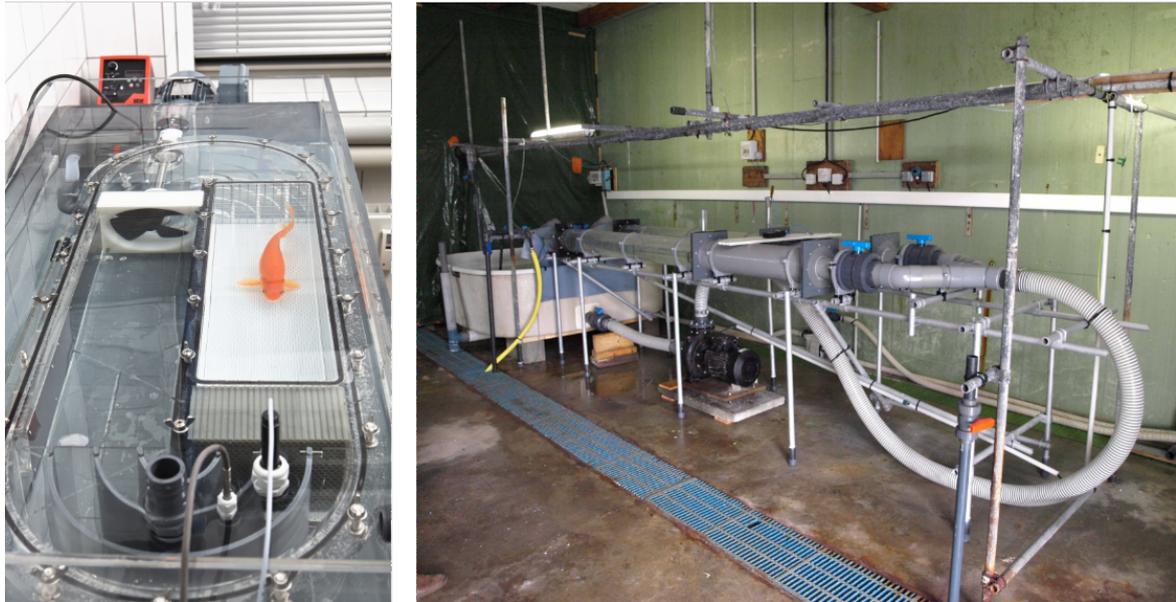


Figure 5: Example of swimming tunnels. Credits: Loligo® Systems (left) and Mauduit (right)

Swimming performance has been used as an integrative biomarker of fish health to assess the effects of abiotic factors such as temperature (Taylor *et al.*, 1997; Farrell, 2007; Rome, 2007), oxygen (Diaz, 2001) or salinity (Brauner *et al.*, 1992, 1994; McKenzie *et al.*, 2001). It has been notably demonstrated that fish swimming performance was maximal at the optimum temperature, and was impaired by reduced habitat quality e.g., hypoxia, food quality and quantity, pollutants including oil and specific petroleum hydrocarbons (Cairns, 1966; Sprague, 1971; Waiwood and Beamish, 1978; McKenzie *et al.*, 2003), PCB (Peterson, 1974), crude oil (Thomas and Rice, 1987) and water soluble fractions of crude oil (Kennedy and Farrell, 2006).

### **3. Dispersant to combat an oil spill in a coastal area**

#### 3.1. Oil spills

Of all the human-induced sources of environmental degradation, large oil spills (> 700 tons) are among the first to come to mind, due to their ecological and socio-economic impacts, and related media coverage. Since the 1970's, important efforts have been devoted to improve safety, security and oil spill prevention during oil extraction and transport. As a result, the number of large spills observed during the early 2000s was less than a tenth of that witnessed during the 1970s (Figure 6). In fact, 86% of the 33,000 tons of oil spilled between 2010 and 2015 were due to only 10 of the 42 accidents recorded during that period (Burgherr, 2007; Kontovas *et al.*, 2010). Although less frequent, large oil spills still represent an acute contamination of the environment affected. For instance, the 2010 "Deepwater Horizon" accident caused the release of 3.19 million barrels (roughly 500 000 m<sup>3</sup>) (US v. BP trial 2015) in the Gulf of Mexico. The spill resulted in oil slicks that cumulatively covered more than 112 000 km<sup>2</sup> of the sea's surface (DWH-NRDA, 2015). The response to that spill consisted in applying approximately 7 000 m<sup>3</sup> of chemical dispersant. This massive oil release and response actions caused injuries to a wide range of habitats (deep ocean-bottom, salt marsh, shoreline, seagrass areas), species (corals, fish, birds, turtles, cetaceans) and ecological functions over a vast area (Beyer *et al.*, 2016).

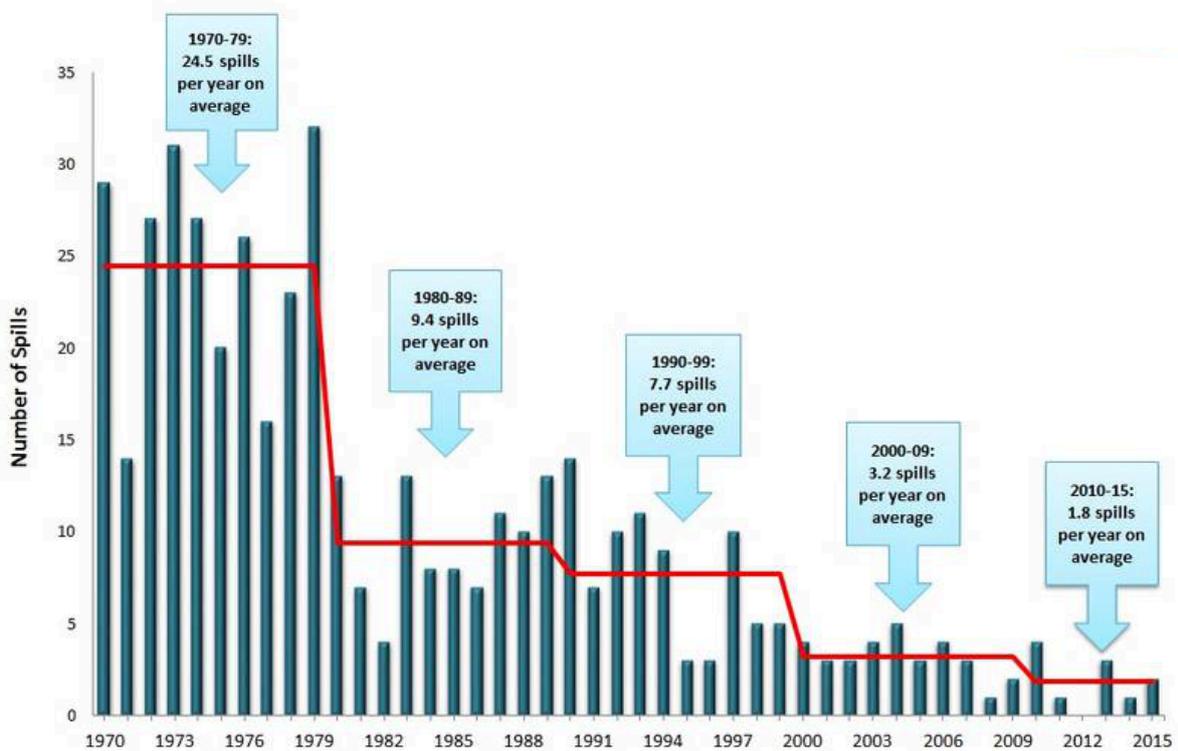


Figure 6: Number of large spills (> 700 tons) recorded in the world since 1970. Credits: ITOFF.

### 3.2. Crude oil and Polycyclic Aromatic Hydrocarbons (PAH)

Crude oil results from the transformation of organic matter in condition of low oxygenation, high pressure and high temperature (Lefebvre, 1978). Its chemical composition varying among oil fields (England et Mackenzie, 1989), oils are identified by the percentages by mass of three families of molecules i.e., saturated hydrocarbons, polar compounds and aromatic hydrocarbons:

- **The saturated hydrocarbons**, mostly present in the lightest crude oils, are compounds which are sparingly soluble in water. They are divided into three groups, n-alkanes or paraffin, branched alkanes or isoparaffins and cycloalkanes or naphthenes.

- **The polar compounds** include the molecules which have atoms other than Carbon and Hydrogen, and are generally of high molecular weight. This category is often divided into two classes: resins and asphaltenes. The higher the percentage of polar compounds within an oil, the heavier it is.
- **The aromatic hydrocarbon family**, which includes Polycyclic Aromatic Hydrocarbons (PAH), consists of molecules based on the benzene ring i.e., from 1 to 7 cycles. These molecules are relatively water-soluble. In 1976, carcinogenic and / or genotoxic property of these compounds led the United States Environmental Protection Agency (US-EPA) to establish a list of 16 PAH that needed to be investigated in priority (Keith, 2015; Figure 7). However, and although studies have shown the toxicity of PAH to aquatic organisms (Ortiz-Delgado *et al.*, 2007, Almroth *et al.*, 2008, Oliveira *et al.*, 2008, Nahrgang *et al.*, 2009), these compounds are not the only determinants of oil toxicity (Barron *et al.*, 1999; González-Doncel *et al.*, 2008).

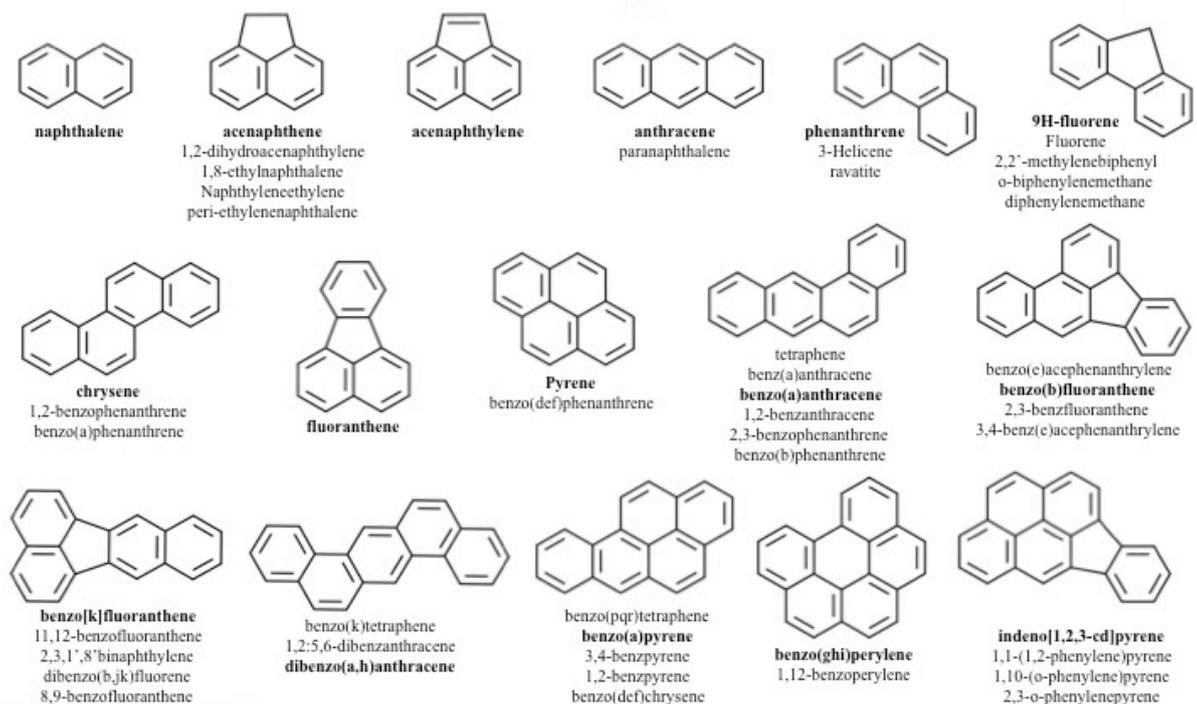


Figure 7: The 16 US-EPA priority PAH compounds.

### 3.3. At sea response strategies

Once oil is spilled at sea it will naturally spread, fragment and disperse under the influence of wind, waves and currents. For spills in coastal waters, the oil will often drift towards the shore and become stranded due to the action of waves and tides. According to the ITOPF, several options can be considered to respond to an oil spill at sea (Figure 8):

- **Containing floating oil within booms for recovery by specialized skimmers** is often seen as the ideal solution to a spill at sea as this aims to physically remove the oil from the marine environment. As a result, it is the primary at-sea response strategy adopted by many governments around the world. However, this strategy is only applicable when drifting floating oil is located, the sea state and weather conditions are calm and when the oil is in a recoverable state.
- **In-situ burning** consists in burning floating oil at sea. A successful burning requires a located oil and an ignition source. This strategy has the potential to remove relatively large amounts of oil from the sea surface. However, the resultant fire and potentially toxic smoke have the potential to impact human health and therefore, it is a technique more suitable for use offshore or away from populated areas.
- **Dispersant application** can quickly remove significant quantities of oil from the sea surface by transferring it into the water column where it is broken down into droplets which are then degraded by natural processes. This technique consists of spraying a mixture of surfactants and solvents onto the oil slick. Surfactant molecules are made up of an oleophilic part (with an attraction to oil) and a hydrophilic part (with an attraction to water). The solvent

transports and distributes the surfactants through an oil slick to the oil/water interface, where they reduce the surface tension and allow small oil droplets to break away from the slick. The droplets thus formed are diluted and more easily degraded by naturally occurring bacteria (Tiehm, 1994; Churchill *et al.*, 1995; Swannell et Daniel, 1999; Davies *et al.*, 2001). This transfer of the oil from the surface to the water column will minimize its impact upon surface dwelling fauna (mammals and seabirds).

There are limitations to the use of dispersants. For instance, they are notably ineffective upon very viscous oils, as they tend to run off the oil into the water before the solvent can penetrate. Therefore, decision regarding their use must be made quickly following a spill as the weathering process tends to increase the viscosity of the spilled oil.

The use of dispersant to treat an oil spill continues to stir up controversy and, at times, generates widespread debates in society. This controversy initially stemmed from the high toxicity of the first generation of dispersants. Their use after the *Torrey Canyon* shipwreck in 1967 in the UK led to some severe additive environmental impacts (Nelson-Smith, 1968). Since that time, however, dispersant toxicity has been significantly reduced, as a result of improvements in their formulation. The reluctance that persists nowadays among responders to consider dispersants as one of the oil spill response option rises from the increased bioavailability of oil compounds that results from their use (Ramachandran *et al.*, 2004). This wariness is particularly of concern in nearshore areas where the dispersed oil might come into contact with sensitive resources before dilution can take place.



Figure 8: Illustration of different at sea response strategies. Containment and recovery of oil (left), in-situ burning (middle) and dispersant spraying (right). Credits: U.S. Coast Guard, AMSA, respectively.

#### 3.4. Effects of crude oil and dispersant-treated oil upon fish

A great deal of research has been conducted to understand the effects of crude oil on the physiology and survival of aquatic organisms. Regulatory guidelines for aquatic pollutants in natural ecosystems have been traditionally based on acute lethality tests such as the 96 H LC50 (USEPA, 2001; CCME, 2002). Although useful to compare toxicants' acute toxicity, lethality tests ignore sub-lethal, often delayed effects. Assessing these effects is, however, crucial as they can also affect fitness determining traits such as reproduction and compromise the long-term success of the population (Kime, 1995). Therefore, a toxicant may have detrimental ecological consequences at concentration well below those causing mortality (Scott and Sloman, 2004). Understanding the effect of sub-lethal dose upon organisms is therefore crucial to characterize the toxicity of a pollutant.

Substantial effort has been devoted by the scientific community to better assess the consequence of an exposure to relevant doses of hydrocarbons. With fish, a large part of this research has focused upon fish embryo (Carls *et al.*, 1999, 2008; Incardona *et al.*, 2004, 2005, 2008, 2014; Brette *et al.*, 2014). It was notably reported that exposure to oil can cause mortality, deformities, cardiac dysfunction and edemas. It has been suggested that fish are more sensitive to oil exposure during

their early-life stages (i.e. embryo or larvae) due to the higher capacity of juveniles and adults to metabolize PAH and detoxify (Hawkins *et al.*, 2002) and due to their thicker skin that prevents diffusion into internal tissues. Because of this supposed higher resistance to crude oil exposure, information about later life stages (i.e. juveniles or adults) are less available in fish. Yet, it has been reported that an exposure to oil is associated with reduced swimming performances (Kennedy and Farrell, 2006; Mager *et al.*, 2014; Stieglitz *et al.*, 2016), immunological alteration (Kennedy and Farrell, 2008; Danion *et al.*, 2011;) changes in maximal or routine metabolic rates (Davoodi and Claireaux, 2007; Klinger *et al.*, 2015), loss of repeatability at hypoxia and temperature challenge tests, impairment of survival in the field (Claireaux *et al.*, 2013) and reduction in cardiac performances (Milinkovitch *et al.*, 2013). However, apart from that of Claireaux *et al.* (2013), studies performed on juvenile or adult fish essentially report effects observed from few minutes to a few days post-oil exposure. There is therefore a lack of information regarding long-term effects of an exposure upon fish health.

#### **4. Study model**

Studies presented in this thesis were conducted using the European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). Well-known throughout history, the European seabass has always been a target species for fishermen. Seabass has been the subject of a number of monograph, notably Barnabé (1976), Pickett and Pawson (1994), and more recently Vázquez and Muñoz-Cueto (2014). The following description aims to present the main biological and ecological characteristics of this species.

#### 4.1. Nomenclature

The specific name of the common bass has evolved considerably since the first known descriptions, which date from Antiquity (Barnabé, 1976). Currently, it is commonly referred to as *Dicentrarchus labrax* (Linnaeus, 1758), but its systematic position is not totally fixed. The European genus *Dicentrarchus* is geographically opposed to the neighboring genera *Morone* and *Roccus*. Some authors group these three genera within the family Serranidae. But others place the seabass in that of the Moronidae, notably because of genetic criteria (Table 1).

<b>Class</b>	Actinopterygii
<b>Order</b>	Perciformes
<b>Suborder</b>	Percoidei
<b>Family</b>	Moronidae
<b>Genus</b>	<i>Dicentrarchus</i>
<b>Species</b>	<i>D.labrax</i>

Table 1: Nomenclature of European seabass

#### 4.2. Morphology

As in all the Perciforms, the body of the common seabass is symmetrical. The seabass, *Dicentrarchus labrax* has a silvery elongated body, with two clearly differentiated dorsal fins and a rather high caudal fin. The opercular bone has two flat spines and a range of spines are visible in the lower part of the preopercular bone, pointing in the direction of the mouth. The vomer presents teeth with a crescent shape. This species has cycloid scales in the interorbital region. The lateral line is

visible as a dark line with 62-80 cycloid scales. The first dorsal fin has 8-10 spiny rays, and the second dorsal fin 12-13 rays of which the first is spiny. The anal fin has 3 spiny rays and 10-12 soft rays. The color is dark grey on the back, passing to grey-silver on the sides, while it is white-silver on the abdomen. Specimens from the sea show a much clearer colour than fish from lagoons and estuarine environments. On the opercula bone, there is a dark spot. The juveniles show a livery with little dark spots, mainly on the front or only on the head, which disappear with age (Morretti *et al.*, 1999).

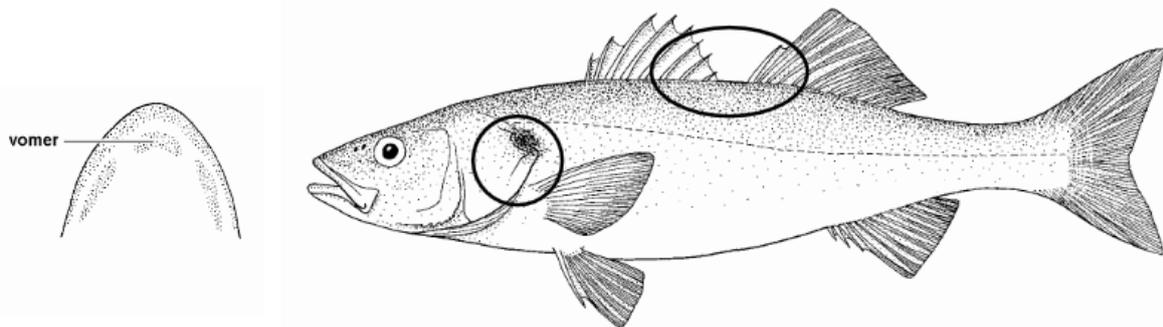


Figure 9: Schematic representation of European seabass *Dicentrarchus labrax* (Linnaeus, 1758). Main characteristics of this species are highlighted: spiny opercular bone, double dorsal fins (spiny and soft, respectively), crescent-shaped vomer. Adapted from: Quero *et al.* (2003)

Sexual dimorphism is not very pronounced in *D. labrax*. Barnabé (1976) identified only a few criteria with which to sex a fish, such as head length and pre-dorsal length, which are slightly higher in females. Their heads would thus be longer and sharper than that of the males.

#### 4.3. Distribution and habitat

The total area of distribution of *Dicentrarchus labrax* extends from 30 ° N (coast of Morocco) to 60 ° N (South of Norway) in the North-East Atlantic. It is present in the Irish Sea, North Sea and Baltic Sea, and it colonizes all the Mediterranean Sea as well as the Black Sea. It can be found up to a hundred meters deep, and up to about 80 km from the coasts. The seabass is an eurytherm and euryhalin fish capable of withstanding temperature from 2 °C to 32 °C and salinity from 0.5 ‰ to 40 ‰. It occurs therefore in brackish waters, at the mouth of estuaries, as well as in coastal areas or in the open sea. However, it particularly occurs in oxygen-rich (Figure 10) and therefore preferentially located along rocky coasts beaten by the sea, and sand beaches with surging waves (Gallet and Cazaubon, 1998).



Figure 10: Fishermen longlining for seabass in the rough sea from Raz de Sein (Brittany, France)

#### 4.4. Feeding

The diet of post-larvae, seabass juveniles and adults in the natural environment has been studied by numerous authors. Their findings indicate that the younger stages feed mainly on Mysidaceae, Amphipods such as Gammarus or Corophium, Copepods, and Decapoda and Cirripede larvae while the diet of adult seabass is

dominated by Decapods, Brachyours, and fish (Boulineau-Coatanea, 1969; Arias, 1980; Roblin, 1980; Ferrari and Chieregato, 1981; Aprahamian and Barr, 1985).

#### 4.5. Life cycle

Seabass eggs are pelagic, relatively small (around 1300  $\mu\text{m}$ , Kennedy and Fitzmaurice, 1972) and take between two and five days to hatch. At hatching, the larvae are about 4 mm and spend about 30 days in the coastal zone (Barnabé, 1976; Kennedy and Fitzmaurice, 1968). At that time, larvae reach the stage of development corresponding to a length of 10 mm which allows them to penetrate the estuarine zones and develop there (Jennings and Pawson, 1992). Juveniles stay in estuaries and coastal habitats for at least the first three years of their lives. Adult seabass have a slow growth, a late sexual maturity (from two to four years), and a long-life span (more than twenty years). However, all these biological parameters vary according to environmental conditions, particularly water temperature. Growth rate increases from north to south, thus, at 5 years of age, the average size of females is 54 cm in the Mediterranean, 40 cm along the Brittany coast and 35 cm in Ireland, while the average size of males is 48 cm in the Mediterranean, 39 cm along the Brittany coast, and 33 cm in Ireland (Gallet and Cazaubon, 1998). It is generally considered that the maximum length of seabass is 100 cm.

#### 4.6. Fisheries and aquaculture

##### 4.6.1. *Capture fisheries*

Seabass capture fluctuated between 8,500 and 11,900 tons between 2000–2009 and it is estimated that recreational fishing accounts for 25% of seabass catches. Most of the reported catches originate from the Atlantic Ocean, with France typically reporting

the highest catches. In the Mediterranean, Italy used to report the largest catches, but has been surpassed by Egypt in recent years (FAO). However, seabass stocks are threatened by overfishing and pollution. Indeed, stocks have been declining since 2005, total biomass in 2011–2012 being 32% lower than the total biomass in the three previous years. In France, the legal harvesting size is 42 cm for recreational and professional fishermen. In 2015, European Union has banned open water trawling of seabass during the spawning season (January to April) in order to ensure the stock's survival. Also, from June 23th 2015 to December 31th 2015, vessels had to respect a fishing restriction from one to three tons per month depending on the fishing technique (Council Regulation (EU) 2015/960).

#### *4.6.2. Farming*

European seabass were one of the first species of fish to be farmed commercially in Europe. It was historically cultured in coastal lagoons and tidal reservoirs, before mass-production techniques were developed in France in the 1970's. Seabass farming production experienced a very fast and very recent growth in Europe. Whereas there was almost no production in 1980, more than 50,000 tons was produced as early as 2000. It is the most important commercial fish widely cultured in the Mediterranean (Figure 11). The most important farming countries are Greece, Turkey, Italy, Spain, Croatia, and Egypt and annual production was more than 120,000 tons in 2010 (FAO, 2012).

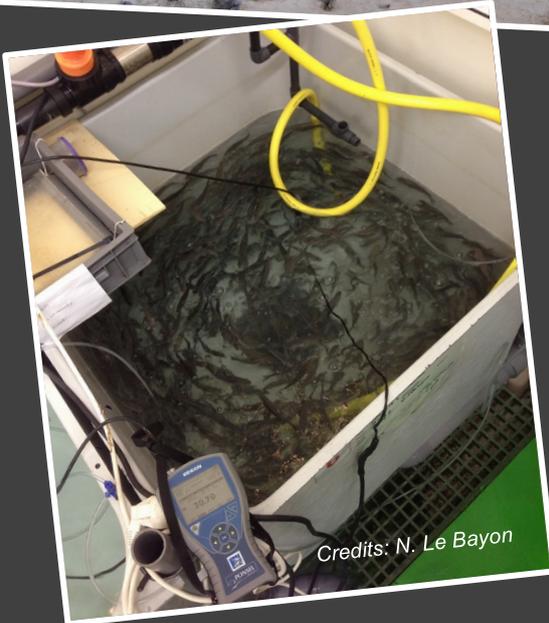


Figure 11: Mediterranean cage farming of seabass. Credits: J. Carnot

#### 4.7. Model species in ecotoxicology

As its biology is well known, seabass have become over the years a test subject in ecotoxicology. For instance, seabass are used as a reference fish in the guidelines of the OECD (No. 203, 1992) concerning acute toxicity tests on fish and the AFNOR standard (NF T 90-307 1985) to determine the acute toxicity of a substance upon fish. Furthermore, European seabass have been employed as a model species in academic ecotoxicology studies since numerous decades. These studies include assessment of various toxicant impacts such as cadmium, copper, PAH, mercury, PCB or crude oil (Romeo *et al.*, 2000; Bado-Nilles *et al.*, 2009; Giari *et al.*, 2008; Claireaux *et al.*, 2013).

## Chapter II: Development of an ecologically relevant methodology to assess fish health



## **1. Introduction**

The first objective of my thesis was to develop a fish health assessment methodology that could be applied to various scenarios including environmental impact assessment and management. For this, the recent rejuvenation of the concept of animal health provides a valuable conceptual basis to bridge the gap between what is desirable, i.e., assessing effects at ecologically relevant organizational level such as population or ecosystem, and what is typically undertaken, i.e., measurements at low organizational level such as molecule or cell. Indeed, modern concepts of animal health include the capacity of individuals to adapt to, respond to, or control life's challenges and changes (Frankish *et al.* 1996). In fish, hypoxia tolerance, temperature susceptibility and swimming capacity are recognized as indicators of functional integrity (Fry, 1947, Tierney and Farrell, 2004, McKenzie *et al.*, 2007, Claireaux *et al.*, 2005, Claireaux *et al.*, 2013 and Castro *et al.*, 2013). Based on the definition of health from Frankish *et al.* (1996) they can also be considered as biomarkers of fish health.

On this basis and by combining measurements of hypoxia tolerance, thermal susceptibility and swimming performance, the purpose of the study was to design a methodology to assess the health of a fish population in the form of an integrated evaluation of individuals' capacity to face everyday life challenges. Using a population of over 700 European seabass juveniles (*Dicentrarchus labrax*), the specific objectives were 1) to verify the temporal stability of individuals' performances, 2) to test the ecological relevance of performances measured by monitoring individuals' growth and survival in a semi-natural field mesocosm and, 3) to verify the ability of the traits to

inform about the history (diagnosis) as well as about the future (prognosis) of the individuals concerned.

## 2. Paper 1 (published in *Aquatic Toxicology*)

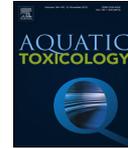
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### Assessing chronic fish health: An application to a case of an acute exposure to chemically treated crude oil



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#### ABSTRACT

Human alteration of marine ecosystems is substantial and growing. Yet, no adequate methodology exists that provides reliable predictions of how environmental degradation will affect these ecosystems at a relevant level of biological organization. The primary objective of this study was to develop a methodology to evaluate a fish's capacity to face a well-established environmental challenge, an exposure to chemically dispersed oil, and characterize the long-term consequences. Therefore, we applied high-throughput, non-lethal challenge tests to assess hypoxia tolerance, temperature susceptibility and maximal swimming speed as proxies for a fish's functional integrity. These whole animal challenge tests were implemented before (1 month) and after (1 month) juvenile European sea bass (*Dicentrarchus labrax*) had been acutely exposed (48 h) to a mixture containing 0.08 g L<sup>-1</sup> of weathered Arabian light crude oil plus 4% dispersant (Corexit® EC9500A), a realistic exposure concentration during an oil spill. In addition, experimental populations were then transferred into semi-natural tidal mesocosm ponds and correlates of Darwinian fitness (growth and survival) were monitored over a period of 4 months. Our results revealed that fish acutely exposed to chemically dispersed oil remained impaired in terms of their hypoxia tolerance and swimming performance, but not in temperature susceptibility for 1 month post-exposure. Nevertheless, these functional impairments had no subsequent ecological consequences under mildly selective environmental conditions since growth and survival were not impacted during the mesocosm pond study. Furthermore, the earlier effects on fish performance were presumably temporary because re-testing the fish 10 months post-exposure revealed no significant residual effects on hypoxia tolerance, temperature susceptibility and maximal swimming speed. We propose that the functional proxies and correlates of Darwinian fitness used here provide a useful assessment tool for fish health in the marine environment.

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#### 1. Introduction

Human population densities in coastal regions are about three-times higher than global average (Small and Nicholls, 2003). This demographic concentration and associated activities put considerable pressure on marine ecosystems, as revealed by indices of ecological imbalances such as eutrophication, increased number and severity of hypoxic events, loss of habitat and loss of biodiversity (UNEP, 2006). To preserve the ecological and economical services provided by coastal ecosystems, it is crucial that our current understanding of their functioning be improved and partic-

**Abbreviations:** CONT, Control; CT<sub>max</sub>, Critical thermal maximum; DISP, Dispersant; GC-MS/MS, gas chromatography-tandem mass spectrometry; ILOS, incipient lethal oxygen saturation; NEBA, Net environmental benefit analysis; OIL, Oil; OIL+DISP, Chemically dispersed oil; PAH, Polycyclic aromatic hydrocarbons; TE, Time to exhaustion; TLOE, Time to loss of equilibrium; TPH, Total petroleum hydrocarbon; U<sub>max</sub>, Maximal swimming speed.

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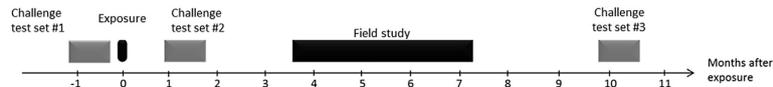


Fig. 1. Study timeline.

ularly that the resilience of the species that occupy them be better assessed.

In human medicine, health is viewed as a cumulative outcome that informs clinicians about the present capacity of a patient to do what she/he has to do. Yet, health also informs about the past of that patient, as her/his current status integrates the latent and residual effects of earlier life conditions and experiences. Moreover, health also incorporates elements about the future of that person, in relation with her/his vulnerability and resilience to everyday life conditions and change (Deem et al., 2001; Hertzman et al., 2001; Hanisch et al., 2012; Stephen, 2014).

The notion of health as it is defined in humans provides a valuable conceptual framework for methodologies aimed at assessing the impact of anthropogenic activities, and mitigation strategies, upon marine organisms. Unfortunately, at present time animal health is too poorly defined to be useful in ecotoxicological impact assessment. A review of the literature indeed shows that animal health is generally limited to present time, is mostly concerned with proximate causes of death or disease, rarely accounts for cumulative effects and commonly fails to include the notions of resilience and vulnerability of populations. As a result there is, to date, no integrated indicator of “coping ability” that could be used to gauge one animal’s health.

In fish, common and long-standing knowledge indicates that hypoxia tolerance, thermal susceptibility and swimming capacity are ecologically relevant indicators of functional integrity and are, therefore, useful markers of fish health (Fry, 1947; Tierney and Farrell, 2004; McKenzie et al., 2007; Claireaux et al., 2005, 2013; Castro et al., 2013). For instance, the altered ability to tolerate hypoxic conditions observed in the common sole (*Solea solea*) following fuel exposure (48 h in 1/200 v/v fuel No.2) was associated with reduced growth and survival in semi-natural field mesocosm (Claireaux et al., 2004). Likewise, using the same field site, Handelsman et al. (2010) and Claireaux et al. (2013) reported that hypoxia tolerance, temperature susceptibility and swimming performances determined survivorship in juvenile sea bass.

As recent events continue to show, resorting to chemical dispersants to deal with oil spills in coastal environments is a controversial matter. At the centre of this controversy is whether the risk of ecological effects increases or decreases when chemical dispersants are used, and whether the response strategy should favour the preservation of landscape and surface organisms by using dispersant or should protect the water column by refraining from treating with chemicals. However, lacking for a proper risk assessment is an adequate understanding of the mechanisms through which toxic effects, classically observed at subcellular level, ripple through higher organisational levels to affect fish populations and ecosystems (Brander et al., 2015). Cellular and molecular biomarkers indeed dominate toxicological assessments and it remains common practice to extrapolate effects at higher levels of biological complexity from information derived from these lower level indicators. Yet, it has long been recognized that the best approach for establishing causalities across hierarchical scales is to proceed from observation of emergent properties at a given organisational level through to studying the underlying mechanisms occurring at organisational levels situated below (Rosen, 1969). Implementing such approaches is one key to successfully bridge the gap between toxicology and ecology (Solomon et al., 2008).

To this background, and by combining measurements of hypoxia tolerance, thermal susceptibility and swimming performance, the aim of the present work was to design a methodology to assess the health of a fish population in the form of an integrated evaluation of individual’s capacity to face everyday life challenges. We validated this methodology by monitoring, over a year, and under laboratory and field conditions, the effect of exposure to chemical dispersant, crude oil and a mixture of both upon the health of a population of 718 juveniles European sea bass (*Dicentrarchus labrax*).

## 2. Materials and methods

### 2.1. Animals

Juvenile European sea bass (*Dicentrarchus labrax*; age 1+; N=718) were obtained from a local fish farm (Aquastream, Lorient, France). On arrival in the laboratory (Ifremer, Brest, France) fish ( $11.27 \pm 1.06$  cm in length and  $17.74 \pm 4.96$  g in body mass) were placed in a 2000-L indoor tank and acclimated for two months to local photoperiod, temperature and salinity. They were fed daily *ad libitum* with a commercial diet (Neo Start Coul 2, Le Gouessant, France). Two weeks before experiments started, fish were anaesthetized (phenoxyethanol;  $0.2 \text{ mL L}^{-1}$ ) and individually implanted subcutaneously with an identification tag (RFID; Biolog-id, France). Fish were starved for 24 h before any manipulation or experiment. The animal care protocols were in conformity with current rules and regulations in France.

### 2.2. Experimental protocol

The experimental schedule is outlined in Fig. 1. Fish health was assessed via a suite of challenge tests conducted 1 month before (February,  $T^{\circ}\text{C}=10$ ), 1 month after (May,  $T^{\circ}\text{C}=15$ ) and 10 months after (January,  $T^{\circ}\text{C}=11$ ) a 48-h exposure to one of four experimental treatments (control, dispersant, oil alone and oil + dispersant mixture). During the period June–October (3–7 month post-exposure) fish were held in a field mesocosm to assess the effect of experimental treatments upon fish capacity to thrive under semi-natural conditions. During the entire experimental period, fish were submitted to the natural temperature ( $9\text{--}20^{\circ}\text{C}$ ), photoperiod and salinity (30–32 PSU) seasonal cycles.

### 2.3. Fish transport

Experimental fish were transported between experimental sites in a  $1 \text{ m}^3$  covered tank. Fish were first anaesthetized (phenoxyethanol;  $0.2 \text{ mL L}^{-1}$ ) and transferred to the transport tank where a light dose of anaesthetic was added (phenoxyethanol;  $0.05 \text{ mL L}^{-1}$ ) to reduce stress. Water temperature (seasonal value) and oxygen level ( $>95\%$  air saturation) were monitored continuously during the journeys.

### 2.4. Challenge tests for functional indicators

Hypoxia challenge tests and temperature challenge tests were performed in the rearing tank according to Claireaux et al. (2013). In these tests, fish from the four experimental treatments were tested in one single run.

#### 2.4.1. Hypoxia challenge test

The hypoxia challenge test involved a rapid decrease of water oxygen level from around 100–20% air saturation over 1 h, after which oxygen level was reduced at a much slower rate (2% decrease in air saturation  $\text{h}^{-1}$ ) until the experiment was terminated. Hypoxia was controlled by introducing nitrogen into the tank using a submersible pump. The water oxygen level was monitored using a calibrated oxygen meter (Odeon, Ponsel Mesure, France). As individual fish lost their ability to maintain equilibrium, they were quickly removed from the experimental arena, identified (tag reading) and placed in a fully aerated recovery tank. The corresponding time (time to loss of equilibrium; TLOE) and oxygen level (incipient lethal of oxygen saturation; ILOS) were also recorded.

#### 2.4.2. Temperature challenge test

The temperature challenge test involved an initial rapid increase of water temperature (from ambient temperature to 27 °C in typically 2.5 h), after which temperature was increased more slowly (0.5 °C  $\text{h}^{-1}$ ) until the end of the experiment. Water temperature was controlled using two 3000 W heaters (Profi heater, Netherlands). A submersible pump (Eheim universal pump 2400, Germany) placed in the tank ensured thermal homogeneity. One hundred percent air saturation was maintained by bubbling a controlled mixture of oxygen and air in the tank. As individual fish lost their ability to maintain equilibrium, they were quickly removed from the experimental arena, identified (tag reading) and placed in a fully aerated recovery tank at the ambient temperature. The corresponding time (TLOE) and critical thermal maximum ( $\text{CT}_{\text{max}}$ ) were also recorded.

#### 2.4.3. Swimming challenge test

The swimming challenge test employed a custom-made swimming flume comprising two translucent swimming chambers (length: 2 m, diameter: 20 cm). Water velocity in these swimming chambers was controlled using a pump (80/16-DE, Calpeda) connected to a frequency regulator (Mitsubishi, F700, Japan). During swimming trials water velocity was monitored continuously using a flow meter (HFA, Höntzsch GmbH, Germany). Seawater was supplied to the swimming chambers from a tank containing thermoregulated and aerated water. Water flow in the swimming chambers was made laminar by passage through two honeycomb sections (length = 50 cm) placed upstream from each swimming chamber.

Fish were swum in groups of approximately 60 individuals haphazardly dip-netted from the rearing tank. Following transfer into the swimming flume, fish were habituated for 3 h at slow water velocity (8  $\text{cm s}^{-1}$ ) before the swimming trial was initiated. Swimming trials started by continuously increasing the water velocity from acclimation (8  $\text{cm s}^{-1}$ ) to 40  $\text{cm s}^{-1}$  over approximately 30 min. Water velocity was then increased incrementally by 5  $\text{cm s}^{-1}$  every 10 min until all the fish present in the chamber had reached exhaustion (typically < 120  $\text{cm s}^{-1}$ ). Fish were considered to be exhausted when they would not remove themselves from the grid place downstream from the swim chamber. At that time, they were removed from the flume via a hatch situated above the back grid, identified and placed in a recovery tank. The corresponding time (time to exhaustion; TE) and water velocity were recorded. Maximal swimming speed ( $\text{U}_{\text{max}}$ ; see Farrell, 2008) was calculated according to Brett (1964). No correction for potential blocking effect was applied.

One week was allowed for recovery between each of the above challenge tests. During these periods, no mortality was observed.

#### 2.5. Experimental exposure

In March 2013, fish (mass:  $21.0 \pm 0.2$  g, length:  $12.16 \pm 0.03$  cm) were transported from Ifremer laboratory to the fish rearing facili-

ties of the Centre of Documentation, Research and Experimentation on Accidental Water Pollution (CEDRE, Brest, France; 12 km) where temperature, salinity, photoperiod and feeding conditions were identical to those previously described.

A set of 12 polyethylene tanks (300 L) was used for fish exposure (Milinkovitch et al., 2011). Four experimental conditions were tested in triplicate (190 fish per condition,  $\approx 60$  fish per replicate): control (CONT), 1 g of dispersant per 300 L (DISP), 25 g of oil per 300 L (OIL) and 1 g of dispersant plus 25 g of oil per 300 L (OIL + DISP). Before being used in the exposure set up, Arabian light crude oil was bubbled with air until its mass was reduced by approximately 10% to mimic a 12-h ageing of an oil slick at sea (Nordvik, 1995). The oil concentration used in this study aimed to reproduce conditions encountered in the vicinity of an oil slick (Sammarco et al., 2013). The dispersant (Corexit® EC9500A, Nalco) was used in accordance with the manufacturer's recommendation. Fish were allocated to the various exposure tanks in such a way that no statistically significant difference in  $\text{U}_{\text{max}}$ , ILOS and  $\text{CT}_{\text{max}}$  was found among the tanks.

Exposure tanks were equipped with a custom-made water mixing devices comprising a funnel and a 12 V submersible bilge pump (L450-500GPH; Johnson). These devices were adjusted in such a manner that any surface water and floating oil would be sucked into the funnel, homogenized and delivered to the bottom of the tank. During the 48-h exposure period, the water in the tanks was not renewed and aeration was maintained via air bubbling.

Following the 48-h exposure period, fish were bathed in clean seawater to remove any obvious oil contamination and transferred to their original rearing tank where they remained for one week before being transported back to Ifremer laboratory. Fish from the different treatments were identifiable (RFID-tag) and so they were mixed together in one common-garden 2  $\text{m}^2$ -tank where water and feeding conditions were similar to those described above. No mortality occurred during exposure and the following weeks.

To characterize the exposure conditions, water total petroleum hydrocarbon concentration was measured immediately before and 4, 24 and 48 h after fish introduction into the exposure tanks. Moreover, 9 fish per treatment were sacrificed to measure white muscle PAH concentrations at 48 h and 1 month post-exposure to confirm PAH initial uptake and subsequent elimination.

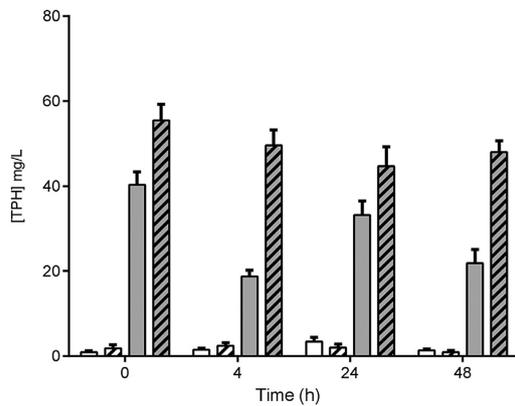
#### 2.6. Chemical analyses

Total petroleum hydrocarbon concentration ([TPH]) in each exposure tank was measured in triplicate with a seawater sample volume of 100 mL taken at 0, 4, 24 and 48 h after the fish were introduced in the tank. The seawater samples were extracted 3 times using 10 mL of dichloromethane Pestipur quality (SDS, Carlo Erba Reagent, France) and the combined organic phases of the extracts were dried by filtering through anhydrous sodium sulfate (pesticide grade). The absorbance of the organic phase was measured using a spectrophotometer at 390 nm (Evolution 600 UV-VIS; Thermo Fisher Scientific) as described in Fusey and Oudot (1976).

The concentrations of 21 polycyclic aromatic hydrocarbons (PAH) (including US EPA compounds) in the white muscle samples taken at 48-h and 1-month post-exposure were assessed by gas chromatography-tandem mass spectrometry (GC-MS/MS) using a procedure described in Lacroix et al. (2014). PAH were extracted from muscles samples using alkaline digestion combined with stir-bar sorptive extraction (SBSE) before analysis by GC-MS/MS. Briefly, samples were individually crushed and 1 g wet weight of minced muscle was added to 10 mL of HPLC-grade anhydrous ethanol (Carlo Erba Reagents, France) containing 10 ng of 7 deuterated internal standards and 0.5 g (0.05  $\text{g mL}^{-1}$ ) of potassium hydroxide (analytical grade). Samples were subsequently digested for 3 h at 80 °C in hermetic flasks. The digested samples

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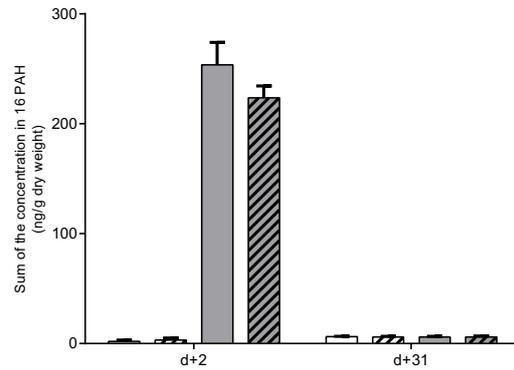
**Fig. 2.** Water total petroleum concentration (mg/L) measured throughout the 48 h-exposure period in tanks from the control (CONT; white bar), dispersant (DISP; white hatched bar), oil (OIL; grey bar) and chemically dispersed oil (OIL+DISP; grey hatched bar) treatments. N=9 (3 samples × 3 replicate tanks). The error bars indicate the calculated SEM.

were then cooled to room temperature before addition of 100 mL of reverse osmosis water. Polydimethylsiloxane stir-bars (Twister 20 mm × 0.5 mm, Gerstel, Germany) were then placed in the solutions and stirred at 700 rpm. After 2 h of extraction, stir-bars were removed from solutions, rinsed with reverse osmosis water and dried over a blot paper. The stir-bars were stored at -20 °C until GC-MS/MS analysis using agilent 7890A coupled to an Agilent 7000 Triple Quadrupole (Agilent, USA). Calibration solutions were obtained by dilutions (in ethanol) of a custom standard solution containing the analytes and provided by Ultra Scientific (USA). PAH levels were quantified relatively to deuterated PAH. Samples water content was determined after drying a sample aliquot for 48 h at 80 °C. Results were expressed as ng PAH/g dry weight.

### 2.7. Field mesocosm exposure

On May 31st 2013 *i.e.*, approximately 3 months after the experimental exposures, fish (N = 680, 170 per treatment) were transported to the “French National Center for Scientific Research (CNRS)” field station in L’Houmeau (450 km from Brest). L’Houmeau mesocosms have been described previously (Nelson and Claireaux, 2005; Claireaux et al., 2007; Handelsman et al., 2010). Briefly, 200-m<sup>2</sup>, 1-m deep earthen ponds are connected to the nearby ocean via a canal which allows partial seawater renewal with each incoming tide. A set of standpipes prevented fish from escaping. Moreover, netting placed above the ponds prevented avian predation. Previous experiments and empirical observations have shown that a natural food web develops in these ponds which can sustain 2–3 kg of fish. A complete description of these earthen pond’s fauna can be found in de Montaudouin and Sauriau (2000). During the experiment, water conditions in the ponds were measured once a week between 11:00 and 14:00.

Immediately upon arrival at L’Houmeau field station, fish were distributed among 7 mesocosm ponds (96 fish per pond, *i.e.*, 24 fish per treatment). To assess survivorship and growth, ponds were individually drained on July 11th, September 9th and finally October 31st when all survivors (N = 470) were transported back to Brest and returned to their original rearing facility at the Ifremer laboratory.



**Fig. 3.** Sum of the concentration (ng/g dry weight) of 21 PAH measured two (d+2) and 31 days post-exposure (d+31) in fish white muscle from the control (CONT; white bar), dispersant (DISP; white hatched bar), oil (OIL; grey bar) and chemically dispersed oil (OIL+DISP; grey hatched bar) treatments. N=9 (3 samples × 3 replicate tanks). The error bars indicate the calculated SEM.

### 2.8. Data analysis and statistics

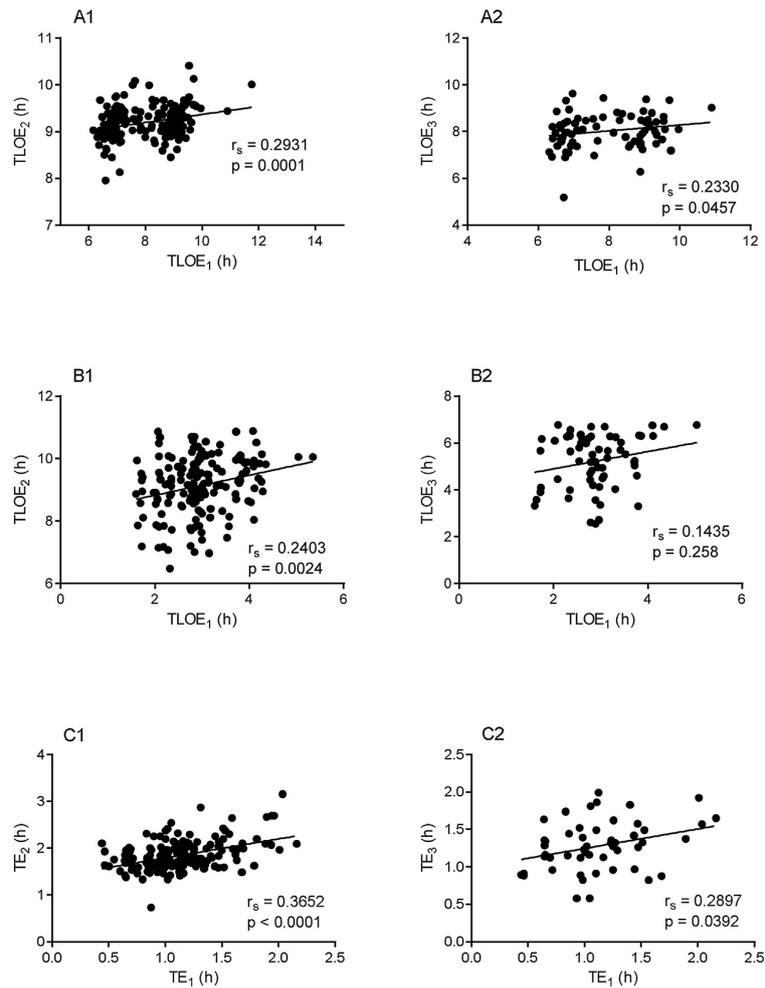
The temporal stability of performance at challenge tests was tested using Spearman rank-order correlation between challenge tests set #1 and #2 (3-months repeatability) and between challenge tests set #1 and #3 (11-months repeatability). Total petroleum hydrocarbon concentration in water and PAH concentrations in white muscle were compared among exposure treatments using an ANOVA. In order to compare the performance at the challenge tests, a Kaplan-Meier survival analysis was conducted. Exposure effect was assessed using log-rank test with the Holm-Šidák method for multiple comparisons. Fish survival in the ponds was analysed using the same procedure. Growth rate measured during the field experiment were analysed using a 2-way mixed ANOVA with pond defined as a random effect. Functional performance of the fish that died and fish that survived the mesocosm exposure were compared using an ANOVA. Values are presented as a mean ± SEM and significance was accepted at p < 0.05. All statistical analyses were performed using R (R Development Core Team, 2008) and SigmaPlot 13 (Ritme).

## 3. Results

### 3.1. Exposure conditions

As expected, water total petroleum hydrocarbon concentration ([TPH]) in the control (CONT) and the dispersant-added (DISP) tanks were below the quantification limit (Fig. 2). On the other hand, [TPH] in the oil (OIL) and oil + dispersant (OIL+DISP) tanks were, respectively, 6- and 10-times higher than the quantification limit and they remained at that level throughout the exposure period.

Similarly, the white muscle tissue concentrations of 21 polycyclic aromatic hydrocarbon compounds ([21 PAH]) measured 2 days post-exposure in fish from the CONT and DISP treatments were below the quantification limit, while concentrations measured in fish from the OIL and OIL+DISP treatments were nearly 250-times that limit (Fig. 3). No significant difference in white muscle tissue [21 PAH] was observed between the OIL and OIL+DISP treatments (ANOVA; p = 0.2565). At 1 month post-exposure, white muscle [21 PAH] was below the quantification limit in all four treatments. It must be noted that among the 21 compounds measured, only four (naphthalene, fluorene, dibenzothiophene,



D

Challenge test	Set	CONT		DISP		OIL		OIL+DISP	
		$r_s$	p value	$r_s$	p value	$r_s$	p value	$r_s$	p value
Hypoxia	1 vs 2	0.2931	0.0001	0.1437	0.0615	0.2412	0.0016	0.2224	0.0035
	1 vs 3	0.233	0.0457	-0.102	0.3226	0.2332	0.0307	0.1234	0.2464
Temperature	1 vs 2	0.2403	0.0024	0.2425	0.0019	0.2306	0.0034	0.1446	0.0681
	1 vs 3	0.1435	0.258	0.06377	0.5436	-0.1387	0.2054	-0.04411	0.6904
Swimming	1 vs 2	0.3652	< 0.0001	0.3524	< 0.0001	0.3217	< 0.0001	0.3146	< 0.0001
	1 vs 3	0.2897	0.0392	0.3021	0.0116	0.2613	0.0386	0.008588	0.9459

**Fig. 4.** Three months (1) and 11 months (2) repeatability of time to loss of equilibrium of control fish at hypoxia (A); temperature challenge tests (B) and time to reach exhaustion at swimming challenge test (C). Performance repeatability of fish from the four treatments is summarized in D. Blank cells: significant correlation ( $p < 0.05$ ), light grey cells: marginally significant correlation ( $p < 0.07$ ); grey cells: no significant correlation.  $r_s$  corresponds to the Spearman's rank correlation coefficient.

phenanthrene) were above the quantification limit (Appendix A). At 1 month post-exposure, white muscle [21 PAH] was below the quantification limit in all four treatments.

### 3.2. Repeatability of individual performance

Individual performance of the CONT fish at the hypoxia challenge test (time to loss of equilibrium, TLOE) was repeatable when this challenge was repeated 3 and 11 months apart (Fig. 4A;  $r_s = 0.2931$ ,  $p = 0.0001$  and  $r_s = 0.2531$ ,  $p = 0.0437$  respectively). Similarly, swimming performance (time to reach exhaustion, TE) of the CONT group was stable over 3- and 11-month periods (Fig. 4C;  $r_s = 0.3652$ ,  $p < 0.0001$  and  $r_s = 0.2897$ ,  $p = 0.0392$ , respectively). Critical temperature (time to loss of equilibrium, TLOE) of the CONT fish was also repeatable after 3 months (Fig. 4B;  $r_s = 0.2178$ ,  $p = 0.0058$ ), but not after 11 months ( $r_s = 0.1377$ ,  $p = 0.2742$ ).

Performance stability over time of fish exposed to dispersant, oil alone or chemically treated oil are summarized in Fig. 4D. Performances of fish from the DISP treatment at hypoxia and temperature challenge test were repeatable or marginally repeatable when these challenges were repeated 3-month apart ( $p = 0.0639$  and  $0.0023$ ). However, performances were not stable over an 11-month period ( $p = 0.2089$  and  $0.5927$ ). Swimming performance of fish exposed to dispersant was stable over 3- and 11-month periods ( $p < 0.0001$ ;  $0.0116$  respectively). Concerning fish exposed to oil, individual performances at hypoxia and swimming challenge tests were repeatable over 3- and 11-month periods ( $p = 0.0016$ ;  $< 0.0001$  and  $0.0307$ ;  $0.0386$  respectively). Temperature susceptibility of these fish was stable over a 3 month period ( $p = 0.0034$ ) but not over 11 months ( $p = 0.2054$ ). Finally, fish from the OIL + DISP treatment displayed stable performance when hypoxia and swimming challenge tests were repeatable over a 3-month period ( $p = 0.0035$ ;  $< 0.0001$ , respectively). Performance repeatability of fish from this treatment at temperature challenge test was also marginally significant ( $p = 0.0681$ ). However, performances of fish exposed to chemically dispersed oil at the three challenge tests were not stable over an 11-month period ( $p > 0.2$ ).

### 3.3. Short-term effects of the treatments on fish performance

Fish performance in the hypoxia challenge tests is summarized in Fig. 5A. Mean time to loss of equilibrium (TLOE) of the control fish was  $9.2 \pm 0.03$  h, which corresponded to a mean ILOS of  $4.00 \pm 0.06\%$  air saturation. No significant difference with the DISP and OIL treatments was observed (Log-rank;  $p = 0.33$  and  $0.13$ , respectively). However, the OIL + DISP fish were significantly less tolerant to hypoxia than the CONT group with a mean TLOE of  $9.10 \pm 0.02$  h (i.e., mean ILOS =  $4.22 \pm 0.05\%$  air saturation;  $p = 0.007$ ).

During the temperature challenge test (Fig. 5B), CONT fish mean TLOE was  $9.16 \pm 0.07$  h, which corresponded to a mean  $CT_{max}$  of  $32.50 \pm 0.04$  °C. Again, no significant difference with DISP, OIL and OIL + DISP treatments was observed (Log-rank;  $p = 0.34$ ).

For the swimming challenge test (Fig. 5C), CONT mean time to exhaustion (TE) was  $1.84 \pm 0.03$  h, which corresponded to a swimming velocity ( $U_{max}$ ) of  $80.4 \pm 0.8$  cm  $s^{-1}$ . No significant difference was observed with the DISP and OIL treatments (Log-rank;  $p = 0.85$  and  $p = 0.17$  respectively). However, the OIL + DISP fish had a significantly lower swimming performance than the CONT group (TE =  $1.72 \pm 0.02$  h;  $U_{max} = 77.7 \pm 0.6$  cm  $s^{-1}$ ; Log-rank;  $p = 0.008$ ).

### 3.4. Field mesocosm study

Water conditions in the earthen ponds over the 5-month field experiment are summarized in Fig. 6. The observed fluctuations in water temperature are typical of this type of ecosystem, temperature being the lowest in early June (15.8 °C) and the highest at the

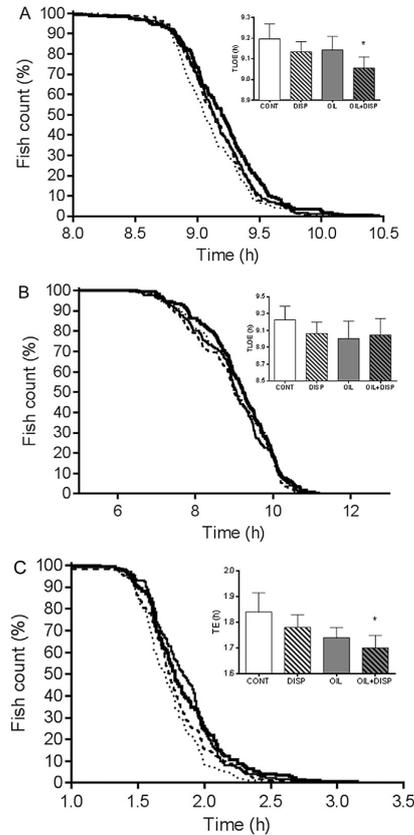


Fig. 5. Fish count throughout the challenge test set #2 (A: Hypoxia challenge test,  $p = 0.01$ ; B: Temperature challenge test,  $p = 0.03$ ; C: Swimming challenge test  $p = 0.51$ ). Solid thick line: CONT; solid thin line: DISP; dashed line: OIL; pointed dashed line: OIL + DISP. Insets: barplots correspond to the median time to loss equilibrium (TLOE) or to reach exhaustion (TE). Error bars indicate the 95% confidence interval calculated with the Kaplan-Meier survival analysis and \* indicate statistical difference.

end of July (25.1 °C). Salinity also fluctuated from a low of 30.8‰ in early June to a high of 40.0‰ in late August. Water oxygen content varied between 8 and 10 mg  $L^{-1}$  during most of the field experiment, except in early August when values as low as 4.6 mg  $L^{-1}$  were measured. Water pH fluctuated within a broad range i.e., 7.5–8.7. All variables considered, August was possibly the most challenging period when high temperature coincided with high salinity, low oxygenation and low pH.

Sea bass growth varied over the 5-month period, but no statistically significant difference was observed among the ponds (ANOVA;  $p < 0.001$  and  $p = 0.12$ , respectively) (Fig. 7a). The summer period (July–August) was associated with best growth. During autumn (September–October), fish growth was markedly suppressed, with fish from ponds 2, 4 and 7 even losing weight. No difference between experimental treatments was noted (ANOVA;  $p = 0.90$ ) (Fig. 7b).

Survivorship was  $>60\%$  at the end of the field experiment and no statistically significant difference was observed among experimental treatments ( $p = 0.63$ ; data not shown).

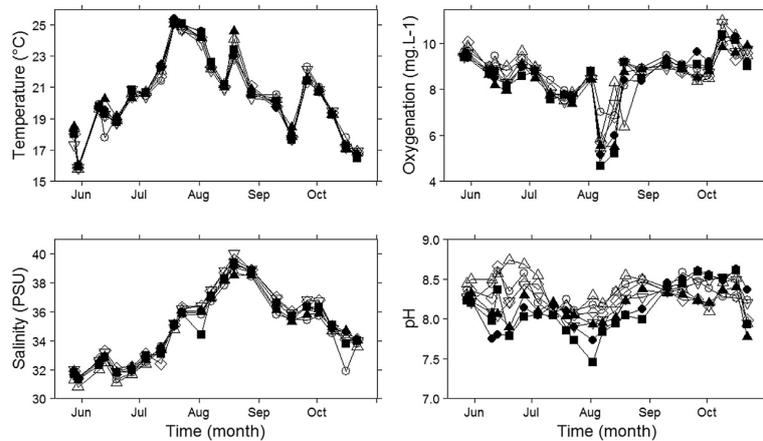


Fig. 6. Water condition in the earthen ponds. Measurements were made weekly in the 7 ponds (○: pond #1, △: pond #2, ◇: pond #3, ▽: pond #4, ■: pond #5, ●: pond #6, ▲: pond #7).

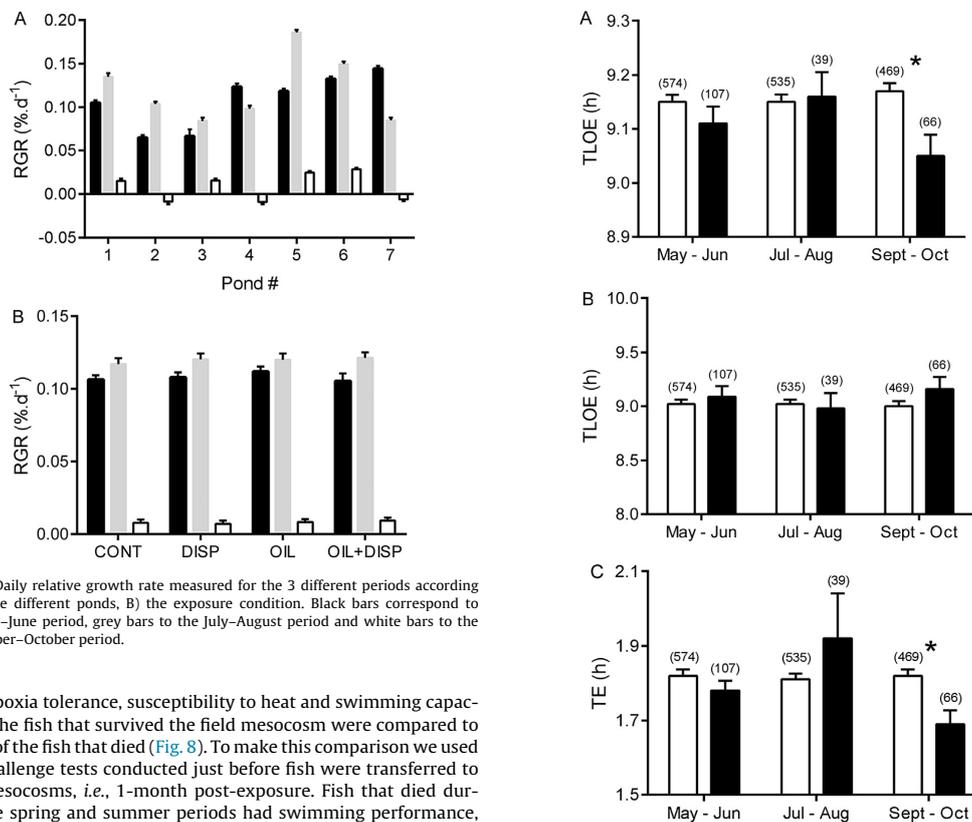


Fig. 7. Daily relative growth rate measured for the 3 different periods according to A) the different ponds, B) the exposure condition. Black bars correspond to the May–June period, grey bars to the July–August period and white bars to the September–October period.

Hypoxia tolerance, susceptibility to heat and swimming capacity of the fish that survived the field mesocosm were compared to those of the fish that died (Fig. 8). To make this comparison we used the challenge tests conducted just before fish were transferred to the mesocosms, *i.e.*, 1-month post-exposure. Fish that died during the spring and summer periods had swimming performance, hypoxia tolerance and thermal susceptibility that were not statistically different from those of the fish that survived (swimming TE: spring  $1.82 \pm 0.02$  vs  $1.78 \pm 0.03$ , summer  $1.81 \pm 0.02$  vs  $1.92 \pm 0.12$ ; hypoxia TLOE: spring  $9.15 \pm 0.01$  vs  $9.11 \pm 0.03$ , summer  $9.15 \pm 0.02$  vs  $9.16 \pm 0.05$ ; temperature TLOE: spring  $9.02 \pm 0.04$  vs  $9.09 \pm 0.10$ ,

Fig. 8. Comparison of fish performances at challenge tests of fish that survived (white bars) or died (black bars) during each period in the ponds. The number of fish present in each group is indicated above the bars. The error bars indicate the calculated SEM and \* indicates statistically significant difference ( $p < 0.05$ ).

summer  $9.02 \pm 0.04$  vs  $8.98 \pm 0.14$ ). During the autumn, on the other hand, fish that did not survive had a lower hypoxia tolerance and lower swimming performance than fish that did survive (swimming TE:  $1.82 \pm 0.02$  vs  $1.69 \pm 0.04$ ; hypoxia TLOE:  $9.17 \pm 0.01$  vs  $9.05 \pm 0.04$ ). No difference in thermal tolerance was, however, observed (TLOE:  $9.00 \pm 0.05$  vs  $9.16 \pm 0.11$ ).

### 3.5. Long-term effects of the treatments and validation of the field observations

Approximately 10 months post-exposure and 4 months after the survivors (460 fish) had returned from the field mesocosm, the suite of challenge tests was repeated. No significant differences were observed among the four treatments (Log-rank; hypoxia challenge test:  $p = 0.760$ ; temperature challenge test:  $p = 0.232$ ; swimming challenge test:  $p = 0.295$ ). Mean time to loss of equilibrium of the control fish was  $7.89 \pm 0.08$  h ( $2.3 \pm 0.1\%$  air saturation) for the hypoxia challenge test and  $5.23 \pm 0.13$  h ( $CT_{max}$ :  $28.12 \pm 0.09^\circ\text{C}$ ) for the temperature challenge test. Mean time to exhaustion for the swimming challenge test was  $1.22 \pm 0.05$  h ( $U_{max}$ :  $62.6 \pm 1.5 \text{ cm s}^{-1}$ ).

## 4. Discussion

Using the chemical treatment of an oil spill and an acute exposure scenario as a case study, this experiment aimed at testing a methodology to evaluate the health status of a fish population following a toxicological insult. We used incipient lethal oxygen saturation (ILOS), critical thermal maximum ( $CT_{max}$ ) and maximal swimming speed ( $U_{max}$ ) as markers of fish ability to cope with natural contingencies, and based on the definition given for humans, we considered that they were proper indicators of fish health. The suite of challenge tests showed that 1 month after exposure, fish from the dispersant alone and oil alone treatments displayed no functional impairments, while fish exposed to chemically dispersed oil had residual effects of reduced hypoxia tolerance and swimming capacity. Transfer of our experimental populations to the field revealed that, under selective feeding condition (autumn), pre-determined swimming performance and hypoxia tolerance were higher in individuals that survived than in individuals that did not, validating the ecological relevance of these biomarkers. However, values of thermal susceptibility were not predictors of individuals' survival during our mesocosm experiment. No difference in field survival and growth was observed among the experimental treatments, suggesting that recovery had occurred at the whole animal level. Correspondingly, when hypoxia tolerance, temperature susceptibility and swimming performance were tested 10 months post-exposure *i.e.*, as surviving fish were returned from the field site into the laboratory, no difference among the treatments was observed, confirming that they had recovered from the functional impairments observed one month post-exposure.

### 4.1. Exposure conditions and white muscle [21 PAH]

Water concentration in total petroleum hydrocarbon ([TPH]) was monitored in all tanks and throughout fish exposure period in order to characterize the exposure conditions and ensure that they were realistic. Mean [TPH] in the oil and oil + dispersant exposure tanks ( $28.5 \pm 5.0$  and  $49.4 \pm 2.3 \text{ mg L}^{-1}$  respectively) corresponded to concentrations reported following oil spills such as Tarut Bay (Saudi Arabia, 1970), Hebei Spirit (South Korea, 2007) and Deepwater Horizon (USA, 2010) ( $1\text{--}750 \text{ mg L}^{-1}$ ; Spooner, 1970; Kim et al., 2010; Sammarco et al., 2013). Moreover, the 30% higher [TPH] measured in the oil + dispersant exposure tanks compared to the oil-alone tanks, indicated that dispersant increased the potential bioavailability of the oil components (Ramachandran et al., 2004).

Polycyclic aromatic hydrocarbon concentrations ([21 PAH]) were measured in fish white muscle 2 days and 1 month post-exposure, the latter corresponding to a few days before the second suite of challenge tests was conducted. Two days post-exposure, fish from the control and dispersant treatments displayed white muscle [21 PAH] below the quantification limits ( $1 \text{ ng g}^{-1}$  dry weight). On the other hand, fish from the oil and chemically dispersed oil treatments displayed white muscle [21 PAH] considerably in excess of the quantification limit ( $253 \pm 20$  and  $224 \pm 11 \text{ ng g}^{-1}$  dry weight, respectively). It must be emphasised, however, that fish can rapidly metabolize PAH and so the muscle tissue samples from 2 days post-exposure may underestimate maximal white muscle PAH concentration (Hellou and Warren, 1997; Varanasi, 1989; Dussauze et al., 2015). Nevertheless, these concentrations are very similar to those measured in the white muscles of the rockfish (*Sebastes schlegelii*) 5 days following the Hebei Spirit oil spill ( $284 \text{ ng g}^{-1}$  dry weight; Jung et al., 2011) and are in line with a previous experiment that investigated similar oil exposure conditions ( $148 \pm 46 \text{ ng g}^{-1}$  dry weight; Danion et al., 2011). A closer look to individual PAH concentrations in fish muscle reveals that tricyclic PAH (fluorene, dibenzothiophene, phenanthrene) represent approximately 98% of the total [PAH]. This is attributable to the weathering process, which is recognized to bring a shift in PAH composition. The fraction of the tricyclic PAH within the total dissolved PAH measured in water indeed increase relative to the smaller two-rings compounds when spilled oil is weathered (Carls et al., 1999; Heintz et al., 1999; Short and Heintz, 1997). One month after exposure, white muscle [21 PAH] concentrations in all four experimental treatments had been restored to a background level.

### 4.2. Repeatability of individual's performance

In order to assess our capacity to standardize the testing protocols, and to demonstrate the ecological relevance of our markers of functional integrity, the long-term temporal stability (3 and 11 months) of the hypoxia tolerance, temperature susceptibility and swimming performance was examined. In the control group, we observed that time to loss of equilibrium (TLOE) at the hypoxia challenge test and time to reach exhaustion (TE) at the swimming challenge test were both repeatable after 3-month and 11-month periods, whereas TLOE at the temperature susceptibility challenge test was repeatable after a 3-month period but not after 11 months. This observation validates our capacity to standardize the challenge tests. Short-term temporal stability of fish swimming performance has been documented by several authors including Kolok (1992) and Claireaux et al. (2007). Moreover, an earlier study by Claireaux et al. (2013) documented the 2-month repeatability of hypoxia and heat tolerance in sea bass. This temporal stability under changing environmental conditions, combined with the fact that hypoxia tolerance, temperature susceptibility and swimming performance have been shown to be heritable (Garenc et al., 1998; Anttila et al., 2013), substantiate the view that these traits are potential targets for natural selection (Hayes and Jenkins, 1997; Dohm, 2002). It is important to note that Spearman's rank correlation coefficients were lower when considering performance measured 11 months apart than those measured 3 months apart. This suggests a progressive loss of repeatability over time. This observation is explained by the fact that this study was conducted using juveniles. Growth and ageing, and associated rearranging of phenotypic architecture, are processes that are likely to blur repeatability (Killen et al., 2016). Moreover, the 11-month period includes the field experiment during which fish were submitted to a large variety of conditions including a major change in food source. This is liable to affect intra-population variability and, potentially, the degree to which our targeted performances remain stable over time (Killen et al., 2016).

The effect of exposure to dispersant, oil alone or a mixture of both upon individual performances temporal stability was also examined. We observed that repeatability over a 3-month period was maintained in all the four treatments. On the other hand, performances measured 11-months apart were not necessarily repeatable. It is important to note, however, that this loss of repeatability is not readily attributable to the exposure treatments as several confounding factors including ageing, sexual maturation, growth and change in environmental conditions may have contributed in modifying the phenotypic architecture within our experimental populations (Killen et al., 2016).

#### 4.3. One-month post-exposure functional tests

At one month post-exposure, performance in the hypoxia challenge test of the dispersant-exposed fish was not statistically different from that of the control fish. This result is in line with Claireaux et al. (2013), who reported no effect of dispersant alone (Finasol® OSR-52) on fish hypoxia tolerance. Hypoxia tolerance of fish from the oil alone treatment also was not statistically different from that of the control fish. This result of juvenile sea bass to Arabian light crude (CAL; [TPH] =  $56 \pm 5.2 \text{ mg L}^{-1}$ ), is, however, not in line with Davoodi and Claireaux (2007), who reported reduced hypoxia tolerance in juveniles of the common sole (*Solea solea*). In this latter study, however, sole were exposed to a dose of No-2 fuel which was approximately 50-times higher than in the present study ( $4 \text{ g L}^{-1}$  vs  $0.08 \text{ g L}^{-1}$ ).

In our experiment, the deleterious effect of oil exposure on seabass hypoxia tolerance was only revealed in the OIL + DISP treatment, as the increase in water [TPH] induced by the dispersant was associated with reduced ILOS. This observation confirmed that dispersant reinforces the effect of oil in the short term (Ramachandran et al., 2004; Anderson et al., 2009). The few studies that have examined the impact of oil exposure upon hypoxia tolerance suggest a relationship via altered cardio-respiratory function (Claireaux et al., 2004; Davoodi and Claireaux, 2007). This hypothesis is consistent with established histomorphological alterations to the gills, leading to reduced oxygen diffusion across the respiratory epithelium and into the vascular system (McKeown and March, 1978; Engelhardt et al., 1981).

Thermal tolerance of fish from the DISP, OIL and OIL + DISP treatments were not statistically different from fish from the control group. This is in line with Claireaux et al. (2013), who reported no effect of the same exposure conditions upon sea bass temperature susceptibility 4 weeks post-exposure. The underlying mechanisms to temperature susceptibility remain unclear (Beitinger and Lutterschmidt, 2011; Schulte, 2015). However, the oxygen and capacity limited thermal tolerance (OCLTT) theory, even if still debated, suggests that functional integrity at high temperature may be limited by a mismatch between tissues oxygen demand and the capacity of the cardiovascular system to supply them with oxygen (Pörtner and Knust, 2007). Cardiac function is central to both hypoxia and heat tolerance and one could have expected a correlation between an individual's ILOS and  $CT_{\text{max}}$ . The fact that we did not find such correlation (data not shown) suggests that other functions and processes are involved in shaping inter-individual variability in these performance traits, e.g., energy metabolism, anaerobic pathways, oxygen extraction and diffusion at the gill, sensitivity of the central nervous system to anaerobic metabolism by-products (Shoubridge and Hochachka, 1980; Hochachka and Somero, 2002; Nilsson and Östlund-Nilsson, 2008). The lack of relationship between ILOS and  $CT_{\text{max}}$  could also result from the ILOS measuring principle. Incipient lethal oxygen saturation determination is indeed based on exposing fish to an oxygen level which is far below sea bass critical oxygen level (20% of air saturation at  $15^\circ\text{C}$ ). Therefore, ILOS may be an index which reflects

fish anaerobic capacities (Claireaux and Chabot, 2016), while heat tolerance rather reflects oxygen uptake and transport capacities.

Swimming performance of fish exposed to either dispersant or oil alone did not differ from the control. This result is in line with McKenzie et al. (2007), who showed that carp (*Cyprinus carpio*) caged for 3 weeks in sites heavily polluted with cocktails of bioavailable organics including PAH ( $12.4 \mu\text{g}$  of PAH collected on semipermeable membrane) displayed similar swimming capacity as fish from a control, unpolluted site. Our findings are, however, contradictory with Kennedy and Farrell (2006) who reported a 25% decrease in the maximal swimming speed of the Pacific herring (*Clupea pallasii*) 8 weeks after a 4-day exposure to the water-soluble fraction (WSF) of North Slope crude oil ([TPH] =  $120 \mu\text{g L}^{-1}$ ). Likewise, Oliveira et al. (2012) reported reduced swimming performance immediately after a 96-h exposure to pyrene (concentrations ranging from 0.125 to  $1 \text{ mg L}^{-1}$ ). Thomas and Rice (1987) also reported a 12% decrease in swimming performance of juvenile coho salmon (*Oncorhynchus kisutch*) within hours of a 48-h exposure to the WSF of Cook Inlet crude oil [ $>2.5 \text{ mg L}^{-1}$ ]. The detrimental effect of hydrocarbon exposure upon fish swimming performance was confirmed by our observation that chemically treated oil treatment impaired sea bass capacity to swim maximally. This strengthens the view that, although not directly affecting fish performance, dispersants potentiate oil toxicity. The mechanisms by which petroleum hydrocarbon exposure might affect swimming capacity are largely unknown as a large set of physiological processes are potentially involved. It has been shown, for instance, that swimming capacity is sensitive to changes in maximal aerobic capacity, cardiac output, function of red and white muscle fibers, as well as anaerobic metabolism (Farlinger and Beamish 1977; Beamish 1978; Thorarensen et al., 1996; Alsop and Wood 1997; Burgetz et al., 1998). Impaired swimming performance may also be the result of the aerobic cost of the detoxification, resulting in reduced scope for activity (Beamish, 1978; Alsop and Wood, 1997; Gregory and Wood, 1999). Although not specifically examined here, gill damage caused by oil exposure (McKeown and March, 1978; Engelhardt et al., 1981; Khan, 2003; Simonato et al., 2008) could also result in impaired oxygen delivery tissues during swimming exercise.

#### 4.4. Field observations

The field study aimed to ascertain the link between performance in the challenge tests and components of Darwinian fitness, by establishing whether the functional impairments observed shortly after exposure had some subsequent ecological relevance under natural conditions. Water conditions observed in the experimental mesocosms during spring, summer and autumn were in accordance with previous observations (Claireaux et al., 2013). With the noticeable exception of water pH, low inter-pond variability in water conditions was observed. We have no satisfactory explanation for the marked among-ponds variability in water pH, but combined with observations of ponds' vegetal groundcover, it points towards variability in pond ecology, with possible consequences in terms of qualitative and quantitative food availability.

Compared with previous studies, the observed fish survival rate was high overall ( $>60\%$ ). For instance, preceding work using the same field site yielded survival rates ranging between 20 and 30% (Claireaux et al., 2013). As might be expected, these remarkable survival records during spring and summer were associated with the highest growth rates, which ranged between 0.10 and  $0.20 \text{ d}^{-1}$  (Fig. 7a). These growth rates are indeed remarkable if one considers that the growth rate of sea bass under food unlimited, farming conditions is  $0.25 \text{ d}^{-1}$  (Guillaume, 2001).

Despite favourable environmental and feeding conditions, mortality still occurred during spring (16%) and summer (5%).

Examination of the predetermined performances at the challenge tests did not allow a conclusion concerning a possible link with survivorship during these periods. This suggests that, at least during the spring period, the relatively higher mortality recorded possibly resulted from the inability of certain individuals to acclimate following the transfer from the lab to the field. In September–October, on the other hand, food availability decreased and competition for food became selective, as illustrated by the nearly 90% reduction in growth rate observed during that period. Interestingly, fish that died during that period (mortality 12%) displayed lower swimming performance, suggesting that high swimming capacity to capture scarce prey was key to survival. More intriguing is the observation that non-survivors also displayed lower tolerance to hypoxia than survivors. During autumn, oxygenation conditions were optimal and they cannot be considered as a direct source of selective mortality (Fig. 6). Alternatively, it can be hypothesized that the fact that fish that died were less tolerant to hypoxia than fish that survived resulted from a potential link between hypoxia tolerance, routine metabolic rate and starvation tolerance. Dupont-Prinet et al. (2010) investigated the link between tolerance of food deprivation, metabolic rate and growth rate in the European sea bass. They found that fast growing animals were also less tolerant to starvation than the slow growing individuals. However, contrary to the above functional hypothesis, these authors found no difference in routine metabolic rate between the starvation-tolerant and the starvation-sensitive phenotypes. Deciphering the functional basis of the relationship between environmental conditions, growth and survivorship clearly requires further study.

No significant difference in survivorship and growth was observed among the four exposure treatments, even during the period when natural selection was potentially the strongest (September–October). This result suggests that fish recovered from the impairments observed one month after exposure and that no long-term loss in individual fitness occurred in relation with exposure to either dispersant, oil and oil + dispersant mixture.

#### 4.5. Eleven-month post-exposure challenge tests

The motive for the long-term assessment conducted in this study was to verify whether the functional impairments observed shortly after exposure were long lasting. The fact that we observed no significant difference in fish performance 10 months post-exposure is remarkable and cannot be attributed to selective mortality during the field phase of the experiment as survivorship was in excess of 60%. Moreover, a reanalysis of our challenge test data, excluding fish that died in the field, confirmed the effect of chemically dispersed oil observed one month post-exposure (hypoxia challenge test:  $p=0.036$ ; swimming challenge test:  $p=0.0009$ ) and that the lack of treatment-related effects observed 10-month post-exposure resulted from individuals recovering and not, for instance, from the culling of the most intoxicated ones during the field stage.

## 5. Conclusion

The main purpose of the research was to design and validate an ecologically relevant fish health assessment approach that could be applied to an acute toxicological exposure. Even though mild selective pressure was observed during the field exposure, hypoxia tolerance and maximal swimming speed measured in the laboratory were found to be predictors of survival. These results suggest that this methodology is liable to bridge the gap between sub-organismal toxicological disturbances and effects at population level.

We applied our health assessment methodology to a case of an acute oil exposure. Our study highlighted that exposure to dispersant and to oil alone does not affect fish health. Exposure to the oil and dispersant mixture was associated with temporarily impaired health. However, recovery occurred and no ecologically relevant consequences were observed when fish were transferred to a semi-natural field mesocosm. Fish health recovery was confirmed by the absence of long-term effects (almost 1 year post-exposure). We suggest that health-centred approach should be promoted and conducted using more fish species, life stages and environmental contexts in order to develop even further the notion of fish health. Such development would allow bridging the gap between toxicology and ecology and should prove highly valuable for the future safeguarding of the marine environment and the development of legislation for an integrated management of marine resources and services.

#### Author contributions

All authors contributed to the design of the study; FM, CL & GC collected the data; FM & GC analysed data; FM, GC, JLZ, SLF, AF & PD wrote the m/s; PLM, ANK, MW financed the study.

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**Appendix A. Concentration of 21 PAH (alkylated and parents) in fish white muscle from the four treatments measured 2 and 31 days post-exposure. The 21 PAH represent the 16 US-EPA PAH and five supplementary PAH (benzo[b]thiophene, biphenyl, dibenzothiophene, benzo[e]pyrene, perylene). LOQ: Limit Of Quantification.**

PAH	Concentration $\pm$ sem (ng g <sup>-1</sup> ) at 2 and 31 days post-exposure							
	d + 2				d + 31			
	CONT	DISP	OIL	OIL + DISP	CONT	DISP	OIL	OIL + DISP
Naphtalene	<LOQ	<LOQ	6 $\pm$ 1	6 $\pm$ 1	<LOQ	<LOQ	<LOQ	<LOQ
Benzothioephene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Biphenyl	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Acenaphthylene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Acenaphthene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Fluorene	<LOQ	<LOQ	23 $\pm$ 7	8 $\pm$ 3	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzothioephene	<LOQ	<LOQ	203 $\pm$ 17	185 $\pm$ 11	<LOQ	<LOQ	<LOQ	<LOQ
Phenanthrene	<LOQ	<LOQ	28 $\pm$ 3	25 $\pm$ 2	<LOQ	<LOQ	<LOQ	<LOQ
Anthracene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Pyrene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzoanthracene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Chrysene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[b]fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[k]fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[e]pyrene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[a]pyrene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Perylene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Indeno[1,2,3-cd]pyrene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[a,h]anthracene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzoperylene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Mean of individual's [21 PAH]	/	/	259 $\pm$ 24	224 $\pm$ 33	/	/	/	/

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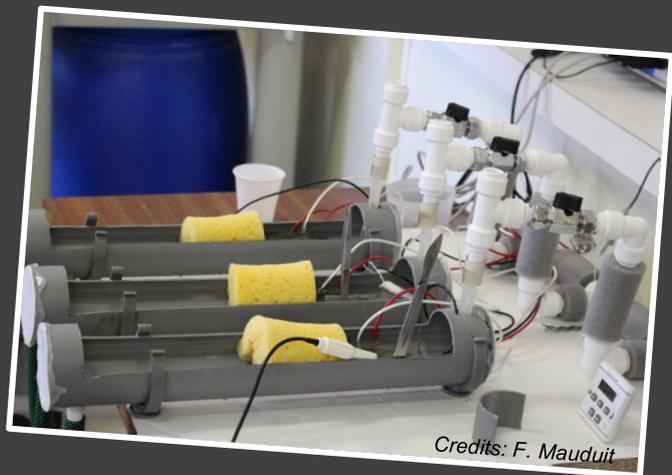
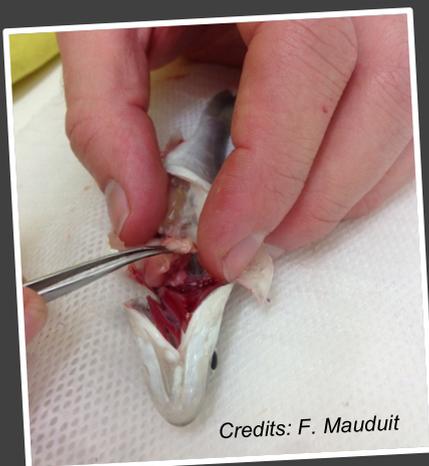
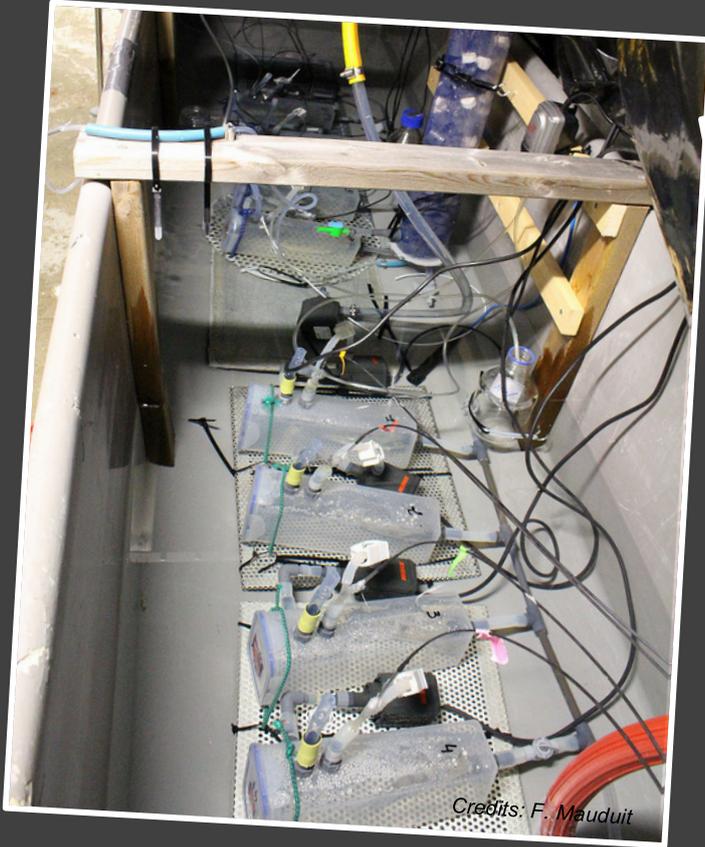
### **3. Conclusion**

Measuring the health of individual organisms is the missing cornerstone in numerous fields within environmental sciences including conservation biology, ecotoxicology and ecophysiology. According to contemporary definitions, indicators that reflect organisms' capacity to adapt to, respond to, or control life's challenges and changes can be considered as proxy of health. We investigated the possibility that complex physiological traits (hypoxia tolerance, temperature susceptibility and swimming performance) are reliable indices of fish health. Key elements that we considered were the ability of the traits to inform about the history (diagnosis) as well as about the future (prognosis) of the individuals concerned.

We found that hypoxia tolerance, temperature susceptibility and swimming performance are all stable over time, validating our capacity to standardize challenge tests and to provide ecologically relevant interpretations of intra- and inter-population variations in these traits. The temporal repeatability of individual's performance under changing environmental conditions, combined with the fact that these traits are heritable (Garenc *et al.*, 1998 and Anttila *et al.*, 2013), substantiates the view that they are potential targets for natural selection (Hayes and Jenkins, 1997 and Dohm, 2002). The ecological relevance of these biomarkers is also strengthened by the fact that even though mild selective pressure was observed during the field exposure, hypoxia tolerance and maximal swimming speed measured in the laboratory were found to be predictors of survival. Therefore, we can consider that measuring these traits allows establishing a prognosis about a fish's capacity to thrive under natural conditions to be established.

Furthermore, we found that fish exposed to a pollutant displayed impaired hypoxia tolerance and maximal swimming speed. Therefore, we can assume that these traits inform about the individuals' past experiences and that they allow us also to establish a diagnosis.

### ***Chapter III: Identification of the confounding factors and the mechanistic basis of performances measured***



## **1. Introduction**

The second objective of my thesis was to examine the underlying mechanisms and to identify the confounding factors influencing the performances measured to assess fish health. A better understanding of these elements is vital for a reliable interpretation of biomarkers' values and/or variations (van der Oost *et al.*, 2003).

Although impairments at organisms, populations or communities' levels have greater ecological relevance, responses at these higher levels of organization are more complex and often lack a mechanistic explanation. The cardiorespiratory function plays a central role in hypoxia tolerance, temperature susceptibility and swimming performances. Therefore, to examine the underlying mechanisms of performances measured, our investigation focused on this key function.

Furthermore, it is acknowledged that confounding factors can affect biomarkers' response and therefore distort their interpretation. Among these factors, environmental history is likely to influence potential markers of fish health and therefore understanding this influence is important. Indeed, when an environmental disturbance such an oil spill occurs, biomarkers' responses recorded in the exposed population are classically compared to observations made in population collected from an uncontaminated site. The compatibility of our markers of fish health and this approach was therefore assessed. Also, because complex physiological traits are the cumulative outcome of numerous underlying mechanisms, regulatory mechanisms can occur and mask the potential effect of a disturbance. To confirm this point, we verified if the lack of observed

long-term effect upon fish health after an exposure to oil was confirmed by measures at a lower level, i.e., the cardiac function.

## 2. Paper 2 (in prep.)

### 1 Environmental shaping of phenotypic diversity

2

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11

#### 12 1 Abstract

13

14 Mechanisms by which fish populations will respond to global warming are unclear.

15 Evolution by natural selection occurring over generations, it will probably be too slow

16 to provide an evolutionary solution. The environmental influence upon gene

17 expression during ontogenesis is a well-documented source of phenotypic diversity.

18 Less documented, however, is phenotypic plasticity at later life stages. To clarify this

19 point, the objective of the present study was to verify whether recent environmental

20 history determined the distribution of hypoxia tolerance and thermal susceptibility

21 within a fish population. To reach this objective a population of 134, tagged

22 *Dicentrarchus labrax* was screened using standardized challenge tests and

23 individuals' hypoxia tolerance and thermal susceptibility noted. The population was

24 then divided in 2 subgroups. One subgroup (L) was maintained under laboratory

25 conditions while the second subgroup (M) was transferred to semi-natural

26 mesocosmes. Five month later, the field population was recovered (70 % survival)  
27 and brought back into the laboratory. Fish populations (L, M) were then mixed and  
28 maintained under common garden conditions during an additional 4-month period. At  
29 that point, the standardized challenge tests were repeated and the distributions of  
30 hypoxia and temperature tolerance within each population were examined.  
31 Comparison of L and M subgroups indicated that the transfer to the field had resulted  
32 in improved hypoxia and heat tolerance which was still observable after the 4-month  
33 common garden period.

## 34 **2 Introduction**

35

36 Growing human pressures, including climate change, are having multiple and  
37 profound consequences for marine ecosystems. The primary direct consequences  
38 include increasing ocean temperatures (Jones *et al.* 2007) and increased frequency,  
39 intensity and duration of hypoxia episodes in shallow areas (Diaz, 2001) with  
40 cascading effects at all levels of biological organization (Wood and McDonald, 1996;  
41 Diaz, 2001; Wu 2002). To respond to the projected changes in their environment,  
42 marine organisms may migrate, acclimate or adapt. However, because of habitat  
43 fragmentation, many species will not be able to track their preferred environment in  
44 space and must, therefore, acclimate or adapt to avoid extinction. Acclimation  
45 involves phenotypically plastic responses in physiology, morphology or behavior that  
46 can help maintain fitness under the new environmental conditions (Angilletta, 2009).  
47 In contrast, adaptation involves the selection of genetic variations that contribute to  
48 shifting the average phenotype toward the species' fitness peak (Munday, 2014).  
49 Although species have adapted to environmental changes throughout their

50 evolutionary history, a primary concern with regard to contemporary environmental  
51 trends is the rate of the changes (Palumbi, 2001). Adaptation occurs through  
52 selection over generations and in many species, particularly those of commercial  
53 interest, generation times are too long for evolution by natural selection to provide  
54 them with adaptive solutions. In that context, the question is whether phenotypic  
55 plasticity could be an emergency source of phenotypes liable to preserve fitness  
56 pending the development of long term, evolutionary adaptation (Williams, 2008).

57

58 Phenotypic plasticity in response to non-optimal thermal and oxygenation conditions  
59 provides a physiological response that allows animals to compensate for  
60 environmental changes (Wilson and Franklin, 2002; Schaefer and Ryan, 2006). For  
61 instance, acclimation allows fish to preserve internal homeostasis across a wide  
62 range of temperature, and allows them to cope, for example, with marked seasonal  
63 fluctuations (Schulte *et al.*, 2011, Sandblom *et al.*, 2014). That said, it is also  
64 important to note that acclimation corresponds to a set of reversible plastic  
65 adjustments as opposed to fixed plasticity which takes place during ontogenesis  
66 (Burggren and Muller 2015; Schulte *et al.*, 2011, Kelly *et al.*, 2012). This fixed  
67 plasticity consists in betting that environmental conditions experienced during  
68 ontogenesis will be those encountered in lifelong (DeWitt *et al.*, 1998). In the context  
69 of changing environment, this bet appears as risky. Furthermore, to be considered as  
70 adaptive, plasticity has to result in the production of a phenotype that is in the same  
71 direction as the optimal value favored by selection in the new environment (Conover  
72 *et Schultz*, 1995).

73

74 In the context of the changes that are affecting our planet, understanding the  
75 capacity of phenotypic plasticity to maintain fish performances and downstream  
76 fitness is a critical concern. To document this issue, the present study aimed at  
77 verifying the effect environmental history on fish hypoxia tolerance and temperature  
78 susceptibility. To this objective, a population of 135 individuals was screened for  
79 hypoxia tolerance and temperature susceptibility. This population was then divided in  
80 two subgroups, one was maintained in the laboratory throughout the experiment  
81 (subgroup-L), while the second spent 5 months in semi-natural field mesocosm  
82 (subgroup-F). On their return from the field experiment, fish from the subgroup-F  
83 were mixed with fish from the subgroup-L and their hypoxia and heat tolerance were  
84 compared after 4 and 11 months of common garden conditions.

### 85 **3 Materials and methods**

86

#### 87 **3.1 Animals**

88 One hundred and thirty-five, one year old sea bass (*Dicentrarchus labrax*) were  
89 obtained from a local fish farm (Aquastream, Lorient, France). Upon arrival in the  
90 laboratory (Ifremer, Brest, France) fish ( $12.87 \pm 0.04$  cm;  $26.7 \pm 0.4$  g) were  
91 anaesthetized (MS-222; 100 mg L<sup>-1</sup>), implanted subcutaneously with an identification  
92 tag (RFID; Biolog-id, France) and placed in 400-L, indoor tanks. Fish were fed daily  
93 *ad libitum* with a commercial diet (Neo Start Coul 2, Le Gouessant). They were unfed  
94 for 24 H before any manipulation or experiment. The protocol was in conformity with  
95 current rules and regulations in France.

96

97 **3.2 Experimental protocol**

98 The experimental schedule is summarized in Fig.1. Fish individual's performances at  
99 challenge tests were assessed 1 month before dividing the experimental population  
100 into 2 subgroups (May 2014). One group was maintained in the laboratory  
101 (subgroup-L; N=35), while the second (subgroup-F; N=100) was transferred into  
102 semi-natural field mesocosms for a 5-month period. At the end of the field period, fish  
103 from the subgroup-F were brought back into the laboratory and mixed with fish from  
104 subgroup-L. Performances at challenge tests were assessed after 4 and 11 months  
105 under common garden conditions. Conditions in the laboratory followed the local  
106 temperature (11-20 °C), photoperiod and salinity (30-32 PSU) seasonal cycles.  
107 Water oxygenation and temperature were monitored daily in laboratory rearing tanks  
108 and hourly in the field mesocosms.

109 **3.3 Fish transport**

110 The experiment mobilized the resources of two experimental sites and it was  
111 necessary to transport fish between them. Most of fish rearing phase was conducted  
112 in Ifremer rearing facilities (Plouzané; France). The mesocosm experiment took place  
113 in L'Houmeau (France), 450 km away from Plouzané. Fish were transported between  
114 the sites in a 1 m<sup>3</sup>-covered tank. Before transfer into the transport tank, fish were  
115 anaesthetized (MS-222; 100 mg L<sup>-1</sup>) and a maintenance dose of anesthetic (MS-  
116 222; 25 mg L<sup>-1</sup>) was maintained during the travel (5 h). Water temperature and  
117 oxygen level were monitored continuously and maintained at seasonal values. No  
118 mortality was recorded during fish transport.

119 **3.4 Challenge tests**

120 Challenge tests have been fully described in Mauduit *et al.*, (2016). They were  
121 performed directly in the fish rearing tank. In these tests, fish from the two  
122 experimental subgroups were tested all together in one single run.

123

124 *3.4.1 Hypoxia challenge test*

125 Hypoxia challenge tests consisted in a rapid decrease of water oxygenation (from  
126 nearly 100% to 20% air saturation in 1 h) followed by of slower descent (2 % air  
127 saturation per hour) until the end of the experiment. Hypoxia was obtained by  
128 bubbling nitrogen in the admission of a submersible pump placed in the experimental  
129 arena. Water oxygenation was monitored using an oxymeter (ODEON, Ponsel  
130 Mesure, France). When a fish lost its ability to maintain balance, it was quickly  
131 removed from the experimental arena, identified (RFID tag reading) and placed in a  
132 fully aerated tank. The corresponding time and oxygenation level were also recorded.  
133 Experiment ended when the last fish was recovered.

134

135 *3.4.2 Temperature challenge test*

136 Temperature challenge tests (TCT) consisted in a first phase of rapid increase of the  
137 water temperature (from ambient temperature to 27 °C in 2.5 h), followed by a much  
138 slower increase (0.5 °C per hour) until the end of the experiment (Claireaux *et al.*,  
139 2013). Water temperature was controlled using two 2500 W heaters (GERVEN,  
140 Netherland). A submersible pump placed in the tank ensured water homogeneity and  
141 air saturation was maintained by controlling the bubbling of a mixture of oxygen and  
142 air in the tank. As fish progressively lost their ability to maintain their equilibrium, they  
143 were recovered from the experimental tank, identified and placed in a recovery tank

144 at acclimation temperature. The corresponding time and temperature were also  
145 recorded.

146

147 A one week recovery period was allowed between challenge tests and no mortality  
148 was observed following these tests.

149

### 150 **3.5 Field study**

151 On June 26<sup>th</sup> 2014, 406 fish were transported to the field station of Fédération de  
152 Recherche en Environnement pour le Développement Durable situated in  
153 L'Houmeau (450 km from Brest). L'Houmeau experimental mesocosms' set-up has  
154 been described on various occasions (Nelson and Claireaux, 2005; Claireaux *et al.*,  
155 2007; Handelsman *et al.*, 2010; Mauduit *et al.*, 2016). Concisely, it is composed of  
156 200-m<sup>2</sup>, 1-m deep earthen ponds connected to the nearby ocean via a canal. The  
157 water in these ponds is partially renewed at each incoming tide and sets of  
158 standpipes prevent fish from escaping. Moreover, bird nets avoid avian predation.  
159 Previous experiments and empirical observations have shown that the natural food  
160 web that develops in these ponds can sustain 2 - 3 kg of fish. A complete description  
161 of these earthen pond's fauna can be found in de Montaudouin and Sauriau (2000).

162

163 Upon arrival at L'Houmeau field station, fish were immediately distributed among 4  
164 ponds ( $\approx$ 100 individuals per pond). During the following 5 months, ponds were  
165 drained monthly (July 16<sup>th</sup>, August 27<sup>th</sup>, September 24<sup>th</sup>) to assess survivorship and  
166 growth. The final fish monitoring was conducted on November 3<sup>rd</sup> when survivors  
167 were transported back to Brest and returned to their original rearing facility. Water

168 conditions in the ponds were measured hourly using a multi-parameter probe (MPx,  
169 NKE, France) recording water oxygenation level, salinity and temperature.

170

### 171 **3.6 Data analysis and statistics**

172 Among sub-groups differences in fish body mass were compared using an ANOVA.  
173 Performances at challenge tests of the two subgroups were compared using a  
174 Kaplan-Meier survival analysis. Throughout the manuscript values are given as mean  
175  $\pm$  SEM and significance was accepted at  $p < 0.05$ . All statistical analyses were  
176 performed using R (R Development Core Team, 2016).

## 177 **4 Results**

### 178 **4.1 Fish initial characteristics**

179 Before transfer to the field, laboratory (L) and field (F) subgroups displayed similar  
180 body mass (Fig.2 A; ANOVA,  $p=0.867$ ), hypoxia tolerance (Fig.2 B; Log-rank,  
181  $p=0.210$ ) and temperature susceptibility (FIG 2C; Log-rank,  $p=0.267$ ).

182

### 183 **4.2 Fish environmental history**

184 Water temperature and oxygenation experienced by the fish throughout the  
185 experiment are summarized on the figure 3 (A and B, respectively).

#### 186 *4.2.1 Laboratory subgroup*

187 Highest water temperature monitored in fish rearing facility was 18.3 °C (early August  
188 2014) and the lowest 9.4 °C (early February 2015). Within this range, large seasonal  
189 variations were observed. Temperature indeed increased from February 2014 (10°C)  
190 to July 2014 (18 °C). Then, temperature stabilized until September 2014 and started

191 to decrease to reach 9.4 °C in February 2015. At that time, water temperature rose  
192 again to reach 18 °C in July 2015 and then stabilized until the end of the experiment  
193 in September 2015.

194 Water oxygen level measured in laboratory facilities was between 95 and 100 % of  
195 air saturation throughout the study.

#### 196 4.2.2 Field subgroup

197 Fish from the subgroup-F experienced the same environmental conditions than fish  
198 from the subgroup-L, excepted during the period from June to November 2014 where  
199 they were transferred to semi-natural mesocosm. During this period, the highest  
200 recorded temperature was 29.8 °C (end of July) and the lowest was 13.8 °C (end of  
201 October). Within this entire period, two patterns were observed. From the end of  
202 June to the end of July, mean daily temperature increased from 22 °C to 27 °C.  
203 Then, from early August to October, temperature decreased from 24 °C to 15 °C.  
204 Also, this seasonal pattern of water temperature was accompanied by diurnal  
205 variations with night time temperature being approximately 5°C lower than during the  
206 daytime.

207 Water oxygen levels measured in the mesocosm, did not reveal any seasonal  
208 variation. However, broad diurnal fluctuations were recorded ranging from 170 - 260  
209 % air saturation during daytime to 40 - 50 % air saturation at the end of the night  
210 periods.

211

### 212 4.3 Field experiment

213 Fish survival and growth in the mesocosm were monitored monthly. Survivorship was  
214 in excess of 70 % at the end of the field experiment (Fig.4). Sixty five percent of the  
215 mortality observed during the field experiment occurred between June 26<sup>th</sup> and July

216 17<sup>th</sup>. Sea bass growth decreased continuously over the 5-month period (ANOVA,  $p <$   
217 0.001). Growth was indeed maximal during the period June 26<sup>th</sup> to July 16<sup>th</sup> (1.5 %  
218 body mass  $d^{-1}$ ; Fig.5) and then progressively declined along the summer and autumn  
219 to be markedly suppressed by the end of the experiment.

220

#### 221 **4.4 Effect of recent environmental history**

##### 222 *4.4.1 Fish body mass*

223 Monitoring of fish body mass throughout the experiment revealed that while fish had  
224 similar body mass before the transfer to the field, fish from the subgroup-F displayed  
225 lower body mass than fish from the subgroup-L ( $73.8 \pm 0.9$  g vs  $93.8 \pm 1.7$  g) on their  
226 return to the laboratory in November 2014 (Fig.6). This 21 % difference in body mass  
227 then decreased progressively to 9 % in January ( $115.3 \pm 1.7$  g vs  $125.7 \pm 2.6$  g) and  
228 then to 3% March 2015 ( $124.3 \pm 1.7$  g vs  $128.5 \pm 2.8$  g). In July and September  
229 2015, mean body mass of subgroup-F was higher than that of the subgroup-L ( $179.2$   
230  $\pm 2.2$  g vs  $154.3 \pm 3.2$  g and  $260.7 \pm 3.2$  g vs  $216.3 \pm 4.5$  g, respectively).

231

##### 232 *4.4.2 Fish hypoxia tolerance and temperature susceptibility*

233 Four months after they had returned to the laboratory, mean time to loss of  
234 equilibrium (TLOE) of subgroup-L at hypoxia challenge test was  $8.61 \pm 0.16$  h while it  
235 was  $7.68 \pm 0.16$  h in the subgroup-L (Log-rank,  $p = 0.005$ ). This corresponded to  
236 incipient lethal oxygen saturations (ILOS) of  $4.8 \pm 0.08$  and  $5.5 \pm 0.08$  % air  
237 saturation respectively (Fig.7). Mean time to loss of equilibrium at the temperature  
238 challenge test were  $3.17 \pm 0.05$  and  $4.30 \pm 0.13$  h for the L and F subgroups

239 respectively (Log-rank,  $p < 0.000$ ). This corresponded to upper critical temperatures  
240 ( $CT_{max}$ ) of  $27.1 \pm 0.3$  °C and  $27.8 \pm 0.5$  °C respectively.

241

242 Eleven months after the end of the field experiment, experimental population was  
243 submitted a third time to the suite of challenge tests. Individual's responses are  
244 summarized in Fig. 8. Regarding the hypoxia challenge test (Fig.8 A), mean TLOE of  
245 fish from the subgroup-L was  $3.28 \pm 0.14$  h which corresponded to  $8.1 \pm 0.3$  % air  
246 saturation. After eleven month under common garden condition with the subgroup-L,  
247 fish from the subgroup-F still displayed higher hypoxia tolerance than fish from the  
248 laboratory subgroup (Log-rank,  $p = 0.000$ ; mean TLOE =  $4.27 \pm 0.16$  h). Regarding  
249 temperature challenge test, mean TLOE in the subgroup-L was  $5.43 \pm 0.10$ , which  
250 corresponded to  $28.4 \pm 0.4$  °C. Temperature susceptibility in fish from the subgroup-  
251 F was significantly lower than in fish from the subgroup-L (Log-rank,  $p = 0.000$ ; mean  
252 TLOE =  $5.79 \pm 0.07$  h) (Fig.8 B).

253

## 254 **5 Discussion**

255

256 To improve our knowledge about fish capacity to cope with rapid changes in their  
257 environmental conditions, the present study aimed at verifying if plasticity in hypoxia  
258 tolerance and temperature susceptibility was adaptive and reversible in juveniles of  
259 sea bass. To reach this objective, a population of 134 individuals was screened for  
260 hypoxia tolerance and temperature susceptibility. Then, this population was divided  
261 in two subgroups, one was maintained in the laboratory throughout the experiment  
262 (subgroup-L,  $n = 34$ ), while the second one experienced a 4-month period in semi-  
263 natural mesocosm (subgroup-F,  $n = 100$ ). At the end of this field experiment, fish

264 from the subgroup-F were brought back into the laboratory and placed in common  
265 garden with fish from the subgroup-L. Hypoxia tolerance and temperature  
266 susceptibility of the 2 subgroups were then assessed a second and a third time, after  
267 a 4 and 11-month period of common garden, respectively. Our results revealed that  
268 environmental history influence fish hypoxia tolerance, thermal susceptibility and  
269 growth. Fish from the subgroup-F displayed a higher hypoxia tolerance, a lower  
270 temperature susceptibility and higher body mass than fish from the subgroup-L.

271

### 272 **5.1 Fish environmental history**

273 Water temperature observed in the laboratory is typical of the thermal conditions in  
274 the Bay of Biscay. Briefly, lowest water temperature (8-10 °C) occurred during the  
275 winter and highest (15-22 °C) during the summer period (PREVIMER data). Water  
276 oxygenation was maintained within optimal range for sea bass capacities (90-100 %  
277 air saturation). On the other hand, from June to September fish in the field  
278 experienced temperature at their upper limit of sea bass tolerance range.  
279 Temperature above 22–24 °C is indeed, detrimental to sea bass as it is accompanied  
280 by a rapid decrease in aerobic metabolic scope (Claireaux and Lagardère, 1999),  
281 impaired cardio-vascular performance (Farrell *et al.*, 2007), reduced growth (Person-  
282 Le-Ruyet *et al.*, 2004), reduced swimming performance (Claireaux *et al.*, 2006) and  
283 by avoidance behavior (Claireaux and Lefrançois, 2007). During the field experiment,  
284 fish from the subgroup-F also experienced strong daily variation in water  
285 oxygenation, from 170 - 260 % air saturation (daytime) to 40 - 50 % air saturation at  
286 the end of the night periods. However, even associated with the high temperature  
287 values recorded from June to August, these transients low oxygen levels remain

288 within sea bass range capacity for regulation. For instance, at 25°C, 50% of air sat  
289 allow a metabolic scope of 90% (Claireaux and Lagardère, 1999).

290

291 Survivorship observed at the end of the field period reached 74%. Compared with  
292 previous studies, this survival rate is rather exceptional. For instance, preceding  
293 studies using same field sites yielded survival rates ranging between 20 and 60 %  
294 (Claireaux *et al.*, 2013; Mauduit *et al.*, 2016). However, it must be emphasized that  
295 71% of the recorded mortality occurred during the first three weeks of the field  
296 experiment. This suggests that at that period and despite abundant food availability,  
297 the relatively higher mortality recorded possibly resulted from the inability of certain  
298 individuals to acclimate (e.g., catch live prey, to deal with important diurnal variation  
299 in oxygen and temperature) following the transfer from the laboratory to the field.  
300 From the end of July to the end of the field experiment in early November, food  
301 availability decreased and intra-specific competition for food increased which may  
302 explain the sparse mortality observed during that period. This hypothesis is  
303 supported by the 15-times decrease in growth rate observed all along the field  
304 experiment, from 1.5 % body mass d<sup>-1</sup> at the beginning to 0.1 % body mass d<sup>-1</sup> at the  
305 end of the field experiment.

306

## 307 **5.2 Fish Growth**

308 The monitoring of fish body mass throughout the experiment revealed that while it  
309 was similar between both subgroups before transfer to the field, body mass was  
310 significantly different between F- and L-subgroups on the return of the former to the  
311 laboratory. During autumn (September–November), growth of fish from subgroup-F  
312 was markedly suppressed while fish from subgroup-L continued to grow. This

313 difference ( $73.8 \pm 0.9$  g vs  $93.8 \pm 1.7$  g) can be explained by the decrease in food  
314 availability observed in the ponds. When fish from the subgroup-F were brought back  
315 into the laboratory and maintained under common garden conditions with fish from  
316 the subgroup-L, the difference in body mass tended to decrease,  $115.3 \pm 1.7$  g vs  
317  $125.7 \pm 2.6$  g in January 2015,  $124.3 \pm 1.7$  g vs  $128.5 \pm 2.8$  g in March. In July and  
318 September 2015, mean body mass of subgroup-F even exceeded that of the  
319 subgroup-L ( $179.2 \pm 2.2$  g vs  $154.3 \pm 3.2$  g and  $260.7 \pm 3.2$  g vs  $216.3 \pm 4.5$  g,  
320 respectively). This observation suggests compensatory growth with an  
321 overcompensation. Compensatory growth is a phase of accelerated growth when  
322 favorable conditions are restored after a period of starvation (Ali *et al.*, 2003). This  
323 phenomenon is well documented in fish, including salmon (Maclean and Metcalfe,  
324 2001; Mortensen and Damsgard, 1993; Damsgard and Arnesen, 1998), seabass  
325 (Sahin *et al.*, 2000; Dosdat *et al.*, 2003; Terova *et al.*, 2006) and charr (Miglav and  
326 Jobling, 1989; Jobling *et al.*, 1993). Overcompensation was observed in *Lepomis*  
327 hybrids by Hayward *et al.* (1997), but seems to be a rare outcome. Such a  
328 compensatory gain could involve trade-offs in energy allocation leading, for instance,  
329 to reduced locomotor activity (Sogard and Olla, 2002).

330

### 331 **5.3 Hypoxia tolerance and temperature susceptibility**

332 Following the field experiment, fish that survived ( $n = 74$ ) were brought back into the  
333 laboratory and maintained under common garden conditions with fish from the  
334 laboratory subgroup for an additional period of 4 months. Performances at challenge  
335 tests conducted at that time revealed that fish from the subgroup-F had higher  
336 hypoxia tolerance and lower temperature susceptibility than fish from the subgroup-L.  
337 Moreover, this increased coping ability observed in fish from the subgroup-F was

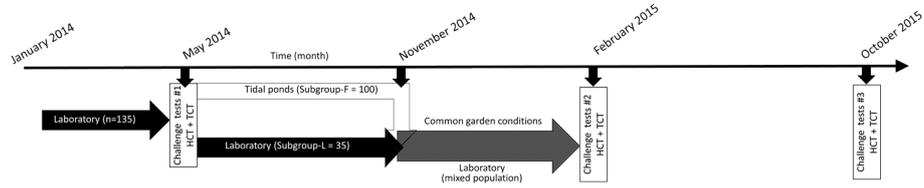
338 still present when challenge tests were repeated a third time after 11 months of  
339 common garden. These results demonstrate that hypoxia tolerance and temperature  
340 susceptibility are plastic and indicate that compensatory growth observed was not  
341 associated with trade-offs for environmental adaptation. It has been reported that  
342 acclimation to high temperature and rearing in environment with variable temperature  
343 lead to increased critical thermal maximum (Feldmeth *et al.*, 1974; Bennet and  
344 Beitinger, 1997; Beitinger *et al.*, 2000; Schaefer and Ryan, 2006). Similarly, it has  
345 been reported that preconditioning to sub-lethal hypoxia lead to increased hypoxia  
346 tolerance (Rees *et al.*, 2001; Yang *et al.*, 2013).

347 Observing plasticity in hypoxia tolerance and temperature susceptibility is therefore  
348 not surprising. However, more striking is the timing of these observations.  
349 Improvements in hypoxia tolerance and temperature susceptibility were indeed  
350 observed after a 4 and 11-months period of common garden. Such a timescale was  
351 chosen to avoid attributing a possible change in phenotypic traits to a training effect  
352 (Nelson et Lipkey, 2015). Therefore, improved coping ability observed could be  
353 fixed. Fixed plasticity is however, often associated to an alteration of the phenotype  
354 occurring during the development of the organism. For instance, one of the most  
355 significant examples of such plasticity is the temperature sex determination in fish. It  
356 has been demonstrated that sea bass exposed to low water temperature (13-15 °C)  
357 during the embryonic and larval stages affects sex ratio in favor of females (Pavlidis  
358 *et al.*, 2000). However, to the best of our knowledge this is the first time that such  
359 plasticity of hypoxia tolerance and temperature susceptibility is observed on sub-  
360 adult fish.

361 **6 Conclusion**

362 The main purpose of this research was to investigate if phenotypic plasticity can  
363 provide an adaptive solution to preserve fitness in juveniles in a world marked by  
364 global increase of water temperature and increased frequency, intensity and duration  
365 of hypoxia episodes in shallow areas. We found that fish that have been exposed to  
366 highly variable semi-natural conditions displayed a higher hypoxia and temperature  
367 tolerance than fish that have been maintained in the laboratory conditions all along  
368 the experiment. Moreover, we found that this increased tolerance for hypoxic and  
369 warm environment was observed 11 months after fish have been brought back into  
370 the laboratory and maintained under common garden conditions. Therefore, changes  
371 in these traits are beneficial, and maintained overtime, indicating that hypoxia  
372 tolerance and temperature susceptibility are adaptive traits. Such improvement in fish  
373 capacities for environmental adaptation is striking and it could provide an emergency  
374 solution to a rapid change in organisms' environment. In the future, additional studies  
375 should be conducted to understand the underlying mechanisms of the improved  
376 capabilities for environmental adaptation.

377



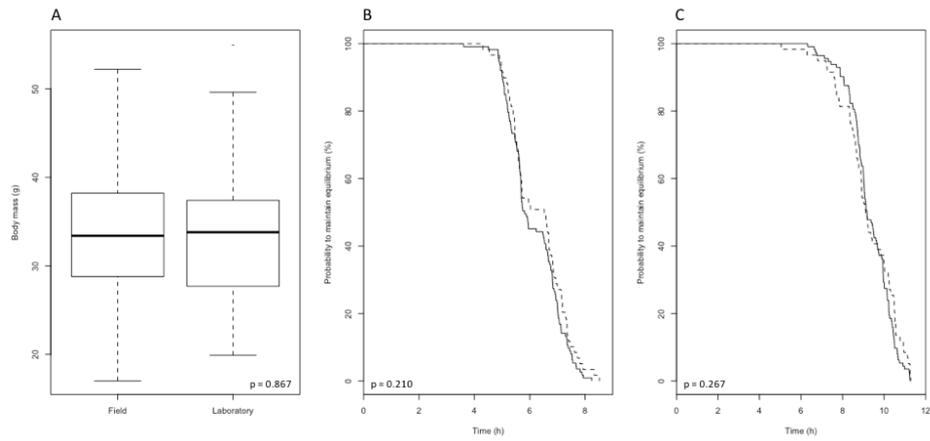
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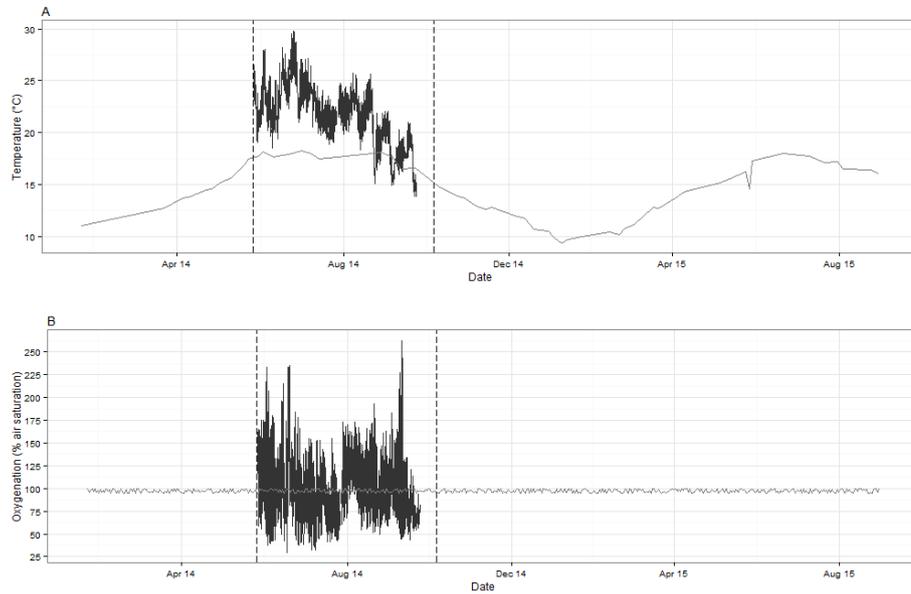
Figure 1: Study timeline.



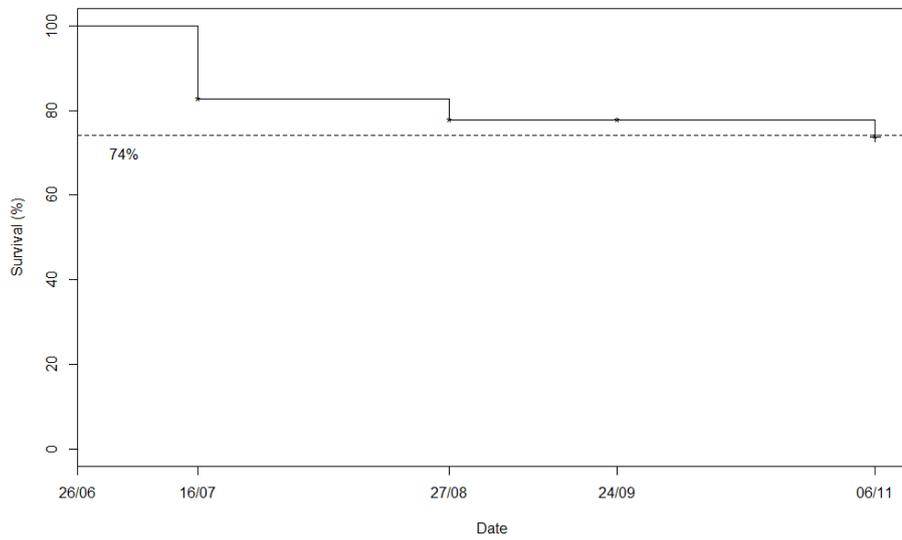
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383 Figure 2: Initial body mass (A) and performances at hypoxia (B) and temperature (C)  
384 challenge test #1.

385

386



387  
388 Figure 3: Water temperature (A) and oxygenation (B) monitored in the laboratory  
389 (grey line) and in the field (black line). Vertical dashed lines indicate the beginning  
390 and the end of the field experiment.  
391

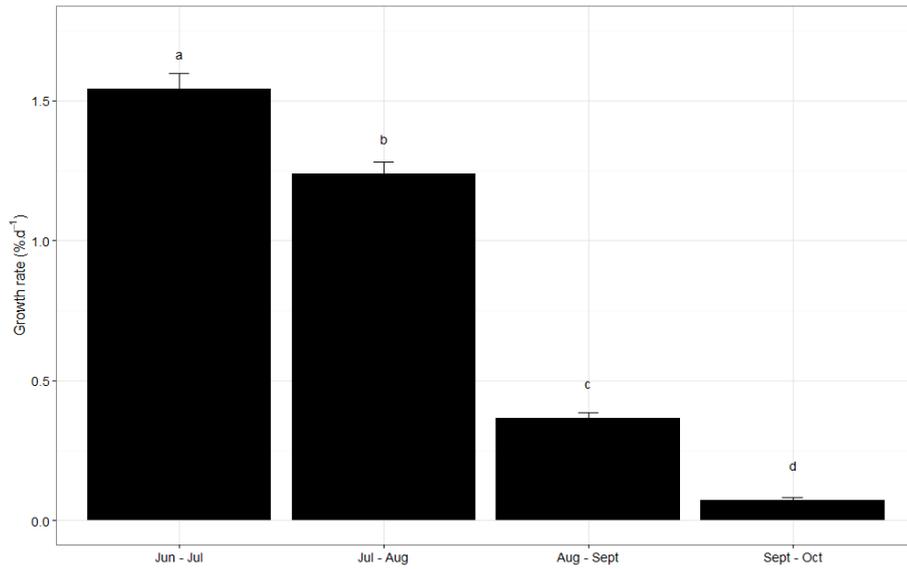


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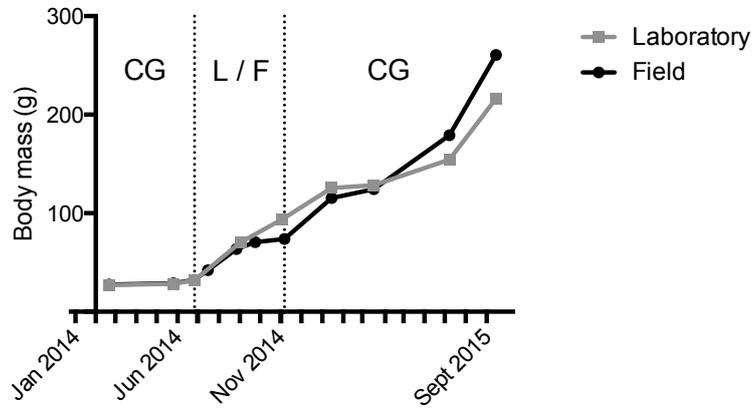
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Figure 4: Fish survival during the field experiment. \* indicate sampling date.

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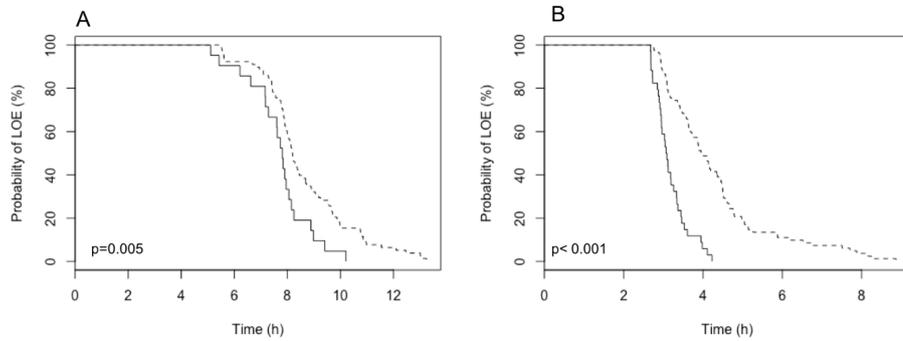
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396 Figure 5: Daily relative growth rate throughout the field experiment. Letters indicate  
397 statistically different means (Tukey;  $p < 0.05$ ).  
398



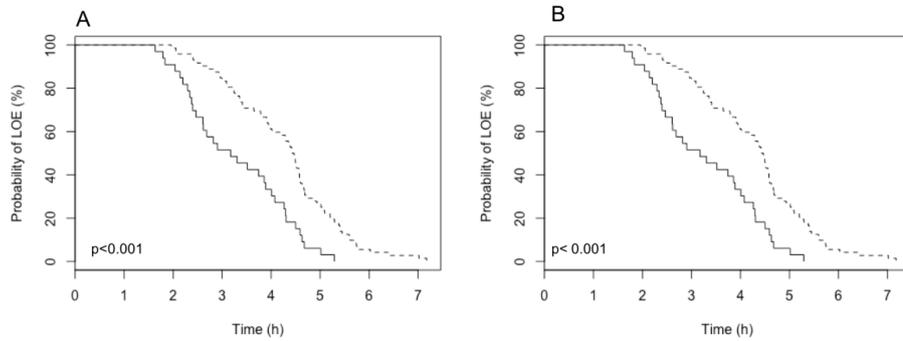
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400 Figure 6: Body mass of fish from the laboratory (grey line) and field subgroup (black  
401 line) throughout the study. Vertical dashed lines correspond to the beginning and the  
402 end of the field experiment.

403



404  
405 Figure 7: Performances at hypoxia (A) and temperature (B) challenge test #2 (4  
406 months after the end of the field experiment). Solid line: Laboratory subgroup;  
407 dashed line: field subgroup. p values indicate the statistical differences at Log-rank  
408 test.  
409



410  
411 Figure 8: Performances at hypoxia (A) and temperature (B) challenge test #3 (11  
412 months after the end of the field experiment). Solid line: Laboratory subgroup;  
413 dashed line: field subgroup. p values indicate the statistical differences at Log-rank  
414 test.  
415

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#### Physiology

## Individual variation in whole-animal hypoxia tolerance is associated with cardiac hypoxia tolerance in a marine teleost

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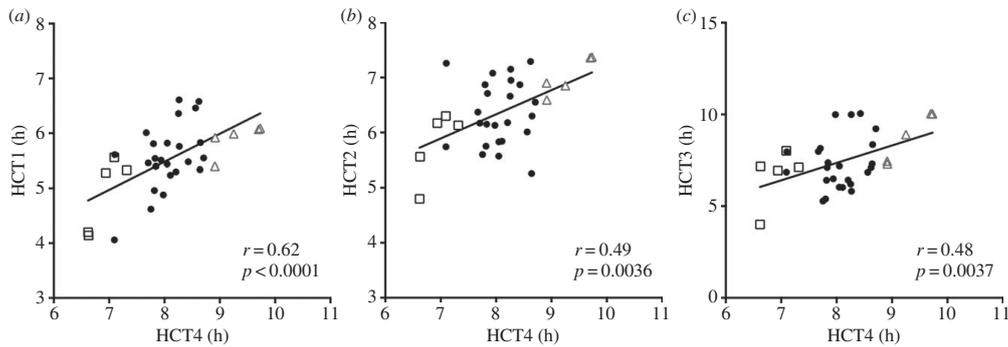
Hypoxia is a pervasive problem in coastal environments and is predicted to have enduring impacts on aquatic ecosystems. Intraspecific variation in hypoxia tolerance is well documented in fish; however, the factors underlying this variation remain unknown. Here, we investigate the role of the heart in individual hypoxia tolerance of the European sea bass (*Dicentrarchus labrax*). We found individual whole-animal hypoxia tolerance is a stable trait in sea bass for more than 18 months (duration of study). We next examined *in vitro* cardiac performance and found myocardial muscle from hypoxia-tolerant individuals generated greater force, with higher rates of contraction and relaxation, than hypoxic-sensitive individuals during hypoxic exposure. Thus, whole-animal hypoxia tolerance is associated with cardiac hypoxia tolerance. As the occurrence of aquatic hypoxia is expected to increase in marine ecosystems, our experimental data suggest that cardiac performance may influence fish survival and distribution.

### 1. Introduction

Oxygen availability is a key driver of animal physiology, behaviour and ecology. Low levels of environmental oxygen (hypoxia) are a growing concern, especially in coastal waters, where the duration, frequency and severity of hypoxic events have increased in recent years [1,2]. Reduced oxygen availability negatively impacts fish, affecting their growth [3], embryonic development [4], sex ratio [5], predator–prey relationships [6], swimming activity [7] and niche utilization [8]. The European sea bass (*Dicentrarchus labrax*) is known to exhibit pronounced intraspecific variation in hypoxia tolerance which directly relates to survivorship in semi-natural tidal ponds [9]. This suggests that hypoxia tolerance is a determinant of individual fitness in sea bass. However, the factors underpinning intraspecific variation in hypoxia tolerance in this animal remain unclear.

Previous studies have drawn associations between measures of cardiac performance and organismal performance traits in fish [10,11]. For example, the cardiac power output of rainbow trout with exceptional swimming performance is significantly higher than that of poor swimmers [12]. Moreover, cardiac oxygen supply in many fish species, including sea bass, is precarious because they do not have a coronary blood supply but instead rely on the residual oxygen in returning venous blood [13]. Thus, cardiac hypoxia tolerance and whole-animal hypoxia tolerance should be strongly correlated in fish; although this hypothesis has not been directly tested.

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**Figure 1.** Individual whole-animal hypoxia tolerance is a stable trait in sea bass. Black squares ( $n = 5$ ) represent fish subsequently designated HCT hypoxia-sensitive fish and grey triangles ( $n = 5$ ) represent fish designated as HCT hypoxia-tolerant fish for myocardial preparation experiments.  $R$ -values and significance determined by Pearson's correlation coefficient ( $N = 34$ ). HCT1 (May 2012); HCT2 (June 2012), HCT3 (January 2013) and HCT4 (December 2013).

Whole-heart contraction and relaxation is dependent on the cycling of  $\text{Ca}^{2+}$  in the muscle cells (cardiomyocytes) that constitute the heart.  $\text{Ca}^{2+}$  cycling is an energy-consuming process and hypoxia is known to depress contractility due to reduced respiration and adenosine triphosphate (ATP) turnover [14]. It is therefore possible that differences in myocardial  $\text{Ca}^{2+}$  handling underlie intraspecific variation in cardiac hypoxia tolerance. Here we test the hypothesis that individual variation in cardiac hypoxia tolerance is associated with individual variation in whole-animal hypoxia tolerance. Our second hypothesis is that superior heart performance during hypoxia is supported mechanistically through enhanced cellular  $\text{Ca}^{2+}$  handling, which we investigated pharmacologically.

## 2. Material and methods

Thirty-four European sea bass (*Dicentrarchus labrax*) (mass  $286 \pm 12$  g, male) were obtained from a commercial supplier (Aqua-stream, Ploemeur, France) and maintained at *Institut Français de Recherche pour l'Exploitation de la Mer* (Ifremer) in Brest, France, where they were implanted with passive integrated transponders (PIT) to identify individual fish (see [9] for details).

Individual hypoxia tolerance was assessed using a hypoxia challenge test (HCT) which has been described in detail [9]. Briefly, nitrogen was used to decrease oxygenation from 100 to 20% air saturation over 90 min and by approximately 2% per hour thereafter. Once fish lost equilibrium, they were identified via PIT tag, and removed to recover in a fully aerated tank. The time taken to lose equilibrium and the corresponding oxygenation level was recorded. HCTs took place on 25th May 2012 (HCT1), 7th June 2012 (HCT2), 15th January 2013 (HCT3) and 12th December 2013 (HCT4). Directly following HCT4, the five most hypoxia-tolerant fish and the five most hypoxia-sensitive fish were used for the functional cardiac analyses.

Fish were killed with a blow to the head in accordance with local animal care protocols. The heart was removed, transferred to physiological saline and four myocardial muscle strip preparations were dissected from the ventricle with a razor blade. Each strip was hung between two vertical clips; the uppermost was attached to a force transducer, while the other provided a stable anchor. The muscle preparations were lowered into individual organ baths containing physiological saline maintained at  $12^\circ\text{C}$ , and stimulated to contract (10 ms, 70–85 V, 0.2 Hz). Two of the muscle strips were oxygenated and designated 'normoxic' and two were aerated to implement hypoxia. Our hypoxic conditions were designed to mimic the fivefold decrease in blood

oxygen known to occur in sea bass exposed to 24 h of hypoxia [15]. See [16], and electronic supplementary material and methods for full experimental details.

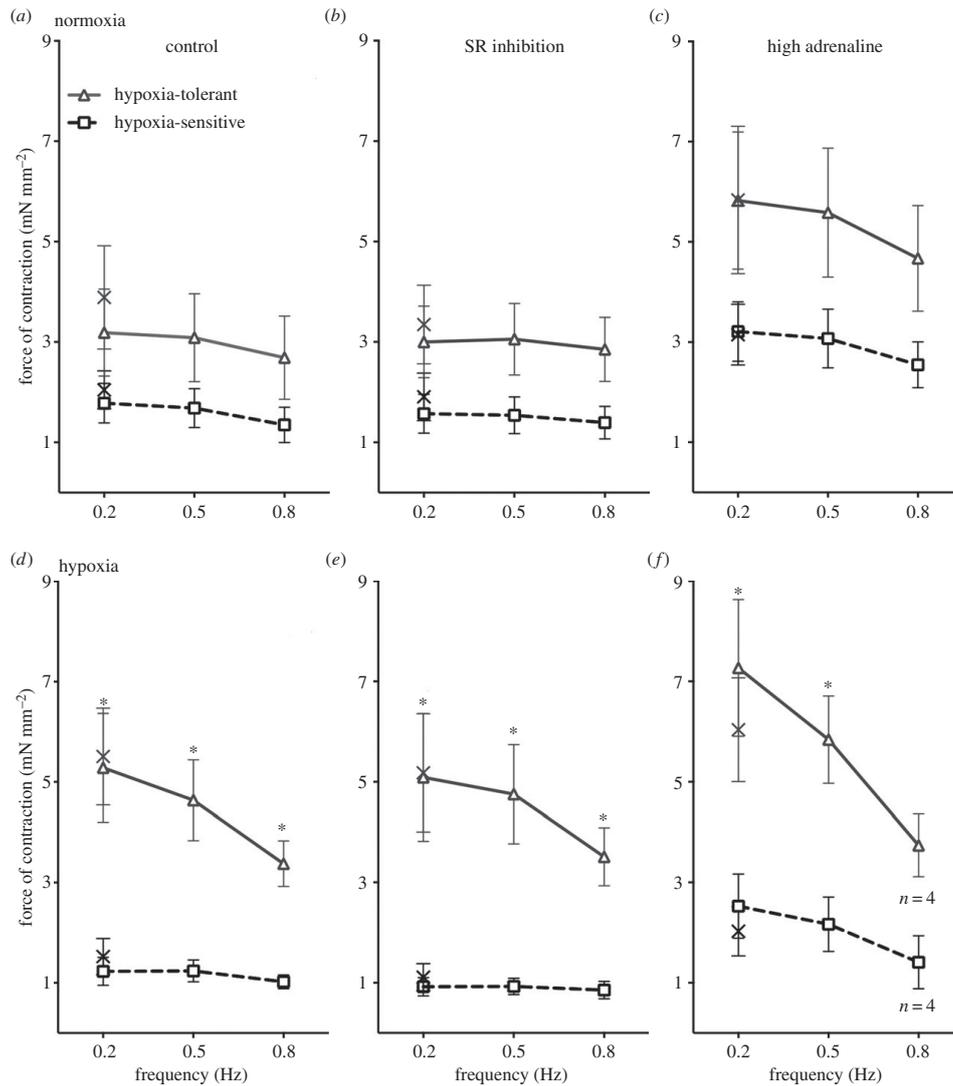
All muscle preparations underwent three consecutive force–frequency trials. The first force–frequency trial was conducted in the presence of 1 nM adrenaline [17] and served as the control condition. In the second force frequency trial, one normoxic and one hypoxic preparation were treated with agents (10  $\mu\text{M}$  ryanodine and 2  $\mu\text{M}$  thapsigargin, [16]) that inhibit intracellular  $\text{Ca}^{2+}$  cycling through the sarcoplasmic reticulum (SR). The other muscle preparations served as time- and oxygenation-matched controls. For the third force frequency trial, all muscle preparations were exposed to a high dose of adrenaline (1  $\mu\text{M}$ ; [18]) which increases extracellular  $\text{Ca}^{2+}$  influx across the cell membrane. This drug treatment protocol was designed to test our second hypothesis and establish the role of intracellular  $\text{Ca}^{2+}$  (SR) and extracellular  $\text{Ca}^{2+}$  cycling in myocardial contractility during hypoxia.

HCT repeatability was assessed with the Pearson product-moment correlation coefficient. The myocardial force and kinetics were calculated as previously described [16]. Linear mixed-effects models (GLM) were used to analyse the relationship between whole-animal hypoxia tolerance and myocardial force of contraction, with force as the fixed factor, heart ID as a random factor and stimulation frequency as a categorical variable. Tukey's post-hoc test was used to determine significance ( $p < 0.05$ ). All mean values are  $\pm$  s.e.m.

## 3. Results

Figure 1 shows individual hypoxia tolerance for each fish in the last HCT (HCT4) plotted against individual hypoxia tolerance in the previous three tests (HCT1–3). Individual hypoxia tolerance was temporally stable for the duration of the study (567 days) with certain fish consistently withstanding hypoxia longer (the 'hypoxia-tolerant' fish) and certain fish consistently succumbing to hypoxia earlier (the 'hypoxia-sensitive fish'). HCT hypoxia-tolerant fish withstood HCT4 for greater than or equal to 8.9 h, whereas all HCT hypoxia-sensitive fish lost equilibrium within less than or equal to 7.5 h. The incipient lethal oxygen saturation for HCT hypoxia-tolerant fish was less than or equal to 3.2% air saturation, while it was greater than or equal to 3.8% air saturation for HCT hypoxia-sensitive fish.

In normoxia, we found no significant difference in the force produced by muscle preparations from HCT hypoxia-tolerant fish and hypoxia-sensitive fish under control conditions



**Figure 2.** Myocardial muscle from hypoxia-tolerant fish outperforms that from hypoxia-sensitive fish under acute hypoxic exposure. Figure shows contractile force of cardiac muscle preparations from HCT hypoxia-tolerant fish and hypoxia-sensitive fish under normoxic conditions (*a–c*) and hypoxic conditions (*d–f*). (*a,d*) Myocardial force across physiologically relevant contraction frequencies under control conditions. (*b,e*) The effect of inhibiting intracellular Ca<sup>2+</sup> cycling. (*c,f*) The effect of increasing extracellular Ca<sup>2+</sup> influx with adrenaline (1  $\mu$ M). The crosses show contractile force contraction upon return to 0.2 Hz after the frequency challenges. Values are means  $\pm$  s.e.m. of  $n = 5$  preparations. Asterisks denote difference between hypoxia-tolerant and hypoxia-sensitive fish (GLM).

(figure 2*a*), after SR inhibition (figure 2*b*) or in the presence of high adrenergic stimulation (figure 2*c*). Likewise, rates of contraction or relaxation did not differ between HCT hypoxia-tolerant and hypoxia-sensitive fish at any frequency or with drug treatments under normoxia (table 1). However, under hypoxia, cardiac muscle preparations from hypoxia-tolerant fish produced significantly more force than hypoxia-sensitive fish under all conditions (control, figure 2*d*; after SR inhibition, figure 2*e*; and in the presence of high adrenaline, figure 2*f*). The difference occurred across all contraction frequencies (0.2, 0.5 and 0.8 Hz) but was most striking at the slow frequencies which are expected to occur *in vivo* during hypoxic

bradycardia [19]. The stronger myocardial contractions under hypoxia in the HCT hypoxia-tolerant fish were accompanied by faster rates of contraction and relaxation than the HCT hypoxia-sensitive fish (table 1).

#### 4. Discussion

Inter-individual variation in hypoxia tolerance remained stable for the duration of our study (567 days) indicating that acute hypoxia tolerance is a stable trait and a potential target for natural selection [20]. Indeed, fish mass increased fourfold

**Table 1.** Contractile kinetics under normoxia and hypoxia in ventricular muscle preparations from European sea bass fish in a given condition. Control saline contained 1 nM adrenaline, SR inhibition was achieved with 10  $\mu$ M ryanodine and 2  $\mu$ M thapsigargin and high adrenaline contained 1  $\mu$ M adrenaline. In total, 0.8 Hz is representative of the *in vivo* heart rate of sea bass at 12°C and bradycardia (0.2 Hz) occurs during hypoxia.  $n = 5$  in all cases except where marked with a dagger, in which  $n = 4$ . Bold typeface and asterisks denote a significant difference between hypoxia-tolerant fish and hypoxia-sensitive fish in a given condition (GLM).

	control						SR inhibition						high adrenaline						
	hypoxia-tolerant			hypoxia-sensitive			hypoxia-tolerant			hypoxia-sensitive			hypoxia-tolerant			hypoxia-sensitive fish			
	0.2 Hz	0.8 Hz	0.8 Hz	0.2 Hz	0.8 Hz	0.8 Hz	0.2 Hz	0.8 Hz	0.8 Hz	0.2 Hz	0.8 Hz	0.2 Hz	0.8 Hz	0.8 Hz	0.2 Hz	0.8 Hz	0.2 Hz	0.8 Hz	
normoxia																			
rate of contraction ( $\text{mN mm}^{-2} \text{s}^{-1}$ )	5.71 ( $\pm 1.39$ )	6.43 ( $\pm 1.78$ )	3.21 ( $\pm 0.64$ )	3.23 ( $\pm 0.80$ )	5.74 ( $\pm 1.21$ )	7.48 ( $\pm 1.48$ )	2.83 ( $\pm 0.70$ )	3.42 ( $\pm 0.74$ )	10.17 ( $\pm 2.18$ )	11.88 ( $\pm 2.50$ )	5.84 ( $\pm 1.01$ )	5.74 ( $\pm 1.21$ )	7.48 ( $\pm 1.48$ )	2.83 ( $\pm 0.70$ )	3.42 ( $\pm 0.74$ )	10.17 ( $\pm 2.18$ )	11.88 ( $\pm 2.50$ )	5.84 ( $\pm 1.01$ )	
rate of relaxation ( $\text{mN mm}^{-2} \text{s}^{-1}$ )	6.35 ( $\pm 1.72$ )	6.63 ( $\pm 1.99$ )	3.49 ( $\pm 0.60$ )	3.08 ( $\pm 0.83$ )	6.31 ( $\pm 1.53$ )	7.34 ( $\pm 1.56$ )	3.05 ( $\pm 0.66$ )	3.15 ( $\pm 0.68$ )	11.37 ( $\pm 2.70$ )	10.30 ( $\pm 2.40$ )	6.47 ( $\pm 0.98$ )	6.35 ( $\pm 1.53$ )	7.34 ( $\pm 1.56$ )	3.05 ( $\pm 0.66$ )	3.15 ( $\pm 0.68$ )	11.37 ( $\pm 2.70$ )	10.30 ( $\pm 2.40$ )	6.47 ( $\pm 0.98$ )	
resting tension ( $\text{mN mm}^{-2}$ )	2.45 ( $\pm 0.85$ )	2.37 ( $\pm 0.83$ )	1.05 ( $\pm 0.49$ )	1.02 ( $\pm 0.47$ )	2.23 ( $\pm 0.84$ )	2.28 ( $\pm 0.85$ )	0.79 ( $\pm 0.38$ )	0.79 ( $\pm 0.36$ )	2.24 ( $\pm 0.85$ )	2.27 ( $\pm 0.86$ )	0.64 ( $\pm 0.36$ )	2.45 ( $\pm 0.85$ )	2.37 ( $\pm 0.83$ )	1.05 ( $\pm 0.49$ )	1.02 ( $\pm 0.47$ )	2.23 ( $\pm 0.84$ )	2.28 ( $\pm 0.85$ )	0.79 ( $\pm 0.38$ )	
<b>hypoxia</b>	<b>0.2 Hz</b>	<b>0.8 Hz</b>	<b>0.2 Hz</b>	<b>0.8 Hz</b>	<b>0.2 Hz</b>	<b>0.8 Hz</b>	<b>0.2 Hz</b>	<b>0.8 Hz</b>	<b>0.2 Hz</b>	<b>0.8 Hz</b>	<b>0.2 Hz</b>	<b>0.8 Hz</b>	<b>0.2 Hz</b>	<b>0.8 Hz</b>	<b>0.2 Hz</b>	<b>0.8 Hz</b>	<b>0.2 Hz</b>	<b>0.8 Hz</b>	
rate of contraction ( $\text{mN mm}^{-2} \text{s}^{-1}$ )	8.59 ( $\pm 1.76$ )	7.78 ( $\pm 1.08$ )	<b>2.16</b> ( $\pm 0.47$ )*	<b>2.68</b> ( $\pm 0.34$ )*	8.89 ( $\pm 2.14$ )	8.97 ( $\pm 1.57$ )	<b>1.71</b> ( $\pm 0.33$ )*	<b>2.15</b> ( $\pm 0.42$ )*	12.19 ( $\pm 2.29$ )	9.24 ( $\pm 1.48$ )	3.38 ( $\pm 1.05$ )	8.59 ( $\pm 1.76$ )	7.78 ( $\pm 1.08$ )	<b>1.71</b> ( $\pm 0.33$ )*	<b>2.15</b> ( $\pm 0.42$ )*	12.19 ( $\pm 2.29$ )	9.24 ( $\pm 1.48$ )	3.38 ( $\pm 1.05$ )	
rate of relaxation ( $\text{mN mm}^{-2} \text{s}^{-1}$ )	10.54 ( $\pm 1.85$ )	7.98 ( $\pm 0.95$ )	<b>2.77</b> ( $\pm 0.64$ )*	<b>2.71</b> ( $\pm 0.51$ )*	10.24 ( $\pm 2.49$ )	14.88 ( $\pm 2.54$ )	<b>1.59</b> ( $\pm 0.34$ )*	<b>2.09</b> ( $\pm 0.40$ )*	14.88 ( $\pm 2.54$ )	8.57 ( $\pm 1.42$ )	3.44 ( $\pm 1.35$ )	10.54 ( $\pm 1.85$ )	7.98 ( $\pm 0.95$ )	<b>1.59</b> ( $\pm 0.34$ )*	<b>2.09</b> ( $\pm 0.40$ )*	14.88 ( $\pm 2.54$ )	8.57 ( $\pm 1.42$ )	3.44 ( $\pm 1.35$ )	
resting tension ( $\text{mN mm}^{-2}$ )	1.74 ( $\pm 0.88$ )	1.77 ( $\pm 0.90$ )	0.37 ( $\pm 0.36$ )	0.38 ( $\pm 0.37$ )	1.41 ( $\pm 0.83$ )	1.44 ( $\pm 0.82$ )	0.29 ( $\pm 0.40$ )	0.35 ( $\pm 0.41$ )	1.31 ( $\pm 0.79$ )	1.39 ( $\pm 1.08$ )	0.06 ( $\pm 0.39$ )	1.74 ( $\pm 0.88$ )	1.77 ( $\pm 0.90$ )	0.37 ( $\pm 0.36$ )	0.38 ( $\pm 0.37$ )	1.41 ( $\pm 0.83$ )	1.44 ( $\pm 0.82$ )	0.29 ( $\pm 0.40$ )	

between HCT1 and HCT4, indicating profound growth and phenotypic remodelling, while inter-individual hypoxia tolerance remained consistent. Hypoxia tolerance is a determinant of sea bass ecological performance under field conditions [9] and has been shown to have a genetic basis in other fish species (e.g. Atlantic salmon, [10]). We have shown separately [21] that robust performance under one environmental challenge test does not necessarily correlate with success in another (thermal tolerance and swimming capacity; and also see [9]). Moreover, there was no correlation between HCT performance and swimming performance in a preliminary study conducted on this cohort of sea bass (data not shown). In other words, although not directly tested here, the hypoxia-tolerant fish were not just better 'all-rounders' than the hypoxia-sensitive fish. Although still to be identified, functional trade-offs with other performance traits are likely, which helps explain why both hypoxia-tolerant and hypoxia-sensitive fish persist over time in a population.

Myocardium from hypoxia-tolerant sea bass performed better under hypoxia than myocardium from hypoxia-sensitive sea bass, linking individual variability in hypoxia tolerance with individual variability in intrinsic cardiac performance. We reasoned that superior contractility under hypoxia would be due to greater involvement of the intracellular  $\text{Ca}^{2+}$  stores of the SR because the SR has been implicated in augmenting  $\text{Ca}^{2+}$  delivery to the heart during periods of stress [22]. However, we found no support for this hypothesis as inhibiting the SR did not affect force production or contractile kinetics under normoxia or hypoxia. Without  $\text{Ca}^{2+}$  cycling through the SR, the sea bass heart would depend wholly on extracellular  $\text{Ca}^{2+}$  for contraction. We show that increasing transmembrane  $\text{Ca}^{2+}$  flux with adrenergic stimulation ( $1 \mu\text{M}$ ) increased force production, but did not explain the difference in individual performance during hypoxia.

Because we assessed myocardial performance *in vitro*, the mechanism underlying superior contractile performance of the hypoxia-tolerant fish in hypoxia must be intrinsic to the heart (i.e. not involving autonomic control). It is possible that variation in cardiac metabolism or myoglobin content may be related to hypoxic performance as fish with a high cardiac myoglobin concentration perform better during hypoxia [10,23].

This study suggests variation in cardiac hypoxia tolerance could, at least in part, underlie variation in whole-animal hypoxia tolerance. Although this finding is based on a small sample size, when it is considered in combination with the finding that hypoxia-tolerant fish have higher survivorship in semi-natural conditions [9], we suggest that the heart may be an important target for natural selection in a future where marine environments are oxygen-stressed. Indeed, we speculate that similar to growing evidence for temperature-tolerance [11], hypoxia tolerance of the fish cardiovascular system may be important in determining fish distribution and survival in the changing oceans.

**Ethics.** All animal procedures were in accordance with the French and EU guidelines for animal research (CGP151019-FISHHEALTH).

**Data accessibility.** Raw data have been deposited in Dryad.

**Authors' contributions.** W.J., K.O., G.C. and H.A.S. designed the study; W.J., K.O., F.M., H.O. and G.C. conducted the study; W.J. and K.O. analysed the data and W.J., K.O. and H.A.S. wrote the manuscript. All authors approve the final version and agree to be accountable for the work in the manuscript.

**Competing interests.** The authors declare they have no competing interests.

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## Chapter III: Identification of the confounding factors and the mechanistic basis of performances measured

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#### 4. Paper 4 (in prep.)

1 Exposure of European sea bass (*Dicentrarchus labrax*) to  
2 chemically dispersed oil has a chronic residual effect on  
3 hypoxia tolerance but not aerobic scope

4

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19 Key words: dispersed oil exposure, PAH, aerobic capacity, hypoxia tolerance, hypoxia  
20 resistance, scope for survival, accumulated oxygen deficit.

21

22 **Abstract**

23 We tested the hypothesis that the chronic residual effects of an acute exposure  
24 of European sea bass (*Dicentrarchus labrax*) to chemically dispersed crude oil is  
25 manifest in indices of hypoxic performance rather than aerobic performance. Sea bass  
26 were first pre-screened with a hypoxia challenge test to establish their incipient lethal  
27 oxygen saturation (ILOS), but on discovering a wide breadth for individual ILOS values  
28 (2.6-11.0 % O<sub>2</sub> saturation), fish were subsequently treated as either hypoxia sensitive  
29 (HS) or hypoxia tolerant (HT) phenotypes, traits that were shown to be experimentally  
30 repeatable. The HT phenotype was characterized by a lower ILOS and critical oxygen  
31 saturation (O<sub>2crit</sub>) compared with the HS phenotype and was subsequently found switch  
32 to glycolytic metabolism at a lower dissolved oxygen even though both phenotypes  
33 eventually accumulated lactate and glucose to the same plasma concentrations at  
34 ILOS. We discovered no residual effect of oil on any of the indices of aerobic  
35 performance, independent of the phenotype, as hypothesized, but contrary to our  
36 hypothesis oil exposure had no residual effect on any of the indices of hypoxic  
37 performance in the HS phenotype. Oil exposure, however, had residual effects on the  
38 HT phenotype because it impaired the repeatability of hypoxic survival traits of the and  
39 also increased O<sub>2crit</sub> by 24 %, scope for oxygen deficit by 40 %, the factorial scope for  
40 oxygen deficit by 17 % and accumulated oxygen deficit by 57 %. Thus, sea bass with a  
41 HT phenotype remained chronically impaired for a minimum of 167 days following an  
42 acute 24-h oil exposure even though the HS phenotype did not. We reasoned that  
43 impaired oxygen extraction at gill due to oil exposure activates glycolytic metabolism at  
44 a higher dissolved oxygen level, conferring an inferior hypoxia resistance for the HT  
45 phenotype, which might eventually compromise the ability to survive hypoxic episodes.  
46

47 **1. Introduction**

48 Forecasting the consequence of an exposure to crude oil on the health status of  
49 a fish population remains a challenging exercise due to a number of issues that must be  
50 properly addressed if projections are to be of some operational value (Allen and Moore,  
51 2004; Fodrie et al., 2014; Chang et al., 2014). Among these issues, the mode of  
52 exposure (acute *versus* chronic), the duration of the exposure (short *versus* long) and  
53 the length of time over which potential impacts are evaluated (immediate *versus* latent  
54 effects) are of enormous importance. At first glance, one might think that such matters  
55 have been resolved a long time ago. Unfortunately, this is not the case and, although  
56 not new, they remain topical (Wiens, 2015). A review of the literature indeed shows that  
57 available information regarding the toxicological impacts of oil exposure on fish is highly  
58 skewed toward studies combining acute exposure, short exposure duration and  
59 immediate assessment, generally at low biological organization levels. While these  
60 types of studies continue to fill gaps in our mechanistic understanding of contamination  
61 and decontamination processes, and associated functional impairments (Incardona et  
62 al., 2004, 2005, 2007, 2015), they are of limited help when addressing issues such as  
63 the impact on the resilience, health, production and recruitment of the affected  
64 populations (Forbes et al., 2006; Brander et al., 2015).

65 Gills are inherently susceptible to being affected by water-borne contaminants  
66 and, despite their key role in shaping a fish's aerobic capacity and activities, little is  
67 known about the long-term effects (beyond a month) of even brief contacts with oil upon  
68 fish respiratory capacity and downstream performances. For instance, lifting and  
69 hypertrophy of secondary lamellar epithelium (Engelhardt et al., 1981; Moles and

70 Norcross, 1998; Stephens et al., 2000), aneurysms (Claireaux et al., 2004; Farrell et al.,  
71 2004) and even disappearance of secondary lamellae (Kochhann et al., 2015) have  
72 been reported immediately following an acute oil exposure. Even though these  
73 alterations are expected to cause osmoregulatory disorders, hypoxemia and  
74 compensatory changes in gill ventilation and perfusion, together with changes in cardiac  
75 output and scope for aerobic metabolic activities (Perry, 1998; Farrell and Richards,  
76 2009; Gamperl and Driedzic, 2009), the residual consequences of such acute  
77 impairment is poorly studied. Moreover, available information about the short-term  
78 consequences of an acute exposure to crude oil upon fish respiratory and metabolic  
79 performance is contradictory. For instance, while residual impacts on metabolic demand  
80 (Claireaux et al. 2004; Davoodi and Claireaux 2007; Claireaux and Davoodi, 2010;  
81 Klinger et al., 2014) and swimming performance (Kennedy and Farrell, 2006; Mager et  
82 al., 2014) were observed over days to weeks post-exposure by some authors, they  
83 were not observed by others (e.g., Milinkovitch et al., 2012; Lucas et al., 2016).

84         Only a handful of published studies have tackled the issue of the long-term  
85 influence of oil exposure on fish capacity to meet the challenges of everyday life. For  
86 example, Claireaux et al. (2004) and Gilliers et al. (2012) showed that a severe  
87 exposure to crude oil of group 0<sup>+</sup> and group 1<sup>+</sup> common sole (*Solea solea*) had lasting  
88 residual effects (3-6 months post-exposure) on survival, otolith and somatic growth, as  
89 well as on energy storage (triacylglycerol to free sterol ratio). Similarly, Hicken et al.  
90 (2011) observed a latent 18 % reduction in critical swimming speed of zebrafish (*Danio*  
91 *rerio*) 1 year after a 48-h oil exposure. However, Claireaux et al. (2013) showed that 1  
92 month post-exposure to a mild dose of oil or oil plus dispersant mixture, the mean

93 tolerance time (time to loss of equilibrium) of a population of European sea bass  
94 (*Dicentrarchus labrax*) exposed to a standardized hypoxia challenge test or to a  
95 temperature challenge test was unaffected. Instead, they revealed that individual pre-  
96 versus post-exposure performance repeatability was altered in the exposed fish,  
97 suggesting an inter-individual variability in the impact of the crude oil treatments. This  
98 suggestion was supported by transferring these fish to highly selective semi-natural field  
99 mesocosmes ( $\approx 30\%$  overall survival after 6 months) in which survivors were those  
100 individuals who had displayed the best performance in the pre-transfer challenge test.  
101 Compared to the control, unexposed population, this yielded a significantly lower growth  
102 rate in the sea bass population exposed to chemically dispersed oil and to a higher  
103 mortality in the populations exposed to oil or to chemically treated oil. More recently,  
104 Mauduit et al. (2016) reported that fish acutely exposed to chemically dispersed oil  
105 continued to display lower hypoxia tolerance and swimming capacity for 1 month post-  
106 exposure but not beyond as oil-exposed populations grew and survived with similar  
107 rates than control sea bass in the aforementioned field mesocosmes but under mild  
108 selective pressure ( $\approx 65\%$  overall survival after 4 months). Moreover, at 10 months  
109 post-exposure, no residual effects on hypoxia tolerance, temperature susceptibility and  
110 maximal swimming speed were found (Mauduit et al., 2016). This suggested that fish  
111 had recuperated at least part of their physiological functions, but whether or not this  
112 recovery is complete remains a question, and is a focus of the present study.

113         While aerobic metabolic capacity is a key determinant of a fish's ability to make  
114 to most of the resources available in its habitat (Fry, 1971; Claireaux and Lefrançois,  
115 2007; Farrell 2016), fish routinely encounter many environmental situations where

116 metabolic demand exceeds the immediate capacity to supply oxygen to tissues (e.g.,  
117 environmental hypoxia, excessive temperature, high water velocities, chasing,  
118 escaping). In such circumstances, survival is determined by individual's capacity to  
119 produce ATP anaerobically and/or to behaviorally or physiologically reduce metabolic  
120 demand (Nilsson and Östlund-Nilsson, 2008). Despite this key role, the latent  
121 consequences of oil exposure on fish anaerobic performance have received very little  
122 attention (Mauduit et al., 2016).

123         The present study therefore designed a physiological paradigm that entailed a  
124 suite of respiratory indices to comprehensively characterize the aerobic and anaerobic  
125 performance of fish, and coined it as integrated respiratory assessment paradigm  
126 (IRAP). The paradigm was implemented to investigate the variation of hypoxia tolerance  
127 in fish and the residual effects of an acute exposure to chemically dispersed crude oil  
128 upon fish capacity to respond to reduced oxygen availability. Specifically, we used a 3  
129 to 4-day experimental protocol to derive well-established indices of respiratory  
130 capabilities for normoxia and hypoxia [i.e., standard metabolic rate (SMR), maximum  
131 rate of oxygen uptake ( $\dot{M}O_{2max}$ ), absolute aerobic scope (AAS), factorial metabolic  
132 scope (FAS), excess post-exercise oxygen consumption (EPOC), critical oxygen  
133 saturation ( $O_{2crit}$ ) and incipient lethal oxygen saturation (ILOS)]. While the difference  
134 between standard metabolic rate ( $O_{2crit}$ ) and the minimum metabolic rate (ILOS) helps  
135 characterize a fish's scope for survival under unfavorable hypoxic conditions  
136 (Hochachka, 1990), we derived three new indices to better define the impacts of  
137 hypoxia (Claireaux and Chabot, 2016) [i.e., scope for oxygen deficit (SOD;  $O_{2crit}$  minus  
138 ILOS), factorial scope for oxygen deficit (FSOD;  $O_{2crit} \text{ ILOS}^{-1}$ ) and accumulated oxygen

139 deficit (AOD): see section 2.5 for calculations]. Of these new indices, AOD should be  
140 the most sensitive in revealing the residual consequences of oil exposure on fish  
141 anaerobiosis, if any exist, because it assesses glycogen depletion and accumulation of  
142 anaerobic metabolic end products by taking time and dissolved oxygen (DO) into  
143 account (Nilsson and Östlund-Nilsson 2008; Mandic et al., 2013). Therefore, we tested  
144 the prediction that IRAP would reveal chronic residual effects of an acute oil exposure in  
145 the indices of hypoxic performance rather than those of aerobic performance, in the  
146 form of elevated  $O_{2crit}$ , SOD, FSOD and AOD in oil-treated fish.

147

## 148 **2 Materials and methods**

### 149 **2.1 Animals**

150 Sea bass were obtained from a commercial farm (Aquastream, Lorient, France)  
151 and were housed at Ifremer laboratory (Brest, France) in a 2000-L indoor tank where  
152 they were fed a commercial diet (Neo Start Coul 2, Le Gouessant, France) via an  
153 automatic feeder (1.5 % body weigh daily). Water temperature (10 – 20 °C),  
154 photoperiod and salinity (30 – 32 PSU) varied seasonally. Within two weeks upon  
155 arrival, fish were anaesthetized (MS-222; 100 mg L<sup>-1</sup>) and implanted subcutaneously  
156 with a radio frequency identification tag (RFID; Biolog-id, France). Fish were unfed for  
157 48 h before any experiment. All procedures followed current animal care rules and  
158 regulations in France.

### 159 **2.2 Overall experimental approach**

160 A two-phase experimental approach was followed. During the first phase, we  
161 used 2-year old fish to examine the range of inter-individual variation of hypoxia

162 tolerance in sea bass and established hypoxia-tolerant (HT) and hypoxia-sensitive (HS)  
163 phenotypes. This phase of the work also investigated whether HT- and HS-phenotypes  
164 accumulated plasma lactate and glucose at different rates during a hypoxia challenge  
165 test (HCT). During the second phase, the residual effects of an acute exposure to  
166 dispersed crude oil were assessed in 1-year old individuals. During this phase, fish were  
167 first submitted to a HCT, followed by a 48-h exposure to chemically treated oil (exposed  
168 fish) and, 50 days later, by a second HCT for both exposed (N=X) and control (N=Y)  
169 fish, which assessed the repeatability of hypoxia tolerance for individual fish. Moreover,  
170 7 months post-exposure, a respirometry study on a sub-sample of exposed and control  
171 fish compared the long-term effects of chemically treated oil upon the aerobic and  
172 anaerobic capacities of HS and HT phenotypes. Figure 1 explains the different events  
173 pertaining to Phases one and two.

174

## 175 **2.3 Methodologies**

### 176 **2.3.1 Hypoxia challenge test**

177 The primary means for examining hypoxic performance and establishing  
178 individuals' hypoxic phenotype was a hypoxia challenge test (HCT), which has been  
179 fully described in Claireaux et al. (2013). Briefly, water oxygenation was rapidly (~40  
180 min) decreased to 20 % air saturation (% sat) by introducing nitrogen gas into the  
181 experimental arena via a submersible pump, after which a slower decrease (0.2% sat  
182 per 10 min) ensued until all fish had lost the capacity to maintain their equilibrium  
183 (typically 6-8 h below 20% sat). Water oxygenation was monitored with an oxygen  
184 meter (Ponsel Mesure, Caudan, France; accuracy:  $\pm 0.2\%$  sat) and regulated by a

185 controller and solenoid valve (Oxy-REG; Loligo Systems, Tjele, Denmark). Each time a  
186 fish lost its equilibrium, the corresponding oxygenation level was recorded as ILOS and  
187 the fish was quickly removed from the experimental arena, identified (RFID tag reading)  
188 and assigned as either a HT or HS phenotype according to the time of ILOS relative to  
189 other fish. These fish were then either immediately sampled (phase 1) or recovered in  
190 an aerated aquarium (phase 2).

191

### 192 **2.3.2: Respirometry**

193         Respirometry trials lasted 2-3 days and 8 fish were tested simultaneously using 8  
194 intermittent-flow respirometers (volume ~2.1 L) submerged in a thermoregulated ( $16.5 \pm$   
195  $0.5^\circ\text{C}$ ; Teco, Seachill TR20) an aerated water reservoir (2 m x 0.6 m and 0.4 m deep).  
196 Fish were fasted for 72 h to reach a post-absorptive stage prior to respirometry (Dupont-  
197 Prinnet et al., 2010; Chabot et al., 2016).

198         The water from the reservoir was re-circulated to each of the respirometers using  
199 computer-controlled flush pumps (Compact 600, EHEIM, Germany), relays and  
200 software (AquaResp, University of Copenhagen, Helsingør, Denmark). Each  
201 respirometry chamber also had its own circulation loop in which an optical oxygen probe  
202 (Robust Oxygen Probe OXROB3, Pyroscience, Germany & Dipping probe oxygen  
203 minisensor, PreSens, Germany) continuously monitored DO inside the chamber. Each  
204 oxygen probe was calibrated to 0 %  $\text{O}_2$  saturation (% sat; sodium sulfite saturated  
205 water) and 100 % sat (fully aerated water) prior a respirometry trial. Each trial began by  
206 hand chasing fish to exhaustion in a 20-L bucket for 10-min and air exposed for 2-min.  
207 Exhausted fish were then placed in a respirometer and the  $\dot{M}\text{O}_2$  measurement cycle

208 immediately started. The first  $\dot{M}O_2$  value was typically available after 250 ~~370~~ s.  
209 Measurement cycles consisted of an opened period, during which respirometers were  
210 replenished with water from the surrounding reservoir, and a sealed period, when the  
211 flushing pumps were turned off and the decline in DO due to fish  $\dot{M}O_2$  was followed.  
212 Note that DO was kept > 85 % sat at all time and that the first 30 s of the sealed period  
213 (stabilization period) was not used to calculate fish  $\dot{M}O_2$ . For the two hours that followed  
214 the introduction of the fish in the respirometers, measurement cycles were 120 s flush,  
215 30 s stabilization and 220 s measurement. This allowed the capture of the highest  $\dot{M}O_2$   
216 values, which were assigned  $\dot{M}O_{2max}$ . After 2 h, a longer measurement cycle (60 s flush,  
217 30 s stabilization and 440 s measure) was used while fish were left undisturbed for 2 or  
218 3 days in a shaded, quiet room so that  $\dot{M}O_2$  would reach SMR (Chabot et al., 2016). On  
219 the last day of each respirometry trial, hypoxic water was generated by pumping  
220 (Compact 600, EHEIM, Germany) reservoir water to the top of a gas equilibration  
221 column where it trickled down through nitrogen gas and into the reservoir and, during  
222 the following flush cycle, into the respirometers. Dissolved oxygen progressively  
223 decreased to 10% sat over 4-6 h period while  $\dot{M}O_2$  was measured to determine  $O_{2crit}$   
224 (Claireaux and Chabot, 2016). Fish were then quickly removed from their respirometers  
225 and recovered in fully aerated water at acclimation temperature. Once emptied, the  
226 background  $\dot{M}O_2$  of each respirometer was measured (30-min) and the entire system  
227 was then disinfected with household bleach.

### 228 **2.3.3 Tissues PAH concentration**

229 To quantify PAH bioaccumulation in the fish tissues, and to assess the  
230 detoxification and depuration processes, 3 fish per exposition tank (n = 9 per treatment)

231 were euthanized to measure PAH concentrations in the bile (24 h after exposure) and  
232 liver (24 h and 1 month post-exposure). Liver concentrations of 20 PAH compounds  
233 (including the components listed by United States Environmental Protection Agency)  
234 were assessed as fully described by Lacroix et al. (2014). Briefly, PAH were extracted  
235 from liver samples using an alkaline digestion combined with stir bar and were analyzed  
236 with sorptive extraction-thermal desorption-gas chromatography-mass spectrometry  
237 (SBSE-GC-MS). Bile PAH metabolites were determined semi-quantitatively using a  
238 fluorospectrophotometer (Aas et al., 2000) with a 5 nm slit width on emission and  
239 excitation channels (Jasco FP-6200, Tokyo, Japan). Analyses were performed using  
240 three excitation-emission wavelengths i.e., 295-335 (naphthalene-type metabolites);  
241 343-383 nm (four-ringed compounds) and 380-430 nm (benzo[a]pyrene-type  
242 metabolites) (Krahn et al., 1987; Lin et al., 1996; Aas et al., 2000).

243

## 244 **2.4 Experimental protocols**

### 245 **2.4.1 Phase 1: Comparing the changes in plasma metabolites during a HCT**

246 On December 12<sup>th</sup> 2013 (water temperature 11°C), 2-year-old sea bass ( $33.9 \pm$   
247  $3.1\text{cm}$ ;  $284.6 \pm 12.2\text{ g}$ ;  $n = 35$ ) were subjected to a HCT to assign a hypoxic phenotype  
248 (HS or HT), based on the individual's incipient lethal oxygen saturation (ILOS). A  
249 second HCT was performed 9 days later (December 21<sup>th</sup>, 2013), during which 3-4 fish  
250 per phenotype were euthanized with a blow to the head at 2 h time interval and 1 ml of  
251 blood withdrawn from the caudal vein using heparinized syringes. Plasma samples were  
252 collected after centrifugation (8 min at 8000 rpm; 4°C) and frozen in liquid nitrogen  
253 before being stored at -20 °C until they were assayed for lactate and glucose using

254 commercial kits (Biomérieux; reference: 61.192 and 61.269, respectively) and a  
255 spectrophotometer (Uvikon 923;  $\lambda = 505$  nm).

256

257 **2.4.2 Phase 2: Residual effects of crude oil exposure**

258         Thirty-three days prior to the oil exposure, a pre-exposure HCT (February 27<sup>th</sup>,  
259 2014) was conducted on 1-year old juveniles (n = 161) at 10°C to identify HT and HS  
260 phenotypes. On April 1<sup>st</sup>, 2014, all 161 fish ( $11.3 \pm 1.1$  cm,  $17.7 \pm 5.0$  g) were then  
261 transported 12 km to the CEDRE for the 48-h exposure to chemically treated oil. At  
262 CEDRE fish were habituated in 2 m<sup>3</sup> tanks supplied with open flow seawater. Water  
263 temperature, salinity and photoperiod were similar as in their Ifremer rearing facility.  
264 Fish were off-feed. Fish exposition set-up consisted in a set of polyethylene tanks  
265 (volume = 300 L) equipped with home-made oil/water mixing devices comprising a  
266 funnel and a submersible bilge pump (Milinkovitch et al., 2011). The treatment groups  
267 were control and chemically dispersed oil (250 g of oil mixed with 10 g of dispersant)  
268 and each one was tested in triplicate. The oil exposure protocol started with bubbling  
269 the oil with air overnight to mimic a 12-h aging of an oil slick at sea (Nordvik, 1995;  
270 Mauduit et al., 2016). Fish were then allocated to the various exposure tanks in a way  
271 that avoided statistical difference in ILOS among the tanks (27 fish per tank). Crude oil  
272 and dispersant (Finasol OSR 52, Total Fluides) was mixed in a bottle in accordance  
273 with manufacturer's recommendation before being poured into 3 of tanks. During the  
274 48-h exposure period, DO in the tanks was maintained above 85 % sat. Following a 48-  
275 h exposure period, fish were briefly bathed in clean seawater to avoid cross-  
276 contamination between the treatment groups and transferred to their original rearing

277 tanks, where they recovered for one week before being transported back to Ifremer.  
278 Control fish went through the identical processes without being exposed to oil. No fish  
279 mortality occurred during transport, oil exposure or the intervening recovery.

280 The second HCT was conducted on 96 fish ( $28.9 \pm 0.7$  g) 49 days after oil  
281 exposure (May 20<sup>th</sup>, 2014) and at ambient temperature (15°C) to test for the individual  
282 repeatability of ILOS. Respirometry trials on 28 fish ( $68.8 \pm 2.6$  g) were started 167 days  
283 post-oil exposure and lasted 18 days. These trials were performed at  $16.5 \pm 0.5^\circ\text{C}$  on a  
284 sub-group of fish that fully represented the breath of ILOS.

285

## 286 **2.5 Data analysis**

287 For each measurement cycle, the slope of declining DO in the respirometer was  
288 calculated by linear regression and  $\dot{M}O_2$  determined using the R statistical language (R  
289 Core Team, 2015). Only DO recordings with a linear decline in DO and an  $R^2 > 0.94$   
290 were accepted. Typically, the first  $\dot{M}O_2$  was designated  $\dot{M}O_{2\text{max}}$  because it was the  
291 highest  $\dot{M}O_2$  value after the exhausted fish was placed into the respirometer (Clark and  
292 Norin, 2016). However, fish were spontaneously active over the next 2-3 days and, in  
293 some cases (n=16), a slightly higher metabolic rate was recorded. When this occurred,  
294 this higher value was assigned to  $\dot{M}O_{2\text{max}}$ . Standard metabolic rate (SMR; minimal  
295 maintenance in post-absorptive and inactive states; Fry and Hart, 1948; Chabot et al.,  
296 2016) was determined using a quantile method (q = 0.2) by applying an R script  
297 (Chabot et al., 2016) to the 2-3 days of continuous  $\dot{M}O_2$  measurements after excluding  
298 the  $\dot{M}O_2$  values in the first 12 h of recovery and hypoxic exposure (326 – 489 individual  
299  $\dot{M}O_2$  measurements per fish). Excess post-exhaustion oxygen consumption (EPOC)

300 was individually determined by integrating  $\dot{M}O_2$  values during recovery until SMR plus  
301 10% was reached (note that EPOC could not be calculated reliably in 6 fish due to their  
302 spontaneous activities). An R script (Claireaux and Chabot, 2016) derived  $O_{2crit}$  from the  
303 intersection of SMR and a regression line for the  $\dot{M}O_2$  measurements as a function of  
304 DO during a hypoxic exposure (Ultsch et al., 1978; Claireaux and Chabot, 2016).  
305 Absolute aerobic scope (AAS) was calculated as  $\dot{M}O_{2max} - SMR$  (Fry, 1971; Claireaux  
306 et al., 2005; Pörtner and Farrell, 2008) and factorial aerobic capacity (FAS) was  
307 calculated as  $\dot{M}O_{2max} / SMR$  (Clark et al., 2005). The ILOS in respirometry trial was  
308 extrapolated using  $\dot{M}O_2$  and DO when fish lost its up-right equilibrium in the previous  
309 HCT (Joyce et al., 2016). Then, the relationship of  $\dot{M}O_2$  and DO obtained from the  $O_{2crit}$   
310 analysis (Figure 2) was used to calculate the corresponding  $\dot{M}O_2$  for each DO values in  
311 HCT of respirometry trials. Moreover, the magnitude of the depletion of glycogen stores  
312 was further assessed with the scope for oxygen deficit (SOD; % sat.;  $O_{2crit} - ILOS$ ), the  
313 factorial scope for oxygen deficit (FSOD; dimensionless;  $O_{2crit} ILOS^{-1}$ ) and accumulated  
314 oxygen deficit (AOD;  $mg O_2 kg^{-1}$ ), an integration of  $\dot{M}O_2$  as a function of time for the  
315 area bounded by SMR and the calculated  $\dot{M}O_2$  from  $O_{2crit}$  to ILOS.

316 ILOS values for the HT and HS phenotypes of the phase 1 were statistically  
317 compared with a Student's t-test, while the plasma lactate and glucose were compared  
318 with a two-way (time  $\times$  phenotype) analysis of covariance (ANCOVA) using body mass  
319 as covariate and a Tukey-Kramer *post-hoc* test. Bile fluorescence (phase 2) was  
320 compared for each wavelength (290-355 nm, 343-383 nm and 380-430 nm) between  
321 control and oil-exposed groups using a Student's t-test. The sum of 20 PAH  
322 components in liver tissues were compared between control and oil-exposed groups at

323 day 2 and day 31 using pairwise t-tests with a *Holm-Šidák* correction. Repeatability of  
324 hypoxic survival traits in both control and oil-treated fish was assessed with Spearman  
325 rank-order correlation coefficient for the ILOS measured pre- and post- exposure HCT,  
326 as well as the  $O_{2crit}$  determined with respirometry and the ILOS extrapolated in  
327 respirometry trial.

328 In order to correct heterogeneity of variance in  $O_{2crit}$ , log transformations were  
329 applied. To test for a dispersed crude oil treatment effect, a Welch one-way ANOVA  
330 compared the SMR,  $\dot{M}O_{2max}$ , AAS, FAS,  $\log O_{2crit}$ , ILOS, SOD, FSOD and AOD values  
331 between control and oil-exposed groups. To test the phenotypically specific responses  
332 to dispersed oil, a Welch one-way ANOVA with a Tukey's Kramer *post-hoc* was applied  
333 to compare the same suite of indices among control HT, control HS, oil HT and oil HS  
334 groups. Statistical analyses were conducted in R (ver.3.2.2; R Development Core  
335 Team, 2015) and SPSS v. 23 (SPSS Inc., Chicago, IL, USA). Statistical significance  
336 was assigned to  $\alpha \leq 0.05$ . Values were presented as mean  $\pm$  s.e.m.

337

### 338 **3 Results**

#### 339 **3.1 Phase 1: Metabolite accumulation in HT and HS phenotypes**

340 The incipient lethal oxygen saturation (ILOS) of 2-year old fish (n=35) ranged  
341 between 4.5 and 7.0 % sat (Figure 3a). As the HCT progressed, sea bass became  
342 progressively hypoxemic, as indicated by the increases in plasma lactate (Figure 4a)  
343 and plasma glucose (Figure 4b) concentrations which denote the switch to glycolytic  
344 metabolism and the mobilization of glycogen reserves. In the hypoxia tolerant (HT)  
345 phenotype (ILOS < 5.6 % sat), plasma lactate and glucose increased at significantly

346 lower rate compared with the hypoxia sensitive (HS) phenotypes (ILOS > 5.6 % sat). As  
347 a result, when DO reached 5.7 % sat (~7 h into the HCT), HT phenotypes had  
348 significantly ( $p \leq 0.004$ ) lower plasma lactate and glucose concentrations than HS  
349 phenotypes (lactate:  $5.8 \pm 0.8$  mM vs  $16.8 \pm 2.4$  mM; glucose:  $1.1 \pm 0.10$  g L<sup>-1</sup> vs  $1.9 \pm$   
350  $0.10$  g L<sup>-1</sup>, respectively). Nevertheless, both phenotypes reached similar peak values for  
351 plasma lactate and glucose (lactate: HS =  $14.7 \pm 1.1$  mM; HT =  $16.8 \pm 2.4$  mM; glucose:  
352 HS =  $1.88 \pm 0.10$  g L<sup>-1</sup>; HT =  $1.95 \pm 0.16$  g L<sup>-1</sup>; Figure 4) at the conclusion of the HCT  
353 when sea bass lost equilibrium at ILOS, even though ILOS was at lower DO and took  
354 20 % longer to reach for the HT than for the HS phenotype.

355

### 356 **3.2 Phase 2: Variability in hypoxia tolerance in control, unexposed fish**

357 Like oil-exposed fish, control fish were subjected to two HCT (Feb. 27 and May  
358 20 2014), but were not exposed to oil between the tests, so that the repeatability of their  
359 ILOS can be tested. ILOS had a wider range during the first HCT (2.6 – 9.9 % sat.) than  
360 during the second HCT (5.3 – 10.1 % sat.; Figure 3b-c). Only one control individual had  
361 a drastic shift in hypoxia tolerance during the 7-month period that separated the two  
362 HCT (Figure 5; pairwise correlation tests:  $r \geq 0.673$ ,  $p \leq 0.023$ ; Table 1).

363 During the second HCT, control fish of HT phenotype used for respirometry  
364 experiments had a 31 % lower ILOS compared with control HS phenotype ( $6.2 \pm 0.1$  %  
365 sat vs  $9.0 \pm 0.2$  % sat;  $p < 0.001$ ). Respirometry trials revealed that the control HT  
366 phenotype had a 24 % lower  $O_{2crit}$  than control sea bass with the HS phenotype ( $14.0 \pm$   
367  $0.3$  % sat vs.  $18.4 \pm 0.6$  % sat;  $p = 0.007$ ). In control sea bass, SOD, FSOD and AOD  
368 were the same for the two phenotypes ( $p \geq 0.25$ ; Figure 6).

369

370 **3.3 Phase 2: Dispersed oil exposure**

371 **3.3.1 Bio-accumulation and excretion of PAH**

372 As expected, the liver PAH concentration measured 2 days after exposure was  
373 26-fold higher for oil-treated fish than control fish ( $1508.9 \pm 158.6 \text{ ng g}^{-1} \text{ dw}$  vs.  $58.9 \pm$   
374  $6.3 \text{ ng g}^{-1} \text{ dw}$ ;  $p < 0.001$ ). Similarly, oil-treated sea bass displayed significantly higher  
375 PAH levels in their bile than control fish across all three wavelengths at 2 days post-  
376 exposure (Figure 7). Specifically, the reading at 343-383 nm for oil-treated fish was a  
377 21-fold higher ( $1444 \pm 50.9$  vs.  $69.6 \pm 3.3$  arbitrary units,  $p < 0.001$ ), 9-fold higher at  
378 380-430 nm ( $223.1 \pm 9.9$  vs.  $24.0 \pm 1.3$  arbitrary units,  $p < 0.001$ ) and 2-fold higher at  
379 290-335 nm ( $771.2 \pm 82.8$  vs.  $383.2 \pm 12.7$  arbitrary units,  $p < 0.001$ ). Detoxification and  
380 PAH excretion during the 31-day post-exposure recovery period reduced liver PAH  
381 concentration in oil-treated fish by 47-fold. Although PAH values measured 31 days  
382 post-exposure were similar to that of the pre-exposure control fish ( $4.0 \pm 0.7 \text{ ng g}^{-1} \text{ dw}$   
383 vs.  $31.9 \pm 11.7 \text{ ng g}^{-1} \text{ dw}$ ,  $p = 0.017$ ), these values were significantly higher than those  
384 measured in the control fish post-exposure ( $4.0 \pm 0.7 \text{ ng g}^{-1} \text{ dw}$ ;  $p = 0.02$ ;

385 Figure 8).

386

387 **3.3.2 Chronic residual effects of chemically dispersed oil**

388 A 48-h exposure to dispersed oil had no effect on the respirometry metabolic  
389 indices measured on the 1-year old sea bass population (SMR,  $\dot{M}O_{2\text{max}}$ , AAS, FAS,  
390 EPOC;  $p \geq 0.21$ ; Figure 8a; Table 2), regardless of fish's hypoxic phenotype (HT or HS).  
391 However, chemically dispersed oil abolished the repeatability of hypoxia resistance as

392 measured by the HCT ( $r = 0.355$ ,  $p = 0.162$ ; Table 1). Specifically, while ILOS  
393 measured 49 days post-exposure and extrapolated from respirometry (167 days post-  
394 exposure) was repeatable ( $r = 0.988$ ,  $p < 0.001$ ; Figure 7b; Table 1), ILOS and  $O_{2crit}$   
395 measured with respirometry were no longer correlated ( $r = 0.142$ ,  $p = 0.586$ ) as  
396 observed in the oil-treated fish (Figure 5a; Table 1). This residual effect of exposure to  
397 dispersed crude oil was also evidenced in the significantly 24 % higher  $O_{2crit}$  in the oil-  
398 treated compared with the control HT phenotype ( $17.4 \pm 0.7$  % sat. vs.  $14.0 \pm 0.3$  %  
399 sat.;  $p = 0.034$ ), despite similar ILOS values ( $6.5 \pm 0.2$  % sat. vs.  $6.2 \pm 0.1$  % sat.,  
400 respectively;  $p = 0.8$ ; Figure 8b). As a result, oil-treated HT and HS phenotypes had the  
401 same  $O_{2crit}$  ( $p = 0.673$ ), even though ILOS of the oil-treated HT phenotypes was 25 %  
402 lower than the HS counterpart ( $6.5 \pm 0.2$  % sat. vs.  $8.7 \pm 0.1$  % sat.,  $p < 0.001$ ; Figure  
403 8b). Chronic residual effects that were specific to the HT phenotype were also evident in  
404 the 57 % elevation of AOD ( $291.1 \pm 28.9$  mg  $O_2$   $kg^{-1}$  vs.  $185.8 \pm 13.6$  mg  $O_2$   $kg^{-1}$ ;  $p =$   
405  $0.008$ ), the 40 % elevation of SOD ( $10.9 \pm 0.5$  % sat. vs.  $7.8 \pm 0.3$  % sat.;  $p = 0.035$ ),  
406 and the 17 % elevation of FSOD ( $2.7 \pm 0.04$  vs.  $2.3 \pm 0.06$ ;  $p = 0.003$ ; Figure 8b). In  
407 contrast, oil exposure of HS fish had no chronic residual effect on  $O_{2crit}$ , SOD, FSOD  
408 and AOD ( $p > 0.226$ ; Figure 8b).

409

#### 410 **4 Discussion**

411 The general objective of using IRAP in this work was to examine the residual  
412 effects of an acute exposure to chemically dispersed oil on the aerobic and anaerobic  
413 performance of the European sea bass and its ability to respond to reduced oxygen  
414 availability. To better reveal the effect of dispersed oil-exposure, the distribution of

415 hypoxia resistance within our experimental populations (yearling and two-year-old) was  
416 determined prior to exposition and then taken into consideration in our analyses.  
417 Experimental populations were prescreened using a hypoxia challenge test (HCT;  
418 Claireaux et al., 2013) and individuals situated at the tail were assigned as hypoxia  
419 tolerant (HT) phenotype, whereas those situated at the head of the distribution were  
420 assigned as hypoxia sensitive (HS) phenotype. We confirmed the wide breadth of the  
421 distribution of hypoxia tolerance within sea bass populations. Furthermore, we provided  
422 mechanistic evidence to support the distinction between HS and HT phenotypes by  
423 showing that the former switched on glycolytic metabolism at a higher DO than the  
424 latter. As a result, the HT phenotype accumulated plasma glucose and lactate at faster  
425 rate than the HS phenotype during progressive hypoxia. This result confirmed the need  
426 to distinguish responses specific to individual phenotypes when exploring the effect of  
427 dispersed oil-exposure. Because our study lasted 7 months, we also examined the long-  
428 term repeatability of hypoxia tolerance. We revealed that exposure to chemically treated  
429 oil diminished that repeatability and abolished the correlation between the minimum  
430 oxygen level required to sustain SMR ( $O_{2crit}$ ) and the incipient lethal oxygen saturation  
431 (ILOS). It means that hypoxic compensatory mechanisms (Farrell and Richards, 2009),  
432 anaerobic sources reserves (Vornanen et al., 2009) and metabolic arrest (Hochachka  
433 and Guppy, 1987; Nilsson and Renshaw, 2004) can occur at or below  $O_{2crit}$  and  
434 potentially affect a fish's survival in severe hypoxia (Regan et al., 2016). Hence, to  
435 further explore performance below  $O_{2crit}$  (Claireaux and Chabot, 2016), we derived new  
436 indices [the scope for oxygen deficit (SOD), factorial scope for oxygen deficit (FSOD)  
437 and the accumulated oxygen deficit (AOD)] that provide insight into glycolytic capability

438 and compensatory mechanisms. In doing so, we discovered a residual effect of the  
439 exposure to chemically treated oil as evidenced by the elevations of  $O_{2crit}$ , SOD, FSOD  
440 and AOD, but only in the HT phenotype. These effects were not observed in the HS  
441 phenotype. The elevation in  $O_{2crit}$  in the HT phenotype is a particularly interesting result,  
442 because it suggests that following oil-exposure their advantage with hypoxia tolerance  
443 has been lost as they now switch to glycolytic metabolism at the same DO as the HS  
444 phenotype. The fact that this residual impact on the HT phenotype was observed 6-  
445 month post-exposure is also of great ecological importance as it suggests a differential  
446 loss in Darwinian fitness between HS and HT phenotypes under suboptimal oxygen  
447 conditions (Mauduit et al., 2016).

448

#### 449 **4.1 Inter-individual variation and repeatability of hypoxia resistance, HS and HT** 450 **phenotypes**

451

452 Both experimental populations (yearling and two-year-old) displayed large inter-  
453 individual variations in ILOS. This broad distribution of hypoxia resistance/tolerance has  
454 been reported on a number of occasions in sea bass (e.g., Claireaux et al., 2013; Joyce  
455 et al., 2015; Mauduit et al., 2016). Variation of hypoxia tolerance among closely related  
456 species has been reported in sculpin, with  $LOE_{50}$  (time for 50% of the experimental  
457 population to lose equilibrium) in the silverspotted sculpins (*Blepsias cirrhosus*) being  
458 37% shorter than in the staghorn sculpins (*Leptocottus armatus*) and tidepool sculpins  
459 (*Oligocottus maculatus*). Interestingly, although these species had different  $LOE_{50}$ , the  
460 levels of lactate in the brain, liver and muscle tissues at LOE were similar in the three

461 species of sculpins (Speers-Roesch et al., 2013). This mirrors our own observation that  
462 HT and HS phenotypes accumulate the same levels of plasma lactate at their  
463 respective ILOS.

464         Despite the inter-individual variation in hypoxia tolerance observed in the present  
465 experiments, hypoxia tolerance in unexposed control fish was repeatable when  
466 measured in seasonally acclimatized fish (pre-exposure: 10 °C *versus* post-exposure:  
467 15 °C;  $r = 0.691$ ,  $p = 0.019$ ; Table 1, Figure 5). This temporal stability has been reported  
468 previously in European sea bass (Claireaux et al., 2013; Joyce et al., 2016; Mauduit et  
469 al., 2016) and is one of the prerequisites to be the target of natural selection (Arnold,  
470 1983; Bolnick et al., 2003; Sears et al., 2009). Also, hypoxia tolerance measured in the  
471 European sea bass in the laboratory was found to be a determining factor of individual  
472 fitness in the field (Claireaux et al., 2013; Mauduit et al., 2016).

473         However, factors underpinning intraspecific variation in hypoxia tolerance still  
474 remain unclear (Joyce et al., 2016). To investigate these underlying mechanisms, we  
475 distinguished between HT and HS phenotypes and compared the onset of glycolytic  
476 metabolism during a progressive hypoxia. We found that in the HS phenotype, lactate  
477 and glucose started to accumulate in the plasma at a higher DO than in the HT  
478 phenotype. The origin of that difference in phenotype's response is difficult to establish  
479 as current understanding of the determinants of metabolite fluxes during hypoxia is still  
480 relatively underdeveloped. The accumulation of lactate in the circulation during hypoxia  
481 is caused by the uncoupled lactate production and disposal, the latter resulting from  
482 increased lactate oxidation combined with its stimulated use as a gluconeogenic  
483 substrate (Hamman et al., 1997; Omlin and Weber, 2010). The use of lactate as a

484 precursor for glucose synthesis, together with a possible imbalance between stimulated  
485 hepatic glucose production and downstream utilization, have been suggested as  
486 responsible for the hyperglycemic response which also occurs during hypoxia (White  
487 and Fletcher, 1989; Wright et al., 1989; Hamman et al., 1997; Omlin and Weber, 2010).  
488 More information about the regulation of the rates of appearance and disposal (fluxes)  
489 of lactate and glucose is however required before phenotypic differences in the time  
490 course of plasma metabolites during hypoxia are interpretable. How oil contamination  
491 might affect the rates of lactate appearance and disposal in these phenotypes is also  
492 worthy a further study.

493

#### 494 **4.2. Aerobic capacity**

495 Examination of the residual effect of oil exposure upon sea bass aerobic  
496 performance was conducted using the framework proposed by Claireaux and Chabot  
497 (2016). This framework is based on the concept of limiting oxygen level curve (LOL-  
498 curve; Neill and Bryan, 1991; Neill et al., 1994) and outlines the response of a fish to a  
499 developing hypoxia (Figure 9). It particularly describes the limiting effect of ambient  
500 oxygen level on aerobic metabolic scope. Using this construct, we found that an acute  
501 exposure to chemically dispersed oil did not chronically affect SMR,  $\dot{M}O_{2max}$ , AAS and  
502 FAS of juvenile European sea bass, regardless of their hypoxia resistance phenotypes.  
503 Observed values for SMR (90 & 92 mg O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup>) and  $\dot{M}O_{2max}$  (388 & 392 mg O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup>)  
504 of control and oil-treated fish respectively were comparable to SMR (89 mg O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup>)  
505 and  $\dot{M}O_{2max}$  (306 mg O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup>) calculated for resting, and then swum, 100 g fasted  
506 sea bass (Claireaux et al., 2006).

507 Gills are particularly susceptible to exposition to water-borne contaminants  
508 (Engelhardt et al., 1981; Moles and Norcross, 1998; Stephens et al., 2000; Claireaux et  
509 al., 2004; Farrell et al., 2004; Kochhann et al., 2015). Accordingly, shortly after exposure  
510 to oil or to some of its compounds, consequences upon fish respiratory capacity and  
511 proceeding performances have been reported (Claireaux et al., 2004; Kennedy and  
512 Farrell, 2006; Davoodi and Claireaux, 2007; McKenzie et al., 2007; Claireaux and  
513 Davoodi, 2010; Hicken et al., 2011; Klinger et al., 2014; Mager et al., 2014). Conversely,  
514 SMR,  $\dot{M}O_{2max}$  and AAS measured shortly after acute exposure to petroleum  
515 hydrocarbons were unaffected in the golden grey mullet (*Liza aurata*) (Milinkovitch et  
516 al., 2012) and in the embryonic/larval mahi-mahi (*Coryphaena hippurus*) (Mager et al.,  
517 2014). Chronic (2-6 months) sub-lethal ingestion of PAH in juvenile (2-month-old) and  
518 adult (6-month-old) also did not affect SMR,  $\dot{M}O_{2max}$  and AAS of zebrafish (*Danio rerio*)  
519 (Lucas et al., 2016). A more recent study on sea bass revealed, however, that while a  
520 residual effect of acute oil exposure persisted 1 month post-exposure in the form of  
521 increased susceptibility to hypoxic and thermal challenges, these impairments were no  
522 longer observed 11 months post-exposure, suggesting a full recovery (Mauduit et al.,  
523 2016). The present study complements the previous results as, 6 months post-  
524 exposure, fish exposed to chemically dispersed oil displayed similar SMR,  $\dot{M}O_{2max}$ , and  
525 hence, a comparable AAS and FAS as control fish, also indicating a recovery of aerobic  
526 capacity.

527

528 **4.3. The transition between aerobic and anaerobic domains ( $O_{2crit}$ )**

529         Nearly 6 months after oil exposure, a residual effect of chemically dispersed oil  
530 was evident in the form of a 24 % elevation of  $O_{2crit}$  in the HT phenotype, but not in the  
531 HS phenotype. This increase in  $O_{2crit}$  is similar to those (+52 % & +64 %) reported in  
532 common sole (*Solea solea*) when measured just a few days after an acute exposure to  
533 a severe dose of fuel No. 2 (Claireaux et al., 2004; Davoodi and Claireaux, 2007).

534         All the components of the oxygen cascade system, from gas exchange at the gill  
535 through to ATP production at mitochondria, are potential determinants of  $O_{2crit}$   
536 (Richards, 2011) and each of these components can potentially be affected by oil  
537 exposure. However, elements of our dataset help better identifying targets for future  
538 investigations. For instance, fish exposed to the chemically treated oil had the same  
539 oxygen demand for maintenance (SMR) than the control fish, suggesting no difference  
540 in minimum oxygen requirement between fish groups. Control and oil exposed fish also  
541 displayed similar  $\dot{M}O_{2max}$  which suggests that they also had comparable capacity to  
542 circulate oxygen maximally. This observation corroborates a recent study by Anttila et  
543 al. (submitted), which shows that 6 months post-exposure to chemically treated oil, the  
544 cardiac performance of sea bass was not different than in control fish. These elements  
545 suggest that the reduced  $O_{2crit}$  observed in the oil-exposed probably cannot be  
546 explained by a dysfunctional heart. Under normoxic conditions, oxygen transfer across  
547 gill epithelia is perfusion-limited (Randall, 1982; Malte and Weber, 1985; Schmidt et al.,  
548 1988) and, as argued above, it is unlikely that oil-exposed fish suffered from impaired  
549 cardiac performance. Under hypoxia, on the other hand, the diffusion across the gill  
550 epithelium is most likely the limiting factor of oxygen flux (Perry et al., 1985; Gillooly et

551 al., 2016). As mentioned above, oil exposure is typically associated with alterations of  
552 the gill epithelium (Mallatt, 1985; Claireaux et al., 2004) and it would be worthy exploring  
553 whether oil-exposed fish, and particularly the HT phenotype, developed thicker gill as a  
554 protective barrier (Mitchell and Cech 1983; Post 1983; Farrell et al., 2004) that persisted  
555 7 months later. One could indeed hypothesize that the increased  $O_{2crit}$  observed in  
556 control HT phenotype resulted from the thickening of an initially thin gill epithelia,  
557 increasing the water-blood distance and bringing overall oxygen extraction and  
558 transport capacity of the HT phenotype to HS level.

559

#### 560 **4.4. Oxygen deficit, the indices that quantify glycogen depletion**

561 As the consequence of diminished repeatability of hypoxic survival traits in oil-  
562 treated fish and the susceptibility of gill to oil exposure in HT phenotype, we discovered  
563 that  $O_{2crit}$ , other than ILOS, made a difference in estimated magnitude of glycolytic  
564 metabolism in hypoxia, resulting in the 57 % increase in AOD, 40 % increase in SOD,  
565 and 17 % increase in FSOD of oil-treated HT phenotype. Thus, the dispersed oil  
566 chronically impaired the ability to extract oxygen from water at a low DO, as previously  
567 documented in bald notothenid (*Pagothenia borchgrevinki*) immediately after a 7-day oil  
568 exposure (Davison et al., 1993). Henceforth, we presented theoretical LOL-curves (

569 Figure 9) to illustrate that  $O_{2crit}$  can be right-shifted without affecting SMR and  
570 ILOS in the oil-treated HT phenotype, while substantially increasing AOD, SOD and  
571 FSOD. We anticipated that the oil-treated HT phenotype switched on glycolytic  
572 metabolism at a higher DO than the control counterparts, and the same group was  
573 resistant to hypoxia below  $O_{2crit}$  for the longest duration. These collectively led to the

574 highest SOD, FSOD and AOD in oil-treated HT phenotype, and therefore the result  
575 meant that oil-treated sea bass of HT phenotype had the deepest depletion of glycogen  
576 stores and perhaps the highest lactate accumulation in either plasma or tissues.

577         Furthermore, our results indicate that  $O_{2crit}$  and ILOS quantify different  
578 physiological mechanisms both involved in hypoxia tolerance. This has been shown  
579 previously in sculpin species where critical oxygen tension has a tight range of 26 – 54  
580 torr, whereas  $LOE_{50}$  vary over a much broader range of 25 – 538 min (Mandic et al.,  
581 2013). The critical oxygen tension measures one individual's ability to sustain SMR  
582 under hypoxic condition. It is therefore an indicator of the capacity of that fish to extract  
583 oxygen from the water into the arterial blood (Chapman et al., 2002; Richards, 2011). In  
584 situations where ambient DO is  $< O_{2crit}$  anaerobic glycolysis must compensate for the  
585 energy supply (MacCormack et al., 2006; MacCormack and Driedzic, 2007), which  
586 leads to an accumulation of plasma lactate to a peak threshold level (Pörtner and  
587 Grieshaber, 1993), as observed in present study and in related sculpin species (Speers-  
588 Roesch et al., 2013). If hypoxic condition continues to worsen, ILOS will eventually be  
589 reached (Richards et al., 2007; Nilsson and Östlund-Nilsson, 2008). The determinants  
590 of ILOS can be the depletion of brain ATP reservoirs (Speers-Roesch et al., 2013),  
591 lactate accumulation and depleted glycogen stores in a variety of tissues (Vornanen et  
592 al., 2009; Figure 4).

593         We coined SOD, FSOD and AOD to better quantify a physiological dynamic  
594 when fishes experience a severe hypoxia environment. While SOD and FSOD can  
595 estimate an anaerobic scope of fish surviving in the environment where DO is below  
596 their  $O_{2crit}$ , AOD is a bioenergetics index that quantifies the cost of glycolytic metabolism

597 using oxygen as a common energy currency and taking time into account. This  
598 potentially enables a comparison, across individuals, populations and species, of the  
599 magnitude of oxygen deficit. Although, no direct comparison study is available for SOD,  
600 FSOD and AOD, cumulative oxygen deficit (severity of hypoxic exposure x time) applied  
601 the same ideology that found that striped bass (*Morone saxatilis*) swam at 50%  $U_{crit}$   
602 were over 5-fold less resistant to hypoxia than resting fish (Nelson and Lipkey, 2015).  
603 Based on present study, we propose that a more accurate quantification of glycolytic  
604 metabolism should account for both magnitude of glycolysis and its effective time, which  
605 is extremely important to assess the full potential of fishes to survive in hypoxia (Fry,  
606 1971; Hochachka, 1990; Claireaux and Chabot, 2016).

607         The absence of metabolic arrest is a prerequisite for AOD to precisely measure  
608 the magnitude of glycolytic metabolism. However, SMR is recommended to derive  $O_{2crit}$   
609 while acknowledging that metabolic arrest could easily affect its value (Claireaux and  
610 Chabot, 2016). AOD could help in this case because if metabolic arrest occurs, AOD  
611 should be larger than excess post-hypoxia oxygen consumption, the area bounded by  
612  $\dot{M}O_2$  and SMR when a fish is recovering in normoxia from a hypoxic event (Svendsen et  
613 al., 2012) and warrants further study.

614

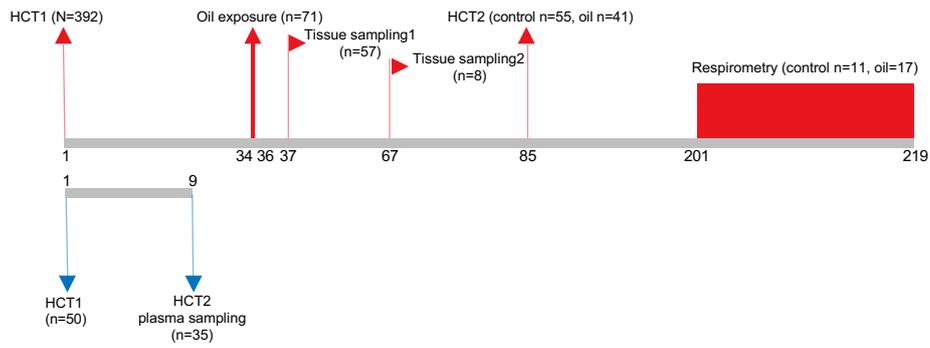
615         In conclusion, the wide breadth of ILOS distribution discovered here for  
616 European sea bass represents divergent phenotypic traits of hypoxic survival because  
617 the HS phenotype clearly starts to accumulate lactate at a higher DO than the HT  
618 phenotype. A residual effect of an acute 48-h exposure to chemically dispersed oil was  
619 not seen for the aerobic indices of sea bass, regardless of their hypoxic phenotypes.

620 Residual effects after a 167-day recovery were evident, however, as a diminished  
621 repeatability of the individual's hypoxic phenotype and as the elevations of  $O_{2crit}$ , SOD,  
622 FSOD and AOD in the HT phenotype, but not HS phenotype. It is worth noting that, if  
623 the data of two phenotypes were pooled, the subtle residual effects would not be  
624 revealed. From an ecological perspective, the HT phenotype of sea bass contains an  
625 important gene pool for this species to thrive in periodically hypoxic habitats. Even  
626 subtle residual effects on the HT phenotype are certainly of great concern for the  
627 population dynamics, recruitment and production, when the HT sea bass might not pay  
628 off a larger oxygen deficit aerobically during hypoxic episodes. Lastly, if we had not  
629 used IRAP and the new indices of anaerobiosis, the subtle residual effects of oil  
630 exposure on the respiratory system would not have been discovered. Despite the  
631 successful application of IRAP in the present study, it is still unknown whether or not  
632 IRAP is effectual at addressing respiratory performance and bioenergetics of fishes at  
633 any other scenarios, and thus the development of IRAP will and must be continued.

634

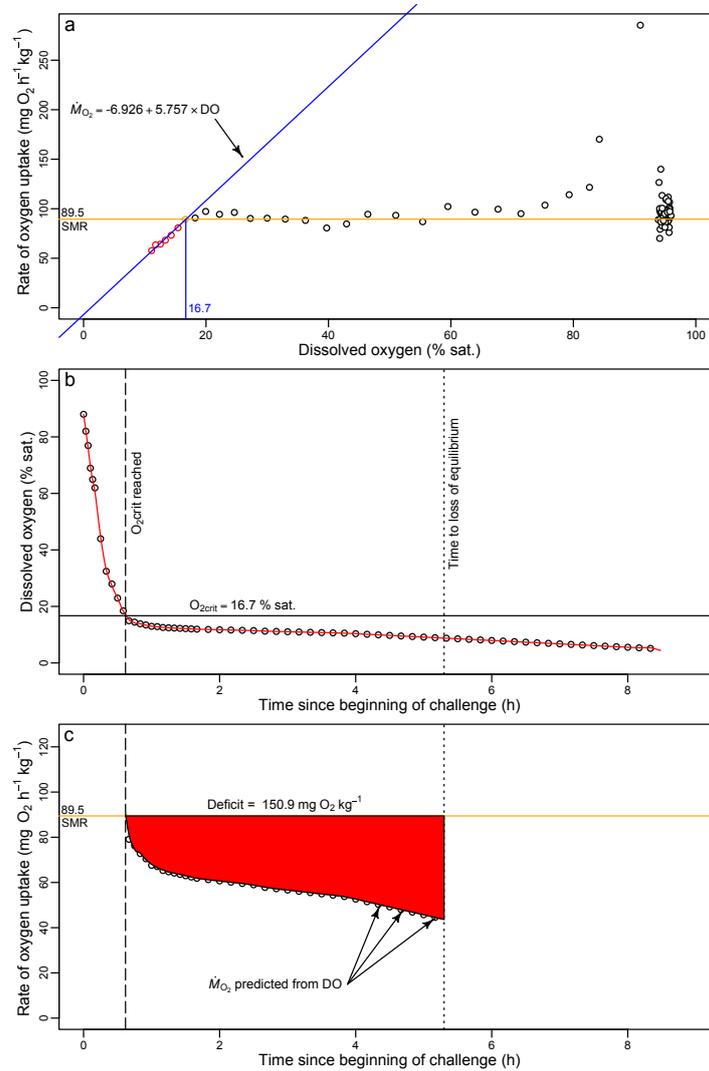
### 635 **Acknowledgements**

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638 Canada Research Chair Tier I.



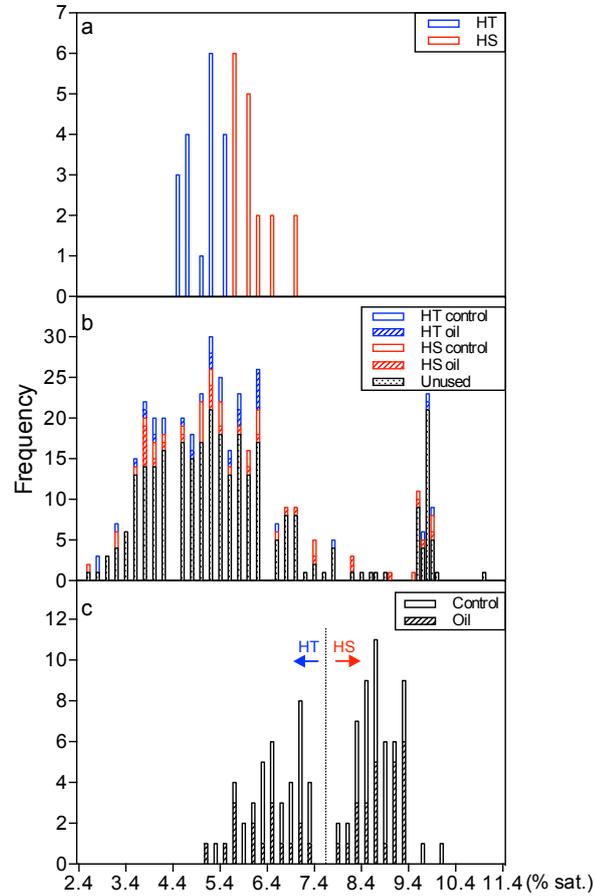
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640 Figure 1. Timeline of the experiments in the 2-year-old (blue) population of European  
641 sea bass (*Dicentrarchus labrax*) for plasma glucose and lactate sampling and the  
642 yearling population of European sea bass for oil contamination study (red). HCT refers to  
643 hypoxia challenge test.



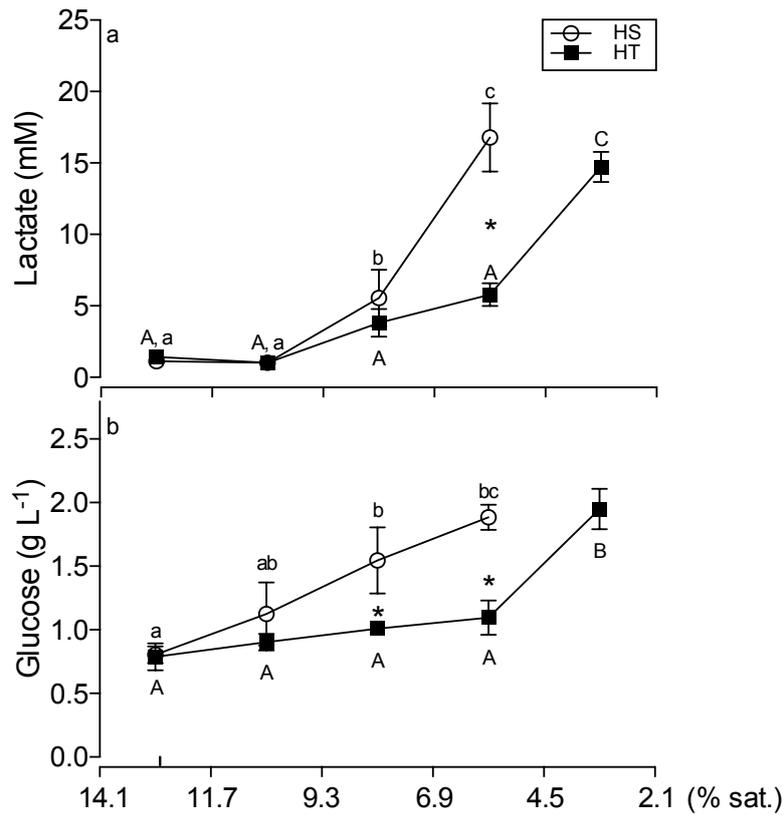
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645 Figure 2. Representative calculation of accumulated oxygen deficit (AOD) of a  
 646 European sea bass (*Dicentrarchus labrax*) in a hypoxia challenge test (HCT). The  $\dot{M}_{\text{O}_2}$   
 647 ~ DO regression equation obtained from (a) used dissolved oxygen level in (b) to  
 648 calculate the rate of oxygen uptake ( $\dot{M}_{\text{O}_2}$ ) in (c). AOD was then an integration of  $\dot{M}_{\text{O}_2}$   
 649 as a function of time for the area bounded by standard metabolic rate (SMR) and the  
 650 estimated  $\dot{M}_{\text{O}_2}$  between critical oxygen level ( $\text{O}_{2,\text{crit}}$ ) and incipient lethal oxygen  
 651 saturation (ILOS).



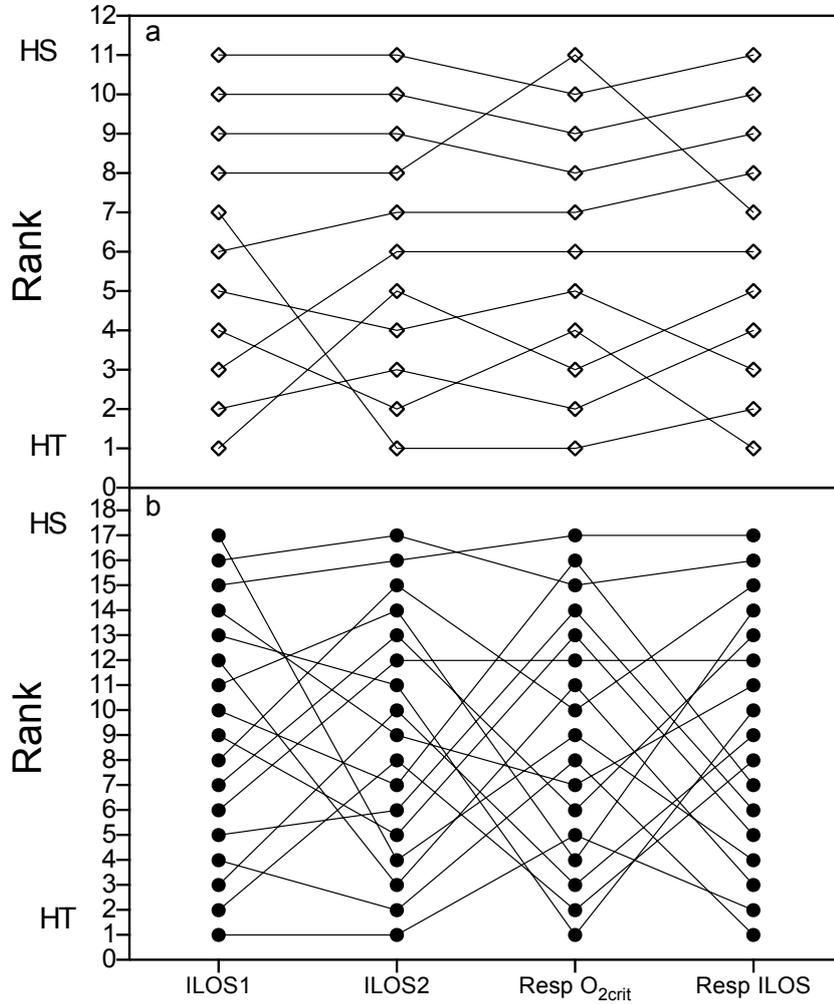
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653 Figure 3. Frequency distribution of incipient lethal oxygen saturation (ILOS) of European  
 654 sea bass (*Dicentrarchus labrax*) measured in plasma metabolites sampling (a, n = 35,  
 655 December, 2013), pre- (b, N = 392, February 2014) and post- (c, n = 96, May 2014)  
 656 chemically dispersed oil exposure. In plasma metabolites sampling, we categorized the  
 657 hypoxia sensitive (HS) phenotype as the first 18 fish that lost their equilibrium (a, ILOS >  
 658 5.6 % sat.), and the remaining 17 fish were hypoxia tolerant (HT) phenotype (a, ILOS <  
 659 5.6 % sat.). The breadth of ILOS in yearling population during the pre-exposure HCT  
 660 were represented in respirometry sampled fish as HT control, HT chemically dispersed  
 661 oil exposed (oil), HS control and HS oil. The HT and HS phenotypes were categorized  
 662 by the left (c, ILOS < 8.25 % sat.) and right distributions (c, ILOS ≥ 8.25 % sat.) in post-  
 663 exposure HCT, respectively, for both control and oil fish.

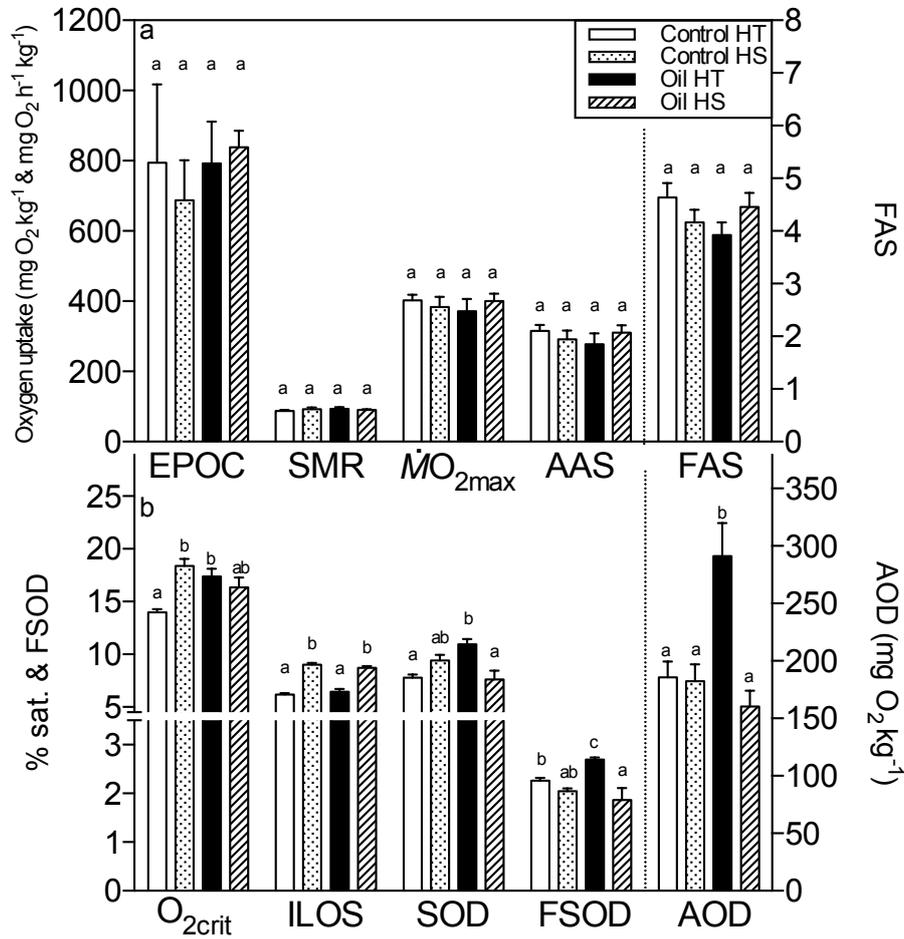


664

665 Figure 4 a) Plasma lactate and b) plasma glucose concentrations of European sea bass  
 666 (*Dicentrarchus labrax*) as a function of water air saturation (% sat.) during a hypoxia  
 667 challenge test (HCT) on December 21, 2013. The fish were randomly sampled every 2  
 668 hours. To identify HT (n=17) and HS (n=18) phenotypes. Different upper case letters  
 669 indicate the statistical significance of sampling time in hypoxia tolerant (HT) phenotype.  
 670 Different lower case letters indicate the statistical significance of sampling time in the  
 671 hypoxia sensitive (HS) phenotype. Asterisk indicate the significant differences between  
 672 HT and HS phenotypes at the same sampling interval. Statistical significance ( $P < 0.05$ )  
 673 was detected by two-way ANOVA followed by Tukey's Kramer *post-hoc* comparisons.  
 674 N-values were 2–7 and error bars represent s.e.m.  
 675

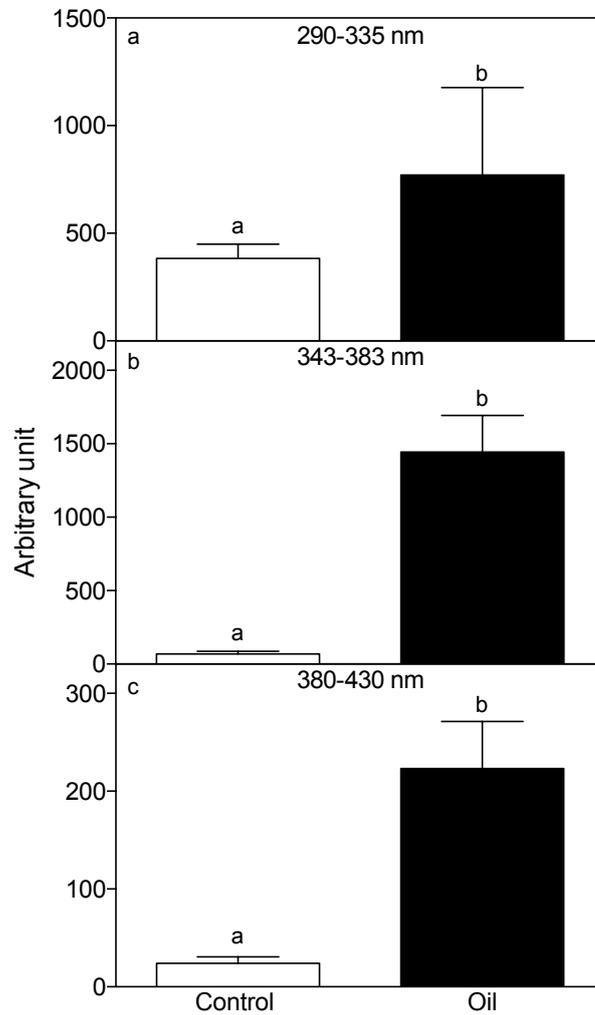


677  
 678 Figure 5. Individual repeatability of hypoxic survival traits in control (a) and chemically  
 679 dispersed crude oil exposed (b) European sea bass (*Dicentrarchus labrax*). The hypoxia  
 680 sensitive (HS) and hypoxia tolerant (HT) fish were measured for incipient lethal oxygen  
 681 saturation (ILOS) in pre- and post- exposure hypoxia challenge test (ILOS1 and ILOS2),  
 682 as well as the extrapolated ILOS and critical oxygen level ( $O_{2crit}$ ) measured in  
 683 respirometry (Resp). The ILOS in respirometry trial was extrapolated from the previous  
 684 HCT.



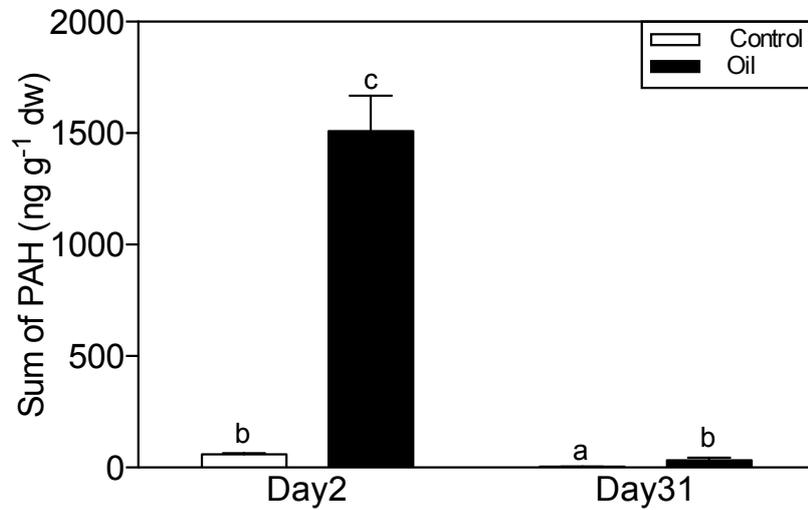
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687 Figure 6. A) Excess post-exercise oxygen consumption (EPOC), standard metabolic  
 688 rate (SMR), maximal rate of oxygen uptake ( $MO_{2max}$ ), absolute aerobic scope (AAS),  
 689 factorial aerobic scope (FAS); B) critical oxygen level ( $O_{2crit}$ ), incipient lethal oxygen  
 690 saturation (ILOS), scope for oxygen deficit (SOD), factorial scope for oxygen deficit  
 691 (FSOD) and accumulated oxygen deficit (AOD) of control hypoxia tolerant (HT) (n=5;  
 692 n=2 for EPOC), control hypoxia sensitive (HS) (n=6; n=5 for EPOC), dispersed oil  
 693 contaminated (oil) HT (n=7; n=6 for EPOC) and oil HS (n=10; n=9 for EPOC) European  
 694 sea bass (*Dicentrarchus labrax*). Different letters indicated the significant differences (P  
 695 < 0.05) between groups within a respiratory index by one-way Welch ANOVA Tukey's  
 696 Kramer *post-hoc*. Values were mean  $\pm$  s.e.m



697

698 Figure 7. Fluorescence measured (arbitrary unit) polycyclic aromatic hydrocarbons at a)  
699 290-355 nm b) 343-383 nm c) 380-430 nm wavelengths in bile of control (n=27) and  
700 chemically dispersed oil exposed (oil) (2 days post-exposure) (n=24) European sea  
701 bass (*Dicentrarchus labrax*). Different letters indicated the significant differences ( $P <$   
702 0.05) between control and chemically-dispersed oil (oil) groups by Student's t-test.  
703 Values were mean  $\pm$  s.e.m.



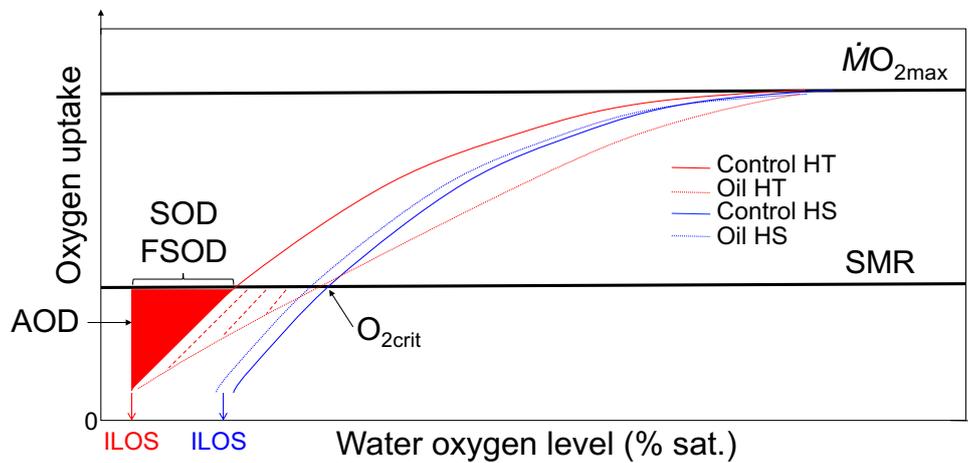
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706 Figure 8. Sum of 20 polycyclic aromatic hydrocarbons components including the  
707 components listed by United States Environmental Protection Agency, in the livers of  
708 control and chemically dispersed oil (oil) exposed European sea bass (*Dicentrarchus*  
709 *labrax*) in day 2 and 31. Different letters indicated the statistical differences ( $P < 0.05$ )  
710 by pairwise t-tests with *Holm-Šidák* correction. N-values were 3–5 and error bars  
711 represent s.e.m.

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716 Figure 9. Schematic illustration of the theoretical limiting oxygen level (LOL) curves  
 717 would distinguish the two phenotypes of hypoxia resistance (hypoxia tolerant, HT;  
 718 hypoxia sensitive, HS) demonstrated here for European sea bass (*Dicentrarchus*  
 719 *labrax*). Control (solid lines) and after exposure to chemically dispersed oil (Oil, dash  
 720 lines) illustrated how we envisage  $O_{2crit}$  (the intersection of standard metabolic rate  
 721 and the curves) without  $MO_{2max}$ , SMR and ILOS changing as a result of the exposure. Scope  
 722 for oxygen deficit (SOD), factorial scope for oxygen deficit (FSOD) and accumulated  
 723 oxygen deficit (AOD) were labeled.

724 Table 1. Spearman's correlation for critical oxygen level ( $O_{2crit}$ ) and incipient lethal  
 725 oxygen saturation (ILOS) of the yearling population European sea bass (*Dicentrarchus*  
 726 *labrax*) over 7-month experiments, including pre- & post- exposure hypoxia challenge  
 727 test (HCT1 & HCT2) and respirometry (Resp).  
 728

		HCT1 ILOS	HCT2 ILOS	Resp ILOS	Resp $O_{2crit}$
HCT1 ILOS	Control		*0.691 P=0.019	*0.673 P=0.023	*0.718 P=0.013
	Oil-treated		0.355 P=0.162	0.375 P=0.138	0.365 P=0.149
HCT2 ILOS	Control			*0.973 P<0.001	*0.900 P<0.001
	Oil-treated			*0.988 P<0.001	0.120 P=0.646
Resp ILOS	Control				*0.809 P=0.003
	Oil-treated				0.142 P=0.586

For each correlation, Spearman's correlation coefficient is shown in the first row, P value is in the second row.

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733 Table 2. The summary of chemically dispersed oil as a main effect on standard  
734 metabolic rate (SMR), maximal metabolic rate ( $\dot{M}O_{2max}$ ), absolute aerobic scope (AAS)  
735 and factorial aerobic scope (FAS), critical oxygen level ( $O_{2crit}$ ), incipient lethal oxygen  
736 saturation (ILOS), scope for oxygen deficit (SOD), factorial scope for oxygen deficit  
737 (FSOD) and accumulated oxygen deficit (AOD) between control and dispersed oil  
738 exposed (oil) 1-year old European sea bass (*Dicentrarchus labrax*). The indices were  
739 from pre- and post- hypoxia challenge test (Pre- and Post-HCT), as well as respirometry  
740 experiment (Resp). Statistical significance was assigned to  $P < 0.05$  in Student's t-test  
741 and Welch one-way ANOVA. Values were mean  $\pm$  s.e.m.

		Dimensions	Control	Oil	F-value	p-value
Pre-HCT	ILOS	% sat.	5.8 $\pm$ 0.1	-	-	-
10 °C	N=392	hour	6.1 $\pm$ 0.1	-	-	-
Post-HCT	ILOS	% sat.	7.7 $\pm$ 0.2	7.8 $\pm$ 0.2	-	0.7
15 °C	n=96	hour	6.2 $\pm$ 0.1	6.1 $\pm$ 0.2	-	0.7
	SMR	mg O <sub>2</sub> h <sup>-1</sup> kg <sup>-1</sup>	90.1 $\pm$ 3.0	91.8 $\pm$ 2.3	0.22	0.65
	$\dot{M}O_{2max}$	mg O <sub>2</sub> h <sup>-1</sup> kg <sup>-1</sup>	392.1 $\pm$ 16.8	388.4 $\pm$ 18.9	0.02	0.89
Resp	AAS	mg O <sub>2</sub> h <sup>-1</sup> kg <sup>-1</sup>	302.0 $\pm$ 15.8	296.6 $\pm$ 18.0	0.05	0.82
16.5 °C	FAS		4.4 $\pm$ 0.2	4.2 $\pm$ 0.2	0.28	0.60
control n=11	$O_{2crit}$	% sat.	16.4 $\pm$ 0.8	16.8 $\pm$ 0.6	0.18	0.68
oil n=17	SOD	% sat.	8.7 $\pm$ 0.4	9.0 $\pm$ 0.7	0.18	0.67
	FSOD		2.1 $\pm$ 0.1	2.2 $\pm$ 0.1	0.26	0.62
	AOD	mg O <sub>2</sub> kg <sup>-1</sup>	183.9 $\pm$ 9.6	214.2 $\pm$ 21.2	21.7	0.21

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- 1095

## 5. Paper 5 (submitted to Environmental Science and Pollution Research)

1 **Influence of crude oil exposure on cardiac function and thermal tolerance of**  
2 **juvenile rainbow trout and European seabass**

3

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16

### 17 **Abstract**

18

19 Oil spills pose a threat to aquatic organisms. However, the physiological effects of crude oil on  
20 cardiac function and on thermal tolerance of juvenile fish are still poorly understood.  
21 Consequently, in this paper we will present results of two separate experiments where we  
22 exposed juvenile rainbow trout and European seabass to crude oil and made cardiac thermal  
23 tolerances and maximum heart rate ( $f_{Hmax}$ ) measurements after one week (rainbow trout) and  
24 6-month recovery (seabass). In both species the  $f_{Hmax}$  was lower in crude oil-exposed fish than  
25 in control ones at temperatures below the optimum but this difference disappeared at higher

26 temperatures. More importantly, the oil-exposed fish had significantly higher Arrhenius break  
27 point temperature for  $f_{Hmax}$ , which gives an estimate for optimum temperature, than control fish  
28 in both species even though the exposure conditions and recovery times differed between  
29 species. The results indicate that exposure of juvenile fish to crude oil did not have a significant  
30 negative impact upon their cardiac performance in high temperatures and upper thermal  
31 tolerance increased when the fish were tested 1 week or 6 months after the exposure. Our  
32 findings suggest that adaptive phenomena upon recovery can reverse the functional  
33 impairments observed during direct exposure of cardiac cells to oil.

34

35 **Key words:** Arrhenius break point temperature, critical thermal maximum, CTmax, fish, heart  
36 rate, oil spill, PAH

37

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39

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46 **Introduction**

47

48 Accidental oil spills and crude oil exposure represent a threat to aquatic environments  
49 worldwide, affecting not only aquatic animals but also human activities (e.g. fisheries,  
50 aquaculture and tourism) (Endersen et al. 2003; Meski and Kaitaranta 2014). Previous studies  
51 have shown that fish embryos and larvae are particularly sensitive to crude oil and its  
52 components, especially polycyclic aromatic hydrocarbons (PAH). For instance, the heart is one  
53 of the most vulnerable organs in fish and crude oil exposures has been associated with  
54 malformations such as reduced cardiac looping and pericardial edema in developing fish  
55 (Thomaz et al. 2009; Incardona et al. 2009, 2012, 2014; Jung et al. 2013). Cardiac dysfunctions  
56 such as reduced ventricular contractility (Jung et al. 2013), increased occurrence of (especially  
57 atrial) arrhythmias and increased variability of heart rate have also been reported in embryos  
58 (Incardona et al. 2009, 2012, 2014, Jung et al. 2013; Sørhus et al. 2016; Khursigara et al. 2017).  
59 Furthermore, it has been shown that a year after embryonic stages had been exposed to  
60 petroleum hydrocarbon, young fish still exhibited misshaped heart (reduced ventricular length-  
61 to-width ratio) and lower critical swimming velocity ( $U_{CRIT}$ ) than unexposed control fish  
62 (Hicken et al. 2011).

63

64 When comparing embryos to juveniles or adult fish, it seems that juveniles and adults might  
65 be less dramatically affected by exposure to petroleum hydrocarbons than younger life stages,  
66 although a large interspecific variability has been reported (e.g. Vosyliene et al. 2005; Davoodi  
67 and Claireaux 2007; Claireaux and Davoodi 2010; Milinkovitch et al. 2012; Claireaux et al.  
68 2013). For example, shortly after an exposure to crude oil (less than 24h), common sole (*Solea  
69 solea*) showed reduced basal metabolic rate (Davoodi and Claireaux 2007) and cardiac output  
70 as well as impaired cardio-respiratory responses to acute warming (Claireaux and Davoodi

71 2010). Similarly, reductions in stroke volume and cardiac output have also been observed in  
72 juvenile mahi-mahi (*Coryphaena hippurus*) shortly after a 24h-exposure to PAH (Nelson et al.  
73 2016). Brette et al. (2014) exposed isolated cardiomyocytes of juvenile bluefin tuna (*Thunnus*  
74 *orientalis*) and yellowfin tuna (*Thunnus albacares*) to dilutions (20%, 10% and 5%) of high-  
75 energy water-soluble fraction (WAF) of oil samples collected from the *Deep Water Horizon*  
76 spill. These authors observed impaired cardiomyocyte function during the exposure, with  
77 reduced amplitude and tail current of the delayed rectifier potassium current, resulting in  
78 prolonged action potential duration (increased repolarization time). Reduced amplitude of  
79 calcium currents was also observed suggesting impaired excitation-contraction coupling  
80 (Brette et al. 2014). It must be noted, however, that lacks of effect have also been reported in  
81 the literature. For instance, aerobic scope, basal and active metabolic rates as well as  $U_{CRIT}$   
82 were unchanged in juvenile golden grey mullet (*Liza aurata*) 24h after exposure to  $60.1 \mu\text{g L}^{-1}$   
83  $\Sigma\text{PAH}$  (Milinkovitch et al. 2012). Claireaux et al. (2013) showed that 1 week after an exposure  
84 to crude oil or to chemically dispersed oil, juvenile European seabass (*Dicentrarchus labrax*)  
85 displayed reduced hypoxia tolerance (incipient lethal oxygen saturation) and thermal  
86 sensitivity (critical thermal maximum,  $CT_{MAX}$ ) compared to unexposed, control fish. These  
87 differences were, however, no more observed 4 weeks (Claireaux et al. 2013) or 10 months  
88 post-exposure (Mauduit et al. 2016). Beyond the fact that reported consequences of exposure  
89 to crude oil are less consistent across studies in juveniles, it is still poorly known whether these  
90 consequences persist over time or if recovery occurs upon return to a clean environment, which  
91 is the natural situation after oil spills. One objective of the present study is to address this issue.  
92  
93 Besides exposure to crude oil, fish are also exposed to other sources of environmental  
94 constrains in nature. One of these sources is the ongoing climate change. Anthropogenic  
95 activities are changing the earth's climate and water temperature has progressively increased

96 around the world. This increase is directly affecting the physiology of ectothermic fish as they  
97 cannot regulate their body temperature and it is expected to have detrimental effects if  
98 populations cannot migrate to new areas or increase their thermal tolerance via phenotypic  
99 plasticity or adaptation through evolution by natural selection (Parmesan 2006; BACC Author  
100 team 2008; Belkin 2009; Pörtner 2010; Marshall et al. 2014). The question is, thus, to examine  
101 whether exposure to petroleum hydrocarbon compounds is liable to affect the capacity of fish  
102 to face warming events, such as heat wave for instance, the occurrence of which is predicted  
103 to increase with climate change (e.g. Teng et al. 2016). In this paper we will present results of  
104 two totally separate experiments about how crude oil-exposure influences cardiac function and  
105 thermal tolerance of juveniles of two ecologically different fish species, the freshwater rainbow  
106 trout (*Oncorhynchus mykiss*) and the seawater seabass (*Dicentrarchus labrax*). These species  
107 were chosen since they are important fisheries species and are living/reared at areas where the  
108 threat for oil accident is especially high (BACC Author team 2008; Marshall et al. 2014).  
109 Rainbow trout was studied 1 week post exposure while seabass was examined 6 months post  
110 exposure.

111

112 The first aim of the studies was to examine whether exposure to crude oil, followed by a  
113 significant recovery period in a clean environment, influenced the upper critical thermal  
114 tolerance ( $CT_{MAX}$ ) of fish. This was done by exposing control and oil-treated fish to acutely  
115 increasing temperature until the fish could no longer keep an upright position. The value of  
116  $CT_{MAX}$  indicates the upper thermal tolerance of a fish beyond which survival is time-limited  
117 (Pörtner 2010). The second aim of the study was to measure how the cardiac function in intact  
118 animals changes when animals are exposed to increasing water temperature and if oil exposure  
119 influences these cardiac responses. To reach the second objective, maximum heart rate ( $f_{Hmax}$ )  
120 was recorded in anaesthetized and atropinized control and oil-exposed fish submitted to a

121 progressive increase of water temperature. By recording the heart rate during incremental  
122 warming, we were also able to estimate the upper thermal tolerance of cardiac function. The  
123 temperature where  $f_{Hmax}$  stops increasing exponentially with increasing temperature (Arrhenius  
124 break point temperature,  $T_{AB}$ ) has been connected to optimum temperature of aerobic scope  
125 and growth in several species (rainbow trout: Anttila et al. 2013a; coho salmon, *Oncorhynchus*  
126 *kisutch*: Casselman et al. 2012; Atlantic salmon, *Salmo salar*: Anttila et al. 2014; sockeye  
127 salmon, *O. nerka*: Chen et al. 2013; chinook salmon, *O. tshawytscha*: Muñoz et al. 2014; Arctic  
128 cod, *Boreogadus saida*: Drost et al. 2014 and goldfish, *Carassius auratus*: Ferreira et al. 2014).  
129 Thus, in the current study  $T_{AB}$  was calculated and used as a proxy of the optimum temperature  
130 of fish ( $T_{OPT}$ ). We also measured the temperature ( $T_{PEAK}$ ) where the highest heart rate ( $f_{Hpeak}$ )  
131 was found and the temperature where cardiac arrhythmias were observed ( $T_{ARR}$ ). These  
132 temperature values are indicative the upper thermal limit for cardiac function (Casselman et al.  
133 2012; Anttila et al. 2013a). These measuring techniques i.e.  $CT_{MAX}$  and heart rate recordings  
134 were used as a screening tools and both techniques provided estimates of upper thermal  
135 tolerance even though, in both cases, the rate of increase of temperature was higher than in  
136 natural situations.

137

## 138 **Materials and Methods**

139

### 140 **Experiment 1. Rainbow trout**

141 The experiments with rainbow trout (age 1+) were conducted at University of Turku, Finland  
142 during summer 2014. The experiments were approved by Finnish Animal Experiment Board  
143 (ESAVI/4068/04.10.07/2013). The rainbow trout were obtained from a nearby commercial fish  
144 farm (The College of Fisheries and Environment, Kirjala, Finland) and the fish were  
145 transported to University of Turku 4 weeks before experiments. Fish were maintained under

146 natural photoperiod and temperature (16°C) conditions in their acclimation tanks (500 L). Fish  
147 were fed daily with commercial pellets (Raisio Group, Raisio, Finland) and the feeding was  
148 ceased 24h before any experiments/manipulations. The mean size of fish did not differ  
149 significantly between experimental groups and the weights and lengths were  $4.6\pm 0.5$  g and  
150  $8.2\pm 0.2$  cm (fork length) for control rainbow trout,  $4.4\pm 0.3$  g and  $8.1\pm 0.1$  cm for oil-exposed  
151 rainbow trout.

152

### 153 *Exposures*

154 Russian Export Blend medium crude oil was acquired from Neste Oil (Raisio, Finland). The  
155 crude oil was weathered in a separate bottle before experiment by bubbling air through the oil  
156 layer until approximately 10% of the oil mass was lost. The exposures were conducted  
157 according to Milinkovitch et al. (2011). The fish were divided into two subgroups (oil-exposed  
158 and control, N=60 fish per group) and transferred from their acclimation tanks to identical,  
159 triplicate polyethylene tanks (185 L) 48 h before exposures (biomass per tank  $1.46$  g L<sup>-1</sup>). The  
160 exposure tanks were equipped with airstones and custom-made mixing system, which allowed  
161 full homogenization of water column and kept oxygen level above 80% of air saturation  
162 throughout the exposures (see Milinkovitch et al. (2011) for further details about mixing  
163 system). The temperature of exposure tanks was kept at the acclimation temperature of fish  
164 (16°C). The exposure was started by pouring 12.5 g of weathered crude oil to the surface of  
165 the tanks (i.e. the oil concentration was  $0.07$  g L<sup>-1</sup>). The exposure lasted 48h. One liter water  
166 samples was taken from the middle of the water column briefly after the exposure started and  
167 at the end of the exposure. The water samples were analyzed by Novalab Oy (Karkkila,  
168 Finland) with the analysis methods described in supplementary materials. Following exposure  
169 rainbow trout were briefly bathed in clean water containing 70 ppm buffered MS-222 and the  
170 adipose fin cut to identify the fish later on. After bathing, fish were returned to their initial

171 acclimation tanks so that both control and exposed fish were in same tank. Control fish  
172 followed the same protocol except that no chemicals were added to their tank. No mortalities  
173 were observed during the exposure and the following week.

174

175 The pH of the water did not change during the exposure ( $7.5 \pm 0.1$ ) and nitrogen waste  
176 concentrations stayed below detection limits (nitrite  $< 0.25 \text{ mg L}^{-1}$ , nitrate  $< 10 \text{ mg L}^{-1}$  and  
177 ammonium  $< 0.5 \text{ mg L}^{-1}$ ). The oxygen concentration and water temperatures were  $9.2 \pm 0.1 \text{ mg}$   
178  $\text{L}^{-1}$  and  $16.3 \pm 0.05^\circ\text{C}$ , respectively.

179

#### 180 *CT<sub>MAX</sub> experiments*

181 Fish were allowed to recover from the exposures 7 days before any experiments. The  $CT_{MAX}$   
182 measurements were done according to Anttila et al. (2013b). Briefly, both control and exposed  
183 rainbow trout ( $n = 15$  per group) were transferred to experimental tank (100 L,  $16^\circ\text{C}$ ) 1 hour  
184 before the experiment started. After the acclimation period in the experimental tank, the  
185 temperature of the water was increased at a constant rate of  $0.3^\circ\text{C min}^{-1}$  up to  $24^\circ\text{C}$  and by  
186  $0.1^\circ\text{C min}^{-1}$  thereafter until the fish lost equilibrium. Water temperature was controlled with a  
187 circulating 2500 W heater (RC6, Lauda, Lauda-Königshofen, Germany). Water oxygenation  
188 and homogeneity was assured by bubbling air vigorously into the tanks and using aquarium  
189 pumps to circulate the water and keeping the oxygenation level above 80% of air saturation  
190 throughout the experiments. Once a fish lost equilibrium, the water temperature was recorded  
191 and fish was quickly removed from the tank and placed in a recovery tank (all the fish had their  
192 own 10 L recovery tanks) at the acclimation temperature. After the experiment, the rainbow  
193 trout were identified and killed with 200 ppm MS-222 buffered with sodium bicarbonate. The  
194 mass and length of the fish were recorded.

195

196 *Heart rate measurements*

197 Maximum heart rate of rainbow trout was measured a week after the exposure ( $n = 12$  per  
198 group, different fish from those in  $CT_{MAX}$  measurements). The measurements were done  
199 according to Casselman et al. (2012) with slight adjustments in drug concentrations. Briefly,  
200 the fish were anesthetized in buffered 80 ppm MS-222 for transfer to the experimental setup  
201 after weighing. The anesthetized state was maintained in the setup with bicarbonate-buffered  
202 70 ppm MS-222. The water for the system was obtained from the rearing tank of the fish. The  
203 fish were placed to experimental chamber (PVC-pipe with sealed ends and top removed) so  
204 that the fish were completely submerged. The water flow to chamber was achieved with  
205 chiller/heater and part of the water flow was directed via pipe-mouthpieces through the mouth  
206 for gill irrigation. Water temperature was controlled with a recirculating chiller/heater (RC6,  
207 Lauda, Lauda-Königshofen, Germany). Two silver electrodes were located underneath the fish  
208 to detect a field electrocardiogram (ECG), which was amplified and filtered by a Grass P122  
209 AC/DC Strain Gage Amplifier (Grass Technologies, Warwick, U.S.A.). ECG recording and  
210 analysis were performed with BIOPAC data acquisition unit (MP100) and Acknowledge  
211 software (ver. 3.9.1.).

212

213 The fish were equilibrated at the initial temperature (12°C) for 1 h, after which they were given  
214 an intraperitoneal injection of atropine sulphate (1.8 mg kg<sup>-1</sup>, Sigma-Aldrich Chemie GmbH,  
215 Munich, Germany) to completely block vagal tone and 15 min later an isoproterenol injection  
216 (4 µg kg<sup>-1</sup>, Sigma-Aldrich Chemie GmbH, Munich, Germany) to maximally stimulate cardiac  
217  $\beta_1$ -adrenoceptors. Both drugs were dissolved in saline (0.9% NaCl). These drug concentrations  
218 had been tested in preliminary experiments to ensure that they produced a maximum heart rate.  
219 Once heart rate had stabilized (15 min after the isoproterenol injection), water temperature was  
220 increased in 1°C increments at a rate of 10°C h<sup>-1</sup>. After every temperature increment, heart rate

221 was allowed to stabilize before recording a value. Warming continued until cardiac arrhythmias  
222 developed, after which the fish were rapidly removed from the apparatus and killed with cranial  
223 percussion and weight and length of the fish were measured.

224

225 For each individual fish the temperatures for  $T_{ARR}$  and  $T_{PEAKT}$  were recorded and the  $T_{AB}$  was  
226 calculated with Arrhenius plots according to Yeager and Ultsch (1989). Two-segment linear  
227 regression lines were fitted to an Arrhenius plot [natural logarithm of  $f_{Hmax}$  and inverse of  
228 temperature (in Kelvin)] and the intersection of the lines was calculated ( $=T_{AB}$ ).

229

### 230 **Experiment 2. Seabass**

231 The seabass (age 1+) for the experiment were obtained from Aquastream Lorient, France and  
232 transferred to Unité de Physiologie Fonctionnelle des Organismes Marins, Ifremer, France 8  
233 weeks before any experiments. On the arrival to the laboratory the fish were  $11.3 \pm 1.1$  cm long  
234 and their mass was  $17.7 \pm 5.0$  g. The fish were maintained under natural photoperiod and  
235 temperature conditions ( $10^{\circ}\text{C}$  at time of arrival) at their rearing tanks (2000 L). Fish were fed  
236 daily with commercial pellets (Le Gouessant, Saint-Martin de Valgagues, France) and the  
237 feeding was ceased 24h before any experiments/manipulations. Two weeks, before  
238 experiments started, seabass were anaesthetized (MS-222;  $100 \text{ mg L}^{-1}$ , Sigma-Aldrich Chimie  
239 GmbH, Munich, Germany) and individually implanted subcutaneously with an identification  
240 tag (RFID; Biolog-id, Bernay, France). All the experiments were approved by Ministère  
241 Délégué à l'Enseignement Supérieur et à la Recherche.

242

243 The exposures were conducted as in experiment 1, apart from a different crude oil (Arabian  
244 light crude oil) and concentration ( $0.8 \text{ g L}^{-1}$ ). This is because it has been previously shown that  
245 a lower concentration ( $0.07 \text{ g L}^{-1}$ ) does not have an effect on the thermal tolerance of seabass

246 (Claireaux et al. 2013; Mauduit et al. 2016). Furthermore, in the experiment with seabass, crude  
247 oil was mixed with dispersant (Finasol OSR 52, 0.017 g L<sup>-1</sup>) while in the experiment with  
248 rainbow trout weathered oil was only mechanically dispersed in water. The oil-dispersant  
249 mixture was made in a bottle, directly poured in the tanks and then bubbled with air overnight  
250 to mimic a 12-h aging of an oil slick at sea (Nordvik 1995). The number of exposed seabass  
251 per treatment was 190. Exposure started when sixty fish were transferred for 48h into triplicate  
252 300 L exposure tanks that contained only sea water (control group) or dispersant treated oil (oil  
253 group; total biomass per tank was 4.2 g L<sup>-1</sup>). The pH of the water did not change during the  
254 exposure (8.1±0.1) and the oxygen concentration was maintained above 80% of air saturation.  
255 Water samples were taken briefly after the exposure started and at the end of the exposure to  
256 analyze the total hydrocarbon content in water. The water samples were taken from the middle  
257 of the water column using a bottle and were analyzed at CEDRE (Brest, France). Nine seabass  
258 were also sacrificed 24h after the exposure in order to measure the PAH concentrations in liver  
259 (see Supplementary material for the methods of chemical analyses).

260

#### 261 *CT<sub>MAX</sub> experiments*

262 Seabass were allowed to recover from the exposures 4 weeks before any experiments.  
263 The CT<sub>MAX</sub> protocol for seabass (*n* = 20 per group) differed slightly from the protocol for  
264 rainbow trout in that the exposures were done in the rearing tank of the fish and temperature  
265 was increased from acclimation temperature to 27°C in 2.5 hours and thereafter the heating  
266 rate was lowered to 0.5°C h<sup>-1</sup> until the fish lost equilibrium (Claireaux et al. 2013). The water  
267 temperature was controlled with JULABO F10, 2500 W heater (Seelbach, Germany). After  
268 seabass lost equilibrium the water temperature was recorded, the PIT tag was read and the fish  
269 were transferred to a recovery tank for 2 h before being returned to their original rearing tank.  
270 The percentage mortality after the CT<sub>MAX</sub> experiment was below 1%. Thereafter the fish were

271 reared in natural photoperiod and temperature conditions until heart rate measurements (6  
272 months later). There were no differences in mortality between groups.

273

#### 274 *Heart rate measurements*

275 Maximum heart rate of seabass ( $n = 18$  per group, the same fish as in  $CT_{MAX}$  measurements)  
276 was measured 6 months after the exposure (water temperature at that time was  $17^{\circ}C$ ). The  
277 mean size of fish did not differ significantly between experimental groups and the weights and  
278 lengths were  $66.4 \pm 4.1$  g and  $17.2 \pm 0.3$  cm for control seabass and  $71 \pm 3.7$  g and  $17.5 \pm 0.3$  cm  
279 for oil-exposed seabass, respectively, at the time of heart rate measurements. The protocol was  
280 exactly the same as with rainbow trout expect that anesthesia solution did not contain  
281 bicarbonate because of the pH buffering capacity of seawater (no significant change in water  
282 pH was observed after MS-222 was added to water). Salinity level of the water was 32-33 ppt.  
283 The water temperature was controlled with a recirculating chiller/heater (F10, JULABO,  
284 Seelbach, Germany) and the ECG was recorded and analyzed with BioPac MP36R (BIOPAC  
285 Systems Inc, Essen, Germany) which had in-built amplification system. The starting  
286 temperature of the measurements for seabass was  $17^{\circ}C$ . The concentration of atropine sulphate  
287 was  $3 \text{ mg kg}^{-1}$  (Sigma-Aldrich Chemie GmbH, Munich, Germany) and concentration of  
288 isoproterenol was  $3.2 \text{ } \mu\text{g kg}^{-1}$  (Sigma-Aldrich Chemie GmbH, Munich, Germany).

289

#### 290 **Statistical analyses of both experiments**

291 Data normality and homogeneity were tested with Kolmogorov-Smirnov and Levene tests,  
292 respectively. Two-way repeated measures ANOVA was used to calculate the differences in  
293  $f_{Hmax}$  values between oil-exposed and control fish during warming (temperature and treatment  
294 as factors) and was followed by Holm-Sidak *post hoc* test. Both species were tested separately  
295 because of differences in starting temperatures and treatments. Two-way repeated measures

296 ANOVAs were also used to analyze 1) the differences in  $f_{Hpeak}$  and scope for heart rate  
297 (difference between lowest  $f_{Hmax}$  and  $f_{Hpeak}$ ) between exposed and control animals and 2) the  
298 differences in  $T_{AB}$ ,  $T_{PEAK}$ ,  $T_{ARR}$ ,  $CT_{MAX}$  between exposed and control animals (exposure and  
299 measurement as factors). However, since the individuals for  $CT_{MAX}$  experiment were different  
300 from individuals used in heart rate experiment for rainbow trout, the differences in  $CT_{MAX}$   
301 between exposed and control rainbow trout were analyzed with t-test. All the statistical  
302 analyses were performed with SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA).  
303 Statistical significance for comparisons of mean values was set at  $\alpha=0.05$ . The values are  
304 presented as means  $\pm$  SEM.

305

## 306 **Results**

307

### 308 **Experiment 1. Rainbow trout**

309 Rainbow trout exposed to  $0.07 \text{ g L}^{-1}$  of weathered Russian export blend medium crude oil  
310 experienced  $3.8 \pm 1.1 \text{ mg L}^{-1}$   $\Sigma$ hydrocarbons. The sum of PAHs was  $4.5 \pm 0.14 \text{ } \mu\text{g L}^{-1}$ . However,  
311 it needs to be noted that only naphthalene, acenaphthalene, fluorene and phenantrene  
312 concentrations were above detection limits. The list of PAH compounds are shown in  
313 Supplementary Table. There were no mortalities after exposures.

314

#### 315 *Heart rate*

316 The exposure to crude oil reduced the heart rate at temperatures below and near optimum  
317 temperature (12-18°C, optimum around 17°C, Anttila et al. 2013a) ( $F_{1,6}=6.3$ ,  $P=0.013$ , Fig. 1a)  
318 while there were no differences between oil-exposed and control fish at temperatures  $>18^\circ\text{C}$ .  
319 Oil exposure did not affect the highest  $f_{Hmax}$  recorded ( $f_{Hpeak}$ ) ( $108 \pm 5.1 \text{ bpm}$  and  $116 \pm 6.7 \text{ bpm}$   
320 for control and oil-exposed rainbow trout, respectively;  $P=0.29$ ). The difference between

321 lowest recorded  $f_{Hmax}$  and the highest recorded  $f_{Hmax}$  (difference=scope for heart rate) were  
322 50.2±4.1 and 64.5±6.5 bpm in control and oil-exposed rainbow trout, respectively, and were  
323 not statistically different from each other ( $P=0.088$ ).

324

#### 325 *Indices of upper thermal tolerance*

326 Oil exposure had a significant influence on rainbow trout thermal tolerance (Fig. 2a). One week  
327 post exposure,  $T_{AB}$  was significantly higher in oil-exposed (16.3±0.4°C) than in control  
328 (15.1±0.4°C) fish ( $P=0.036$ , Fig. 1a, 2a). Similarly, all the indices of upper thermal tolerances  
329 (i.e.  $T_{PEAK}$ ,  $T_{ARR}$  and  $CT_{MAX}$ ) were higher in oil-exposed than in control rainbow trout: the  
330 differences between oil-exposed and control fish were 1.8°C, 2.3°C and 0.8°C, for  $T_{PEAK}$ ,  $T_{ARR}$   
331 and  $CT_{MAX}$ , respectively (Fig. 2a) ( $T_{PEAK}$ :  $P=0.002$ ;  $T_{ARR}$ :  $P=0.033$ ;  $CT_{MAX}$ :  $P=0.005$ ).  $CT_{MAX}$ ,  
332  $T_{ARR}$ ,  $T_{PEAK}$  and  $T_{AB}$  differed significantly from each other ( $P<0.001$ ).

333

#### 334 **Experiment 2. Seabass**

335 Seabass experienced 141±7.8 mg L<sup>-1</sup>  $\Sigma$ hydrocarbons, when exposed to 0.8 g L<sup>-1</sup> of weathered  
336 Arabian light crude oil mixed with 0.017 g L<sup>-1</sup> of dispersant (Finasol OSR 52). The sum of  
337 PAHs in liver of seabass was 34 299 ± 8 926 ng g<sup>-1</sup> when measured two days post-exposure.  
338 However, it needs to be noted that not all the PAH present in the water were detected in the  
339 fish liver. The concentrations in PAHs are detailed in Supplementary table.

340

#### 341 *Heart rate*

342 The exposure to crude oil caused similar effects in seabass as observed in rainbow trout  
343 although experimental designs and post-exposure recovery times were different. For example,  
344 in seabass, as in rainbow trout, the oil-exposed fish had lower  $f_{Hmax}$  values ( $F_{1,5}=4.8$ ,  $P=0.03$ )  
345 than the control fish when measured below the optimum temperature (17-22°C, optimum

346 around 22-24°C, Claireaux and Lagardère 1999), (Fig. 1b). However, there were no differences  
347 between oil-exposed and control seabass at temperature > 24°C. As in rainbow trout the oil  
348 exposure did not influence the  $f_{Hpeak}$  values ( $P=0.91$ ). The values for  $f_{Hpeak}$  were  $125\pm 6.7$  bpm  
349 and  $126\pm 4.9$  bpm for control and oil-exposed seabass, respectively. The scope for  $f_{Hmax}$  were  
350  $34.0\pm 5.0$  and  $35.7\pm 5.8$  for control and oil-exposed seabass, respectively, and the groups did  
351 not differ from each other ( $P=0.83$ ).

352

### 353 *Indices of upper thermal tolerance*

354 Again, as in rainbow trout, the oil-exposed seabass had higher  $T_{AB}$  (1.0°C difference) compared  
355 to control seabass ( $P=0.05$ ). The  $T_{AB}$  values were  $19.3\pm 0.3$ °C and  $20.3\pm 0.3$ °C for control and  
356 oil-exposed seabass, respectively (Fig. 1d, 2b). Furthermore, the  $T_{PEAK}$  was higher ( $P=0.03$ ,  
357 difference 1.3°C, Fig. 2b) in oil-exposed seabass than in the control. The  $T_{PEAK}$  values were  
358  $21.8\pm 0.4$ °C and  $23.1\pm 0.4$ °C for control and oil-exposed seabass, respectively. However, in  
359 seabass the  $T_{ARR}$  and  $CT_{MAX}$  did not differ statistically between oil-exposed and control fish  
360 after a 6-month recovery ( $T_{ARR}$ :  $P=0.62$ ;  $CT_{MAX}$ :  $P=0.94$ , Fig. 2b).

361

### 362 **Discussion**

363

364 Recent studies have demonstrated that embryonic fish exposed to hydrocarbon compounds  
365 display various cardiac malformations and functional disorders (e.g. Thomaz et al. 2009;  
366 Incardona et al. 2009, 2012, 2014; Jung et al. 2013; Sørhus et al. 2016; Khursigara et al. 2017).  
367 However, there is very limited literature available about influences on later life stages. The aim  
368 of the current study was, thus, to measure, in experiments involving two different fish species,  
369 how exposure to crude oil, followed by a significant recovery period in a clean environment,  
370 influenced the upper thermal tolerance and cardiac function of juvenile fish. We found that a

371 week (trout) to months (seabass) post exposure, crude oil-exposed fish displayed higher (trout)  
372 or unchanged (seabass) upper critical thermal tolerance than control, unexposed fish. We also  
373 found that crude oil did not have a significant chronic residual influence on the cardiac  
374 performance of our juvenile fish at high temperatures and the estimate for optimum temperature  
375 of the fish even increased after exposure in both species.

376

377 The upper critical temperature ( $CT_{MAX}$ ) corresponds to temperature at which a fish, exposed to  
378 a progressive and controlled increase in water temperature, is no longer able to maintain an  
379 upright position. When the  $CT_{MAX}$  is reached survival is time-limited and, thus,  $CT_{MAX}$  is used  
380 as a proxy for upper critical thermal tolerance of fish (Pörtner 2010). In the current study we  
381 observed that the oil-exposed rainbow trout had significantly higher  $CT_{MAX}$  than control ones.  
382 In seabass, on the other hand, no difference in  $CT_{MAX}$  was observed between control and oil-  
383 exposed fish. These values ( $29.0 \pm 0.2^\circ\text{C}$  for rainbow trout acclimated at  $16^\circ\text{C}$  and  $31.3 \pm 0.3^\circ\text{C}$   
384 for seabass acclimated to  $17^\circ\text{C}$ ) were similar to those reported earlier ( $29.4^\circ\text{C}$  and  $28-35^\circ\text{C}$ ,  
385 Beitinger et al. 2000; Claireaux et al. 2013). It has previously been found in seabass that  
386 exposure to crude oil ( $0.07 \text{ g L}^{-1}$ ) did not affect their upper critical thermal tolerance ( $CT_{MAX}$ )  
387 (Mauduit et al. 2016). The present study, using 12.5 times higher oil concentration, confirms  
388 that exposure to petroleum hydrocarbons has no significant, long-term effect on upper critical  
389 thermal tolerance in this species. Furthermore, although in the present experiment seabass was  
390 exposed to ten times higher concentration than trout, it displayed remarkably efficient recovery  
391 as indicated by the preserved upper critical thermal tolerance observed 6 months post-exposure.

392

393 Optimal temperature ( $T_{OPT}$ ) of rainbow trout and seabass are  $16.5-17^\circ\text{C}$  and  $22-24^\circ\text{C}$ ,  
394 respectively (Jobling 1981; Claireaux and Lagardère, 1999). In both species we observed that  
395 at suboptimal temperatures, maximum heart rate ( $f_{Hmax}$ ) of oil-exposed individuals was lower

396 than in control specimens. This result is consistent with earlier study by Milinkovitch et al.  
397 (2013a) who found in juvenile golden grey mullet exposed to crude oil ( $0.07 \text{ g L}^{-1}$ ,  $\Sigma\text{PAH } 3.3\text{-}$   
398  $60.1 \mu\text{g L}^{-1}$ ) that the capacity of ventricular muscle strips to generate force at suboptimal  
399 temperature ( $14.5^\circ\text{C}$ ) and high stimulation frequency ( $1.2 \text{ Hz}$ ) was not affected when measured  
400 directly after exposure. Furthermore, these authors reported that oil exposure did not  
401 significantly affect the force-frequency relationship established in control, unexposed fish.  
402 Similarly, Nelson et al. (2016) found that a 24h exposure to PAHs ( $9.6 \mu\text{g L}^{-1}$ ) did not influence  
403 the routine heart rate of juvenile mahi-mahi when measured directly after exposure. However,  
404 a significant decrease in routine cardiac output was reported by these authors, probably  
405 resulting from a decreased stroke volume. Claireaux and Davoodi (2010) found that juvenile  
406 common sole had reduced cardiac output, as well as impaired cardio-respiratory responses to  
407 an acute warming, 24h after exposure to crude oil ( $\Sigma\text{PAH } 39 \text{ ng L}^{-1}$  of water and  $15\text{-}71 \text{ ng g}^{-1}$   
408 dry liver tissues). These authors found that 24h post-exposure there was no statistically  
409 significant difference in routine heart rates between oil-exposed and control sole at  
410 temperatures between  $15\text{-}25^\circ\text{C}$  (optimal temperature in sole is  $19^\circ\text{C}$ ; Lefrançois and Claireaux  
411 2003). However, a difference was observed as temperature reached  $30^\circ\text{C}$ , with the cardiac  
412 output of the oil-exposed common soles not being able to match the temperature driven  
413 increase in metabolic oxygen demand (Claireaux and Davoodi 2010). There are no other  
414 studies about the effect of crude oil or PAHs on heart rate in non-embryonic fish. However,  
415 work on embryonic fish indicates that exposure conditions affect the cardiac response to oil  
416 compounds. For example, developing zebrafish (*Danio rerio*) exposed to oiled gravel effluent  
417 displayed reduced routine heart rate, while exposure to water-accommodated fractions  
418 containing dispersed oil microdroplets had no effect (Jung et al. 2013). In Pacific herring  
419 (*Clupea pallasii*), oiled-gravel exposure increased the variability of routine heart rate and  
420 generally reduced it (Incardona et al. 2009). A similar response was observed in herring

421 embryos exposed to bunker fuel after *Cosco Busan* collision in San Francisco Bay (Incardona  
422 et al. 2012), as well as in haddock (*Melanogrammus aeglefinus*) embryos exposed  $0.7\text{--}7\ \mu\text{g L}^{-1}$   
423  $\Sigma\text{PAH}$  (Sørhus et al. 2016) and in rainbow trout larvae exposed to  $1.6\ \text{g L}^{-1}$  of crude oil  
424 (Vosyliene et al. 2005). An exposure to  $\Sigma\text{PAH} \sim 2\text{--}5\ \mu\text{g L}^{-1}$  (which is similar to the PAH  
425 concentration in our experiment with rainbow trout) reduced routine heart rate in embryos of  
426 bluefin and yellowfin tuna (Incardona et al. 2014). In larval red drum (*Sciaenops ocellatus*) the  
427 exposure to crude oil ( $2.6\ \mu\text{g L}^{-1}\ \Sigma\text{PAH}$ ) reduced the routine stroke volume but did not affect  
428 heart rate (Khursigara et al. 2016). In the current study, the reduction of  $f_{\text{Hmax}}$  at suboptimal  
429 temperatures in the oil-exposed fish may appear rather small ( $\sim 4$  bpm). However, it could have  
430 some negative consequences on cold tolerance of fishes. This is because the critical lower limit  
431 of heart rate to support the oxygen delivery to tissues could be reached at higher temperatures  
432 in oil-exposed fish as compared to control ones, a situation observed e.g. between warm and  
433 cold acclimated rainbow trout the warm acclimated ones having lower heart rate at cold  
434 temperatures and, thus, reduced tolerance for cold temperatures (e.g. Currie et al. 1998; Aho  
435 and Vornanen 2001). However, this aspect should be investigated in future studies with oil-  
436 exposed and control fish.

437

438 Despite the fact that  $f_{\text{Hmax}}$  was lower in exposed fish than in control fish at temperatures below  
439 the optimal temperature ( $T_{\text{OPT}}$ ) of the species considered, this difference disappeared at  
440 temperature above  $T_{\text{OPT}}$ . As a result, the highest  $f_{\text{Hmax}}$  values were similar in exposed and  
441 control fish. This is an important finding, as at high temperatures the maximum cardiac  
442 function is critical for fish survival through preserving aerobic capacity and upper thermal  
443 tolerance (Steinhausen et al. 2008; Farrell 2009; Eliason et al. 2013). Indeed, in both species  
444 Arrhenius break point temperature ( $T_{\text{AB}}$ ) which indicates the optimum temperature of fish (e.g.  
445 Casselman et al. 2012; Anttila et al. 2013a; Ferreira et al. 2014) was higher in oil-exposed

446 animals than in control ones. Furthermore, the other indices of upper thermal tolerance of  
447 cardiac function i.e.,  $T_{PEAK}$  (temperature where highest  $f_{Hmax}$  was observed) and  $T_{ARR}$   
448 (temperature where arrhythmias were observed) were higher in exposed rainbow trout than in  
449 control trout. In seabass,  $T_{PEAK}$  was also higher in exposed than in control seabass. A 4-month  
450 field experiment recently illustrated the ecological significance of these results as the survival  
451 and growth of oil-exposed seabass was shown to be similar to that of control fish although they  
452 were exposed to naturally high summer temperatures (Mauduit et al. 2016).

453

454 The heart rate and upper thermal tolerance values of cardiac function observed in the current  
455 study lie within the range of published values from these animals. Rainbow trout swimming at  
456 maximum sustainable velocity at 15°C has a heart rate of 96 bpm after vagotomy (Priede 1974).  
457 This value compares nicely with the values of 80 bpm observed in the present study (see also  
458 Mercier et al. 2000). The maximum heart rate of exercising seabass has been measured at  
459 around 90 bpm at 20°C (Chatelier et al. 2005) which agrees relatively well with the current  
460 finding of 90 bpm at 17°C. The  $T_{AB}$  values ( $15.1 \pm 0.4^\circ\text{C}$  and  $19.3 \pm 0.3^\circ\text{C}$  for rainbow trout and  
461 seabass, respectively) are somewhat lower, but near the optimum growth temperatures of these  
462 species ( $16.5\text{-}17^\circ\text{C}$  and  $\sim 22\text{-}24^\circ\text{C}$ , Jobling 1981; Claireaux and Lagardère 1999).

463

464 The two experiments that are reported here were conducted separately and differences in  
465 experimental conditions and design must be considered i.e., PAH composition of the oil tested,  
466 duration of the recovery post-exposure, concentration of crude oil tested, usage of dispersant  
467 and availability of tissues PAH concentrations.

468

469 Crude oil used in the seabass and trout experiments was of different origins and this is  
470 particularly reflected in their composition in PAH, the most potent oil compounds from a

471 cardiac standpoint. In embryonic fish it has been shown that PAHs are particularly detrimental  
472 to the cardiac function although reported effect are compound-specific (see review by  
473 Incardona and Scholz 2016). For instance naphthalene, chrysene, anthracene and  
474 benzo[k]fluoranthene, abundant PAHs in both tested oils, are known for having little effects  
475 on the physiology (conduction properties) and anatomy/pathology (pericardial edema) of the  
476 heart (Incardona et al. 2004; 2011). On the other hand, dibenzothiophene and phenanthrene  
477 have been shown to cause pericardial edema and to reduce heart rate by blocking  
478 atrioventricular conduction in a concentration-dependent manner (Incardona et al. 2004).  
479 Furthermore, benzo[e]pyrene and benzo[a]pyrene have also been shown to induce pericardial  
480 edema (Incardona et al. 2011). In the current experiments phenantherene was present in the  
481 water and fish tissues and dibenzothiophene was even the most abundant PAH in the seabass  
482 experiment.

483

484 Another difference between the two experiments is the duration of the post-exposure recovery  
485 period in clean water. The rainbow trout measurements were done one week after the exposure  
486 while seabass were measured 6 months post-exposure. The short-term effects of oil exposure  
487 upon the cardiac function are well documented (e.g. Incardona et al. 2009, 2012, 2014;  
488 Claireaux and Davoodi 2010; Jung et al. 2013; Milinkovitch et al. 2013a; Brette et al. 2014;  
489 Nelson et al. 2016; Sørhus et al. 2016; Khursigara et al. 2017). A review of the literature shows,  
490 however, that published studies of the toxicological impacts of oil exposure on fish  
491 predominantly investigated acute exposure, short exposure duration and immediate  
492 assessment, mostly at low biological organization levels. While these studies contribute to  
493 increase our mechanistic understanding of contamination and decontamination processes (e.g.  
494 Ramachandran et al. 2004; Milinkovitch et al. 2013a; Danion et al. 2014; Dussauze et al. 2014;  
495 Sadauskas-Henrique et al. 2016; Sandrini-Neto et al. 2016; Sanni et al. 2016), they are of

496 limited help when addressing issues such as, for instance, impact on the resilience, health,  
497 production and recruitment of the affected populations (Forbes et al. 2006). From this  
498 perspective, the present experiments are remarkable as, although providing little additional  
499 information about the well documented patterns of PAH bioaccumulation and metabolization  
500 in fish (Claireaux and Davoodi 2010; Milinkovitch et al. 2013a; Nelson et al. 2016), they  
501 provide evidence in favor of the absence of chronic latent effects of oil exposure on fish thermal  
502 tolerance and cardiac performance. These results substantiate the recovery process reported by  
503 Claireaux et al. (2013) and Mauduit et al. (2016) regarding swimming capacity, tolerance to  
504 heat and to hypoxia. Interestingly, although the two sets of experiments involved two different  
505 fish species with quite different exposures and at two different times post-exposure, very  
506 similar response patterns were observed. This is an important result, because it shows that  
507 cardiac recovery could be relatively general phenomenon in juvenile fish. However, in order  
508 to evaluate the recovery process more thoroughly, more time points should be examined in  
509 future.

510

511 One other difference between the two studies was that we didn't use dispersant in the trout  
512 study and that tissue PAH concentrations were not measured in that species. Nevertheless,  
513 nominal concentrations tested in present studies are representative of the concentrations  
514 measured following an accidental spill (Boehm and Fiest, 1982; Milinkovitch et al. 2011),  
515 which have been shown to result in detrimental effects on cardiac function when measured  
516 within days-weeks post-exposure (e.g. Claireaux and Davoodi 2010; Incardona et al. 2014;  
517 Sørhus et al. 2016; Khursigara et al. 2017).

518

519 In conclusion, exposure to crude oil did not have a significant impact on maximum heart rate  
520 and cardiac function of juvenile fish at temperatures above their optimum when the

521 measurements were done 1 week (trout) to 6 months (seabass) after the exposure. We observed  
522 that exposure may even improve the upper thermal tolerance of fish. The reason for this  
523 unexpected result is unknown, thus, future studies should focus on this issue. Our findings also  
524 suggest that recovery processes exist that can reverse the functional impairments observed  
525 shortly following direct exposure. This is also an important finding for the fisheries as both of  
526 these species are important human food species and are living/reared at areas where the threat  
527 for both oil accidents and warming surface waters is particularly high.

528 **Compliance with ethical standards**

529

530 **Conflict of Interest**

531 The authors declare that they have no conflict of interest.

532

533 **References**

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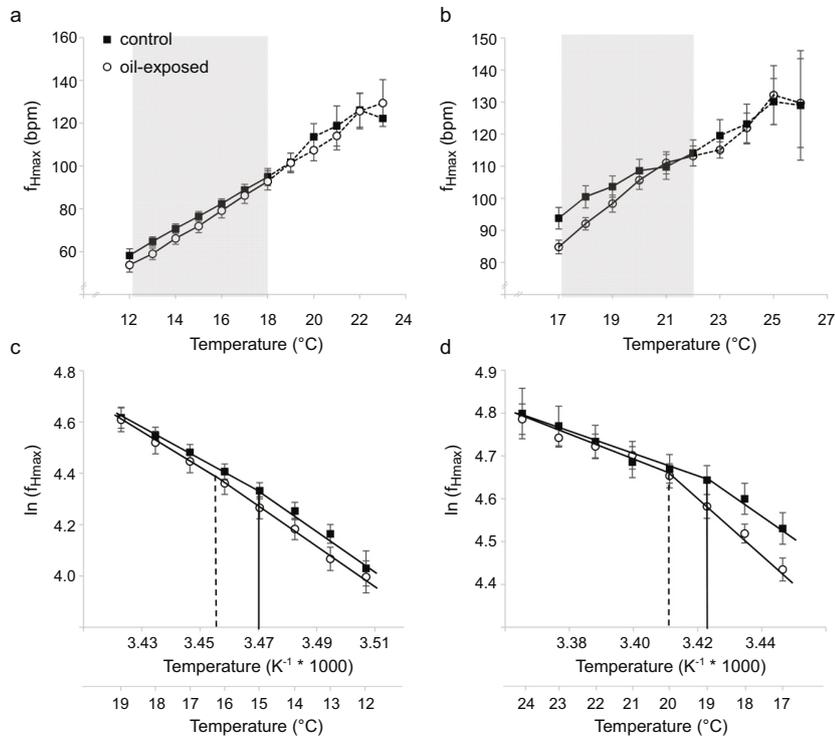
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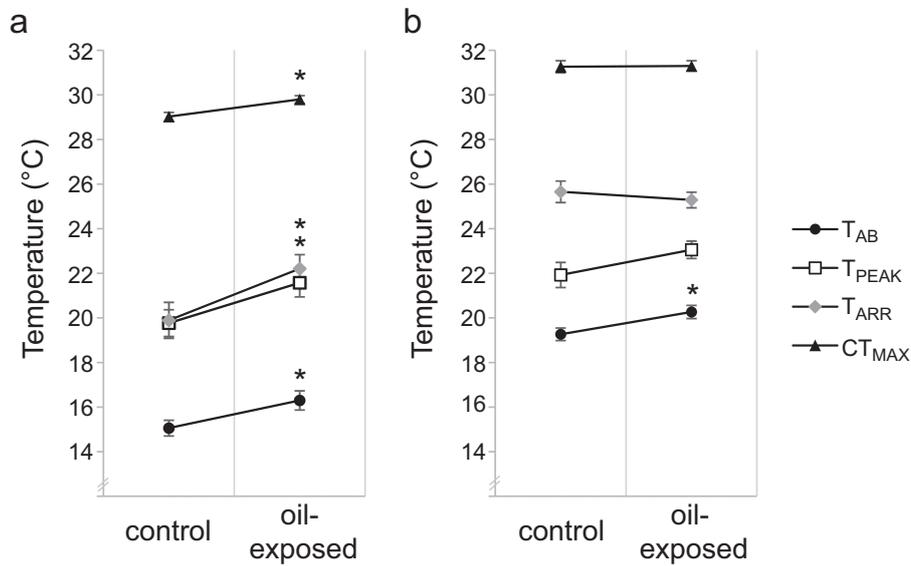
767 **Figures**



768

769 **Figure. 1** The cardiac responses to increasing temperature in control and oil-exposed fish after  
 770 one-week (rainbow trout) and six-month (seabass) recovery. The effect of increasing  
 771 temperature on the maximum heart rate ( $f_{Hmax}$ ) of **a** juvenile rainbow trout and **b** European  
 772 seabass. The grey area indicates the temperature window where there are significant differences  
 773 between control and oil-exposed fish. The dotted lines indicate temperatures where some of  
 774 the fish already had arrhythmias. The Arrhenius plots of  $f_{Hmax}$  responses to increasing  
 775 temperature for rainbow trout are given in **c** and for seabass in **d**. The vertical lines indicate an  
 776 Arrhenius break point temperature ( $T_{AB}$ ).  $n = 12$  for rainbow trout and  $n = 18$  for seabass

777



778

779 **Fig. 2** The temperature tolerance values of control and oil-exposed fish. Arrhenius break point  
 780 temperature ( $T_{AB}$ ), temperature for peak maximum heart rate ( $T_{PEAK}$ ), temperature where  
 781 cardiac arrhythmias are observed ( $T_{ARR}$ ) and critical upper temperature tolerance ( $CT_{MAX}$ ) of  
 782 **a** rainbow trout and **b** European seabass. \* indicates statistically significant differences between  
 783 oil-exposed and control fish.  $n = 12$  for rainbow trout (except for  $CT_{MAX}$  measurements  $n = 15$ )  
 784 and  $n = 18$  for seabass (except for  $CT_{MAX}$  measurements  $n = 20$ )

785

786 **Supplementary materials and methods**

787

788 **Water and tissue chemistry**

789

790 **Experiment 1. Rainbow trout**

791 All the water chemistry analyses from experiment 1 were conducted by Novalab Oy, Karkkila,  
792 Finland. The water samples (1 liter) were taken from each tank at the start and end of oil  
793 exposure, treated with 2 mL of 37% HCl and stored at +4°C before analyses. The analyses  
794 were conducted within two days after sampling. Total hydrocarbon content (C10-C40) and  
795 concentration of 16 different PAH compounds (US-EPA PAHs) were measured. For the total  
796 hydrocarbon analyses the water samples were extracted with heptane. The extract was cleaned,  
797 dried and concentrated before analyses. The C10-C40 content was analyzed with gas  
798 chromatography-flame ionization detector (GC-FID). The results were calculated dividing the  
799 hydrocarbons into middle heavy and heavy hydrocarbons and into the sum of them (>C10 -  
800 C21, >C21 - <C40, and >C10 - <C40). The functionality of the method was checked during  
801 every measurement with external control samples and blank samples. The C10-C40 content of  
802 water samples were corrected for blank values and calculated with external standard curve.

803

804 The analysis of 16 PAH compounds was performed on toluene extracts by GC-mass  
805 spectrometry (GC-MS) with selected ion monitoring (SIM) technique. The extract was cleaned,  
806 dried and concentrated before analyzing. The results were calculated using two inner standards  
807 for each compound. The functionality of the method was checked during every measurement  
808 with control samples and blank samples. Limits of detection were <0.05 mg L<sup>-1</sup> for C10-C40  
809 and 0.1 µg L<sup>-1</sup> for PAH compounds. In determinations of sum of PAHs and sum of total  
810 hydrocarbons, only values above the limit of quantification were included. Novalab Oy  
811 participates in intercalibrations regularly.

812

813 **Experiment 2. Seabass**

814 The analyses of concentration of total hydrocarbons of water samples and PAH content (21  
815 different PAH compounds i.e. 16 US-EPA PAHs and five supplementary PAH:  
816 benzo[b]thiophene, biphenyl, dibenzothiophene, benzo[e]pyrene and perylene) of liver of  
817 seabass were conducted at CEDRE (Brest, France). The details of methods are presented in  
818 Maudit et al. (2016). Briefly, the water samples (100 mL) were extracted 3 times with  
819 dichloromethane (Carlo Erba Reagents, France). The extract was cleaned, dried and

820 concentrated before analyses. The absorbance of organic phase (i.e giving the sum of total  
821 hydrocarbons in water samples) was measured with Evolution 600 UV-VIS spectrophotometer  
822 (Thermo Fisher Scientific) at 390 nm and the absorbance was compared to control samples and  
823 corrected with blank.

824

825 GS-SM technique was used to measure the PAH content of liver. The liver samples (100 mg)  
826 were digested with ethanol and potassium hydroxide and the PAH compounds were extracted  
827 with stir-bar sorptive method using polydimethylsiloxane stir-bars (Twister 20 mm \* 0.5 mm,  
828 Gerstel, Germany) (more details in Maudit et al. 2016). After the extraction, the stir bars were  
829 cleaned and dried and PAH content was analysed with GC-MS. 7 inner standards were used in  
830 the analyses and the PAH levels of samples were quantified relative to standard PAHs. The  
831 water content of liver samples were analysed by drying the aliquot samples at 48°C for 48h.  
832 The results are presented as ng PAH g<sup>-1</sup> dry weight of liver. The limits of detection were 4 ng  
833 g<sup>-1</sup> for dibenzothiophene and fluoranthene, 8 ng g<sup>-1</sup> for fluorene, phenanthrene, anthracene,  
834 pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene,  
835 benzo[e]pyrene and 40 ng g<sup>-1</sup> for rest of the 21 PAH compounds.

836

837

838 **Supplementary Table** Concentration of 16 PAHs in water samples of rainbow trout exposure  
 839 tanks (experiment 1) and 21 PAHs in seabass liver samples (experiment 2) from both control  
 840 and oil- treatments. The 16 PAHs represent the 16 US-EPA PAHs and the five supplementary  
 841 PAHs are: benzo[b]thiophene, biphenyl, dibenzothiophene, benzo[e]pyrene and perylene. The  
 842 limit of quantification = LOQ.

	Experiment 1, rainbow trout		Experiment 2, seabass	
	Concentration $\pm$ sem ( $\mu\text{g L}^{-1}$ ) during exposure		Concentration $\pm$ sem ( $\text{ng g}^{-1}$ ) 2 days after exposure	
	control	oil	control	oil
Naphtalene	<LOQ	$3.2 \pm 0.85$	<LOQ	$1952 \pm 611$
Benzo[b]thiophene	-	-	<LOQ	$446 \pm 59$
Biphenyl	-	-	<LOQ	$2267 \pm 405$
Acenaphthylene	<LOQ	<LOQ	<LOQ	$2164 \pm 420$
Acenaphthene	<LOQ	$0.15 \pm 0.07$	<LOQ	$370 \pm 73$
Fluorene	<LOQ	$0.4 \pm 0.2$	<LOQ	$1327 \pm 605$
Dibenzothiophene	-	-	<LOQ	$20708 \pm 3529$
Phenanthrene	<LOQ	$0.75 \pm 0.4$	<LOQ	$5090 \pm 1356$
Anthracene	<LOQ	<LOQ	<LOQ	224
Fluoranthene	<LOQ	<LOQ	<LOQ	9
Pyrene	<LOQ	<LOQ	<LOQ	21
Benzo[a]anthracene	<LOQ	<LOQ	<LOQ	<LOQ
Chrysene	<LOQ	<LOQ	<LOQ	43
Benzo[b]fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[k]fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[e]pyrene	-	-	<LOQ	<LOQ
Benzo[a]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Perylene	-	-	<LOQ	<LOQ
Indeno[1,2,3-cd]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[a, h]anthracene	<LOQ	<LOQ	<LOQ	<LOQ
Benzoperylene	<LOQ	<LOQ	<LOQ	<LOQ

843  
844

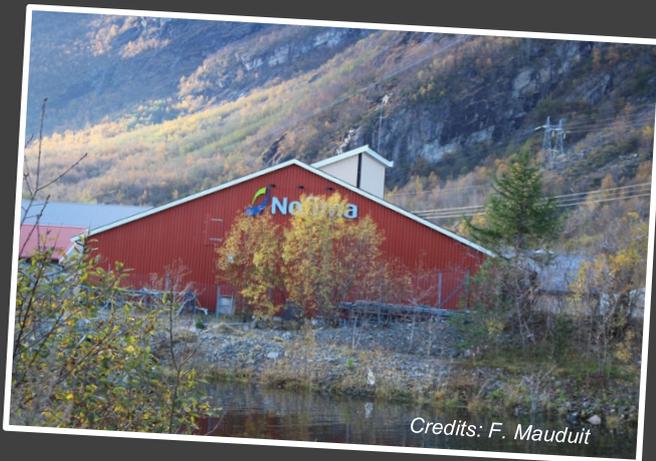
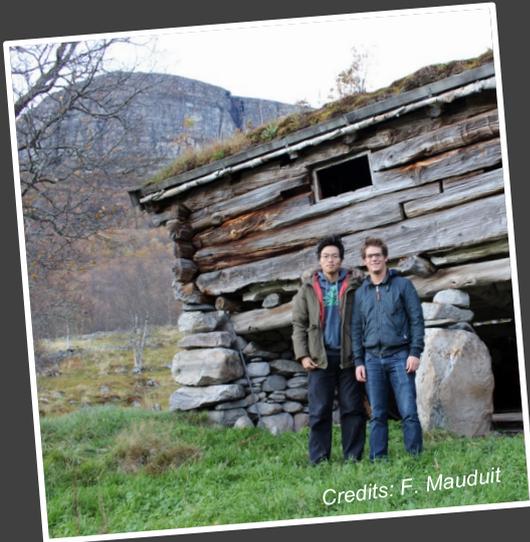
## **6. Conclusion**

This chapter aimed to document the underlying mechanisms and confounding factors of the performances measured to evaluate fish health. A better understanding of these elements is indeed required for a reliable interpretation of biomarkers' response.

Examination focused on the cardiorespiratory function. We notably found that hypoxia tolerance was not related to fish aerobic capacity but was driven by the capacity to preserve the working capacity of the myocardium (contractility and stroke volume) under hypoxic condition. We can also consider that the ability to extract and use oxygen anaerobically contribute to that performance.

Investigation of the potential confounding factors revealed that environmental history can strongly influence response of our potential markers of fish health. Comparison of markers' response in two populations with different environmental history could therefore lead to erroneous conclusions. Furthermore, to validate our interpretation of biomarker response, we verified that the lack of long-term effect reported after an exposure to oil was not due to compensatory or regulatory mechanisms. No effect of oil on cardiac function was observed, supporting our initial interpretation of the potential markers of health. Therefore, we can state that the lack of effect observed is not due to the masking effect of regulatory and compensatory mechanisms.

## Chapter IV: Case studies



## **1. Introduction**

Health of fish populations is an important indicator of their ecosystems' condition and a factor that directly relates to human wealth and health. Fish are typically at the top of the aquatic food chain and, therefore, are likely to accumulate a variety of contaminants and pass them on to human beings through food, which in turn may cause chronic or acute diseases (Mozaffarian and Rimm, 2006). Assessing fish health is therefore the cornerstone in several domains, including public health safety, conservation biology, environmental impact assessment and fisheries. Despite this broad interest, methodologies that provide reliable evaluations of fish health are lacking. The third objective of my thesis was therefore to fill this gap by proposing a methodology to improve current management strategies and practices by the industry, resource managers and conservation practitioners. To reach that objective we particularly verified that the methodology was generalizable by testing it in two different contexts, the treatment of an oil spill and the evaluation of farmed fish condition. We also paid special attention to the fact that the methodology provided operational information easily transferable to socio-economic sectors and general public. Lastly, we ensured that the methodology required minimum resources in term of equipment and manpower.

The first experiment examined the consequences for juveniles of seabass of being exposed to a dispersant-treated oil spill. The second experiment aimed at verifying the consequences of domestication upon the robustness of farmed Atlantic salmon. Even though these case studies may seem quite different, they both share the same objective: improving current practices regarding preservation and management of aquatic species.

The use of dispersants to combat oil spills still raises controversy and, at times, generates widespread debate in society. The reluctance to consider dispersants as a valuable oil spill response option rises from the increased bioavailability of oil compounds that results from their use (Ramachandran *et al.*, 2004). To provide ecologically relevant information regarding dispersant-treated oil toxicity for fish, we assessed the effect of an exposure upon hypoxia tolerance and temperature susceptibility.

The Atlantic salmon farming industry is faced with a persistent challenge that relates to the survival of smolts following their transfer to the sea cages ( $\approx 16\%$  of annual loss). For decades, Norwegian Atlantic salmon breeding programs have focused improving on-growing procedures. There is, however, growing concerns that this strategy has contributed to a compromise in fish robustness and phenotypic plasticity. Our study aimed to verify if this domestication and breeding strategy has affected the capacity of farmed salmon to respond to a training protocol that aimed at improving athleticism. Towards this objective a suite of indices for respiratory performance and hypoxia tolerance were measured in two strains of Atlantic salmon, the domesticated *Bolaks* and the wild *Lærdal*.

## 2. Paper 6 (submitted to Environmental pollution)

1 **Assessing the effect of exposure to dispersant-treated oil on fish health using**  
2 **hypoxia tolerance and temperature susceptibility as ecologically relevant**  
3 **biomarkers**

4

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22 France

23

24

25

26 **Author Contributions**

27 All authors contributed to the design of the study; FM, CL, NL & GC collected the  
28 data; FM & GC analysed data; FM, GC, JLZ, SLF, AF & PD wrote the m/s; PLM,  
29 ANK, MW financed the study.

30

31 **Abbreviation**

32 CONT: Control

33 CT<sub>max</sub>: Critical thermal maximum

34 HCT: Hypoxia challenge test

35 ILOS: Incipient lethal oxygen saturation

36 O25D1: 25 g of oil + 1 g of dispersant

37 O125D5: 125 g of oil + 5 g of dispersant

38 O250D10: 250 g of oil + 10 g of dispersant

39 PAH: Polycyclic aromatic hydrocarbons

40 SBSE-GC-MS/MS: stir bar sorptive extraction-thermal desorption-gas  
41 chromatography-tandem mass spectrometry

42 SIMA: Spill Impact Mitigation Assessment

43 TCT: Temperature challenge test

44 TLOE: Time to loss of equilibrium

45 TPH: Total petroleum hydrocarbon

46

47 **Abstract**

48 Spill Impact Mitigation Assessment (SIMA) requires a reliable assessment of the  
49 ecological consequences of dispersant use upon marine animals, including fish. To  
50 this objective, environmental and fishery scientists continually strive to develop  
51 methods to assess fish health. In this sense, complex physiological traits are valuable  
52 tools as they are the integrative outcomes of numerous underlying mechanisms and  
53 therefore reflect the functional integrity of a fish and its capacity to face natural  
54 contingencies. In fish, hypoxia tolerance and temperature susceptibility are promising  
55 biomarkers. However, their use in impact assessment has been limited by several  
56 knowledge gaps, which include an incorrect understanding of the baseline fluctuation  
57 in these biomarkers' response and of the relationships between biomarker response  
58 and pollutant exposure. The purpose of the present study was therefore to fill these  
59 gaps by 1) verifying the extend of inter-population variability of hypoxia tolerance and  
60 temperature susceptibility, in juveniles of European sea bass and 2) testing the effect  
61 of an exposure to 3 different concentrations in dispersant-treated oil upon fish health  
62 to document the causal relationship between exposure to dispersant-treated oil and  
63 biomarkers response. Our results revealed a high inter-population variability in  
64 hypoxia tolerance and temperature susceptibility, which highlighted that using a  
65 population as a control to assess the effect of a stressor upon another one, could  
66 lead to false negative or false positive results. Furthermore, assessment of fish  
67 sensitivity to dispersant treated oil revealed that the exposure did not lead to any  
68 ecologically relevant impairment, suggesting that juvenile sea bass are able to  
69 tolerate concentrations of dispersed oil typically associated with large-scale oils spills.

70 **Capsule**

71 Using hypoxia tolerance and temperature susceptibility as biomarkers of fish health,  
72 no latent effect of dispersant-treated oil was observed.

## 73 **1 Introduction**

74 While oil reserves remains the world's leading source of energy (IEA 2015), supply  
75 and demand are rarely located in the same place. Thus, efficient transport is vital to  
76 ensure that a continuous flow of oil sustains distant energy needs. Maritime transport  
77 is recognized as a cost-effective means to connect petroleum supply and demand  
78 areas. Indeed, sea-borne oil trade in 2016 amounted to around 10 000 billion ton-  
79 miles (ITOPF, 2016).

80

81 Although increased oil trade is generally associated with increased risk of accidental  
82 oil release, the number of large spills (> 700 tons) has decreased over the last 40  
83 years. For instance, the number of large spills observed during the early 2000s is  
84 less than a tenth of that witnessed during the 1970s (ITOPF, 2016). Yet, despite a  
85 reduction in number, large spills remain highly relevant, both economically and  
86 ecologically. In fact, 86% of the 33,000 tons of oil spilled between 2010 and 2015  
87 were due to only 10 of the 42 accidents recorded (ITOPF, 2016)

88

89 Resorting to dispersants is one of the response strategies available during an oil spill  
90 and dispersants have been widely utilized since the landmark *SS Torrey Canyon*  
91 shipwreck in 1967 in the UK. However, the 'first generation' dispersants used in the  
92 late 1960s and the 1970s, while very effective in breaking up oil slicks at sea, were  
93 also highly toxic to aquatic life and were responsible for some severe additive  
94 environmental impacts (Smith, 1968). Nevertheless, scientific investigations  
95 conducted since then have shown that the observed toxicity was essentially induced  
96 by the solvents (oil fractions composed of aromatic hydrocarbons with low boiling  
97 points) rather than the surfactants (Le Floch, 2016).

98

99 Nowadays, third generation oil dispersants are mixtures containing a high  
100 concentration of surfactants in a water miscible solvent, which together facilitate the  
101 breakup of oil slicks into small droplets, thus enhancing their biodegradation by  
102 bacteria (Lessard and DeMarco, 2000). The reluctance that persists among  
103 responders to consider dispersants as one of the oil spill response strategies stems  
104 from the increased bioavailability of oil compounds that results from their use and in  
105 their potential toxicity for marine species (Ramachandran *et al.*, 2004). Adverse  
106 effects of dispersant use is of particular concern in nearshore areas where the low  
107 dilution potential of an oil slick may expose ecologically sensitive ecosystems to high  
108 concentrations of petroleum compounds.

109

110 To identify the response actions that minimize environmental impacts, decision-  
111 makers resort to Spill Impact Mitigation Assessment (SIMA, formerly known as  
112 NEBA, Net Environmental Benefit Analysis). SIMA is a consensus-based tool that  
113 allows decision-makers to use inputs from stakeholders, subject matter experts,  
114 regulators and responsible parties to assess the trade-offs associated with the  
115 various response options and to select those in line with agreed-upon preservation or  
116 mitigation objectives. Following an oil spill, trade-offs are inevitable between limiting  
117 impacts to landscape and to surface dwelling resources (wetlands, birds, marine  
118 mammals, turtles) *versus* preserving resources that exist in the water column and on  
119 the bottom (fish, corals). Therefore, to establish a comprehensive framework for  
120 dispersant use in nearshore areas, information about its potential impact on marine  
121 resources is required.

122

123 In ecotoxicology, the ability to diagnose the effects of toxic chemicals often hinges on  
124 biomarkers. A biomarker is a substance, a structure or a process that can be  
125 measured accurately and reproducibly, and which reflects the interactions between a  
126 biological system and its environment. According to this definition, a group of genes,  
127 a biochemical pathway, an organism, a population or an ecosystem can be  
128 considered as a biological system. However, the literature shows that most  
129 biomarkers lie at the sub-organismal level of organization (molecules, cells or  
130 tissues), whereas SIMA is largely applied at the ecosystem level (Paniagua-Michel  
131 and Olmos-Soto, 2016). Although functional links exist between organizational levels  
132 (McCarthy *et al.*, 1991), the lack of integrated mechanistic models describing how  
133 toxic effects cascade through organizational levels currently limits our ability to  
134 extrapolate from toxicity at sub-organismal level to impact at SIMA-relevant levels  
135 (e.g. population or ecosystem) (Depledge *et al.*, 1993; van der Oost *et al.*, 2003;  
136 Forbes *et al.*, 2006).

137

138 To fill the gap between what is desirable (assessing the consequences at high  
139 organizational level) and what is generally measured (sub-organismal biomarkers),  
140 several authors have recommended merging current bottom-up approaches of  
141 ecotoxicology (from cellular to population level) with top-down approaches aimed at  
142 resolving ecologically relevant issues (Rosen *et al.*, 1969; Beketov and Liess, 2012;  
143 Rohr *et al.*, 2016). In fish, these issues mostly relate to fisheries management. The  
144 sustainable management of fish stocks which implies, for instance instance, a reliable  
145 assessment of fish health status for proper projections of the stock dynamics and  
146 production. To assess fish health, complex physiological traits may be valuable tools  
147 as they are the integrative outcome of numerous underlying mechanisms and so

148 reflect the functional integrity of fish and their capacity to face natural contingencies  
149 (Brett, 1958; Sprague, 1971; McKenzie *et al.*, 2007, Claireaux *et al.*, 2013; Mauduit *et*  
150 *al.*, 2016). These tools may be, therefore, of immediate ecological relevance.

151

152 To evaluate the potential offered by high-organizational level biomarkers to assess  
153 fish health, a set of six criteria has been proposed (McKenzie *et al.*, 2007):

- 154 1. *the assay to quantify the biomarker should be reliable (with quality assurance),*  
155 *relatively cheap and easy to perform;*
- 156 2. *the biomarker response should be sensitive to pollutant exposure and/or*  
157 *effects in order to serve as an early warning signal;*
- 158 3. *data about baseline fluctuations in the biomarker value should be available in*  
159 *order to distinguish between natural variability (noise) and contaminant-*  
160 *induced stress (signal);*
- 161 4. *confounding factors to the biomarker response should be identified;*
- 162 5. *the mechanistic basis of the relationships between biomarker response and*  
163 *pollutant exposure (dosage and time) should be established;*
- 164 6. *the ecological significance of the biomarker, e.g., the relationships between its*  
165 *response and the (long-term) impact to the organism, should be established.*

166

167 In fish, hypoxia tolerance and temperature susceptibility are integrated physiological  
168 traits that fulfill most of the above criteria. It has been demonstrated, for instance, that  
169 these traits are temporally stable (criterion #1; Joyce *et al.*, 2016; Mauduit *et al.*,  
170 2016), are sensitive to sub-lethal concentrations of a wide range of pollutants  
171 (criterion #2; Becker and Wolford, 1980; Mauduit *et al.*, 2016) and are potential early  
172 indicators of impact at a high organizational level (criterion #6; Mauduit *et al.*, 2016).

173 Moreover, confounding factors to consider when interpreting these biomarkers have  
174 been identified such as temperature, pH, feeding, ageing and habituation (criterion  
175 #4; i.e., Claireaux *et al.*, 2007; Nilsson and Östlund-Nilsson, 2008; Roze *et al.*, 2013).  
176 However, baseline fluctuations in biomarker values in healthy populations are poorly  
177 documented, as are the underlying mechanisms that link biomarkers response and  
178 pollutant exposure conditions (criteria #3 and #5, respectively). If the natural variation  
179 of a given biomarker is low in a reference population, any change induced by the  
180 toxicant will be easy to detect. On the other hand, a high natural variation in a  
181 biomarker could conceal a toxicant-induced effect (Cairns, 1992). Thus, determining  
182 the sources and the extent of variability in biomarkers is essential to improve our  
183 capacity to discriminate signal from noise.

184

185 The purpose of the present study was, using the European sea bass (*Dicentrarchus*  
186 *labrax*) as a model system 1) to verify the extent of inter-population variability of  
187 hypoxia tolerance and temperature susceptibility in order to improve our ability to use  
188 these biomarkers as health assessment tools; and 2) to document the causal  
189 relationship between exposure to dispersant-treated oil and biomarkers response by  
190 testing 3 different concentrations of dispersant-treated oil.

191

## 192 **2 Materials and methods**

193

### 194 **2.1 Animals**

195 Four hundred and six, one-year-old sea bass (*Dicentrarchus labrax*) were obtained  
196 from a local fish farm (Aquastream, Lorient, France). Upon arrival in the laboratory

197 (Ifremer, Brest, France) fish ( $12.87 \pm 0.04$  cm;  $26.7 \pm 0.4$  g) were placed in 400-L,  
198 indoor tanks and acclimated for two months to laboratory conditions under natural  
199 photoperiod and temperature (salinity 30-32 ‰). They were fed daily *ad libitum* with a  
200 commercial diet (Neo Start Coul 2, Le Gouessant). Two weeks before the experiment  
201 started, fish were anaesthetized (MS-222;  $100 \text{ mg L}^{-1}$ ) and implanted subcutaneously  
202 with an identification tag (RFID; Biolog-id, France). Fish were unfed for 24 h before  
203 any manipulation or experiment. All protocols were in conformity with current rules  
204 and regulations in France.

205

## 206 **2.2 Experimental protocol**

207 The experimental schedule (fully described in Mauduit *et al.*, 2016) is summarized in  
208 Fig. 1. Briefly, fish health was assessed via a suite of challenge tests conducted 1  
209 month before (March), 1 month after (May) and again 10 months after (March) a 48-h  
210 exposure to one of four experimental treatments (Control, 25 g of oil + 1 g of  
211 dispersant, 125 g of oil + 5 g of dispersant, 250 g of oil + 10 g of dispersant). During  
212 the period June - November (3 to 7 months post-exposure) fish were held in a field  
213 mesocosm to assess the effect of experimental treatments upon fish capacity to  
214 thrive under semi-natural conditions. During the entire experimental period, fish were  
215 submitted to the natural seasonal cycles for temperature ( $11 - 20$  °C), photoperiod  
216 and salinity (30-32 PSU).

217

## 218 **2.3 Fish transport**

219 The experiment mobilized the resources of three experimental sites and it was  
220 necessary to transport fish among them. Most of the fish rearing was conducted at

221 Ifremer rearing facilities, while experimental oil exposure was conducted at Centre of  
222 Documentation, Research and Experimentation on Accidental Water Pollution  
223 (Cedre), about 12 km from Ifremer laboratory. In contrast, the mesocosm experiment  
224 was located 450 km from Brest. Fish were transported among all sites in a 1 m<sup>3</sup>-  
225 covered tank. Before transfer into the transport tank, fish were anaesthetized (MS-  
226 222; 100 mg L<sup>-1</sup>) and a maintenance dose of anesthetic (MS-222; 25 mg L<sup>-1</sup>) was  
227 maintained during the travel. Water temperature and oxygen levels were monitored  
228 continuously and maintained at seasonal values.

229

#### 230 **2.4 Experimental exposure**

231 In April 2014, fish were transported to the Cedre and placed in 2 m<sup>3</sup> tanks supplied  
232 with open flow seawater. Temperature, salinity and photoperiod followed seasonal  
233 variations and fish were unfed.

234

235 A set of 12 polyethylene tanks (300 L) equipped with homemade mixing devices  
236 comprising a funnel and a submersible bilge pump was used for the fish exposure  
237 treatment (Milinkovitch *et al.*, 2011). Four experimental conditions were tested in  
238 triplicate *i.e.* control (labelled CONT), 25 g of oil plus 1 g of dispersant (O25D1), 125  
239 g of oil added with 5 g of dispersant (O125D5) and 250 g of oil added with 10 g of  
240 dispersant (O250D10). Dispersant (Finasol OSR 52, Total Fluides) was used in  
241 accordance with manufacturer's recommendation (Dispersant/oil ratio of 4%). The oil-  
242 dispersant mixture was made in a bottle, directly poured in the tanks and then  
243 bubbled with air overnight to mimic a 12-h aging of an oil slick at sea (Nordvik, 1995).  
244 Fish were allocated to the various exposure treatments (102 fish per treatment, 34  
245 per tank) in such a manner that no statistically significant difference in hypoxia

246 tolerance and temperature susceptibility measured in the first challenge was found  
247 among treatments (Log-rank,  $p = 0.184$  and  $0.881$ , respectively). In this way,  
248 differences in performance among treatments could be attributed to a treatment  
249 effect. Following the exposure period (48 h), fish from the four treatments were briefly  
250 bathed in clean seawater to avoid cross-contamination and recovered in their original  
251 rearing tank for one week before being returned to the Ifremer laboratory.

252

## 253 **2.5 Challenge tests**

254 The two challenge tests (hypoxia and temperature) performed on the sea bass have  
255 been fully described in Mauduit *et al.* (2016). They were performed 1 month before  
256 (March), 1 month after (May) and again 10 months after (March) a 48-h oil exposure  
257 in the rearing tanks to reduce fish handling, with a one-week recovery period  
258 between the hypoxia and temperature challenge tests.

259

### 260 *2.5.1 Hypoxia challenge test*

261 The hypoxia challenge test (HCT) consisted of an initial, rapid decrease in water  
262 oxygenation (from near 100% to 20% air saturation, over 1 h) and followed by a  
263 slower decrease ( $2\% \text{ air saturation h}^{-1}$ ) until all the fish lost equilibrium. Hypoxia was  
264 achieved by bubbling nitrogen through a submersible pump placed in the  
265 experimental arena. Water oxygenation was monitored using an oxymeter (Ponsel  
266 Mesure). When a fish lost its ability to maintain balance, it was quickly removed from  
267 the experimental arena, identified (RFID tag reading) and placed in a fully aerated  
268 tank to recover. The corresponding time and oxygen level were also recorded. All fish  
269 recovered.

270

## 271 2.5.2 Temperature challenge test

272 The temperature challenge test (TCT) consisted of an initial, rapid increase in water  
273 temperature (from ambient temperature to 27 °C) over 2.5 h, followed by a slower  
274 increase (0.5 °C h<sup>-1</sup>) until all the fish lost equilibrium (Claireaux *et al.*, 2013). Water  
275 temperature was 10 °C in March and 15 °C in May (controlled using two 2500 W  
276 heaters; GERVEN). A submersible pump placed in the tank ensured homogeneity of  
277 water temperature and air saturation (controlled by bubbling of a mixture of oxygen  
278 and air in the tank). When a fish lost its ability to maintain balance, it was quickly  
279 removed from the experimental arena, identified (RFID tag reading) and placed in a  
280 fully aerated tank at acclimation temperature to recover. The corresponding time and  
281 temperature were also recorded. All fish recovered.

282

283 **2.6 Chemical analyses**

284 To assess the severity of fish exposure and document the detoxification process,  
285 liver concentrations of 21 polycyclic aromatic hydrocarbons (including the 16 PAHs  
286 used by US-EPA in fish contamination studies) were assessed 24 h and 1 month  
287 post exposure by GC–MS using a procedure fully described in Lacroix *et al.* (2014).  
288 Briefly, PAHs were extracted from liver samples using an alkaline digestion combined  
289 with stir bar sorptive extraction–thermal desorption–gas chromatography–tandem  
290 mass spectrometry (SBSE–GC–MS/MS). Also, the presence of PAH metabolites in  
291 bile was measured 24 h post-exposure. The concentration of bile polycyclic aromatic  
292 hydrocarbon metabolites was determined semi-quantitatively using fluorescence  
293 response (Aas *et al.*, 2000). It consisted of measuring the fluorescence with a  
294 spectrophotometer with a 5 nm slit width on emission and excitation channels (Jasco  
295 FP-6200, Tokyo, Japan). Analyses were performed using three excitation-emission

296 wavelengths i.e., 295-335 (naphthalene-type metabolites); 343–383 nm (four-ringed  
297 compounds including pyrene-type metabolites) and 380–430 nm (benzo[a]pyrene-  
298 type metabolites) (Krahn *et al.*, 1987; Lin *et al.*, 1996; Aas *et al.*, 2000).

299

## 300 **2.7 Mesocosm experiment**

301 On May 26<sup>th</sup> 2014, 400 fish (100 per treatment group) were transported to the  
302 “National Center for Scientific Research (CNRS)” field station in L’Houmeau (450 km  
303 from Brest). The L’Houmeau mesocosms set-up has been described on various  
304 occasions (Nelson and Claireaux, 2005; Claireaux *et al.*, 2007; Handelsman *et al.*,  
305 2010). Briefly, it has numerous 200-m<sup>2</sup>, 1-m deep earthen ponds that are connected  
306 to the nearby ocean via a canal, which allows partial seawater renewal in the ponds  
307 with each incoming tide. Standpipes prevent fish from escaping and bird nets cover  
308 the ponds to prevent avian predation. The fish must forage for food. Previous  
309 experiments and empirical observations have shown that the natural food web that  
310 develops in these ponds can sustain 2-3 kg of fish per pond. A complete description  
311 of the fauna of these earthen ponds is found in de Montaudouin and Sauriau (2000).  
312 Water conditions in the ponds (oxygenation, salinity and temperature) were  
313 measured hourly using a MPx multi-parameter probe (NKE).

314

315 Upon arrival at L’Houmeau field station, fish were immediately distributed among 4  
316 ponds (25 fish per treatment per pond). Ponds were drained monthly (July 16<sup>th</sup>,  
317 August 27<sup>th</sup>, September 24<sup>th</sup>) to assess survivorship and growth during the following  
318 5 months, with a final assessment on November 3<sup>rd</sup> when survivors were transported  
319 back to their original rearing facility in Brest .

320

## 321 **2.8 Data analysis and statistics**

322 Performance in challenge tests was analyzed using a Kaplan-Meier survival analysis,  
323 followed by a log-rank test. Fish survival in the ponds was analyzed using the same  
324 procedure. Growth rate measured in the mesocosms was analysed using a 2-way  
325 ANOVA. PAH concentrations in fish liver and bile fluorescence of different exposure  
326 treatments were compared using ANOVA, followed by Tukey's post-hoc test. Values  
327 are presented as a mean  $\pm$  SEM and statistical significance was accepted at  $p <$   
328 0.05. All statistical analyses were performed using R (R Development Core Team,  
329 2015).

330

## 331 **3 Results**

### 332 **3.1 Inter-population variability**

333 The frequency distributions of individual pre-exposure performances in the initial pre-  
334 exposure challenge tests were compared to those reported previously (Mauduit *et al.*,  
335 2016) for fish of a similar age and at the same temperature. Mean time to loss of  
336 equilibrium (TLOE) at HCT was  $6.43 \pm 0.06$  h in the present study (Fig. 2A) and was  
337 significantly lower than the group tested previously ( $8.15 \pm 0.03$  h;  $p < 0.001$ ).  
338 Similarly, the TCT performance was significantly lower in the present study than  
339 previously (Fig. 2B;  $2.98 \pm 0.03$  h vs  $3.92 \pm 0.03$  h;  $p < 0.001$ ).

### 340 **3.2 Oil Exposure**

341 The polycyclic aromatic hydrocarbon concentration ([PAH]) measured 1 day post-  
342 exposure in the liver of sea bass exposed to 25 g of oil treated with 1 g of dispersant  
343 (O25D1 =  $3.7 \pm 0.4 \mu\text{g g}^{-1}$  dw) was 5-times higher than the control treatment ( $0.79 \pm$

344 0.04  $\mu\text{g g}^{-1}$  dw) (Fig. 3). Likewise, a 5-times greater oil exposure (125 g of oil treated  
345 with 5 g of dispersant) resulted in a similar amplification of the [PAH] (O125D5 =  
346  $23.7 \pm 2 \mu\text{g g}^{-1}$  dw), while a 10-times greater oil exposure (250 g of oil plus 10 g of  
347 dispersant) resulted in a 40-times higher liver [PAH] (O250D10 =  $34.3 \pm 9 \mu\text{g g}^{-1}$  dw)  
348 than in the treatment control. Tricyclic PAH compounds (fluorene, dibenzothiophene,  
349 and phenanthrene) represented almost 80% of the total [PAH] measured (appendix  
350 1). Nonetheless, by 31 days post-exposure, liver [PAH] concentrations measured in  
351 fish from all four treatment groups ( $0.03 - 0.46 \mu\text{g g}^{-1}$  dw) had returned to  
352 background concentrations, which meant that latent effects were measured at least 4  
353 months after a liver PAH burden could be measured.

354

355 Bile concentration in fluorescent aromatic compounds (FAC) in fish sampled 1 day  
356 post-exposure was significantly influenced by chemical exposure conditions at the 3  
357 wavelengths tested ( $p < 0.001$ ; Fig. 4). Analysis revealed that at 295–335 nm (2/3-  
358 ring compounds; Fig. 4A), FAC levels measured in fish from the lowest experimental  
359 oil exposure (O25D1) were significantly higher than in fish from the treatment control  
360 ( $p < 0.0001$ ). Similarly, FAC levels observed in fish from the O125D5 and O250D10  
361 were also significantly higher than in fish from treatment CONT but these levels were  
362 statistically lower than in fish from O25D1. Concerning the 343–383 nm (4-ring  
363 compounds; Fig. 4B) and 380–430 nm (5-ring compounds; Fig. 4C) wavelengths, the  
364 O25D1, O125D5 and O250D10 treatments resulted in significantly higher  
365 fluorescence levels than sea bass from the co-treatment CONT ( $p < 0.0001$ ).  
366 Moreover, fluorescence levels were higher in fish from treatments O125D5 and  
367 O250D10 than in fish from treatment O25D1 ( $p < 0.0001$ ). No significant difference

368 between the O125D5 and O250D10 treatments was found ( $p = 0.9990$  at 343–383  
369 nm;  $p = 0.1739$  at 380–430 nm).

370

### 371 **3.3 Functional tests performed 1 month post-exposure**

372 The responses to two challenge tests are summarized in Table 2. Mean time to loss  
373 of equilibrium in the HCT for the control fish was  $7.065 \pm 0.035$  h, which  
374 corresponded to  $4.82 \pm 0.04$  % air saturation, and did not differ significantly ( $p >$   
375  $0.999$ ) from the OIL + DISP treatment. Similarly, TLOE for the control fish in the TCT  
376 was  $9.274 \pm 0.090$  h, which corresponded to  $31.41 \pm 0.05$  °C and did not differ from  
377 the treatment CONT ( $p = 0.455$ ). Therefore, consistent with the depuration of PAH  
378 from the tissues, there was no evidence of latent effect of oil exposure on the  
379 performance of sea bass during the challenge tests performed 1 month post-  
380 exposure.

381

### 382 **3.4 Mesocosm experiment**

383 Environmental conditions were similar in the different experimental ponds of the  
384 mesocosm (Fig. 5). Daily and seasonal patterns existed for the water temperature.  
385 Independent of the season, a 5°C difference between nighttime minimum and  
386 daytime maximal temperatures was generally observed. The highest recorded  
387 temperature was 29.8 °C (July 25<sup>th</sup> at 18:37) and the lowest recorded temperature  
388 was 13.8 °C (October 24<sup>th</sup> at 9:55; Fig. 5A). While water oxygen level displayed no  
389 seasonal trend, broad daily variations existed, with an evening maximum ranging  
390 between 170 - 260 % air saturation (a supersaturation state that reflected  
391 photosynthetic activity) and an early morning minimum ranging between 30 and 50 %  
392 air saturation (that reflected biotic respiration) (Fig. 5B). Water salinity, however,

393 fluctuated between 29 to 39 PSU without either a seasonal or a diurnal pattern (Fig.  
394 5C). Breaks in the time series corresponded to water renewal that occurred at spring  
395 tides or when ponds were drained for fish monitoring.

396

397 Fish growth was maximal between June 19<sup>th</sup> and July 16<sup>th</sup> (1.5 % d<sup>-1</sup>; Fig. 6A).  
398 However, growth performance progressively decreased as summer progressed, with  
399 little fish growth occurring by the end of the experiment. No significant difference in  
400 growth existed among the four treatment groups ( $p = 0.524$ ; data not shown).

401

402 Fish mortality occurred mainly during the first period of the field experiment (15%  
403 between June 26<sup>th</sup> and July 17<sup>th</sup>) despite the better growth conditions. Survivorship at  
404 the end of the field experiment exceeded 70% in all treatment groups (Fig. 6B), with  
405 no significant difference in survival among the treatment groups ( $p = 0.779$ ).

406

407

### 408 **3.5 Functional tests performed 11 month post-exposure**

409 The responses in the final set of challenge tests are summarized in Table 3. TLOE  
410 for the control fish for the HCT was  $9.088 \pm 0.2$  h, which corresponded to  $4.2 \pm 0.1$  %  
411 air saturation. No significant difference ( $p > 0.999$ ) was observed with any of the  
412 dispersant-treated oil treatments. TLOE for the control fish for the TCT was  $4.293 \pm$   
413  $0.2$  h, which corresponded to  $27.8 \pm 0.1$  °C. No significant difference ( $p = 0.455$ ) was  
414 observed with any of the dispersant-treated oil treatments.

415

**416 4 Discussion**

417 Complex physiological traits are promising tools to assess fish health. The literature  
418 indeed shows that hypoxia tolerance and temperature susceptibility meet most of the  
419 criteria that define a reliable and suitable biomarker (McKenzie *et al.*, 2007; Claireaux  
420 *et al.*, 2013; Mauduit *et al.*, 2016), in part because these traits are stable over time,  
421 sensitive to sub-lethal concentrations of a wide range of pollutants and are potential  
422 early indicators of impact at high organizational level (Becker and Wolford, 1980;  
423 Mauduit *et al.*, 2016). Moreover, some confounding factors ,such as temperature, pH,  
424 feeding, ageing and habituation, have been documented for these biomarkers. These  
425 strengths, however, come with weaknesses that need to be addressed before these  
426 biomarkers can be operationalized. Lacking are, for instance, natural fluctuations in  
427 these biomarkers in healthy individuals, as well as a fuller understanding of their  
428 mechanistic relationships with pollutant exposures. The purpose of the present study  
429 was, therefore, to help fill these gaps for sea bass by verifying the extent of inter-  
430 population variability of hypoxia tolerance and temperature susceptibility, and testing  
431 the effect of an exposure to increasing concentrations of dispersant-treated oil on  
432 these traits of performance. Challenge tests revealed a high inter-population  
433 variability in hypoxia tolerance and temperature susceptibility. Also, the challenge  
434 tests revealed no functional impairment at 1 month and 11 months post-exposure to  
435 dispersant-treated oil. Moreover, fish survival and growth in a semi-natural  
436 mesocosom with a highly variable environment (daily and seasonal) confirmed no  
437 latent effects of oil exposure.

438

**439 4.1 Inter-population variability**

440 Fish performance in the pre-exposure hypoxia and temperature challenge tests was  
441 different to that measured previously (Mauduit *et al.*, 2016). Specifically, fish tested in  
442 the present study (born in 2013) had a significantly lower hypoxia tolerance and a  
443 higher temperature susceptibility than those tested in 2014 (born in 2012) ( $6.43 \pm$   
444  $0.06$  h vs  $8.15 \pm 0.03$  h and  $2.98 \pm 0.03$  h vs  $3.92 \pm 0.03$  h, respectively). This is a  
445 surprising observation, especially given that these populations were obtained from  
446 the same fish farm and brood stock (but possibly with a different parental gene pool),  
447 had the same age, experienced similar rearing conditions and were acclimatized to  
448 the same temperature (11 °C).

449

450 The extent of inter-population variability in fish performances observed above is an  
451 important result that must be considered in impact assessment processes because a  
452 biomarker for a population from a contaminated area is typically compared to that of  
453 a population from an uncontaminated site (Stein *et al.*, 1992; Stagg *et al.*, 2000). This  
454 approach assumes that any observed difference is fully attributable to the  
455 contamination and not to an inter-population difference in biomarkers response /  
456 value. Our results demonstrate that this assumption clearly can be false. Had we  
457 compared the 2013 population with that of the 2014 population without using a  
458 control treatment for oil exposure in either year, we would have wrongly attributed an  
459 effect to the oil-exposure. Conversely, high variability could lead to false-negative  
460 effects. To circumvent such issues, Clutton-Brock and Sheldon (2010) recommend  
461 longitudinal studies modelled after the Athlete Biological Passport developed for an  
462 anti-doping testing program. Such approach implies using challenge tests to monitor  
463 year-to-year performance and, by so doing, defining a population's reference range.

464 Any value falling outside this reference range would indicate an impairment of fish  
465 coping ability and consequently a risk to the health of a fish stock.

466

467

## 468 **4.2 Oil exposure**

469 The lowest oil-dispersant mixture concentration tested in the present work (25 g of oil  
470 + 1 g of dispersant) is known to mimic the conditions that fish are liable to encounter  
471 when an oil spill is treated with dispersant (Mauduit *et al.* 2016). The other two  
472 concentrations tested here (125 g of oil + 5 g of dispersant and 250 g of oil + 10 g of  
473 dispersant) corresponded to 5 and 10-times this realistic concentration and were  
474 used to test the capacity of sea bass to withstand, and possibly recover from, a  
475 severe exposure to chemically dispersed oil. One day and one month post-exposure,  
476 fish from the CONT treatment displayed background [PAH] ( $0.79 \pm 0.04$  and  $0.03 \pm$   
477  $0.04 \mu\text{g g}^{-1}$  dw, respectively), similar to values reported *in natura* ( $0.014 - 1.09 \mu\text{g g}^{-1}$   
478 dw; Baumard *et al.*, 1998; Arias *et al.*, 2009). By contrast, 1 day post-exposure, fish  
479 from the O25D1 treatment displayed liver [PAH] 5 times higher than in the CONT  
480 treatment ( $3.7 \pm 0.4 \mu\text{g g}^{-1}$  dw) confirming the PAH exposure and uptake. Moreover,  
481 body burden of PAH increased almost in proportion (5- and 9-fold) with exposure  
482 concentration (5- and 10-fold). However, fish can rapidly metabolize and eliminate  
483 PAH (Lee *et al.*, 1972; Varanasi *et al.*, 1989; Hellou and Warren, 1997), which means  
484 1 day post-exposure may not represent the peak tissue PAH concentration (Varanasi  
485 *et al.*, 1989; van der Oost *et al.*, 1994; Law and Hellou, 1999). A closer look to PAH  
486 concentration in fish liver reveals that tricyclic PAHs (fluorene, dibenzothiophene,  
487 phenanthrene) represented approximately 80% of the total [PAH] in the liver  
488 (appendix 1). PAH metabolites are excreted predominantly in the bile (Krahn *et al.*,

489 1982; Varanasi *et al.*, 1989). Fluorescence intensity measured 1 day post-exposure  
490 at 290-335 nm (2-ring compounds) was significantly higher in fish exposed to  
491 chemically dispersed oil than in fish from the control treatment. However,  
492 fluorescence levels were higher in fish exposed to the lowest concentration of oil-  
493 dispersant mixture (25 g of oil + 1 g of dispersant) than in the two others (125 g of oil  
494 + 5 g of dispersant and 250 g of oil + 10 g of dispersant, respectively). Surprising as it  
495 may seem, this result can be also explained by the weathering process which  
496 enhances the loss of low-molecular-weight PAH (Carls *et al.*, 1999; Heintz *et al.*,  
497 1999; Short and Heintz, 1997). Most of 2-ring PAH were probably eliminated during  
498 the weathering process and what was measured corresponded to background and  
499 probably irrelevant concentrations. This hypothesis is supported by the low ratio of  
500 naphthalene to total PAH measured in sea bass liver samples at 1 day post-exposure  
501 ( $\approx 6\%$  of total [PAH], appendix 1). When measured at 343–383 nm (4-ring  
502 compounds) and 380–430 nm (5-ring compounds), fluorescence intensity was  
503 significantly higher in fish exposed to the oil + dispersant mixtures than in fish from  
504 the control treatment. Also, the level of fluorescent aromatic compounds (FAC)  
505 increased according to the nominal concentration of oil in the O25D1 and O125D5  
506 treatments. However, FAC in bile of fish from the O250D10 treatment did not differ  
507 from those measured in bile of fish from the O125D5. Therefore, this suggests a  
508 hyperbolic relationship between the nominal concentration of dispersant-treated oil in  
509 the exposure tanks and fish intake of 4 and 5-ring PAH.

510

### 511 **4.3 Functional tests performed 1 month post-exposure**

512 The present result that 1 month post-exposure sea bass performed as well as control  
513 fish in challenge tests (hypoxia and temperature) is in line with Claireaux *et al.*

514 (2013), who also reported no impaired hypoxia tolerance and temperature  
515 susceptibility in sea bass, 1 month post-exposure to chemically treated oil (CAL -  
516 Finasol, OSR-52). However, this result is inconsistent with the impairment of hypoxia  
517 tolerance observed by Mauduit *et al.* (2016) 1 month post-exposure to similar  
518 conditions. These conflicting observations could be explained by either a difference in  
519 the dispersant used (Corexit 9500 rather than Finasol OSR-52) or the inter-  
520 population variability in performance (Fig. 1). Indeed, Dussauze *et al.* (2015),  
521 reported a higher probability of sea bass survival after exposure to crude Arabian  
522 Light treated with Finasol OSR-52 compared with Corexit 9500. In addition, the  
523 apparently lower ability of sea bass to cope with hypoxia and temperature compared  
524 with the population tested by Mauduit *et al.* (2016) may preclude quantitative  
525 comparisons between the studies. Very clearly, these inconsistencies contribute to  
526 shed new light on the need to better explore physiological diversity to allow the  
527 transition from toxicology to operational ecological toxicology.

528

#### 529 **4.4 Mesocosm experiment**

530 The mesocosm study aimed to establish a linkage between performance in the  
531 challenge tests and components of Darwinian fitness, by verifying whether the lack of  
532 functional impairments observed shortly after exposure were confirmed under natural  
533 conditions. Water conditions observed in the experimental mesocosms during spring,  
534 summer and autumn were in accordance with previous observations (Claireaux *et al.*,  
535 2013; Mauduit *et al.*, 2016) and did not differ considerably among ponds. Moreover,  
536 water temperature during late-June to early-August was clearly near the upper limit of  
537 sea bass tolerance range as temperature above optimal (22-24 °C) decreases  
538 metabolic scope (Claireaux and Lagardère, 1999), impairs cardiac performance

539 (Farrell *et al.*, 2007), reduces growth (Person-Le-Ruyet *et al.*, 2004) and reduces  
540 swimming performances (Claireaux *et al.*, 2006). However, extreme temperatures  
541 were only transiently observed (less than 3 h), typically at the end of the day when  
542 water tended to be hyperoxic (170 -200 % air saturation). In contrast, cooler  
543 overnight temperatures were associated with hypoxia (40-50 % air saturation).  
544 Oxygen levels below 60-70% air saturation for sea bass reduce aerobic metabolic  
545 scope, constrain digestion and prevent fish from feeding maximally (Claireaux and  
546 Lagardère, 1999, Pichavant *et al.*, 2001), even though critical air saturation in 15°C-  
547 acclimated sea bass is 15-20 % (Claireaux and Lagardère, 1999). Thus, sea bass  
548 can adjust their physiology to cope with ephemeral stressful environmental  
549 conditions.

550

551 Growth is generally viewed as an integrative biomarker of Darwinian fitness (Biro *et*  
552 *al.*, 2004; Amara *et al.*, 2007) and an indicator of the ability of a population to renew  
553 itself (Depledge *et al.*, 1995). Our results revealed that fish exposed to chemically  
554 treated oil grew and survived just as well as control fish. This result confirmed those  
555 of the hypoxia and temperature challenge tests, reinforcing the discovery that  
556 exposure to chemically treated oil did not affect the ability of sea bass to cope with  
557 environmental contingencies. This result is also in line with a previous experiment  
558 that observed no effect of exposure to chemicals upon growth and survival in the  
559 mesocosom (Mauduit *et al.*, 2016).

560

#### 561 **4.5 Functional tests performed 11 months post-exposure**

562 Eleven months post-exposure, performance in challenge tests was again similar in  
563 control and oil-treated fish, confirming the lack of impairment due to exposure to the

564 oil-dispersant mixture observed earlier in challenge tests and under the semi-natural  
565 environmental conditions of the field mesocosm. This is also in line with a previous  
566 experiment (Mauduit *et al.*, 2016) where no long-term effects upon sea bass health  
567 were reported.

568

569 Possible adverse long-term effects (i.e. cancer) in fish exposed to PAH have been  
570 reported in numerous published reports (*e.g.*, Stegeman and Lech, 1991; Boström *et*  
571 *al.*, 2002) but they were not observed over this study. However, it has been  
572 demonstrated that the relative risk of neoplasm occurrence in English sole in Puget  
573 Sound and croaker increased directly with age (Rhodes *et al.*, 1987; Baumann,  
574 1992). The age of earliest occurrence of neoplasms in both species ranged, from 6 to  
575 10 years. Therefore, adverse long-term effects in fish exposed to chemically  
576 dispersed oil could still occur but at a time-scale larger than the one tested in the  
577 present study.

578

## 579 **5 Conclusion**

580 The main purpose of this research was to complement existing knowledge on fish  
581 health assessment using complex physiological traits as biomarkers. Complex  
582 physiological traits such as hypoxia tolerance and temperature susceptibility are  
583 known as promising biomarkers of fish capacity to cope with natural contingencies.  
584 Objectives of the present study were first to verify the extent of inter-population  
585 variability of hypoxia tolerance and temperature susceptibility and secondly, to verify  
586 the effect of an exposure to 3 increasing concentrations of dispersant-treated oil upon  
587 fish health. We found that hypoxia tolerance and temperature susceptibility were

588 highly variable from one population to another. This expands our knowledge on the  
589 use of complex physiological trait as biomarker because it improves our ability to  
590 identify the difference between signal and noise. Such inter-population variability is  
591 indeed a major source of noise and this highlights that using a population as control  
592 to assess the effect of a stress upon another one, could lead to false negative or  
593 false positive results. This is an important step forward to widespread the use of  
594 complex physiological traits as biomarkers in ecological risk assessments. Such  
595 assessments remain to be performed on a number of populations to account for the  
596 intraspecific variability found in the present study. Furthermore, assessment of fish  
597 sensitivity to dispersant treated oil reveals that the exposure did not lead to any  
598 ecologically relevant impairment, suggesting that juveniles of sea bass are able to  
599 cope with the typical concentrations of dispersed oil occurring during large-scale oil  
600 spills. Such a result provides a potentially important input to the SIMA process and  
601 can assist in identifying those response actions that result in the least environmental  
602 impacts. Finally, this study highlights that a proper assessment of ecologically  
603 relevant consequences of an oil spill upon fish health calls for 1) the identification of  
604 at risk populations; 2) a regular measurement of the performance of individuals from  
605 those populations through challenge tests; and 3) a determination of the natural  
606 variability of these performance measures to define their natural variability in healthy  
607 fish. In this way, possible ecologically relevant impairments upon fish health  
608 attributable to an oil spill would be reliably observable and measurable.

609

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849 **Legends**

850

851 Figure 1: Study timeline.

852

853 Figure 2: Kaplan-Meier analysis of time to loss of equilibrium during hypoxia (A) and  
854 temperature (B) challenge test #1. Solid line: performances reported in Mauduit *et al.*,  
855 2016; dashed line: performances of fish tested in the present study.

856

857 Figure 3: Sum of the 21 polycyclic aromatic hydrocarbons (including the 16 US-EPA  
858 listed) concentrations measured in fish liver 1 day and 1 month post-exposure. Black:  
859 control fish (CONT); blue: fish exposed to a mixture of 25 g of oil + 1 g of dispersant  
860 (O25D1); green: 125 g of oil + 5 g of dispersant (O125 + D5); red: 250 g of oil + 10 g  
861 of dispersant (O250D10). Different letters in superscript indicate statistically  
862 significant difference ( $p < 0.05$ ).

863

864 Figure 4: Fluorescent aromatic compounds (FAC) measured 1 day post-exposure in  
865 fish bile at three wavelengths (top panel: 290-335nm; middle panel: 343-383 nm;  
866 bottom panel 380-430 nm) in control fish (CONT), fish exposed to a mixture of 25 g  
867 of oil + 1 g of dispersant (O25D1), 125 g of oil + 5 g of dispersant (O125 + D5), 250 g  
868 of oil + 10 g of dispersant (O250D10). Different letters in superscript indicate  
869 statistically significant difference ( $p < 0.05$ ).

870

871 Figure 5: Temperature (A), water oxygenation (B) and salinity (C) monitored hourly in  
872 the different ponds throughout the different ponds. Ponds are identified by different  
873 colors. Breaks in the time-series correspond to water renew and monthly drainage to  
874 recover fish.

875

876 Figure 6: Growth (A) and survival (B) monitoring over the field experiment. Black bars  
877 / line: CONT; blue bars / line: O25D1; green bars / line: O125D5; red bars / line:  
878 O250D10.

879

880 Table 1: Mean time to loss of equilibrium (h) during hypoxia challenge test and  
881 temperature challenge test #1 realized 1-month pre-exposure for fish from the  
882 different exposure sub-treatments.

883

884 Table 2: Mean time to loss of equilibrium (h) during hypoxia challenge test and  
885 temperature challenge test #2 realized 1-month post-exposure for fish from the  
886 different exposure sub-treatments.

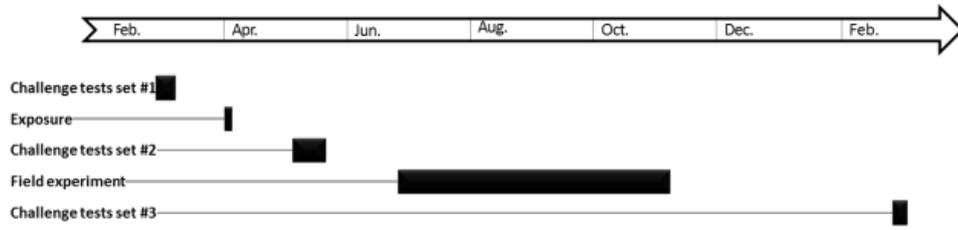
887

888 Table 3: Mean time to loss of equilibrium (h) during hypoxia challenge test and  
889 temperature challenge test #3 realized 11 months post-exposure for fish from the  
890 different exposure sub-groups.

891

892 **Figures**

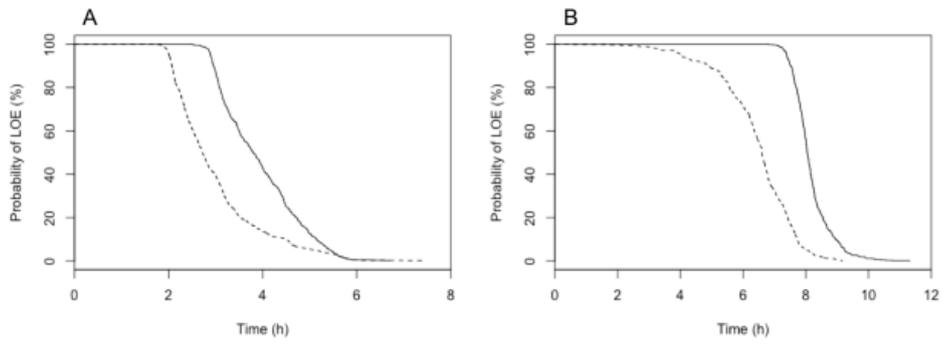
893 Figure 1:



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896 Figure 2:

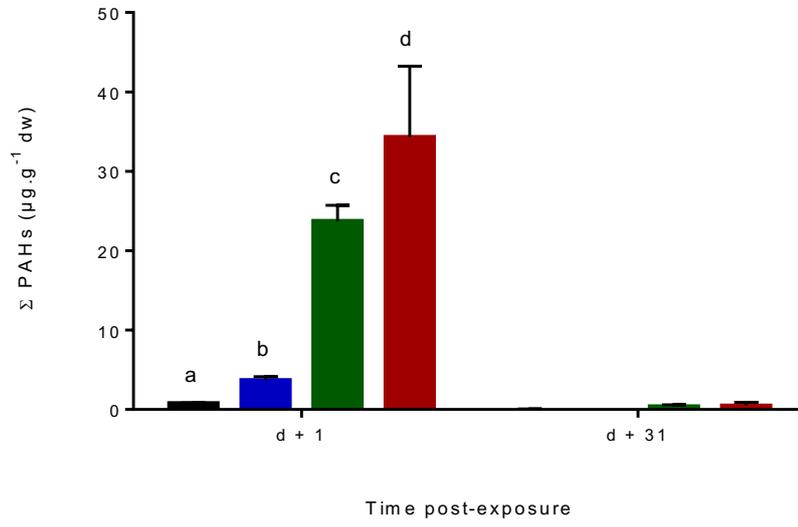


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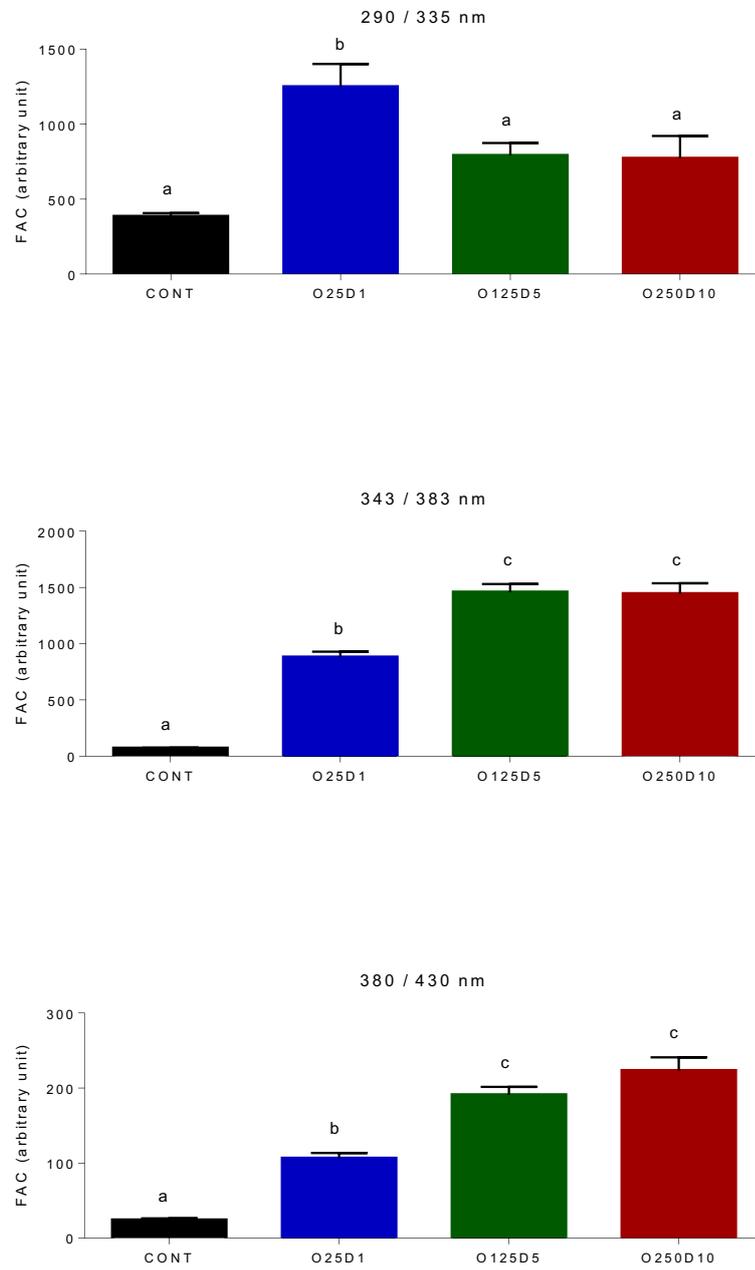
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900 Figure 3:

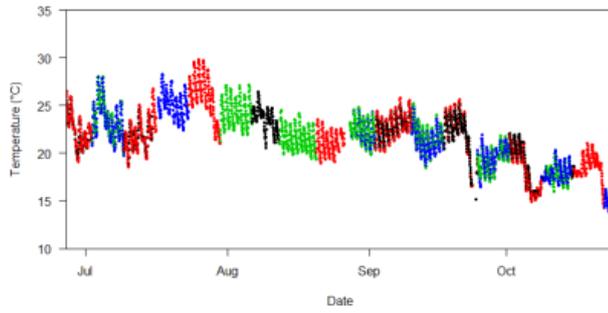


915 Figure 4:



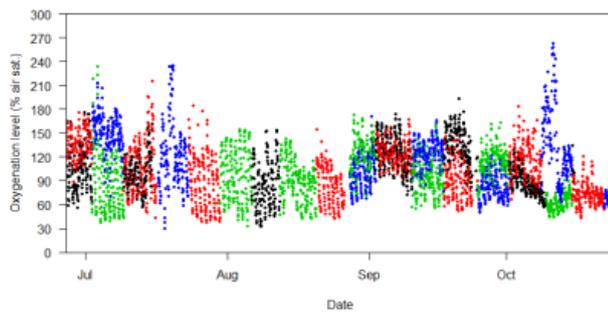
917 Figure 5:

918 A



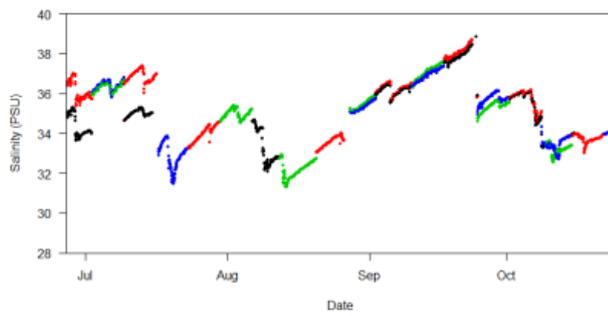
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920 B



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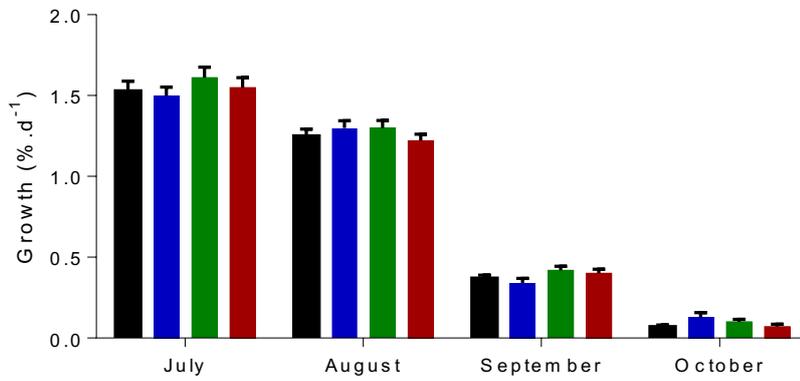
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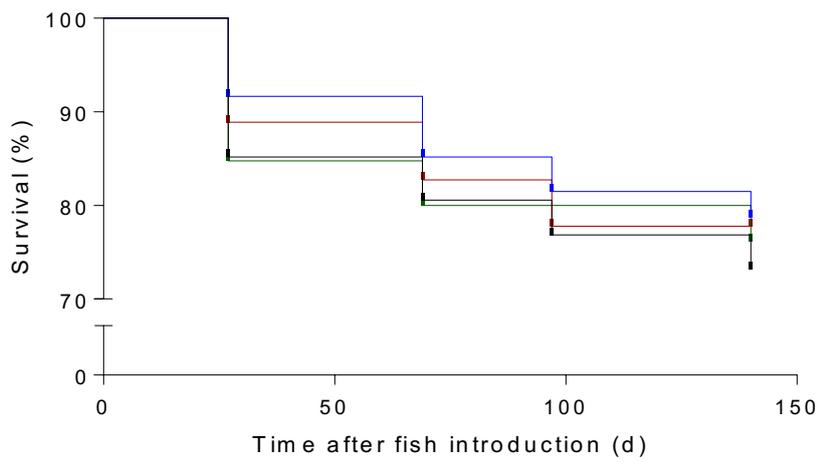
924 Figure 6:

925 A



926

927 B



928

933 Table 1:

Exposure condition	HCT		TCT	
	Mean TLOE	SEM	Mean TLOE	SEM
Control	6.11	0.17	2.93	0.09
Oil 25 g + Dispersant 1 g	6.21	0.17	2.96	0.09
Oil 125 g + Dispersant 5 g	6.01	0.17	2.88	0.08
Oil 250 g + Dispersant 10 g	6.56	0.18	2.88	0.10

HCT: hypoxia challenge test; TCT: temperature challenge test; TLOE: time to loss equilibrium

934

935

936 Table 2:

Exposure condition	HCT		TCT	
	Mean TLOE	SEM	Mean TLOE	SEM
Control	7.065	0.035	9.274	0.090
Oil 25 g + Dispersant 1 g	7.101	0.033	9.434	0.089
Oil 125 g + Dispersant 5 g	7.059	0.040	9.446	0.092
Oil 250 g + Dispersant 10 g	7.099	0.041	9.404	0.102

HCT: hypoxia challenge test; TCT: temperature challenge test; TLOE: time to loss equilibrium

937

938

939 Table 3:

Exposure condition	HCT		TCT	
	Mean TLOE	SEM	Mean TLOE	SEM
Control	9.088	0.228	4.293	0.159
Oil 25 g + Dispersant 1 g	9.074	0.204	4.348	0.137
Oil 125 g + Dispersant 5 g	8.881	0.207	3.817	0.109
Oil 250 g + Dispersant 10 g	9.426	0.255	3.998	0.138

HCT: hypoxia challenge test; TCT: temperature challenge test; TLOE: time to loss equilibrium

940

941

## 942 Appendix 1:

PAH	Concentration $\pm$ sd (ng.g-1) at 1 and 31 days post-exposure							
	<i>d + 1</i>				<i>d + 31</i>			
	Control	Oil 25 + Disp 1	Oil 125 + Disp 5	Oil 250 + Disp 10	Control	Oil 25 + Disp 1	Oil 125 + Disp 5	Oil 250 + Disp 10
naphthalene	256 $\pm$ 156	216 $\pm$ 31	2151 $\pm$ 1313	1952 $\pm$ 1058	<LOQ	<LOQ	<LOQ	<LOQ
benzothiophene	<LOQ	<LOQ	467 $\pm$ 158	446 $\pm$ 102	<LOQ	<LOQ	<LOQ	122
biphenyl	111 $\pm$ 6	209 $\pm$ 60	1270 $\pm$ 85	2267 $\pm$ 701	<LOQ	<LOQ	<LOQ	74
acenaphthylene	<LOQ	154 $\pm$ 35	1339 $\pm$ 90	2164 $\pm$ 728	<LOQ	<LOQ	<LOQ	82
acenaphthene	<LOQ	94	329 $\pm$ 14	370 $\pm$ 127	<LOQ	<LOQ	<LOQ	<LOQ
fluorene	83 $\pm$ 21	320 $\pm$ 46	1724 $\pm$ 55	1327 $\pm$ 1047	21	<LOQ	54 $\pm$ 25	63 $\pm$ 26
dibenzothiophene	323 $\pm$ 90	2433 $\pm$ 235	13401 $\pm$ 1769	20708 $\pm$ 6112	30 $\pm$ 26	33 $\pm$ 4	314 $\pm$ 185	294 $\pm$ 206
phenanthrene	53 $\pm$ 16	304 $\pm$ 28	3019 $\pm$ 496	5090 $\pm$ 2349	<LOQ	<LOQ	27 $\pm$	26
anthracene	<LOQ	<LOQ	<LOQ	224	<LOQ	<LOQ	<LOQ	<LOQ
fluoranthene	<LOQ	<LOQ	18 $\pm$ 5	9	<LOQ	<LOQ	14 $\pm$	<LOQ
pyrene	<LOQ	<LOQ	<LOQ	21	<LOQ	<LOQ	<LOQ	<LOQ
benzo(a)anthracene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
chrysene	<LOQ	<LOQ	26 $\pm$ 8	43	<LOQ	<LOQ	<LOQ	<LOQ
benzo(b+k)fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
benzo(e)pyrene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
benzo(a)pyrene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
perylene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
indeno(123-cd)pyrene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
dibenzo(ah)anthracene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
benzo(ghi)perylene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

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## Domestication compromises athleticism and respiratory plasticity in response to aerobic exercise training in Atlantic salmon (*Salmo salar*)



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## ABSTRACT

Commercially selective breeding of Atlantic salmon (*Salmo salar*) primarily for rapid growth may compromise cardiorespiratory robustness and its related phenotypes. Therefore, a suite of respiratory indices was used to evaluate aerobic capacity and hypoxia tolerance to test the hypothesis that exercise training can improve the athletic robustness in both domesticated and wild strains of Atlantic salmon, but with the domesticated strain having a less cardiorespiratory plasticity and a lower athletic robustness than the wild strain. We also tested a second hypothesis that a constant acceleration screening protocol should segregate fish according to athletic robustness based on their swimming ability. These hypotheses were tested with parr from *Bolaks* (domesticated) and *Lærdal* (wild) strains of Atlantic salmon that were reared under identical hatchery conditions. After screening into either inferior (bottom 20%) or superior (top 20%) swimmers, the four groups of fish (two strains and two swimming performance levels) either were given an 18-day exercise-training regime (an incremental water current of 2.0–2.8 fork lengths  $s^{-1}$ ), or were maintained at the control water current (0.5 fork lengths  $s^{-1}$ ) for 18 days. Subsequently, fish were sampled for metabolic enzyme analysis in red and white swimming muscles (citrate synthase, CS, and lactate dehydrogenase, LDH;  $n = 15$  from each group) and their individual respiratory capacities were comprehensively assessed by measuring the standard metabolic rate (SMR), maximum rate of oxygen uptake ( $MO_{2max}$ ), absolute aerobic scope (AAS), factorial aerobic scope (FAS), excess post-exercise oxygen consumption (EPOC), critical oxygen level ( $O_{2crit}$ ) and incipient lethal oxygen saturation (ILOS). Contrary to our expectations, the inferior and superior swimmers were indistinguishable in either strain and these data were pooled. While exercise training produced several tangible benefits for the wild fish, it produced very few for the domesticated fish. For example, the wild strain, but not the domesticated strain, had a significantly higher  $MO_{2max}$ , AAS and EPOC as a result of training. Also, CS activity in red muscle increased after training to a larger extent in the wild strain than in the domesticated strain. When compared with the wild strain, the domesticated strain had a significantly lower  $MO_{2max}$ , AAS, FAS and CS activity in white muscle. Thus, the domesticated strain appeared to be athletically less robust than the wild strain. These results imply that approximately ten generations of selective breeding for rapid growth in commercial aquaculture have reduced the overall athletic robustness of domesticated salmon as compared to their wild conspecifics, and given the success in improving athletic robustness of the wild strain, it still remains to be seen whether an exercise training protocol can be developed that will provide benefits to the salmon aquaculture industry.

**Statement of relevance:** This manuscript fits perfectly with the scope of aquaculture. We address the possibility that the Norwegian Atlantic salmon (*Salmo salar*) breeding program that extensively focuses on commercial benefits traits may be compromising the cardiorespiratory system, which may contribute to the mortality of smolts after seawater transfer. We hypothesized that a combination of exercise-screening and exercise-training protocols could select for superior cardiorespiratory performance. This hypothesis was tested by comparing domesticated and wild Norwegian Atlantic salmon strains, and using comprehensive measurements of athletic and hypoxia performance in individual fish.

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We believe this paper will be of specific interest to aquaculture professionals who are seeking the enhanced husbandry approaches for achieving higher survival rate over seawater transfer as well as general physiologists. To our knowledge our study is the first to comprehensively illustrate athleticism of domesticated Atlantic salmon from biochemical and cardiorespiratory system levels. Furthermore, we believe this is the first study to show the promising results of enhancing cardiorespiratory system of domesticated Atlantic salmon strain in a hatchery setting.

We used a suite of respiratory indices to evaluate athletic robustness and hypoxia performance in individual fish. We discovered that a domesticated strain of Atlantic salmon demonstrated a reduced athleticism and less plasticity in response to a short exercise-training regime compared with a wild strain of Atlantic salmon. We conclude that commercial aquaculture practices may trade off the robustness of the cardiorespiratory system, but not to an extent of completely losing the potential of benefiting from exercise training.

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## 1. Introduction

Freshwater production of Atlantic salmon smolts in commercial aquaculture is characterized by high intensity production (Kristensen et al., 2009, 2012a) and several handling procedures, such as grading, vaccination and transport, which are physiologically challenging. A particularly critical production procedure is the transfer of smolts to seawater transfer because it is associated with significant mortality (Maxime et al., 1986, 1990; Jørgensen and Jobling, 1994; Iversen et al., 2005). In Norway alone, a 15–20% mortality rate still accompanies salmon grow-out in sea at the first 90-day, which amounts to 40–50 million fish annually (Kristensen et al., 2012a). Both the osmo-respiratory compromise (McCormick and Saunders, 1987; Randall and Brauner, 1991; Gallagher et al., 2001; Sardella and Brauner, 2007) and lower cardiorespiratory fitness (Castro et al., 2011) have been implicated as underlying mechanisms for this mortality, with secondary disease outbreaks as a diagnosed cause of death (Aunsmo et al., 2008).

A fish's activities are ultimately governed by the capacity of its cardiorespiratory system to supply oxygen to working tissues, making this system a determining factor of a fish's ability to face environmental contingencies, whether of natural or anthropogenic origin. It therefore seems reasonable to assume that within a population, those individuals with the greatest cardiorespiratory capacity will be less likely to suffer energy budgeting conflicts as they have a greater capacity to multitask (Neill et al., 1994). In farmed Atlantic salmon, this advantage could translate into improved survival during seawater transfer of smolts and other challenging aquaculture procedures, e.g., chemical delousing procedures. Here we used a suite of established cardiorespiratory indices, ones that can be reliably measured in individual fish over a period of several days, to define the general athletic robustness of a salmon. Collectively, these indices measured the fish's aerobic capacity for sustained swimming, ability to recover from exhaustion, ability to produce ATP aerobically at muscle tissue level, and ability to tolerate hypoxia and temporarily support metabolic demands with anaerobic metabolism.

Selective breeding for rapid growth, delayed maturity and high fecundity (as well as rearing in a sheltered environment with an unlimited food source and the absence of predators) appears to have reduced athletic robustness in farmed fish. For instance, cultured salmonids characteristically have a high body condition factor, a rounded cardiac ventricle and a low plasma level of atrial natriuretic peptides (Poppe et al., 2003; Claireaux et al., 2005; Kristensen et al., 2012b). They also display a higher incidence of cardiac aberrations (e.g., a misaligned bulbus arteriosus; Mercier et al., 2000), which have been associated with reduced capacities for cardiac pumping and oxygen delivery (Poppe and Taksdal, 2000; Poppe et al., 2002, 2003; Gamperl and Farrell, 2004; Claireaux et al., 2005). Domesticated fish also have lower swimming muscle contraction capacity and lower enzymatic capacity for aerobic energy production as compared to wild fish (Anttila et al., 2008a; Anttila and Mänttari, 2009). Poorer swimming performance associated with domestication (McDonald et al., 1998; Jonsson and Jonsson, 2006) could reflect a reduced aerobic capacity (Fry,

1971; Claireaux et al., 2005). At an extreme, the rapid growth rate of growth hormone transgenic salmonids is similarly associated with reduced aerobic capacity and hypoxia tolerance (Farrell et al., 1997; Stevens et al., 1998; Cook et al., 2000). Thus, intensive selection for beneficial traits during commercial rearing (e.g., rapid growth) appears to have negatively impacted the physiological integrity of farmed fish (McKenzie et al., 2007), with possible consequences on smolt's ability to handle the additional energy costs and osmotic stress that accompany transfer into seawater (Usher et al., 1991).

In intensive aquaculture, exercise training can benefit fish, provided the exercise regime is not too severe, i.e., plasma stress hormones are not greatly elevated, ionic/osmotic homeostasis is not disrupted and blood flow to the digestive system is not diverted to skeletal muscle (Farrell et al., 1991; Thorarensen et al., 1993; Kiessling et al., 1994; Davison, 1997; Kieffer, 2000; McKenzie et al., 2003). Indeed, aerobic training in fish is viewed by some as an integrated approach to improve animal wellbeing (Nelson, 1989), in part because training is known to enhance oxygen extraction efficiency and supply, anaerobic metabolism, the capacity to recover from exhaustion, and reduces the osmo-respiratory compromise (Kieffer, 2000; Gallagher et al., 2001; Kieffer, 2010). Also, aerobic training can enlarge muscle fibers in swimming muscles and mitochondria in cardiac myocytes (Davison, 1997; Castro et al., 2013), and increase oxidative enzyme activities in both red and white skeletal muscles, while reducing their anaerobic enzymes activities (Anttila et al., 2006, 2008b). Lipid utilization (McClelland et al., 2006; Anttila et al., 2010) and the capacity to deplete muscle glycogen similarly increase after aerobic training (Hochachka, 1961; Poston et al., 1969; Pearson et al., 1990; Gamperl et al., 1994; Milligan, 1996). Furthermore, aerobic training of Atlantic salmon parr before sea transfer enhanced gene expression for infectious pancreas necrosis virus resistance and seawater relevant ion-transportation pathways (Castro et al., 2011, 2013; Esbaugh et al., 2014). Consequently, the aquaculture industry has growing interests in identifying appropriate exercise regimes that would strengthen stress tolerance, growth and survival before and after sea transfer (Grisdale-Helland et al., 2013).

For the present study, we tested the hypothesis that exercise training of domesticated and wild strains of Atlantic salmon parr would improve their athletic robustness measured at biochemical and whole animal levels. We also reasoned that by screening swimming performance ahead of training with a constant acceleration test, superior and inferior swimmers could be segregated according to their athletic robustness (Anttila et al., 2014a). Thus, after the screening protocol, Atlantic salmon parr were given an 18-day incremental aerobic exercise training regime in a hatchery setting followed by measurements of a suite of respiratory indices to evaluate athletic robustness. We further hypothesized that the domesticated *Bolaks* strain should have a lower athletic robustness and less cardiorespiratory plasticity in response to exercise training than the wild *Lærdal* strain. To ensure a broad characterization of athletic robustness, we measured each fish's standard metabolic rate (SMR, the minimal maintenance metabolic rate of an aquatic ectotherm in a post-absorptive and inactive states; Fry and Hart, 1948) and maximum rate of oxygen uptake ( $\dot{M}O_{2\max}$  as an estimate proxy of

maximum metabolic rate; Fry, 1947; Brett and Groves, 1979), as well as the absolute aerobic scope (AAS) and factorial aerobic scope (FAS), which are indices of the aerobic capacity for activities (Pörtner and Farrell, 2008; Pörtner, 2010; Clark et al., 2013). The capacity for both aerobic and anaerobic energy production in red and white swimming muscles were assessed by measuring the citrate synthase (CS) and lactate dehydrogenase (LDH) activities, respectively. Hypoxia tolerance and anaerobic capacity were characterized by measuring, respectively, the critical oxygen level ( $O_{2crit}$ , the capability to extract dissolved oxygen; Ultsch et al., 1978) and the incipient lethal oxygen saturation (ILOS, the minimal oxygen saturation that aquatic animal maintaining equilibrium; Claireaux et al., 2013). Lastly, we measured excess post-exercise oxygen consumption (EPOC; Lee et al., 2003) to quantify the ability to recover from exhaustion.

## 2. Materials and methods

### 2.1. Experimental animals and rearing conditions

The *Bolaks* (domesticated) strain was generated from eggs of 7 females that were fertilized by 2 males and the fertilized eggs were incubated at 7 °C in SalmoBreed (Bergen, Norway) until 396 degree days (dd). The *Lærdal* (wild) strain were generated from eggs of 5 females that were fertilized by 2 males and the fertilized eggs were incubated at 7 °C on site until 410 dd. Eyed eggs were then transported to Nofima research station, Sundalsora, Norway and incubated in 5–6 °C freshwater until hatching using side-by-side incubators (463–487 dd for *Bolaks* and 513–518 dd for *Lærdal*). Emergent fry were similarly reared under identical standard conditions and fed the same diet (Skretting, Stavanger, Norway) in side-by-side 5.3 m<sup>3</sup> circular fiberglass tanks (approximately 25 kg m<sup>-3</sup> stock density). Rearing temperature was progressively increased to 12 °C in accordance with Norwegian aquaculture industry standards and maintained at 12 °C throughout the experiment. At 3 g size (bulk weighed), fish were graded to obtain homogenous populations with respect to body mass/fork length and maintain stocking density (35 kg m<sup>-3</sup>). At 25 g size and two weeks prior to swim screening, 600 fish per strain were selected to limit the variance in body mass and fork length to  $\pm 3$  g and  $\pm 1$  cm, respectively, and individually tagged with a passive integrated transponder (Jojo Automasjon AS, Sola, Norway). Each stock was then reared in five replicate circular tanks (0.1 m<sup>3</sup>, n = 120 per strain, 36 kg m<sup>-3</sup> stock density) until the fish were screened for their swimming performance. Throughout, water exchange and current were set and routinely adjusted to self-clean the tanks in accordance to standard procedures and to provide a nominal water current that was slightly lower [0.2–0.3 fork lengths (FL) s<sup>-1</sup>] than that used later for the control fish (see Section 2.2). Specific growth rates were 3.40 and 3.07 in the *Bolaks* and *Lærdal* strains, respectively, during this period. Therefore, to minimize the size dichotomy, the faster growing *Bolaks* strain were screened, trained and tested two months ahead (September 2014) of the *Lærdal* strain (November 2014). The experiments were approved by the National Animal Research Authority, according to the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (EST 123).

The *Lærdal* and *Bolaks* strains are characteristic Norwegian wild and domesticated Atlantic salmon, respectively. The *Lærdal* strain, from the Norwegian *Lærdal* River (61°N), was selected because of its relatively large spawning population size, genetic purity, genetic stability and a limited genetic influence from aquaculture escapees (Glover et al., 2012, 2013; Finstad et al., 2013; Johnsen et al., 2013). Its freshwater habitat has a large water discharge and low water temperature, which are typical conditions for wild Atlantic salmon in Norway (Brooks et al., 2006; Urke et al., 2013). The *Lærdal* strain consists mainly of late maturing (2 and 3 sea-winter) spawners and the migration route passes through the longest (>200 km) Norwegian fjord system, Sognefjorden. The domesticated *Bolaks* strain originates from and is primarily

produced as broodstock in Western Norway. The two founding strains were from the Vosso (60°N) and Årøy (61°N) Rivers. For the first five generations until 2000, selective breeding with other strains focused on promoting characteristics of rapid growth and delayed sexual maturation, after which a family-based breeding program was established to enhance specific disease resistance, fillet quality and coloration as selective breeding traits in addition to growth and delayed sexual maturation.

### 2.2. Screening and training protocols

The experimental design is summarized in Fig. 1. For each strain, 480 parr were initially screened using the following protocol. Batches of 120 parr were fasted for 1 day before being transferred to a pair of side-by-side transparent PVC tunnels (60 fish per tunnel; stocking density = 29 kg m<sup>-3</sup>). The swimming section was 2 m long and 20 cm in diameter, which allowed 4 fish to easily swim abreast, if required. Fish were habituated for 4 h, while maintaining station in the water current (0.5 FL s<sup>-1</sup>) without tail beats. Water velocity was incremented by 5 cm s<sup>-1</sup> every 10 min until all the fish in a tunnel had reached exhaustion (typically  $\leq 145$  cm s<sup>-1</sup>). Fatigued fish, which were too refractory to remove themselves from the net at the rear of the swimming section, were manually removed via a hatch situated above the back grid and scanned for their pit-tags. Their body mass, fork length, final water speed ( $U_{max}$ ; Farrell, 2008) and swimming duration were recorded.  $U_{max}$  for each fish was calculated from the proportion of the time period spent at the final velocity increment (Brett, 1964). Both swim tunnels received rearing tank water (12  $\pm$  0.5 °C) from a 7 m<sup>3</sup> reservoir via a pump (VAKI Heathro Self Priming 6" pump, VAKI Aquaculture Systems Ltd., Kópavogur, Iceland). Water flow from each pump and to each swim tunnel was independently controlled with a Cubix remote controller (HBC-radiomatic GmbH, Crailsheim, Germany) and a Micronics Portaflow 300 ultrasonic flowmeter that monitored water flow in the swim tunnel (Micronics Ltd., Buckinghamshire, UK). The design of the apparatus was based on a Brett-type swimming respirometer, which has been extensively and critically tested since its conception over 50 years ago (Brett, 1964). We improved on Brett's original design by inserting flow straightening devices in the section situated upstream from the swimming section to ensure lamellar flow and a proper cross-sectional water velocity profile. The maximum water velocity was approximately 145 cm s<sup>-1</sup>.

For each population and test, the first 20% and last 20% of each group of 120 fish to reach fatigue were categorized as inferior and superior swimmers (Fig. 2A & B). The respirometry tests (Section 2.3) and enzyme activity assays (Section 2.4) were performed only on these subgroups to maximize our chances of segregating athletic robustness. The remaining fish were returned unused to their original rearing tanks.

For the exercise-training regime, each swim tunnel contained 40 inferior and 40 superior swimmers (stock density = 38 kg m<sup>-3</sup>). The training lasted 18 days, followed by a 2-day recovery period (Fig. 1). One swim tunnel (water velocity of 0.5 FL s<sup>-1</sup>) was used for control fish, which would spread themselves along the length of the swim tunnel and only swim occasionally (using slow and small-amplitude tail beats to move forward). The other swim tunnel was used for aerobic exercise training, which involved maintaining the water velocity at 2 FL s<sup>-1</sup> for the first 7 days, at 2.4 FL s<sup>-1</sup> for next 7 days and at 2.8 FL s<sup>-1</sup> for the last 4 days. Again, fish spread themselves along the length of the swim tunnel and typically maintained station in the water current with only occasional changes in location. Fish were fed a daily ration of 2% biomass through a hatch situated above honeycomb grid at the front of the swim tunnels, which was connected to an automatic belt feeder. A technician monitored feeding twice daily to ensure the pellets were evenly spread and fish were feeding. After the 18-day period, fish were segregated into 8 groups of 40 fish, from which up to 12 fish per group were subsequently used for the respirometry testing and 15 fish per group were used to measure enzyme activities. The

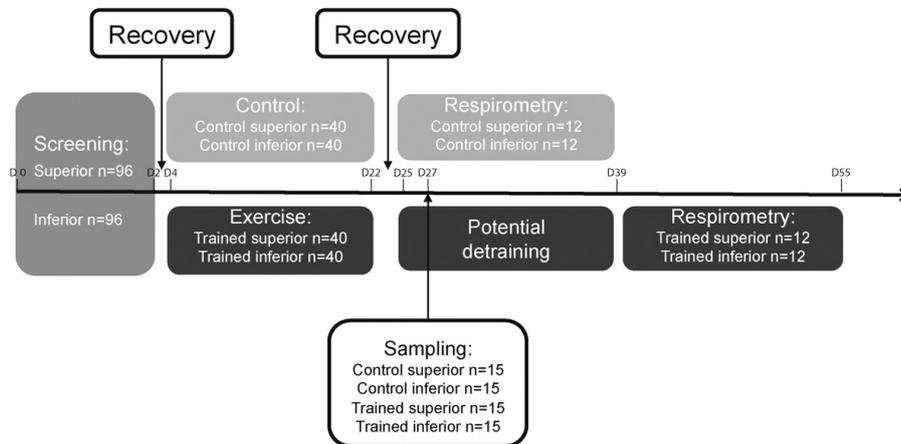


Fig. 1. Experimental design and timeline of screening, training, tissue sampling for enzyme activity assays, and respirometry conducted on *Lærdal* (wild) and *Bolaks* (domesticated) Atlantic salmon (*Salmo salar*) parr. An identical experimental design was practiced on the two strains.

rest of the fish were used for other purposes that were separate from current experiments.

### 2.3. Respirometry trials

Prior to testing, the fish groups were returned to their rearing tanks for 3 days where they were held on a 24-h photoperiod with a limited diet to prevent smoltification and further growth. The control group were tested first to allow the exercised group some recovery, a practice that would likely be needed for an industry application prior to sea transfer. Each batch of respirometry trials simultaneously examined 8 fish that had been fasted for 3 days using intermittent-flow respirometers (water volume = 2.1 L each) were submerged in a water reservoir (3 m × 0.5 m and 0.3 m deep), where aerated water (12 ± 0.5 °C) flowed continuously. Water flow through the respirometers was regulated using computer-controlled flush pumps (Compact 600, EHEIM, Germany) and relays (AquaResp, University of Copenhagen, Helsingør, Denmark). Continuously mixing of the respirometry chamber was assured by a circulation loop, into which an optical oxygen probe (Robust Oxygen Probe OXROB3, Pyroscience, Germany & Oxygen Minisensor, PreSens, Germany) that continuously monitored dissolved oxygen saturation (% sat.). Prior to every respirometry trial, the oxygen probes were calibrated to 0% sat. (water saturated with sodium sulfite) and 100% air saturation (fully aerated water).

A respirometry trial began with a chasing protocol, during which each fish was individually hand chased in a 10-L bucket for 10-min and then given a 2-min air exposure. At the end of air exposure, the fish was placed immediately in a respirometer during flushing mode. An oxygen uptake rate ( $\dot{M}O_2$ ) measurement cycle consisted of flush, stabilization and measurement periods. Only oxygen saturation values obtained during the measurement periods were used to calculate  $\dot{M}O_2$ . Oxygen saturation was then monitored using a measuring cycle comprised of a 30 s flush, a 45 s stabilization and a 105 s measurement at the first 2 h to capture the maximum  $\dot{M}O_2$ , which was assigned  $\dot{M}O_{2max}$ . The computer recorded the first  $\dot{M}O_2$  value about 150 s to 180 s after the fish was placed in the respirometer. As  $\dot{M}O_2$  decreased toward SMR, the measurement cycle was changed to a 120 s flush, a 80 s stabilization and a 400 s measurement to guarantee that oxygen saturation was >97% at the start of the measurement period. These measurements continued for 2 or 3 days in a dark and quiet environment, allowing the fish to fully adjust to the respirometer and reach a minimal maintenance  $\dot{M}O_2$  (SMR; Section 2.5).

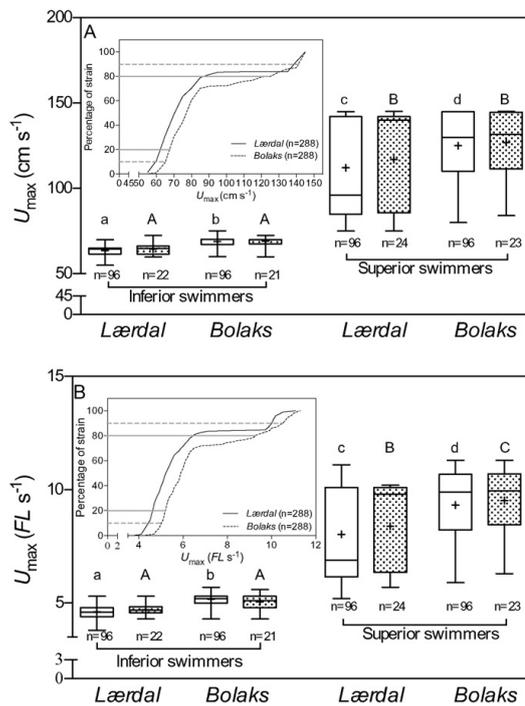
$O_{2crit}$  and ILOS were determined at the end of a trial by introducing hypoxic water into the respirometers. Water was pumped (Compact 600, EHEIM, Germany) from the water reservoir to the top of a gas equilibration column where it trickled down through nitrogen gas that was injected into the bottom. Air-saturation of the water decreased progressively to 10% over an 8-h period, during which the  $\dot{M}O_2$  measurement cycles were a 60 s flush, a 45 s stabilization and a 195 s measurement. When a fish lost its equilibrium, the air saturation was noted as the ILOS.  $O_{2crit}$  was determined with a post-experiment calculation (Section 2.5). Fish were immediately resuscitated with well-aerated water at 12 °C (survival rate was 94%). To eliminate microbial respiration, the entire system was disinfected with Virkon S (Lilleborg Profesionell, Oslo, Norway) for 6 h on completion of every respirometry trial.

### 2.4. Enzyme activity assays

Fish were weighed, measured and euthanized 5 days after the 18-day training regime. Blocks of red and white muscles were removed from the mid-line at the mid-point between the adipose fin and tail fin and frozen immediately and separately in liquid nitrogen for storage at -80 °C until analyzed. For enzymatic analysis, each muscle sample was homogenized separately in 19 vols of homogenization buffer (0.1% Triton, 50 mM Hepes, 1 mM EDTA, pH 7.4). Citrate synthase (CS, EC 2.3.3.1) and lactate dehydrogenase (LDH, EC 1.1.1.27) activities were measured according to Dalziel et al. (2012) at 21 °C. The measurements were done with EnSpire 2300 Multilabel Reader (Perkin Elmer, Turku, Finland) and the final substrate concentrations followed the optimizations were done by Dalziel et al. (2012). For CS the concentrations were 0.15 mM DTNB, 0.15 mM acetyl CoA and 0.5 mM oxaloacetic acid in 50 mM Tris (pH 8.0) and for LDH 0.27 mM NADH and 25 mM sodium pyruvate in 50 mM Tris (pH 7.4). Assays were performed in triplicate for each sample and a background reaction rate was subtracted. The activities of enzymes were calculated  $g^{-1}$  tissue. All the reagents were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

### 2.5. Data analysis

Only % air saturation data with a linear decrease and  $R^2 > 0.8$  during the measurement cycle were accepted for an accurate calculation of  $\dot{M}O_2$ . SMR was determined from between 288 and 432 measurement cycles per fish. In the absence of a fully accepted method to estimate



**Fig. 2.** A) Absolute and B) relative maximum swimming capacity ( $U_{\max}$ ) of inferior and superior swimmers of the Lærdal (wild) and Bolaks (domesticated) Atlantic salmon (*Salmo salar*) parr. The results for the screening test ( $n = 96$ ) and the sub-sample used for respirometry ( $n = 21$ – $24$ ) are presented separately. Each boxplot shows the interquartile range and 95% coefficient variation with the mean is shown as '+'. Different lowercase letters indicate significant differences ( $p < 0.05$ ) in screening test and different uppercase letters indicate significant differences in the fish sampled by respirometry, detected by two-way ANOVA Tukey HSD *post-hoc*. There were no significant differences ( $p > 0.05$ ) between fish tested by screening protocol and the fish sampled by respirometry in respective swimming groups, tested by Student's *t*-test. The insets show the cumulative frequency polygons for  $U_{\max}$  of both strains. The inferior or superior 10% and 20% of swimmers are indicated as horizontal dash and solid lines respectively.

SMR (see Chabot et al., 2016), we used an R script (Chabot et al., 2016) to determine two estimates: a) a quantile of 0.2 ( $q_{0.2}$ ), and b) the mean of the lowest 10  $MO_2$  values (Low10) after removing the lowest 2% of the dataset.  $MO_{2\max}$  was not always the first  $MO_2$  measurement made after chasing and was as late as the 5th  $MO_2$  measurement, but averaged the 2nd  $MO_2$  measurement.  $AAS = SMR - MO_{2\max}$ , while  $FAS = MO_{2\max} / SMR$ . EPOC was calculated from the integral of the area bounded by the actual  $MO_2$  measurement and SMR. However, we only used the recovery period until  $MO_2$  remained at or below  $SMR + 10\%$  for at least 3 measurement cycles, rather than using SMR per se. This strategy limited the obvious overestimate of EPOC and its duration due to spontaneous fish activity. We acknowledge that while our approach slightly underestimates EPOC and its duration, the effect of spontaneous activity on EPOC would be numerically much greater. Yet, even with this modification, EPOC could not be calculated reliably in 20 individuals given the extent of their spontaneous activity during the recovery period.  $O_{2crit}$  was derived using R script from the intersection of a regression line for  $MO_2$  versus oxygen saturation and the SMR (Claireaux and Chabot, 2016).

Statistical comparisons of SMR,  $MO_{2\max}$ , AAS, FAS, EPOC,  $O_{2crit}$ , ILOS, CS and LDH activities were made using a two-way (strain & training)

multivariate analysis of variance (MANOVA) with Pillai's trace test to correct for unbalanced sample size, followed by multiple ANOVAs and Tukey HSD *post-hoc* (Zar, 1996).  $\log_{10}$  transformations were applied on  $MO_{2\max}$  and EPOC in Low10 dataset to achieve the assumption of multivariate normality. Statistical comparisons of  $U_{\max}$ , regarding strain and initial swimming capacity, were made using a two-way ANOVA with Tukey HSD *post-hoc*. Statistical analyses were conducted in R (ver. 3.2.2; R Development Core Team, 2015) and SigmaPlot (ver. 12.5). Statistical significance was assigned when  $\alpha < 0.05$ .

### 3. Results

#### 3.1. Swimming capacity and growth

Prior to respirometry, body mass was 9.2% larger ( $p = 0.002$ ) in the domesticated Bolaks strain and their fork length was 2.1% longer than in the Lærdal strain ( $p = 0.001$ ) (Bolaks:  $35.6 \pm 0.8$  g,  $14.4 \pm 0.1$  cm; Lærdal:  $32.6 \pm 0.5$  g,  $14.1 \pm 0.1$  cm) (Table S1). Regression analysis found no significant correlations either between respiratory indices and body mass, or between enzymatic variables and body mass within either strain or swim groupings (Figs. S1 & S2).

The cumulative frequency polygons demonstrated that neither absolute nor relative  $U_{\max}$  had normal distributions (see insets in Fig. 2) and that the distribution patterns were similar for each screening trial and for both strains (Figs. S3a, S3b, S4a & S4b). Superior swimmers swam on average almost twice as fast as inferior swimmers using absolute swimming speed (entire fish population:  $117.0 \pm 1.9$  vs.  $65.1 \pm 0.3$  cm  $s^{-1}$ ;  $p < 0.001$ ; only fish used for respirometry:  $121.9 \pm 2.6$  vs.  $66.8 \pm 2.7$  cm  $s^{-1}$ ;  $p < 0.001$ ; Fig. 2A) as well as relative swimming speed (entire fish population:  $8.6 \pm 0.1$  vs.  $4.8 \pm 0.1$  FL  $s^{-1}$ ;  $p < 0.001$ ; only fish used for respirometry:  $9.0 \pm 0.2$  vs.  $4.9 \pm 0.2$  FL  $s^{-1}$ ;  $p < 0.001$ ; Fig. 2B). Unexpectedly, the wild Lærdal strain had a significantly lower  $U_{\max}$  than the domesticated Bolaks strain for both superior and inferior swimmers (Fig. 2).

#### 3.2. Respirometry indices (based on Low10 SMR values)

Contrary to the initial hypothesis, screening had no main effect on the metabolic and enzymatic indices (three-way MANOVA:  $p = 0.7$ ; Table S2). Therefore, the data of superior and inferior swimmers were pooled for subsequent analyses of effects of training and strain. Training and strain (two-way MANOVA: training  $p < 0.001$ ; strain  $p < 0.001$ ; Table 1), as well as the interaction (two-way MANOVA:  $p = 0.002$ ; Table 1), significantly influenced several of the metabolic and enzymatic indices.

Overall SMR ( $n = 90$ ) was  $65.8 \pm 1.6$  mg  $O_2$   $h^{-1}$   $kg^{-1}$ . Neither training, nor strain had independent effects on SMR ( $p \geq 0.19$ ; Table 1). Nevertheless, strain significantly influenced  $MO_{2\max}$ , with wild strain displaying a 24% higher  $MO_{2\max}$  than domesticated strain ( $392.8 \pm 10.7$  vs.  $317.8 \pm 7.3$  mg  $O_2$   $h^{-1}$   $kg^{-1}$ ,  $p < 0.001$ , Table 2). Moreover, there was an interaction between training and strain ( $p = 0.036$ , Table 2), with training increasing  $MO_{2\max}$  by 16% in wild fish ( $p = 0.045$ ) but not in domesticated fish ( $p = 0.98$ , Fig. 3).

Since AAS and FAS are derived from SMR and  $MO_{2\max}$ , the training and strain effects on AAS and FAS were similar to those for  $MO_{2\max}$ . Training increased AAS by 12% ( $306.2 \pm 12.2$  vs.  $272.7 \pm 7.9$  mg  $O_2$   $h^{-1}$   $kg^{-1}$ ,  $p = 0.003$ , Table 1) and AAS was 30% higher for wild fish than domesticated fish ( $327.1 \pm 10.9$  vs.  $251.4 \pm 5.9$  mg  $O_2$   $h^{-1}$   $kg^{-1}$ ,  $p < 0.001$ , Table 1). Wild fish had a 17% higher AAS after training ( $p = 0.009$ ), whereas AAS was unresponsive to training in domesticated fish ( $p = 0.96$ , Fig. 3). Wild fish had a 24% higher FAS than domesticated fish ( $6.2 \pm 0.2$  vs.  $5.0 \pm 0.2$ ,  $p < 0.001$ , Table 1) and training increased FAS by 7% ( $5.8 \pm 0.2$  vs.  $5.4 \pm 0.2$ ,  $p = 0.005$ ; Table 1). There was an interaction between strain and training ( $p = 0.012$ ), but neither the domesticated nor wild fish reached statistical significance for an effect of training on FAS (Fig. 3).

**Table 1**

A summary of the main factor effects and significant interactions (F-values and p-values) of strain and training on respiratory variables of Atlantic salmon (*Salmo salar*). Standard metabolic rate (SMR) was calculated using two different SMR methods (Low10 and q0.2). Absolute aerobic scope (AAS), factorial aerobic scope (FAS), excess post-exercise oxygen consumption (EPOC) and critical oxygen level ( $O_{2crit}$ ) also had two values corresponding with the two SMR calculation methods.

	Low10		q0.2	
	F-value	p-value	F-value	p-value
<b>MANOVA</b>				
Strain	9.5	<0.001	9.7	<0.001
Training	6.8	<0.001	6.1	<0.001
Strain × training	3.2	0.002	3.2	0.002
<b>SMR</b>				
Strain	0.77	0.38	0.45	0.51
Training	1.8	0.19	0.85	0.36
<b>AAS</b>				
Strain	37.5	<0.001	36.8	<0.001
Training	9.7	0.003	8.9	0.004
Strain × training	9.3	0.003	9.9	0.003
<b>FAS</b>				
Strain	15.4	<0.001	12.6	<0.001
Training	8.4	0.005	3.8	0.056
Strain × training	6.0	0.012	2.7	0.10
<b>EPOC</b>				
Strain	0.01	0.92	0.015	0.90
Training	9.1	0.002	7.5	0.008
Strain × training	1.0	0.32	0.18	0.67
<b><math>O_{2crit}</math></b>				
Strain	9.7	0.003	9.7	0.003
Training	16.0	<0.001	14.8	<0.001
Strain × training	4.5	0.038	5.6	0.028

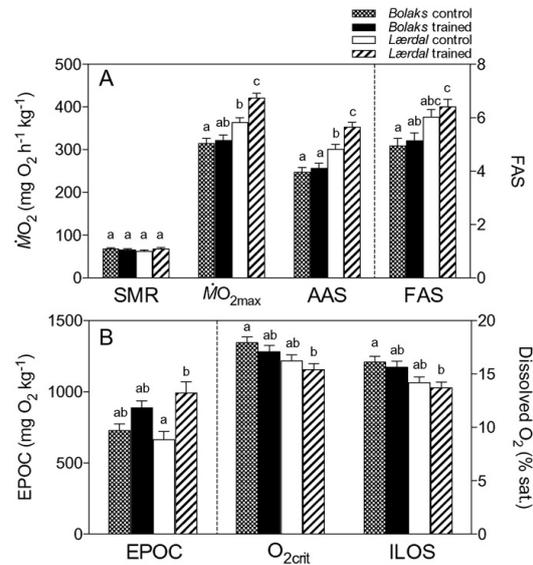
Training significantly increased EPOC by 30% ( $914.0 \pm 55.7$  vs.  $704.1 \pm 25.0$  mg  $O_2$  kg $^{-1}$ ,  $p = 0.002$ , Table 1), but strain ( $p = 0.92$ , Table 1) had no independent main effect on EPOC. There was no interaction between training and strain ( $p = 0.32$ , Table 1) and while EPOC increased significantly ( $p = 0.02$ ) by 49% as a result of training in the wild fish, there was no training effect ( $p = 0.3$ ) on EPOC in the domesticated fish (Fig. 3).

Training and strain significantly impacted  $O_{2crit}$  and ILOS. Training decreased overall  $O_{2crit}$  by 6% ( $16.2 \pm 0.4$  vs.  $17.2 \pm 0.3\%$  sat.,  $p < 0.001$ , Table 1) and  $O_{2crit}$  was 9% lower in wild fish than

**Table 2**

A summary of the main factor effects and significant interactions (F-values and p-values) of strain and training on aerobic and anaerobic capacities of Atlantic salmon, illustrated by maximum rate of oxygen uptake ( $\dot{M}O_{2max}$ ), lethal oxygen saturation (ILOS), citrate synthase (CS) and lactate dehydrogenase (LDH) in red and white muscles.

Factor	F-value	p-value
<b><math>\dot{M}O_{2max}</math></b>		
Strain	19.1	<0.001
Training	2.7	0.1
Strain × training	4.6	0.036
<b>ILOS</b>		
Strain	10.8	0.002
Training	11.2	0.001
Strain × training	5.4	0.024
<b>CS red muscle</b>		
Strain	0.23	0.64
Training	31.7	<0.001
Strain × training	8.4	0.005
<b>CS white muscle</b>		
Strain	17.8	<0.001
Training	1.2	0.29
<b>LDH red muscle</b>		
Strain	0.83	0.36
Training	3.3	0.073
Strain × training	5.3	0.025
<b>LDH white muscle</b>		
Strain	10.9	0.002
Training	1.6	0.22



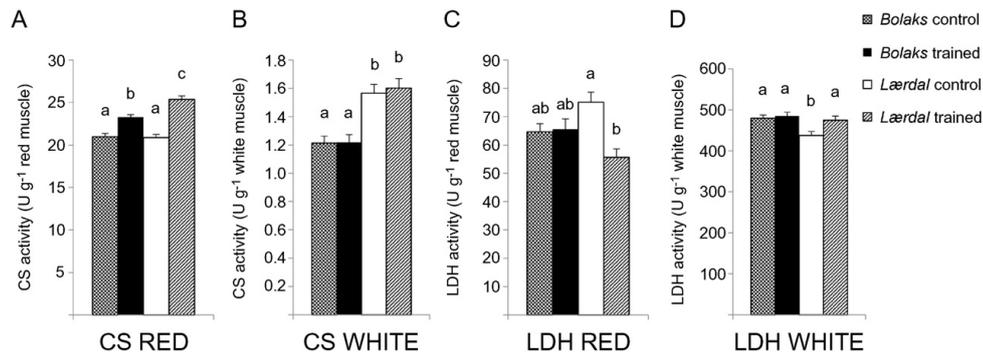
**Fig. 3.** Interactive effects of domestication and exercise training on A) standard metabolic rate (SMR), maximal metabolic rate (MMR), absolute aerobic scope (AAS) and factorial aerobic scope (FAS); B) excess post-exercise oxygen consumption (EPOC), critical oxygen level ( $O_{2crit}$ ) and incipient lethal oxygen saturation (ILOS) of Atlantic salmon (*Salmo salar*) parr. Different letters indicate significant differences between groups within a respiratory index ( $p < 0.05$ ) by two-way ANOVAs and Tukey HSD post-hoc. Values are mean  $\pm$  s.e.m.,  $n = 24$  in Bolaks untrained, 21 in Bolaks trained, 23 in Lærdal untrained, 22 in Lærdal trained.

domesticated fish ( $15.9 \pm 0.5$  vs.  $17.5 \pm 0.3\%$  sat.,  $p = 0.0028$ , Table 1). Training also decreased overall ILOS by 5% ( $14.6 \pm 0.5$  vs.  $15.3 \pm 0.4\%$  sat.,  $p = 0.001$ , Table 2) and ILOS was 12% lower in wild fish than domesticated fish ( $14.0 \pm 0.5$  vs.  $15.9 \pm 0.3\%$  sat.,  $p = 0.002$ , Table 2). Although there were interactions between strain and training for both  $O_{2crit}$  and ILOS ( $p \leq 0.038$ ), the training effect on  $O_{2crit}$  and ILOS did not reach statistical significance for either strain (Fig. 3).

Aerobic ATP production capacity, as measured by CS activity, was 16-times higher in red than in white swimming muscle (Fig. 4A & B; see Table S3 for morphometrics), as anticipated. Training had significant effects on CS activity that were more pronounced in wild than in domesticated fish. In red muscle, training significantly increased CS activity by 22% in wild fish ( $p < 0.001$ ) and by 11% in domesticated fish ( $p < 0.001$ , Table 2, Fig. 4A). In white muscle, CS activity was 30% higher in wild compared with domesticated fish ( $p < 0.001$ ), but training did not influence the CS activity of white muscle in either strain (Table 2, Fig. 4B). LDH activity, an index of anaerobic capacity, was 7-times higher in white swimming muscle than in red swimming muscle (Fig. 4C & D), as expected. Training reduced LDH activity in red muscle by 35% ( $p < 0.001$ ) in wild fish but not in the domesticated fish (Table 2, Fig. 4C). In white muscle, LDH activity was 6% significantly lower in wild fish compared with domesticated fish ( $p = 0.0016$ ), and training significantly increased LDH activity by 8% ( $p = 0.048$ ) in wild fish but not in domesticated fish after training (Table 2, Fig. 4D). Therefore, in terms of key metabolic enzymes, wild fish generally had higher aerobic enzyme activity levels and a higher plasticity that responded to aerobic training than domesticated fish.

### 3.3. Respirometry indices (based on q0.2 SMR values)

By definition, the q0.2 estimate of SMR will always generate numerically higher value compared with the Low10 estimate. The q0.2



**Fig. 4.** The activity of A) citrate synthase (CS) in red muscle; B) CS in white muscle; C) lactate dehydrogenase (LDH) in red muscle and; and D) LDH in white muscle of Atlantic salmon (*Salmo salar*) parr. Different letters indicate significant differences ( $p < 0.05$ ) between groups within muscle types (two-way ANOVAs and Tukey HSD *post-hoc*). Values are means  $\pm$  s.e.m.,  $n = 15$  per group.

estimates (data not shown) were always  $< 11\%$  numerical higher than the Low10 estimates (Table S2). Even so, the outcomes of the comparisons for training and strain main effects were no different (Table 1). For clarity, our figures only present the Low10 estimate of SMR.

#### 4. Discussion

The general goal of this work was to examine if generations of selective breeding for growth (on average a 14% enhancement per generation for the first six generations; Gjedrem and Baranski, 2009) have influenced the athletic robustness and cardiorespiratory plasticity of domesticated *Bolaks* Atlantic salmon strain. This goal was pursued using a suite of indices for respiratory performance and hypoxia tolerance, along with measurements of muscle enzymatic activities that collectively characterized aerobic and anaerobic capacities and which we collectively term athletic robustness. Plasticity in response to aerobic exercise training was examined and the comparison between the domesticated *Bolaks* strain and the Norwegian *Lærdal* wild strain provided additional insights into the effects of domestication. Lastly, by experimentally separating each strain into inferior and superior swimmers, we hoped to establish an association between athletic robustness and swim performance during a simple screening test. The most important potential of this work for aquaculture is the insight into how selective breeding for growth has produced negative and possibly unforeseen effects on athletic robustness and cardiorespiratory plasticity. The domesticated strain certainly had a reduced athletic robustness and also less plasticity in response to a short exercise-training regime compared with a wild strain. Further, the potential of domesticated fish benefiting from exercise training was not completely lost but remained below that for the wild strain, providing further insight into the trade off the athletic robustness resulting from commercial aquaculture practices when compared with cardiorespiratory performance of a wild strain. Thus, the potential of using exercise protocols to train fish to reduce seawater transfer mortality in aquaculture and providing physiological basis for selective breeding to regain athletic robustness and cardiorespiratory plasticity warrant further investigation.

##### 4.1. Training effects

The potential benefits of exercise training in fish are well established (Davison, 1997; Kieffer, 2010). Therefore, we anticipated that an 18-day regime would be sufficiently long and intense enough to improve athletic robustness (defined generally here as a high aerobic capacity, rapid recovery from exhaustion and superior hypoxia tolerance). This proved to be true for the wild *Lærdal* strain to a much greater extent than for the domesticated *Bolaks* strain. Training induced increases in

$MO_{2max}$ , AAS, EPOC, CS activity in red muscle and LDH activity in white muscle in the wild *Lærdal* strain, effects that were absent in the domesticated *Bolaks* strain. Training only enhanced CS activity in red muscle in the *Bolaks* strain.

These observations support our hypothesis that the domesticated *Bolaks* strain had less cardiorespiratory plasticity than the wild *Lærdal* strain in response to exercise training. This important discovery means that commercial aquaculture practices might need to adopt a longer duration of training than used here to sufficiently exploit the full plasticity of the cardiorespiratory system in domesticated strains (Davison, 1997; Gamperl and Farrell, 2004), because previous long-term, but less intensive ( $1\text{--}1.5 \text{ FL s}^{-1}$ ) exercise regimes have induced training effects, i.e., improved growth, greater ventricle mass and enhanced disease resistance in both domesticated and wild Atlantic salmon (Castro et al., 2011, 2013; Grisdale-Helland et al., 2013; Anttila et al., 2014a). It is also possible that our trained fish underwent some detraining prior to the start of the respirometry measurements and potentially the domesticated strain may have detrained faster. A recovery period post-training is inevitable given the need to properly measure the respirometry indices and the industry requirement for transferring smolts to seawater. The diverse training effects observed for the wild strain after 17 days clearly illustrates the retention of training effects despite this recovery period, as well as the practical potential for the aquaculture industry. Indeed, previous studies report retention of training effects well beyond 17 days. For example, following a 48-day aerobic training regime (up to  $1.0 \text{ FL s}^{-1}$ ), 119 days were needed before juvenile Atlantic salmon lost the growth benefit and 48 days to lose the gene expression for the acquired resistance to infectious pancreas necrosis virus (Castro et al., 2011). Coho salmon smolts exercised for 40 days (at  $1.5 \text{ FL s}^{-1}$ ) retained enhanced swimming endurance for 60 days (Besner and Smith, 1983), while striped bass fry exercised for 60 days (at either  $1.5\text{--}2.4 \text{ FL s}^{-1}$  or  $2.4\text{--}3.6 \text{ FL s}^{-1}$ ) retained enhanced swimming performance for 56 days (Young and Cech, 1993). Nevertheless, the current training regime was shorter than previous studies, which might lead to faster detraining. The relatively short training duration used here was aimed to discover a minimum training period (and associated economic cost) to enhance physiological robustness. Atlantic salmon aquaculture may benefit from water velocities in the range of  $1\text{--}2 \text{ FL s}^{-1}$  and a longer training duration than used here. Of course, once suitable protocols are established for training intensity, duration and timing for different life-stages, the ultimate goal is to measure marine survival of trained smolts during commercial grow out. Alternatively, a different genetic selection program may be needed to prevent the trade off of cardiorespiratory robustness.

Exercise training enhanced anaerobic glycolytic capacity (LDH activity) and utilization in white muscle of wild strain, an effect not reflected

in either  $O_{2crit}$  or  $ILOS$  of these two strains. Instead, it was reflected in EPOC, which is an index of a) the rapid recharging oxygen storage on hemoglobin and myoglobin (~20% of total; Burnett et al., 2014), b) the rapid resynthesis of creatine phosphate and adenosine triphosphate, and c) the slower lactate clearance and glycogen resynthesis (Gaesser and Brooks, 1984; Scarabello et al., 1991; Wood, 1991) and possibly restoring ionic imbalances due to the osmo-respiratory compromise (Gallaughier et al., 2001). A higher CS activity in red skeletal muscle of trained fish, as observed previously (Davison, 1997; Kieffer, 2000; Gallaughier et al., 2001; Anttila et al., 2006, 2008a, 2008b), would not only aid aerobic ATP production for swimming, but would benefit aerobic ATP production during recovery. A higher LDH activity in white skeletal muscle could enhance lactate clearance (Milligan et al., 2000), something of less important in red skeletal muscle, as indicated by a reduction of LDH activity in trained wild fish here and previously (Anttila et al., 2006, 2008b).

#### 4.2. Strain differences

The present comparison between a domesticated strain and a wild strain was intended to provide insight into the effects of domestication. Indeed, we found support for our hypothesis that the domesticated *Bolaks* strain would have less cardiorespiratory plasticity and a lower athletic robustness than the wild *Lærdal* strain. However, precisely separating out the effect of domestication (i.e., genetic selection for growth, plentiful food, lack of predators, habituation to aquaria and people, limited environmental extremes etc.) by simply comparing a wild and a domesticated strain is impossible, especially given the mixed stock origins of the *Bolaks* strain following ten generations selective breeding for growth. Also, the genetic divergence between the two strains remains undetermined, even if single-nucleotide polymorphism analysis shows a 0.30–0.32 genetic dissimilarity when compared with themselves and only a 0.31–0.38 genetic dissimilarity when compared against each other (Nick Robinson, pers. comm.). Thus, the differences in performance between strains observed here could be due to: a) genetic selection of desirable traits, such as a faster growth rate for the *Bolaks* strain, b) selective pressures encountered only by the wild fish, c) random genetic drift (possibly exacerbated by population bottlenecks), or d) pre-existing genetic differences between the *Lærdal* strain and the base populations that founded the *Bolaks* strain. We propose that the observed differences between *Bolaks* and *Lærdal* strains are a combination of strain and domestication since the two strains of experimental fish were grown from eyed eggs in the identical hatchery conditions, and that northern and southern strains of European wild juvenile Atlantic salmon had an almost indistinguishable cardiac physiological response to warming after common garden rearing from eggs (Anttila et al., 2014b).

Support for domestication reducing the aerobic capacity of *Bolaks* strain comes from the lower  $MO_{2max}$ , AAS, FAS and CS activity in white muscle when compared with the wild strain. Importantly, enhancement of AAS and FAS in the wild strain was solely through  $MO_{2max}$  with no change in SMR. Superior cardiorespiratory and muscular capacity of wild fish is likely the result of natural selection and adaptation to habitat, as observed in sockeye salmon (*Oncorhynchus nerka*; Eliason et al., 2011) and tropical killifishes (*Aphyosemion* sp.; McKenzie et al., 2013). Conversely, compared with wild or semi-wild hybrid strains, domesticated fish can have up to 9-times lower aerobic enzyme activities in their swimming muscle (Anttila et al., 2008a; Anttila and Mänttari, 2009), a lower swimming speed (McDonald et al., 1998; Reinbold et al., 2009) and a 25–84% lower holding velocity than wild yearlings (Rimmer et al., 1985), but a higher lipid concentration in swimming muscles (McDonald et al., 1998). Underpinning the differences in swimming capacity might be the cardiac malformations common to domesticated fish (Poppe and Taksdal, 2000; Mercier et al., 2000; Poppe et al., 2003; McKenzie et al., 2007; Kristensen et al., 2012b), but hearts were not examined here simply because it is unlikely

that a routine aquaculture practice prior to smolt transfer will be cardiac sampling.

#### 4.3. Segregation of superior and inferior swimmers with an exercise screening protocol

Despite good evidence that a more robust cardiorespiratory system positively correlates with a superior swimming capacity in salmonids (e.g., Keen and Farrell, 1994; Claireaux et al., 2005; Farrell, 2007; McKenzie et al., 2007), we found no support for our hypothesis that superior swimmers (top 20% of each strain and despite an almost 2-fold difference in their  $U_{max}$  compared with bottom 20% of each strain) benefitted from a higher aerobic capacity at the biochemical and whole animal levels, a more rapid recovery from exhaustion and a better hypoxia tolerance. Even an exploratory three-way MANOVA model restricted to just the fastest 10% swimmers ( $140 \leq U_{max} \leq 145 \text{ cm s}^{-1}$ ) and slowest 10% swimmers ( $60 \leq U_{max} \leq 67.5 \text{ cm s}^{-1}$ ) yielded no significant differences for any respiratory index. Previous screening protocols for Atlantic salmon smolts have similarly failed to segregate inferior and superior swimmers according to their routine metabolic rate,  $MO_{2max}$  and aerobic scope, despite a 55% difference in their swimming capacity (Anttila et al., 2014a). Theoretically, fish cannot swim faster or longer without a metabolic cost, unless swimming is mechanically more efficient, a possibility not tested here. Interestingly, the numerical difference in EPOC between top and bottom 10% of both strains almost reached statistical significance ( $898.5 \pm 63.8$  vs.  $729.8 \pm 57.4 \text{ mg O}_2 \text{ kg}^{-1}$ , ANOVA:  $F = 3.9$ ,  $p = 0.06$ ), without any interaction between strain and swimming capacity. Therefore, perhaps the constant acceleration screening protocol used here does a better job of distinguishing anaerobic rather than aerobic capacity by segregating aerobic swimmers and burst-and-coast swimmers, two swimming phenotypes previously observed in wild European sea bass (*Dicentrarchus labrax*) (Marras et al., 2010). Fish use different gaits (burst and sprint) and muscle groups (glycolytic white skeletal muscle) to swim faster and this is why a prolonged swimming test uses incremental water velocities lasting > 10 min (Jones, 1982; Rome, 1992; Hamner, 1995; Burgetz et al., 1998; Martínez et al., 2003). Our screening protocol was adapted from constant acceleration test to measure  $U_{max}$ , which is approximately 33% higher than the prolonged swimming speed (Farrell, 2008). Swimming behaviours and willingness to swim also play a role in determining  $U_{max}$  (Anttila et al., 2014a), and these factors could help explain the observed results. For example, weaker swimmers in a group of 60 fish in a 2-m long swimming tunnel could position themselves behind better swimmers to use slipstream and reduce the energetics of swimming (Bell and Terhune, 1970; Anttila et al., 2014a) and therefore mask differences in cardiorespiratory indices for the truly superior swimmers. Future studies could focus, therefore, on alternative screening protocols that do a better job of assessing aerobic performance and using a commercial scale screening apparatus that can accommodate larger numbers of fish. With regard to the former, transitions in swimming gait (Peake and Farrell, 2004, 2005, 2006) could be used to highlight different swimming modes.

#### 4.4. SMR estimation method

SMR is at the foundation of many of the indices reported in the current dataset (AAS, FAS, EPOC and  $O_{2crit}$  all depend on SMR). According to the definition of SMR, which is the metabolism that supports basic homeostasis without any input into locomotion, digestion, growth and reproduction, SMR should locate at the left side of the distribution curve for a range of  $MO_2$  values measured over several days in quiescent, post-absorptive fish (Steffensen, 1989; Steffensen et al., 1994; Chabot et al., 2016). However, effectively isolating true SMR values from active  $MO_2$  due to minor locomotory activity is not a trivial task. Therefore, to be objective, we provided two estimates of SMR ( $q_{0.2}$  and the Low10) and their derivatives (Table 1). The Low10 estimate will always be

lower than the  $q_{0.2}$  estimate of SMR because it averages the lowest 10 values (here we also eliminated the lowest 2% of  $MO_2$  measurements as a compensation for any measurement errors that would underestimate SMR). Given the size of the  $MO_2$  dataset used here, these lowest 10 values would approximate a  $q_{0.12}$ .

In conclusion, this study demonstrated that cultivation of Atlantic salmon has likely compromised athletic robustness and plasticity. The wild *Lærdal* strain had a significantly higher  $MO_{2max}$ , AAS, FAS and CS activity in white muscle when compared with domesticated *Bolaks* strain. The wild *Lærdal* strain showed plasticity in response to aerobic exercise training through improvements to  $MO_{2max}$ , AAS, EPOC and CS activity in red muscle, as well as LDH activity in white muscle, whereas training only improved CS activity in red muscle of domesticated *Bolaks* strain. These results suggest that the salmon breeding companies should further investigate the implications of their strong focus on growth performance because it appears to have negative consequences on athletic robustness.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.aquaculture.2016.05.015>.

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#### **4. Conclusion**

This fourth chapter presents two cases studies where our approach to assessing fish health was implemented. The common objective of these studies was to verify the applicability of our methodology in various contexts in relation with environmental management and conservation strategies. For this, two experiments were conducted. The first experiment aimed to provide information regarding the toxicity for marine life of treating an oil spill with dispersant while the second experiment verified the consequences of domestication on Atlantic salmon robustness. Even though these two studies may seem quite different, they both required a reliable measure of fish health.

Fish health assessment demonstrated that dispersant treated oil did not have long term, ecologically relevant impact on fish juvenile and that salmon's domestication has led to reduced athleticism and plasticity of the cardiorespiratory system in response to a short exercise-training regime. These results provide a scientific basis for new guidelines regarding environmental management and aquaculture practices, demonstrating that fish health assessment can shed new light on various environmental issues. We hope that these two examples will promote the use of health assessment in environmental impact studies.

## *General conclusion and perspectives*



## **1. General conclusion**

The resources and amenities provided by coastal areas are crucial to the societal and economic needs of the global population. Yet, these areas are also under increasing pressure from human use and associated activities. While representing less than 20% of the land surface, coastal zones are important repository for biodiversity and ecosystems that support the function of the Earth's systems. Moreover, they are essential to secure human population's wealth and health, and they should be, therefore, crucial targets for renewed preservation, sustainable exploitation and management strategies and policies. This implies, however, that potential anthropogenic impacts on these systems are properly assessed and their consequences reliably evaluated. Unfortunately, current approaches and use of biomarkers largely fails to provide ecologically relevant information regarding human impact upon marine organism and a gap between what is desirable (information at ecologically relevant levels) and what is realized (measures at sub-organismal level) still exists. To fill this gap, the notion of health provides a valuable theoretical basis to develop a methodology.

Common and long-standing knowledge indicates that performance traits such as hypoxia tolerance, thermal susceptibility and swimming capacity can be used as biomarkers of fish functional integrity (Tierney and Farrell 2004; McKenzie *et al.*, 2007, Roze *et al.*, 2012, Claireaux *et al.*, 2013). Based on the ties established by Frankish *et al.*, (1996) between functional integrity and health, we investigated the possibility of using these traits to inform about fish health. For this, we combined laboratory and field experiments which is an innovative approach in relation to traditional practices in biomarkers development. Moreover, we examined the

underlying mechanisms and identified the confounding factors influencing the performances measured and their interpretation as biomarkers. Finally, we validated that methodology through two case studies which allowed identifying the strength and weaknesses of these markers as indicators of health. This was done using the 6 criteria proposed by van der Oost *et al.* (2003).

**1. the assay to assess fish health should be reliable (with quality control), relatively cheap and easy to perform;**

Assessing fish health involves manipulating live animals and it is very important that harming and stressing animals is avoided in order to provide reliable and interpretable biomarker responses. In this regard, we observed a significant repeatability of our markers over time spanning from 3 to 11 months. We also demonstrated that our markers' responses correlated with individuals' performances in the field (Paper 1). Furthermore, setups required to perform challenge tests are inexpensive (less than 10 000 €), they are easily built and can be operated following modest personnel training. Moreover, after the initial expenditure in equipment, these setups require little running costs compared to those mobilized by biochemical and molecular markers analyses.

**2. biomarker responses should be sensitive to stressors in order to serve as an early warning signal of health impairment;**

We demonstrated that shortly after exposure to dispersant-treated oil fish displayed decreased hypoxia tolerance and swimming performances which persisted during 4 weeks post-exposure (Paper 1). We also revealed that

individuals' performances were predictive of fish survival in the field, validating the capacity of our markers to serve as an early warning signal of ecologically relevant disorders.

**3. data about baseline fluctuations in the biomarkers' values should be available in order to distinguish between natural variability (noise) and health impairment (signal);**

In our different experiments, we documented individual and population diversity in biomarkers' responses (Papers 1 and 6) and highlighted that these sources of variability must be considered when designing studies and interpreting biomarkers' response. For instance, this variability implies measuring markers in a large number of individuals to increase the power of statistical analysis.

**4. confounding factors to the biomarker response should be identified;**

Confounding factors of hypoxia tolerance, temperature susceptibility and swimming performances are well documented. It is for instance admitted that these performances are influenced by acclimation temperature, nutritional status, habituation, body size and life stage (Killen *et al.*, 2016). In the present study, we found that while performances varied over time due to changes in acclimation temperature and fish size, fish rank was stable over time, *e.g.*, a good swimmer remains a good swimmer and a poor swimmer remains a poor swimmer (Paper 1). We also identified that environmental history influences the responses of our markers of fish health, precluding direct comparison of populations from different sites (Paper 2).

**5. the mechanistic basis of the relationships between biomarker response and health impairment should be established;**

It is admitted that this criterion is the most difficult to meet for complex physiological trait such as hypoxia tolerance, temperature susceptibility and swimming performances (McKenzie *et al.*, 2007). Complex physiological traits are indeed the cumulative outcome of numerous underlying mechanisms. Nonetheless, we identified several links between performances during hypoxia challenge tests and the cardiorespiratory function. Performance during these challenge tests is, for instance, associated with the capacity of ventricular muscle strips to preserve contractility under hypoxic conditions (Paper 3) and it also correlates with individual's  $O_{2crit}$  (Paper 4). However, the mechanistic basis of performances observed during temperature and swimming challenge tests remains unclear and should be investigated further.

**6. the ecological significance of the biomarker, e.g., the relationships between its response and the (long-term) impact to the organism, should be established**

While most low-organizational level biomarkers fail to meet this criterion, it represents the primary strength of our approach. We demonstrated that two of our markers of health (hypoxia tolerance and swimming performance) were predictive of fish success in the field (growth and survival) (Paper 1). We can therefore consider that their measures are ecologically significant.

According to these 6 criteria, our methodology to assess fish health is liable to shed a new light on persistent issues regarding ecological impact assessment and management. Application of this methodology to concrete case studies has demonstrated that our approach is generalizable to different contexts and that it provides operational information easily transferable to socio-economic sectors and general public (Papers 6 and 7). Therefore, we believe that our approach is a valuable tool to assess anthropogenic impacts on ecosystems.

## ***2. Perspectives***

In this thesis, we tested a methodology aiming at assessing fish health. Although promising results were obtained, improvements are still needed in order to address the weaknesses that we have identified. The main limitation identified is related to the mechanistic bases of the relationship between our biomarkers' responses and pollutant exposure. In this regard, the cardiorespiratory system is an appropriate target as it plays a central role in the performances measured. Recent progresses in biologging offer promising avenues for future research through, for instance, micro wireless heart rate loggers. Such loggers would allow monitoring individual's heart rate during the challenge tests. Comparison of individual's hypoxia tolerance, temperature susceptibility and swimming performance with their heart rate could allow deciphering the links between the heart function and biomarkers' response.

We also demonstrated that high inter-population variability and the influence of environmental history on our markers of health clearly preclude direct comparison of populations as classically practiced during impact assessment procedure, *i.e.*, a population from a contaminated site is compared to a population from a clean site used as a control. To tackle this issue, we recommend conducting longitudinal studies. This approach is notably used in human medicine to detect doping. To detect doping, athlete's biological passport consists in monitoring biological markers overtime. From these data and population epidemiology, upper and lower limits are defined. A value laying outside these limits could be indicative of doping or of an underlying health issue. This approach allows to establish a causal relationship between an event and a change in the biologic system studied. In fish, challenge tests developed in this thesis are cheap, easy to use, transportable in the field and

therefore fit perfectly with longitudinal studies approach. To validate this hypothesis, it would be necessary to target at risk or model cohorts in the wild (from a marine protected area or close to an offshore oil field for instance) and then to monitor over time their response at hypoxia, temperature and swimming challenge tests in order to define a reference range for each of our markers of health. Once the reference range defined, the monitoring of cohort's response to challenge test allow to assess its health status. When a value lay outside the reference range, this could indicate a health impairment and therefore a threat for the entire fish population (Figure 12).

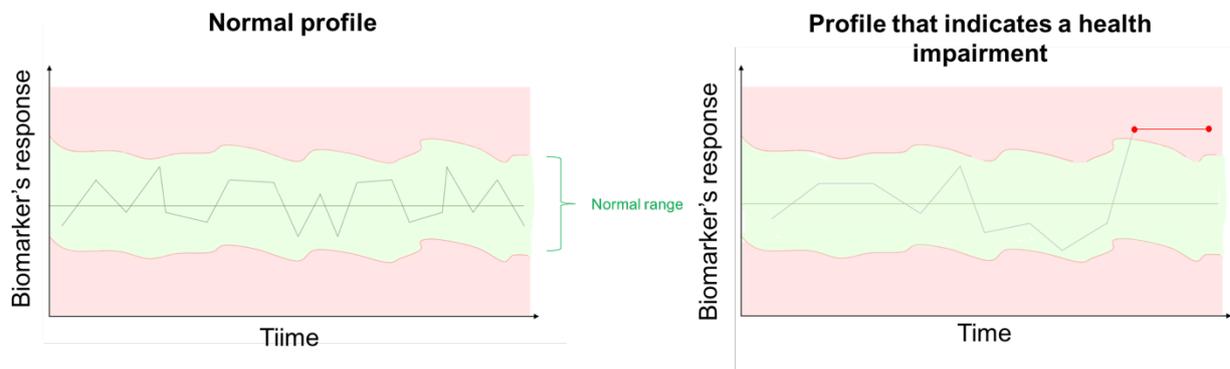


Figure 12: Example of a biomarker's response that indicates a normal profile (left) and a health impairment (right).

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## **English version**

**Title:** Assessing fish health: the missing cornerstone in ecological risk assessment and management

**Keywords:** fish, health, ecology, functional integrity, biomarker, hypoxia, temperature, swimming, oil spill

**Summary:** Growth of human population and associated intensification of human activities is putting considerable pressure on coastal marine ecosystems. To assess anthropogenic impact on these ecosystems, substantial efforts have been devoted to the development of biomarkers. Despite a potential for a broad application range, most commonly used biomarkers target effects at sub-organismal organizational level (molecules, cells or tissues) and very few are liable to indicate impacts at organism or higher organizational levels. On the other hand, although too far removed from causal events to constitute early warning signals of environmental stress, high-level organizational biomarkers are of considerable relevance to human activities, economy and well-being. They are indeed the integrative result of the environmental history of an organism and, at the same time, reflect their vulnerability and resilience to changes in their living conditions. This view is corroborated by the recent revision of the concept of animal health, which nowadays incorporates the latent effects of past living conditions, the consequences of past exposures and experiences and the cumulative consequences of these effects. The joint use of high organizational levels markers and the concept of health could help overcome the failure of current biomarkers to provide ecologically relevant information. In this context, the objectives of my thesis were 1) to develop a methodology to assess fish health; 2) to investigate the underlying mechanisms and confounding factors of the performances measured and 3) to verify the applicability of our approach through two case studies. Throughout these researches, we demonstrated that hypoxia tolerance, temperature susceptibility and swimming performances are promising biomarkers of fish health. Their responses are stable over time, predictive of fish survival in the field and sensitive to an exposure to dispersant-treated oil. Also, application of this methodology to case studies demonstrated that our approach is generalizable to different contexts and that it provides operational information easily transferable to socio-economic sectors and general public.

## **Version française**

**Titre :** Evaluer l'état de santé des poissons : la pierre angulaire manquante dans l'évaluation et la gestion des risques écologiques

**Mots clés :** poissons, santé, écologie, intégrité fonctionnelle, biomarqueur, hypoxie, température, nage, marée noire

**Résumé :** La croissance de la population humaine et l'intensification des activités associées exercent une pression considérable sur les écosystèmes marins côtiers. Afin d'évaluer l'impact des activités humaines sur ces écosystèmes, des efforts considérables ont été consacrés au développement de biomarqueurs. En dépit d'un potentiel pour une large gamme d'applications, les biomarqueurs les plus couramment utilisés ciblent des effets situés à des niveaux organisationnels faibles (molécules, cellules ou tissus) et très peu sont susceptibles d'indiquer des impacts sur des niveaux organisationnels supérieurs (organisme, population, écosystème). Or, ces derniers sont d'une pertinence considérable pour les activités humaines, l'économie et le bien-être. Ils sont en effet le résultat intégratif de l'histoire environnementale d'un organisme et, en même temps, ils reflètent leur vulnérabilité et leur résilience aux changements de leurs conditions de vie. Cette opinion a été corroborée par la révision récente du concept de santé animale qui dorénavant, intègre les effets latents des conditions de vie antérieures, les conséquences des expositions et des expériences passées, ainsi que les conséquences du cumul de ces effets. L'utilisation conjointe des marqueurs de haut niveaux organisationnels et du concept de santé pourrait donc aider fournir des informations écologiquement pertinentes grâce à l'utilisation de biomarqueurs. Dans ce contexte, les objectifs de ma thèse étaient 1) de développer une méthodologie pour évaluer la santé des poissons, 2) d'étudier les mécanismes sous-jacents des performances mesurées et 3) de vérifier l'applicabilité de cette approche à de nombreux contextes en s'appuyant sur deux études de cas. Au cours de ces recherches, nous avons démontré que la tolérance à l'hypoxie, la sensibilité à la température et les performances de nage sont des biomarqueurs fiables de la santé des poissons. Leurs réponses sont en effet stables dans le temps, prédictives de la survie des poissons dans leur milieu naturel et sensibles à une exposition à un polluant. De plus, l'application de cette méthodologie à des études de cas a démontré que notre approche est généralisable à différents contextes et qu'elle fournit des informations opérationnelles facilement transférables aux secteurs socio-économiques et au grand public.