Insights into Marine Fish Physiology in a Changing World

From biochemical to behavioural effects

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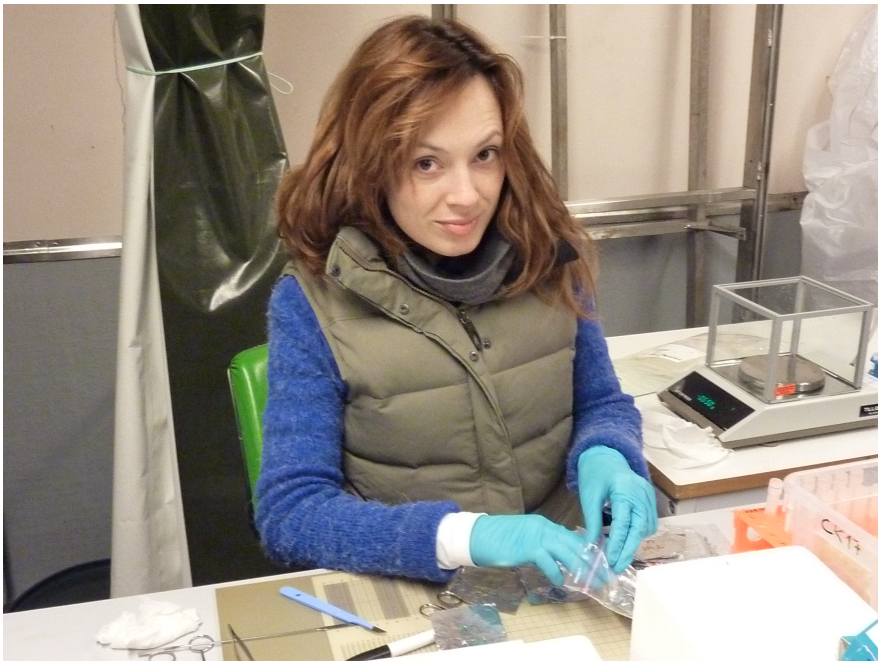
Front-page photo: Atlantic halibut (*Hippoglossus hippoglossus*) (adapted from commons-wikimedia.org).



The author collecting samples of Atlantic halibut tissue
(photo by Ingibjörg Einarsdottir)

Supervisors: Prof. Lars Förlin and Associate Prof. Joachim Sturve
Examiner: Prof. Michael Axelsson

To my parents, Julieta and Erni, my brother Juliano, and to my dear Thomas

"*There is pleasure in the pathless woods,*
there is rapture in the lonely shore,
there is society where none intrudes,
by the deep sea,
and music in its roar;
I love not Humanity the less,
but Nature more."

Lord Byron
(1788 – 1824)

Abstract

Ocean acidification and global warming are largely caused by increased levels of atmospheric CO_2, and marine fish are exposed to both these stressors simultaneously. Although the effects of temperature on fish have been investigated over the last century, the effects of moderate CO_2 exposure and the combination of both stressors are not well-understood, especially long-term effects.

In **Papers I, II** and **III** we investigated the protein expression and biochemical parameters in gills, blood plasma, and liver of Atlantic halibut (*Hippoglossus hippoglossus*) exposed to temperatures of 5, 10, 12 (control), 14, 16, and 18 °C (impaired growth) in combination with control (400 μatm) or elevated CO_2 (1000 μatm) levels for 3 months.

Paper I shows the protein expression in gills and blood plasma of halibuts exposed to elevated CO_2 at 12 °C and 18 °C. Elevated CO_2 induced the regulation of immune system-related proteins in plasma of fish from both temperature treatments. Gills from fish exposed to elevated CO_2 at control temperature show modulation of energy metabolism proteins, as well as indications of increased cellular turnover and apoptosis signalling; while gills from fish exposed to both elevated CO_2 and elevated temperature indicate increased expression of energy metabolism proteins. In conclusion, moderate CO_2-driven acidification, alone and combined with increased temperature, can elicit biochemical changes that may affect fish health.

To further investigate the findings in **Paper I** we analysed non-specific immune components in blood plasma (**Paper II**), and examined the occurrence of oxidative stress in liver (**Paper III**) of Atlantic halibut exposed to elevated CO_2 at 5, 10, 12, 14, 16, and 18 ºC. **Paper II** reveals that both measured immune components (lysozyme and complement system) had increased activities in response to elevated CO_2, which is consistent with the findings of **Paper I**. These changes represent an additional energetic cost for fish.

Paper III indicates the occurrence of oxidative stress, which can damage macromolecules such as DNA, membranes, and enzymes. Protein carbonyls were consistently higher in the elevated CO_2-treated fish at all studied temperatures, while the antioxidant enzymes did not show the same results, suggesting that the exposure to elevated CO_2 increased reactive oxygen species (ROS) formation, with consequent oxidative damage that bypasses the antioxidant defence system of the cells. The consequent oxidative stress might be connected to the increased expression of energy metabolism proteins seen in **Paper I.**

Paper IV provided an overview of elevated CO_2 effects at whole organism-level through behavioural studies. Elevated CO_2 exposure for 20 and 40 days, caused several behavioural disturbances, including the reduction of boldness, exploratory behaviour, lateralization, and learning in the three-spined stickleback (*Gasterosteus aculeatus*). The effects were present throughout the exposure period and increased in effect size with exposure time. Given the severity of disturbances, our findings suggest that elevated CO_2 could pose a serious problem for sticklebacks.

This thesis provides significant insights into how marine fish can be affected by near-future elevated CO_2 and temperature. The CO_2 levels estimated to occur at the end of this century can pose physiological challenges to marine fish, and have the potential to negatively impact fish populations if acclimation fails to occur.

Keywords: ocean acidification, carbon dioxide, temperature, global warming, *Hippoglossus hippoglossus*, *Gasterosteus aculeatus*, teleost fish, gills, plasma, liver, immune system, energy metabolism enzymes, oxidative stress, proteomics, behaviour.

List of Papers

This thesis is based in the following papers, referred to in the text by roman numerals as follows:

I. **Bresolin de Souza K**, Jutfelt F, Kling P, Sturve J (2014) Effects of increased CO_2 on fish gill and plasma proteome. *PLoS ONE* 9(7): e102901.

II. **Bresolin de Souza K**, Asker N, Förlin L, Sturve J. Non-specific immunity of Atlantic halibut exposed to elevated CO_2 at six different temperatures. *Submitted to Fish Shellfish Immunology.*

III. **Bresolin de Souza** K, Almroth BC, Sturve J. Biochemical effects of elevated CO_2 levels and different temperatures in the Atlantic Halibut. *Manuscript.*

IV. Jutfelt F*, **Bresolin de Souza K***, Vuvlsteke A, Sturve J (2013) Behavioural disturbance in a temperate fish exposed to sustained high-CO_2 levels. *PLoS ONE* 8(6): e65825. **Contributed equally to the study*

The article's/manuscript's respective supplementary materials are appended at the end of the thesis and are reproduced with permission from the respective journals.

Definitions and Abbreviations

AChE: Acetylcholinesterase

BChE: Butyrylcholinesterase

Carbon pump: Biological ocean's sequestration of carbon from the atmosphere to the deep sea.

Climate change: Long-term change in the Earth's climate.

DNA: Deoxyribonucleic acid, which is the carrier of genetic information.

EROD: Ethoxyresorufin-O-deethylase (EROD), an index of CYP1A enzymatic activity

GABA: Gamma-aminobutyric acid, main inhibitory neurotransmitter in adult vertebrate central nervous system.

Global change: Planetary-scale changes in the Earth's system.

GPx: Glutathione peroxidase

GR: Glutathione reductase

GST: Glutathione S-transferase

GSH/GSSG: Glutathione (reduced/oxidized)

HCO3-: Bicarbonate, a physiological pH buffer.

LC_{50}: Median lethal concentration or population critical concentration 50, used to compare toxicities.

MS: Mass spectroscopy

NADP(H): Nicotinamide adenine dinucleotide phosphate (oxidized/reduced)

Ocean acidification: Reduction in the pH of the ocean over an extended period of time, caused by the uptake of carbon dioxide (CO_2) from the Earth's atmosphere.

PC: Protein carbonyls

***p*CO_2**: Partial pressure of carbon dioxide

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

SOD: Superoxide dismutase

TCA: Tricarboxylic acid

2DE: Two-dimensional gel electrophoresis

Table of Contents

1 Introduction

1.1 Importance of fish to ecosystems and human populations

Humanity has a high socio-economic and cultural dependency on marine ecosystems, largely based in fundamental ecosystem services provided by fish populations. For example, the ocean provides 11% of global animal protein consumed by humans (FAO 2014), being of large importance for the food security of millions of people. In the past, the oceans were considered inexhaustible in terms of resources to feed the growing human population. However, ever-increasing population growth, together with industrialized fishing methods, is driving the depletion of wild fish stocks (Tidwell and Allan 2001; MacCauley et al. 2015). Awareness of damaging fishing methods is compelling a shift in fisheries management, from management based on yield optimization to stock assessments and management; the main reason for this being evident overexploitation of global fish stocks (King et al. 2014). The European Commission estimated that three-quarters of commercial marine stocks are overfished; and without doubt, subsidies for fisheries implemented in recent decades has created an unsustainable imbalance between fish resources and fishing capacity in the European Union (Cordón and García 2014).

Marine fish are one of the cornerstones of biodiversity in marine ecosystems, and biodiversity is increasingly being recognized as a proxy of healthy ecosystems worldwide (Laurila-Pant et al. 2015). The loss of biodiversity as a result of human activities can potentially reduce important interactions between trophic levels leading to deleterious trophic cascade consequences (Österblom et al. 2007; Laurila-Pant et al. 2015). The importance of biodiversity has been recognized by the European Union through policy drivers such as the EU Biodiversity Strategy and through a biodiversity descriptor named Good Environmental Status in the Marine Strategy Framework Directive (MSFD 2008; European commission 2008). These frameworks exemplify the a growing awareness of the importance of biodiversity, which plays important roles in ecosystem services as a regulator of ecosystem processes, as a final ecosystem service and as a good; with important ecological, socio-cultural and economic implications (Mace et al. 2012; Laurila-Pant et al. 2015). Besides their importance for biodiversity maintenance, fish also contribute to nutrient cycling, food chain dynamics, and have cultural and aesthetic importance, among other functions (Holmlund and Hammer 1999; Grant et al. 2013).

1.2 Anthropogenic marine stressors

"The ocean moderates anthropogenic climate change at the cost of profound alterations of its physics, chemistry, ecology, and services; and the management options to address ocean impacts narrow as the ocean warms and acidifies" (Gattuso et al. 2015)

Stressors can be defined as casual factors or stimuli that evoke a physiological response in the organism's external or internal environment (Atkinson et al. 2015). For marine organisms

there are natural stressors associated with daily, seasonal, or life cycles; and other unpredictable stressors that can derive from anthropogenic activities such as pollution, debris, noise, fisheries interactions, etc. There are also stressors derived indirectly from human activities e.g. climate change, ocean acidification, temperature increase, hypoxia, and disease outbreaks (Atkinson et al. 2015). Independently from the stressor, there are costs associated with the responses, which can lead to physiological dysfunctions in fish (Barton 2002).

The interactions between stressors can have three different outcomes: (1) additive effects, in which stressors affect the organism in an independent way and the combined effects are simply the sum of individual effects, (2) antagonistic effects, in which stressors offset the effects of each other, and (3) synergistic effects, where stressors interact in a way that their combined effects are larger than the sum of individual effects (Przeslawski et al. 2015). Regarding the combined effects of acidification and warming on marine biota, synergistic effects are more common than additive or antagonistic interactions according to a meta-analysis study (Harvey et al. 2013).

On a global scale, the main threat to marine fish biodiversity is fishing (Hiddink et al. 2008), driven by fishing practices that damage marine ecosystem and an increasing demand for resources as the human population grows. However, other factors are also intensifying the damage of fishing pressure, such as climate change, ocean acidification, habitat loss, invasive species, debris, eutrophication, pollution, and other ecosystem changes. The deterioration of global oceanic conditions is accelerating the decline of marine fish populations and also inhibiting their recovery (Brander 2012), and fish protection programs so far had none of very small success in reducing the decline of fish populations (Hutchings and Reynolds 2004; FAO 2014).

Current research estimates that global change will impact fish stocks directly by causing pronounced geographic shifts in fish abundance and distribution in the coming 50-100 years, and recent evidence has shown that such shifts have already happened in benthic community composition and Arctic fish distribution, in association with ocean warming (Barker and Knorr 2007; McBride et al. 2014). The direct and indirect impacts of climate change in fisheries are expected to have extensive economic implications for the fisheries sector (Brander 2012; McBride et al. 2014).

There are strong indications that ocean acidification amplifies the effects of pollution, especially in coastal ecosystems. Many pollutants reduce the photosynthesis rate in marine photosynthetic organisms, which in turn, reduces carbon dioxide (CO_2) uptake by phytoplankton (Zeng et al. 2015). The inhibition of primary production caused by pollutants can reduce the efficiency of CO_2 removal from the atmosphere, reducing the climate change mitigation exerted by the oceans (Macdonald et al. 2005). Another important problem related to ocean acidification is that it can increase the toxicity of aquatic pollutants, such as heavy metals by changing their speciation and bioavailability (Doney et al. 2009). Water pH can also influence the toxicity of ionisable pharmaceuticals (Boström and Berglund 2015). In addition, the process of ocean acidification reduces the concentrations of OH^- and CO_3^{2-}, which can form strong bonds in seawater with divalent and trivalent cations, and the consequent anions reduction can change the speciation of metal ions, converting them to forms that are more toxic to biota (Zeng et al. 2015).

There is a continuous influx of pollutants into the oceans from sources such as fossil fuel combustion and other industrial activities, which apart from emitting CO_2, are also sources of heavy metal contamination (Zeng et al. 2015). Mercury and lead, for example, are very common contaminants in coastal ecosystems and are often detected in high concentrations in water and sediments (Doney et al. 2009). Heavy metals also bioacumulate and are passed through the food chain, reaching humans and threatening human health through contaminated food products from the ocean (e.g. fish and sea-birds) (Elliott and Elliott 2013). Oil pollution, which is also a source of heavy metals, can originate from e.g. tank cleaning, shipwrecks, and oil-producing platform accidents (Hazen et al. 2010). Oil slicks on water surfaces have the potential to limit gas exchange and reduce light penetration into the water, affecting phytoplankton and photosynthesis rates, and potentially affecting the carbon pump (González et al. 2009). To add to the problem, ocean acidification also changes the rate or organic degradation *via* alteration of metal speciation and nutrient availability (Zeng et al. 2015).

Anthropogenic pollutants other than CO_2 also make a significant contribution to acidification in coastal regions since respiration of organic matter (e.g. from eutrophication) can severely acidify coastal and estuarine waters (Feely et al. 2010). This type of acidification is a substantial problem especially in bottom waters where water exchange is limited (Cai et al. 2011). This type of acidification is becoming more concerning than in open oceans because it has a more rapid and direct effect on near-shore ecosystems (Waldbusser et al. 2011), with consequent impacts on local economies and several environmental implications. For instance, a long-term study shows a pH decrease of 0.006-0.012 units $year^{-1}$ in the Chesapeake Bay, a much faster pH decrease in comparison to open ocean (0.0017 $year^{-1}$) during the same period of time (1985-2010) (Waldbusser et al. 2011).

Global change affects marine biota through three main environmental changes: temperature increase, which is a major driver of variances (Pörtner and Knust 2007; Pörtner 2010); a progressive carbon dioxide accumulation, which increasingly acidifies the ocean (Caldeira and Wickett 2003); and hypoxia, caused by enhanced water stratification and increased oxygen demand of organisms in warmed/acidified areas (Stramma et al. 2008; Couturier et al. 2013). Moreover, these stressors do not occur individually in nature, but together (Pörtner 2010), and sometimes with other changes, such as in salinity levels (due to freshening, stratification, or drought), diverse types of pollution (Sokolova and Lanning 2008), changes in sea level, vertical stratification, changes in ocean circulation (Crozier and Hutchings 2014), etc. The aforementioned changes can also affect and interact with the variables described earlier, but these changes are not so widespread, often remaining limited to certain areas of the world (Lanning et al. 2008). Therefore, the most straightforward effects of global change in marine fish are expected to be related to direct physiological stress, associated with factors such as: lowered pH, increased temperature, and lowered oxygen levels (Crozier and Hutchings 2014).

1.2.1 Elevated CO_2 levels and increased temperature

"Ocean acidification and warming are considered two of the greatest threats to marine biodiversity, yet the combined effects of these stressors on marine organisms remains largely unclear"
(Harvey et al. 2013)

Business-as-usual scenarios are leading to a continued input of CO_2 into the Earth's atmosphere, changing the oceans chemistry at a rate never observed before. The acidification of the oceans is caused by the continuous absorption of CO_2 emissions, a phenomenon named Ocean Acidification (Solomon et al. 2009). CO_2 emissions are mainly produced by anthropogenic activities (Fig. 1), and according to estimates the CO_2 already present in the atmosphere is enough to continue the ocean's pH reduction (Fig. 2) even if the CO_2 emissions are strongly reduced (Solomon et al. 2009; Orr 2011). The increase of CO_2 concentration in oceans worldwide is increasing its partial pressure in open waters, and shifting the equilibrium of the following reaction to the right: $H_2O + CO_2 \_ H_2CO_3 \_ H+ + HCO_3$, increasing the production of hydroxonium ions (H_3O+), which in turn reduces the ocean's pH (Claiborne et al. 2002). Current oceanic pH levels are ca. 8.1 pH units, a reduction of 0.1 units compared to pre-industrial levels, and are expected to decrease to 7.7 pH units in the year 2100 (Caldeira and Wickett 2003), if no concrete actions are taken to change the current situation.

At the beginning of the industrial revolution, in the 18th century, atmospheric CO_2 concentration was 280 ppm (Feely et al. 2004), while today it exceeds 400 ppm (Dlugokencky and Tans 2015, NOAA 2015). Further increases to 420-940 ppm are predicted to occur by the end of this century, depending on the rate of anthropogenic emissions and land use (Cubasch et al. 2013). CO_2 in the ocean is increasing at approximately the same rate as in the atmosphere, since atmospheric and ocean surface pCO_2 are kept in equilibrium (Doney 2010). Oceans are a sink for inorganic carbon because of its buffering capacity, together with the carbon fixation provided by oceanic photosynthetic plants and algae. It is estimated that the oceans stand for about half of the net primary production on earth, resulting in approximately 45 gigatons of fixed carbon per year, of which 16 gigatons are distributed into the oceans (Falkowski et al. 1998). Although CO_2 itself is a non-detrimental gas, which is necessary to support life, the amount of atmospheric CO_2 is one of the main contributors to the Earth's temperature regulation system (Cubasch et al. 2013).

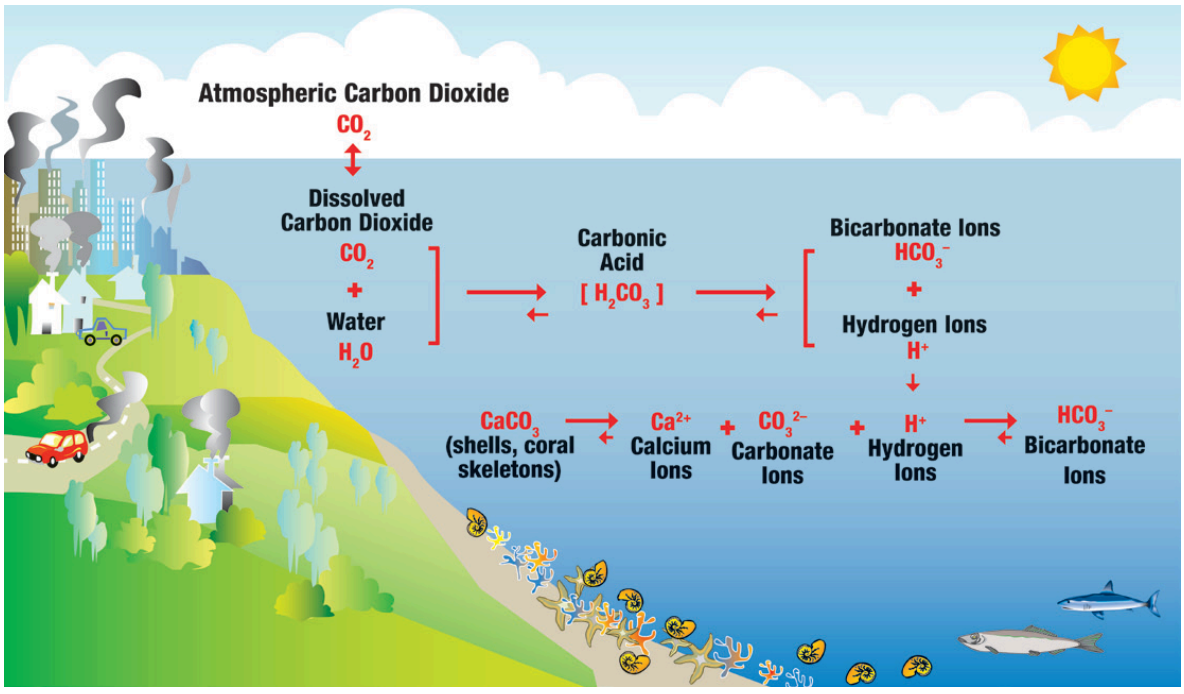


Figure 1. An overview of the main changes in ocean chemistry associated with the dissolution of CO_2 into the oceans (National Research Council, 2013).

Therefore, the rise in atmospheric CO_2 levels is driving ocean acidification and global temperature increase, and is predicted to cause dramatic changes in marine ecosystems in the coming decades (Doney et al. 2012).

Rising atmospheric CO_2 levels increase the global surface temperature, which is now an average of 0.76 °C warmer than it was at the start of the industrial revolution (Solomon et al. 2007). Furthermore, the rise in atmospheric CO_2 concentrations are likely to continue with a temperature increase of 2 to 6 °C by the year of 2099 (Solomon et al. 2007; Sokolov et al. 2009), accompanied by de-oxygenation of seawater, since temperature and oxygen are predicted to change in parallel with CO_2 levels (Pörtner et al. 2005). In addition to the direct effects of a temperature rise, warming also adds thermic energy to the oceanic system enhancing the effects of other disturbances and increasing environmental variability (MacNeil et al. 2010).

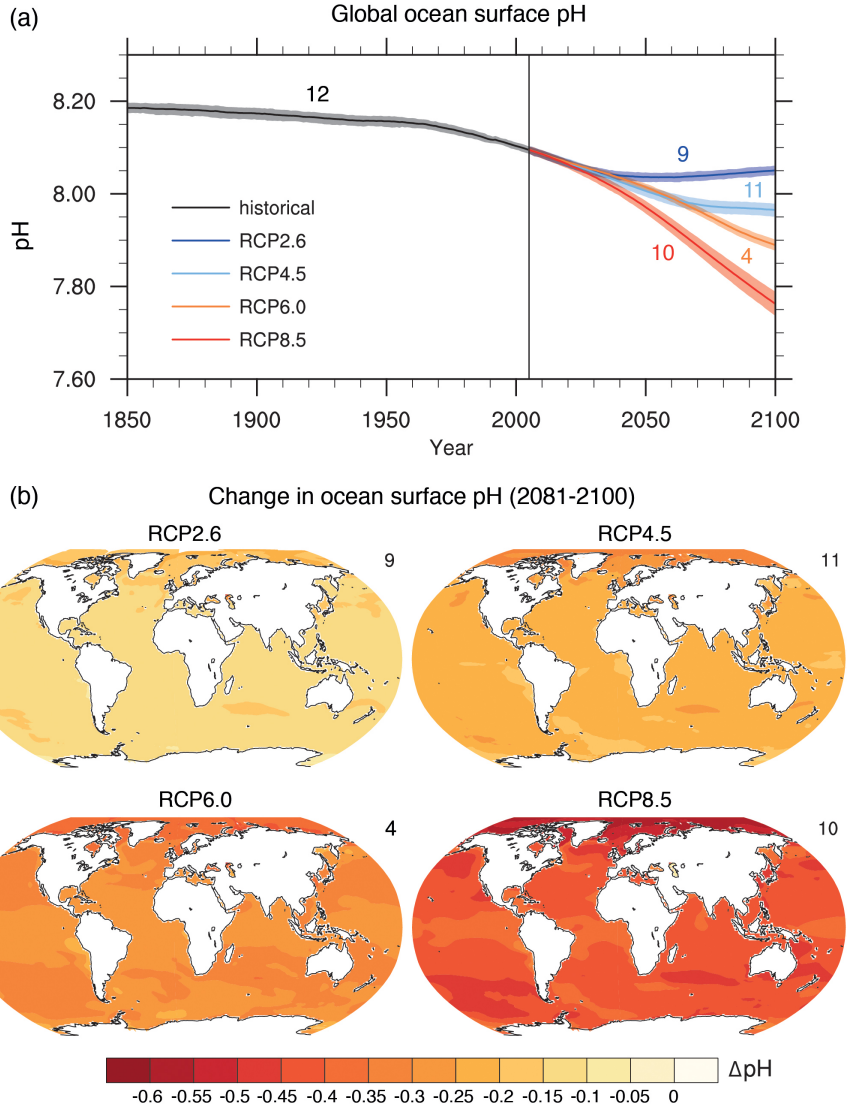


Figure 2. Modeled average changes in ocean surface pH between 1986-2005 and 2081-2100 following the four IPCC scenarios from the lowest (RCP2.6) to the highest (RCP8.5). The number of CMIP5 models to calculate the multimodel means is indicated in the upper right corner of each panel. Reproduced from Stocker et al. (2013) with permission.

1.3 Effects of elevated CO_2 and increased temperature on marine biota

Ocean acidification is a widespread stressor to marine biota, along with potential major ecological shifts. Changes in the ocean's CO_2 concentrations can impact marine organisms directly, through e.g. osmoregulation, or indirectly by affecting their sensitivity to other environmental variables, such as temperature (Pörtner et al. 2005). Responses of marine organisms to ocean acidification are projected to become progressively more apparent in the next 50-100 years, and most studies suggest that these responses are expected to vary because marine organisms have different responses and levels of resilience (Harvey et al. 2013). In general, higher levels of CO_2 dissolved in seawater diffuse more easily across animal cell walls in contact with it, often decreasing physiological pH levels. The main cellular mechanisms to deal with internal acidification include passive buffering of cellular fluids, transport of relevant ions, transport of CO_2 via respiratory pigments, and metabolic suppression (Clairborne et al. 2002; Seibel and Walsh 2003; Pörtner et al. 2004).

To further understand possible impacts of ocean acidification, a wide range of biological responses have been measured across a variety of marine taxa. A meta-analysis study compiling the existent data on the effects of ocean acidification shows that the primary negative effects on biota are impacts on survival, calcification, growth, and reproduction (Kroeker et al. 2010). One widely studied subject is the effect of ocean acidification on the reduction of calcium carbonate saturation and its impact on a wide range of marine organisms, including plankton, benthic molluscs, echinoderms and corals (Zeng et al. 2015). Ocean acidification also alters phytoplankton abundance and carbon fixation rates in both calcifying and non-calcifying photosynthetic organisms (Doney et al. 2009). There are also studies reporting immune modulation elicited by acidification in bivalves (Bibby et al. 2008; Matozzo et al. 2012) and echinoderms (Hernroth et al. 2011).

Environmental stress is a constant challenge for aquatic organisms, and stress can cause molecular, biochemical, and behavioural changes (Barton 2002). Physiological responses to stress can be classified as primary, which involves endocrine changes such as increased levels of circulating catecholamines and corticosteroids; secondary, which comprises of changes in features associated with metabolism, hydro-mineral balance, cardiovascular, respiratory and immune functions; and tertiary, which refers to whole-animal responses such as in growth, disease resistance, behaviour, and survival (Barton 2002, and references therein).

Alongside ocean acidification, ocean warming is also predicted to affect marine organisms. Research shows that animal performance decreases below optimum levels during cooling and warming, and that thermal stress can negatively impact not only growth, reproduction, foraging, the immune system, and behaviour (Pörtner and Farrell 2008), but also reproductive success, among other features (Donelson 2015; Gattuso et al. 2015). As temperature increases above optimum, ectotherms experience rise in resting metabolic rate and a reduction in metabolic scope for aerobic activity. Changes in aerobic capacity are expected to reduce the ability to perform activities like swimming, foraging, growth, and energy storage (Donelson 2015).

Among the most sensitive groups to ocean warming are tropical ectotherms, because they have evolved in relatively stable environments (Tewksbury et al. 2008; Donelson 2015). Existing research indicates that tropical species are already living close to their thermal

optimum, and any future temperature increase is expected to exert a negative impact (Tewksbury et al. 2008; Donelson 2015). On the other hand, deep sea and polar organisms also appear to be vulnerable to changes in temperature since they have limited or no options to migrate and might already be living close to their thermal tolerance, due to cold adaptation through millions of years in stable cold ecosystems (Przeslawski et al. 2015). These results suggest that increased temperatures should favour species with wider thermal windows (Pörtner and Farrell 2008).

For ectotherms in general, substantial temperature increase represents cellular stress due to the inability of proteins to fold properly, as well as due to loss of the integrity of cell membranes, because the excess thermal energy breaks their weaker bonds (Donelson 2015, and references therein). Proteins lose both their structure and function through this process. The response of marine ectotherms to temperature increase is expected to be largely determined by the rate of cellular processes and resulting physiological effects; and proper oxygen delivery to the cells is proposed as an important mechanism to enabling marine organisms to cope with warming (Pörtner and Knust 2007; Donelson 2015).

Research to date suggests that individual marine species, as well as ecological processes are expected to exhibit diverse responses to global change (Harvey et al. 2014). This is especially true under long-term exposure, as oxidative, thermal and osmotic stress threaten to damage cells because mechanisms of cellular protection and repair have the capacity to function for a limited period of time (Pörtner 2010). In addition, mechanisms of protection, such as anti-oxidative defence, anaerobic metabolism or the heat-shock response, can show variable capacities (Pörtner 2010). In response to long-term stress, adaptation should take place at a certain degree and compartments, and should be restricted by the acclimatization capacity of the species and the ecological niche occupied by such species (Pörtner 2010; Crozier and Hutchings 2014). For example, organisms living in a more stable environment adapt to these conditions and the variations specific to their habitat, and are therefore more sensitive to changes outside the usual range.

There are several cellular processes that respond to environmental stress. A common effect of most types of stress is that they damage cell macromolecule structures during the acute phase, activating the response of a common set of cellular responses, such as repairing DNA damage and redox balance regulation (Kültz 2005; Tomanek 2015). Another typical effect of environmental stress is the increased production of reactive oxygen and nitrogen species (ROS and RNS), which can also damage cell structures (Haliwell and Gutteridge 2006; Tomanek 2015). However, a rise in the abundance of oxidative stress proteins does not necessarily means that ROS levels increased enough to damage cell structures, although it is an indication that ROS has increased sufficiently to signal an increase in defence against ROS (Tomanek 2015). Research shows that heat stress induces an increase in oxidative stress dependent antioxidant enzymes, and that this is likely to be a consequence of increased ROS production and proteins related to the generation of reducing equivalents needed to increase defences against ROS (e.g. NADPH) (Tomanek 2014; Tomanek 2015). The cells respond to ROS production by decreasing aerobic metabolism, reducing ROS production through NADH-producing pathways, and with the subsequent oxidation of NADH along the electron transport chain (Tomanek 2014; Tomanek 2015). Cellular defence against ROS also includes mechanisms that raise the efficiency of the defence system by inducing the production of antioxidant enzymes and increasing levels of molecular antioxidants such as glutathione

(Nimse and Pal 2015). It is important to highlight that the consequences of stress and ROS production are not necessarily detrimental, since they are part of the adaptive mechanism, allowing organisms to cope with changing environmental conditions (Barton and Iwama 1991).

Traditionally, comparative physiology studies have investigated elevated CO_2 and temperature separately, even though studies of the interaction of these factors are necessary to understand realistic conditions, to relate cause and effect, and to predict effects at ecosystem-level (Pörtner 2010). Future scenarios indicate that synergistic effects of ocean acidification with other variables such as temperature, threaten marine biota. A good example of this is that CO_2 is usually elevated in expanding oxygen minimum water layers, causing hypoxia and hypercapnia to occur together (Pörtner 2010). A meta-analysis study about the interactive effects of ocean acidification and warming in marine organisms shows negative synergistic effects of acidification and warming in reproduction and survival of marine biota (Harvey et al. 2013). Another meta-analysis study comprising 128 studies representing 119 species from different phyla summarizes the effects of multiple abiotic stressors on marine embryos and larvae (Przeslawski et al. 2015). This study shows that with interactions of temperature and pH there were more synergistic interactions (71%) than additive (15.5%) or antagonistic (8.5%) interactions.

1.3.1 Effects on marine fish

The effects of global change on marine fish are generally expected to be related to direct physiological stress associated with factors such as lowered pH, increased temperature, and lowered oxygen levels, and these factors can contribute to higher disease incidence and morbidity (Crozier and Hutchings 2014). Besides negative effects on growth and survival (Baumann et al. 2012), currently available data about the effects of elevated CO_2 on fish shows significant changes in other crucial physiological functions, such as respiration (Cruz-Neto and Stevenson 1997), blood circulation (Lee et al. 2003), central nervous system (Söderstrom and Nilsson 2000), metabolism (Perry et al. 1988), and behaviour (Ross et al. 2001) together with neurotransmission alterations (Nilsson et al. 2012). The acidification of tissues and body fluids trigger compensation mechanisms, which are not likely to be sustainable over longer periods of time without any physiological damage (Teien et al. 2004; Pörtner 2010). Recent studies strongly emphasize that commercially important fish species are already experiencing phenological and geographical shifts as a consequence of increased temperature, and that their responses will depend on species-specific thermal tolerance and the degree of warming (Gattuso el al. 2015). Increased temperature also causes shifts in community composition, therefore reducing food availability for fish (Pörtner and Farrell 2008).

Anthropogenic-driven environmental challenges like ocean acidification and temperature stress are occurring with increasingly higher frequency and intensity, and mobile animals such as fish, can display behaviours that could help to avoid or escape unfavourable environments and possibly prevent extra energetic costs associated with sub-optimal areas (Cooke and Philipp 2009). However, this is not always possible, depending on the physiological and geographical limitations of each species. The behaviour of marine animals exposed to elevated CO_2 can change in several ways, and there are two main mechanisms that

might explain such changes. First, shifts in energy allocation where substantial physiological costs cause a reduction in available energy for other important biological functions, such as acid-base maintenance; and second, the disruption of the ability to gather, assess and process information from the surroundings, which can impact a fish's decision-making during crucial survival situations (Briffa et al. 2012; Munday et al. 2013). Research on two coral reef fish, cardinal and damselfish fish, show that riso of several degrees above average summer water temperature is enough to cause a reduction in these fishes performances (Nilsson et al. 2008; Munday et al. 2009; Nilsson et al. 2010), which adds to the evidence that tropical fish are sensitive to future temperature increase (Tewksbury et al. 2008; Donelson 2015). Another consequence of temperature rise is that marine fish are extending their territory range polewards (Sandersfeld et al. 2015).

The behaviour of fish is determined by multiple external and internal sensory responses that can be altered by climate change. When such alterations occur they uncover changes in physiological conditions. Studies show that elevated CO_2 impairs the olfactory system of fish, affecting key behaviours such as boldness, lateralization, and learning ability in both reef fish and sticklebacks (Briffa et al. 2012; Näslund et al. 2015). Changes in water pH modify the charge of odour molecule receptor sites, preventing either their recognition or their proper binding to receptors, which could disrupt the perception and avoidance of localized disturbances (Briffa et al. 2012; Munday et al. 2013). In addition, auditory and visual threat responses were also shown to be impaired in marine fish, suggesting a systemic effect of elevated CO_2 on brain function and cognition (Briffa et al. 2012; Chivers et al. 2014).

Increased levels of CO_2 are reported to interfere with sensory inputs and neuronal performance of fish by changing GABA-A system functioning in larval clownfish and damselfish. This mechanism was proposed and demonstrated by Nilsson et al. (2012), showing that the GABA-A neurotransmission was disturbed in larvae and juvenile damselfish exposed to elevated CO_2 (Chivers et al. 2014). The GABA-A receptor is a major inhibitory neurotransmitter receptor in the vertebrate brain. The exposure to elevated CO_2 induces ionic changes in related chloride (Cl-) and bicarbonate ($HCO3^{-}$), suggesting an alteration in the gradient of these anions in neurons of fish exposed to elevated CO_2 (Nilsson et al. 2012; Chivers et al. 2014).

2 Aims of this thesis

The main objectives of this doctoral thesis were to increase information about: 1) long-term physiological and behavioural effects of elevated CO_2 exposure on marine fish species, and 2) the effects of this stressor combined with temperature. These objectives were addressed using: 1) proteomics as a screening tool to give a wide overview of possible outcomes of elevated CO_2 at a cellular-level, combined or not with increased temperature, 2) biochemical analysis to further investigate the results found with proteomics, and 3) behavioural studies to search for effects at organism-level. The approach involving elevated CO_2 combined with a series of temperatures, ranging from above to below optimum, increases ecological realism and contributes to the understanding of possible interactions between the variables. To the present moment, very few studies about the possible effects of ocean acidification and climate change were conducted at long-term, especially with fish species.

3 Methodology

3.1 Fish models

3.1.1 Atlantic halibut

The Atlantic Halibut (*Hippoglossus hippoglossus*) (Fig. 3) is a benthic marine fish widely distributed around the northern part of the Atlantic Ocean and in parts of the Arctic Ocean (Haug 1990). This long-lived fish is the largest flatfish, reaching weights of more than 300 kg and living more than 50 years (Haug 1990). They are "sit-and-wait" ambush predators with remarkable morphology and camouflage, and they feed on benthic crustaceans and fish (Nilsson et al. 2010).

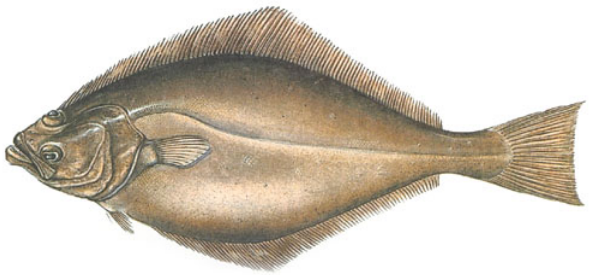


Figure 3. The Atlantic halibut (*Hippoglossus hippoglossus*).
Source: http://www.greenpeace.org/seafood/red-list-of-species/

Early life stages of the Atlantic halibut are not well understood, although it is known that both eggs and larvae drift with ocean currents for substantial distances and that juveniles adopt a benthic life after metamorphosis. Juveniles stay in coastal areas of 20-60 m depth before migrating to distant areas of both shallow and deep waters (Glover et al. 2006).

Atlantic halibut were heavily overfished in the 19th century. After that it became an important cold-water species for aquaculture. Today, the Atlantic halibut is listed as a species of concern (NOOA 2015) due to the slow rate of growth and previous overfishing. Currently there is a large effort in the development of efficient and successful ways to supply healthy juveniles for aquaculture purposes (Gomes et al. 2014).

The Atlantic Halibut was a good model for our studies **(Papers I, II** and **III)** because it is an important species from an ecological point of view, and it has a wide distribution in the Northern hemisphere. Since the Arctic marine biomes are warming twice as fast as the global average (Fossheim et al. 2015), and changes in biota distribution and ecosystem functioning are already being reported (Kortsch et al. 2015), the Atlantic halibut physiology and distribution are susceptible to impact.

3.1.2 Three-spined stickleback

The three-spined stickleback (*Gasterosteus aculeatus*) (Fig. 4) is a small teleost fish (5-10 cm), native to coastal zones of the Northern hemisphere (Wootton 1976). Sticklebacks are closely related to pipefish and seahorses, and despite being ancestrally marine, they

colonized freshwaters after the retreat of Pleistocene glaciers (Barber 2013, and references therein).

Figure 4 The three-spined stickleback (*Gasterosteus aculeatus*).
Source: http://www.semois-chiers.be/infos-pratiques/peche/

Studies of the three-spined stickleback behaviour were started in the early 20th century by European ethologists, and with time they became an iconic species in the fields of animal behaviour and behavioural ecology (Huntingford and Ruiz-Gomez 2009), especially in the reproductive behaviour of males (Wootton 2009). Sticklebacks are considered behaviourally robust since they settle rapidly after a disturbance; they also rely strongly on vision for social interactions and other survival activities (Huntingford and Ruiz-Gomez 2009), which include behavioural displays that facilitate observations from the human point of view.

The three-spined stickleback is a widely used fish model for many reasons: it lives in a wide geographical range of habitats coupled to broadly divergent habitat types, it has a varied diet, it occupies a central position in food webs, has long being studied, and is highly suited for laboratory studies since it usually adapts to captivity within a week (Barber 2013). These features made the three-spined stickleback a good model species for our behavioural study (**Paper IV**).

3.2 Analytical methods

3.2.1 Proteomics

The proteome of an organism is a dynamic and complex system that can respond quickly to environmental changes (Tomanek 2014). Proteins express the biochemical apparatus of an organism under a set of circumstances, and can reflect how organisms respond to a changing environment. Their properties provide insights into molecular phenotypes that represent functional adaptations to environmental change (Albertsson et al. 2007; Tomanek 2014). Since living organisms deal with simultaneous stressors in nature, the study of environmental changes should be done in an extensive way to allow the discovery of complex physiological alterations, such as those caused by elevated CO_2 and temperature Proteomics is a highly suitable research approach in this context. The study of proteins reveals functional disturbances, because proteins are the final product of many redundant gene expression processes (Fig. 5), making protein level results very specific for a tested variable and treatment (Albertsson et al. 2007). A practical advantage of proteomics compared to genomics is that proteins are usually more stable than nucleotides during sample preparation (Anderson and Anderson 1998).

The 2DE (two-dimensional gel electrophoresis) is a high-resolution method able to separate complex protein mixtures by molecular charge in the first dimension and by mass in the second dimension, and can provide several types of information about hundreds of proteins investigated simultaneously (Chandramouli and Qian 2009). The 2DE method is often followed by mass spectroscopy (MS) to identify interesting regulated or modified spots. In **Paper I**, the combination of 2DE and MS were appropriate tools to study the proteome of Atlantic halibut, which had not its genome fully sequenced at the time we completed the study. The 2DE method separates complete and intact proteins with all their modifications (e.g. post-translational), and this is of major importance when the filiation of the protein is crucial information, such as in the case of samples from non-sequenced species (Rabilloud et al. 2010). Also, 2DE is the most preferred technique for parallel quantitative expression profiling of complex protein mixtures such as whole cell and tissue lysates (Gorg et al. 2004), which was the case for our samples.

Regardless of the proteomic separation technique, a mass spectrometer is always used for subsequent protein identification. Mass spectrometers consist of an ion source and an ion detection system that analyse the proteins. This occurs in three main steps (1) protein ionization and generation of gas-phase ions, (2) separation of ions according to their mass to charge ratio, and (3) ion detection (Mann et al. 2001). After MS analysis the derived peptide masses are compared with peptide fingerprints of know proteins in the database using search engines (e.g. mascot), and in this way the proteins are identified.

Quantitative image analysis determines regulated spots, which show changes in biological processes. These changes are often accompanied by individual biological variability. This variability is connected to the plasticity of the proteome, or how long it takes for the cells to

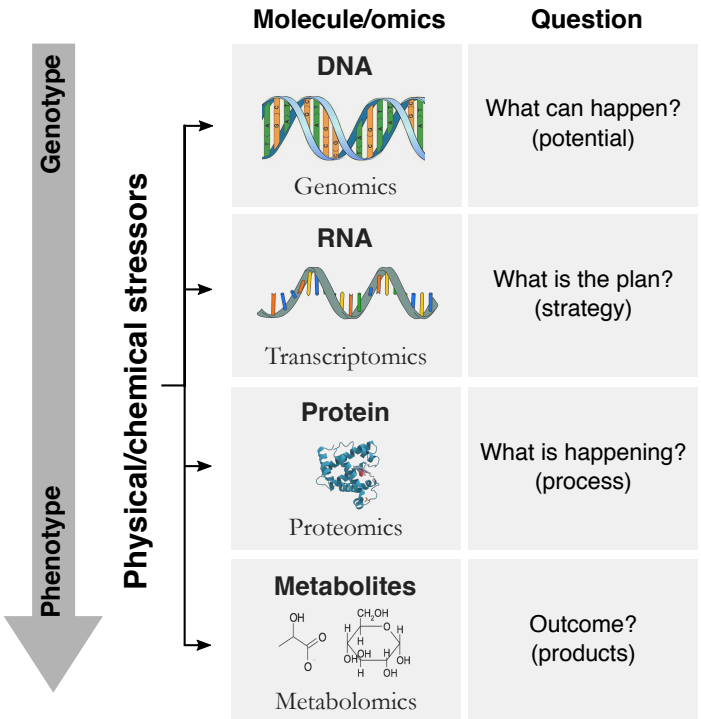


Figure 5. Chart of different "omics" methods and the questions they aim to answer. Modified from Blanco and Ruiz-Romero (2012).

adapt and change (e.g. prokaryotic cells do this faster than mammalian cells), and also the physiological/genetic heterogeneity, as *in vitro* systems are less variable than *in vivo* systems. In **Paper I**, we observed higher individual variation in samples from fish exposed to both environmental stressors (elevated temperature and CO_2) when compared to fish exposed to only elevated CO_2, the first presenting larger standard deviations. The long-held concept of low variability among conspecifics is being contested, while the idea of the importance of individual variability for survival, niche expansion and demographic stochasticity reduction is rising (Macheriotou et al. 2015, and references therein).

3.2.2 Enzymatic assays

The nonspecific immunity of fish is a fundamental defence mechanism against external insults, and is influenced by various external and internal factors such as temperature and stress (Uribe et al. 2011). The complement system and lysozyme are part of the innate immune system; the former can be stimulated by several triggers, playing a range of roles in inflammation, as well as in lysing and opsonizing foreign cells (Li and Leatherland 2012), while the latter is a bacteriolytic enzyme (Uribe et al. 2011). The analysis of immune system components is used as an indicator of fish immune status.

Antioxidants (Fig. 6) are at the frontline of cellular defence, reducing or preventing oxidative stress (Winston and DiGuilio 1991) by either scavenging superoxides and free radicals, or stimulating cellular detoxification mechanisms resulting in increased detoxification of free radicals (Matés 2000). In fish the main antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) (Filho 1996). Since cells under any sort of stress can present increased antioxidant defences and increased oxidative stress, levels of antioxidant enzymes are used as indicators of this physiological state (Stoliar and Lushchak 2012). In addition, the quantification of protein carbonylation is also a proxy of oxidative stress damage, because proteins are important targets of free radical damage in cells (Almroth et al. 2008), and studies of oxidative stress dynamics show that proteins can be oxidized before lipids or DNA in cells with excess ROS (Du and Gebicki 2004).

The enzymatic assays (**Paper II** and **III**) were performed at room temperature in spite of the temperature treatments given to the fish, which is a standard procedure to preserve the enzyme's integrity and activity that would be lost if measured at the same temperature treatments given to the fish during the exposure period. Also, it is a standard procedure to perform enzymatic assays in identical conditions for all samples to allow comparison. According to the literature, the pH has an affect on the activity of enzymes. However, in our case the samples used in **Papers II** and **III** (blood plasma and liver) were not directly in contact with the external environment, being therefore protected from the water pH achieved to simulate ocean acidification. This can be confirmed by our ionic measurements (**Paper II**), which show that most of the fish used in our exposure were able to maintain a proper ionic balance.

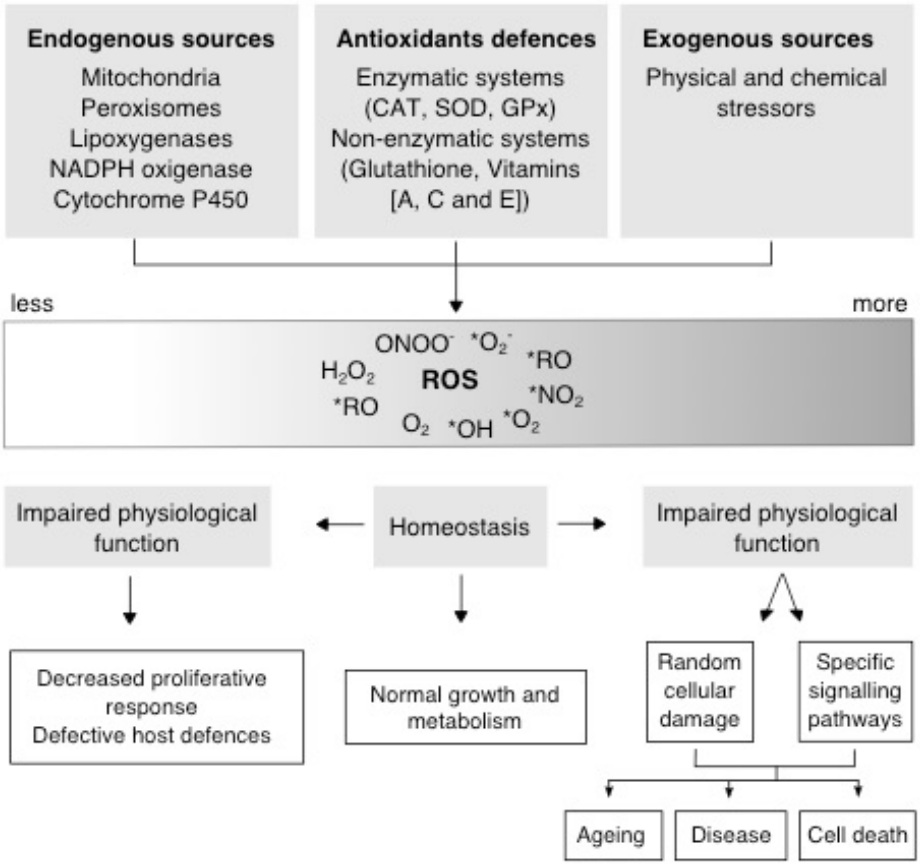


Figure 6. Cellular responses to reactive oxygen species (ROS). Several external agents can trigger ROS production, but oxidants are also produced from the normal intracellular metabolism in mitochondria, peroxisomes, and many cytosolic enzyme systems. Enzymatic and non-enzymatic antioxidants neutralizes and regulates ROS levels to maintain physiological homeostasis. Modified from Finkel and Holbrook (2000).

Changes in protein abundance are not directly proportional or directly related to changes in the enzymatic activity of that same protein. Protein abundance depends on factors such as transcript levels, the rate of translation and the rate of protein degradation (Belle et al. 2006), while the biological activity of a protein depends on processes such as differential splicing, proteolysis, subcellular targeting, assembly into complexes, post-translational modifications, and the levels of substrate and effectors (Stitt and Gibon 2014), among others. Therefore, through enzymatic assays (**Papers II** and **III**) we have measured their activity, not necessarily their abundance, while in **Paper I** we have measured a proxy of protein quantity by using proteomics. The analyses of the proteome and enzyme activity are complementary, since proteomics refers to protein quantity and enzymatic assays provide functional information (Stitt and Gibon 2014).

3.2.3 Fish behaviour

We performed our behavioural experiments (**Paper IV**) at a time when very few studies were published on the behaviour of marine fish exposed to elevated CO_2, especially multiple stressor/long-term studies like ours. Our interest on behaviour began because it can reflect effects at the organism-level (Fig. 7), possibly giving important insights for future studies. In addition, the sensitivity of behaviour as a tool to investigate effects of an exposure can be 10-

1000 times higher than survival tests (e.g. conventional LC_{50}) in the case of a toxicological assessment (Robinson 2009; Hellou 2011). Since the behavioural repertoire of fish (especially sticklebacks) can be very wide, it gave us several options regarding the type of behaviour to be investigated.

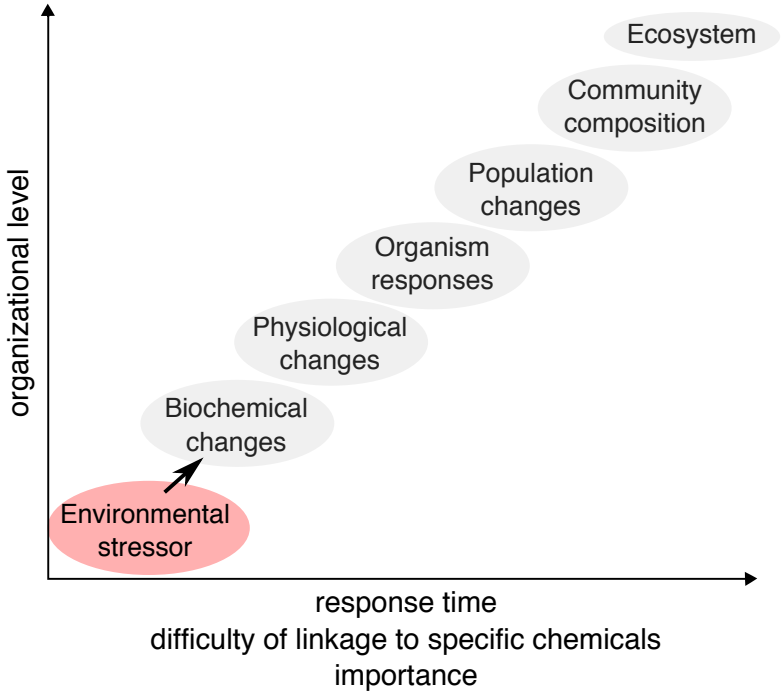


Figure 7. Relationship between responses at different organizational levels. For instance, by investigating behaviour (organism responses) it is possible to look at a wide range of effects and to predict effects at higher organizational levels. However, the higher in the organizational level we investigate more challenging is to relate to the exact causes.

Behavioural studies can be used as early warning signals, and are a proxy used to evaluate possible ecological consequences (Hellou 2011). In **Paper IV** we investigated simple behaviours in response to elevated CO_2 exposure to provide a broad assessment of possible changes. The behavioural tests chosen were based on possible traits that could be affected by elevated CO_2 exposure. *Behavioural lateralization* reflects brain functional asymmetries, and testing this capacity is a powerful proxy of brain function. Brain lateralization is a crucial feature for various decision-making tasks involving left *versus* right responses to environmental stimuli, and this trait has been shown to change in tropical fish exposed to elevated CO_2 (Domenici et al. 2012). The *novel object* test explores the shy-bold axis of behaviour by observing how fish respond to unfamiliar objects or situations, where shy individuals are expected to flee, retreat and become cautious or inactive, while bold individuals are expected not to show these responses or show the opposite behaviour under the same circumstances (Toms et al. 2011). Boldness is an important trait in fish, influencing decision making in situations such as territory defence and predator interaction. The *escape challenge* test was first described in **Paper IV**, and was designed to assess exploratory behaviour due to an improvement in escape time from day 20 to day 40 in the control group, we believe that this procedure also tested memory.

4 Findings and Discussion

The importance of the studies enclosed in this thesis is related to the following: 1) long-term studies regarding the effects of elevated CO_2 and increased temperature on marine fish are scarce; 2) most studies have taken into consideration only one stressor (elevated CO_2 or temperature); 3) research on the effects of elevated CO_2 on marine fish are scarce, since calcifying organisms have received most of the attention due to the ubiquitous effects of lower water pH on their calcium fixation (Wernberg et al. 2012).

4.1 Paper I: Effects of increased CO_2 on fish gill and plasma proteome

The purpose of this study was to use proteomics to provide an overview of the physiological changes in Atlantic halibut exposed to elevated CO_2 and increased temperature, and to give a direction for future studies. The proteomics approach provided an overview of cellular metabolism and pathway changes after long-term exposure to elevated CO_2 and increased temperature, as well as the effects of such stressors combined. This is one of the first studies to be performed with fish to investigate the effects of ocean acidification using proteomics.

The findings in gills (Table 1) show that energy-generating enzymes are up-regulated in fish exposed to both elevated CO_2 and increased temperature. The up-regulation of these enzymes (*ATP synthase*, *malate dehydrogenase*, *malate dehydrogenase thermostable*, and *fructose-1,6-bisphosphate aldolase*) was possibly caused by a higher energy demand and/or increased protein synthesis (Koschnick et al. 2008; Deigweiher et al. 2010). This result indicates changes in the energetic balance, possibly towards acid-base regulation (Melzner et al. 2009) and perhaps other coping mechanisms not investigated here. After elevated CO_2 exposure, gills at control temperature also show modifications in proteins related to energy generation (*enolase-α*, up-regulated; and *glyceraldehyde 3-phosphate dehydrogenase*, down-regulated), although these proteins are multifunctional and also have other cellular functions.

Our findings in gills also suggest increased cellular turnover, supported by the up-regulation of two proteins from each temperature treatment, which are involved in apoptosis and cell proliferation (Hershey 1991; Boersma et al. 2005; He and Sun 2007). At control temperature *annexin (5 and max 1)* and *eukaryotic translation elongation factor 1γ (EF1γ)* were up-regulated, while at 18 °C *receptor for activated protein kinase C* and *putative ribosomal protein S27* were up-regulated. These results indicate that exposure to elevated CO_2 can increase the turnover of gill cells, probably as part of the stress response and cell protection mechanisms. This evidence is reinforced by the down-regulation of the structural protein *tropomyosin* (Claiborne et al. 2002; Choi et al. 2012) in gills at control temperature, since several studies have related the down-regulation of *tropomyosins* with loss of normal structure and cell membrane disturbance (Cooper et al. 1985), a typical effect in gills with increased ionic exchange.

In blood plasma we found higher expression of different isoforms of *complement component C3 (CC3)* and *fibrinogen β-chain precursor* for both elevated-CO_2-exposed

Table 1. Effects of elevated CO_2 exposure at two different temperatures (12 and 18 °C). Spots named as "up" -regulated are those containing increased amounts of protein; "down" -regulated spots are those with reduced amounts of protein; spots named as "high-CO_2" are those detected only in the high-CO_2 group (but not in the control group). The accession number is an identifier given to the protein according to the NCBI database, and the score is the sum of the unique ion scores. "Pep." represents number of peptides. Mann-Whitney U-rank test and p-value ≤ 0.05. n=12, six controls and six exposed.

Matchset	Spot	p-value	Regulation	Protein Name	Species	Proposed Function	Pep.	Accession No.	Score
Gill 12 ˚C				Annexin 5	*Anoplopoma fimbria*	Apoptosis Signalling	6	229366222	569
	1	0.002	Up	Annexin max1	*Epinephelus bruneus*	Apoptosis Signalling	2	328677117	337
				Annexin 5B	*Danio rerio*	Apoptosis Signalling	2	160773369	130
				Cytoskeletal tropomyosin	*Coturnix coturnix*	Cellular Structure and Motility	14	833603	3011
	2	0.005	Down	Tropomyosin 4-α	*Takifugu rubripes*	Cellular Structure and Motility	11	28557136	1686
				Tropomyosin 4-α	*Salmo salar*	Cellular Structure and Motility	11	213515262	1575
	3	0.041	Down	Glyceraldehyde 3-phosphate dehydrogenase	*Acanthopagrus schlegelii*	Multifunctional/Energy Metabolism	2	89147695	129
				Enolase 1- α	*Salmo trutta*	Multifunctional/Energy Metabolism	8	11999265	1061
	4	0.022	Up	Enolase 1- α	*Gillichthys mirabilis*	Multifunctional/Energy Metabolism	9	226441951	775
				Enolase- α	*Acipenser baerii*	Multifunctional/Energy Metabolism	10	98979415	773
	5	0.023	High-CO_2	Eukaryotic translation elongation factor 1 γ	*Pseudopleuronectes americanus*	Protein Biosynthesis/Apoptosis Related	3	28394501	150
Gill 18 ˚C	1	0.047	Up	Fructose-1,6-bisphosphate aldolase	*Sphoeroides nephelus*	Energy Metabolism	6	2828145	1698
	2	0.007	Up	Receptor for activated protein kinase C	*Oreochromis mossambicus*	Inflammation Mediator/Apoptosis Related	9	37498964	1844
	3	0.038	Up	Malate dehydrogenase 1A	*Danio rerio*	Energy Metabolism	7	41053939	1356
				Malate dehydrogenase thermostable	*Sphyraena idiastes*	Energy Metabolism	8	14583129	1169
	4	0.041	Up	ATP synthase subunit α	*Salmo salar*	Energy Metabolism	6	209151440	505
	5	0.046	Up	Putative ribosomal protein S27	*Oncorhynchus mykiss*	Protein Biosynthesis/Apoptosis Signalling	2	14787421	79
Plasma 12 ˚C	1	0.049	Up	Fibrinogen β chain precursor	*Larimichthys crocea*	Inflammation Regulator/Immune System Related	4	218665023	384
				Fibrinogen β chain precursor	*Paralichthys olivaceus*	Inflammation Regulator/Immune System Related	3	146447341	213
	2	0.041	Down	IgM heavy chain constant region	*Hippoglossus hippoglossus*	Immune Response Mediator	8	7769631	804
	3	0.009	High-CO_2	Apolipoprotein AI precursor	*Platichthys flesus*	Immune System Effector/Lipid Carrier	4	60417202	686
	4	0.043	Down	Module-substituted chimera haemoglobin β-α	-	-	6	4929993	395
	5	0.048	High-CO_2	Complement component C3	*Paralichthys olivaceus*	Immune System Effector	8	6682835	365
	6	0.045	Up	Complement component C3	*Dicentrarchus labrax*	Immune System Effector	4	339269297	481
Plasma 18 ˚C	1	0.040	Up	Fibrinogen β chain precursor	*Larimichthys crocea*	Inflammation Regulator/ Immune System Related	6	218665023	1228
	2	0.008	Up	Complement component C3	*Hippoglossus hippoglossus*	Immune System Effector	9	58373439	627

groups (12 °C and 18 °C; Table 1), which suggests that elevated CO_2 alone was the cause. Both proteins have a role in the immune system: *CC3* supports the activation of all three pathways of the complement system in teleost fish: the classical, alternative, and lectin pathways (Holland and Lambris 2002); and fibrinogen regulates inflammation in several tissues (Davalos and Akassoglou 2012). In addition, the down-regulation of *IgM heavy chain constant region* (control temperature) is consistent with these findings, since it mediates immune responses (Tian et al. 2010). These results suggest that elevated CO_2 exposure induced immune activation in the Atlantic halibut.

Conclusions

Exposure to elevated CO_2 caused the up-regulation of proteins related to cellular turnover and apoptosis in gills, as well as the modulation of proteins related to energy metabolism. Exposure to both stressors, elevated CO_2 and increased temperature, caused an up-regulation of several proteins related to energy-generation, which was not observed at control temperature. Therefore, this result is a consequence of both stressors combined. There are also indications of immune system modulation in response to elevated CO_2 exposure at both temperature treatments, suggesting a CO_2-effect on the immune system. To our knowledge, this is the first indication of immune system changes in marine fish in response to elevated CO_2 exposure.

4.2 Paper II: Non-specific immunity of Atlantic halibut exposed to elevated CO_2 at six different temperatures

This study is in agreement with the findings of **Paper I** and shows that the innate immunity of the Atlantic halibut was stimulated by elevated CO_2, since *lysozyme* and *complement system* activities were induced. These results are one of the first indications that elevated CO_2 exposure leads to immune modulation in marine fish.

Lysozyme activity (Fig. 8) was stimulated by exposure to elevated CO_2 in most temperature treatments (except at 14 and 18 °C). This result might be associated to a general stress response, because *lysozyme* activity has been shown to rise in stressed fish (Caipang et al. 2008), and also to increase in several fish species exposed to a number of environmental stressors (Li and Leatherland 2012). The *alternative complement* activity, which plays a central role in *complement system* activation, was stimulated by elevated CO_2 in all temperature treatments (Fig. 8).

The results from **Paper I** and **II** indicate a CO_2-related effect in the activation of the *complement system*. In teleost fish, the *complement system* components play a range of roles in inflammation, as well as in lysing and opsonizing of foreign cells (Li and Leatherland 2012). In addition, in **Paper II** the *complement* activity was not affected by temperature, a result that has been demonstrated before (Magnadottir 2006).

The increase in *lysozyme* activity followed the temperature increase, which is in agreement with previous studies with the Atlantic halibut (Langston et al. 2002). Interestingly, at 18 °C there was a shift in *lysozyme* activity (higher activity in control group) that might be a result of the combination of stressors, because this group was exposed to the highest temperature

combined with elevated CO_2 treatment. Multiple stressors were able to supress lysozyme in the Atlantic salmon (*Salmo salar*; Jokinen et al. 2011).

Plasma [Na^+] and [Cl^-] differ from controls (same temperature) only in fish kept at 10 and 18 °C (Table 2), showing that most of the experimental fish were able to maintain homeostasis after three months of exposure to elevated CO_2. The majority of fish species studied to date had reduced plasma [Cl^-] after exposure to elevated CO_2, though in most studies the exposures were shorter (Brauner and Baker 2009; Esbaugh et al. 2012) than in our study, and might be the reason why we obtained different results. The existing literature on the influence of temperature on plasma ions and osmolality shows variations related to fish species, seasons and other factors (Nordlie 2009), and there are no clear mechanistic studies to explain these variations.

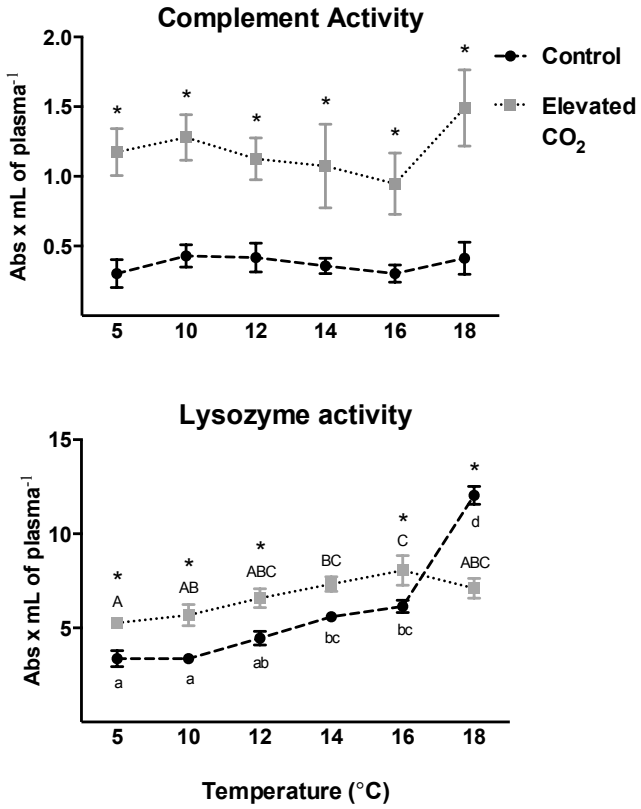


Figure 8. Complement system and lysozyme activities. Asterisks (*) indicate statistical signify-cance between elevated CO_2-treated and control groups at the same temperature. Letters indicate statistically similar means; high and lower case letters represent elevated CO_2 and control groups, respectively. Values as means ± SEM, $P \leq 0.05$ and n = 8.

The lactate levels of most fish exposed to elevated CO_2 do not differ from those of the controls (same temperature) in most groups (Table 2), apart from the fish kept at 16 °C. This finding means that most of our experimental fish were not experiencing lactic acidosis after three months of exposure, which does not necessarily mean that there was no anaerobic energy supply, because it is possible that after long-term exposure the pathways associated with lactate removal were activated.

Table 2. Plasma ions and lactate. Asterisks (*) indicate significant differences within the same temperature treatment. Letters indicate similar means for comparisons between temperatures. Values in bold were calculated from n = 3 and were therefore excluded from statistical analyses. Absent values are from groups of fish with no sufficient blood plasma to perform all measurements. $P \leq 0.05$, n = 5 to 8. Values are presented as means ± SEM.

Temperature (°C)	K^+ (mmol/L)		Na^+ (mmol/L)		Ca^{++} (mmol/L)		Cl^- (mmol/L)		Lactate (mg/dL)	
	Control	Elevated CO_2	Control	Elevated CO_2	Control	Elevated CO_2	Control	Elevated CO_2	Control	Elevated CO_2
5	**7.1 ± 1.8**	**5.0 ± 0.4**	**179 ± 9.7**	**169 ± 0.3**	**1.5 ± 0.20**	**1.2 ± 0.01**	-	-	-	-
10	4.1 ± 1.3^{ab}	4.6 ± 0.1*	162 ± 1.9	171 ± 4.0*	1.3 ± 0.04	1.5 ± 0.09^{a}	146 ± 2.6	134 ± 3.8*	4.4 ± 0.4^{a}	4.9 ± 0.5
12	4.8 ± 0.4^{a}	4.6 ± 0.2	174 ± 2.8	172 ± 1.1	1.3 ± 0.06	1.5 ± 0.07^{ab}	148 ± 3.6	134 ± 1.1	4.8 ± 0.3^{ab}	4.9 ± 0.4
14	4.0 ± 0.1^{ab}	4.7 ± 0.2*	171 ± 1.7	167 ± 2.7	1.3 ± 0.07	1.2 ± 0.03^{ab}	146 ± 1.6	148 ± 2.7	5.8 ± 6.0^{b}	6.0 ± 2.7
16	4.0 ± 0.1^{b}	4.4 ± 0.1*	168 ± 1.3	172 ± 1.3	1.2 ± 0.07	1.2 ± 0.05^{b}	147 ± 1.5	144 ± 1.6	5.1 ± 1.5^{ab}	7.7 ± 1.6*
18	4.3 ± 0.1^{ab}	4.4 ± 0.1	171 ± 3.9	160 ± 1.6*	1.4 ± 0.10	1.3 ± 0.04^{ab}	143 ± 1.1	136 ± 2.9*	5.0 ± 1.1^{ab}	5.4 ± 2.9

Conclusions

This study is in agreement with the findings of **Paper I** and show that the innate immunity of Atlantic halibut was stimulated by elevated CO_2, since *lysozyme* and *complement system* activities were increased. These results are one of the first indications that exposure to near-future elevated CO_2 concentrations can lead to immune modulation in marine fish.

The stimulation of *lysozyme* and *complement system* activities in response to elevated CO_2-exposure (**Paper II)** is perhaps a consequence of non-optimal conditions combined with general physiological stress. These results add to the evidences of immune changes, which are in agreement with findings on **Paper I** that show the regulation of *complement component C3 (CC3)*(activates all immune response pathways), *fibrinogen β-chain precursor* (regulates inflammation in several tissues), and *IgM heavy chain constant region* (mediates immune responses).

Most of the experimental fish were able to maintain ionic homeostasis, which could translate into increased energetic costs, supporting the results from **Paper I** regarding increased energy demand in elevated CO_2-exposed fish.

4.3 Paper III: Biochemical effects of elevated CO_2 levels and different temperatures in the Atlantic Halibut

Oxidative stress can damage macromolecules such as DNA, membranes, and enzymes (Pastore et al. 2003). Levels of oxidized proteins such as protein carbonyls are indicators of oxidative stress (Dalle-Donne et al. 2003; Almroth et al. 2005), and were consistently increased in the elevated CO_2-exposed fish at all temperatures. However, the analysis of antioxidant enzymes does not show the same results, suggesting that elevated CO_2 exposure increased ROS formation, with consequent oxidative damage that bypasses the antioxidant defence system (Fig. 9).

Glutathione Reductase (GR) was suppressed at 12 and 14 °C in response to elevated CO_2, which may indicate an overconsumption of glutathione in other cellular reactions. In contrast, glutathione peroxidase (GPx), which is responsible for cellular protection against lipid peroxidation (Winston and Di Giulio 1991), was more affected by temperature, especially at the extremes (5 and 18 °C), than by elevated CO_2. It is possible that there was lipid peroxidation at those temperature treatments. Superoxide dismutase (SOD) and catalase (CAT) correlate significantly with one another in control but not in elevated CO_2-treated fish. This mismatch in these enzymes can result in increased leakage of ROS, allowing ROS to interact with other molecules, causing oxidative damage. Overall, the activities of antioxidant enzymes did not increase linearly with temperature increase, and this has been observed previously in similar studies with fish (Madeira et al. 2013; Vinagre et al. 2014). The proposed hypothesis in those studies was that oxidative stress-related enzymes might increase in activity with temperature increase until reaching a peak, beyond which enzyme activity might decrease with further increase in temperature. The temperature of this peak depends on the thermal niche of each species and is species-specific (Tomanek 2014). Metabolic changes resulting from exposure to elevated CO_2 are shown to occur in these experimental fish (from the same exposure) in a previous study (Bresolin de Souza et al. 2014) showing the modulation of proteins such as *glyceraldehyde 3-phosphate dehydrogenase*, *fructose-1,6-phosphate aldolase*, and *malate dehydrogenase*. Similar changes, connecting an increased expression of metabolic enzymes and increased oxidative stress have been shown in the Pacific oyster (*Crassostrea gigas*) exposed to elevated CO_2 (Timmins-Schiffman et al. 2014). Acetylcholinesterase (AChE) activity is sensitive to stress and inflammation (Ming et al. 2015) and serum butyrylcholinesterase (BChE) quantification is an indicator of systemic inflammation in humans decreasing in plasma with increase in in-

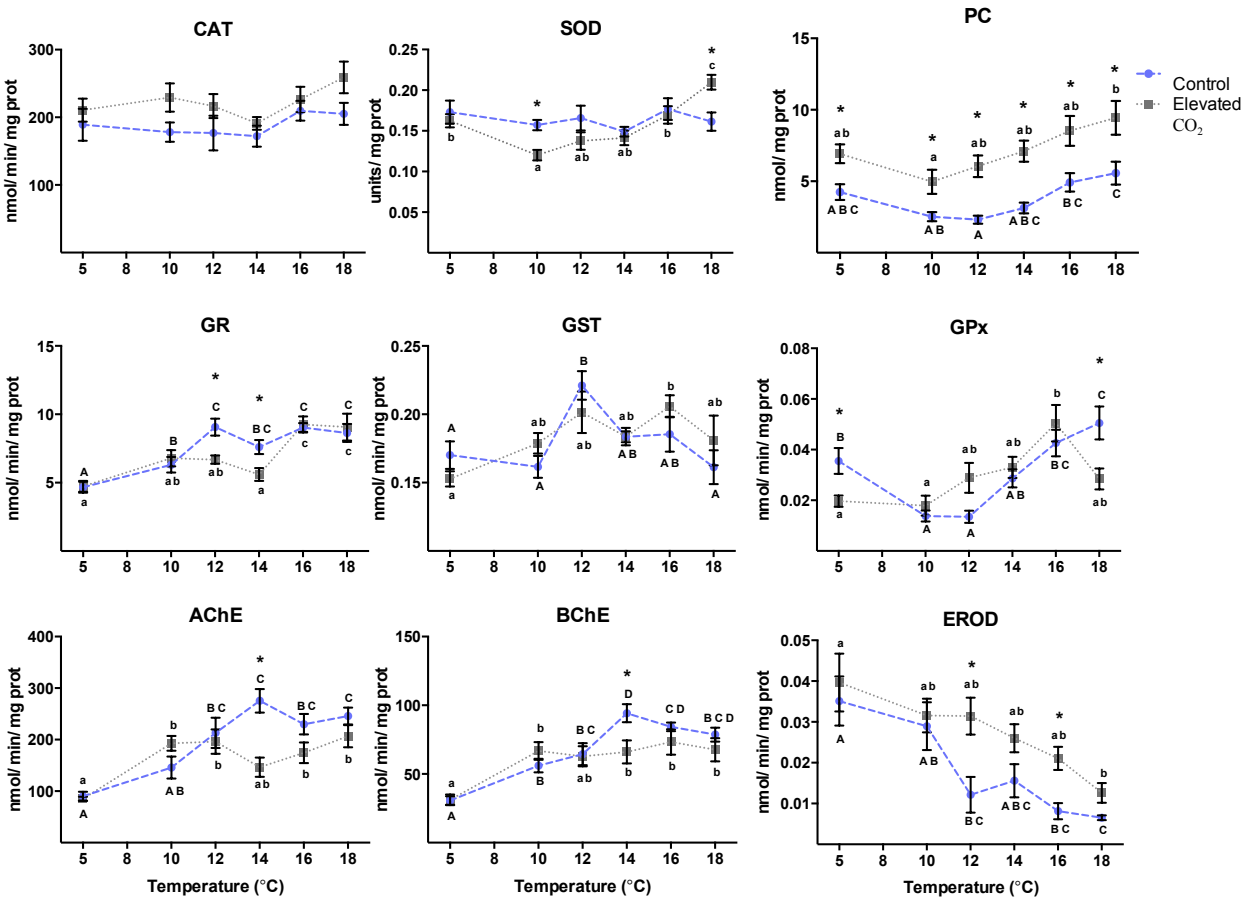


Figure 9. Biochemical parameters measured in liver of elevated CO_2-treated fish exposed to different temperatures. From top left to right down: catalase (CAT), superoxide dismutase (SOD), protein carbonyls (PC), glutathione reductase (GR), glutathione S-transferase (GST), glutathione peroxidase (GPx), acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and ethoxyresorufin *O*-deethylase (EROD). Values of elevated CO_2-treated groups significantly different from controls are indicated by *. Letters indicates significant differences in temperature, high caption letters indicate control and low caption letters indicate elevated CO_2 values. Error bars as ± SEM and confidence interval of 95%.

flammation (Zivkovic et al. 2015). In the present study, both AChE and BChE were inhibited by elevated CO_2 treatment at 14 °C, which may reflect changes in the immune system and is in line with previous studies (Bresolin de Souza et al. 2014; Bresolin de Souza et al., submitted) showing innate immune system modulation in response to elevate CO_2 exposure.

Ethoxyresorufin O-deethylase (EROD) was induced in the elevated CO_2-treated fish at all studied temperatures, thus indicating that there is a CO_2 effect on the CYP1A activity, which is independent of xenobiotic exposure. As in mammals, the induction of CYP1A in fish requires the activation of cytosolic aryl hydrocarbon receptors (AhR) and posterior synthesis of proteins, which includes CYP1A and many phase II enzymes (Whyte et al. 2000, and references therein). Hence, the observed CO_2-dependent increase of CYP1A activity in the current study cannot be due to a xenobiotic induction, but to rather a post-translational or kinetic regulation of the enzyme. In addition, EROD induction has been negatively correlated with temperature, which was not the case for the antioxidant enzyme activities, indicating that these proteins have different regulatory pathways linked to temperature (Solé et al. 2015;

Regoli and Giuliani 2014). Previous studies show the same negative correlation between EROD induction and temperature acclimation in fish (Solé et al. 2015; Whyte et al. 2000). Since CYP1A is membrane-bound it may be more affected by temperature than cytosolic enzymes due to ubiquitous membrane properties.

Conclusions

Elevated CO_2 exposure led to oxidative damage at all temperatures, with levels of protein carbonyls being consistently higher in this treatment, leading to the conclusion that elevated CO_2 alone was the cause. The antioxidant enzyme assays show that elevated CO_2 exposure can generate more ROS than the cells can handle. These findings might be associated to the cellular cytoskeletal disturbances found in gills of the elevated CO_2-exposed fish (**Paper I**), since the cytoskeleton is a major target for ROS and RNS (Dalle-Donne et al. 2001), but might also be a result of increased ionic exchange (**Paper I**). The relationship between elevated CO_2-exposure, oxidative stress, and cytoskeleton disturbances have also been demonstrated in the eastern oyster (*Crassostrea virginica*; Tomanek 2011).

The increased activity of the studied cholinesterases (AChE and BChE) could indicate an inflammatory state, a result that is in line with the findings of **Papers I** and **II**. The EROD induction is possibly a result of a post-translational or kinetic regulation of CYP1A enzymes.

4.4 Paper IV: Behavioural disturbances in a temperate fish exposed to sustained high-CO_2 levels

This study shows robust effects in all of the behavioural tests performed with the three-spined stickleback, where several behavioural features were affected by elevated CO_2 exposure. In the lateralization experiment, performed at the 40th day of exposure, control fish turned to their preferred side 70% of the time on average, while elevated CO_2-exposed fish did not show a preference and turned to each side 50% of the time (Figs. 10 and 11), an effect larger than at the 20th day of exposure. A similar reduction in lateralization has previously been described in a coral reef fish larvae exposed to elevated CO_2 (Domenici et al. 2012; Nilsson et al. 2012), suggesting that elevated CO_2 might affect lateralization of fish from different environments and life stages. The importance of behavioural lateralization for fish can be exemplified in a range of situations, such as multi-tasking, orientation, and escaping harmful situations (Dada 2006; Nepomnyashchikh and Izvekov 2006), and any disturbance affecting this feature may reduce fitness.

The difference in escape time at the 40th day of exposure was a reduction of escape time by the control group. This experiment was not specifically planned to test learning and memory, yet the improvement of the control fish in escape time from the 20th to the 40th day is possibly because the fish remembered the previous trial. The elevated CO_2-exposed fish showed no improvement at the second test, which indicates decreased learning capacity. Learning and memory are vital for fish survival, such as learning new possible predators and remembering new feeding grounds. Fish can learn about their environment through their own experience, through cues from injured conspecifics, and from observing other fish (Sloman et al. 2006), therefore any learning impairment may affect fish survival.

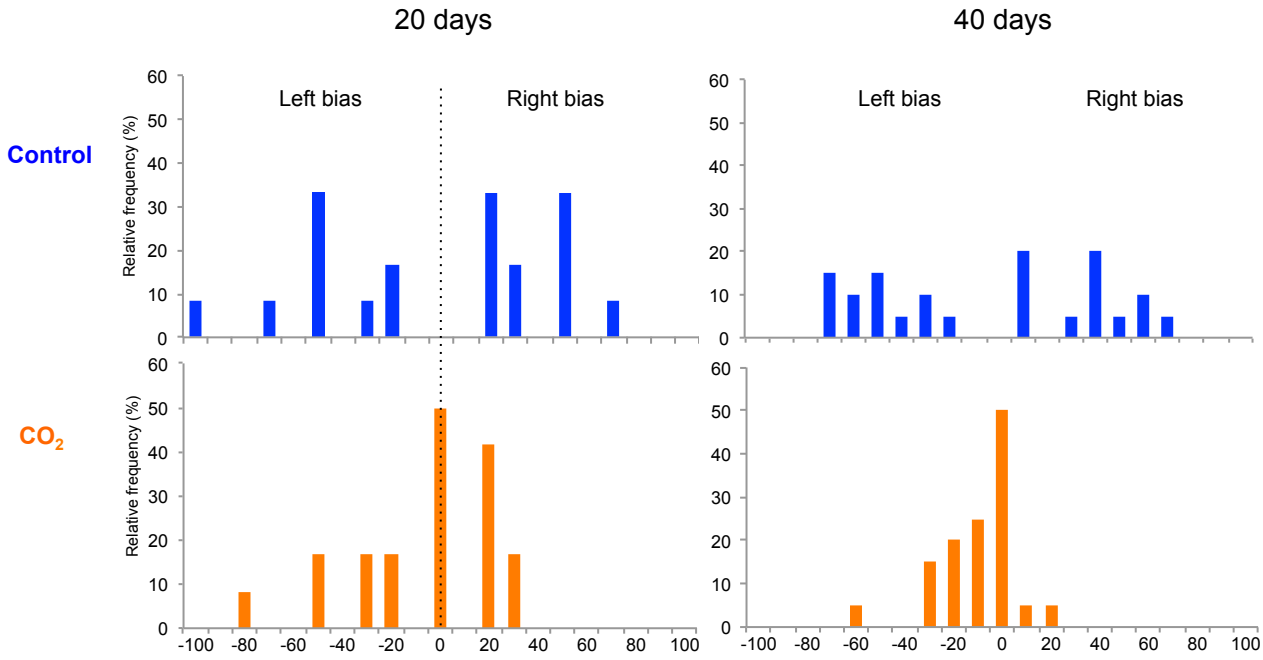


Figure 10. Relative lateralisation index of fish exposed to control water or CO_2-enriched water for 20 and 40 days. The histograms show the frequency of fish with each side preference from -100 to 100, where -100 indicates that all turns were to the left, 0 indicates that half of the turns were to the left and half were to the right, and 100 indicates that all turns were to the right. n = 20-25.

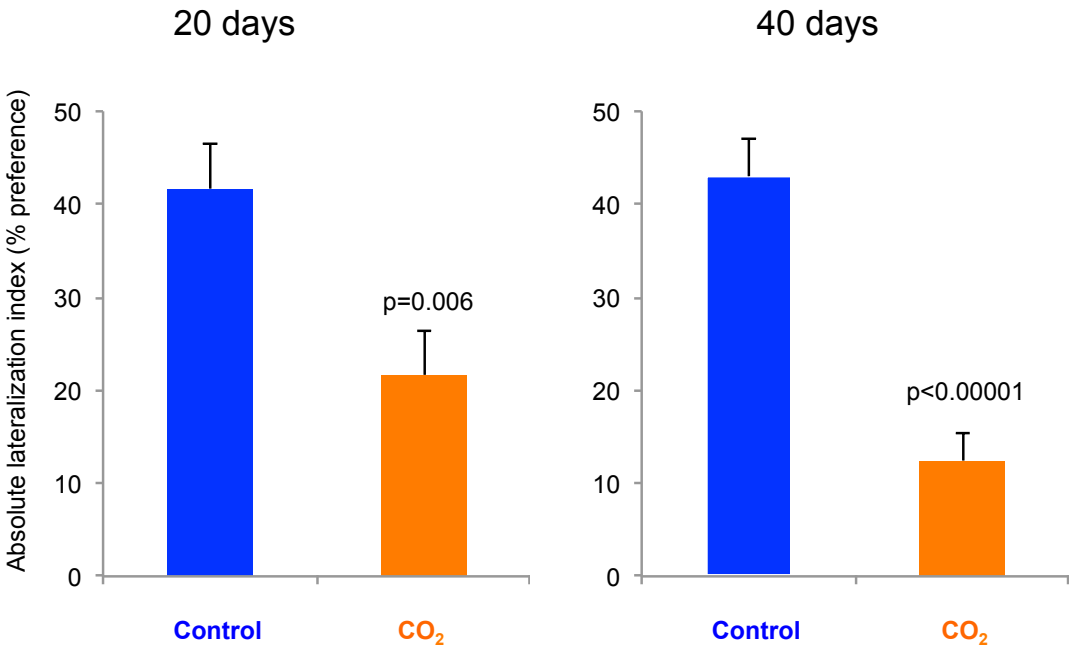


Figure 11. Absolute lateralisation of fish exposed to control water or CO_2-enriched water for 20 and 40 days. N=20-25. Data are presented as means ± standard error of the means (SEM), and *p*-values indicate statistical significance (*t*-test).

Sticklebacks are physiologically plastic and have a great potential for acclimation to environmental changes (Pottinger et al. 2002; Östlund-Nilsson et al. 2006; Barrett et al. 2011), they also have short life spans of 3 years on average. Thus, a 40-day exposure is considered long-term compared to the life span of sticklebacks and should allow acclimation to a large extent. However, our results show that acclimation mechanisms, if present, failed to restore all tested behaviours, and the cause of which is unknown.

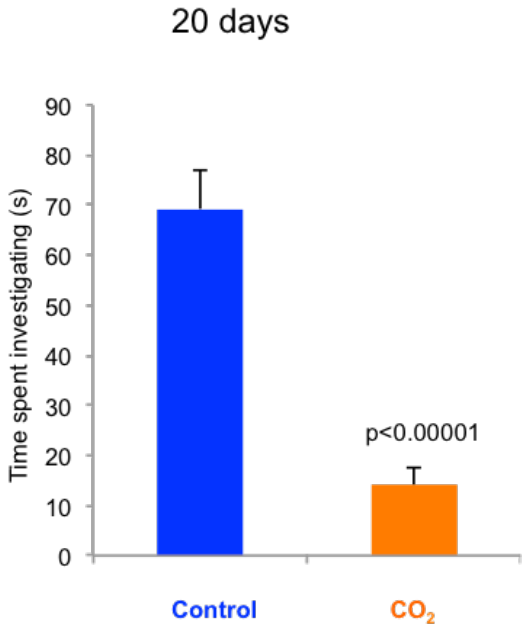


Figure 12. Novel object test. Bars show the average time spent investigating a novel object after 20 days of control or CO_2 exposure. N=20 for each treatment. Data are presented as means ± SEM, and the *p*-value indicates statistical significance (*t*-test).

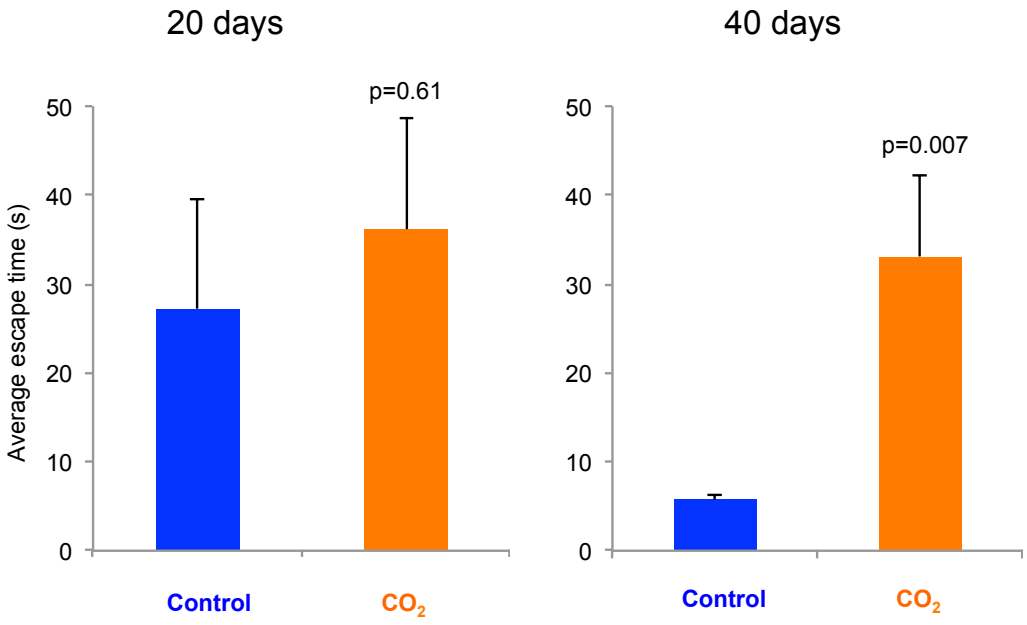


Figure 13. Average escape time from a chamber by fish exposed to control water or CO_2-enriched water for 20 and 40 days. N=20 for each treatment. Data are presented as means ± SEM, and *p*-values indicate statistical significance (*t*-test).

Since performance in all three tests (and learning) was severely impaired, it is possible that other aspects of the behavioural repertoire may also have been affected by elevated CO_2 exposure. From a physiological viewpoint, ubiquitous changes in behaviour are consistent with the proposed neurophysiological mechanism involving altered GABA-A function (Nilsson et al. 2012), since GABA-A receptors are omnipresent in the central nervous systems of vertebrates and any disturbance to the GABA system would affect several behaviours. Thus, complex behaviours such as prey capture, predator avoidance, and mating rituals would potentially be disturbed in a natural setting with subsequent negative fitness consequences.

Conclusions

Ocean acidification could adversely affect temperate fish behaviour this century. Despite sticklebacks being highly tolerant to many other environmental factors, acclimation to counter the effects of elevated CO_2 exposure was not detected in the behavioural endpoints investigated. If the adaptation of tolerance to elevated CO_2 is slower than the rate of CO_2 increase the ecological consequences of ocean acidification could be severe.

5 Thesis Conclusions

The studies in this thesis provide significant insights regarding how fish are affected by near-future elevated CO_2 concentrations and temperature. It also shows physiological changes possibly related to detrimental effects and/or acclimation mechanisms that could allow fish to cope with the conditions in our experimental exposures. The initial proteomics approach (**Paper I**) was a source of valuable information when very little was known about possible effects of near-future ocean acidification on fish, especially in combination with increased temperature.

In **Paper I** elevated CO_2 exposure modulated the expression of energy metabolism proteins, and up-regulated proteins involved in cell apoptosis and turnover in gills. There were also indications of immune system modulation related to CO_2-exposure. The results of **Paper I** were further investigated in **Paper II** and **III**. **Paper II** shows that elevated CO_2 exposure stimulated *lysozyme* and *complement system* activities of fish, possibly as part of a protective mechanism, and perhaps related to the oxidative stress observed in **Paper III,** since immune and oxidative stress responses are often related. The stimulation of innate immune components (*lysozyme* and *complement system)* in response to elevated CO_2 exposure (**Paper II)** is possibly a response to non-optimal conditions combined with general physiological stress, which includes increased oxidative stress (**Paper III**).

There is possibly a connection between the modulation of *complement component C3 (CC3)*(activates all immune response pathways), *fibrinogen β-chain precursor* (regulates inflammation in several tissues), and *IgM heavy chain constant region* (mediates immune responses) seen in **Paper I**, together with the stimulation of *total complement* and *lysozyme activities* (innate immunity constituents) in **Paper II,** and the reduced activity of AChE and BChE (both related to inflammation) in **Paper III.** These are all components of protective mechanisms triggered by elevated CO_2 exposure, since they are all related to the immune system and many play a role on inflammatory disorders. These results are the first indications of immune modulation as a result of elevated CO_2 exposure in marine fish.

Most of the studied fish were able to keep ionic homeostasis (**Paper II**), however the active acid-base maintenance is energetically costly for fish. Shifts in energy allocation towards ionic homeostasis maintenance, immune system modulation, oxidative damage reduction, increased cell turnover (**Paper I**), and perhaps other protective mechanisms not investigated here, represent extra energetic costs for fish, which have the potential to reduce fitness. Higher energy allocation towards these processes, are possibly related to results from **Paper I** regarding the modulation of energy metabolism proteins (mostly up-regulation) in elevated CO_2-exposed fish at both studied temperatures. **Paper I** also shows that the exposure to both elevated CO_2 and increased temperature raised the expression of several proteins related to energy-generation, suggesting that these effects are a consequence of both stressors combined.

Paper III shows that elevated CO_2 exposure can generate more ROS than the cells can handle, and the consequent oxidative stress might affect the mitochondria's capacity. Several pieces of evidence, among present (**Paper III**) and previous (**Papers I** and **II**) findings, indicate a CO_2-effect on mitochondria. Apart from oxidative stress, the mitochondria are

involved in metabolism, apoptosis, and innate immunity (Shadel and Horvath 2015), and all these systems were affected by elevated CO_2 exposure in our studies. **Paper I** shows that elevated CO_2 exposure affected gill cell metabolism and apoptosis signalling, as well as innate immunity (blood plasma) in the Atlantic halibut (**Paper I and II**); while **Paper III** shows evidence of CO_2-related oxidative stress in liver of the same fish.

In **Paper IV,** elevated CO_2 concentrations were shown to affect temperate fish behaviour, reducing boldness, exploratory behaviour, lateralization, and learning. These are crucial behaviours for fish survival, and were consistently reduced throughout the exposure period and increased in effect size with exposure time. Despite sticklebacks being highly tolerant to many other environmental factors, their behaviour was strongly affected by elevated CO_2 exposure. The loss of normal behaviour can represent serious negative effects on both physiology and on the behavioural repertory necessary for fish survival. If the evolution of tolerance to elevated CO_2 is slower than the rate of CO_2 increase, the ecological consequences of ocean acidification could be severe.

The results of this thesis show that moderate CO_2-driven acidification, predicted to occur at the end of this century, can elicit biochemical and behavioural changes that can impair fish health and survival if acclimation fails to occur or fails to be sustained. The combination of elevated CO_2 and increased temperature will possibly cause larger shifts in energy allocation towards protective and coping mechanisms, which can translate into even greater physiological challenges to marine fish.

6 Outlook

Understanding long-term impacts of global change is challenging because most of the experimental research to date focuses on the short-term and individual stressors in isolation. The field of ocean acidification, climate change, and fish physiology would benefit from more studies about a combination of stressors, which are more ecologically realistic. It is also very important to combine ocean acidification and/or temperature studies with other non-CO_2 environmental pollutants, since very little is known about this subject. Contaminants are ubiquitous in the environment and their impacts are of increasing concern due to human population growth and the generation of deleterious effects in aquatic species.

In research, there should be more studies originated from the same experiments and/or with the same studied subjects (such as **Papers I, II,** and **III** of this PhD thesis). Studies like these were a source of more information and insight than if they had been isolated studies, and it is also a good way to apply the 3R's in science. Multiple studies where information from common studied subjects, can be built and connected are rare. Isolated studies, as are the rule in research, generate information that is more difficult to understand and interpret, which also uses more time and resources.

Additional long-term studies are necessary to evaluate to what extent marine fish can acclimatize to elevated CO_2 and temperature increase. Future research should investigate if the observed physiological changes will persist after a longer exposure period, as well as their consequences to teleost fish. Mechanism-based investigations are also needed to further understand possible implications at population level. Also, the focus should be on vulnerable species, species that live in areas of rapid changing, and species with little data available.

Awareness of the capacity of teleost fish to adjust and possibly adapt to future environmentally relevant CO_2 concentrations and temperature is fundamental in order to predict and perhaps remediate biological impacts. Only through research it is possible to estimate potential impacts on marine fish populations and therefore enable the creation of management strategies.

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